An Investigation into Factors Affecting Condensin Association with Mitotic Centromeres

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Statement

I hereby declare that this thesis has not been, and will not be submitted in whole or in part to another university for the award of any other degree.

Signature:.....

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Doctor of Philosophy (Genome Damage and Stability)

An Investigation into Factors Affecting Condensin Association with Mitotic Centromeres

SUMMARY

The SMC protein family (Structural Maintenance of Chromosomes) consists of a group of highly conserved protein complexes, central to chromosome dynamics and key cell cycle events. Condensin is a member of the SMC protein family, best known for its role in chromosome condensation and segregation in mitosis. The condensin complex becomes enriched at specific chromosome loci in a cell-cycle specific manner. However, the details of how it becomes associated with chromatin remain unclear. A particular area of interest regarding condensin association and activity is at the centromeres and pericentromeres, where condensin has been consistently shown to be enriched specifically during mitosis.

This work is comprised of four results chapters, investigating factors affecting condensin association with mitotic centromeres in *Saccharomyces cerevisiae*, using chromatin immunoprecipitation (ChIP). We started by establishing a robust ChIP assay suitable for probing condensin enrichment at the centromeric regions. We conducted genetic control experiments to ensure the functionality of the experimental technique. In the next chapter we explored the importance of the kinetochore with regards to condensin enrichment, and found that perturbing the budding yeast kinetochore results in a loss of centromeric condensin association during mitosis. We then used condensin phosphorylation site and mitotic kinase mutants to examine the role of condensin subunit phosphorylation in its association with chromatin. Our results showed that Ipl1 (Aurora B kinase) and condensin phosphorylation is important for its enrichment at the centromere, but rather surprisingly that Cdc5 (polo-like kinase) a known activator of condensin does not appear to be. The final chapter investigates the function of condensin's intrinsic ATPase activity, and we found that ATP-binding activity but not ATP-hydrolysis is important for condensin association with chromatin.

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1 Introduction

1.1 Organising chromosomes in cells

If the DNA in just one human cell were stretched out end to end, it would extend to a length of about 2 metres. However, the nucleus containing these nucleotide polymers is only ~6 μm in diameter. This is the equivalent of squeezing 24 miles worth of very fine thread into a tennis ball (Alberts B 2002). To facilitate the organisation of these long molecules in such a relatively small compartment, DNA is packaged into a DNA-protein structure known as chromatin. Histone proteins bind to DNA and generate folds, loops and coils to provide an organised chromatin structure that can be contained within the nucleus, yet still easily accessed by enzymes and other proteins important for cellular processes such as transcription, DNA replication and cell division.

For cell division to occur, DNA packaged as chromatin must undergo another major compaction step in mitosis. During this chromosome condensation, long thread-like chromatin is condensed to form well defined, rod-shaped mitotic chromosomes (Kschonsak and Haering 2015, Antonin and Neumann 2016, Piskadlo and Oliveira 2016). Chromosome condensation was first observed over 130 years ago by a cytologist named Walther Flemming. Using salamander eggs dyed with coal tar, and a basic brightfield microscope, Flemming observed and documented the process of chromosome formation and segregation in mitosis. The mechanisms of mitotic chromosome organisation are still poorly defined. However, the activity of the SMC complexes, particularly the condensin complex are closely linked to this process. The aptly named condensin complexes are members of this SMC protein family and play a pivotal part in chromosome condensation and resolution in mitosis.

Although condensins were discovered almost 3 decades ago and have been under the scrutiny of research scientists worldwide since then, it is still unknown how condensin

operates at a molecular level to achieve genome-wide chromosome condensation. Improper chromosome condensation and segregation can lead to genomic instability, aneuploidy and cell death. Miss-regulation of chromosome condensation machinery has been linked to human disorders such as primary microcephaly (Hirano 2012) and cancer (Ham *et al.* 2007, Strunnikov 2010). Therefore, information regarding regulation of condensin, including factors affecting its association with chromatin could prove useful in further understanding its role in human disease.

1.2 Chromatin structure and Chromosome condensation

The basic unit of chromatin is the canonical nucleosome, which is comprised of 147 bp of DNA, tightly wrapped in a left-handed manner 1.65 times around histone proteins H2A-H2B and H3-H4, which are arranged in an octomeric complex (Richmond and Davey 2003). Each nucleosome is 11 nm in diameter, and commonly associates with the linker histone H1. H1 wraps a further 20 bp of DNA resulting in two full turns around the octomer, forming a structure called the chromatosome (Bustin *et al.* 2005). Nucleosomes are spaced about 20 bp apart throughout the length of a chromosome (however this varies between species and developmental stages), and the DNA in-between them is referred to as linker DNA. Each chromosome is therefore a long chain of nucleosomes and linker DNA, which appear as 'beads on a string' when observed using EM (electron microscopy) (Olins and Olins 1974).

For the accurate partition of genetic information from a mother cell into two daughters, the process of cell division in mitosis necessitates a further condensation of thread-like chromatin into rod-shaped mitotic chromosomes. To provide an idea of the scale of this condensation stage, DNA packaged as interphase chromatin is already quite significantly compressed with a compaction ratio of approximately 1000-fold. However, in the formation of mitotic chromosomes the compaction ratio in vertebrates is estimated to be 2-3 times more than in interphase, as assayed by

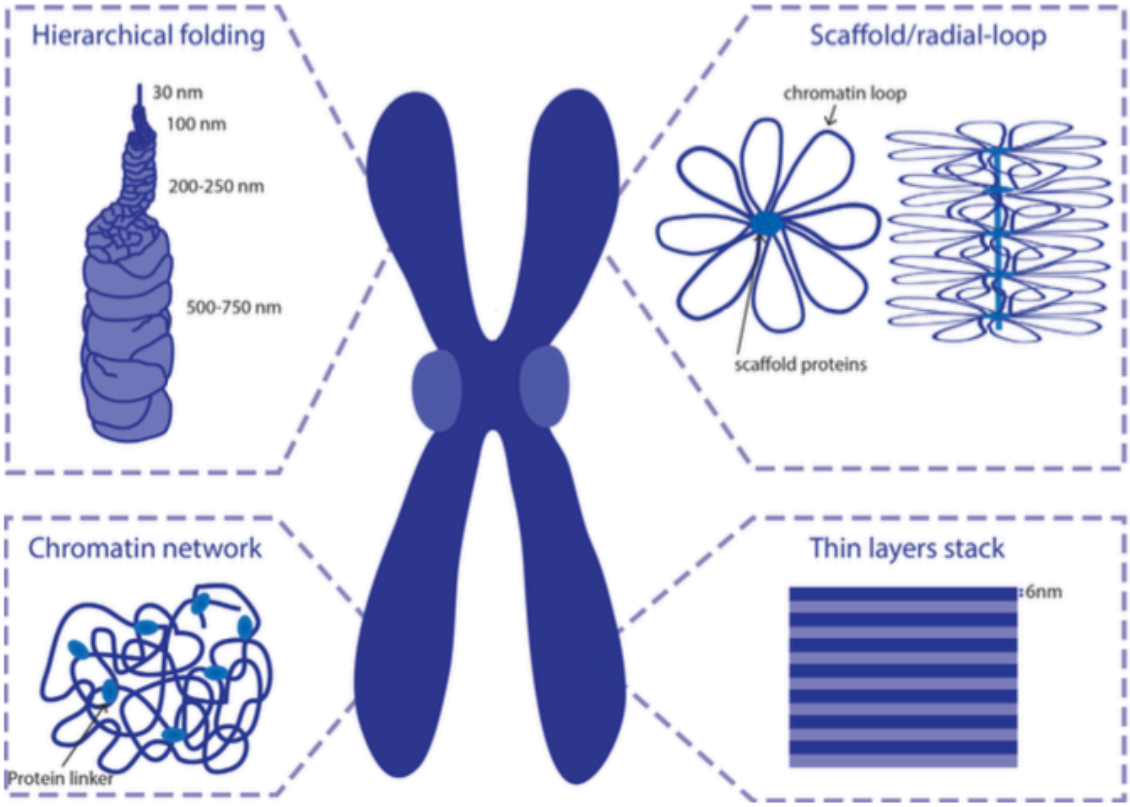


Figure 1.1 Schematic representation of current models for mitotic chromosome condensation.

Taken directly from (Piskadlo and Oliveira 2016). This figure demonstrates four current models for mitotic chromosome condensation (described in the text): Hierarchical folding, scaffold/radial loop, chromatin network and Think layers stack.

chromatin volume measurements (Mora-Bermudez *et al.* 2007, Martin and Cardoso 2010) and FRET analysis between histone proteins (Lleres *et al.* 2009).

Spatial compaction of chromatin is not the only important feature of mitotic chromosome condensation. The structural changes during mitotic condensation also facilitate the resolution of identical sister chromatids, so they can be equally distributed into two cells. For this to occur, entanglements between chromatids arising during interphase must be resolved. Furthermore, mitotic compaction of chromatin is also required to generate chromosomes with appropriate levels of stiffness and elasticity so that they're able to withstand pulling forces from mitotic spindles in anaphase (Piskadlo and Oliveira 2016).

The study of chromatin, chromosome structures and how DNA can cycle between the two states of compaction, has proven difficult over the years. Several factors have

contributed to the challenging nature of these investigations: The multi-scale nature and dynamic properties of chromatin as well as the complexity of its molecular composition have made it difficult for structural biologists, biochemists and geneticists to elucidate the interactions and components of chromatin that determine how it is organised (Belmont *et al.* 1999, Luger and Hansen 2005, Woodcock and Ghosh 2010). Whilst the processes of chromosome condensation are still unclear in molecular terms, various theories have been proposed in an attempt to explain mitotic chromosome organisation. These theories can be grouped into four main models of chromosome condensation (see Figure 1.1). The first of these models proposes that DNA is hierarchically folded into structures of increasingly high order by helical coiling, to achieve chromosome compaction (Belmont *et al.* 1987). The idea of hierarchical folding of chromosomes was long accepted, based on the *in vitro* evidence of coiling and folding of lower levels of chromatin. While the basic unit of chromatin is known and widely acknowledged, details regarding the higher order structure are highly debated. It has been generally accepted for some time that the histone H1 stabilises the folding of nucleosomes and linker DNA into solenoidal 30 nm chromatin fibres as (Horn and Peterson 2002) seen by electron micrographs of eukaryotic chromosomes (Finch and Klug 1976). There have been multiple hypotheses regarding the arrangement of the nucleosomes in the proposed 30 nm chromatin fibre; current models include a one-start interdigitated solenoid (Robinson *et al.* 2006) and two-start zigzag models (Song *et al.* 2014). However recent attempts to identify the presence of 30 nm chromatin fibres *in vivo* have been inconclusive (Eltsov *et al.* 2008, Nishino *et al.* 2012), Indeed, no repeating chromatin structures larger than the 10 nm nucleosome fibre have been identified in these recent studies, leading to alternative ideas emerging regarding chromatin structure.

The second model suggests that mitotic chromatin forms a series of radial loops of several ten kilo bases (kb) in length, which are folded around a central axial protein scaffold which acts as a ‘glue’ to maintain the chromosome structure (Paulson and Laemmli 1977, Marsden and Laemmli 1979, Hansen 2002). This model is based on a study demonstrating that upon removal of histone proteins, chromosomes maintain a scaffold or axis, surrounded by loops of chromatin attached to the inner core,

visualised by EM (Paulson and Laemmli 1977, Marsden and Laemmli 1979). These first two models are not mutually exclusive; it is possible for example, that hierarchically folded chromatin could be looped around a central axis, as suggested in a unified model of chromosome structure proposed by (Kireeva *et al.* 2004).

In contrast, experiments have shown that DNA digestion, but not protease treatment removes the elasticity of chromosomes (Poirier and Marko 2002) (Pope *et al.* 2006). These findings appear to rule out the possibility of a central protein scaffold (Poirier and Marko 2002). The pattern of protease-induced chromosome unfolding observed is thought to be consistent with a cross-linked mitotic chromosome. Therefore the chromatin network model was proposed. In this model, chromatin is the axial component of chromosomes, cross-linked intermittently with protein linkers. Finally, the most recently proposed model suggests that the banding patterns observed in chromosome karyotyping, can be explained by stacks of thin planar layers of chromatin (6 nm) that sit perpendicular to the chromosome axis (Daban 2015).

The extent and timings of chromosome compaction have been investigated in multiple organisms. Studies in live cells have shown that condensation begins in early prophase, with maximal compaction apparent in late anaphase, and de-compaction beginning in telophase (Mora-Bermudez *et al.* 2007, Lleres *et al.* 2009, Petrova *et al.* 2013). The extent of chromosome condensation that occurs upon entry into mitosis has been seen to vary between organisms. For example, distance measurements between chromosome markers in *Saccharomyces cerevisiae* have shown that unlike the global extensive chromosome condensation observed in human cells, only certain chromosome regions (such as the ribosomal DNA repeats) exhibit significant condensation events during mitosis (Vas *et al.* 2007). The extent of chromosome condensation in *S. cerevisiae* was shown to be dependent on chromosome size; the longer the chromosome polymer, the more it is condensed. It is thought that this positive correlation between chromosome length and the extent to which it is compacted, may be down to a ‘mid-zone’ ruler (Ladouceur *et al.* 2011). The additional chromosome-size-dependent compaction was observed in anaphase (Neurohr *et al.* 2011). Cell size reduces dramatically during metazoan development while genome size

remains unchanged, and it is thought that a conserved 'midzone ruler' mechanism may be in place to ensure appropriate levels of chromosome compaction are maintained throughout development.

Differences have also been observed in the timings of chromosome condensation. In most metazoans, there is a pre-NEBD (nuclear envelope breakdown) compaction (Maddox *et al.* 2006, Minocherhomji *et al.* 2015). This is followed by a further compaction after NEBD, facilitated by the influx of multiple cytoplasmic proteins involved in condensation. Live cell fluorescence microscopy in *Caenorhabditis elegans* revealed an unusual bi-phasic chromosome condensation (Maddox *et al.* 2006), during which two compaction stages occur prior to NEBD. Despite these inter-species differences observed in the process of chromosome condensation, and the uncertainty surrounding the structure of mitotic chromosome, factors have been identified that are universally required for mitotic compaction of chromatin; the primary effector of which is the aptly named condensin complex of the SMC protein family.

1.3 Structural Maintenance of Chromosomes – The SMC protein family

The SMC proteins (Structural Maintenance of Chromosomes) are a highly conserved set of ATPase protein complexes, which are central to the processes of chromosome dynamics throughout the cell cycle. In eukaryotes there are three core SMC complexes: condensin, cohesin and Smc5/6. These SMCs play important roles in chromosome condensation, sister chromatid cohesion and also DNA repair, transcription and replication (recently reviewed in (Jeppsson *et al.* 2014). SMC protein complexes consist of an SMC heterodimer (Smc2/Smc4 for condensin, Smc1/Smc3 for cohesin, and the Smc5/Smc6), and a set of complex-specific non-SMC proteins (see Figure 1.2). Although none of these canonical SMC family members have been found in prokaryotes, functionally equivalent and structurally similar SMC homodimers can be found in most organisms (Cobbe and Heck 2004). SMC proteins consist of two globular

domains joined by a long stretch of anti-parallel coiled-coil. Each SMC protein folds over on itself along the coiled coil region, and N- and C- termini meet to form an ATPase head domain. The fold of both SMC proteins meet at the top of the complex to form a hinge at the apex of a V-shaped dimer. The SMC complexes are relatively large; the coiled coil arms stretch approximately 50nm in length (the equivalent of 150 bps of dsDNA). All three SMCs have a Kleisin subunit that interacts with the ATPase head domains, and Condensin (I and II) and Cohesin each have two accessory HEAT domain proteins. The HEAT proteins are predicted to be mainly composed of α-helical HEAT (Huntingtin, elongation factor 3, the A subunit of PP2A TOR lipid kinase) repeat motifs. The Smc5/6 complex does not include HEAT proteins, but instead has two associating KITE subunits (Kleisin interacting tandem winged-helix elements) (Palecek and Gruber 2015).

Data obtained using atomic force microscopy (AFM) and EM have shown the SMC dimers and complexes to form mainly V, O and rod-shaped configurations (Melby *et al.* 1998, Anderson *et al.* 2002, Haering *et al.* 2002, Matoba *et al.* 2005, Soh *et al.* 2015). However, the coiled-coil arms of SMC complexes have also been observed to extend from the hinge in almost opposite directions from each other in some prokaryotes (Haering *et al.* 2002, Li *et al.* 2010). The variety in SMC complex configurations observed may be down to intrinsic structural flexibility, differences between complexes, or experimental artefacts. It is likely that SMC complexes undergo conformational changes during their interactions with chromatin, and is it thought that these changes are tightly linked with the ATPase activity of these complexes (Soh *et al.* 2015). Indeed, a recent paper uses EM and FRET (fluorescence resonance energy transfer) analysis to demonstrate a transformation from a rod to ring configuration of Smc-ScpAB prokaryotic condensin. A process which is dependent on ATP and DNA binding (Soh *et al.* 2015).

Whilst best known for their essential roles in chromosome condensation and segregation (condensin), sister chromatid cohesion (cohesin) and DNA repair (Smc5/6), the range of cellular functions involving these SMC complexes is a list that continues to expand. In fact, SMCs are involved in most DNA-based processes. All three complexes

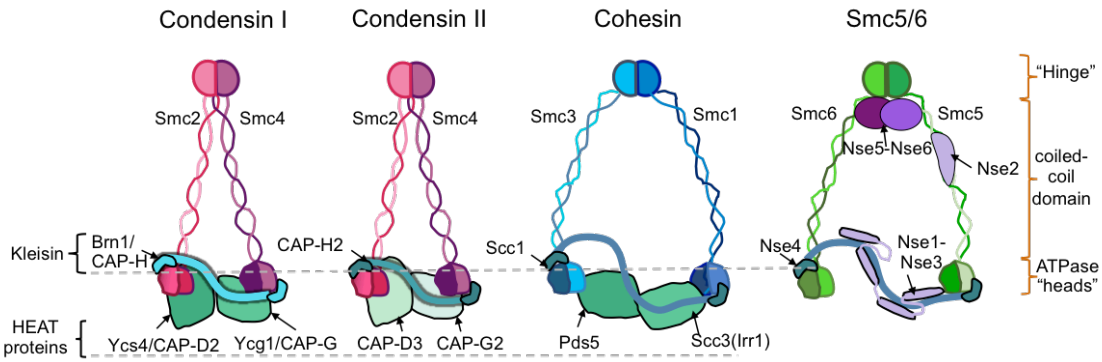


Figure 1.2 The SMC protein family.

The eukaryotic family of SMC proteins, adapted from (Haering and Gruber 2015). SMC protein complexes consists of an SMC heterodimer connected at a hinge domain, each with an extended coiled-coil extending down to the where the C and N-terminals meet to form the ATPase head domains. Complex-specific, non-SMC proteins accompany each heterodimer; a kleisin subunit associates with both SMC head domains, and in Condensin (I and II) and Cohesin, two HEAT domain subunits. Instead of HEAT proteins, Smc5/6 has a tandem-WHD, tandem-WHD E3 ligase and a SUMO ligase. *S. cerevisiae* protein names have been used, and additionally the mammalian names for condensin I and II.

are essential for viability; however, they have some over-lapping roles. Condensin and cohesin both play a part in DNA repair, and cohesin also contributes to chromosome condensation (Uhlmann 2016). Although the molecular details of exactly how these proteins carry out their functions are still debated, what is clear is that their association with chromosomal DNA is fundamental to the process.

1.4 Condensins

1.4.1 The protein complexes

Condensins are highly conserved, large pentameric protein complexes, which play a central role in chromosome condensation and segregation in all three domains of life. Most eukaryotes possess two condensin complexes, condensin I and condensin II, which are more than half a megadalton in size (Piazza *et al.* 2013). Both condensin complexes share the core SMC subunits Smc2 and Smc4 (I will refer to subunits using *S. cerevisiae* nomenclature unless otherwise specified), but differ in their non-SMC subunits (**Table 1.1**). Whilst condensin I is conserved from yeast to humans, condensin

II has had a more complex evolutionary path. Phylogenetic analysis suggests that condensin II has been lost multiple times from species of different kingdoms independently throughout evolution; there seems to be no correlation between genome complexity and the presence or absence of condensin II.

Condensin I and II differ only in their non-SMC subunits; in human cells, the SMC2/SMC4 heterodimer of condensin I associates with CAP-H, CAP-D2 and CAP-G and condensin II contains CAP-H2, CAP-D3 and CAP-G2. Interestingly, in the model organism *C. elegans,* a third condensin complex condensin I^{DC} has been identified in addition to the presence of the two canonical complexes. Condensin I^{DC} is involved specifically in the dosage compensation of the sex chromosomes, and differs from condensin I by just one subunit; the SMC4 is replaced by DPY-27 (Csankovszki *et al.* 2009).

Most bacteria and archaea species contain a single condensin-like complex known as SMC-ScpAB, consisting of three different subunits; SMC, ScpA and ScpB. Some members of the subclass of γ-proteobacteria (Including *Escherichia coli*) contain the distinct but functionally analogous MukBEF complex, composed of MukB MukE and MukF (Hirano 2012). Unlike the SMC heterodimers of eukaryotes, the core of these prokaryotic condensin-like complexes consists of an SMC homodimer (SMC/MukB). The ScpAs and MukF are predicted to act as kleisins (Schleiffer *et al.* 2003) and ScpB/MukE are KITE winged-helical domain proteins similar to those associated with the eukaryotic Smc5/6 complex (Palecek and Gruber 2015).

The structure of the condensin protein complexes is similar to other members of the eukaryotic SMC family (Figure 1.2). The Kleisin subunit bridges the ATPase head domains of the SMC heterodimer via N-terminal domain interactions, forming a closed tripartite ring. Structural studies of prokaryotic condensin revealed that the N- and C-termini of the kleisin subunits interact with the ATPase heads in fundamentally

	SMC subunits		Kleisin	HEAT-repeat	
S. cerevisiae	Smc2	Smc4	Brn1	Ycs4	Ycg1/Ycs5
S. pombe	Cut14	Cut3	Cnd2	Cnd1	Cnd3
D. melanogaster	Smc2	Smc4 (gluon)	Barren (Cap-H)	Cap-D2	Cap-G
			Cap-H2	Cap-D3	-
C. elegans	MIX-1	SMC-4	DPY-26	DPY-28	CAPG-1
			KLE-2	HCP-6	CAPG-2
H. sapien	SMC2 (CAP-E)	SMC4 (CAP-C)	CAP-H	CAP-D2	CAP-G
			CAP-H2	CAP-D3	CAP-G2
				Tandem-WHD KITE proteins	
multiple prokaryote species	SMC		ScpA	ScpB	
E. coli	MukB		MukF	MukE	

Table 1.1 Condensin subunit nomenclature.
Table adapted from Piazza et al. 2013 (Piazza *et al.* 2013) Subunits highlighted in grey represent those associated with condensin II.

different ways; the N-terminus of ScpA binds the Smc coiled coil domain whereas the C-terminus binds the Smc ATPase domain (Bürmann *et al.* 2013). Subsequent studies disclosed a similar asymmetry in the binding of the eukaryotic cohesin kleisin subunit to the Smc1/Smc3 proteins (Haering *et al.* 2004, Gligoris *et al.* 2014, Huis in 't Veld *et al.* 2014), suggesting that this asymmetric binding is a conserved feature of SMC complexes including eukaryotic condensins. The current model is that the N-terminus

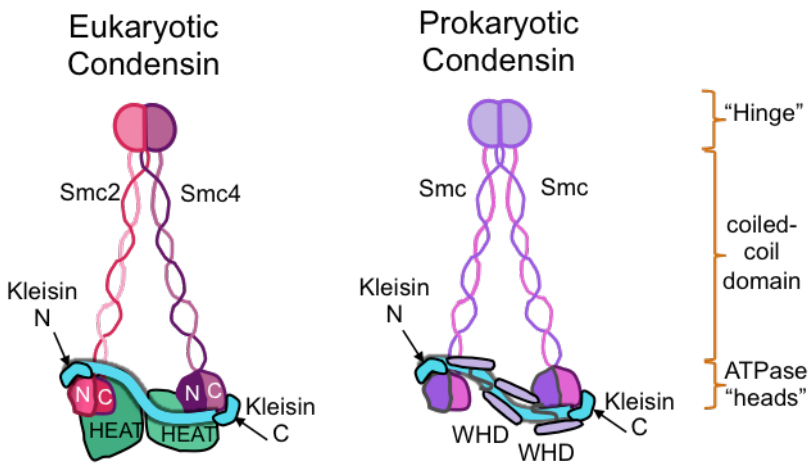


Figure 1.3 The architecture of prokaryotic and eukaryotic condensin.

In eukaryotic and prokaryotic condensin, two SMC subunits form a heterodimer or homodimers respectively via hinge domains which are positions at one end of their 45-nm long intra-molecular anti-parallel coiled- coil region. The N-terminal and C-terminal halves of each SMC subunit are displayed in light and dark shades. In both eukaryotic and prokaryotic condensin, the kleisin subunit is thought to bind to the two ATPase head domains of an SMC dimer and in an asymmetric manner, with the kleisin N-terminus binding one Smc protein (Smc2 in eukaryotes) via an interaction with the coiled-coil domain. The Kleisin C-terminus is thought to bind the other Smc protein via the head domain directly. The kleisin subunit then in turn interacts with two HEAT domain proteins in eukaryotes and copies of a subunit composed of tandem winged-helix domains (WHD) in prokaryotes. The SMC-like MukBEF complexes found in many Y-proteobacteria (not shown) deviate in the arrangement of their kleisin subunits Woo *et al.* (2009). Figure adapted from (Frosi and Haering 2015) .

of Brn1 binds the head domain of Smc2 via an interaction with the coiled-coil domain and the C-terminus binds the Smc4 head domain directly (see Figure 1.3).

Cogs in the machine

Early studies using *Xenopus* cell-free extracts and genetic investigations in *S. cerevisiae* showed that the whole complex is required for condensin function *in vitro* (Kimura and Hirano 1997), and *in vivo* (Freeman *et al.* 2000). However, the subunits within the complex have different roles and activities. Most notably, the two condensin complexes I and II differ only in their non-SMC subunits and yet exhibit distinct cellular locations and functions (Hirano 2012). Further to this, *C. elegans* condensin I^{DC} only differs from canonical condensin I by a single subunit however the complex has a completely separate role in dosage compensation. A DNA-annealing activity was attributed to the Smc2/Smc4 heterodimer during early investigations of condensin in

S. pombe (Sutani and Yanagida 1997) (Sakai *et al.* 2003). The Smc2/Smc4 sub-complex promotes the conversion of complementary ssDNA into dsDNA in the absence of ATP (Sakai *et al.* 2003). The ATPase activity of the two SMC proteins is vital in condensin activity and mutations affecting either ATP-binding or ATP-hydrolysis result in a loss of condensin function *in vivo* (Hudson *et al.* 2008). The non-SMC subunits play their own roles: the kleisin subunit that bridges the Smc proteins acts as a scaffold for the recruitment of the two HEAT domain accessory proteins (Neuwald and Hirano 2000, Onn *et al.* 2007). An investigation in *S. cerevisiae* identified a DNA-binding activity of the HEAT-repeat subunits of condensin (Piazza *et al.* 2014). Further to this, a functional assay for condensin subunits using reconstituted complexes in *Xenopus* egg cell-free extracts, revealed that the two HEAT proteins of condensin I have antagonistic effects on the dynamic assembly of chromosome axes during formation of mitotic chromosomes (Kinoshita *et al.* 2015) (to be discussed further in 1.4.2.1). With these examples in mind, it is important to consider subunit-specific roles of condensin components when examining its functions as a complete complex.

1.4.1.1 DNA-binding and chromatin association

Condensin must associate with chromosomes in order to function as an SMC protein, however it is still unclear how condensin complexes actually interact with DNA and chromatin. The condensin complex has intrinsic DNA binding activity independent of ATP, as shown *in vitro* using *Xenopus* egg extracts (Kimura and Hirano 1997, Kimura *et al.* 1999). However, recent studies in both *Xenopus* egg cell-free extracts (Kinoshita *et al.* 2015) and a chromatid reconstitution system using purified components (Shintomi *et al.* 2015) have shown that targeting of condensin I to chromatin templates requires ATP binding. These findings suggest condensin may undergo both ATP-independent and ATP-dependent binding to DNA/chromatin. The complex as a whole exhibits a binding affinity for dsDNA and structured DNAs such as bent and cruciform DNA *in vitro*. Competitive binding assays revealed a binding preference of condensin for longer (600 bp) DNA fragments rather than shorter (Kimura and Hirano 1997). Recent

in vitro work however, shows that bacterial MuKBEF complex preferentially binds ssDNA (Niki and Yano 2016).

One model for condensin binding is that the Smc2/Smc4 heterodimer exerts the complex's main DNA-binding activity, which is regulated by the non-SMC subunits. In support of this, *S. cerevisiae* and *Schizosaccharomyces pombe* yeast SMC heterodimer subcomplexes have been shown to bind DNA *in vitro* (Kimura and Hirano 2000, Sakai *et al.* 2003, Stray and Lindsley 2003). Additionally, the *S. pombe* SMC heterodimer was reported to bind DNA via its hinge domain, as observed using AFM (Yoshimura *et al.* 2002). The DNA binding activity of the prokaryotic condensin SMC homodimer MukB has been shown to be inhibited by associated subunits MukE and MukF (Petrushenko *et al.* 2006). Together these findings support the idea that the Smc2-Smc4 heterodimer exerts condensin's main DNA-binding activity and that this activity is regulated by the non-SMC subunits (Kimura and Hirano 2000, Kimura *et al.* 2001, Yoshimura *et al.* 2002).

In contrast, *in vitro* DNA-binding assays showed the hinge of the mouse condensin complex to preferentially bind ssDNA (Griese *et al.* 2010), which the authors proposed may be linked to this DNA binding activity having a role in DNA repair rather than condensin's main role in chromosome condensation. Further to this, HEAT domain proteins were reported to have a DNA-binding activity of their own (Piazza *et al.* 2014). Using reconstituted *S. cerevisiae* condensin, Piazza *et al.* demonstrated that the non-SMC subcomplex has DNA binding activity (albeit with less affinity than the complex as a whole) and that this binding was dependent on the presence of both HEAT proteins. Importantly, the same study reported that the non-SMC complex preferentially bound dsDNA over ssDNA. Therefore, it seems that the DNA-binding activity of condensin is more complex than first imagined.

Another way that condensin is thought to associate with chromosomes is through topological entrapment of chromatin. It is hypothesised that the tripartite ring formed by Smc2-Smc4-kleisin, topologically encircles chromatin fibres in a manner analogous to that of the SMC complex cohesin (reviewed in (Gligoris and Lowe 2016)).

Interestingly, recent work suggest prokaryotic condensin complexes may bind DNA in a topological manner (Niki and Yano 2016) (Wilhelm *et al.* 2015). In further support of the entrapment model, following chemical cross-linking of the three ring interfaces of Smc-ScpAB, the prokaryotic condensin complex was shown to remain bound to intact chromosomes isolated under protein-denaturing conditions (Wilhelm *et al.* 2015). Additionally, the linearization of circular DNA *in vitro* facilitated the release of DNA from bacterial MukBEF (Niki and Yano 2016). As with eukaryotic condensin, there is also evidence for direct DNA binding of prokaryotic condensins (Petrushenko *et al.* 2006). The DNA-entrapment model is also supported by a study in *S. cerevisiae* that demonstrates that the association between the Smc2-Smc4-Brn1 trimer and circular minichromosomes, can be diminished by either the linearisation of the minichromosomes (*in vitro*) or proteolytic cleavage of the condensin subcomplex (*in vivo*) (Cuylen *et al.* 2011). However, the dissolution of condensin from mini chromosomes *in vitro* required significantly higher salt concentrations than that required for removal of cohesin, suggesting that condensin may make additional direct protein-DNA interactions. Further evidence against topological entrapment being condensin's main method of chromosome association, comes from a study in vertebrate cells that reporting that the disruption of the putative condensin ring by conditional cleavage of SMC2 in DT40 cells, does not affect the integrity of the complex or its ability to associate with mitotic chromosomes (Hudson *et al.* 2008).

It has been postulated that condensin associates with chromatin via interactions with histone proteins. The kleisin subunits of human and *S. pombe* condensin complexes have been reported to directly interact with H2A and H2A.Z (Tada *et al.* 2011). However *in vitro* assays of *S. cerevisiae* non-SMC subcomplex actually showed a binding preference for naked DNA over nucleosomal DNA (Piazza *et al.* 2014). Furthermore, there is mounting evidence that condensin preferentially binds nucleosome-free DNA *in vivo* (Sutani *et al.* 2015, Toselli-Mollereau *et al.* 2016). Multiple studies investigating condensin localisation have identified condensin binding at sites of highly transcribed genes (Wang *et al.* 2005, D'Ambrosio *et al.* 2008, Kim *et al.* 2013, Kranz *et al.* 2013) and recent work in *S. pombe* has reported a link between condensin accumulation, and regions depleted of nucleosomes by transcriptional

coactivators Gcn5 (histone acetyltransferase) and RSC (chromatin-remodelling complex) (Toselli-Mollereau *et al.* 2016). The molecular details of how the condensin complexes associate with DNA and chromatin remain unclear, it is likely that the association with chromatin, and the loading and unloading of condensin is a multi-faceted process.

1.4.2 Functions of condensin complexes

Early insights into condensin function *in vitro*

Hirano pioneered the discovery and biochemical analysis of the condensin complex in the mid-to-late 1990s. Using *Xenopus* egg extracts, he first identified the SMC heterodimer (XCAP-C and XCAP-E) and demonstrated its necessity in mitotic chromosome assembly and structural maintenance *in vitro* (Hirano and Mitchison 1994). He then went on to purify the whole condensin complex, and investigate its DNA binding activity. Condensin was found to exhibit a DNA-stimulated ATPase activity and was shown to have the ability to introduce positive supercoils into plasmid DNA in an ATP-dependent manner (Hirano *et al.* 1997) (Kimura and Hirano 1997). In 1998, a mitosis-specific phosphorylation of condensin by CDK1 (Cyclin Dependent Kinase or Cdc28 in *S. cerevisiae*,) was observed. This post-translational modification was shown to be important in the regulation of condensin activity *in vitro* (Kimura *et al.* 1998). At the turn of the century, genetic work in yeast generated *in vivo* evidence of condensin's role in chromosome morphology, and showed that all five subunits of condensin are essential for viability (Sutani *et al.* 1999, Freeman *et al.* 2000). The presence of a mammalian condensin complex was confirmed, and seen to associate with chromosomes in a mitosis specific manner (Schmiesing *et al.* 2000). Hirano then demonstrated that the human condensin complex is able to rescue chromosome condensation defects observed in *Xenopus* egg extracts depleted of endogenous condensin (Kimura *et al.* 2001), suggesting that condensin function is highly conserved. These early studies of condensin provided an insight into the molecular activities of

the condensin complexes, and on-going research seeks to elucidate how these activities translate into large-scale changes in chromosome morphology.

Condensin is vital for chromosome condensation and segregation

For accurate cell division, long expanses of newly replicated sister chromatids must be condensed and segregated from each other into two daughter cells. Condensin complexes have been shown to be vital in this process, as revealed by studies in multiple model systems. Inactivation by mutation of any condensin subunit in yeast, and depletion of condensin I or II in flies, worms and vertebrate cells causes severe chromosome defects that are often characterised by the failure to compact chromosomes, and the formation of anaphase bridges due to a failure to resolve chromosome arms (Hirano 2012) (Hudson *et al.* 2009). Condensin I was originally identified by its ability to reconstitute metaphase chromosomes *in vitro* in *Xenopus* cell-free egg extracts. Biochemical analysis of these assembled chromosomes highlighted the condensin complex as being a key feature (Hirano and Mitchison 1994). Subsequent anti-body blocking experiments of the Smc4 (CAP-C) subunit showed that the formation and maintenance of condensed chromosomes visualised in the *Xenopus in vitro* system was condensin-dependent (Hirano and Mitchison 1994). Obvious chromosome condensation defects were also observed *in vivo* when condensin was perturbed in *S. cerevisiae* and *S. pombe* (Strunnikov *et al.* 1995) (Saka *et al.* 1994). In *S. cerevisiae*, spores containing SMC2 gene deletions proved inviable, and so an in-depth analysis of the temperature sensitive mutant allele *smc2-6* was conducted to assess the biological function of condensin. At restrictive temperatures, *smc2-6* strains were seen to arrest in mitosis at the second cell division. At the first cell division, 15% of cells observed failed to separate chromatin into two separate masses despite a fully elongated mitotic spindle; a morphology consistent with a chromosome condensation defects. The authors then confirmed chromosome condensation defects in the *smc2-6* strain by using a FISH (fluorescent in situ hybridisation) condensation assay in which distance between fluorescent loci were measured in mitosis, and found to be increased in the condensin mutant compared to wild-type (Strunnikov *et al.* 1995). Similar phenotypes were observed in *S. pombe* strains containing temperature

sensitive mutant alleles of *cut14-208* (SMC2) and *cut3-477* (SMC4). These condensin mutant strains exhibited cell cycle arrests and/or loss of viability following a round of abnormal division in which chromosomes were not properly condensed and thus not segregated (Saka *et al.* 1994). Further investigations in *S. cerevisiae* used colony-sectoring experiments to provide evidence that condensin mutants (*smc2-8* and *smc4-1)* can cause genomic instability by mis-segregation and chromosome loss (specifically chromosomes containing rDNA sequences). The authors proposed that this is due to an entanglement of sister chromatids arising from incomplete condensation (Freeman *et al.* 2000). Together these studies in yeast demonstrate the importance of condensin in chromosome condensation and segregation.

Interestingly, whilst severe chromosomal defects were observed upon condensin inactivation by immunodepletion in *Xenopus* cell-free extracts, and by temperature-sensitive mutant alleles in yeasts, only relatively modest chromosomal phenotypes were apparent upon condensin depletion by RNAi (RNA interference) in mammalian cells (Hirota *et al.* 2004) and transcriptional repression in chicken DT40 cells (Hudson *et al.* 2003). These studies lead to the assumption that condensin was not essential for chromosome condensation and segregation in higher eukaryotes. On the contrary, two recent studies have provided evidence that condensin is indeed important for these chromosome processes in vertebrates (Houlard *et al.* 2015) (Shintomi *et al.* 2015). Houlard *et al.* used mouse oocytes to demonstrate the requirement of condensin II in chromosome compaction. Cre recombinase expression was used to delete floxed kleisin subunits of both condensin I and II, and in doing so it was revealed that condensin II provides the main condensation activity and determines the morphology of meiotic chromosomes. Furthermore, proteolytic cleavage of condensin II kleisin leads to an unravelling of previously condensed metaphase chromosomes, suggesting it has a key role in maintaining the condensed state (Houlard *et al.* 2015).

The second study revisited the *Xenopus* cell-free system from which condensin I was first isolated (Hirano and Mitchison 1994). Shintomi *et al.* used fractionation to isolate and identify the minimal set of factors required to successfully reconstituting mitotic chromatids *in vitro (Shintomi et al. 2015).* The study identified the minimal

requirements to be to be a chromatin substrate mixed with six purified factors: core histones, three chaperones (FACT, Nap1 and nucleoplasmin), topoisomerase II and condensin I. In the absence of condensin I, formless chromatin masses were observed in the place of structured and resolved chromosomes. Together, these two studies validate the notion that condensin is the primary molecular effector of chromosome condensation. It is thought that the absence of severe chromosome defects observed in studies employing RNAi or transcriptional repression of condensin can be attributed to the incomplete inactivation of the complex.

1.4.2.1 Condensin in chromosome condensation

How does condensin achieve genome-wide chromosome compaction?

The mechanism by which condensin operates to compact and maintain condensed chromosomes remains highly debated and poorly understood. There are two main predictions for how condensin works to compact chromosomes. One envisions condensin as a structural linker of chromatin, operating via topological entrapment of DNA to induce compaction by tethering sections of chromatin together. The second predicts that condensin works enzymatically in supercoiling, loop-extrusion and/or knotting to drive compaction; the two concepts are not mutually exclusive and the mechanism is most likely a mixture of the two.

The model proposing topological entrapment of DNA, was initially invoked by the discovery that the condensin-related cohesin complex generates sister chromatid cohesion by entrapment of both chromatids within the SMC-kleisin tripartite ring (Haering *et al.* 2008). As discussed in 1.4.1.1, there is *in vivo* and *in vitro* evidence supporting the idea of topological entrapment of DNA for *S. cerevisiae* and bacterial condensin complexes (Cuylen *et al.* 2011, Wilhelm *et al.* 2015, Niki and Yano 2016). As a physical linker of chromatin, condensin could form a proteinaceous scaffold to anchor the DNA loops by physical tethering (see Figure 1.1), or by stochastically crosslinking freely diffusing 10 nm fibres (Cuylen *et al.* 2011, Thadani *et al.* 2012). The

entrapment of chromatin within the complex is thought to facilitate crosslinking of long-range intra-chromosomal interactions. Long-range intrachromosomal interactions could translate into compaction by the sequential entrapment of two DNA helices by a single condensin ring (Cuylen *et al.* 2011). Alternatively condensin may entrap individual segments of chromatin, which are then brought together by multimerisation of the complex in a manner similar to the handcuff configuration proposed for cohesin (Zhang and Pati 2009). One proposed model that combines both ideas, is based on *in vitro* data in which the Smc2/4 heterodimer was seen to form long filamentous structures and uniformly sized compact rings upon binding to DNA. The model suggests that Smc proteins chirally compact DNA by binding more than one DNA duplex, and additionally by lateral interactions between complexes (Stray *et al.* 2005). Bacterial studies provide support for the concept of multimeric condensin assemblies. AFM analysis revealed the existence of higher-order multimeric structures formed by the Smc-ScpAB complex *in vitro* (Fuentes-Perez *et al.* 2012) Furthermore, electron micrographs have shown purified *Escherichia coli* MukBEF as an oligomer, forming extended fibres and rosette-like configurations (Matoba *et al.* 2005). Other than the clustering of GFP-tagged MukB observed in living *E. coli* cells (Ohsumi *et al.* 2001), there has yet been no evidence of multimerisation of condensin complexes *in vivo.* Therefore, it is unclear how or whether interactions between condensin complexes contribute to chromosome condensation.

In the case of topological entrapment of chromosomes, it is unknown how condensin could selectively generate intra-chromosomal links rather than linking separate chromosomes. *In vitro* studies have shown the eukaryotic condensin complex to preferentially bind structured DNA (Kimura and Hirano 1997) and the *E. coli* MukB to prefer right-handed DNA crossings (Petrushenko *et al.* 2010); perhaps such binding preferences could play a role in discriminating between intra- and inter-chromosomal interactions.

A length of DNA when supercoiled is more compact than when relaxed; this is reflected by a faster migration by centrifugation or electrophoresis (Weil and Vinograd 1963). Condensin purified from *Xenopus* and yeast cells has an ATP-dependent ability

to introduce positive supercoiling of circular plasmid DNA, in the presence of topoisomerase I (Hirano *et al.* 1997, Kimura *et al.* 1998, St-Pierre *et al.* 2009), and positive (right-handed) knotting in a nicked plasmid with topoisomerase II (Hirano *et al.* 1997). These findings contribute to the hypothesis that condensin may alter chromosome morphology through introducing global positive writhe (Kimura *et al.* 1999). Interestingly, prokaryotic MukB also generates right-handed knotting in the presence of type-2 topoisomerase, however the net supercoiling stabilized by the *E. coli* condensin complex is negative (Petrushenko *et al.* 2006). The introduction of positive supercoiling by condensin in eukaryotes provides a mechanism for chromosome-wide condensation, however it is unclear how the supercoiling activity observed in closed circular DNA would translate to the much longer, linear chromosomes of eukaryotes.

Another model proposes DNA-loop extrusion as a possible mechanism by which condensin compacts chromosomes (Alipour and Marko 2012, Burmann and Gruber 2015). The loop extrusion model envisages a DNA loop being threaded through the condensin complex and either pushed or pulled through the ring in a manner that extends the loop. This loop extension would bring two distal regions of the same chromosome close together at the origin of the loop and in theory also the DNA on either side of the loop. Although there is no mechanistic evidence for condensin being able to extrude DNA, the theory is supported by symmetrical DNA contacts that originate from condensin loading sites in bacterial chromosomes, revealed by Hi-C and super-resolution imaging (Marbouty *et al.* 2015, Wang *et al.* 2015). This model could explain how condensin preferentially generates intrachromosomal links.

The uncertainty surrounding how chromatin is structured within mitotic chromosomes (as discussed in 1.2) lends further ambiguity to how condensation is achieved; future advancements in this field will undoubtedly shed some light on condensin's mode of action. The answer to how condensin achieves genome-wide compaction is likely to be a mixture of both structural and enzymatic activity, with condensin generating both intra-chromosomal links and positive writhe to condense individual chromosomes.

Contributions of condensin I and condensin II

Most eukaryotes are in possession of two condensin complexes: condensin I and condensin II. The two complexes exhibit different subcellular locations and chromosomal positions (see 1.4.3), and are non-redundant. Whilst condensin I was the first to be identified in *Xenopus* cell-free extracts, condensin II was also present in this system, however in much less abundance than condensin I (there is a 5:1 ratio of condensin I to condensin II in this system) (Ono *et al.* 2003). In line with the relative quantities of each condensin complex in this cell-free system, depletion of each complex revealed condensin I to play a predominant role.

The shape of metaphase chromosomes varies widely from organism to organism (Losada and Hirano 2001), and the abundance ratio of the two complexes appears to play a critical role in shaping them (Shintomi and Hirano 2011). It is thought that the two condensin complexes have differing roles in conferring axial and longitudinal compaction. Quantitative immunodepletion experiments in the *Xenopus* cell free extracts were used to directly investigate the contribution of each condensin complex (Shintomi and Hirano 2011). By reducing the abundance ratio of condensin I and II from 5:1 to 1:1, the chromosome morphology changed to become shorter and thicker. Whereas when condensin II was completely depleted (1:0 ratio) the chromosomes became longer. These results suggest that condensin I primarily acts in radial compaction of chromosomes, whilst condensin II acts to shorten them (Linear compaction). Work *in vivo* also supports this notion; RNAi knockdown of condensin I in HeLA cells produces swollen chromosomes, whilst knockdown of condensin II results in a lengthening of them (Ono *et al.* 2003, Hirota *et al.* 2004). Similar phenotypes were also observed for condensin I- and condensin II-depleted chicken DT40 cells. The authors propose that condensin II acts to provide rigidity to the chromosome axis, around which condensin I arranges loops of chromatin to compact chromosomes laterally (Green *et al.* 2012). A more recent model proposed by Hirano (Hirano 2016) has condensin II initiating loop formation through mechanisms such as chiral looping (Hirano 2012) or loop extrusion (Alipour and Marko 2012), followed by axial shortening of chromosomes by intra-chromosomal cross-linking (Kschonsak and Haering 2015) or

condensin multimerisation. Condensin I then contributes to lateral compaction by axes reinforcement and further organising the loops via its supercoiling activity (Hirano 2016).

It is unclear how two very similar complexes can act so differently with regards to localisation, and contribution to chromosome composition. It is also important to note that condensin I and II also have over-lapping functions. Further insight into recruitment and regulatory mechanisms of condensin are needed to clarify the driving force behind these differences.

Contributions of Cap-D2 and Cap-G

Condensin subunit CAP-G has recently been shown to be required for recruitment of condensin onto chromosomes in yeast and human cells (Piazza *et al.* 2014). However, its role in condensin activity continues after recruitment. CAP-D2 and CAP-G subunits of condensin I have been shown to exhibit antagonistic functions in the dynamic assembly of chromosome axes (Kinoshita *et al.* 2015). By reconstituting condensin complexes lacking either the CAP-D2 or CAP-G and adding them to *Xenopus* egg cell-free extracts; Kinoshita *et al.* have demonstrated marked differences between the chromosome formations achieved by each tetramer. Neither tetramer could support proper assembly of mitotic chromosomes. In the absence of CAP-D2 clearly discernable chromosomes were not detected. In contrast the CAP-G delete generated chromosome structures with highly characteristic abnormalities. DAPI staining and fluorescent tagging revealed these chromosomes to have Smc4 present on a DNA-dense axial structure with a condensin-free fuzzy chromatin mass surrounding the axis. Sequential add-back experiments revealed more about the distinct activities of the two subunits. Adding the CAP-G delete tetramer to pre-assembled chromosomes caused an elongation of the structure, whereas adding the CAP-D2 delete tetramer destabilized pre-assembled chromosomes. The model proposed from the study is that CAP-D2 plays a primary role in assembling chromosome axes, whereas the CAP-G works to antagonize the action of CAP-D2. So in the absence of CAP-G, the balance is lost and results in chromosomes with abnormally thin axes, and conversely in the

absence of CAP-D2 the complex binds but fails to induce axis formation. The dynamic nature of chromosome structure and the necessity of condensin in its maintenance, are highlighted by the changes occurring upon addition of mutants after chromosome establishment in this study.

1.4.2.2 Condensin in sister-chromatid segregation

DNA intertwines: Condensin and Topoisomerase II in chromosome segregation

The role of condensin in mitosis can be split into two distinguishable activities: compaction of chromosomes, and their segregation in anaphase. Condensin inactivation in *S. cerevisiae* results in chromosome arms 1.5 times their normal length in mitosis (Guacci *et al.* 1994). This elongation should only negate separation of the longest chromosomes, however resolution of most chromosomes is impaired (Strunnikov *et al.* 1995, Bhalla *et al.* 2002, D'Ambrosio *et al.* 2008). Additionally, Aurora B kinase (a known condensin regulator – see 1.4.4.2) is required for condensin-dependent condensation but not resolution of the rDNA locus in *S. cerevisiae* (D'Amours *et al.* 2004, Sullivan *et al.* 2004). Crucial to this chromosome-segregation activity, is the interplay between condensin and topoisomerase II (Baxter *et al.* 2011) (Charbin *et al.* 2014) (D'Ambrosio *et al.* 2008).

The topoisomerases are a family of enzymes that act in the regulation of DNA topology (Wang 2002). There are two main types of topoisomerases: topo I and topo II. Both operate by transiently nicking DNA, which can allow the relaxation of topological stress. Topo I nicks just one strand of DNA to allow the passage of the other strand through the break, before re-annealing. Topo II however, can make cuts through both strands of a DNA double helix and pass another segment of the duplex though the cut before re-sealing. In this way, topoisomerase II can catalyse the catenation (linking) and decatenation (unlinking) of two different DNA duplexes in an ATP-dependent manner (Lodish H 2000). Topological stress can arise during any cellular event that requires unwinding of a section of DNA, such as gene transcription, and DNA

replication. During S-phase the progression of replication forks leads to overwinding and positive supercoiling ahead of the fork, and underwinding generating negative supercoiling behind the fork. The supercoiling can be resolved by both topo I and topo II. However, upon completion of replication, many links are converted into double-stranded DNA intertwines (catenanes) between sister chromatids (Sundin and Varshavsky 1980). These interlinking DNA double helixes can only be resolved by topo II (Holm *et al.* 1985). This decatenation activity continues right up until anaphase and is dependent on the mitotic spindle (Holm *et al.* 1985, Uemura *et al.* 1987, Baxter and Diffley 2008).

The notion that condensin and topo II work together in the same functional pathway, first arose from the observation that the chromosome segregation defects observed in condensin mutant strains (Hirano *et al.* 1986), were similar to those seen following topo II inactivation (Uemura and Yanagida 1984). Cut14 and Cut3 (Smc2 and Smc4) in *S. pombe* are named after the 'cut' phenotype noted in a genetic screen for cytological abnormalities of temperature-sensitive mutants (Hirano *et al.* 1986). The 'cut' (cell untimely torn) phenotype is characterised by cell division occurring without nuclear division, and was first described in a paper investigating *S. cerevisiae TOP2* mutants (Uemura and Yanagida 1984). Due to the uncoupling of nuclear and cellular division, anaphase bridges of stretched unresolved chromosomes can be miss-segregated resulting in loss of genome integrity and aneuploidy. The similar phenotypes prompted investigation into potential genetic interaction between condensin and topo II. Double mutant strains displayed genetic interaction by synthetic lethality in *S. pombe* (Saka *et al.* 1994). Since then, multiple studies have demonstrated the co-dependency of condensin and topo II in chromosome segregation (Coelho *et al.* 2003, Baxter *et al.* 2011, Charbin *et al.* 2014).

How do condensin and topo II work together to achieve chromosome resolution? One idea is that condensin directly stimulates topoisomerase activity. The *Drosophila* condensin complex was shown to stimulate topo II DNA-relaxation activity *in vitro (Bhat et al. 1996).* Interestingly, as the concentration of condensin was increased, topo II activity was inhibited. This finding suggests that condensin promotion of topo II

activity is more complex than direct stimulation. More recent studies point to a model in which condensin can stimulate resolution of sister chromatid intertwines, by generating a DNA substrate geometry that promotes topo II access to and/or recognition of catenanes (Baxter *et al.* 2011, Charbin *et al.* 2014). Positively supercoiled DNA has been shown to be the preferred substrate of topo II (Crisona *et al.* 2000). Baxter *et al.* demonstrated that centromeric plasmids undergo a dramatic topological change during mitosis, in which DNA becomes positively supercoiled in a manner dependent on both condensin and mitotic spindles. The authors went on to show that whilst topo II rapidly relaxes positive supercoiling on decatenated plasmids, its activity switches to prioritise decatenation of positively supercoiled plasmids when catenanes are present (Baxter *et al.* 2011). These data show that the positive supercoiling activity dependent on condensin, drives the decatenation of plasmids in mitosis by topoisomerase II, and it is thought that this is the mechanism by which sister chromatids are resolved in mitosis.

Sister-chromatid cohesion: condensin-dependent removal of cohesin in chromosome segregation

The cohesin complex is essential for sister chromatid cohesion throughout S-phase and mitosis. Through topological entrapment, cohesin keeps sister chromatids physically connected to one another from their point of replication (Uhlmann and Nasmyth 1998) right up until anaphase onset (Uhlmann *et al.* 1999). For chromatids to segregate, two sequential steps in metazoans achieve cohesion disestablishment. The first is the prophase pathway in which the bulk of cohesin is removed from chromosome arms, leaving cohesion between sister chromatids at centromeres only. This pathway is regulated by the phosphorylation of cohesin SA1 and SA2 (HEAT accessory proteins) by PLK1 (polo-like kinase) and cohesin associated protein Wapl1. The second is achieved by the cleavage of the cohesin kleisin subunit by separase at the metaphase-anaphase transition (Waizenegger *et al.* 2000). The prophase pathway of removal is not evident in *S. cerevisiae* and cohesin remains bound to chromosome arms right up until APC (anaphase promoting complex) activation (Alexandru *et al.* 2001).

Condensin has been shown to be required for complete removal of cohesin in human and yeast cells (Hirota *et al.* 2004) (Yu and Koshland 2005) (Renshaw *et al.* 2010). Depletion of condensin I (but not condensin II) in HeLA cells results in a small amount of residual cohesin detectable on chromosome arms in cells arrested with nocodazole (Hirota *et al.* 2004). How might condensin work to remove cohesin? Phosphorylation of cohesin subunit Scc1 by PLK1 (Cdc5) in *S. cerevisiae* makes it a more desirable substrate for cleavage by separase (Alexandru *et al.* 2001). One theory is that condensin recruits the kinase to facilitate the removal of cohesin. The finding that meiotic chromosome enrichment of Cdc5 and phosphorylation of cohesin were both reduced in condensin mutants supports this idea (Yu and Koshland 2005).

Alternatively, condensin may destabilize cohesin binding by changing chromosome structure. In a study using fluorescently labeled yeast chromosome loci to probe anaphase movement dynamics, Renshaw *et al.* found that sister chromatid separation in *S. cerevisiae* progresses gradually from centromeres to telomeres (Renshaw *et al.* 2010). During this separation, chromosomes stretch due to the presence of residual cohesin, and subsequently recoil to in a manner that aids its removal. This recoiling activity is significantly impaired in condensin mutants. It is thought that condensin-dependent recoiling activity facilitates chromosome segregation in a manner distinct from its role in chromosome decatenation. This may be achieved by generating force to break cohesin bridges, or by conformation changes that might make cohesin more accessible for cleavage by separase.

Centromere structure: generating tension at the centromere

Centromeres are specialised regions of chromosomes, required for the proper distribution of genetic information between dividing cells in mitosis. Protein structures known as kinetochores assemble at centromere sites, acting as docking points for mitotic spindles, which pull sister chromatids to opposite ends of the cell during anaphase (Biggins 2013). Despite the common functionality of centromeres as a site for kinetochore assembly, the actual physicality of centromeric regions varies greatly between different organisms (Malik and Henikoff 2009). In higher eukaryotes,

centromeres consist of large regions of heterochromatic satellite DNA (tandem repeats of non-coding DNA). Human centromeres can span up to 4 Mb in length (Willard 1990, Verdaasdonk and Bloom 2011) and each one facilitates approximately 15 kinetochore-microtubule interactions (McEwen *et al.* 2001). *S. cerevisiae* however, have point centromeres, defined by specific DNA sequences. Point centromeres make only one kinetochore-microtubule attachment per chromosome (Peterson and Ris 1976). A third classification of centromere is found on *C. elegans* holocentric chromosomes. These holocentromeres are organised as discretely localised centromeres, dispersed along the length of a chromosome (Steiner and Henikoff 2014). A defining feature of centromeres is the presence of cenH3-containing nucleosomes, and while point centromeres are defined by a specific DNA sequence, regional and holocentromeres are epigenetically defined by the location of these specialised nucleosomes.

For accurate chromosome segregation to occur, sister chromatid kinetochores need to be positioned in such a way as to facilitate bioriented amphitelic chromatid attachments (Etemad and Kops 2016). This is achieved when a stable kinetochore attachment of each sister chromatid is made with a microtubule from opposite poles of the cell (from two different SPBs/centrosomes). In metaphase, pulling forces caused by kinetochore-spindle microtubule (MTs) interactions are resisted by tension generated by the stretching of centromeric chromatin. This tension and stretch at centromeres is important for chromosome alignment (McIntosh *et al.* 2002), the back-to-back orientation of sister kinetochores (Lončarek *et al.* 2007), the stabilisation of kinetochore-MT interactions and also spindle checkpoint signalling to prevent early progression into anaphase (Etemad and Kops 2016). There are three factors important in generating centromeric tension: the elastic properties of chromatin (Chien and van Noort 2009), sister chromatid cohesion (Yeh *et al.* 2008), and the higher order structure of centromeric chromatin (Bloom 2014).

Condensin and cohesin are enriched at centromeres and pericentromeres (Tanaka *et al.* 1999, Glynn *et al.* 2004, Eckert *et al.* 2007, D'Ambrosio *et al.* 2008, Kim *et al.* 2013), and perturbing either SMC complex results in loss of proper tension-sensing and error correction in mitosis (Yong-Gonzalez *et al.* 2007, Ng *et al.* 2009). Condensin is

important in generating a stable rigid chromosome state required for spindle attachment (Gerlich *et al.* 2006). RNAi depletion of condensin I (but not condensin II) in HeLA cells resulted in normally compacted chromosomes that were mechanically labile and unable to withstand spindle forces. There are several studies that suggest condensin is important in generating elasticity and tension, specifically at centromeric regions in several different organisms. Condensin I depletion in *Drosophila* leads to distortion of kinetochores and a loss of elasticity of chromatin at the centromere, suggesting condensin plays an important role in the structural integrity of centromeric heterochromatin during mitosis (Oliveira *et al.* 2005). Furthermore, depletion of condensin reduces the stiffness of centromeric chromatin by 50% in chicken DT40 cells. Interestingly the ATPase activity of SMC2 is required for normal stiffness of centromeric chromatin (Ribeiro *et al.* 2009). A more recent study in *S. cerevisiae* suggests condensin works alongside cohesin to form an intramolecular loop at the centromere that functions as a mitotic chromatin spring (Stephens *et al.* 2011). The authors showed condensin to lie proximal to the centromere, from where they propose it contributes to the chromatin spring, by resisting outward force of the spindle. Cohesin was observed radially and thought to restrict pericentric chromatin to contribute to the spring from a distal position. Furthermore in *S. cerevisiae*, a study has suggested a condensin-dependent mechanism independent of the error-correction activity of Ip1 (Aurora B kinase) in generating a bias for sister kinetochore biorientation (Verzijlbergen *et al.* 2014). Although it remains unknown how condensin contributes to the higher order structure of centromeric chromatin at a molecular level, these studies highlight the importance of condensin activity at these chromosome regions in facilitating accurate chromosome biorientation in metaphase.

1.4.2.3 Condensin in interphase

Maintenance of interphase chromatin

Although condensin is best known for its mitotic activity, there is mounting evidence suggesting that the complex is also active during interphase. In organisms possessing

both condensin complexes, condensin II but not condensin I remains in the nucleus throughout the cell cycle. It is thought condensin II may play a role in chromatin organisation throughout the cell cycle. Although condensin II function in interphase remains unclear, recent studies establish that the complex does have a role in regulating the organisation of interphase chromatin. In mouse embryonic stem (ES) cells, RNAi depletion of condensin II leads to loss of chromatin compaction in interphase (Fazzio and Panning 2010). Furthermore, condensin II has also been shown to be required for maintenance of interphase chromatin in *Drosophila* (Bauer *et al.* 2012, Smith *et al.* 2013). Interphase chromatin exists in a pre-stressed state, and enzymatic de-condensation of chromatin leads to swelling of nuclei (Mazumder *et al.* 2008). Depletion of condensin II subunits in *Drosophila* and HeLa cells leads to an increase in nuclear size, and suggests that the complex also indirectly maintains nuclear structure via its maintenance of interphase chromatin (George *et al.* 2014).

rDNA stability in *S. cerevisiae*

Within the remit of maintenance of interphase chromatin, condensin has been shown to be important in upholding the integrity of rDNA in *S. cerevisiae*. In this organism, condensin I maintains its nuclear location throughout the cell cycle. The ~150 copies of 9.1 kb rDNA units in *S. cerevisiae*, are grouped in a long tandem array on chromosome XII (Petes 1979). Each unit of rDNA comprises a 35s rRNA-coding sequence, a 5S rRNA-coding sequence and two non-transcribed spacers NTS1 and NTS2. During S phase, condensin is recruited to the replication fork barrier (RFB) sequence located in NTS1, and works alongside Fob1 in contributing to the maintenance of the rDNA sequence by preventing the contraction of the rDNA repeats (Johzuka *et al.* 2006). Condensin also appears to protect the rDNA during nutrient starvation. Upon nutrient starvation condensin is loaded onto the rDNA in interphase, leading to condensation and thus protection of chromatin in this region (Tsang *et al.* 2007, Tsang *et al.* 2007). It is thought that this interphase compaction is likely to inhibit intrachromosomal HR (homologous recombination) to protect the integrity of the rDNA locus (Wu and Yu 2012). In the absence of condensin, Rad52 (a protein involved in DSB repair) is erroneously localised to the rDNA (Tsang and Zheng 2009). In nutrient deficient

conditions, deletion of *RAD52* rescues cell lethality caused by condensin inactivation. Therefore condensin-dependent exclusion of Rad52 is thought to be one mechanism by which the stability of rDNA is maintained (Tsang and Zheng 2009).

Regulation of gene expression

It is known that the non-canonical condensin I^{DC} complex in *C. elegans* is key in the dosage compensation of the X chromosome (Chuang *et al.* 1994). Dosage compensation is the process by which the level of X-linked gene expression is equalised between the sexes. Condensin I^{DC} as part of the dosage compensation complex (DCC), binds to both X chromosomes in hermaphrodites to down regulate X-linked gene expression to the same levels as those in males (Csankovszki *et al.* 2009). Emerging studies suggest canonical condensin may also have a role in transcription regulation in interphase. Condensin I and condensin II are both recruited to oestrogen-bound active enhancers, and activate gene expression in response to oestrogen stimulation in human cancer cells (Li *et al.* 2015). Additionally in *Drosophila*, condensin II has been attributed with a role in antagonising transvection, a regulatory process in which gene transcription is activated or repressed by regulatory elements on homologous chromosomes (Hartl *et al.* 2008).

DNA damage response and repair

The first indication of condensin's role in DNA repair came from a study in *S. pombe*, in which a temperature-sensitive mutant of the kleisin subunit Cnd2 showed a sensitivity to DNA damaging agents, and a defect in the DNA damage checkpoint activation (Aono *et al.* 2002). Additionally in *S. pombe*, a point mutation in the hinge domain of Cut14 (SMC2) generated defects in DNA damage repair (Sakai *et al.* 2003). Interestingly, these defects were rescued by a mutation in replication protein A (RPA). RPA coats ssDNA during DNA replication and repair, preventing the DNA from winding back on itself or forming secondary structures (Iftode *et al.* 1999). The authors suggest that condensin's DNA-reannealing activity may work antagonistically against RPA-mediated ssDNA coating.

The two condensin complexes have also been implicated in DNA repair in humans. A study using HeLA cells showed condensin I to have a role in single stranded break repair. This role is potentially facilitated via condensin's interphase-specific interaction with DNA nick-sensor poly (ADP-ribose) polymerase 1 (PARP1), and base excision repair factor XRCC1 (Heale *et al.* 2006) (Kong *et al.* 2011). In contrast human condensin II has a role in double stranded break (DSB) repair though the HR pathway. This function was demonstrated by a defect in HR repair upon condensin II depletion (Wood *et al.* 2008). Further to this, mutations in the CAP-G2 and CAP-H2 subunits of condensin II in *A. thaliana* lead to hypersensitivity to excess boron (Sakamoto *et al.* 2011). The study demonstrated that both excess boron and the condensin II mutations up-regulated the generation of DSBs and DSB-inducible gene transcription. Together the data suggests that boron toxicity involves inducing DSBs and that plant condensin II plays a role in their repair.

1.4.3 Localisation and sites of enrichment

A protein's localisation during the cell cycle can provide insight into its function and regulation. In line with their role in chromosome condensation, condensins have been seen to localise with mitotic chromosomes in every organism studied to date. At the cellular level, Condensin I is found in the cytoplasm throughout interphase and associates with chromatin upon nuclear envelope breakdown at the onset of mitosis. Condensin II however is localised to the nucleus throughout the cell cycle (Hirano 2012). *S. pombe* and *S. cerevisiae* only have one condensin complex and although identified as condensin I by amino acid sequencing, in *S. cerevisiae* it has been shown to localise to the nucleus throughout the cell cycle (Freeman *et al.* 2000). In *S. pombe* however, condensin localises to the nucleus specifically during mitosis (Sutani *et al.* 1999). It is thought that this discrepancy in condensin localisation may be linked to the particularly short G2 phase in the *S. cerevisiae* cell cycle, during which S-phase and M-phase partially overlap.

In higher eukaryotes at the chromatin level, condensin I in has been shown to bind chromosomes as early as prometaphase in cells undergoing open mitosis, and dissociate in late anaphase. Condensin II, although nuclear throughout the cell cycle, only shows a weak interaction with chromatin during interphase. Condensin II becomes stably attached in prophase, with a slow turnover rate of more than 80 minutes as shown by FRAP (fluorescence recovery after photo-bleaching) (Gerlich *et al.* 2006). Condensin I has been shown to have a much more dynamic association with mitotic chromosomes (Gerlich *et al.* 2006, Oliveira *et al.* 2007). Fluorescent tagging of condensin I and II in HeLa cells revealed alternate binding pattern of condensins along chromosome arms in metaphase (Ono *et al.* 2004). The binding signal of condensin I showed a spiral-like pattern, with condensin II displaying a more irregular pattern. Further to this, a unique arrangement of condensin binding is observed at centromeres; the condensin I signal is thinner and more internal than the condensin II signal which appeared as two intense dots predicted to be local to the kinetochores (Ono *et al.* 2004). Interestingly, when either condensin is depleted, the binding pattern of the non-depleted complex is altered, however this may be a secondary effect of altered chromosome structure (Ono *et al.* 2003). Using ChIP, the presence of condensin II on interphase chromatin was observed in *C. elegans,* along with an extensive overlap between condensin binding sites and those of the SCC-2 cohesin loader at sites of transcription (Kranz *et al.* 2013). The same study also shows the preferential binding of condensin I^{DC} to the X chromosome.

Binding sites of condensin have been best characterised in *S. cerevisiae* and *S. pombe* (Wang *et al.* 2005, D'Ambrosio *et al.* 2008, Schmidt *et al.* 2009). The first attempt at globally analysing condensin binding in *S. cerevisiae* was conducted by Wang *et al.* (2005). Using ChIP on chip in a mixed population of cells, they identified condensin binding sites along chromosome arms with an average spacing of 10.7 kb. The number of condensin-enriched loci was seen to directly correlate with chromosome size (Wang *et al.* 2005). Quantitative immunoblotting has shown condensin to be present once every 5 kb (on average) of mitotic DNA in *Xenopus* (MacCallum *et al.* 2002). The difference in binding frequency may be species specific and/or experimental techniques may introduce variation (mixed population versus mitotic). In *S. cerevisiae*

condensin I is bound to chromosomes even in interphase, remaining largely unchanged along chromosome arms throughout the cell cycle (Wang *et al.* 2005, D'Ambrosio *et al.* 2008). The studies in yeast reveal condensin to be enriched at centromeres, in a way that is particularly striking in mitosis (Wang *et al.* 2005, D'Ambrosio *et al.* 2008, Schmidt *et al.* 2009) (Verzijlbergen *et al.* 2014). Interestingly, a more recent study has shown that condensin enrichment at the centromeric region in *S. cerevisiae* relocates to chromosome arms during anaphase (Leonard *et al.* 2015). Other specialised regions of condensin enrichment have been identified at telomeres and at sites of high levels of transcription such as the rDNA and tRNAs (D'Ambrosio *et al.* 2008, Nakazawa *et al.* 2015). Specific enrichment at centromeres and areas of high transcription levels has also been observed in *C. elegans* (Hagstrom *et al.* 2002) (Kranz *et al.* 2013).

1.4.4 Factors affecting condensin localisation, chromatin association and regulation of activity

One approach for understanding how condensin functions in chromosome condensation, segregation, and other aspects of chromosome maintenance, is to investigate the regulatory and recruitment mechanisms of the complex. Many factors have already been implicated in facilitating and regulating condensin association with chromatin, including *cis* elements, recruitment factors, post-translational modifications, and the ATPase activity of the complex itself.

1.4.4.1 Transcriptional control

Transcriptional control of condensin subunits does not appear to be a major regulator of condensin activity. In human cells, quantitative immunoblot analysis revealed that the protein levels of condensin subunits are almost constant throughout the cell cycle (Takemoto *et al.* 2004). Similarly, in *S. cerevisiae*, the expression levels of Smc2 and Smc4 do not vary from interphase to mitosis (Freeman *et al.* 2000). Transcription and protein levels of regulatory subunits Brn1 and Ycs4 are under a mild cell cycle control

and slightly elevated in mitosis (Spellman *et al.* 1998, Freeman *et al.* 2000). In contrast to early studies, it has recently been demonstrated that Ycg1 (Cap-G) is expressed in a cell-cycle-dependent manner, with the protein being down-regulated following mitosis (Doughty *et al.* 2016). The authors suggest that the cyclical expression of Ycg1 maintains it at limiting levels compared to the other condensin subunits and that it's down-regulation in interphase contributes to a reduction in condensin activity.

Interestingly, In fast-replicating cells such as human intestinal and colorectal tumour cells there is a high level of SMC2 protein expression (Dávalos *et al.* 2012). It has been proposed that this high level of expression is due to the direct activation of SMC2 transcription by the binding of β-catenin (of the WNT signalling pathway) to the SMC2 promoter. As such, condensin expression has been identified as a potential therapeutic target in cancer cells (Dávalos *et al.* 2012). Although transcription plays a small part in condensin regulation it is unlikely that expression level is a primary regulatory mechanism responsible for the cell cycle control of condensin activity.

1.4.4.2 Post-translational modifications in regulation

Phosphorylation, the addition of a phosphate group (PO_4^{3-}) to a molecule, is one of the most common and influential post-translational modifications. Phosphorylation and subsequent dephosphorylation alters the activity and function of many protein enzymes, including condensin complexes. The phosphorylation of condensin is thought to modulate its function in two ways during the cell cycle; regulation of chromosomal targeting of condensin by phosphorylation of specific sites, and regulation of the biochemical activity of the complex. Data from high-throughput proteomic studies and site-directed mutations of predicted phosphorylation sites have enabled the assembly of detailed phosphorylation maps for condensin subunits (St-Pierre *et al.* 2009, Bazile *et al.* 2010, Hegemann *et al.* 2011, Kagami and Yoshida 2016). In these studies, large numbers of phospho-serine and –threonine residues were identified on SMC4/CAP-C and the non-SMC subunits, however no phosphorylation sites had been identified on SMC2 in humans until recently and only one has been found in *S. cerevisiae* (Figure

1.4). A recent review lists 11 phosphorylation sites on human SMC2 (Kagami and Yoshida 2016); however, it is unknown how often the sites are phosphorylated or whether they have any functional significance. Indeed, metabolic incorporation of ^{32}P in human cells showed SMC2/CapE to be the only condensin subunit on which phosphorylation was not detectable *in vivo* during M phase (Takemoto *et al.* 2004) (Takemoto *et al.* 2006).

Both yeast and human condensin complexes are heavily phosphorylated in mitosis (Bazile *et al.* 2010). These phosphorylation sites on condensin subunits are often clustered in poorly conserved and structurally disorganised regions (Bazile *et al.* 2010). The positioning of phosphorylation sites in what are predicted to be flexible peptide loops may allow an increased accessibility of target sites for corresponding kinases. While the exact position of phosphorylation sites is not well conserved even in closely related species, their relative position within specific subunits tends to be highly conserved even in species that are distantly related (figure 1.4) (Holt *et al.* 2009). Only a fraction of condensin phosphorylation sites match consensus sequences for well-known mitotic kinases such as CDK and PLK, which fits with the idea that the complex is regulated by multiple kinases and phosphatases. At least seven different enzymes have been found to modify the phosphorylation status of condensin: Aurora B kinase, CDK, PLK, MPS1, Cdc14, casein kinase 2 (CK2) and protein phosphatase A (PP2A). The influence that these kinases and phosphatases have on condensin activity differs between organisms.

Cyclin dependent kinase 1

CDK1 is the primary activator of condensin at the beginning of mitosis. The cyclin dependent kinase is activated at the start of mitosis by the production of mitosis-specific cyclins, and its kinase activity orchestrates the progression of prophase to anaphase. Chromosome condensation is dependent on CDK1 activity (Kimura *et al.* 1998, Sutani *et al.* 1999, McCleland and O'Farrell 2008, McCleland *et al.* 2009), and the kinase has been shown to directly phosphorylate condensin *in vitro* and *in vivo* in a way that alters the activity of the complex (Kimura *et al.* 1998, Sutani *et al.* 1999,

Robellet *et al.* 2015). CDK1-mediated phosphorylation of condensin is required for condensin-dependent supercoiling of DNA in *Xenopus* cell-free extracts and has a critical role in chromosome condensation in *S. cerevisiae* (Kimura *et al.* 1998, Bazile *et al.* 2010, Robellet *et al.* 2015). CDK1 phosphorylation sites were revealed in the N-terminal region of Smc4 (and no other condensin I subunits) by mass spectrometry. The direct phosphorylation of *S. cerevisiae* condensin by CDK1 was confirmed *in vitro* (Robellet *et al.* 2015). Early work identified mitosis specific CDK phosphorylation of T19 Cut3/Smc4 to be required for the relocation of *S. pombe* condensin I from the cytoplasm to the nucleus (Sutani *et al.* 1999). Additionally, work in HeLA cells has shown CDK1 to phosphorylate the Cap-D3 subunit of condensin II, which is important for further phosphorylation by Plk1 (see below) and vital for condensation in early mitosis (Abe *et al.* 2011).

In *S. cerevisiae*, the timing of phosphorylation of Smc4 by Cdk1 was shown to be early in mitosis and much earlier than the phosphorylation of Ycg1 in anaphase (Bazile *et al.* 2010). Cdk1 is down regulated in anaphase (Whitfield *et al.* 1990, Pines and Hunter 1991, Yeong *et al.* 2000), however condensed chromosomes are still maintained by condensin until the late stages of mitosis (Vagnarelli *et al.* 2006) (Guacci *et al.* 1994, Mora-Bermudez *et al.* 2007). Therefore, it is thought that condensin activity is regulated in a step-wise manner during mitosis, and while it is very sensitive to CDK1 activation at the start of mitosis, by anaphase condensin activity is no longer dependent on CDK1 activity.

Polo-like kinase 1

Polo-like kinase is thought to be a major activator of condensin in anaphase. The regulation of condensin by Cdc5/PLK1 has been best characterised in *S. cerevisiae,* in which the kinase is currently thought to be the ultimate effector of condensin phosphorylation in late mitosis (St-Pierre *et al.* 2009). It has been shown that the three non-SMC subunits of condensin are phosphorylated in late mitosis in a Cdc5-dependent manner (St-Pierre *et al.* 2009). Further to this, Cdc5 phosphorylation of condensin *in vitro* leads to hyper-activation of the complex's supercoiling activity, and

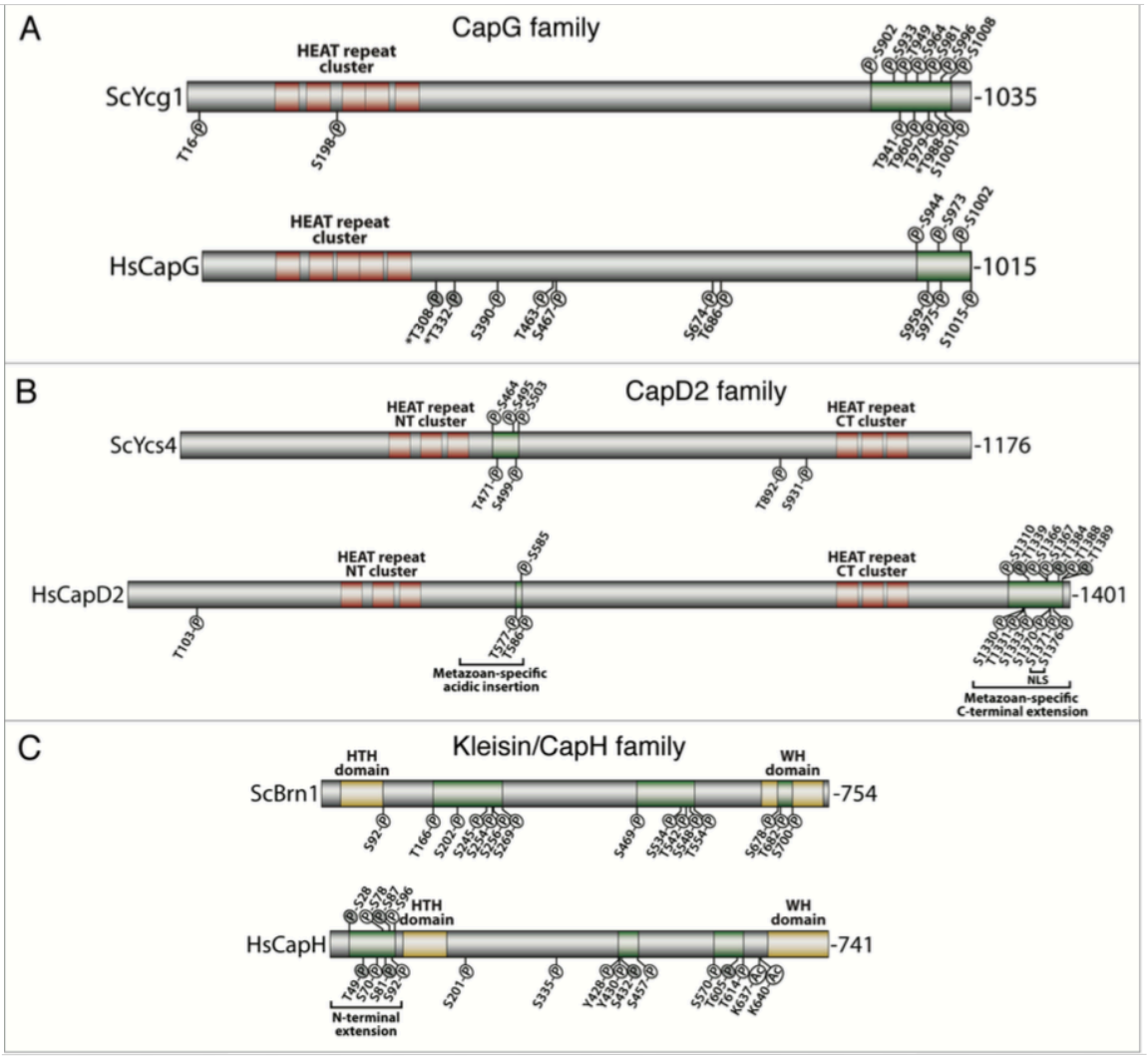


Figure 1.4 Phosphorylation and Acetylation sites in *S. cerevisiae* and human condensin I subunits.
Figure taken directly from (Bazile *et al.* 2010). This figure shows a compilation of phosphorylation and acetylation sites identified on yeast and human condensin subunits isolated from cell extracts. Sites of interest are marked by a bar and "P" for phosphorylation, and "Ac" for acetylation. Phosphorylation sites that conform to the minimal CDK1 consensus are highlighted in grey and residues marked with an asterisk are in vitro CKF1 phosphorylation sites. Green markings represent phosphorylation site clusters, and the red are HEAT repeats and Winged-helical domains (WHDs) as labelled in the figure. This figure compiled by Bazile *et al.* (2010) is non-exhaustive but is included as a point of reference and to show the comparison between human and yeast condensin subunits. **(A)** CapG family members. **(B)** CapD2 family members. (**C)** CapH/Kleisin family members, (figure continues on following page).

the phosphorylation of the proposed Cdc5 target sites is required for proper anaphase condensation and viability *in vivo*. Strikingly, the activation of condensin by Cdc5/PLK1 is dependent on prior phosphorylation of the complex by Cdk1 (St-Pierre *et al.* 2009).

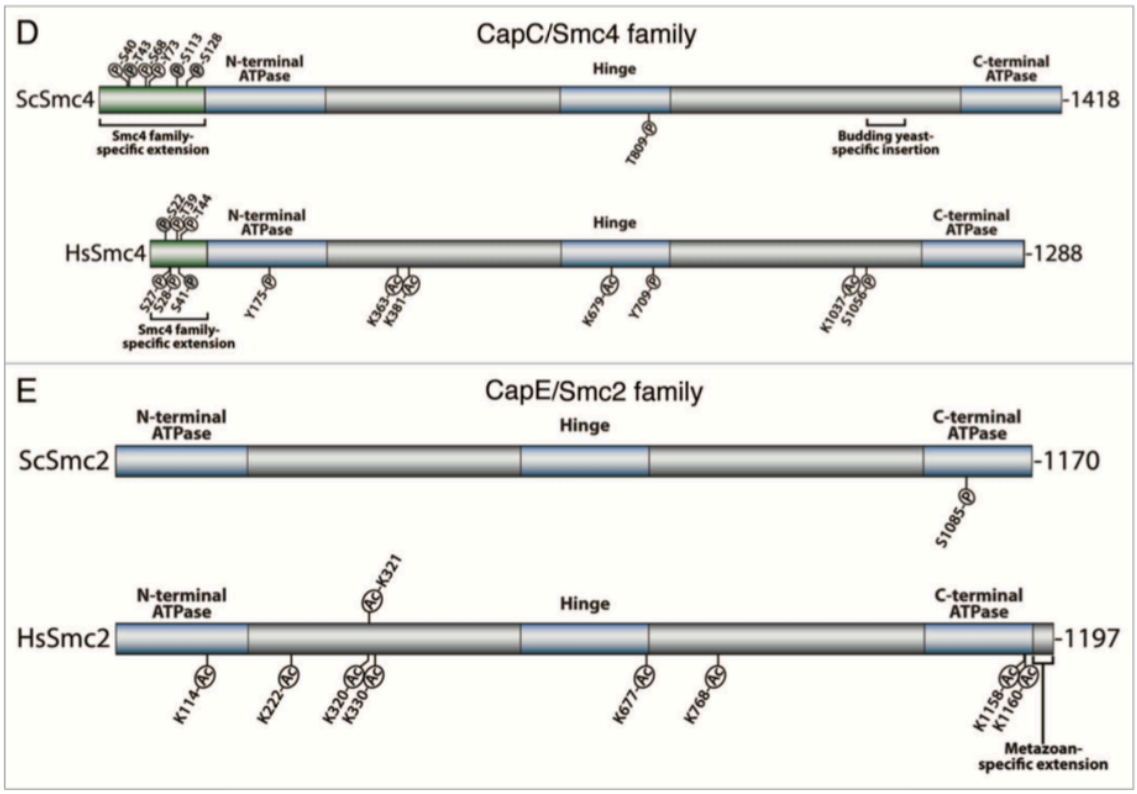


Figure 1.4 continued - Phosphorylation and Acetylation sites in *S. cerevisiae* and human condensin I subunits. (D) CapC family members and **(E)** CapE family members.

It has also been suggested that CDK1 and PLK1 work sequentially to regulate condensin II in human cells (Abe *et al.* 2011). A study in HeLa cells demonstrated that CDK1 phosphorylates the CAP-D3 subunit at Thr1415, which triggers further phosphorylation of the condensin II complex by PLK1. Therefore, phosphorylation sites in condensin subunits are sequentially phosphorylated throughout the progression of mitosis. Interestingly, a condensin mutant in which phosphorylation at Thr1415 is abrogated, impairs chromosome condensation but does not perturb recruitment of condensin II on mitotic chromosomes, hinting at the presence of distinct pathways in the regulation of condensin recruitment and its activation.

Aurora B kinase

Aurora B kinase plays an important role in the establishment of correct kinetochore/microtubule attachments in mitosis (Liu and Lampson 2009). In addition to this role in biorientation, Aurora B has also been shown to have a role in condensin

regulation in numerous organisms. The kinase has been shown to be required for the accumulation of condensin I on chromosomes from prometaphase to anaphase in *D. melanogaster* (Giet and Glover 2001), *C. elegans* (Collette *et al.* 2011), human cells (Lipp *et al.* 2007) and *Xenopus* egg extracts (Takemoto *et al.* 2007). Notably, a ~50% reduction in condensin enrichment was observed upon depletion/inhibition of Aurora B in humans and in *Xenopus* egg extracts (Lipp *et al.* 2007) (Takemoto *et al.* 2007). In coherence with this observation, the non-SMC subunits of human condensin complexes I and II are phosphorylated by Aurora B *in vitro* and are phosphorylated in mitosis *in vivo* in an Aurora B-dependent manner (Lipp *et al.* 2007). In *S. pombe*, phosphorylation of Cnd2 (kleisin) at Ser70 by Ark1/Aurora B kinase is required for the stable association of condensin with chromosomes (particularly centromeres and rDNA) right up until telophase (Nakazawa *et al.* 2008) (Nakazawa *et al.* 2011). In contrast, there has yet been no evidence that condensin association with chromatin is regulated by Ipl1/Aurora B in *S. cerevisiae* (Lavoie *et al.* 2004).

The precise role of Aurora B in the control of condensin activity is a point of contention due to differing results between studies and species. Despite evidence to the contrary, there are examples in which the depletion of Aurora B appeared to have little or no effect on the global levels of condensin association with chromosomes in *C. elegans* (Maddox *et al.* 2006) HeLa cells (Ono *et al.* 2004) and *Xenopus* egg extracts (Losada *et al.* 2002) (MacCallum *et al.* 2002). Also, the role of AIR-2/Aurora B in condensin II centromeric localisation in *C. elegans* (Hagstrom *et al.* 2002) and in human cells (Ono *et al.* 2004) has not been consistently apparent in different studies (Lipp *et al.* 2007) (Maddox *et al.* 2006). Possible explanations for these conflicting results may be that condensin enrichment in different chromosomal regions may be more effected by loss of Aurora B activity than others. For example, loss of condensin enrichment is most evident at centromeric regions upon Aurora B depletion in human cells (Lipp *et al.* 2007). Also, even when a loss of condensin enrichment is observed in response to Aurora B depletion in higher eukaryotes, a significant fraction or the complex remains on chromosomes. Perhaps observing this partial loss of condensin loading requires particular experimental conditions and assay sensitivity.

Another controversial aspect surrounding the involvement of Aurora B kinase in the regulation of condensin activity is the fact that in several organisms the depletion of the kinase does not translate into severe defects in chromosome condensation. For example, in mitotic *Xenopus* egg extracts, chromosome condensation appears unperturbed by Aurora B depletion (Losada *et al.* 2002) (MacCallum *et al.* 2002). The major chromosome compaction step in *C. elegans* and human cells occurs during prophase (Kaitna *et al.* 2002, Hirota *et al.* 2004, Ono *et al.* 2004, Maddox *et al.* 2006) and loss of aurora B activity causes no apparent defects in chromosome condensation during this stage of mitosis in these organisms (Losada *et al.* 2002, MacCallum *et al.* 2002). Conversely, Aurora B contributes greatly to the chromosome condensation that occurs in anaphase in human cells (Mora-Bermudez *et al.* 2007). Interestingly, depletion of Aurora B kinase does impact chromosome condensation in *Drosophila* S2 cells (Giet and Glover 2001), and in both *S. cerevisiae* and *S. pombe* (Morishita *et al.* 2001, Rajagopalan and Balasubramanian 2002, Lavoie *et al.* 2004).

Ipl/Aurora B also appears to be required only for condensation events taking place in late mitosis in *S. cerevisiae*. A study by Lavoie et al. (2004) shows that Ipl1/Aurora B is required for condensin-dependent maintenance of rDNA compaction in late mitosis (Lavoie *et al.* 2004). The same study claims an Ipl1/Aurora B-dependent phosphorylation of condensin non SMC subunits, although the supporting data is not shown (Lavoie *et al.* 2004). Subsequently, some Ipl1-dependent phosphorylation has been observed in *S. cerevisiae*. A delay but not a complete loss of mitotic phosphorylation of the non-SMC subunits was demonstrated by western blot (St-Pierre *et al.* 2009). Cdc5/Plk1 but not Ipl1 is able to phosphorylate condensin subunits *in vitro* and when over-expressed in S-phase. Therefore the current model in *S. cerevisiae* is that Ipl1/Aurora B does not directly phosphorylate condensin, but rather works up stream of Cdc5/Plk1 to regulate condensin in anaphase (St-Pierre *et al.* 2009). More work needs to be done to clarify the role of Aurora B in condensin association with chromatin, and regulation of activity of the complex.

Mps1 kinase

MPS1 (monopolar spindle) kinases are found in most eukaryotes, and regulate a number of steps in mitosis. The most widely conserved function of these kinases is their involvement in chromosome attachment and the SAC (spindle assembly checkpoint) at centromeres/kinetochores (Liu and Winey 2012). Recently it has been shown that chromosomal localisation of condensin II in early mitosis is controlled by Mps1-mediated phosphorylation of CAP-H2 at Ser492 in human cells (Kagami *et al.* 2014). Depletion of Mps1 results in incomplete chromosome condensation during prophase. It is not yet known whether this regulation of condensin II association with chromosomes is conserved in other organisms.

Protein phosphatases

In *S. cerevisiae*, protein phosphatase Cdc14 is thought to regulate condensin activity by both promoting its enrichment in early mitosis (D'Amours *et al.* 2004), and its removal from chromosomes during telophase (Varela *et al.* 2009). There are two waves of Cdc14 release. In the first, Cdc14 is released from the nucleolus by the Cdc-fourteen early anaphase release (FEAR) network. This early wave of Cdc14 is required for enrichment of condensin at the rDNA, and the subsequent segregation of this chromosome region (D'Amours *et al.* 2004). The second wave of Cdc14 release is orchestrated during telophase by the mitotic exit network (MEN) and coincides with condensin disassociation from the rDNA (Varela *et al.* 2009). Condensin disassociation is delayed upon perturbation of the MEN pathway, suggesting Cdc14 may play a role in regulating the removal of condensin from the rDNA in *S. cerevisiae*.

A second phosphatase has also been linked to condensin regulation. Protein phosphatase 2A (PP2A) dephosphorylates condensin *in vitro,* and is thought to dephosphorylate CAP-H2 subunit of condensin II during anaphase (Yeong *et al.* 2003, Takemoto *et al.* 2009). One hypothesis is that this dephosphorylation is a signal for unloading of the complex. However, there is little data to support this theory. Additionally, the chromosomal localisation of condensin II earlier in mitosis is

regulated by protein phosphatase 2A (PP2A). This recruiting activity is inhibited by okadaic acid, but not fostriecin even though both are strong inhibitors of PP2A phosphatase activity. This finding suggests that the protein phosphatase recruits condensin II in a manner that is independent of its catalytic activity (Takemoto *et al.* 2009). Therefore, both Cdc14 and PP2A may have dual roles of positive and negative regulation of condensins in mitosis.

Casein kinase 2

Casein kinase 2 (CK2) is thought to phosphorylate condensin in a way that exerts an inhibitory effect. CK2 has been reported to phosphorylate condensin I in HeLa cells mainly during interphase (Takemoto *et al.* 2006), when condensin activity is at a minimum. CK2-dependent phosphorylation of condensin inhibits the condensin-dependent supercoiling of DNA *in vitro*; therefore, it may negatively regulate condensin in this manner during interphase. If this were the case, the removal of CK2 phosphorylation in mitosis would then serve as an activation mechanism.

Acetylation and SUMO-ylation

Whilst the phosphorylation status of condensin plays a major role in the regulation of the complex during mitosis, there is also evidence of other post-translation modifications of condensin subunits. SUMOylation, the covalent attachment of a member of the SUMO (small ubiquitin-like modifier) family of proteins to specific target proteins via an enzymatic cascade (Wilkinson and Henley 2010), is a major regulator of protein function. In *S. cerevisiae*, early studies showed Smc4, Brn1 and Ycs4 to be sumoylated during mitosis (D'Amours *et al.* 2004, Denison *et al.* 2005, Hannich *et al.* 2005). More recently, in all five condensin subunits have been shown to be sumoylated in a mitosis-specific manner (Takahashi *et al.* 2008). Further to this, potential acetylation sites have been identified on Smc2, Smc4 and Cap-H in human condensins (see **Figure 1.4**) (Bazile *et al.* 2010). Whether the sumoylation or acetylation of condensin subunits is functionally relevant remains to be seen.

1.4.4.3 Cis elements and recruitment factors

Condensin is a cell cycle regulated DNA binding complex. How the cell cycle dependent activity of condensin is targeted to specific loci during mitosis, is a question that lies at the heart of understanding how its localisation and function is regulated. The simplest prediction is that a mixture of cell-cycle cues and *cis* interactors generate local enrichment. As discussed in 1.4.3, from yeasts to mammals, condensins are enriched at centromeres/pericentromeres, telomeres and along chromosome arms proximal to highly transcribed genes. A number of proteins and *cis* elements have been identified as potential recruiting factors of the SMC complex to specific regions on chromosomes.

The rDNA and highly transcribed regions along chromosome arms

As discussed in 1.4.2.3, condensin functions works alongside Fob1 in the maintenance of rDNA repeats in *S. cerevisiae*. The most serious defect in condensin mutants affects the condensation and segregation of these ribosomal RNA genes in *S. cerevisiae* (Freeman *et al.* 2000, D'Amours *et al.* 2004, Lavoie *et al.* 2004, Sullivan *et al.* 2004). Condensin enrichment at the replication fork barrier (RFB) within rDNA repeats has been shown to be Fob1 dependent (Johzuka *et al.* 2006). In a *FOB1* deletion mutant, condensin localisation (by ChIP) is lost at the RFB. However, a small amount of condensin is still present at NTS1 and NTS1, suggesting a Fob1-dependent and Fob1-independent recruitment of the complex. Johzuka *et al.* found evidence that three proteins; Tof2, (and two monopolin subunits) Csm1 and Lrs4 were required for Fob1-dependent recruitment of condensin. These proteins also interact with the RFB site in a Fob1-dependent manner, and are required for repression of recombination within the rDNA and transcriptional silencing at NTS1. It is thought that Tof2, Cms1 and Lrs4 mediate a protein-protein interaction network between Fob1 and condensin, and that the RFB acts as a *cis* element for Fob1-dependent recruitment of condensin (Johzuka and Horiuchi 2009). Likewise In *S. pombe*, the monopolin subunits associate with condensin and assist with its localisation at the rDNA (and also at centromeres, see below) (Tada *et al.* 2011).

In *S. cerevisiae* and *Sordaria fimicola,* cohesin components have been shown to be required for proper condensation in early mitosis (pre-anaphase) (Guacci *et al.* 1997, van Heemst *et al.* 1999, Hartman *et al.* 2000, Lavoie *et al.* 2002, Lavoie *et al.* 2004). Mutations in cohesin factors such as *MCD1/SCC1* (Kleisin) and *PDS5/SPO76* (HEAT repeat subunits) not only perturb sister chromatid cohesion but are also unable to establish and/or maintain chromosome condensation. It has been proposed that cohesins act as *cis* factors for condensation in early mitosis by restricting domains of condensin function (Lavoie *et al.* 2002). As briefly discussed in 1.4.2.1, in higher eukaryotes the bulk of cohesin is removed in prophase. Consistent with this, cohesin mutants do not appear to affect chromosome condensation in vertebrate cells (Sonoda *et al.* 2001). This finding suggests that low levels of cohesin are sufficient to promote condensation, or that cohesin action in condensation is redundant with additional/alternative factors in these systems.

The loading of the cohesin complex *in vivo* at centromeres and chromosome arms is facilitated by Scc2/4 (Ciosk *et al.* 2000). The cohesin loader has also been implicated in having a role in condensin recruitment. Several studies have shown condensin binding sites along chromosomes (also at centromeric regions), to correspond with those of the Scc2/4 complex (Kranz *et al.* 2013) (D'Ambrosio *et al.* 2008, Nakazawa *et al.* 2015). Interestingly, in *S. cerevisiae* upon Scc2/4 depletion, condensin binding at sites on chromosomes arms is reduced by 50% (D'Ambrosio *et al.* 2008). This finding suggests that while Scc2/4 is not necessary for condensin loading, it promotes its full level of association along chromosome arms. Unlike with cohesin, no protein-protein interaction between Scc2/4 and condensin could be detected by co-immunoprecipitation, suggesting that the recruitment activity is indirect. Recent evidence from Uhlmann suggests that Scc2/4 helps to maintain nucleosome-free regions, perhaps this might be important for condensin loading (Lopez-Serra *et al.* 2014). In chicken DT40 cells, the chromokinesin Kif4 has been implicated in the recruitment of condensins (mainly condensin I) to chromosome axes in mitosis (Samejima *et al.* 2012). In human cells AKAP95, a zinc finger protein that interacts with the kleisin subunit of condensin I has been shown to play a role in recruiting the

complex onto chromatin (Steen *et al.* 2000). Furthermore, Rbf1 a member of the RB (retino blastoma) tumour-suppressor protein family, has been shown to directly interact with CAP-D3 and regulate association of condensin II with chromatin in *Drosophila (Longworth et al. 2008)*.

The enrichment of condensin near to highly transcribed genes has led to investigations into links between condensin localisation and features of high gene expression. Several studies in *S. pombe* provide data in support of this. Condensin enrichment is reduced by mutations in RNA pol I, or its up-stream activating complex (Nakazawa *et al.* 2008). Further to this, inhibition of RNA Poll II diminishes condensin association with mitotic chromosomes (Sutani *et al.* 2015). Several transcription factors have been implicated in condensin loading on mitotically active genes. These include Sep1 (Nakazawa *et al.* 2015), and TATA-box-binding protein (TBP) (Iwasaki *et al.* 2015) in *S. pombe* and *S. cerevisiae,* an ectopic B-box element, recognized by TFIIIC, constitutes a minimal condensin-binding site (D'Ambrosio *et al.* 2008). These studies all suggest that high levels of transcription positively regulate condensin association.

There is however, some controversy surrounding the link between condensin function and active gene transcription. In *S. cerevisiae* the transcription of the rDNA repeats by RNA pol I or RNA pol II prevents the stable association of condensin at this region (Johzuka and Horiuchi 2007, Clemente-Blanco *et al.* 2009, Clemente-Blanco *et al.* 2011). As previously discussed (1.4.4.2), Cdc14 phosphatase plays a role in condensin function at the rDNA. It is thought that Cdc14 promotes condensin activity by inhibiting RNA polymerase I transcription through the dephosphorylation of polymerase subunits (Clemente-Blanco *et al.* 2009). Furthermore, transcription is usually repressed during mitosis in most eukaryotes (Gottesfeld and Forbes 1997), when condensin association with chromatin is at its peak. Most recently a study in *S. pombe* yeast suggests that transcriptional coactivators Gcn5 (histone acetyltransferase) and RSC chromatin remodelling complex, locally evict nucleosomes at gene promoters to facilitate condensin binding at these regions (Toselli-Mollereau *et al.* 2016). The authors argue that nucleosomes act as a barrier for the initial binding

of condensins. The role of transcription in condensin association with chromatin remains unclear.

Centromere and pericentromeric regions

While condensin distribution along chromosomes has been shown to remain largely unchanged throughout the cell cycle in *S. cerevisiae*, the centromeric peak of condensin becomes enriched in a mitosis-specific manner (Wang *et al.* 2005, D'Ambrosio *et al.* 2008). Nakazawa *et al.* (2008) have shown kinetochore components of *S. pombe* to be important in localization of condensin to the centromere in mitosis. By expressing GFP-tagged Cut14/Smc2 in *S. pombe* kinetochore mutants, they showed that kinetochore components (MIS6, CNP1, and MIS13) are required for the centromeric localization of condensin. It is possible then, that parts of the kinetochore may be responsible for recruiting condensin to the centromere. It seems likely that this mechanism of condensin accumulation may be conserved, as condensin II also fails to localise at centromeres upon depletion of CENP-I (hMIS6) by RNAi in human cells (Nakazawa *et al.* 2008).

Studies have shown that monopolin complexes in yeast work to facilitate proper microtubule-kinetochore attachments during cell division (Rabitsch *et al.* 2003, Gregan *et al.* 2007). It was originally thought that in *S. cerevisiae* this regulation of attachments was restricted to meiosis I, whilst in *S. pombe* monopolin functioned in both meiosis II and mitosis (Rabitsch *et al.* 2003). However, it has been shown more recently that monopolin is also required for mitosis in *S. cerevisiae* (Brito *et al.* 2010). One theory is that monopolin works to regulate centromere-microtubule interactions by crosslinking kinetochore components (Corbett *et al.* 2010). However, a study in *S. pombe* suggests that their main role is in the recruitment of condensin (Tada *et al.* 2011). As mentioned above, parts of the monopolin complex in *S. cerevisiae* are involved in condensin recruitment to the rDNA in *S. cerevisiae* (Johzuka and Horiuchi 2009). The Pcs1 and Mde4 monopolin subunits recruit condensin to the rDNA and centromeric regions in *S. pombe* (Tada *et al.* 2011). In *Candida albicans* monopolin subunits also recruit condensin to centromeres as shown by ChIP (Burrack *et al.* 2013).

It is as yet unknown whether this mechanism of recruitment is conserved in higher eukaryotes. Although structural and sequence analyses showed that the Pcs1/Mde4 complex shares similar features with the conserved kinetochore complex Spc24/Spc25, no true homologs of monopolin subunits have yet been identified in vertebrates (Rumpf *et al.* 2010) (Tada *et al.* 2011).

Shugoshins are a conserved family of proteins known to be important in chromosome biorientation and protection of cohesion at centromeres/pericentromeres in many organisms (Marston 2015). Shugoshin is thought to aid the establishment of biorientation by promoting the correction of tensionless attachments (Indjeian *et al.* 2005, Huang *et al.* 2007, Kawashima *et al.* 2007). During the course of this study it was reported that the *S. cerevisiae* shugoshin protein, Sgo1 has been shown to work alongside PP2A to recruit both condensin and Ipl1/Aurora B to pericentromeric regions (Peplowska *et al.* 2014, Verzijlbergen *et al.* 2014). Fluorescence microscopy assays showed that the centromeric enrichment of GFP-tagged Ycg1 is almost completely abolished in Sgo1 or Rts1 (PP2A regulatory subunit) deletion mutants (Peplowska *et al.* 2014). More precisely, ChIP assays revealed that Brn1 enrichment in the pericentromere (but not the centromere itself), is diminished in the absence of Sgo1 (Verzijlbergen *et al.* 2014).

1.4.4.4 ATPase activity of the condensin complex and its role in chromatin association

SMC proteins are ATPases, a class of enzymes that can harness energy from catalysing the hydrolysis of ATP into ADP. An accumulation of evidence indicates that the ATPase activity of SMC protein complexes is crucial in their functionality and proper association with chromatin. Although condensin interacts with DNA *in vitro* in the absence of ATP [50, 60], the ability of condensin to confer changes in DNA structure and condense DNA *in vitro* is ATP-dependent (Kimura *et al.* 1999) (Strick *et al.* 2004). This *in vitro* DNA compaction activity can only occur in the presence of hydrolysable ATP, which indicates a separation of the ATP-binding and ATP-hydrolysis function

(Strick *et al.* 2004). Indeed, mutations perturbing the ATPase cycle of condensin in chicken DT40 cells revealed ATP-binding, but not hydrolysis to be essential for stable condensin association with chromosomes *in vivo* (Hudson *et al.* 2008). However, continuous ATP-hydrolysis by *S. cerevisiae* condensin is required for the maintenance of chromosome structure in *Xenopus* cell-free extracts (Kinoshita *et al.* 2015).

Similarly, studies have demonstrated the importance of ATPase activity in the stable association of cohesin (Arumugam *et al.* 2003, Weitzer *et al.* 2003, Ladurner *et al.* 2014, Murayama and Uhlmann 2014) and more recently, the Smc5/6 complex (Kanno *et al.* 2015) with DNA. The Scc2/4 cohesin-loading complex has been shown to stimulate the ATP-hydrolysis activity of cohesin (Ladurner *et al.* 2014, Murayama and Uhlmann 2014), which in turn is required for its stable binding of DNA in the form of topological entrapment (Arumugam *et al.* 2003, Weitzer *et al.* 2003, Ladurner *et al.* 2014, Murayama and Uhlmann 2014). A study using purified *S. cerevisiae* Smc5/6 has shown that the complex requires the binding of ATP to Smc6 in order to generate electrostatic interactions with DNA. The authors also demonstrate that the subsequent topological entrapment of DNA by the complex requires ATP-hydrolysis (Kanno *et al.* 2015). Comparisons are often made between the SMC complexes, and from the data obtained so far it appears that there are both similarities and differences in the role of ATPase activity in their association with chromatin. Stable binding of all three complexes appears to be ATP-dependent. However, whilst cohesin and Smc5/6 undergo ATP-hydrolysis in order to topologically entrap DNA, there is no evidence so far to indicate this to be the case for condensin. Smc5/6 and condensin both require ATP-binding for a stable interaction with DNA (however the Smc5/6 work has only been done *in vitro*) whilst cohesin requires the extra step of hydrolysis. Chromosome spreads revealed ATP-hydrolysis mutants of *S. cerevisiae* cohesin greatly reduced the amount of the complex associated with chromosomes (Arumugam *et al.* 2003), however the fluorescent labelling of condensin ATP-hydrolysis mutants in vertebrate cells showed similar levels of chromosome association as that of wild-type (Hudson *et al.* 2008).

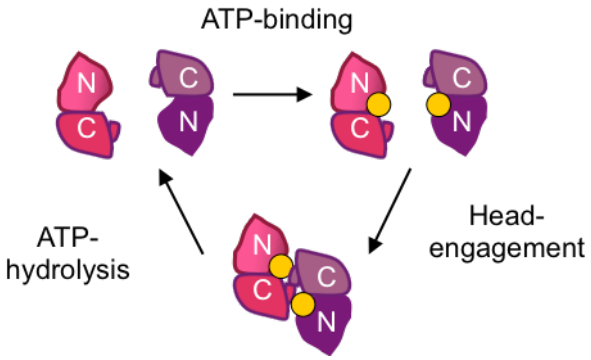


Figure 1.5 ATPase cycle of condensin.

Adapted from (Hirano 2016) A schematic representation of the engagement and disengagement of the two SMC heads domains regulated by ATP-binding and hydrolysis.

What is actually happening at a molecular level during the ATPase cycle of condensin? Each SMC head domain is formed of the N- and C- termini of the protein to generate globular ABC-like ATPase domains. An important feature of ABC-like ATPases is that the ATP molecules bind to one head then interact with signature motifs on the other (Holland and Blight 1999). Therefore the ATPase head domains of SMC subunits are thought to engage upon nucleotide binding, and to disengage upon subsequent ATP-hydrolysis (Lammens *et al.* 2004, Hirano 2005). The hydrolysis of the two ATP molecules sandwiched between two SMC heads is thought to be simultaneous and cooperative (reviewed in (Holland and Blight 1999)). These ATP-dependent interactions between the SMC head domains are hypothesized to drive structural rearrangements of condensin protein complexes, which may play a key role in how they engage DNA (Hirano 2005, Hirano 2016). As mentioned in 1.3, a study by Soh *et al*. shows a transformation from a rod to ring configuration of a bacterial condensin upon binding of ATP by the head domains and DNA by the hinge domain (Soh *et al.* 2015). Further investigation into condensin's ATPase cycle is needed in order to further understand its role in in the association of the complex with chromatin.

1.4.4.5 Summary

Work in the field of condensin so far has cemented our appreciation of its importance in cellular processes; most importantly condensin's role in chromosome condensation

and segregation. There is a multitude of factors implicated in the regulation of condensin in these roles. The phosphorylation status of condensin is controlled by several kinases and phosphatases, and discrete local populations of condensin on chromosomes appear to be regulated by their own melee of *cis* interactions and regulatory factors. At the very core of condensin regulation is the ATPase enzymatic cycle of the complex itself. There are big gaps in the understanding of how certain factors regulate condensin, and one way to gain understanding is to probe whether and how they impact condensins association with chromatin.

1.5 *S. cerevisiae* as a model organism

To investigate factors affecting condensin association with chromosomes I will be making use of the model organism Saccharomyces cerevisiae*. S. cerevisiae,* also known as budding yeast (due to the formation of buds during the cell cycle) serves well as research model because it has a large number of conserved genes and cellular mechanisms; Following Leland Hartwell's work in the 1970s it has been demonstrated that the basic cell cycle machinery is conserved from yeast to humans (Nurse 1990). In a comparison between all yeast protein sequences to mammalian sequences, 31% of yeast protein-coding genes were found to have a mammalian homolog (Botstein *et al.* 1997). Furthermore, approximately 20% of human disease genes have counterparts in yeast (Primrose 2008) highlighting the similarities between the cellular processes involved in homeostasis. Conserved mechanisms and accessible genomic data, combined with the easy and cost-efficient genetic manipulation of yeast, have cemented *S. cerevisiae* as exemplary model organism.

Whilst *S. cerevisiae* cells do not compact their DNA to the same extent as mammalian cell, condensation does occur (Guacci *et al.* 1997), and condensin is absolutely essential in this species. Furthermore, the presence of only condensin I complex may simplify an investigation into factors affecting its association with chromosomes because the binding pattern of the two complexes has been shown to be

interdependent (Ono *et al.* 2003). As discussed throughout this introduction, a multitude of investigations into condensin function and activity have already been conducted in *S. cerevisiae* and have contributed invaluable knowledge to sustain a growing understanding of SMC proteins.

1.6 Experimental research goals

The broad research goal for the work laid out in the following chapters, is to investigate factors affecting condensin association with mitotic chromosomes in *S. cerevisiae*. Studies have consistently shown the centromeric region in *S. cerevisiae* to be increased in condensin enrichment specifically during mitosis. The point centromere of *S. cerevisiae* allows the direct testing of condensin loading over a small and specific area, something that cannot be done by ChIP in systems with regional centromeres that consist of expansive repetitive DNA. As discussed above, *S. cerevisiae* is an excellent genetic tool in which the function of both essential and non-essential genes can be tested.

To fulfil my broad research goal, I worked to achieve four more precise research aims. The first is to set up a functional and efficient chromatin immunoprecipitation (ChIP) assay that is sensitive to changes in the chromatin enrichment of condensin. Once this is achieved I aim to utilise the ChIP assay in my other three research goals. Kinetochore factors have been implicated in the association of condensin with centromere regions in *S. pombe* and vertebrate cells. My second aim is to test whether *S. cerevisiae* kinetochore components play a role in condensin's mitotic enrichment at the centromere. Multiple studies have shown the importance of phosphorylation for condensin activity, however there is still no clear picture of how phosphorylation regulates the complex. Therefore, my third aim is to probe how condensin phosphorylation affects condensin enrichment on chromatin at the mitotic centromere. Finally, the ATPase activity of condensin has been shown to be important for its association with chromatin in vertebrate cells. The work in my last chapter aims to investigate how perturbing the ATPase cycle affects condensin association with chromatin in *S. cerevisiae*.

2 Materials and Methods

2.1 List of strains

All strains used in this study were of W303 background.

Strain number	Strain name (as referenced in text)	Genotype
11	Tet-degron wild-type	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3) leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3*
12	wild-type + *pRS316*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3) leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 pRS316*
278	*smc2-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3) leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)*
333	*ndc80-1*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3) leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 ndc80-1*
335	*ndc80-1/ smc2-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3) leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker) ndc80-1*
358	*ipl1-321/ smc2-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3) leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker) ipl1-321*

360	*ipl1-321*	*MAT**α** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ipl1-321*
362	*cse4-323/* *smc2-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA* *(tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *cse4-323*
364	*cse4-323*	*MAT**α** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *cse4-323*
367	*ndc10-1*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ndc10-1*
488	AID-degron wild-type	*MAT**a** his3-11 leu2-3 trp1-1 ura3-1 can1-100* *ura3-1::GALOsTir1-9myc (URA3)*
507	*ycg1-aid*	*MAT**a** his3-11 leu2-3 trp1-1 ura3-1 can1-100* *ura3-1::GALOsTir1-9myc (URA3)* *YCG1:: ycg1-aid (c-terminal) (KanMX_)*
508	*ycs4-aid*	*MAT**a** his3-11 leu2-3 trp1-1 ura3-1 can1-100* *ura3-1::GALOsTir1-9myc (URA3)* *YCS4:: ycs4-aid (c-terminal) (KanMX_)*
518	*ndc10-1/* *smc2-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA* *(tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *ndc10-1*
705	AID-degron wild-type	*MAT**a** his3-11 leu2-3 trp1-1 ura3-1 can1-100* *ura3-1::GALOsTir1-9myc (URA3)* *brn1-3V5 (natNT2)*
706	*ycs4-aid*	*MAT**a** his3-11 leu2-3 trp1-1 ura3-1 can1-100* *ura3-1::GALOsTir1-9myc (URA3)* *YCS4:: ycs4-aid (c-terminal) (KanMX_)* *brn1-3V5 (natNT2)*
707	*ycg1-aid*	*MAT**a** his3-11 leu2-3 trp1-1 ura3-1 can1-100*

		ura3-1::GALOsTir1-9myc (URA3) *YCG1:: ycg1-aid (c-terminal) (KanMX_)* *brn1-3V5 (natNT2)*
708	Tet-degron wild-type	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *brn1-3V5 (natNT2)* *ycg1-3V5(natNT2)*
711	*ndc80-1*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ndc80-1* *brn1-3V5 (natNT2)*
712	*ndc10-1*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ndc10-1* *brn1-3V5 (natNT2)*
817	*Smc2 o/e*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-WT-SMC2-6HA, TRP1* *brn1-3V5 (natNT2)*
818	*smc2-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *brn1-3V5 (natNT2)*
819	*D1112A*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-D1112A-6HA, TRP1* *brn1-3V5 (natNT2)*
820	*E1113Q*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)*

		leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-E1113Q-6HA, TRP1* *brn1-3V5 (natNT2)*
821	*K38I*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-K38I-6HA, TRP1* *brn1-3V5 (natNT2)*
822	*L567K*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-L567K-6HA, TRP1* *brn1-3V5 (natNT2)*
823	*L665R*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-L665R-6HA, TRP1* *brn1-3V5 (natNT2)*
824	*R58A*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-R58A-6HA, TRP1* *brn1-3V5 (natNT2)*
825	*S1085R*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-SMC2-S1085R-6HA, TRP1* *brn1-3V5 (natNT2)*

858	*cdc20-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *cdc20-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA* *(tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *brn1-3V5 (natNT2)*
1064	*cdc5-10*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *cdc5-10* *brn1-3V5 (natNT2)*
1065	*cdc5-99*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *cdc5-99 (HIS3)* *brn1-3V5 (natNT2)*
1070	*smc4-10A*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc4-10A::3xSTII::HIS3MX6*
1072	*cdc7-1*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *cdc7-1* *brn1-3V5 (natNT2)*
1073	*brn1-570*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *brn1-570::URA3* *brn1-3V5 (natNT2)*
1074	*ycs4-543*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ycs4-543::KanMX* *brn1-3V5 (natNT2)*
1077	*ycg1-521/* *ycs4-543*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ycg1-521::kanMX* *ycs4-543::kanMX*

		brn1-3V5 (natNT2)
1078	*ycg1-521*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ycg1-521::kanMX*
1101	*Smc2 o/e* *ycg1-3V5*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA* *(tetR-VP16)-tetO_2 - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-WT-SMC2-6HA, TRP1* *ycg1-3V5 (natNT2)*
1102	*smc2-td* *ycg1-3V5*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc2-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA* *(tetR-VP16)-tetO_2 - Ub -DHFRts - 3HA-linker)* *ycg1-3V5 (natNT2)*
1107	*cse4-323*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *cse4-323* *brn1-3V5 (natNT2)*
1108	*ipl1-321*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ipl1-321* *brn1-3V5 (natNT2)*
1109	*smc4-10A*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc4-10A::3xSTII::HIS3MX6* *brn1-3V5 (natNT2)*
1110	*ycg1-521*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *ycg1-521::kanMX* *brn1-3V5 (natNT2)*
1111	*smc4-td*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)*

		leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3 *smc4-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *brn1-3V5 (natNT2)*
1112	*Smc4 o/e*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc4-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1-WT-SMC4-6HA, TRP1* *brn1-3V5 (natNT2)*
1113	*smc4_n_termΔ*	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *smc4-td SMC2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO$_2$ - Ub -DHFRts - 3HA-linker)* *trp1-1::SDM-pFA6a-GAL1- smc4del3- -6HA, TRP1* *brn1-3V5 (natNT2)*
1291	Tet-degron wild-type	*MAT**a** ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100* *UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3)* *leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3* *brn1-3V5 (natNT2)*

Table 2.1 List of strains

2.1.1 Additional strain details

All strains kindly sent by other labs for use in this project, were backcrossed to contain the tet-degron genetic components.

- Phospho-mutant Strains 1073, 1074, 1070, 1077, 1109, 1111 and Cdc5 mutant strains (1064, 1065) were backcrossed from strains kindly provided by the D'Amours lab (St-Pierre *et al.* 2009, Robellet *et al.* 2015)
- Strains containing kinetochore mutants *cse4-323* (364, 468, 1107), *ndc10-1* (367, 518, 712) and *ndc80-1* (333, 335, 711) or *ipl1-321* (358, 360, 1108) were backcrossed from strains kindly provided by the Biggins lab.
- The *cdc7-1* strain (1072) was backcrossed from a strain kindly provided by the Zegerman lab

2.1.2 Protein-Tag confirmation

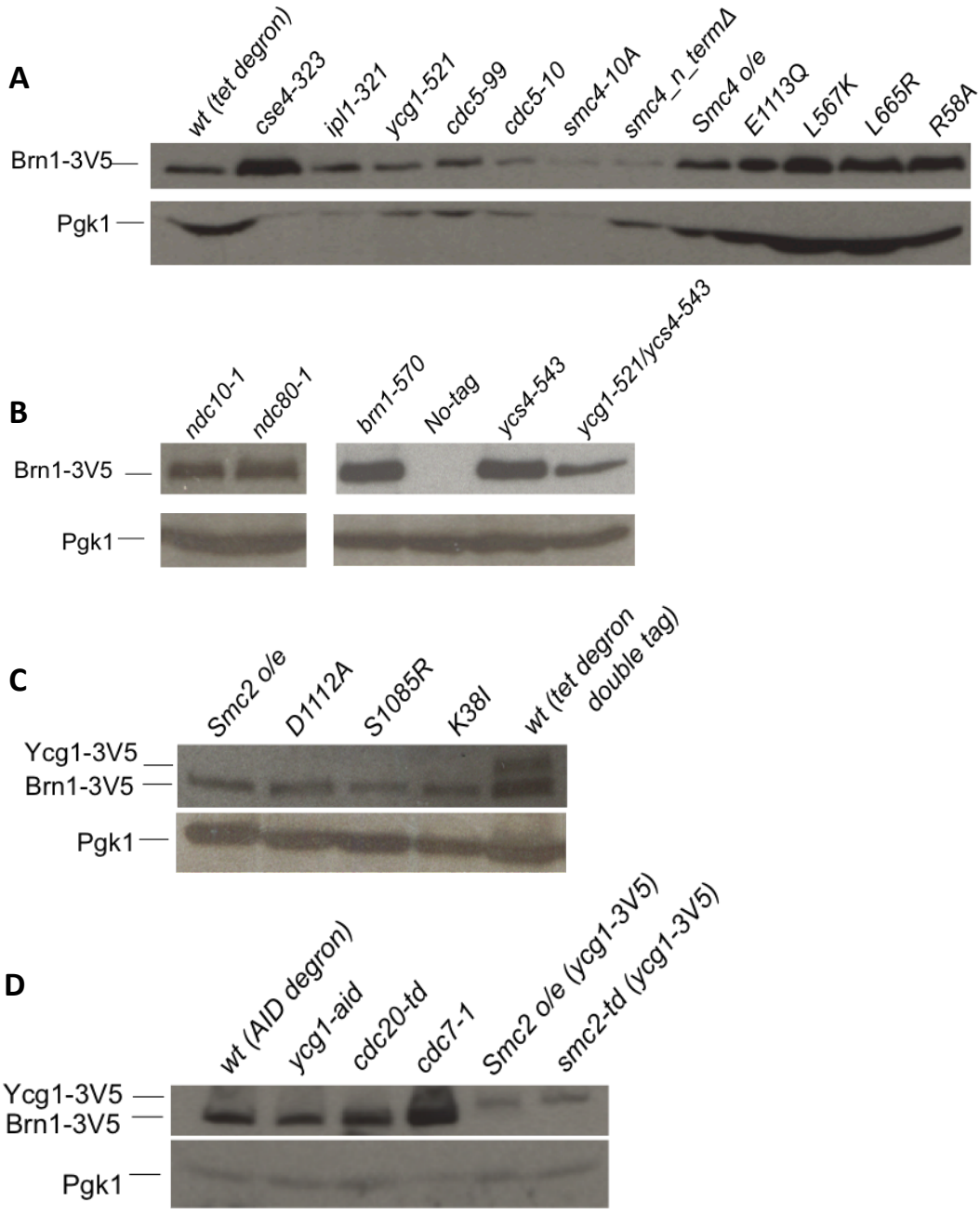


Figure 2.1 Protein-tag (Brn1-3V5 and Ycg1-3V5) confirmation

Western blot analysis demonstrating the presence of Brn1-3V5 and Ycg1-3V5 in strains used for ChIP analysis. Samples of exponentially growing cells were prepared for western blot analysis using whole-cell TCA extraction (detailed in 2.6.1). Western blot was undertaken as detailed in 2.6.2 and samples were run on a 8% polyacrylamide gel. Pgk1 used as a loading control. Protein tag check for strains (from left to right) **A)** 1291, 1107. 1108. 1110, 1065, 1064, 1109, 1113, 1112, 820, 822, 823, 824. **B)** 712, 711, 1073, 1078, 1074, 1077. **C)** 817, 819, 825, 821, 708. **D)** 705, 707, 858 1072, 1101, 1102.

2.2 List of primers

Primer number	Sequence
1	GATGAGAACAACACTGCGAATGACCAATAC
2	CCACCGGGAAGACCAAGACACA
3	AAATGCCTAGACAATCCAGACCAG
4	TTTGCAGGCTCATTATTCAGATTT
5	ACAACCAAAAAATCAACTTAAATAGATGAAGAGCTCGCATATCAATTTTAATCGATGAATTCGAGCTCG
6	AATAACATATAATATAAAACGGAAGAAACGGGTAAACGTCAGTTCGATTAATCGATGAATTCGAGCTCG
7	ACGTCACTGCATTATTGGAGCAAGGTTTCAAGGTTGTATCCGCAAAAGAACGTACGCTGCAGGTCGAC
8	TGTCCATAGATGAAGAAGATAAGGATTCAGAGTCTTTCAGCGAGGTCTGTCGTACGCTGCAGGTCGAC
24	AGGCCTTCCCAAGCAAGAATTTGA
25	CCGCAACTACATTCTGGGCATTATG
26	TCAGTAGCTGCCCCTTTAAAGTCAG
27	GAAGGCCTCCGTTCTTATGGTTATT
28	ATGCAAGCACCAAGCGCTCTTCT
29	AAGGGGCAGCTACTGAAGGTTTTG
30	TTATTCTTCCTTCCGGTTTTATCGT
31	TGAGGTTCATCAACAATTGGATTTT
32	TGCTTGCAAAAGGTCACATGCTTAT
33	CATTTTGGCCGCTCCTAGGTAGTG
34	CAGCGCGTTTCACTTTTAAATCAAA
35	TTGCATGATCAAAAGGCTCAATGTT
36	TCATGCAATTGCTCAAAGCGTAAAA
37	ACCGCTGTATGCAATTTCTTGTGGT
46	ATTGAAAGCTTGGGGATACAAAGGAAAACAAGGAG

47	ATTGAAAGCTTATGTAGTAGTATTGCGAGATG
48	ATTGAGGATCCTATAAAACGGAAGAAACGGGTAA
49	ATTGAGGATCCTGGCCCAAACGGATAACACG
59	GGTATTGTTTTGGATTCCGGTGA
60	TTGCATTTCTTGTTCGAAGTCCA
64	CACACTTGTACTCCATGACTAAACC
65	GACAGAGAGGGCAAAAGAAAA
106	GGCGAGAAAGGAAGGGAAGAAA
107	AGTGAGCGAGGAAGCGGAAGAG
108	GTCAGTCTGAAAATTTTTCA
109	AATATTGTCTTTAAATAAGA
110	AGGTATGATTATAGCGATGAG
111	AGCAGCGGAGGGTTTATGCA
112	ATTTCATAGAAATGTAGTAG
113	ACTAAGCTATTTGATAAGTTC
114	AGATTCCAGGATTCAAATCC
115	ATTGATTGATCAACATGTCA
116	AGCGATCCAATGCTTCAAAG
M.Y-33	CGCGCCTTAATTAATTAAGTGCTATCAAGACCTAGT
M.Y-42	ACAACCAAAAAATCAACTTAAATAGATGAAGAGCTCGCATATCAATTTTAATCGATGAATTCGAGCTCG
M.Y-43	TGTCCATAGATGAAGAAGATAAGGATTCAGAGTCTTTCAGCGAGGTCTGTCGTACGCTGCAGGTCGAC
M.Y-50	CTTTGCAGCACAAAAAAAAAAAAAAAAAAAAAAAAAAGATCATCAACTAATCGATGAATTCGAGCTCG
M.Y-51	ATGACTTGATAGTGAATTATGAGGATCTAGCGACAACACAGGCAGCGTCACGTACGCTGCAGGTCGAC
S.S-270	GCCTTCTACACTTAGCCAATGAGCATGGGT

Table 2.2 List of primers

Primers used for ChIP

24 SMC2 ChIP 1 upper
25 SMC2 ChIP 1 lower
26 SMC2 ChIP 2 upper
27 SMC2 ChIP 2 lower
28 SMC2 ChIP 3 upper
29 SMC2 ChIP 3 lower
30 SMC2 ChIP 4 upper
31 SMC2 ChIP 4 lower
32 SMC2 ChIP 5 upper
33 SMC2 ChIP 5 lower
34 SMC2 ChIP 6 upper
35 SMC2 ChIP 6 lower
36 SMC2 ChIP 7 upper
37 SMC2 ChIP 7 lower

59 ACT1_F
60 ACT1_R
64 SC-39 rDNA
65 SC-40 rDNA

2.3 Media

For use of the following media as agar plates, 2% (w/v) bacto-agar was added. Carbon sources were filter sterilised (Millipore) and added after autoclave and are specified throughout the text. Luria-Bertani (LB) media was used to grow *E. coli.*

2.3.1 YP rich media

1% (w/v) Bacto-yeast extract

2% (w/v) Bacto-peptone

For **YPD** - 2% (w/v) glucose

For **YPRG** - 2% (w/v) raffinose and 2% (w/v) galalactose

For **YPR/ADE** – 2% (w/v) raffinose and 0.4% (w/v) adenine sulphate

2.3.2 Minimal Media

1x YNB (Yeast Nitrogen Base)
2% (w/v) Bacto-agar
2% (w/v) glucose
Supplemented with 0.004% (w/v) adenine sulphate, L-histidine, L-leucine, uracil and L-tryptophan unless otherwise stated.

2.3.3 Rich Sporulation Medium (RSM) agar plates

0.25% (w/v) Bacto-yeast extract
1.5% (w/v) Potassium Acetate
0.1% (w/v) glucose
2% (w/v) Bacto-agar
0.008% (w/v) adenine sulphate, uracil
0.004% (w/v) L-histidine, L-leucine, L-lysine, L-tryptophan, L-methionine, L-arginine
0.0016% (w/v) L-tyrosine
0.02% (w/v) L-phenylalanine

2.3.4 Drugs and chemicals used for selection

Nourseothricin (NAT) - Jena Bioscience, AB-102L
Geneticin disulphite (G-418) - Melford, G0175
Ampicillin sodium salt (Amp) – Sigma, A9518

2.4 Molecular cloning techniques

2.4.1 *E. coli* transformation

DH5α cells (stored at -80°C) were thawed on ice and then incubated with purified plasmid DNA for 30 minutes on ice. The cells were then heat-shocked at 42°C for 1 – 1.5 minutes, then placed back on ice for 2 minutes. Samples were then incubated at

37°C in 1 ml LB media for up to 1 hour. The *E. coli* was then plated onto LB plates containing 100 µg/ml Ampicillin and grown over night at 37°C.

2.4.2 Plasmid extraction from *E. coli* (Miniprep)

E. coli cells were grown overnight in 5 ml LB ampicillin (100 µg/ml) media at 37°C, before being pelleted and resuspended in 250 µl P1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA, QIAGEN Qiaprep®). 250 µl of buffer P2 (200 mM NaOH, 1 % w/v SDS) and 350 µl of buffer N3 (4.2 M Gu-HCl 0.9 M potassium acetate pH 4.8) was added and samples were mixed by inverting the tube 4-6 times. Samples were spun at 13,000 rpm for 10 minutes and the supernatant applied to QIAprep spin columns. Samples were spun for 30-60 seconds and the flow-through discarded. The QIAprep column was washed with 750 µl buffer PE (10 mM Tris-HCl pH 7.5, 80 % ethanol) and eluted into 50 µl buffer EB (10 mM Tris-Cl, pH 8.5) or water.

2.4.3 Restriction digest

Restriction digestion enzymes were purchased from NEB (New England biolabs) and Fermentas and the digestions were carried out according to the manufacturer's instructions.

2.4.4 Plasmid ligation

50 ng of digested vector DNA was incubated on ice with cut insert DNA in a concentration ratio of 1:2. Rapid DNA ligase T4 was purchased from ThermoFisher Scientific (K1422) and used according to manufacturer's instructions.

2.5 Yeast techniques

2.5.1 Genetic crosses

Yeast strains were grown overnight on YPD agar media. A thin layer of each strain were incubated together on YPD to mate the exponentially growing cells. After 24-28 hours,

the diploid yeast cells were selected for by mechanical selection using the singer tetrad dissector (Singer MSM400) or by using auxotrophic and antibiotic plates. Single colonies of diploid cells were then inoculated onto RSM (rich sporulation media) plates and left at 25°C for 3-5 days to sporulate. Once sporulated, the tetrads were dissected and then genotyped using selection plates to identify the desired genetic markers. PCR genotyping was also used where necessary.

2.5.2 Tetrad dissection

A tip-full of sporulated cells were transferred to a clean tube containing 250 µl of water and then incubated with 1 µl of Zymolyase® (120493-1 AMS Biotech) for 5-10 minutes. Cells were then spun down (3000 rpm, for 2 min) and the pellet re-suspended in 250 µl water. 10 µl of the cell suspension was then pipetted onto a YPD plate in the top right corner with the plate at an incline to encourage vertical spreading of the cells. The plate was then left to dry before the tetrads were dissected using the singer tetrad dissector (Singer MSM400).

2.5.3 *S. cerevisiae* transformation

S. cerevisiae cells were grown in 50 ml YP to a concentration of 1 x 10^7 cells/ml. Cells were then pelleted (3500rpm, 5 mins) and washed in 10 ml water before being pelleted again and transferred to micro centrifuge tubes in 1 ml water. Cells were then spun at 8,000 rpm for 1 minute and washed in 1 ml LiAc-TE (0.1 M LiAc, 0.01M Tris-HCl, 0.0001M EDTA pH 7.5) then pelleted and re-suspended in 250 µl LiAc-TE. For each transformation, 5 µl of single stranded sperm DNA (15632-011 UltraPure™ Salmon Sperm DNA Solution, Life technologies) and 1-5 µl of insert DNA (up to 10 µg) and 300 µl 40 % PEG-LiAc-TE (w/v Polyethylene glycol) was added to 50 µl of cell solution and mixed thoroughly. Cells were agitated at room temperature for 45 minutes and then 40 µl DMSO (Dimethyl sulfoxide, D/4121/PB08 Fisher scientific) was added before cells were heat shocked for 15 minutes at 42°C and put on ice for 2 minutes. Cells were then vortexed and spun at 3000 rpm for 2 minutes and left in YPD for 1 hour (or 4

hours to overnight in the case of the integration of antibiotic resistance markers) before being plated onto selective media.

2.5.4 Phenol-Free genomic DNA extraction

10 ml of exponentially growing *S. cerevisiae* cells were pelleted and re-suspended in 500 µl DNA extraction buffer (1 % SDS, 100 mM NaCl, 50 mM Tris_HCl pH 8.0, 10 mM EDTA). 1 µl of lyticase (L2524, Sigma Aldrich) and 4 µl 2-Mercaptoethanol (63689, Sigma Aldrich) was added to the cells before being incubated at 37°C for 5 minutes. 150 µl of 5 M KAC was then added to the cells, which were then incubated for 10 minutes on ice. The samples were spun at 13,000 rpm for 10 minutes and the supernatant transferred to a clean tube. 1 vol of isopropanol was added before being spun again at 4500 rpm for 15 minutes at 4°C. The supernatant was aspirated and the pellet washed with 500 µl of 70 % ethanol, before a final 5-minute spin after which the supernatant was aspirated and the pellet re-suspended in 100 µl TE (10 mM Tris-HCl, 1mM EDTA pH 7.5) and 2 µl RNase (Fisher Bio reagents, DNase-Free).

2.5.5 Colony PCR

A loop of fresh yeast cells was re-suspended in 50 µl of water and heated at 95°C for 10 minutes. Cells were then spun at 13,000 rpm for 20-30 seconds and 5 µl was taken from the top of the supernatant and pipetted into a clean PCR tube containing 20 µl of Taq polymerase reaction mix (1x Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM of each nucleotide, 0.2 µM of each primer and 1.25 units Taq polymerase (Thermo fisher scientific AB- 0192/B)).

2.5.6 Spot tests

Yeast strains were incubated overnight at 25°C in 5 ml YP solution and either 2% raffinose or 2% glucose. Cells were counted using the haemocytometer to confirm exponential growth. Cells were concentrated to 10^8 cells/ml in YP liquid media (with 2% carbon source and 0.1% ampicillin). 10-fold or 5-fold serial dilutions from a starting

concentration of 10^8 cells/ml (unless otherwise specified) were pipetted onto appropriate media plates and incubated for 48 hours.

2.5.7 Cell cycle synchronisation and Protein-DNA crosslinking in preparation for ChIP analysis

2.5.7.1 Alpha-factor block and release into Nocodazole

Cell cultures were grown to log phase at 25°C in YPR/ADE, then arrested in G1 by the addition of alpha factor peptide (GenScript custom synthesis) 10 µg/ml. Once 90% of cells were in G1, galactose (gal) was added to the cell culture (2%) followed by 50 µg/ml doxycycline (or 85 µg/ml for AID degron experiments) 15 minutes later. 30 mins after the addition of galactose, cell cultures were incubated at 37°C for 1 hour (or 2 hours for conditional depletion of Smc2), then released from G1 into depletion media (YPRG/ADE + 50 µg/ml Dox). Time 0 was taken at the re-suspension of cells in the first wash (of three). Nocodazole was added (10 µg/ml) at 40 min and once (80-90 %) cells were arrested in G2/M; then fixed in ~1.5 % formaldehyde for 15 minutes. To quench the crosslinking reaction, 140 µl/ml of 1M glycine was added for 5 minutes. Cell cultures were then washed once in PBS before the pellet was frozen in liquid nitrogen or dry ice and stored at -80°C. (Doxycycline only required in experiments in which conditional protein repression of Smc2 or Smc4 is undertaken, using the tetracycline degradation system). For conditional protein depletion of Ycg1 using the auxin-dependent protein degradation system, 0.5mM NAA (1-naphthaleneacetic acid) was added 15 minutes after the first wash from alpha factor.

2.5.7.2 Exponential growth to Nocodazole block

Alternatively, Nocodazole (10 µg/ml) was added to cells in log phase, 120 minutes later, gal (2%)and dox (50 µg/ml) and Nocodazole (5 µg/ml) was added. At 120 minutes, after the initial Nocodazole dose, the temperature was shifted to 37°C for 1

hour (or 2 hours for conditional depletion of Smc2) before cells were fixed and stored as above.

2.5.8 FACs sample preparation and analysis

0.5 ml of cell culture (approximately $1x10^7$/ml) was washed in PBS then fixed in 0.5 ml 70% EtOH. Cells were stored at 4°C. Pellets were then incubated at 37°C overnight in 1ml 50mM Tris pH 8 with 5µl RNaseA (Fisher Bio reagents, DNase-Free). Cells were again precipitated and then re-suspended in 0.5 ml 5 mg/ml pepsin in 55mM HCL to digest for 30 minutes at 37°C. Cells were then washed once with 1 ml 50mM Tris pH8, then precipitated and finally re-suspended in 0.5 ml 50mM Tris PH 8.0 containing 0.5 mg/ml PI (prodidium iodide). Cells were then sonicated (using the micro-tip ultrasonic processor) at 20% amplitude for 10 seconds before being processed using a BD Accuri C6 sampler and analysed using FCS express 4 flow software (unless otherwise stated).

2.6 Protein Analysis

2.6.1 Whole cell protein extraction for western blot analysis – TCA extraction

10 ml of (approximately) $1x10^8$ cells/ml were pelleted and re-suspended in 200 µl of 20% TCA (w/v Trichloroacetic acid). Approximately 100 µl of 0.5 mm silica beads (thistle scientific) were added and the cells were lysed using a FASTPREP (FastPrep24, MP) machine using 6 rounds of 30 seconds at 6.5 power (with 30 second pauses in-between for cooling). The bottom of the tubes were pierced with a hot needle and spun at 4000 rpm for 2 minute to extricate the lysed cell suspensions from the silica beads, into a clean tube beneath. To ensure the collection of the whole cell lysate, 600 µl of 5% TCA was added to the beads and spun again (4000 rpm 5 mins) into the same collection tube. The samples are pelleted and the TCA removed before being re-suspended in 200 µl 1x sample buffer. 4x sample buffer (250 mM Tris pH6.8, 8 % SDS, 20 % glycerol, 20 % b-ME, 0.4 % bromophenol blue) was adjusted to 1x by adding 2.4

vol water and 0.6 vol 1M pH 8.8 Tris to 1 vol 4x sample buffer. Samples were then boiled for 5 mins, spun down at 12000 rpm for 2 mins and the supernatant transferred to a fresh tube and stored at -20 °C for subsequent analysis by Western blot.

2.6.2 Protein analysis by immunostaining (Western blot)

Protein samples prepared by TCA extraction were separated by SDS-PAGE (Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electophoresis). Polyacrylamide gels for SDS-PAGE were prepared using Protogel (30 % w/v Acrylamide: 0.8 % Bis-Acrylamide Stock solution (37.5:1), ELR-210-010P), according to the solution mixtures shown in the tables below (adapted from (Sambrook *et al.* 1989). Protein samples were run in a polyacrylamide gel in 1x running buffer (0.025M Tris-base, 0.25 M glycine, 0.1 % SDS) using a BIORAD mini-PROTEAN TetraCell system. Protein samples were run through the stacking gel at 80 V and through the separating gel at 80-120 V, alongside PageRuler Plus Prestained Protein Ladder (26619 Thermo scientific).

	6% Gel (5 ml)	**8% (5ml)**
H_20 (ml)	2.7	2.3
30 % Acrylamide mix (ml)	1.0	1.3
1.5M Tris (pH 8.8) (ml)	1.3	1.3
10 % SDS (ml)	0.05	0.05
10 % APS (ml)	0.05	0.05
TEMED (ml)	0.004	0.003

Table 2.3 Western blot gel mix

TEMED: N, N, N’, N’-Tetramethylethylenediamine.

	Stacking Gel (2ml)
H_20 (ml)	1.4
30 % Acrylamide mix (ml)	0.33
1.5M Tris (pH 6.8) (ml)	0.25
10% SDS (ml)	0.02
10% APS (ml)	0.02
TEMED (ml)	0.002

Table 2.4 Western blot stacking gel mix

The 1-dimensionally separated proteins were then transferred onto a nitrocellulose membrane (Amersham™ Protran® Premium Western blotting membranes GE10600004) over 2 hours in 1x transfer buffer (20 mM Tris, 20 % Methanol, 750 mM Glycine) using the XCell SureLock™ Mini-Cell Electrophoresis System. The nitrocellulose membrane was initially assessed for protein content using Ponceau-S protein stain solution (0.2 % w/v Ponceau S, 3 % w/v Trichloro acetic acid) before being washed with PBS-T (Phosphate buffered saline containing 0.1 % Tween 20 Sigma P1379). The membrane was then blocked in 6 % milk/PBS-T (Marvel dried skimmed milk) for a minimum of 1 hour rotating at room temperature, before the primary antibody (see below) was added and left to incubate overnight in 1 % milk/PBS-T at 4°C rotating. Blots were washed at for at least 3 x 15 minutes in PBS-T before being incubated with the secondary antibody (see below) in 1 % milk/PBS-T for 1 hour rotating at room temperature. The membrane was then washed again for a minimum of 3 x 15 minutes in PBS-T and then exposed using Western Lightning® Plus-ECL (Perkin Elmer NEL104001EA). The reaction was detected using an Image Quant LAS 4000 luminescence image analyser, or alternatively by exposing the membrane to Amersham hyperfilm (28900837) and developing the film using a Protec photon imaging system.

2.6.2.1 Primary antibodies used in Western blots:

Anti-V5 antibody (mouse monoclonal MCA1360, abD Serotec®) used at 1:1000 concentration

Anti-HA antibody (12Ca5 mouse monoclonal $IgG_{2\beta K}$. Roche, Fisher scientific 10026563) used at 1:1000

Anti-phospho-serine 4 antibody (rabbit polyclonal, kindly provided by the Damien D'Amours lab) used at 1:2000

Anti-PGK1 antibody (Mouse monoclonal, IgG1 22C5D8, Invitrogen) used at 1: 50,000

2.6.2.2 Secondary antibodies used in Western blots:

Anti-Rabbit antibody (goat**,** P044801-2 Dako) used at 1:1000

Anti-Mouse Antibody (rabbit, P026002-2 **Dako) used at 1:1000**

2.6.3 Protein tagging for ChIP

To generate Brn1-3V5 and Ycg1-3V5 recombinant proteins, the 3V5 tag was PCR amplified from the pYMN21-3V5 plasmid (modified from pYMN21 (Janke *et al.* 2004) using primers M.Y-50, M.Y-51 and M.Y-42 M.Y-43 respectively (see primer list). Cells were transformed with the amplicon (as above) and integration was confirmed using primers M.Y-33 and S.S-270 for Brn1-3V5 and M.Y-33 and 1 for Ycg1-3V5. Tags were further confirmed by doing a Western Blot using V5 antibody (see 2.1.2).

2.6.4 Chromatin Immuno-Precipitation (ChIP)

Cells that have been fixed in 1.5% formaldehyde were defrosted on ice and re-suspended in 100 µl ChIP buffer (see below). Cells were lysed in a FASTPREP machine using 6 rounds of 30 seconds at 6.5 power, (with 30 second pauses in-between for cooling) using approximately 1 ml of 0.5 mm silica beads (thistle scientific). The bottom of the tubes were pierced with a hot needle and spun at 2000 rpm for 1 minute to extricate the lysed cell suspensions from the silica beads into a clean tube beneath. Samples were then spun down (13000 rpm for 10 min at 4°C) and re-suspended in 1 ml

ChIP buffer and then sonicated for 15 minutes (30 seconds on, 30 seconds off) in the Biorupter Pico (diagenode) water bath sonicater. This procedure produces chromosomal DNA fragments of optimum length that range between 100-600 base pairs. From each cell chromatin sample, 100 µl was processed as an input control (see below), 200 µl was incubated with 12.5µg/ml anti-V5 antibody (mouse monoclonal MCA1360, abD Serotec®), and 200 µl was used as a no-antibody control; the latter two were agitated for an hour and a half at 4°C. Following this incubation, the antibody and no-antibody chromatin samples were each then incubated at 4°C for 2 hours, with 45 µl magnetic beads, (Dynabeads protein G – life technologies) that have been washed 3 times in 1ml ChIP buffer. To remove unbound sample, the magnetic beads were then washed four times in ChIP buffer, and a fifth time in ChIP buffer minus the PMSF and protease-inhibiting supplement. To reverse cross-linking, magnetic beads were incubated with 10% Chelex® 100 resin beads (BioRad 142-1253), in purified water at 95°C for 30 min. Samples were spun down and the supernatant kept at -20°C prior to analysis by qPCR.

The input controls were precipitated using 0.1 x vol 3M NaAC pH5.2 and 2.5 x vol 100 % EtOH and then treated with the other samples to reverse cross-linking, before being PCR-purified using a Nucleospin PCR clean up kit and eluted in nuclease-free purified water.

ChIP Buffer

150 mM NaCl

50 mM Tris HCL

5mM EDTA

0.5 % np-40 (IGEPAL)

7 % Triton X-100

Supplemented with PMSF to 1mM and EDTA free protease inhibitor (cOmplete Tablets, Mini EDTA-free EASYpack, Roche) just prior to use.

2.6.5 Quantitative PCR analysis of ChIP

qPCR reaction mix

component	Volume (total 20 µl)	concentration
2X AB-1323/B ABsolute™ QPCR SYBR®Green Low ROX Mix	10µl	1 x
Primers (listed below) - 0.85µm each primer pair stock	7 µl	0.3 µm
DNA	3 µl	n/a

Table 2.5 qPCR reaction mix

The Immunoprecipitated DNA was analysed using the PCR reaction mix listed above and processed in an MX3005p qPCR machine. Data was analysed using the 'Percentage Input Method' where the CT values obtained from the ChIP are divided by the CT values obtained from the Input control samples. Since 1% of starting chromatin was used for each input sample, in order to adjust the CT value of input samples to 100% 6.644 (log2 of 100) was subtracted from it. Then the following formula was used to calculate the percentage input for each IP sample:

2^(adjusted ChIP input CT value – IP CT value) x 100

The percentage of input values obtained from the qPCR analysis of a minimum of three separate ChIP experiments were then averaged. Error bars used for ChIP qPCR histograms represent the standard error of the mean. A two-tailed homoscedastic T test was performed to assess statistical difference between datasets.

Thermal profile

Temperature	Time	Number of cycles
95°C	15:00 min	1 cycle
95°C	00:30 sec	40 Cycles
55°C	01:00 min	
72°C	01:00 min	
95°C	01:00 min	1 cycle
55°C	00:30 sec	
95°C	00:30 sec	

Table 2.6 qPCR thermal profile

3 Setting up an efficient and sensitive chromatin immunoprecipitation assay

3.1 Results

3.1.1 Introduction

Chromatin immunoprecipitation (ChIP) is a commonly used technique for studying protein-chromatin interactions. ChIP has been used multiple times to study the chromatin association of condensin, in *S. pombe* (Nakazawa *et al.* 2008, Tada *et al.* 2011, Sutani *et al.* 2015) *S. cerevisiae* (Freeman *et al.* 2000, Wang *et al.* 2005, Clemente-Blanco *et al.* 2009, Clemente-Blanco *et al.* 2011, Verzijlbergen *et al.* 2014, Leonard *et al.* 2015), and *C. elegans* (Kranz *et al.* 2013). ChIP is the main technique used throughout my investigation and therefore setting up a robust and functional assay was critical.

Condensin has been shown to have mitosis-specific chromatin enrichment at the centromere in yeast (Wang *et al.* 2005, D'Ambrosio *et al.* 2008, Nakazawa *et al.* 2008) and in human cells (Ono *et al.* 2004). Due to its relative simplicity amongst its eukaryotic peers, the budding yeast centromere has been well studied and was the first to be isolated (Clarke and Carbon 1980). The *S. cerevisiae* point centromeres are between 116-120 bp in length and are comprised of three distinct DNA elements found on all 16 chromosomes (Clarke and Carbon 1983, Fleig *et al.* 1995). These DNA subdivisions are known as centromere DNA element I (CDEI), CDEII and CDEIII and have been shown to be sufficient to facilitate chromosome segregation in mitosis, when inserted ectopically into plasmid DNA (Clarke and Carbon 1980, Fitzgerald-Hayes *et al.* 1982). CDEI and CDEIII are short, (8 bp and 26 bp respectively (Verdaasdonk and Bloom 2011)) highly conserved palindromic DNA sequences that sit either side of CDEII; an AT-rich non-conserved region of about 78-86 base pairs (Carbon 1984). CDEII and CDEIII are essential for meiotic and mitotic centromere function (Hegemann and Fleig 1993). CDEI is important for highly accurate chromosome segregation, but is not

essential for centromere function (Mellor *et al.* 1990) (Wilmen *et al.* 1994) (McGrew *et al.* 1986). Condensin is also known to be enriched at the rDNA on chromosome XII (Freeman *et al.* 2000, Wang *et al.* 2005, Nakazawa *et al.* 2008), and so throughout my investigations I also probed a region of the rDNA, and the actin (*ACT1*) gene locus where condensin is not thought to be enriched, for use as points of reference.

3.1.2 ChIP experimental design

As depicted in figure 3.1A, ChIP is based on *in vivo* crosslinking of chromosome-binding proteins, followed by cell lysis, DNA shearing and immunoprecipitation of the protein-DNA complex. The cross-linking reaction fixes the cellular matter at a particular time-point, meaning if the protein of interest is in contact with chromatin, it should remain so throughout the rest of the process up until analysis. After the cells are fixed they are then lysed and the DNA is sheared to an optimal size. Once the DNA is sheared, the protein of interest can be immuno-precipitated using protein-specific antibodies or the protein of interest can be tagged with a moiety for which there are commonly produced and previously tested antibodies. Since there are no commercially available condensin-specific antibodies, for the purposes of our experiments, condensin subunit Brn1 was tagged with a 3V5 moiety to allow for efficient pull-down with an anti-V5 antibody. The antibody-epitope conjugates are then isolated using a secondary antibody attached to magnetic beads. The cross-linking is then reversed to separate the DNA allowing for analysis by qPCR (technical details can be found in 2.6.4 and 2.6.5).

To investigate the enrichment of condensin at mitotic centromeric regions, prior to the crosslinking stage the cell cultures were directed through a defined preparation procedure detailed in 2.5.7 (figure 3.1B). Exponentially growing cells were arrested in

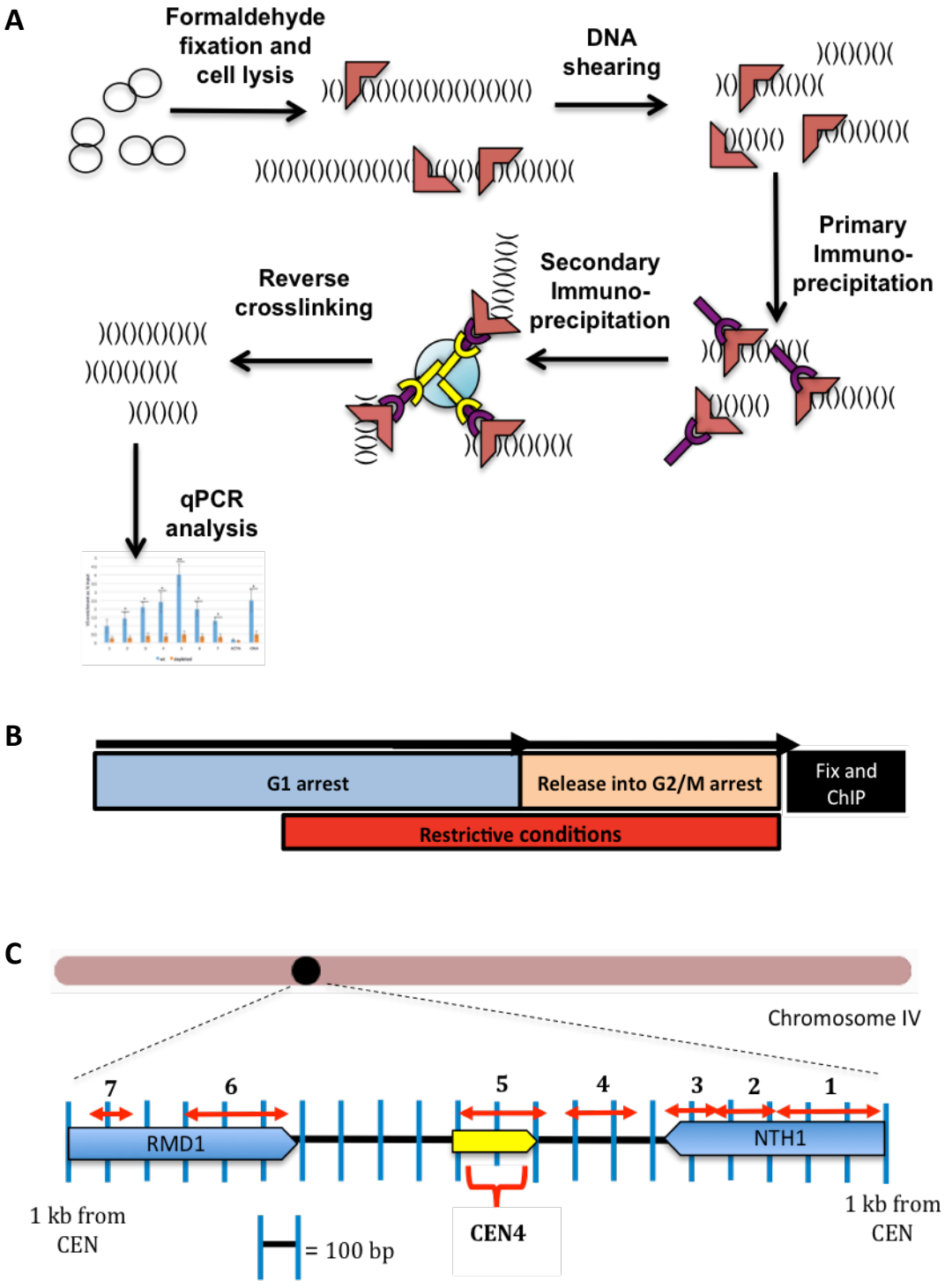


Figure 3.1 ChIP experimental design

A schematic representation of the chromatin immuno-precipitation experimental design. (previous page) **A)** A cartoon depicting the different stages of the ChIP process. Budding yeast arrested in G2/M (double budded), are fixed with formaldehyde, lysed and sonicated to shear the DNA. The protein of interested is immunoprecipitated with primary antibodies and then with secondary antibodies attached to magnetic beads. Cross-linking is reversed and DNA quantities are analysed using quantitative real-time QPCR. **B)** A diagram representing the cell culture preparation procedure designed to synchronise cells prior to cross-linking in mitosis. Cells are first arrested in G1 using alpha factor, restrictive conditions (specific to the experiment) are implemented prior to cells being released from G1 into a G2M arrest using Nocodazole. **C)** Centromeric regions to be investigated using locus specific primers (detailed in 2.2). The primers are designed to probe distal pericentromeric regions (1-3 and 6,7) proximal to the centromere (4) and CEN4 itself (5).

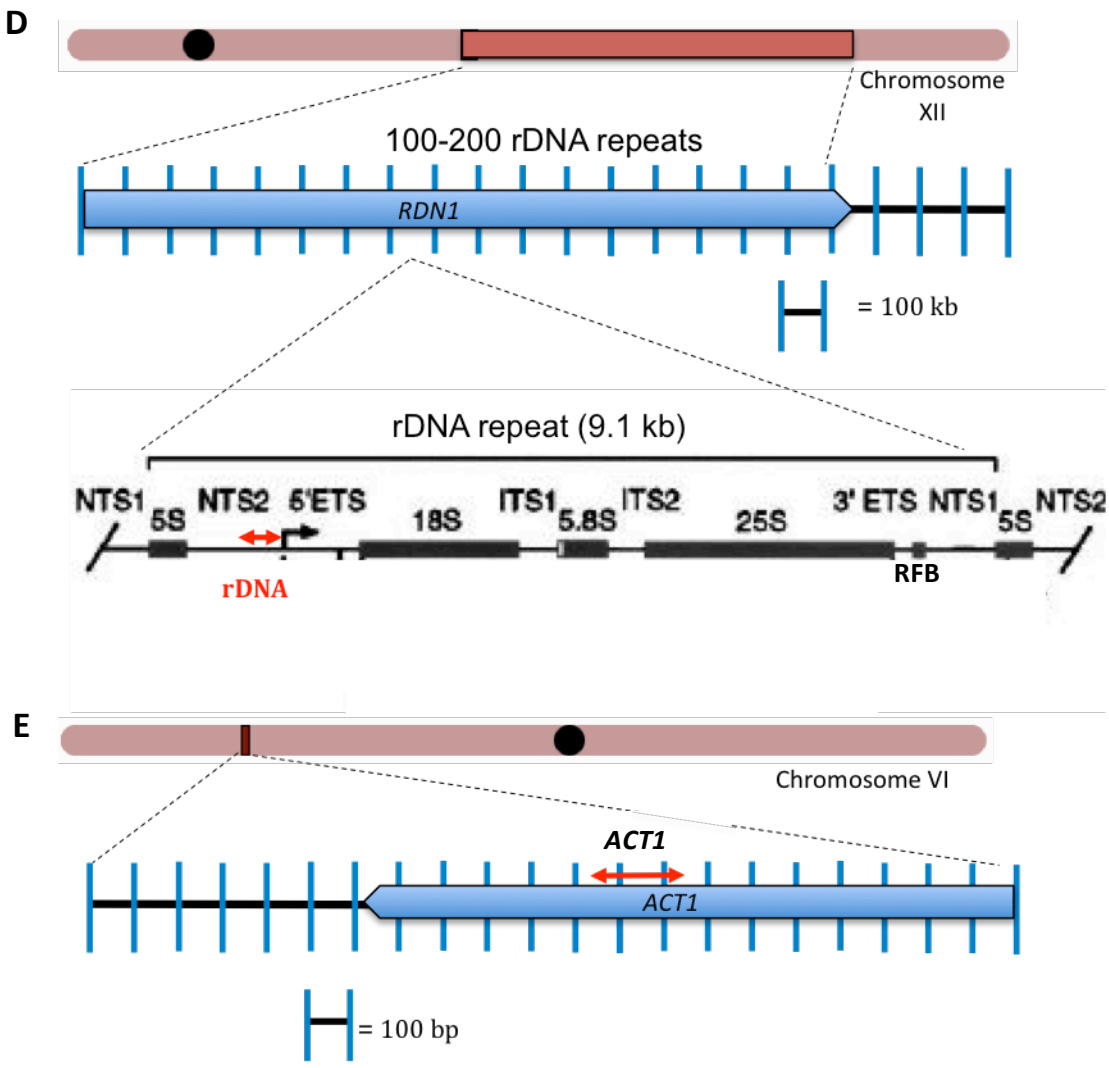


Figure 3.1 continued.

D) A cartoon depicting the approximate location of the rDNA locus probed using primers detailed in 2.2. The bottom section of the figure is an image taken from (Venema and Tollervey 1999). One rDNA repeat contains the genes for 5S, 5.8S, 25S and 18S rRNAs, Internal spacer regions (ITS1 and ITS2), external spacer regions (5' ETS and 3' ETS), and non-transcribing spacer regions (NTS1 and NTS2). **E)** A cartoon depicting the approximate location of the ACT1 locus probed by primers detailed in 2.2.

G1 and switched to restrictive conditions, before being released as a synchronised cell population into a G2/M arrest. This defined preparation protocol ensures as much consistency as possible between repeat experiments and also between comparison yeast strains, as cells are at the same stage of the cell cycle and under the same external conditions when condensin enrichment is assayed. Figure 3.1C shows the centromeric areas probed during the course of my experiments, the primer pairs from 1-7 span an area of approximately 1 kb either side of *CEN4*. Primer pairs 1-3, 6 and 7

span the distal pericentromere and lay in open reading frame areas covering *RMD1* and *NTH1*. Primer pair 4 probes an area of proximal pericentromere, whilst primer pair 5 spans the three CDEs of the centromere. Further to these centromeric regions of interest, we also probed the NTS2 (non-transcribed spacer) region of the rDNA, using primers previously utilised by the Haering lab (Cuylen *et al.* 2011) (figure 3.1D) and the actin gene locus, *ACT1* with primers previously utilised by the Aragón lab (McAleenan *et al.* 2012) (figure 3.1E).

3.1.3 Experiment optimisation

For a successful ChIP experiment, a population of fixed cells must be thoroughly lysed to ensure access to the chromatin within. Cell lysis is achieved using a Fast-prep machine (see 2.6.4), in which tubes containing a concentrated cell suspension mixed with silicon beads, are vigorously shaken to rupture the cells. In order to ensure thorough cell lysis, I conducted a bead titration assay. When visualised using light microscopy, *S. cerevisiae* cells appear much darker upon lysis, in comparison to a light glow emitted from non-ruptured cells, as can be seen in figure 3.2A (left panel). Figure 3.2A shows that with approximately 500 µl of beads, the population of cells are only partially lysed, when approximately 1 ml of beads are used, the population of cells is completely lysed. Therefore, approximately 1 ml of beads was used in the cell lysis process in future experiments.

For accurate qPCR readouts from ChIP, the ideal DNA strand length is between 100-600 base pairs (bp) (as recommended in the Diagenode shearing optimisation users guide PR-Guide-CSh-V1-15_05_12). DNA shearing was carried out using a waterbath sonicator (see 2.6.4) that operates on one power setting. Therefore, in order to find the length of time required to achieve optimal DNA strand length, I ran cell-lysis solutions through 5, 15 and 30 minutes' sonication. To treat as similarly as possible, all three samples were placed in the water bath to begin with, and when removed at their corresponding time point, they were replaced with tubes containing the same volume of water so as not to alter the sonication process for remaining samples. Figure 3.2B

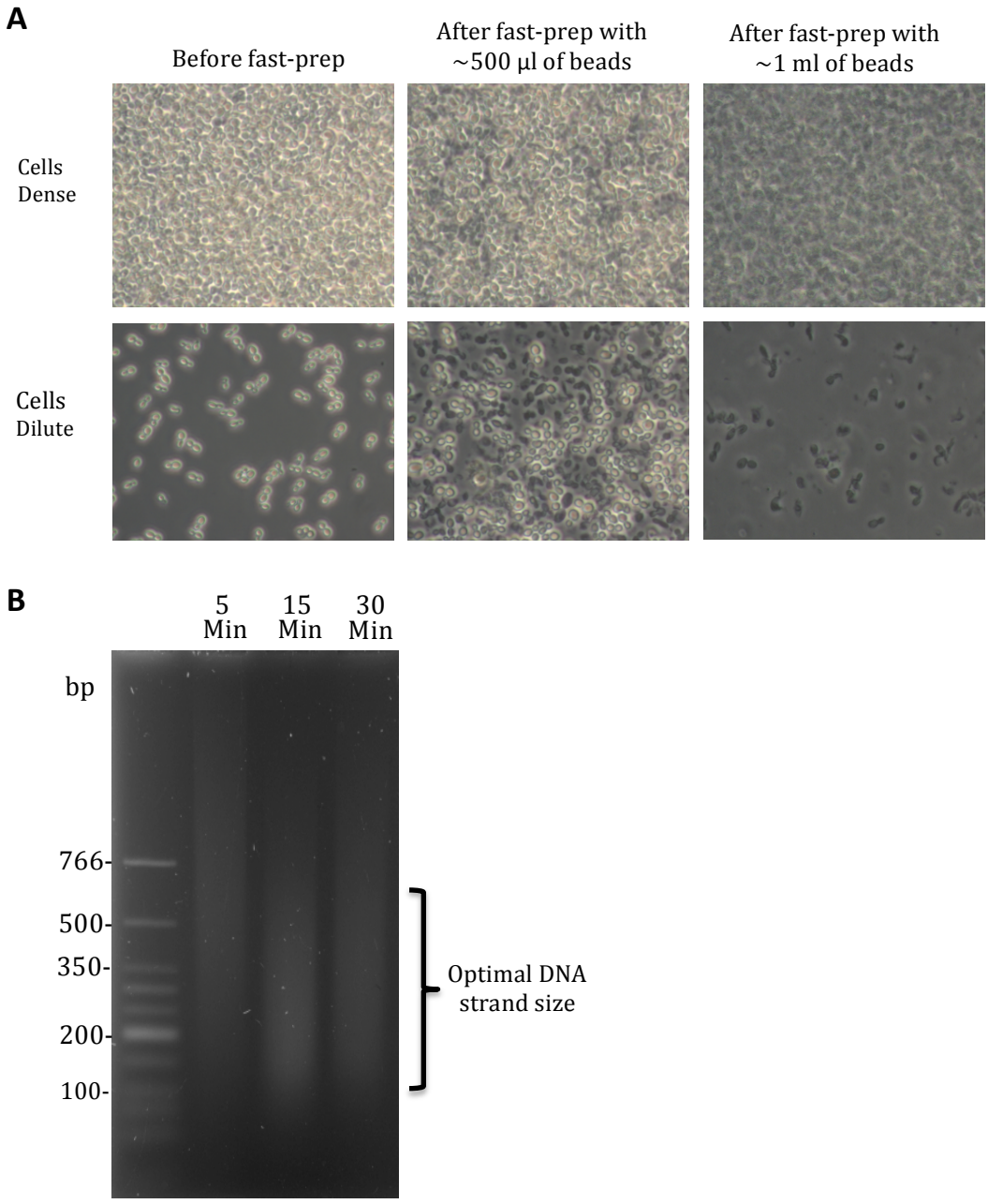


Figure 3.2 Optimisation of ChIP assay.

A) Optimisation of volume of silicon beads used in the Fast-prep cell lysis. Exponentially growing cells were fixed in 1.5% formaldehyde and kept at – 80 °C overnight. Cells were thawed on ice and re-suspended in 100 µl of ChIP buffer. Either~ 500 µl or ~ 1 ml of silicon beads were added to the cell suspensions and they were placed in the Fast-prep for 6 x 30 seconds at full power. For the control panel (left) no beads were added and the sample was not subjected to shaking by the Fast-prep. Cells were examined under the microscope both as concentrated samples, and diluted with ChIP buffer and images obtained using AxioCam ERc 5s. Lysed cells appear as a dark grey colour. **B)** Test to find the sonication time required to obtain optimal the DNA fragment length of 100-600 bps. One 150 ml culture of cells was equally split into 3 separate cultures and processed through fixation and cell lysis as described above (using the optimised ~1 ml of silicon beads). Lysed cells were placed in the diagenode pico biorupter (see 2.6.4) At the appropriate time intervals (5 minutes, 15 minutes and 30 minutes) the corresponding tube was removed and replaced with a tube of the same volume of water. DNA was purified according to the ChIP protocol (2.6.4) and run on a 2% agarose gel alongside a low molecular weight DNA ladder (biolabs N32335). (Figure continues on following page)

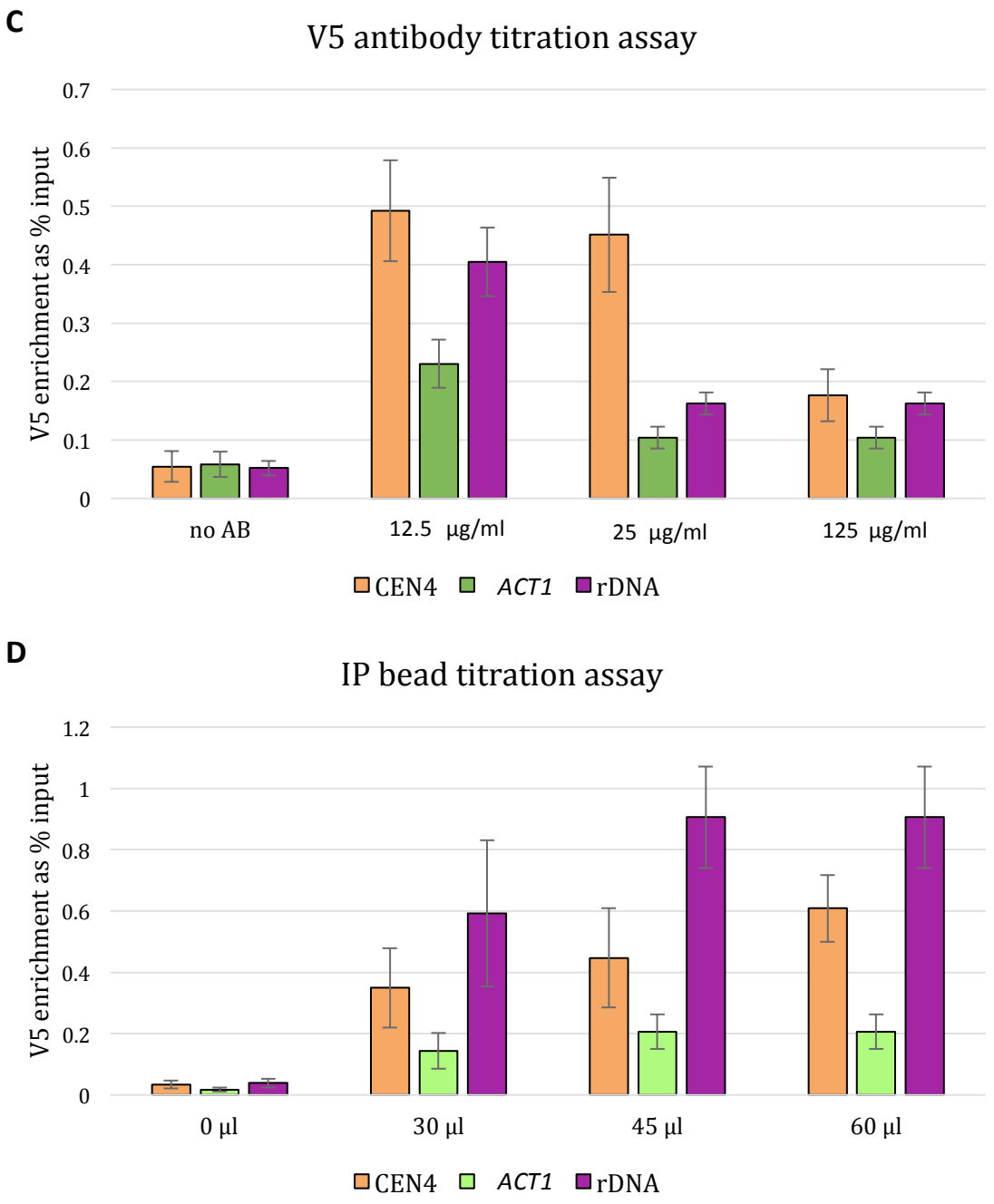


Figure 3.2 continued - C) Histogram showing the levels of Brn1-3V5 enrichment obtained from the V5 antibody titration assay. Chromatin was incubated with 12.5 µg/ml, 25 µg/ml or 125 µg/ml anti-V5 antibody and processed for ChIP analysis. Data shown is the average enrichment values from 3 separate ChIP experiments. Error bars represent standard error of the mean (SEM). Each sample was normalised to its corresponding input signal. **D)** Histogram showing the levels of Brn1-3V5 enrichment obtained from the Protein G-coated magnetic bead titration assay. Chromatin already immunoprecipitated using the primary anti-V5 antibody was incubated with 30 µl, 45 µl or 60 µl of protein G-coated magnetic beads. Data shown is the average enrichment values from 3 separate ChIP experiments. Error bars represent standard error of the mean (SEM).

shows that after 5 minutes of sonication, the sizes of DNA bands ranges broadly from approximately 150 bp all the way up to very large bands near the top of the gel. After 15 minutes and 30 minutes of sonication the majority of the DNA smear falls within the region of optimal DNA strand size. Therefore, 15 minutes of sonication is sufficient for generating the ideal DNA strand length.

Next, to optimise the immunoprecipitation stages of the protocol, I conducted titration assays of the primary antibody and the magnetic beads coated with the corresponding secondary anti-body (figure 3.2C and 3.2D). Ideally the concentration of antibody and the volume of beads used should be kept as low as possible to keep experimental costs to a minimum. The indicator of optimum antibody concentration is the level of condensin enrichment achieved. The highest levels of Brn1-V5 enrichment are preferred because an experimental system should be as sensitive as possible. We expected to see the highest Brn1-3V5 enrichment levels at the *CEN4* and rDNA loci with lower levels of enrichment at *ACT1* where condensin is not thought to bind.

For consistency, cells were subjected to a block and release protocol under restrictive conditions (detailed in 2.5.7.1 and explained in more detail below). Cells were lysed and DNA was sheared according to optimised conditions. Sheared chromatin was incubated for 1.5 hours with no antibody, 12.5 µg/ml, 25 µg/ml or 125 µg/ml of the primary anti-V5 antibody, before being processed through the rest of the ChIP protocol (figure 3.1A). For the purposes of antibody optimisation, only three genomic sites were probed: primer region 4 proximal to *CEN4, ACT1* and rDNA. Figure 3.2C shows that the highest level of Brn1-3V5 enrichment is obtained at each of the three loci (*CEN4*, ACTIN and rDNA), when using 12.5 µg/ml of anti-V5 antibody, compared to the enrichment levels obtained when using either 25 µg/ml or 125 µg/ml of antibody. The level of enrichment of Brn1-3V5 at *ACT1* is also notably lower than at *CEN4* or the rDNA when using 12.5 µg/ml of antibody, as is to be expected from what is known about condensin binding patterns. The level of Brn1-V5 enrichment obtained at *CEN4* is almost equal when the lowest two concentrations of antibody are used. However, the level of enrichment seen at the rDNA is much reduced when 25 µg/ml of antibody was used compared to when 12.5 µg/ml was used. When using 125 µg/ml of anti-V5

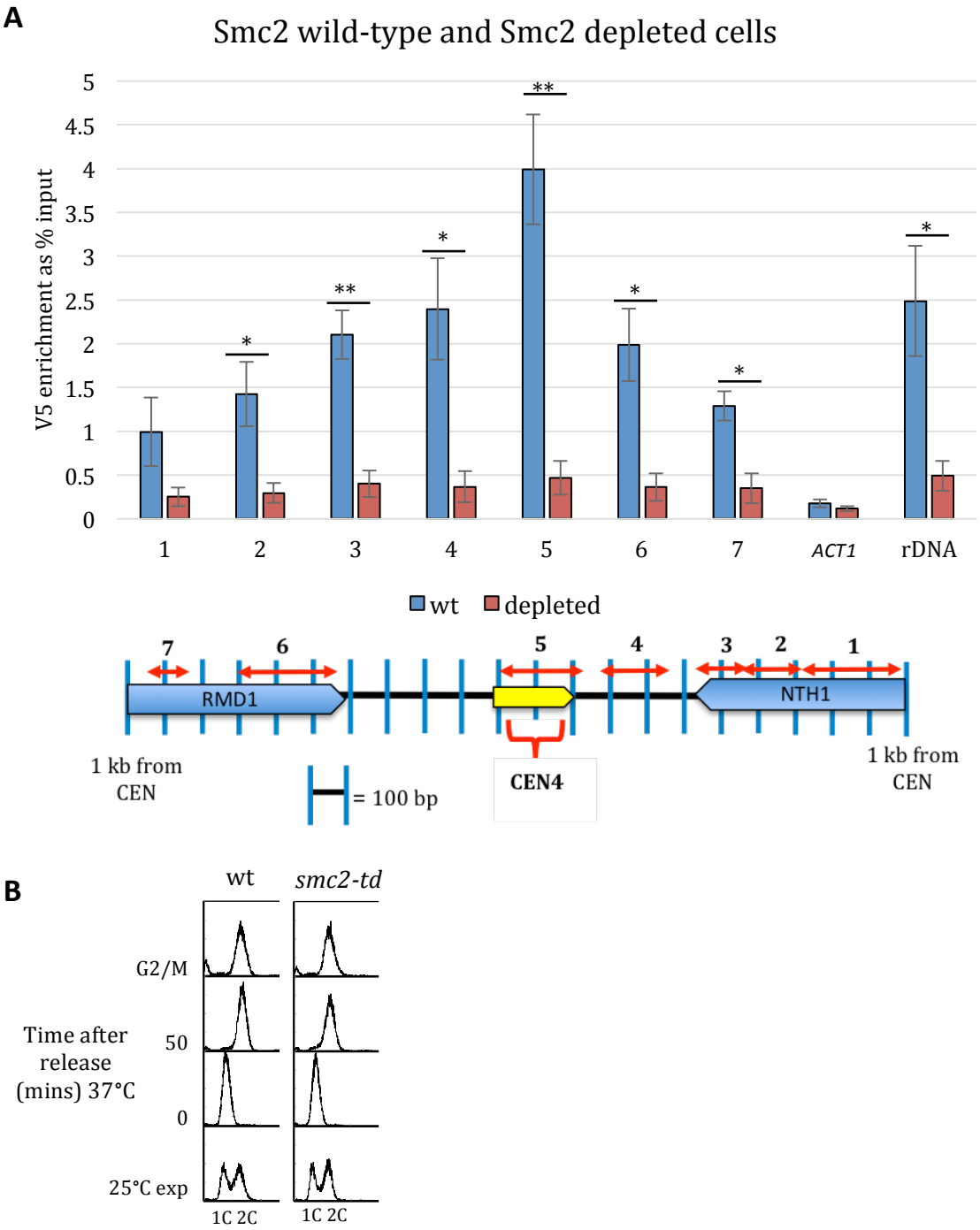


Figure 3.3. Condensin enrichment is significantly reduced in Smc2 depleted cells.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of wild-type (tet-degron wt 1291) and *smc2-td* (818) through the block and release protocol. 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

antibody, enrichment at all three loci is reduced compared to levels obtained using a tenth of the concentration. These data signify that out of the three concentrations of antibody tested, the lowest concentration 12.5 µg/ml is optimum. To find the ideal volume of secondary antibody-coated magnetic beads to use in each ChIP experiment, I conducted a magnetic bead titration assay using different volumes of protein G-coated Dynabeads. Samples that had been immunoprecipitated with the optimised concentration of primary antibody, were incubated for 2 hours with no beads, 30 µl, 45 µl or 60 µl of protein G Dynabeads, and then processed through the rest of the ChIP protocol. As can be seen in figure 3.2D the levels of Brn1-V5 enrichment increase with the volume of beads used.

While the highest level of enrichment is observed when 60 µl of protein G magnetic beads are used, the enrichment levels seen for 45 µl beads is almost identical at the rDNA locus and only marginally lower at the centromere. This slight difference in enrichment was weighed up against the expense incurred by using more beads, and it was decided that 45 µl was optimal to proceed. Importantly, in both figure 3.2C and 3.2D the no-antibody control samples ('no AB' in 3.2C and '0 µl' in 3.2D) showed only negligible levels of Brn1-3V5 enrichment at all three loci. Therefore, there does not appear to be significant background levels of enrichment produced by the ChIP protocol. Following ChIP optimisation, I conducted qPCR analysis using serial dilutions of input chromatin samples to confirm that the CT values used for qPCR analysis fell within the linear range (Data not shown).

3.1.4 ChIP assay is sensitive to depletion of condensin

With the protocol optimised, it was then important to ensure that the chromatin enrichment observed was actually dependent on condensin. To test this, we made use of the tetracycline/doxycycline-inducible degron (here in referred to as tet-degon). The tetracycline-inducible degron system allows three levels of manipulation of target gene expression. Firstly, the target gene (*SMC2*) is placed under the control of the TetR-repressible promoter, TetO. In the absence of doxycycline, TetO is able to

promote constitutive expression of *SMC2.* Upon doxycycline addition, activation of the TetR represses TetO, in turn repressing transcription of *SMC2*. Secondly, the tet-degron construct places *UBR1* (an ubiquitin E3 ligase) under the control of the *GAL1* promoter. Therefore, the presence of galactose causes the cellular concentration of Ubr1 to increase significantly. The target protein is tagged with a degron moiety (*smc2-td*). When the degron moiety is accessible, the surge in E3 ligase levels causes a concomitant increase in polyubiquitination of the degron moiety, and proteasome degradation of the target protein. Thirdly, the degron system is temperature-sensitive. Temperature increase causes unfolding of the degron moiety attached to the protein of interest, exposing internal lysine sites and facilitating its polyubiquitination and protein degradation via the proteasome.

Therefore, the addition of doxycycline, galactose, and an increase of temperature from 25°C to 37°C constitute the restrictive conditions in which the tet-degron system is used. For consistency, strains with a gene under the control of the tet-degron system are always compared against a strain containing both the *GAL:UBR1* and TetR constructs (see table 2.1) but no degron moiety or TetO promoter. This comparison strain is referred to as wild type (wt) or 'tet-degron wt' when referred to in comparison to another degron system.

To investigate whether that the enrichment signal obtained from our ChIP protocol is representing condensin enrichment, we used the tet-degron system to conditionally deplete the Smc2 subunit of condensin during S-phase and up to the G2/M block. In Figure 3.3A we can see that the ChIP results for the strain with wild-type levels of Smc2 (wt) shows a spread of chromatin enrichment across the pericentromere, peaking directly at *CEN4.* There is a similar level of enrichment at the rDNA, and negligible enrichment at the *ACT1*. There is however, a dramatic loss of Brn1-3V5 enrichment in cells depleted of Smc2, compared with wild-type cells at all loci probed (with the exception of the *ACT1*). This loss of ChIP signal in response to condensin depletion provides strong evidence that the signal of enrichment observed, is indeed representing condensin enrichment at these loci.

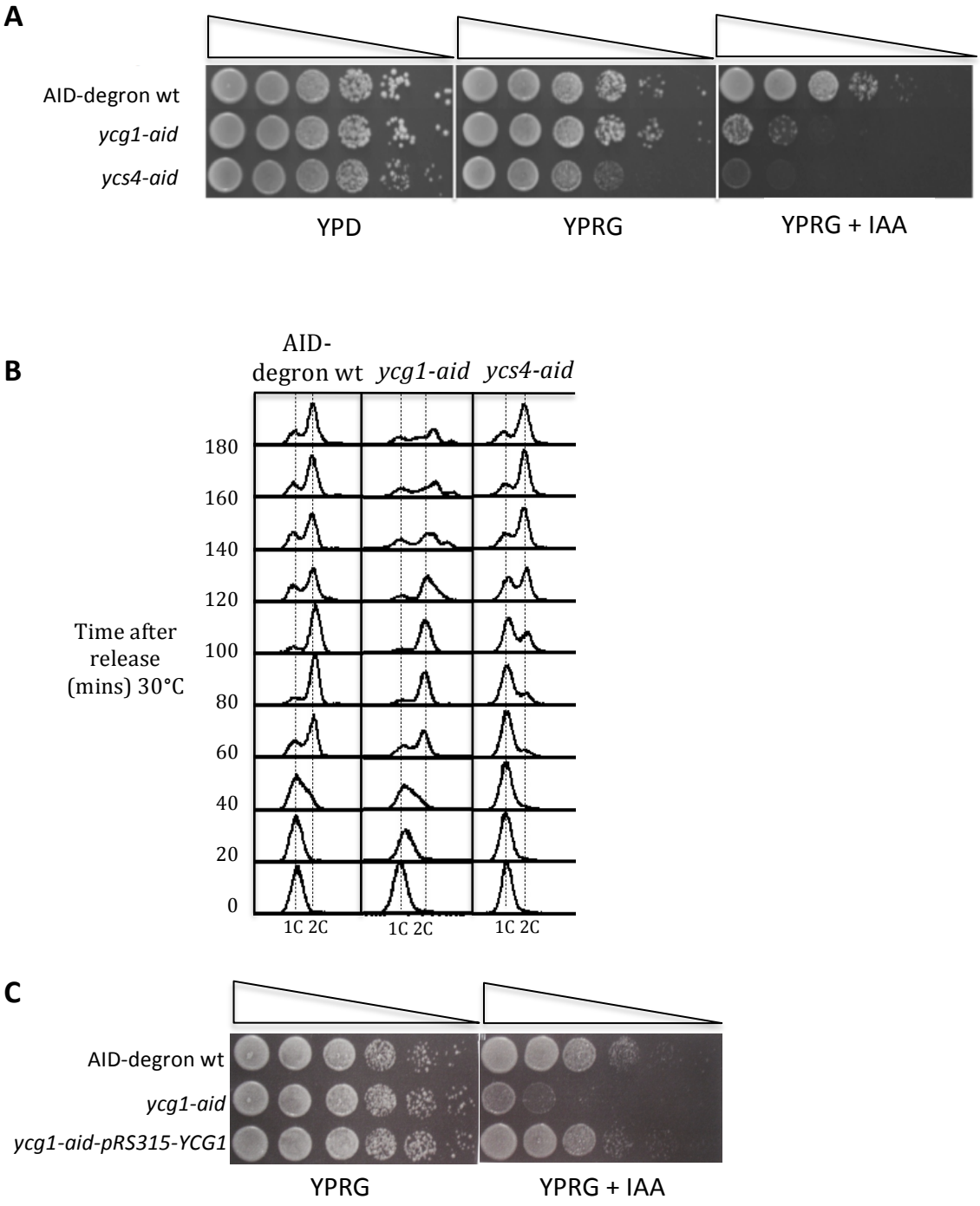
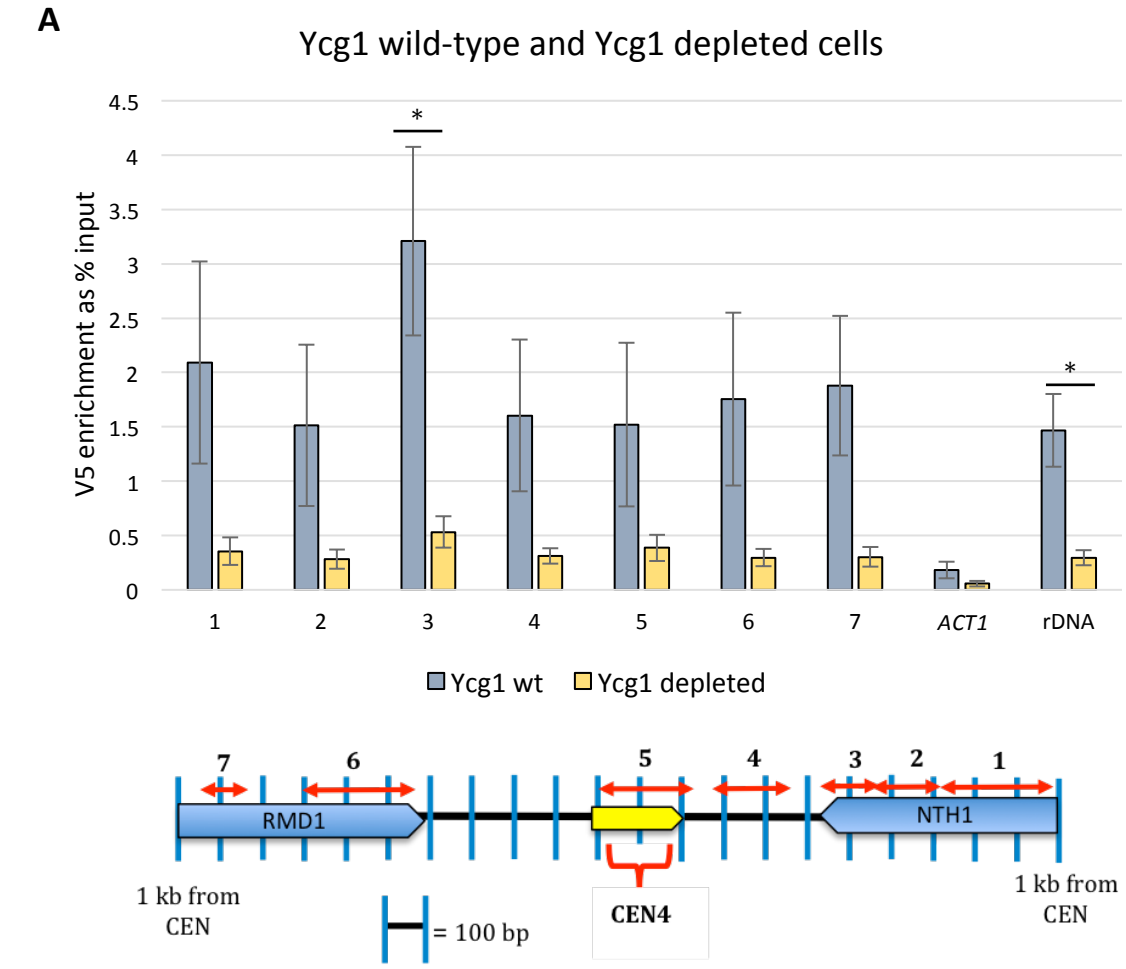


Figure 3.4 Characterisation of condensin AID-degrons.

A) Spot test showing the cell viability of *ycg1-aid* and *ycs4-aid* compared with AID-degron wt under on YPD, YPRG and YPRG + IAA. 10-fold serial dilutions of exponentially growing cells were plated and left to grow at 25°C for up to 48 hours. **B)** FACS data showing the progression of AID-degron wt (488), *ycg1-aid* (507) and *ycs4-aid (508)* through the cell cycle. Exponentially growing cells were arrested in G1 using alpha factor mating pheromone, placed under restrictive conditions (galactose and auxin at 30°C) before being realised to progress through the cell cycle. Cell samples for FACS analysis were taken just before release (0 min) and every 20 minutes after for 3 hours. **C)** Spot test showing the cell viability of AID-degron wt (488), *ycg1-aid* (507) and *ycg1-aid pRS315-YCG1* (507 + PRS315-*YCG1*). 10-fold serial dilutions of exponentially growing cells were plated onto YPRG and YPRG +IAA solid media and incubated at 25°C for up to 48 hours.

During the course of the project, I had another opportunity to test the validity of the ChIP protocol by testing the dependency of signal on the condensin subunit Ycg1.
I made use of the auxin-inducible degron system (AID) (Nishimura *et al.* 2009) to allow the conditional depletion of Ycg1 and Ycs4. The AID-degron makes use of the plant protein degradation pathway, in which the auxin hormone directly induces the degradation of AUX/IAA protein family by an SCF E3 ubiquitin ligase. Other eukaryotes, including *S. cerevisiae* share the SCF degradation pathway but not the auxin response. This allows us to transplant the AID system into *S. cerevisiae* and use the addition of auxin to conditionally deplete selected proteins (Ycg1 and Ycs4). An *Arabidopsis thaliana* protein (the degron) sensitive to the auxin-induced degradation is fused to the target proteins. The *A. thaliana* F-box transport inhibitor response 1 (TIR1) is placed under the control of the *GAL1* promoter and is expressed upon addition of galactose. Auxin hormone (such as indole-3-acetic acid, IAA) binds to the TIR1 protein and promotes the interaction of the E3 ubiquitin ligase SCF-TIR with the degron. SCF-TIR1 recruits an E2 ubiquitin enzyme that polyubiquitylates the degron resulting in rapid degradation by the proteasome. Therefore, in using the AID system, the addition of galactose and IAA should result in the depletion of Ycg1 and Ycs4. For consistency, when investigating these degrons they are compared to mutant strains containing the *GAL1:TIR1* construct but no auxin-inducible degron moiety. This comparison strain is referred to as 'AID-degron wt'.

I conducted a spot test to investigate the growth phenotypes of the AID strains. The degron strains I created, *ycg1-aid* and *ycs4-aid*, as well as the AID-degron wt were viable in non-restrictive conditions (figure 3.4A left panel). The presence of galactose slightly affected growth of all three strains, with *ycs4-aid* most affected (3.4A centre panel). This may be due to galactose being a less efficient carbohydrate source, or the galactose-induced expression of TIR1. The possibility of the latter highlights the importance of comparing degron strains to the AID-degron wt. The addition of auxin hormone (IAA) results in the loss of viability of *ycg1-aid* and complete cell death of *ycs4-aid* (3.4 right panel). We assume the cell death is a direct result of the auxin-induced depletion of these condensin subunits.



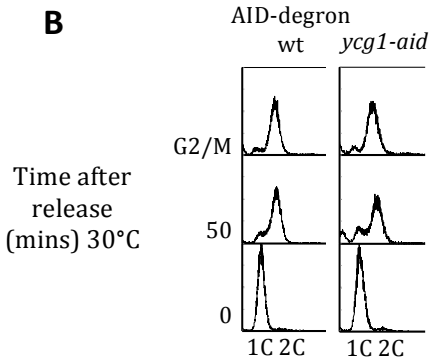


Figure 3.5 Condensin enrichment is significantly reduced in Ycg1 depleted cells.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions (in this case the addition of galactose and 85 µg/ml IAA at 30°C) and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05. B) Representative FACS data showing the progression of AID-degron wt (705) and *ycg1-aid* (707) through the block and release protocol. The '0 mins' time point taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M assessed by budding index.

Chromosome miss-segregation resulting in aneuploidy is a hallmark of many condensin mutants. Miss-segregation is an abnormal mitotic event in which chromosomes are separated into daughter cells erroneously, leading to more of less than the proper complement of chromosomes in each daughter. To investigate whether chromosome segregation defects were contributing to cell death in the *ycg1-aid* and *ycs4-aid*mutants I analysed them using fluorescence-activated cell sorting (FACS). Exponentially growing cells were blocked in G1 and exposed to galactose and IAA before being released synchronously into the cell cycle. Cell samples for FACS analysis were taken every 20 minutes for 3 hours. The first time I conducted the experiment I kept cells at 25°C and the FACs data showed no abnormalities in chromosome segregation during the 3-hour analysis (data not shown). Therefore, I conducted the experiment at 30°C to speed up progression through the cell cycle and thus put more pressure on cell division machinery. In Figure 3.4B the FACs data shows that the AID-degron wt cells began to exit G1 40 minutes after release from alpha-factor, almost all cells having reached G2/M by 80 minutes. By 120 minutes a large subset of cells had continued back through the cell cycle to G1. The Ycg1-depleted (*ycg1-aid)* cells began to exit G1 also by 40 minutes after release, and had also progressed to G2/M by 80 minutes. However, upon progression through mitosis at 120 minutes, *ycg1-aid* cells exhibit miss-segregation of chromosomes resulting in aneuploidy. Aneuploidy is identifiable by the appearance of peaks above the 2C and below the 1C (complements) of DNA. In contrast Ycs4-depleted (*ycs4-aid)* cells began to exit G1 at 60 minutes after release, 20 minutes later than the AID-degron wt and *ycg1-aid* cells. It was not until 120 minutes after release that the majority of cells have reached G2/M, a significant delay compared to the other two strains. Furthermore, whilst the AID depletion of Ycg1 caused miss-segregation there is no evidence of miss-segregation of chromosomes in the *ycs4-aid* mutant. A sub-population of *ycs4-aid* cells failed to exit from the G1 arrest (Figure 3.4B). The unusual G1 arrest of a sub-population of Ycs4-depleted created complications for analysis by ChIP due to the requirement of arresting cells in G2/M for analysis; for this reason, I did not carry out ChIP analysis using this mutant strain.

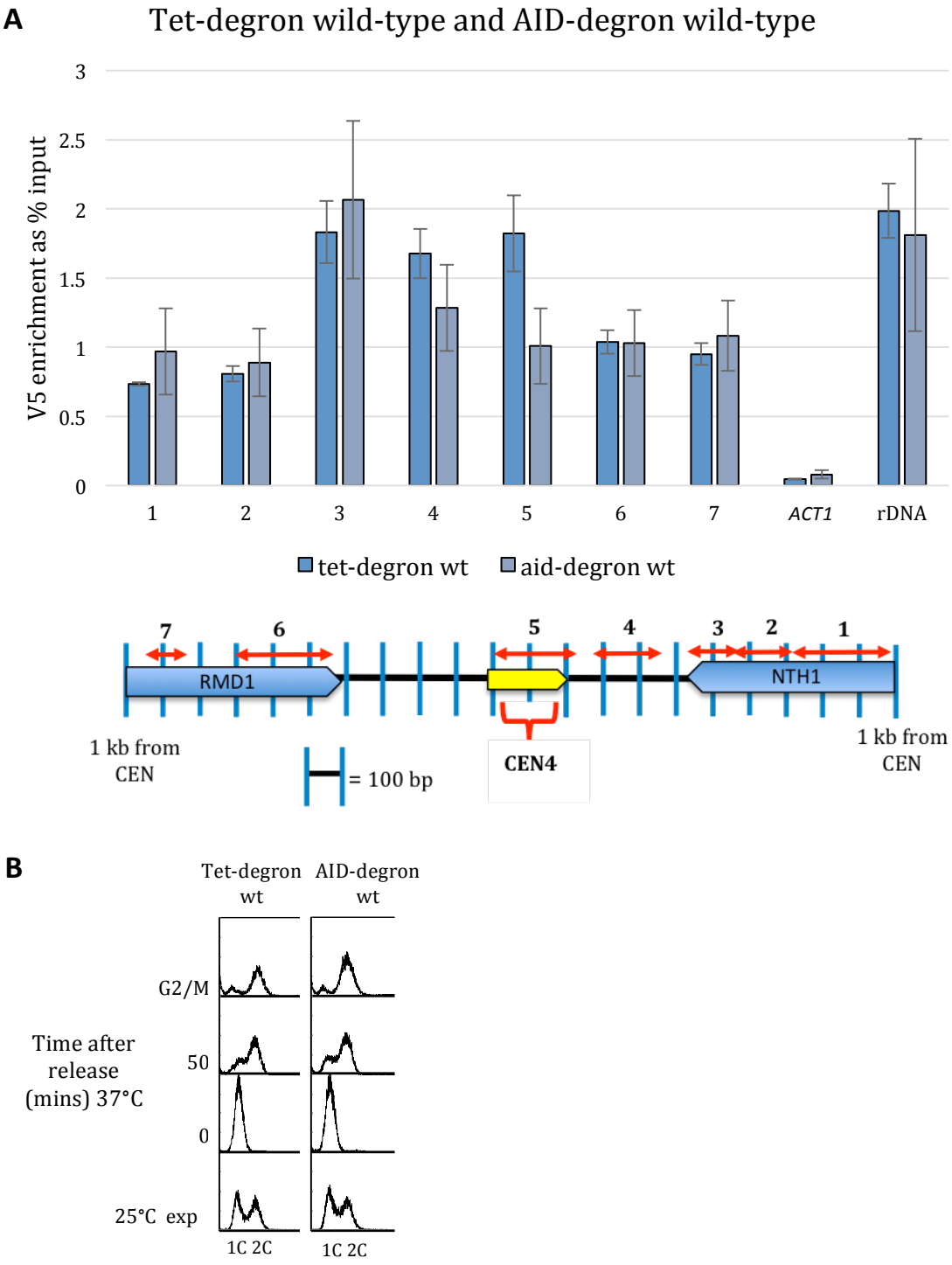


Figure 3.6 Condensin enrichment in the Tet-degron wt and AID-degron wt cells.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **B)** Representative FACS data showing the progression of tet-degron wt (708) and AID-degron wt (705) through the block and release protocol. 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M assessed by budding index.

Before analysing the condensin enrichment of *ycg1-aid* mutant strain by ChIP, I conducted a complementation assay to support the assumption that loss of viability of *ycg1-aid* is due to the depletion of Ycg1. I transfected the *ycg1-aid* strain with a plasmid expressing the *YCG1* wt gene. Using spot test analysis, I then compared the growth phenotype of *ycg1-aid* with and without the wild-type gene (figure 3.4C). In the left panel we can see that all three strains: AID-degron wt, *ycg1-aid* and *ycg1-aid-pRS315-YCG1* grow to a similar extend on the plate containing raffinose. As expected, upon addition of IAA to the media, the growth of *ycg1-aid* cells is significantly inhibited. The *ycg1-aid-pRS315-YCG1* strain however, grows almost as well as the AID-degron wt. Therefore, the expression of wt *YCG1* rescues the cell viability phenotype of *ycg1-aid* providing evidence that the phenotype is a direct effect of Ycg1-depletion. I then conducted a ChIP analysis comparing the AID-degron wt with Ycg1-depleted (*ycg1-aid*) cells (figure 3.5). The Aid-degron wt strain shows condensin to be enriched across the pericentromere and rDNA (however the biggest peak falls on loci 3 instead of 5 – to be discussed). In comparison, for the strain depleted of Ycg1 there is a significant decrease of ChIP enrichment of Brn1-3V5 signal at all loci (there is even a decrease at actin where condensin is not specifically enriched). This provided further evidence that the ChIP signal observed is representative of condensin enrichment and that the ChIP assay is sensitive to loss of condensin association with chromatin.

3.1.5 Consistent levels of condensin enrichment in different genetic backgrounds

Within each ChIP experiment, a control strain must be used as a point of reference for comparison. For consistency throughout the experiments conducted in the rest of this thesis (with the exception of the AID-degron experiments) all strains are based on the tet-degron wt in addition to mutations in factors being investigated as having an effect on condensin association with chromatin. In figures 3.3 and 3.5 the population of cells that are depleted of a protein, are compared with a control strain that have all of the mechanisms available for depletion of the subunit, minus the targeting mechanism. To probe whether the genetic background of these comparison strains (tet-degron wt and

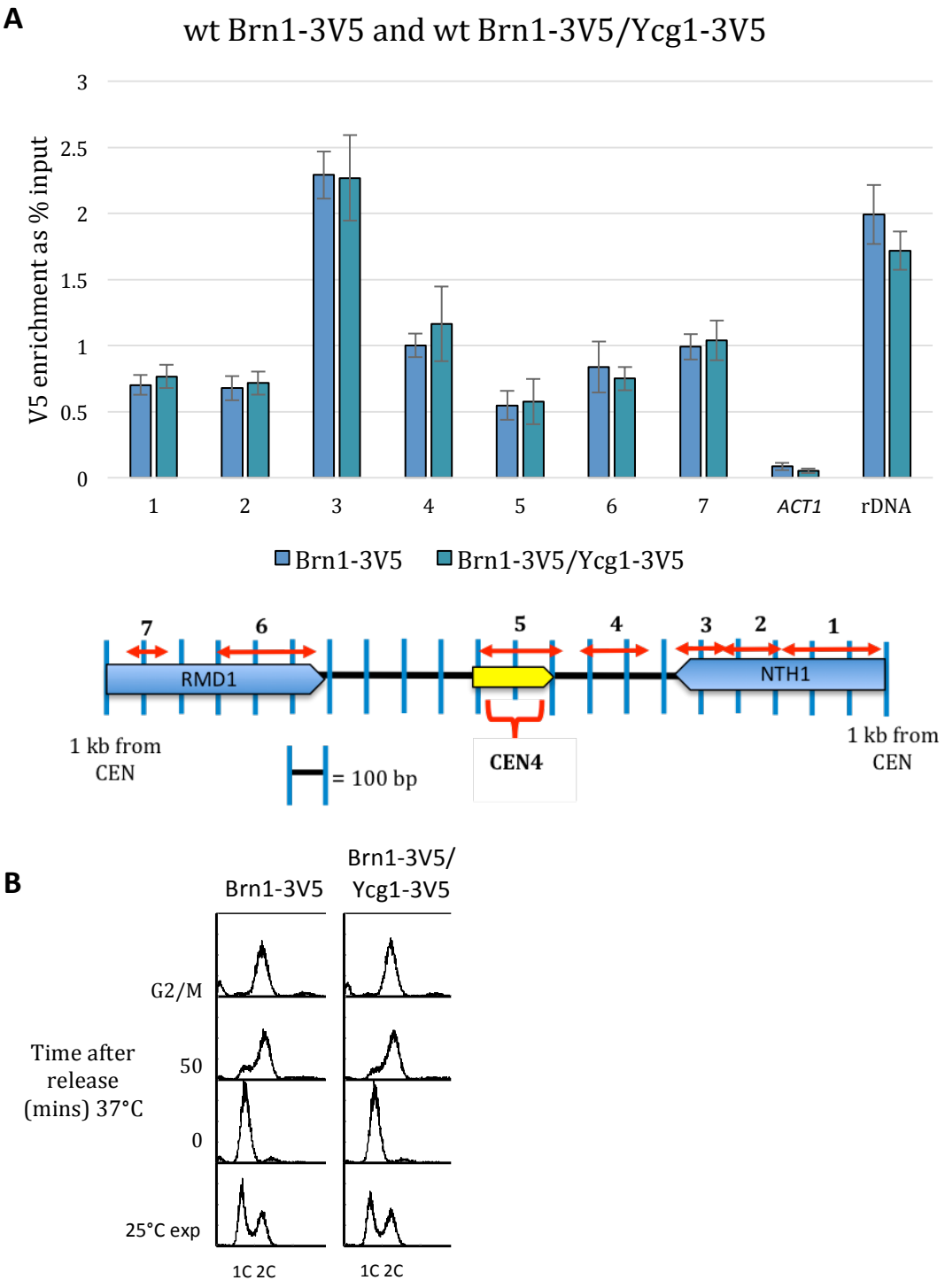


Figure 3.7 Brn1-3V5 enrichment and Brn1-3V5/Ycg1-3V5 enrichment.

A) Histogram showing levels of Brn1-3V5 and Brn1-3V5/Ycg1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using Nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **B)** Representative FACS data showing the progression of tet-degron wt Brn1-3V5 (1291) and Brn1-3V5/Ycg1-3V5 (708) through the block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M assessed by budding index.

AID-degron wt) were adversely affecting condensin enrichment levels, I conducted a side-by-side ChIP analysis of the two control strains (Figure 3.6). Very similar levels of condensin enrichment was observed in both genetic backgrounds at each genomic loci probed, suggesting that there was little to no adverse effect on condensin enrichment by these degron systems.

3.1.6 Immunoprecipitation of Brn1-3V5 subunit is sufficient

Since it remains unknown how condensin interacts with chromatin at the molecular level, it is possible that some subunits are more amenable to immunoprecipitation than others. To see whether we were detecting maximal chromatin association of condensin by immunoprecitating one subunit (Brn1-3V5), we investigated whether the tagging of an additional subunit of condensin (Ycg1-3V5) would have an effect on the levels of enrichment observed from ChIP. We probed whether any difference in enrichment could be seen in strains in which two subunits were tagged and therefore immunoprecipitated. Tet-degron wt strains containing Brn1-3V5 or Brn1-3V5 and Ycg1-3V5 were analysed by ChIP (Figure 3.7) and we observed no difference between the two. This data suggests that immunoprecipitation of one 3V5-tagged subunit was sufficient to represent the population of condensin at these loci.

3.1.7 ChIP assay corroborates mitosis-specific enrichment of condensin

My experimental design is based upon the understanding that condensin is specifically enriched on chromatin during mitosis, when the complex is assumed to be most active. To further assess the functionality and sensitivity of this ChIP assay, we investigated whether we could observe the cell-cycle dependent change in condensin enrichment at the loci we were probing. For this experiment, the first cell culture sample was taken and fixed while synchronised in the G1 block prior to release, and the second was fixed at the usual G2/M block in nocodazole and both were analysed by ChIP (figure 3.8). We observed a clear increase in condensin enrichment for cell cultures fixed in G2/M when compared with those fixed in G1. The increase in condensin enrichment at the G2/M block is evident across the pericentromere and

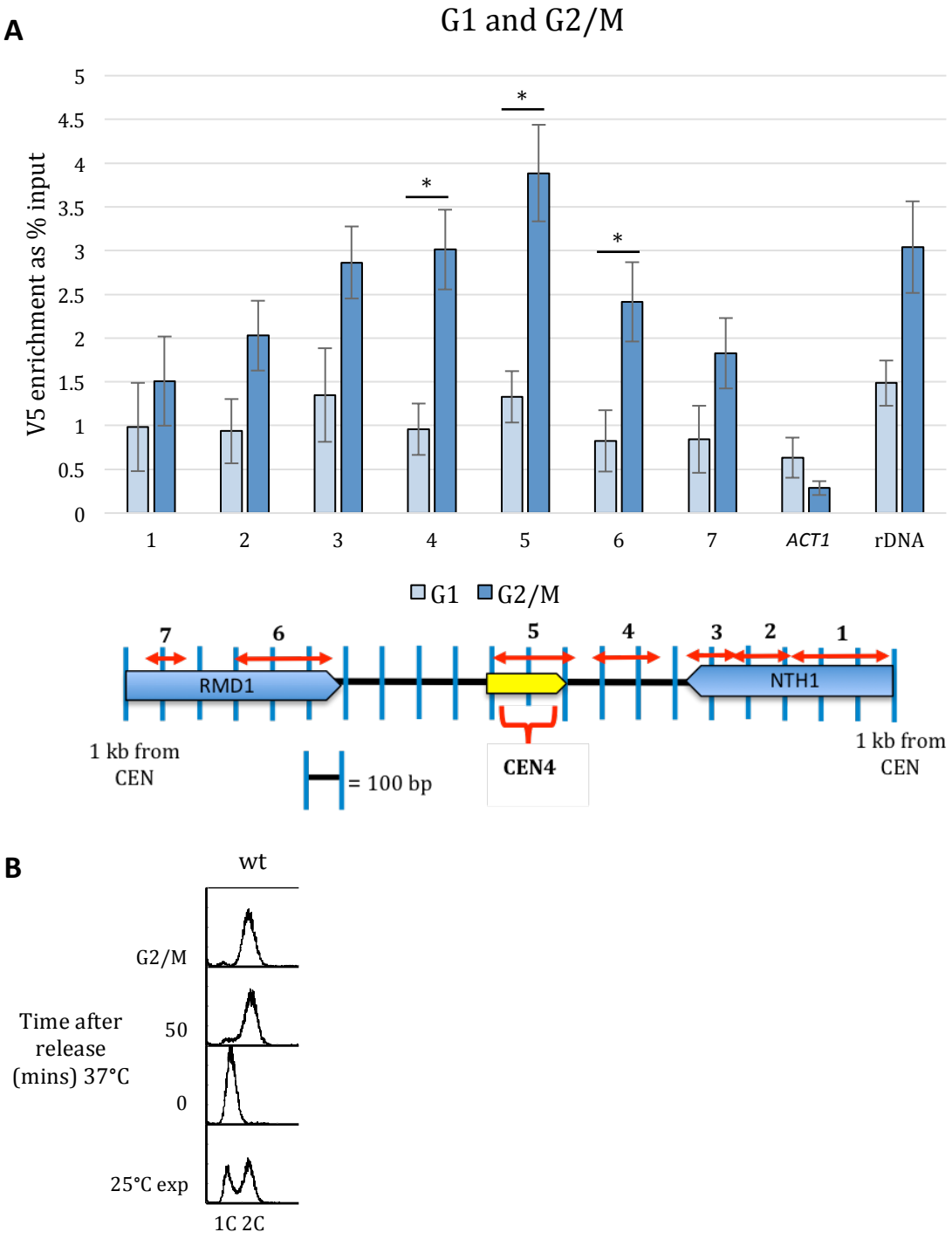


Figure 3.8 Condensin enrichment in G1 and G2/M.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using Nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05. **B)** Representative FACS data showing the progression of tet-degron wt (708) through the block and release protocol. 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M assessed by budding index.

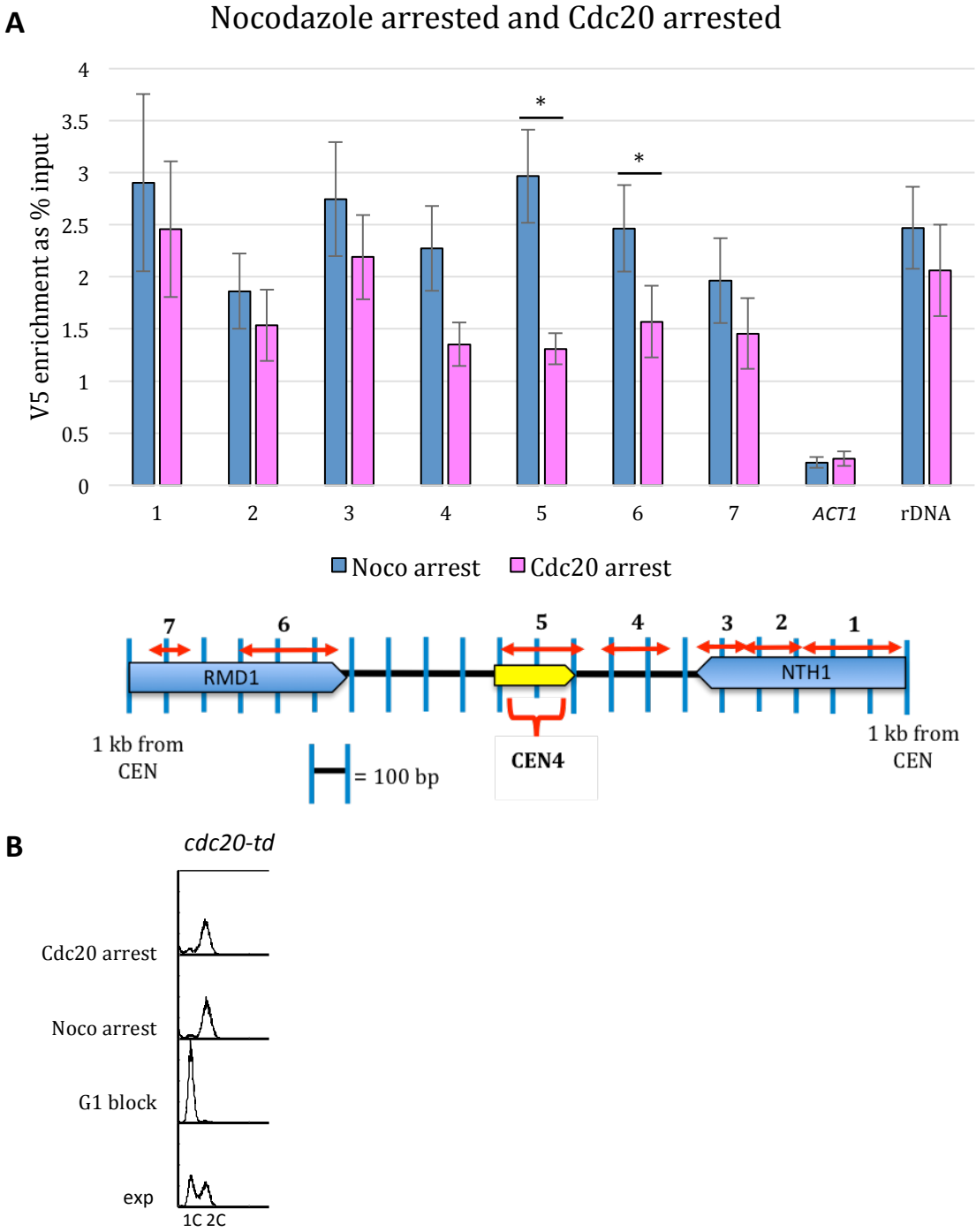


Figure 3.9 Condensin enrichment in cells arrested using nocodazole and cells arrested using Cdc20 depletion

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cells were subsequently released from nocodazole arrest into a Cdc20 arrest. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05.**B)** Representative FACS data showing the progression of *cdc20-td* (858) through the block and release protocol. 'exp' = exponentially growing population.

centromere as expected. The mitosis-specific increase is also present at the rDNA. This data not only confirms the mitosis-specific enrichment on and surrounding *S. cerevisiae* centromeres, as others have observed, but also further underlines the sensitivity and functionality of this ChIP assay for investigating condensin enrichment on chromatin.

Nocodazole is a drug that induces a metaphase arrest by destabilising the mitotic spindles. We wanted to investigate whether a nocodazole arrest was appropriate for use in probing condensin enrichment by ChIP. To test this, I used a tetracycline-repressible degron to conditionally deplete Cdc20. Depletion of Cdc20 generates a metaphase arrest in the absence of nocodazole, to allow the comparison of condensin enrichment in both metaphase arrests. Cells were synchronised in G1 before being released to progress through the cell cycle and subjected to restrictive conditions. A nocodazole arrested was induced and the first cell culture sample was fixed for ChIP analysis. The remaining cells were released from the nocodazole block into the subsequent Cdc20 arrest before being fixed using formaldehyde. The cell samples were analysed using ChIP (figure 3.9). We observed that condensin enrichment was slightly lower in the Cdc20 arrested cells compared with those arrested using nocodazole, most notably at loci closest to the centromere. This finding is consistent with previous studies (Nerusheva *et al.* 2014, Leonard *et al.* 2015). No change in condensin enrichment was observed at the negative control loci at the actin gene and there was very little difference at the rDNA.

3.2 Discussion

The work in this chapter describes the process of setting up and optimising the ChIP protocol for condensin, and goes on to show the functionality of the technique. The process of optimising the ChIP protocol helped to produce accurate and reliable data, and also allowed me to develop a deeper understanding of the different stages of ChIP and where things can go wrong experimentally; invaluable for maintaining accurate and consistent experimental technique throughout.

There are several drawbacks of ChIP analysis in general (discussed in (Jeppsson *et al.* 2014)). Firstly, with genome-wide ChIP-seq and ChIP-on-Chip, the DNA amplification step required before sequencing or application to a DNA microarray means that the data is not quantitative. However, the advantage of probing specific loci and using qPCR analysis means that the data we obtain is semi-quantitative. Secondly, all ChIP analysis is carried out on a population of cells, meaning that high levels of enrichment may signify more protein complexes at a specific locus, or that more cells in the population have the protein complex at this site. By using a cell-synchronising protocol we hope to achieve as standardised a cell population as possible. However, it is important to be aware of this caveat. Furthermore, in *S. cerevisiae* there is a risk of false-positives at highly transcribed genes when using ChIP-seq or ChIP-qPCR (Teytelman *et al.* 2013). Genetic control experiments were carried out to ensure the relevance of the ChIP data acquired, most importantly the loss of ChIP signal upon depletion of condensin subunits shown in figures 3.3 and 3.5 demonstrated that the ChIP signal we observe is directly linked to the condensin complex. Furthermore our ChIP protocol shows condensin enrichment at loci and in a cell-cycle dependent manner that agrees with data already published in the literature; the mitosis specific enrichment at the centromeric regions (Wang *et al.* 2005, D'Ambrosio *et al.* 2008) and at the rDNA (Wang *et al.* 2005), as well as a decrease in centromeric enrichment of condensin in a Cdc20 arrest when compared to a nocodazole arrest (Leonard *et al.* 2015).

Background signals can vary between ChIP experiments. To monitor this, in all ChIP experiments conducted throughout this thesis, a ‘no antibody’ control was in place for each sample and analysed by qPCR at each genome locus investigated. In Figures 3.2C and 3.2D the results from the no-antibody samples are displayed in the histogram. However, for clarity of data presentation, from thereon the enrichment values are excluded from the histograms. The no-antibody samples remained consistently negligible throughout experiments.

If you compare ChIP data between different experiments, you can observe differences in the total enrichment levels at each locus and also in the enrichment patterns of the

same strain across the loci. For example, in figures 3.3 and 3.8 we can see the tet-degron wild type ('wt' in the key of 3.3 and 'G2/M' in the key of 3.8) shows a clear peak right on the primer pair 5 locus at the centromere, whereas this same strain used again in figure 3.6 ('tet-degron wt' in the key) the levels of condensin enrichment are very similar across locus 3, 4, 5 and the rDNA. Further to this, the same strain in figure 3.7 ('Brn1-3V5' in the key), the locus 5 directly on the centromere is actually the least enriched locus apart from actin. The difference in pattern is consistent though, by which I mean the dip in enrichment at locus 5 is reflected in both strains and over three repeat experiments in figure 3.6 and 3.7. From this qPCR data we realised that we can only truly make comparisons between strains at each specific locus, we cannot compare between loci of one strain because this is not consistent between experiments. This does not pose a problem in my analysis of factors affecting condensin association because for each investigation I compare to a control strain that is processed side-by-side with the experimental strain. However, what is clear from this chapter is that condensin enrichment at the actin site is consistently lower than other loci in each experiment.

Something important to remember when considering ChIP data is that it only represents a snap shot of a moment in time; a tableaux of protein-chromatin interaction. We cannot directly infer from ChIP data whether a protein is more or less active temporally or spatially merely because it exhibits a higher or lower level of enrichment. ChIP data needs to be carefully examined in the context of what we already know about a protein. For instance, a chromatin-binding protein that functions enzymatically may exhibit a high level of dynamic turnover, which could appear in ChIP data as having a lower level of enrichment. Conversely, a chromatin-binding protein that has a more structural function, may load and become stationary when active, for example cohesin during S-phase following eco1 acetylation; which in this case an increase in function would appear as a higher level of enrichment in a ChIP read-out. For these reasons in following chapters we are careful when inferring changes in condensin activity from information on how factors effect condensin association with chromatin.

4 Investigating the role of kinetochore components in the association of condensin with mitotic centromeres

4.1 Results

4.1.1 Introduction

Chromosome-microtubule interactions, key to the dynamic process of sister chromatid separation in anaphase, occur via kinetochores present at centromeres. The kinetochore is a conserved complex of centromeric DNA and an associated protein structure that serves as an interface for chromosome attachment to mitotic spindles. To begin investigating factors potentially involved in condensin recruitment to the centromere region, a logical starting point is to look at *cis* factors that may have a significant impact on the local chromatin environment. The kinetochore therefore, being the most notable feature at the centromere, stands to be a good initial area of enquiry. As discussed in 1.4.4.3, work carried out in *S. pombe* by Nakazawa *et al.* (2008) has shown kinetochore components to be important in the localisation of condensin to the centromere in mitosis. By expressing GFP-tagged Cut14/Smc2 in *S. pombe* kinetochore mutant strains, the authors showed that kinetochore components Mis*6*, Cnp1, and Mis13 (homologues of *S. cerevisiae* Ctf3, Cse4 and Dsn1 respectively) are required for the centromeric localization of condensin. It is likely then, that parts of the kinetochore may play a role in condensin enrichment at mitotic centromeres. The authors also show that condensin II fails to localise at centromeres upon depletion of CENP-I (hMIS6) by RNAi in human cells (Nakazawa *et al.* 2008) suggesting that this mechanism of condensin accumulation may be conserved.

Although centromeres vary significantly between different species, the function of the kinetochore and many components of the protein structure are very well conserved (Kitagawa and Hieter 2001). The kinetochore can be split into 2 functional units; the first is the inner kinetochore, which acts as a structural attachment to the centromeric

DNA, and also a scaffold for the assembly of the rest of the kinetochore protein complex. The inner kinetochore includes the components of the constitutive centromere-associated network (CCAN), which is present at the centromere throughout the cell cycle. Secondly, there is the outer kinetochore, which assembles in late interphase/early mitosis in metazoans (Liu *et al.* 2006, Cheeseman and Desai 2008, Cheeseman *et al.* 2008) (but is constitutively localised at the *CEN* in *S. cerevisiae (Goshima and Yanagida 2000)*) and functions as a platform for kinetochore-microtubule interaction. The outer kinetochore consists of the conserved kinetochore-microtubule interface (KMN) network. On top of this, there are two regulatory systems in place, the spindle assembly checkpoint (SAC) and the spindle tension checkpoint which act to ensure that anaphase cannot begin until amphiletic kinetochore-microtubule attachments have been made (Santaguida and Musacchio 2009). Vertebrate kinetochores are much larger than those observed in *S. cerevisiae;* it's thought that the human and other regional kinetochores consist of repeated units of the core functional kinetochore observed in *S. cerevisiae* (Zinkowski *et al.* 1991) (Joglekar *et al.* 2008).

The *S. cerevisiae* kinetochore

As shown in Figure 4.1, the inner kinetochore of *S. cerevisiae* is made up of Cse4, the CBF3, Mif2 and some CTF19 proteins (protein-complex names are in capitals for clarity), which interact with outer kinetochore components of CTF19. The proteins of the CTF19 complex then in turn interface with the KNM network (named after the Knl1 (SPC105), Mis12 (MTW1) and NDC80 complexes in *S. pombe*). The KMN network in *S. cerevisiae* (MTW1/MIND, SPC105 and NDC80) acts not only a receptor for microtubule attachment but also participates in the recruitment of SAC components including the DAM1 complex (Pesenti *et al.* 2016). Despite decades of research, it is still not entirely clear how the kinetochore assembles; however studies looking at the kinetochore protein-protein interactions and mutational studies have provided some ideas.

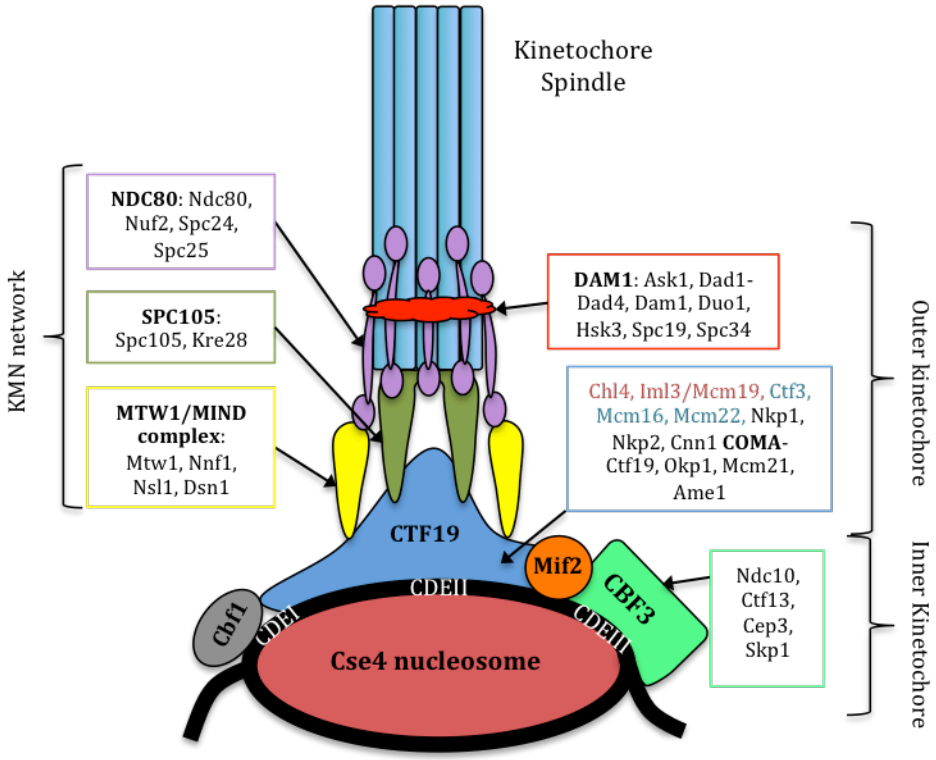


Figure 4.1 The *S. cerevisiae* kinetochore

A schematic representation of the budding yeast kinetochore adapted from (Cieslinski and Ries 2014). The very base of the kinetochore consists of the Cse4-containing nucleosome, which is wrapped with the centromeric DNA CDEI, CDEII and CDEIII. The Cse4 nucleosome is bound by proteins of the Inner kinetochore, which in turn provide a platform for the outer kinetochore and KMN network to assemble. The kinetochore spindle of the mitotic apparatus attaches to the outer kinetochore components and the DAM1 complex works as part of the SAC to ensure proper attachment. Names of complexes are written in upper case for clarity.

Cse4 (CENPA in mammals) is a histone H3 variant protein, found almost exclusively at functional centromeres and is ubiquitous in nearly all eukaryotes (with one exception noted; the parasitic trypanosome protozoa) (Malik and Henikoff 2009). Cse4 is essential for the construction of a functioning kinetochore. Studies in *S. cerevisiae* using ectopic incorporation of the histone H3 variant have shown Cse4 to be necessary *and* sufficient for chromosome segregation (Heun *et al.* 2006). The sequencing of a 123-135 bp nuclease-resistant stretch of centromeric DNA revealed that the *S. cerevisiae* Cse4-containing nucleosome is perfectly positioned to wrap the entirety of the point centromere DNA around itself (Cole *et al.* 2011). The CBF3 inner kinetochore protein-complex works alongside the Scm3 chaperone protein to localise Cse4 to centromere sites. There is however, conflicting evidence for the point at which Cse4 is

incorporated at the centromere in *S. cerevisiae*. There is evidence for incorporation both in S-phase (Aravamudhan *et al.* 2013) and at anaphase (Shivaraju *et al.* 2012). There have been several attempts to identify the number of Cse4-containing-nucleosomes at centromere sites, with recent work providing evidence of two Cse4 molecules per kinetochore (Wisniewski *et al.* 2014).

The CBF3 complex consists of four essential inner kinetochore proteins; Ndc10, Cep3, Ctf13 and Skp1. Ndc10 and Cep3 can directly bind the CDEIII DNA as part of the CBF3 complex and Ndc10 can bind CDEII independently (Espelin *et al.* 2003). The association of Cse4 with centromeric DNA is thought to be through a direct interaction between Ndc10 and Scm3 (Cho and Harrison 2012). Skp1 and Cft13 are important in regulating the CBF3 complex assembly and turnover (Rodrigo-Brenni *et al.* 2004). CBF3 and Cse4 are also required for the centromeric localisation of the inner kinetochore protein Mif2 (CENPC). Mif2 plays the important role of recruiting Iml3 of the CTF19 complex (see below) through direct interaction. Together, Cse4, Mif2 and CBF3 form a platform for the assembly of the kinetochore. Another DNA-binding kinetochore protein in *S. cerevisiae* is the non-essential Cbf1 protein, which binds CDEI and is important for optimal chromosome stability in mitosis (Baker *et al.* 1989).

Along with Cse4 and CBF3, the CTF19 complex is present at the centromere throughout the cell cycle (McKinley and Cheeseman 2016). Along with CBF3 and Mif2, the CTF19 complex is the *S. cerevisiae* equivalent of the CCAN for constitutive centromere associated network in vertebrates. The primary function of the CTF19 complex is to act as a binding interface for Cse4 that allows the establishment of the KMN of the outer-kinetochore. There are several proteins within the CTF19 complex that form stable sub-complexes: the COMA complex, which consists of Ctf19, Okp1, Mcm21 and Ame1; Chl4 and Iml3/Mcm19; and Ctf3, Mcm16 and Mcm22. Of all the proteins in the CTF19 complex, only Okp1 and Ame1 of COMA are essential for viability. The COMA complex is involved in correcting erroneous MT-kinetochore attachments (Knockleby and Vogel 2009). The Okp1-Ame1 has been shown to not only directly bind inner kinetochore protein Mif2 but also to be necessary for the binding of

the MTW1 complex at the kinetochore, and therefore plays an important role in the assembly of the outer kinetochore (Hornung *et al.* 2014). The Chl4-Iml3 sub-complex is localised at the centromere in a Ctf19-dependent manner (although recruited by Mif2), and is also required for Ctf19 interactions with Ctf3 and Iml3 suggesting it is important for kinetochore structure (Pot *et al.* 2003). As its name suggests, Chl4 (chromosome loss 4) is required for chromosome stability (Kouprina *et al.* 1993). Interestingly, Okp1-Ame1, Chl4 and Mcm16-Ctf3 have been shown to have a role in the spindle assembly checkpoint (Matson *et al.* 2012) (Pot *et al.* 2005). Cnn1 of the CTF19 complex interacts directly with the NDC80 and SPC105 complexes of the outer kinetochore (De Wulf *et al.* 2003) and facilitates a functionally competent conformation of the KMN network. Phosphorylated Cnn1 helps to regulate KMN activity by inhibiting MTW1 and SPC105 complexes from interacting with NDC80 (Bock *et al.* 2012).

The KMN network forms the primary interface between the outer kinetochore and the attaching microtubule. MTW1 (also referred to as the MIND complex) consists of Mtw1, Nnf1, Nsl1 and Dsn1. Since CTF19 components and NDC80 components both bind non-competitively to the MTW1 complex, it is a thought to act as a bridge between inner and outer kinetochore components (Hornung *et al.* 2011). NDC80 and SPC105 complexes both have microtubule binding activities and act as the connection point between the outer kinetochore and spindle microtubules. The Spc105 complex is made up of just Spc105 and Kre28, and is also important in the spindle assembly checkpoint. The NDC80 complex is a dumbbell-shaped tetramer composed of two globular heterodimers (Ndc80-Nuf2, Spc24-Spc25,) connected by a long coiled-coil domain (Wigge and Kilmartin 2001, Wei *et al.* 2005). NDC80 is required for kinetochore assembly, the clustering of kinetochores during mitosis and is also important for checkpoint control (Janke *et al.* 2001, McCleland *et al.* 2003). The NDC80-MT interaction appears to be regulated by the phosphorylation of the Ndc80 N-terminal tail by Ipl1 kinase to prevent erroneous kinetochore-microtubule (MT) attachments. Ndc80 also contributes to kinetochore-MT interaction by recruiting the DAM1 complex. The DAM1 complex consists of ten essential subunits, and forms a ring structure around microtubules (Li *et al.* 2005, Westermann *et al.* 2005). The primary

function of DAM1 is thought to be the coupling of chromosome movement to MT depolymerisation and is vital in the separation of sister chromatids in anaphase (Asbury *et al.* 2006).

The work in this chapter investigates the role of the essential kinetochore proteins Cse4, Ndc10 and Ndc80 in the association of condensin with mitotic centromeres. Investigating these three proteins allows an analysis at different levels of the kinetochore; the very base of the protein complex with Cse4 in centromere-specific nucleosomes and a middle level with Ndc10 which is involved in Cse4 incorporation, right up to the very extremity of the kinetochore with Ndc80 which facilitates kinetochore-microtubule attachments.

4.1.2 Loss of Cse4 function does not significantly change condensin enrichment at the mitotic centromere

To investigate whether the H3 histone variant Cse4 has a role in condensin recruitment, we used the temperature sensitive loss of function mutation *cse4-323,* generated and previously described by Sue Biggins *et al.* (Biggins *et al.* 2001). To reiterate, for consistency between experiments all mutants have been crossed to contain the tet-degron wt genetic background, and are compared with the tet-degron wt (wild-type) strain in each experiment. Wild-type and *cse4-323* cells were processed using the G1 block and release protocol and condensin association with chromatin was analysed using ChIP. Interestingly, the ChIP data (figure 4.2A) revealed that the *cse4-323* mutant cells appeared to have no significant difference in levels of condensin enrichment when compared with wild-type. The ChIP data suggests that a fully functioning Cse4 is not important in condensin recruitment to the centromeric chromatin.

Since the *S. pombe* homologue of Cse4, Cnp1 has been shown to be important for condensin localisation to centromeres; it is a rather unexpected result that Cse4 does

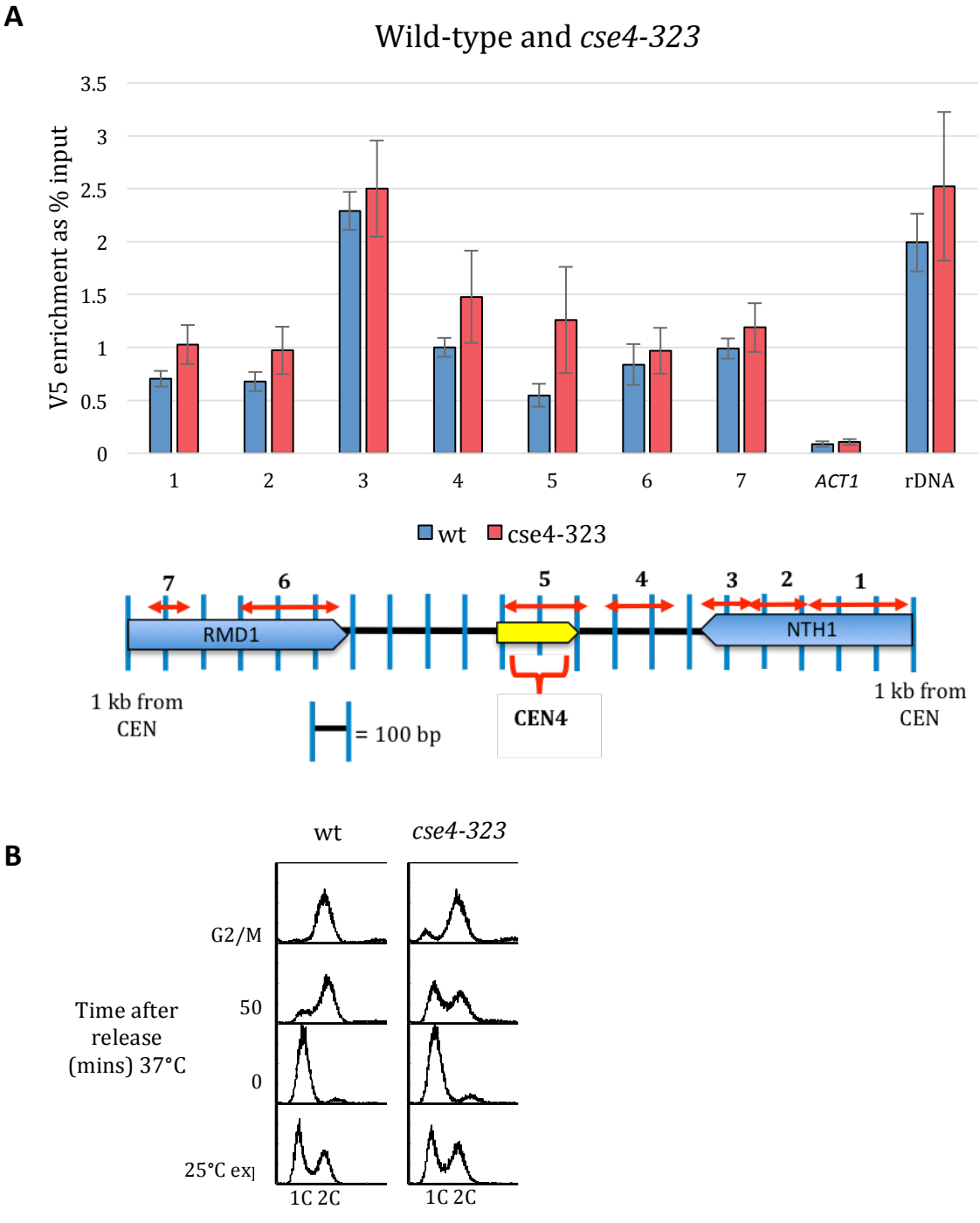


Figure 4.2 Investigating the role of Cse4 in condensin association with chromatin.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions (without doxycycline) and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 1291) and *cse4-323* (1107) through the block and release protocol. 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

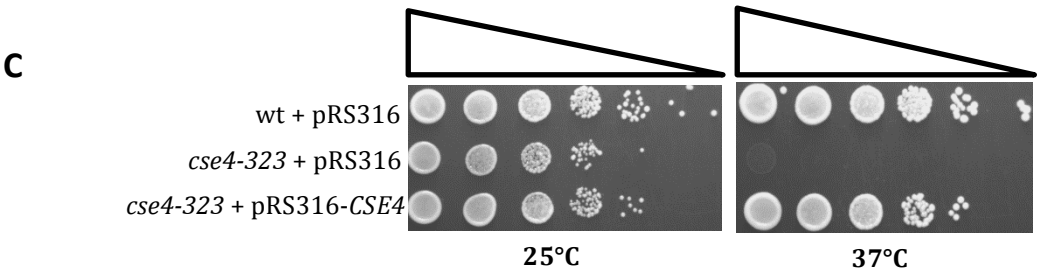


Figure 4.2 continued.

C) Spot test showing the cell viability of wild-type + *pRS316* (12), *cse4-323* + *pRS316* (364 + *pRS316*) and *cse4-323* + pRS316-*CSE4* (467*).* 10-fold serial dilutions of exponentially growing cells were plated onto YPD solid media and incubated at either 25°C or 37°C for up to 48 hours.

not appear to be. We conducted a complementation assay in the form of a spot test (Figure 4.2C) to confirm that Cse4 is indeed responsible for the loss of viability of this *cse4-323* mutant. If a secondary loss of function mutation had come into effect during the cross into the tet-degron wt background it may potentially be evident if wild-type *CSE4* does not rescue the *cse4-323* mutant strain. I cloned the *CSE4* gene and inserted it into the *pRS316* plasmid. I then inserted either the *pRS316-CSE4* or *pRS316* plasmid into the *cse4-323* mutant strain and pRS316 into the wild-type comparison strain. Figure 4.2C shows that at the permissive temperature both *cse4-323* strains containing *pRS316* or *pRS316-CSE4* grow almost as well as wild-type *pRS316*. At restrictive temperatures however, wild-type *pRS316* continues to grow well and *cse4-323 pRS316* is completely inviable. In contrast, the *cse4-323 pRS316-CSE4* grew almost as well as wild-type *pRS316*. Therefore, the *cse4-323* mutant is rescued by expression of the wild-type *CSE4*. The complementation of the *cse4-323* mutant with the expression of wild-type Cse4 does not rule out the possibility of a secondary mutation that does not negatively affect viability. From these data it appears that perturbing Cse4 has no effect on condensin association with centromeric chromatin.

4.1.3 Inner kinetochore protein Ndc10 is important for condensin enrichment at mitotic centromeres

Next we wanted to investigate whether the inner kinetochore protein Ndc10 has a role in the mitotic enrichment of condensin at centromeres. To do so we used the *ndc10-1*

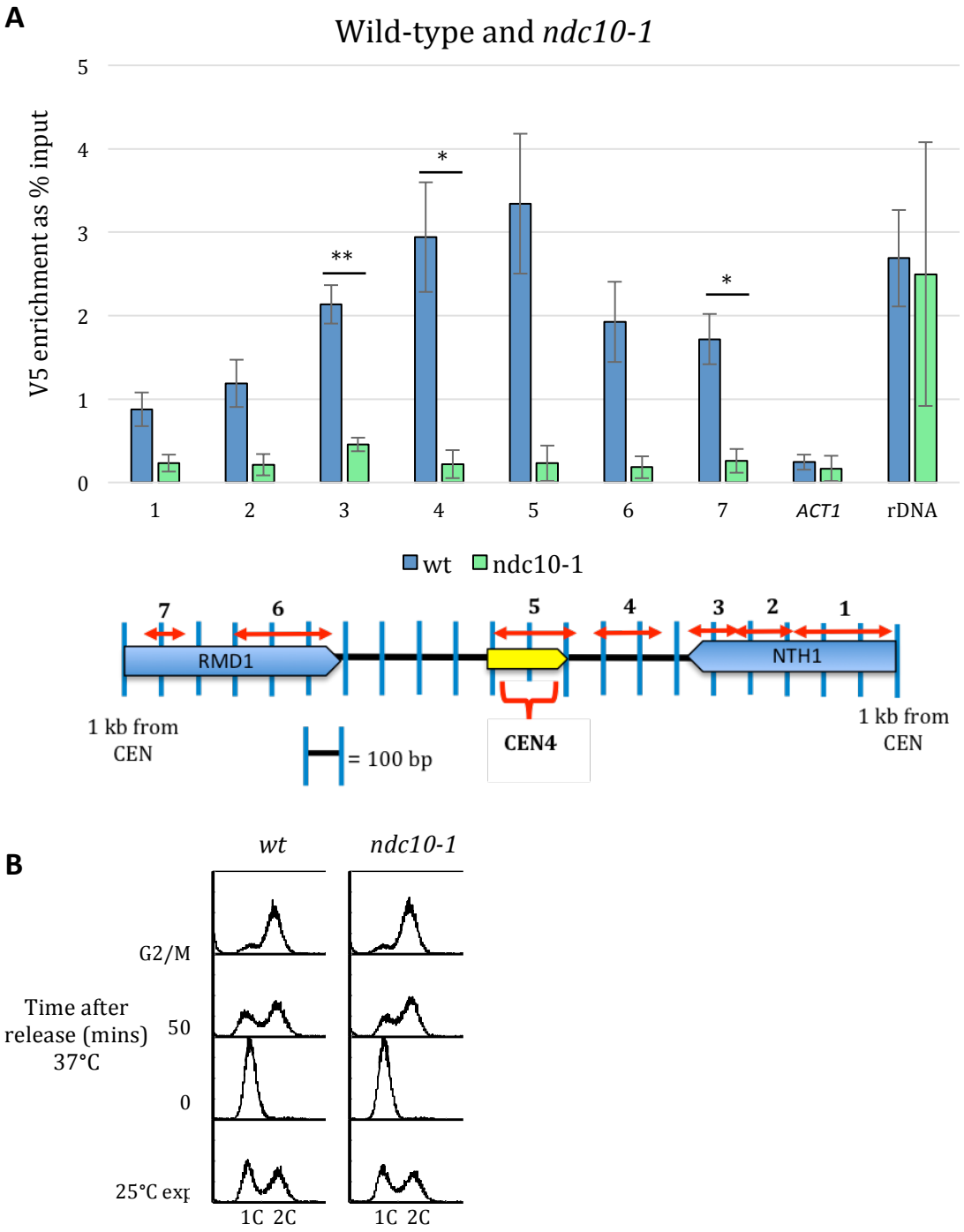


Figure 4.3 Investigating the role of Ndc10 in condensin association with chromatin.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions (without doxycycline) and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *ndc10-1* (712) through the block and release protocol. 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

mutant generated and previously described by (Goh and Kilmartin 1993). Wild-type and *ndc10-1* cells were processed through a G1 block and release protocol into nocodazole, and condensin enrichment was analysed by ChIP. Figure 4.3A shows that compared to wild-type, the *ndc10-1* mutant leads to significant loss of condensin enrichment all across the centromere and pericentromere loci, but importantly not at the *ACT1* locus or the rDNA. This data shows that proper Ndc10 function is important for condensin enrichment at the centromeric regions in mitosis.

4.1.4 Outer kinetochore protein Ndc80 is important for condensin enrichment at mitotic centromeres

To probe the contribution of the outer kinetochore to condensin enrichment at the centromere, we used the *ndc80-1* temperature sensitive mutation of *NDC80* (generated and previously described by (Wigge *et al.* 1998)). Wild-type and *ndc80-1* were directed through a G1 block and release protocol into a nocodazole block where they were fixed in G2/M using formaldehyde. Analysis of the *ndc80-1* mutant strain by ChIP (figure 4.4A) revealed a clear loss of condensin enrichment in comparison to the wild-type strain, all across the centromere and pericentromere, with no significant difference at the two control loci *ACT1* or the rDNA. This data suggests that proper Ndc80 function is required for condensin recruitment to the centromeric regions during mitosis.

4.1.5 Probing for genetic interaction between the kinetochore and condensin

Since perturbing kinetochore components Cse4, Ndc10 and Ndc80 have different effects on condensin association with chromatin we thought that perhaps this difference would be reflected in their genetic interactions. To explore any potential genetic interaction between Cse4, Ndc10, Ndc80 and condensin, I carried out partial depletion spot tests in which I used varying temperatures and concentrations of

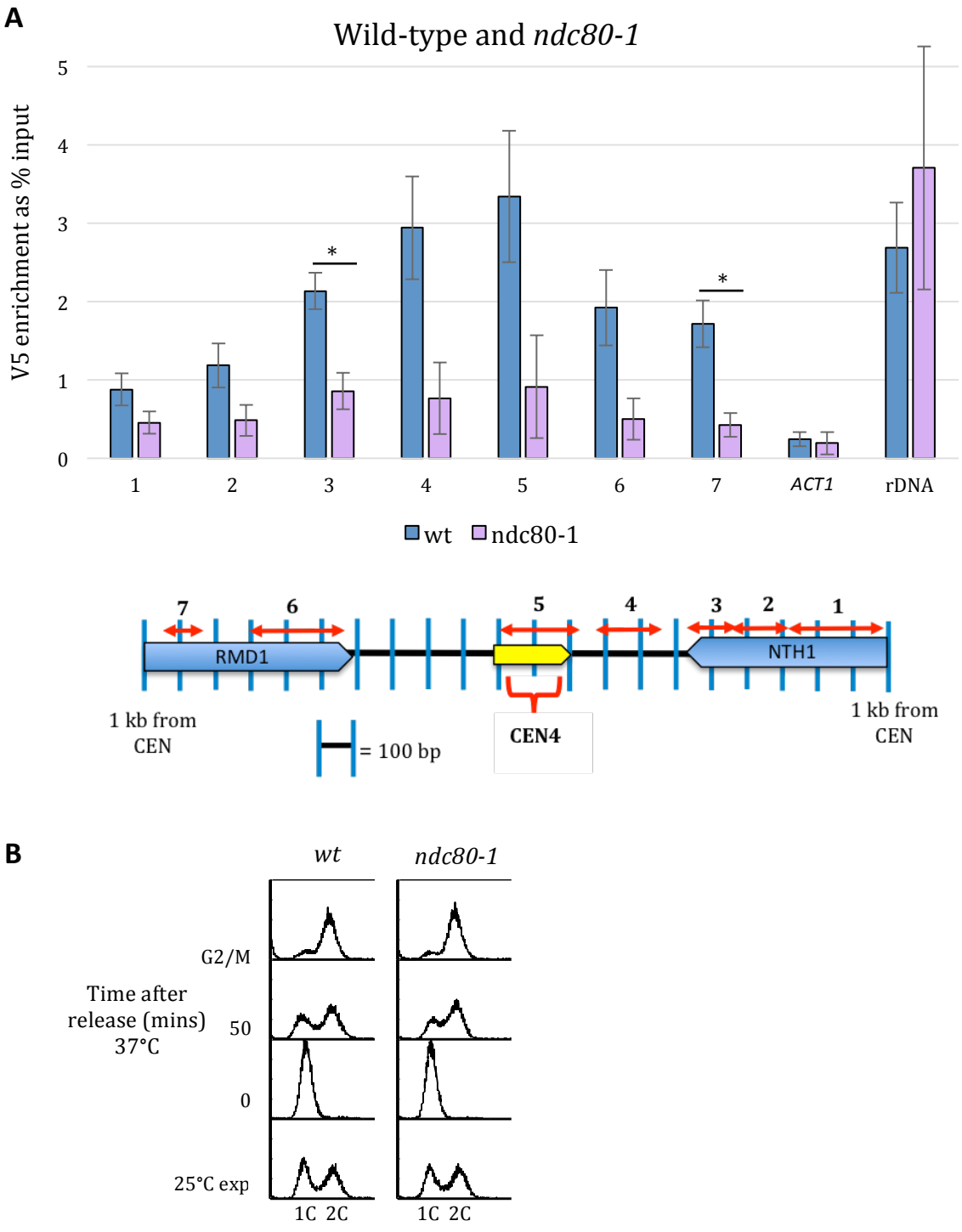


Figure 4.4 Investigating the role of Ndc80 in condensin association with chromatin.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions (without doxycycline) and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in 2.6.4. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *ndc80-1* (711) through the block and release protocol. 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

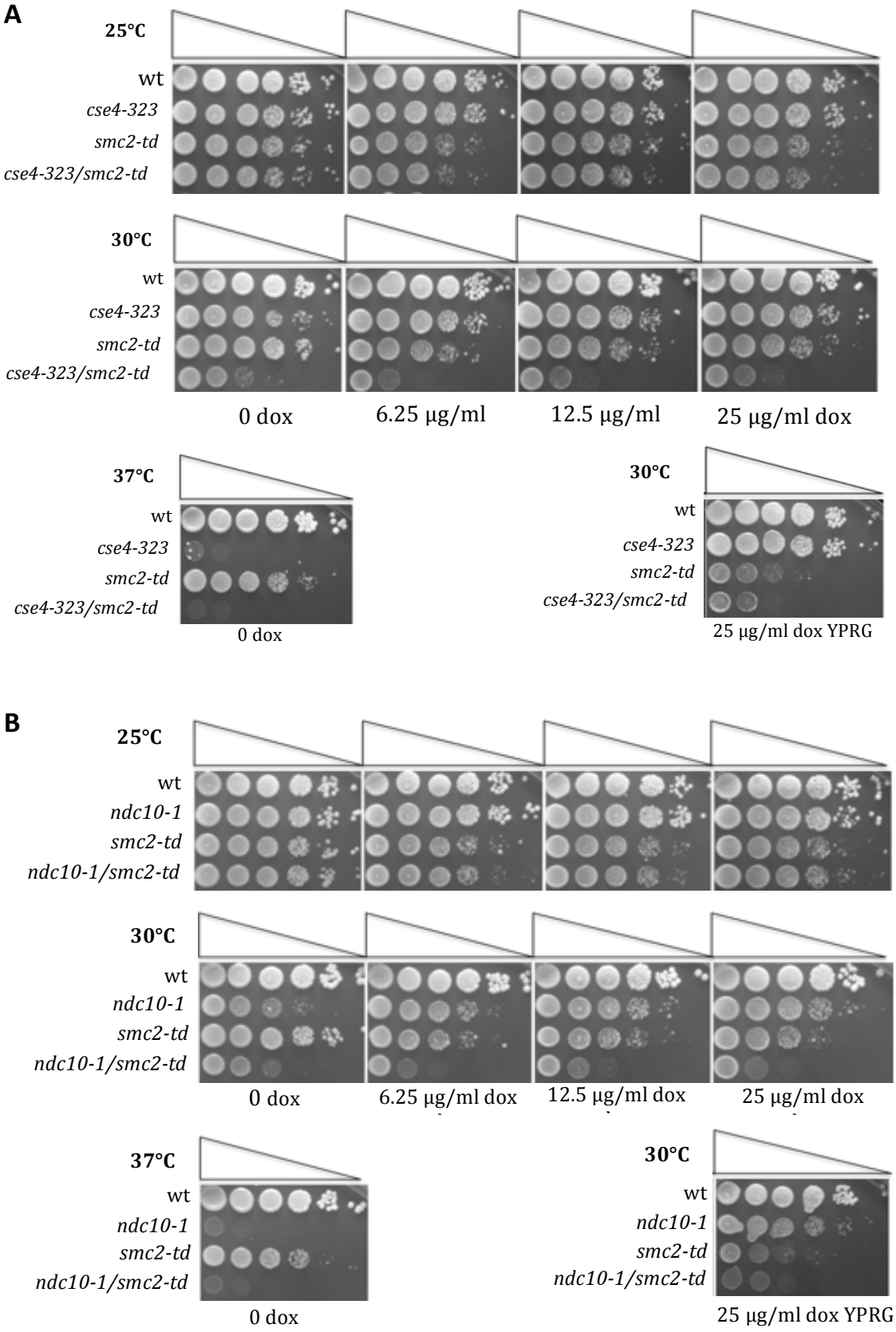


Figure 4.5 Probing for genetic interactions between kinetochore components and condensin

(Figure details on following page.)

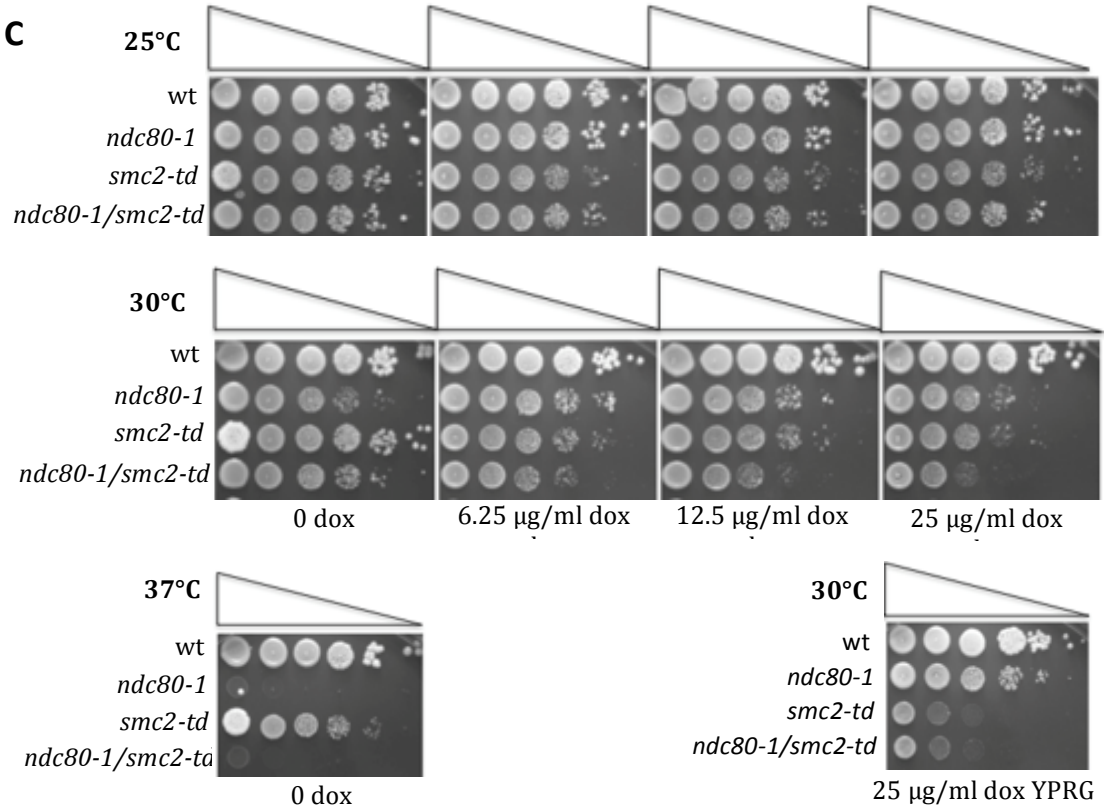


Figure 4.5 continued

Spot test Spot test showing the cell viability of wild-type, **A)** *cse4-323* (364)*, smc2-td* (278) *and cse4-323/smc2-td* (362) **B)** *ndc10-1* (367)*, smc2-td* (278) *and ndc10-1/smc2-td* (518) and **C)** *ndc80-1* (333), *smc2-td* (278) and *ndc80-1/smc2-td* (335) strains under various growth conditions*.* 10-fold serial dilutions of exponentially growing cells were plated onto YPD solid media with no doxycycline, 6.25 µg/ml, 12.5 µg/ml or 25 µg/ml doxycycline, or YPRG with 25 µg/ml doxycycline and incubated at either 25°C, 30°C or 37°C for up to 48 hours.

doxycycline to partially impair protein function of both the kinetochore proteins and Smc2. I made double mutant strains containing one of the kinetochore mutants *cse4-323, ndc10-1 or ndc80-1* and the condensin mutant *smc2-td* and plated these alongside wild-type and the two single mutant strains. The idea is that if the kinetochore components and condensin function in the same pathway then by partially perturbing the function of both proteins the double mutants should be much more severely affected than the single mutants.

As shown by the spot tests in figure 4.5, *cse4-323* and *ndc10-1* and to a much lower extent *ndc80-1* appear to have a synthetic genetic interaction with condensin. So while mutations that affect the kinetochore appear to interact with depletion of condensin,

the severity of the interaction does not correlate with the impact that each mutation has on condensin enrichment.

4.2 Discussion

The work in this chapter demonstrates for the first time that kinetochore factors have a role in condensin association with mitotic centromeres in *S. cerevisiae.* The kinetochore has previously been identified as having a role in condensin enrichment at *S. pombe* and human centromeres (Nakazawa *et al.* 2008). It is interesting this role of the kinetochore is conserved despite the significant differences between regional and point centromeres.

From the ChIP experiments carried out, it is evident that the normal function of the inner kinetochore protein Ndc10 and the outer kinetochore protein Ndc80 are required for condensin enrichment. There are potentially two ways by which this kinetochore-dependent recruitment of condensin may occur. Firstly, Ndc10 and Ndc80 (and potentially others we have not investigated) may act as direct recruiters of the condensin complex. Alternatively, these kinetochore factors may contribute to creating a *cis*-binding platform at centromeric regions that is primed for recruitment of the condensin complex, either through local topological changes in chromatin or by facilitating the localisation of other condensin regulators.

Ndc80 has been shown by yeast two-hybrid and co-immunoprecipitation to have physical interaction with the Smc2 subunit of the condensin complex (Zheng *et al.* 1999). However, a protein-protein interaction is not sufficient evidence for a direct recruitment mechanism. No such interaction has been identified between Ndc10 and condensin. Since the importance of the kinetochore in facilitating condensin association with centromeres is conserved between the *S. pombe* and *S. cerevisiae*, it is logical to assume that it operates to do so using a similar mechanism. In the *S. pombe cut17-275* Cut17/Bir1/Survivin mutant strain, kinetochore components linked

to condensin recruitment were normally localised, but condensin failed to accumulate at the centromere, suggesting that condensin's recruitment is not via a direct interaction with these proteins. It therefore seems unlikely that they are functioning as direct recruiters of condensin.

Bir1/Survivin is a subunit of the chromosomal passenger complex (CPC), and along with Sli15 controls the targeting and activation of Ipl1-aurora B kinase. As discussed in 1.4.4.2 Aurora B kinase is heavily linked to condensin activation and association with chromatin. Ndc10 and Bir1 can be co-immunoprecipitated, and Bir1 has been shown to be important for Ndc10 localisation in mitosis (Widlund *et al.* 2006). Also, post-translational modification of Bir1 in the form of sumoylation has been shown to be dependent on the sumoylation of Ndc10 (Montpetit *et al.* 2006). The kinetochore and the CPC are intricately linked. Perhaps a properly functioning kinetochore is required for Bir1/CPC-dependent localisation of Aurora B and therefore acts to facilitate condensin association through local enrichment of its regulator.

If a normal functioning kinetochore is required for condensin enrichment at centromeres, it is quite surprising that although Cnp1/Cse4 is required for condensin enrichment in *S. pombe (Nakazawa et al. 2008)*, the ChIP data (figure 4.2A) in this chapter suggests that this is not the case in *S. cerevisiae*. Cse4 mutants in general have been shown to have defective kinetochore function and altered centromeric chromatin structure (Stoler *et al.* 1995, Meluh *et al.* 1998). Specifically, the *cse4-323* mutant has been shown to be defective in localising inner kinetochore protein Dsn1 (Pinsky *et al.* 2003), suggesting that this particular mutation is defective in kinetochore assembly and function. It seems counter intuitive that perturbing the very base of the kinetochore does not affect condensin enrichment, while mutations in *NDC10* and *NDC80* do. While the perturbation of Cse4 is contributing to the loss of viability in *cse4-323* mutants as shown by the complementation assay in figure 4.2C, we do not know what this mutation does at the molecular level. If the destabilisation of the kinetochore structure itself negatively affects condensin enrichment to this region, then perhaps *ndc10-1* and *ndc80-1* mutants are more penetrative than *cse4-323* in this regard. While several *CSE4* mutant alleles cause a metaphase arrest with unsegregated

DNA (Stoler *et al.* 1995), *cse4-323* allows progression through anaphase causing miss-segregation defects (Biggins *et al.* 2001). Perhaps in future experiments investigating alternative *CSE4* mutants, or perhaps a cleaner method such as conditional depletion would reveal a more definitive answer about whether condensin enrichment is effected by loss of Cse4 function.

More work needs to be done in order to clarify the role of the kinetochore in condensin association with mitotic centromeres. To investigate the potential direct recruitment, artificial tethering of Ndc10 or Ndc80 to an ectopic region could be used to see if this is sufficient for condensin localisation. For further investigation into whether the kinetochore facilitates recruitment via local enrichment of other condensin regulators, firstly known condensin regulators can be tested to see whether they effect condensin association with centromeric chromatin in *S. cerevisiae*. It could then be investigated whether these regulating factors are absent or present at the centromere in the *ndc10-1* and *ndc80-1* kinetochore mutants.

5 Investigating the role of phosphorylation in condensin association with the mitotic centromere

5.1 Results

5.1.1 Introduction

Post-translational modification of condensin, particularly by phosphorylation, has been shown to be important for its function and association with chromatin both *in vivo* and *in vitro* (as discussed in 1.4.4.2). Phosphorylation by CDK appears to be a universal mechanism of full condensin activation (Kimura *et al.* 1998, Nakazawa *et al.* 2008, Robellet *et al.* 2015). Aurora B kinase and PLK have often been identified as regulators of condensin, however seemingly in a more context-dependent fashion. In *S. cerevisiae*, Ipl1/Aurora B and Cdc5/PLK have been identified as regulators of the condensin complex in late mitosis (Lavoie *et al.* 2004) (St-Pierre *et al.* 2009). Whether this regulation of condensin activity influences the association of the complex with chromatin remains to be seen. Full phosphorylation of the complex is dependent on both Cdc5 and Ipl1. However, Cdc5 but not Ipl1 is competent in phosphorylating condensin subunits *in vitro* (St-Pierre *et al.* 2009). Therefore, the current model in *S. cerevisiae* is that Ipl1 works upstream of Cdc5 to regulate condensin in anaphase.

In this chapter, we investigate the impact of impairing condensin phosphorylation on the enrichment of the complex at the centromere and rDNA during mitosis, in *S. cerevisiae*. We utilise phosphorylation site mutants of the different condensin subunits, and also perturb mitotic kinases known or suspected to play a role in the phosphorylation of the condensin complex.

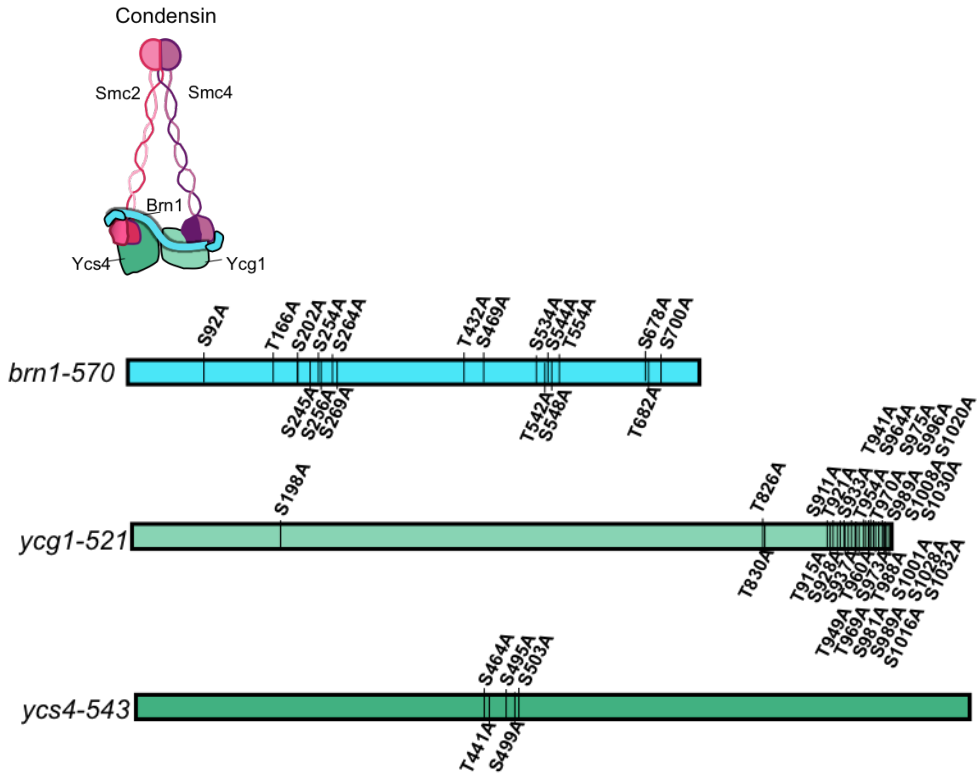


Figure 5.1 Schematic representation of non-SMC phosphorylation site mutants

Schematic representation of phosphorylation site mutants *brn1-570, ycg1-521* and *ycs4-543* generated by (St-Pierre *et al.* 2009). Marker-lines represent approximate position of each putative phosphorylation site. In each case an S (serine) or a T (threonine) has been replaced with an A (alanine).

5.1.2 Mutations in non-SMC condensin subunit phosphorylation sites significantly reduce condensin association with chromatin

Cdc5 has been shown to be important in condensin activation *in vitro* and *in vivo* (St-Pierre *et al.* 2009). Putative Cdc5 phosphorylation sites were identified and mutated on each of the non-SMC condensin subunits (figure 5.1) (St-Pierre *et al.* 2009). We wanted to investigate whether the loss of phosphorylation sites on individual subunits had an effect on condensin enrichment in mitosis. We therefore carried out ChIP analysis of *brn1-570*, *ycs4-543* and *ycg1-521* (kindly provided by the D'Amours laboratory). I also analysed a double mutant of *ycs4-543/ycg1-521,* which I generated by mating *ycs4-543* and *ycg1-521* strains. As in previous experiments in this study, cells

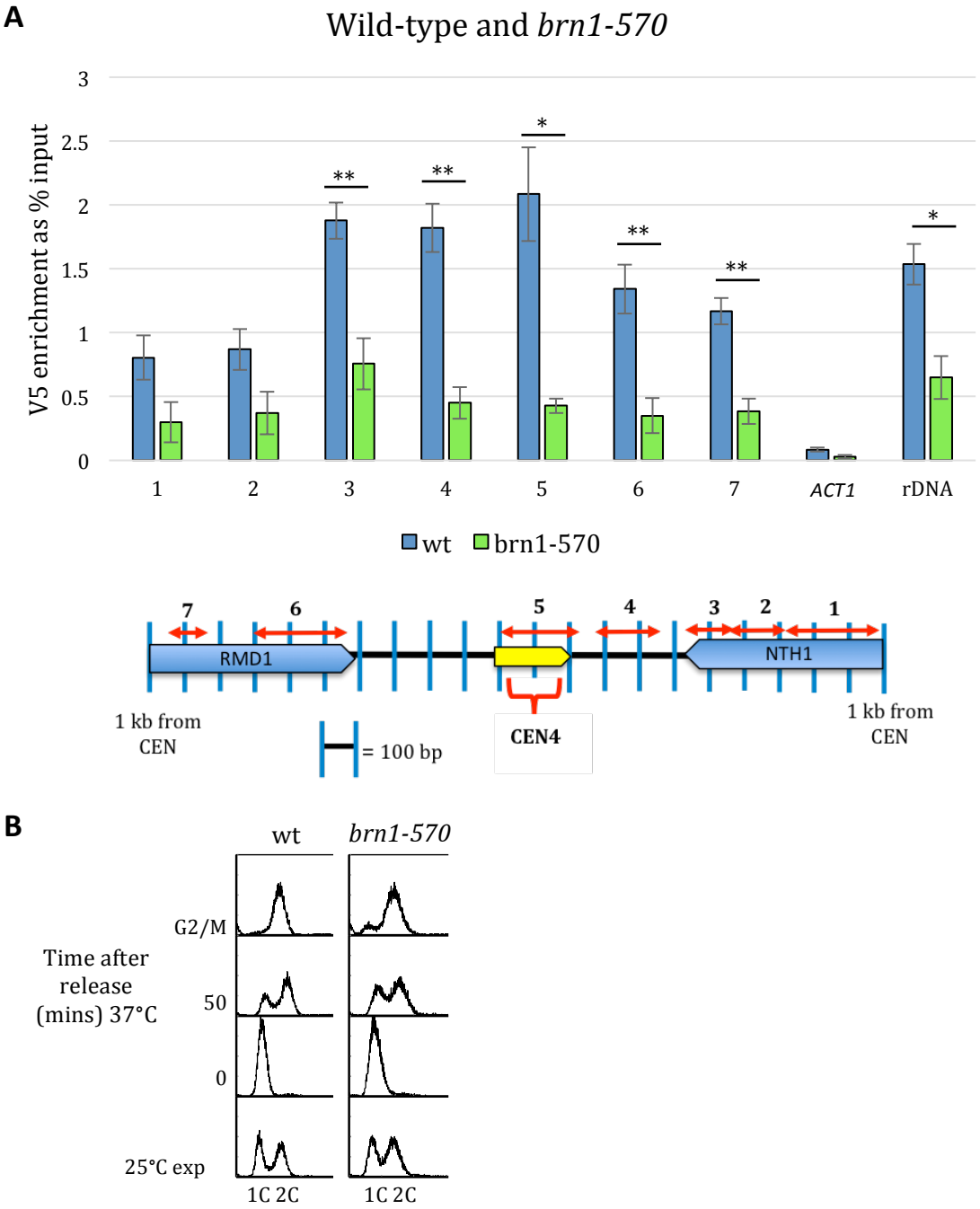
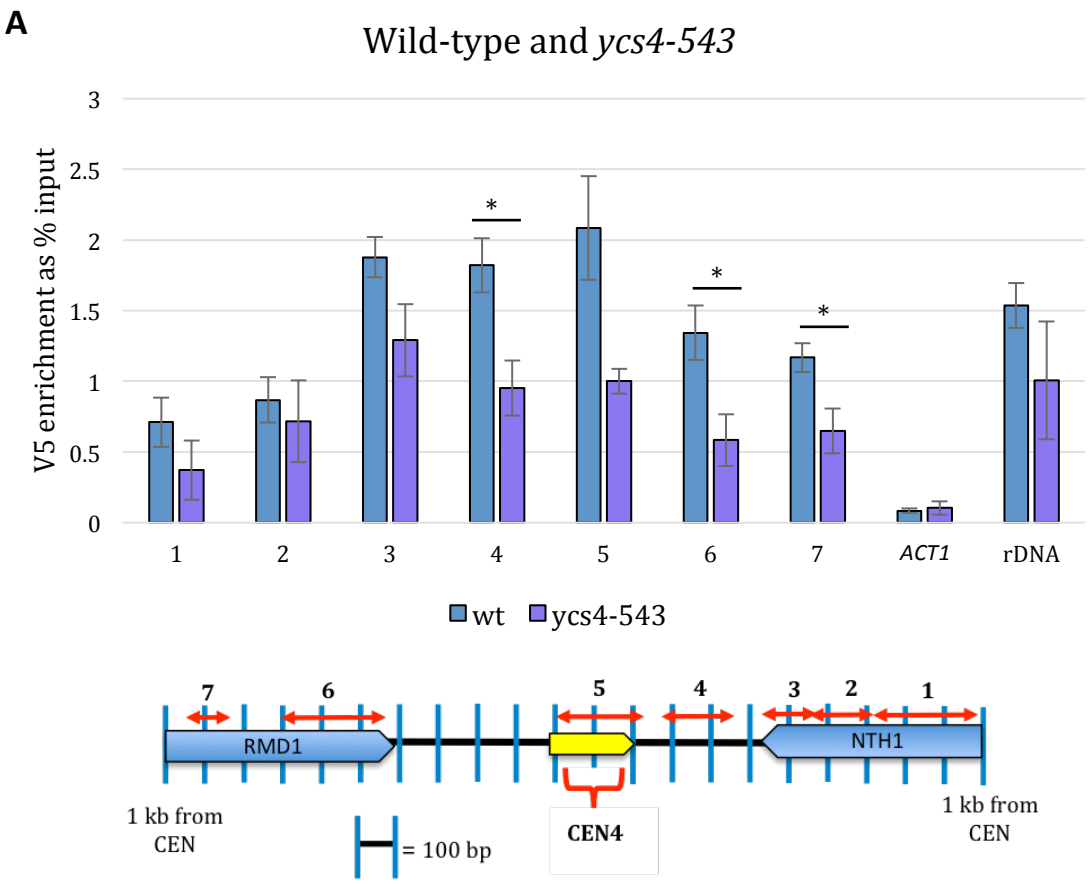


Figure 5.2 Mutations inactivating Brn1 phosphorylation sites results in a significant loss of condensin enrichment on chromatin.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate and ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *brn1-570* (1073) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.



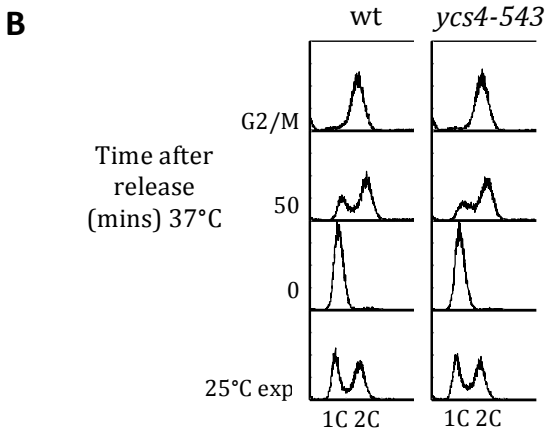


Figure 5.3 Mutations inactivating Ycs4 phosphorylation sites results in a significant loss of condensin enrichment at centromeric regions.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05.**B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *ycs4-543* (1074) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

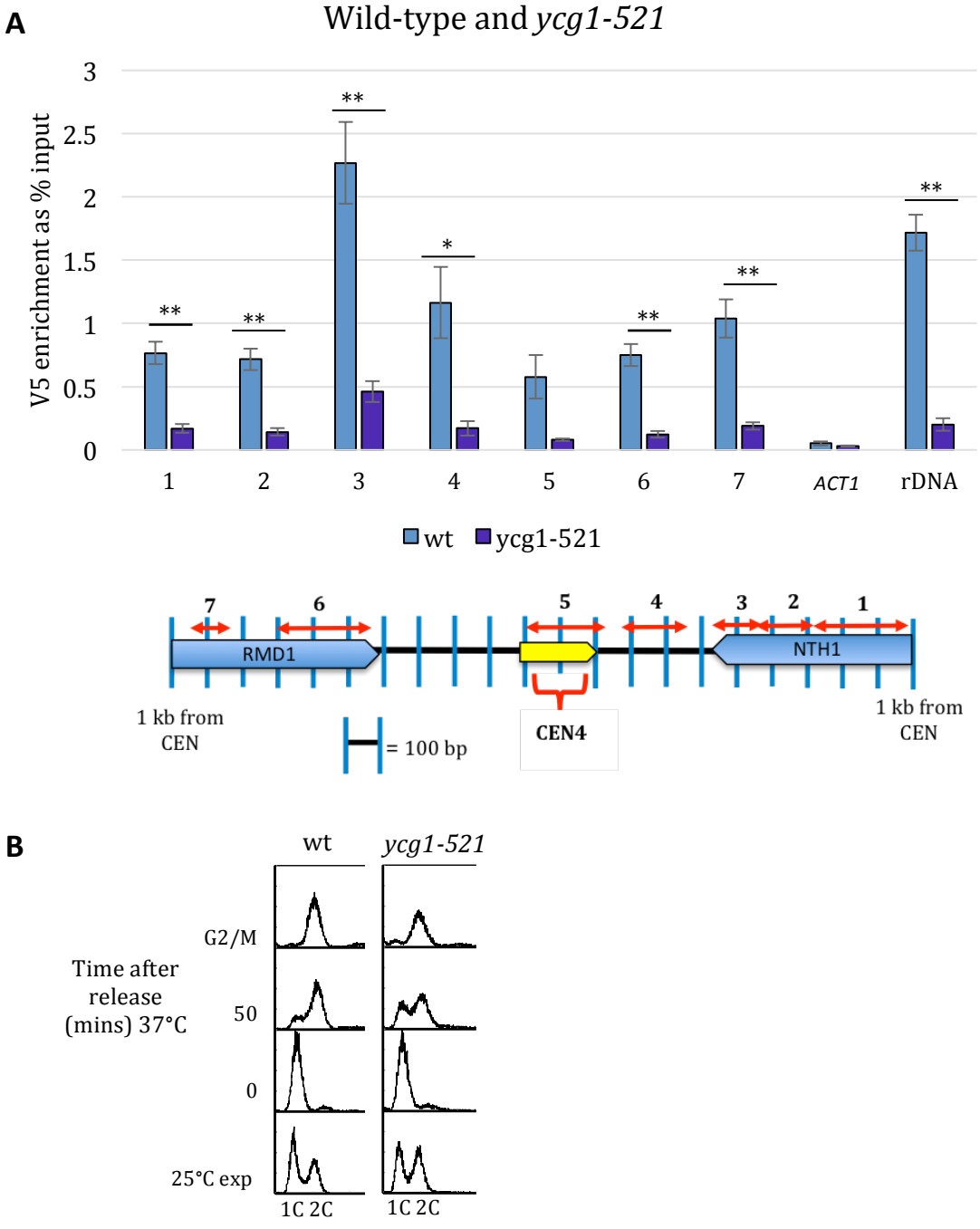


Figure 5.4 Mutations inactivating Ycg1 phosphorylation sites results in a significant loss of condensin enrichment on chromatin.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of wild-type (tet-degron wt 1291) and *ycg1-521* (1110) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

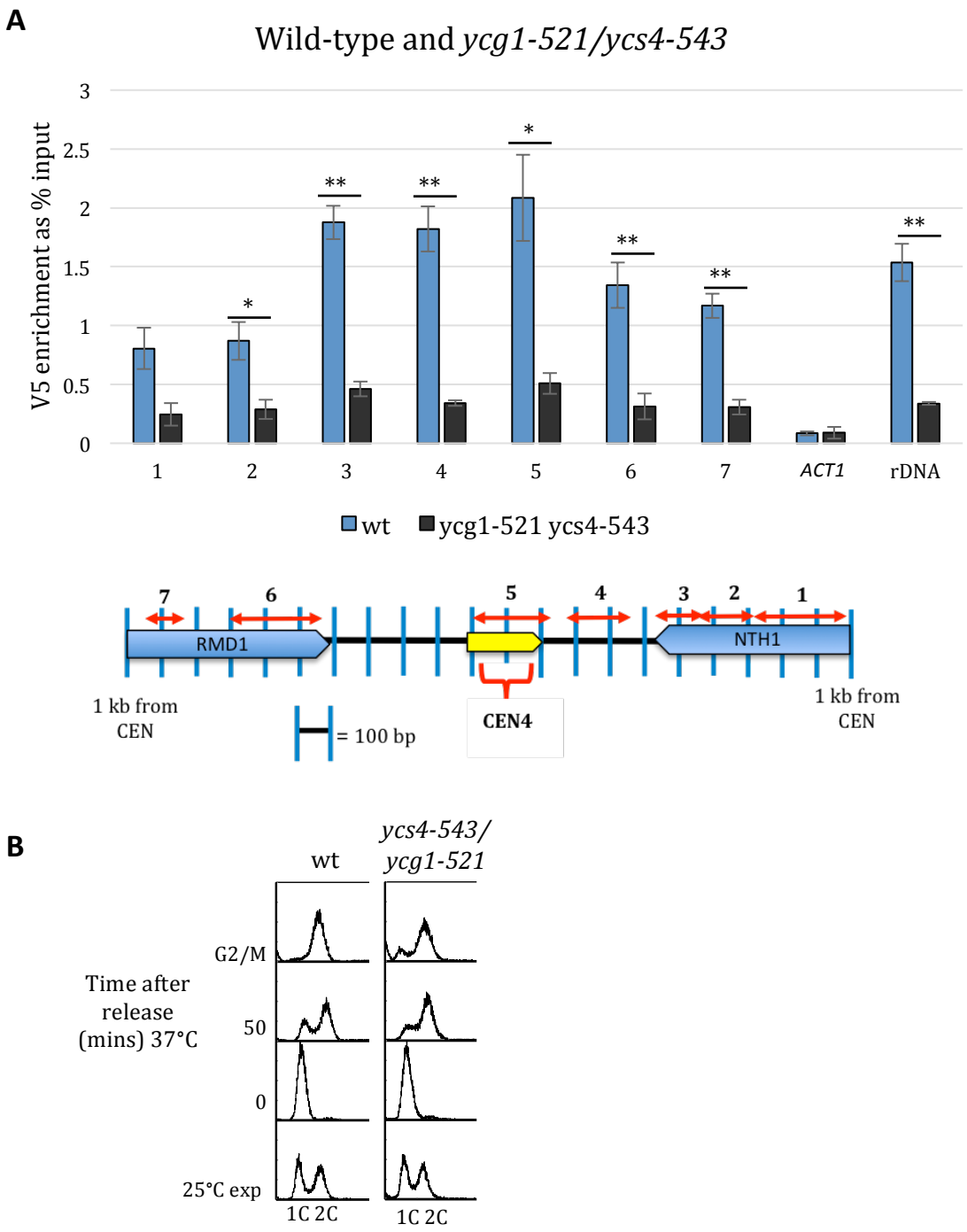


Figure 5.5 Loss of condensin enrichment in the *ycg1-521/ycs4-543* double mutant is similar to the loss of enrichment observed in the *ycg1-521* single mutant.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *ycg1-521/ycs4-543* (1077) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

were processed using a defined preparation procedure (see 2.5.7.1). In figures 5.2, 5.3 and 5.4 the ChIP data for phosphorylation site mutants (*brn1-570, ycs4-543 and ycg1-521 respectively)* all show clear loss of condensin enrichment compared to the enrichment seen in wild-type cells. All three mutants show no loss of enrichment at the negative control *ACT1* gene locus. Interestingly, while the ChIP analysis of the *ycs4-543* mutant (figure 5.3) revealed a clear loss of condensin enrichment across the centromere, there was little difference between the *ycs4-543* and wild-type condensin enrichment levels at primer regions 1 and 2 and at the rDNA. The loss of condensin enrichment is more pronounced in the *brn1-570* and *ycg1-521* mutants than the *ycs4-543*. When examining condensin association with chromatin in the double mutant *ycg1-521/ycs4-543* we expected to see a loss of condensin enrichment at least as severe as that which we observed for the *ycg1-521* mutant. In figure 5.5 we can see that indeed, the loss of enrichment in *ycg1-521/ycs4-543* compared to wild-type is similar to that seen for the *ycg1-521* mutant.

Next, we were interested to see whether the significant loss of condensin enrichment in these phosphorylation site mutants was reflected by a loss of condensin function. Each condensin subunit is essential for viability in *S. cerevisiae*. Therefore, we conducted a spot test to investigate the growth phenotype of these phosphorylation site mutant strains. We can see in figure 5.6A that all mutants are viable at both 25°C and 37°C degrees, with a very slight growth defect seen for the *ycs4-543/ycg1-521* double mutant at 37°C. A common phenotype of condensin mutants is the miss-segregation of chromosomes resulting in aneuploidy. I used FACS analysis to investigate whether this phenotype was present in the non-SMC phosphorylation site mutants (figure 5.6B). Exponentially growing cells were arrested in G1, and released to progress through the cell cycle. Cell samples were taken every 20 minutes for 160 minutes and processed for analysis by FACS. FACS analysis of these mutants (figure 5.6B) revealed a slight delay in exit from G1 for *brn1-570* and *ycg1-521* but no evidence of miss-segregation of chromosomes following mitosis for any of the phosphorylation site mutants. This data shows that condensin activity in these mutants, (including the double mutant) is not impaired to an extent at which we see aneuploidy by FACS.

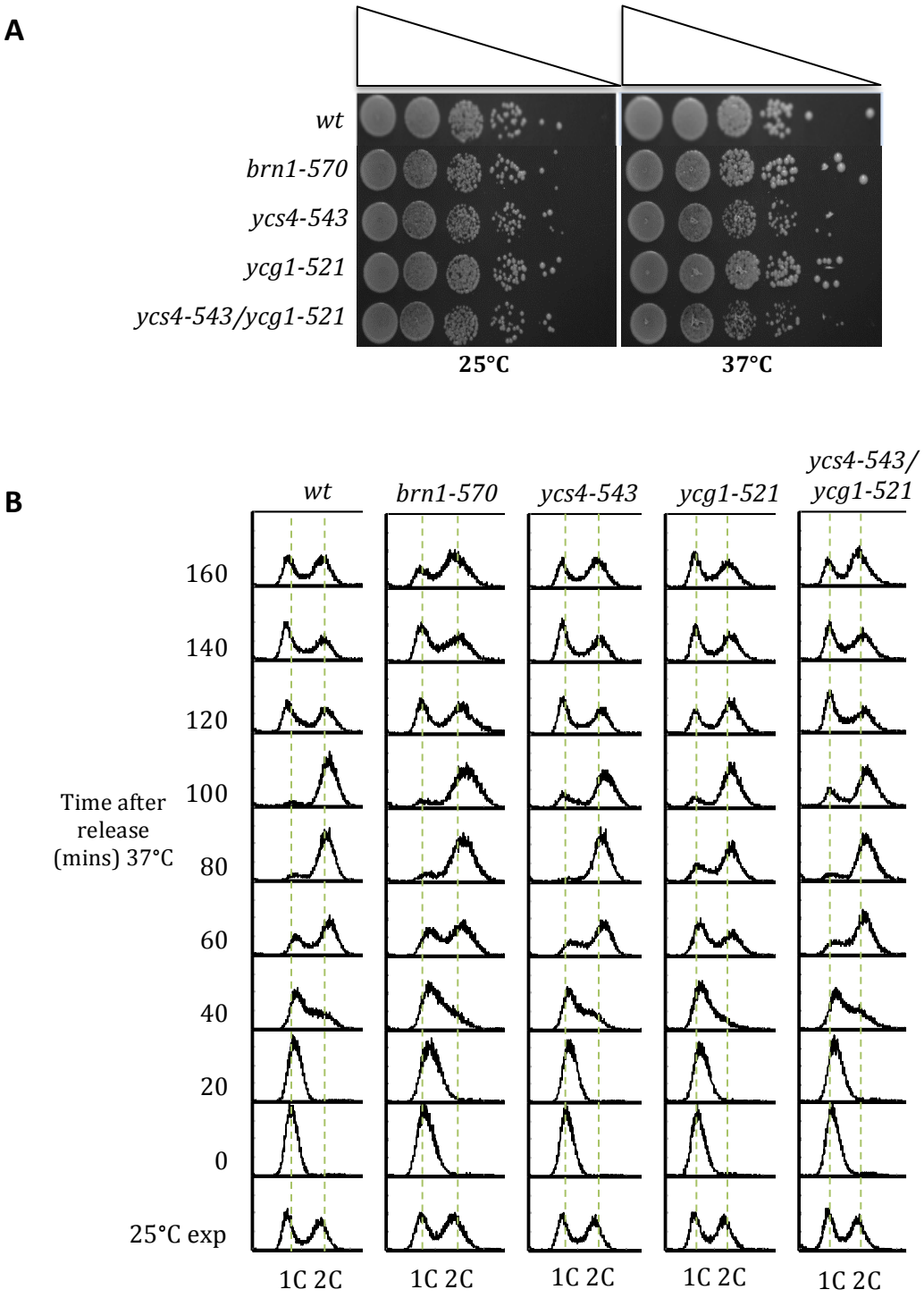


Figure 5.6 Characterisation of non-SMC phosphorylation site mutants

A) Spot test showing the growth phenotype of phosphorylation site mutants. 10-fold serial dilutions of exponentially growing cells were plated onto YPD solid media and incubated at 25°C or 37°C for up to 48 hours. **B)** FACS data showing the progression of wt (708), *brn1-570 (1073), ycs4-543 (1074), ycg1-521 (1078) and ycs4-543/ycg1-521 (1077)* double mutant through the cell cycle. Exponentially growing cells were arrested in G1 using alpha factor mating pheromone, placed under restrictive conditions (galactose at 37°C) before being realised to progress through the cell cycle. Cell samples for FACS analysis were taken just before release (0 min) and every 20 minutes after for 160 minutes.

This finding is in agreement with the original paper which shows there are no obvious rDNA segregation defects in the individual subunit mutants (St-Pierre *et al.* 2009). So, while we know that phosphorylation is important for condensin activity, the loss of these phosphorylation sites on individual subunits, and even on two of these subunits is not enough to cause a loss of condensin function in a way that causes chromosome segregation defects or affects cell viability. The mutation of the phosphorylation sites in condensin non-SMC subunits results in a significant loss of condensin enrichment on chromatin while we see little evidence for loss of condensin function.

5.1.3 Cdc5/polo-like kinase is not required for condensin enrichment on chromatin

We've shown that the putative Cdc5 phosphorylation sites on non-SMC subunits are important for condensin association on chromatin in mitosis, next we wanted to investigate the role of Cdc5. To examine the effect of perturbing Cdc5 kinase on condensin enrichment we used the *cdc5-99* mutant strain (characterised by (St-Pierre *et al.* 2009)) for analysis by ChIP. Pierre *et al.* (2009) have demonstrated that the mitosis-specific phosphorylation-induced gel retardation of Ycg1, Ycs4 and Brn1 was lost in the *cdc5-99* mutant allele, and so accordingly we expected to see a loss of condensin enrichment. Surprisingly, the result from the ChIP analysis showed no significant difference between condensin enrichment in the *cdc5-99* strain when compared with the wild-type, at all loci tested (figure 5.7). Due to the unexpected nature of this finding, we sought to confirm it by using a second Cdc5 mutant allele, *cdc5-10*. As can be seen in figure 5.8, the ChIP analysis of condensin enrichment in *cdc5-10* mutant cells showed that, in agreement with our findings using the *cdc5-99* allele, there is no significant difference in levels of condensin enrichment between wild-type cells and cells in which Cdc5 is perturbed. Therefore, we have demonstrated that Cdc5 kinase is not required for condensin chromatin enrichment at the centromere or rDNA as seen by ChIP in mitosis.

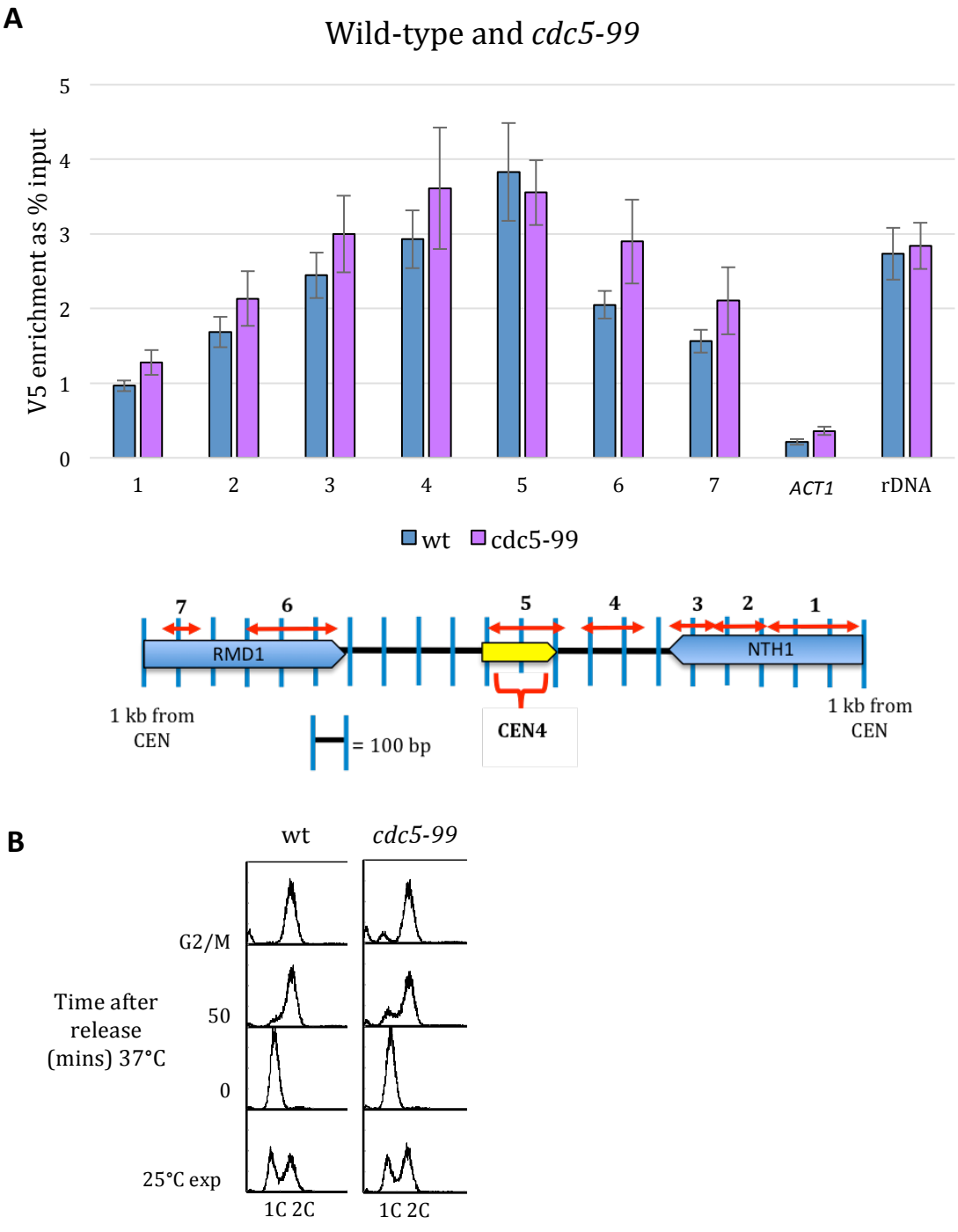


Figure 5.7 Cdc5 is not required for condensin enrichment on chromatin in mitosis (*cdc5-99* mutant allele)

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *cdc5-99* (1065) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

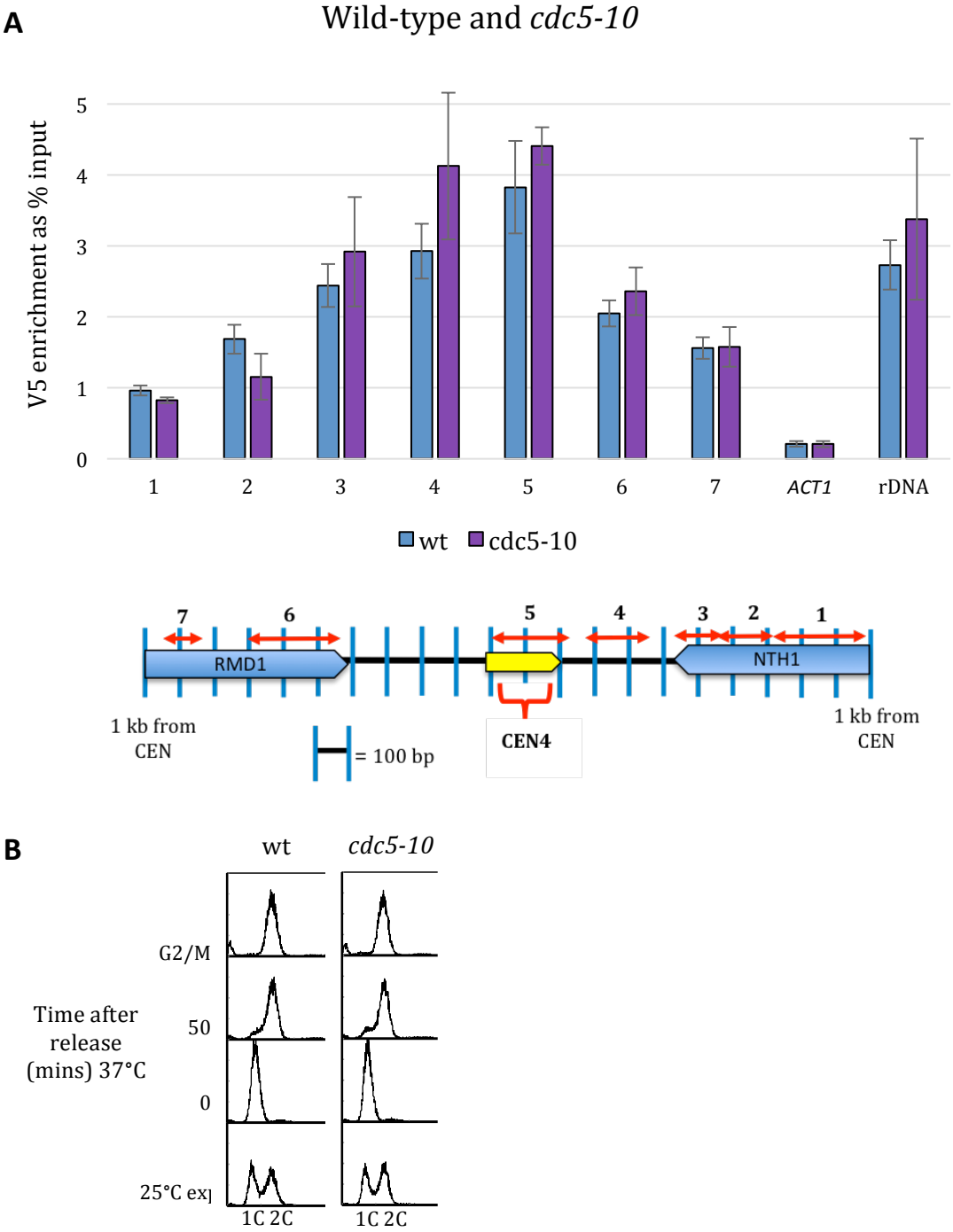


Figure 5.8 Cdc5 is not required for condensin enrichment on chromatin in mitosis (*cdc5-10* mutant allele)

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *cdc5-10* (1064) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

5.1.4 Ipl1/Aurora B kinase is required for condensin association with the pericentromere in mitosis

The current model in *S. cerevisiae* suggests that Ipl1 kinase acts upstream of Cdc5 to facilitate phosphorylation of condensin non-SMC subunits (St-Pierre *et al.* 2009). However, we've shown that the while these phosphorylation sites are important for the mitotic association of condensin with chromatin Cdc5 kinase is dispensable. Our data therefore so far doesn't fit with the model that predicts that Cdc5-dependent phosphorylation of condensin is required for condensin enrichment in *S. cerevisiae* mitosis. Condensin phosphorylation has been shown to be partially dependent on Ipl1 in *S. cerevisiae* (St-Pierre *et al.* 2009). Therefore, we next wanted to test whether perturbing Ipl1 has an effect on condensin enrichment. To gauge the influence that Ipl1 has on condensin enrichment on chromatin, we used the *ipl1-321* mutant (generated and characterised by (Biggins *et al.* 1999)) for analysis by ChIP. As evident in figure 5.9 there is a distinct loss of condensin enrichment for the *ipl1-321* mutant at multiple loci when compared to wild-type. Notably, there is no significant difference between *ipl1-321* and wild-type directly at the centromere (loci 5) and at the rDNA.

We then probed for an indication of a genetic interaction between Ipl1 and condensin. I used partial depletion spot tests (figure 5.9C) in which varying temperatures and concentrations of doxycycline were used in an attempt to incrementally impair protein function. I made double mutant strains containing both the *ipl1-321* and *smc2-td* mutations and plated these alongside wild-type and the two single mutant strains. The idea is that by partially perturbing the function of both proteins, if they act in the same pathway then the double mutants should exhibit a growth defect or loss of viability far greater than that of the single mutants. The spot test revealed no indication of a genetic interaction between Ipl1 and condensin.

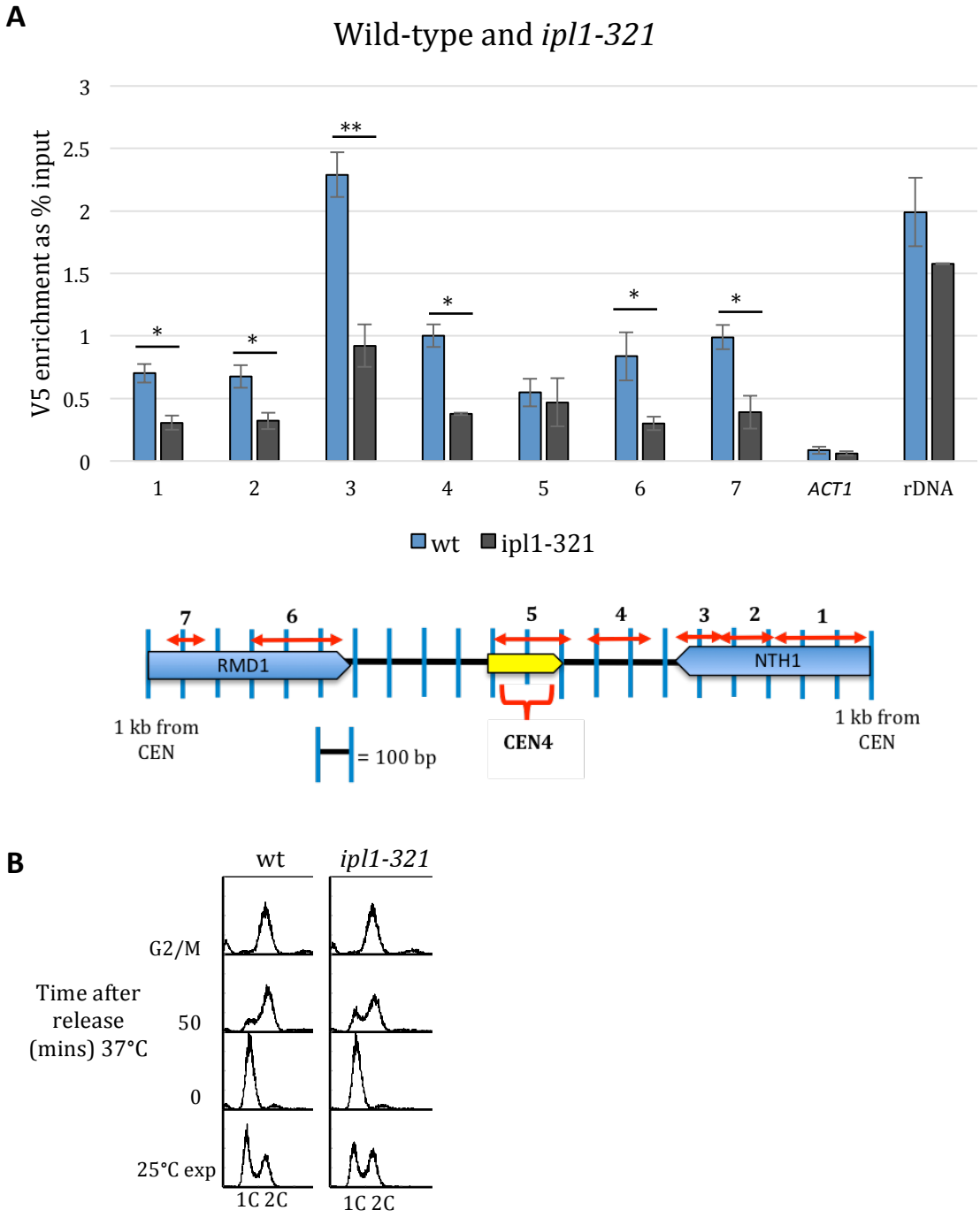


Figure 5.9 Ipl1 is required for the mitotic enrichment of condensin at the pericentromere

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 1291) and *ipl1-321* (1108) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

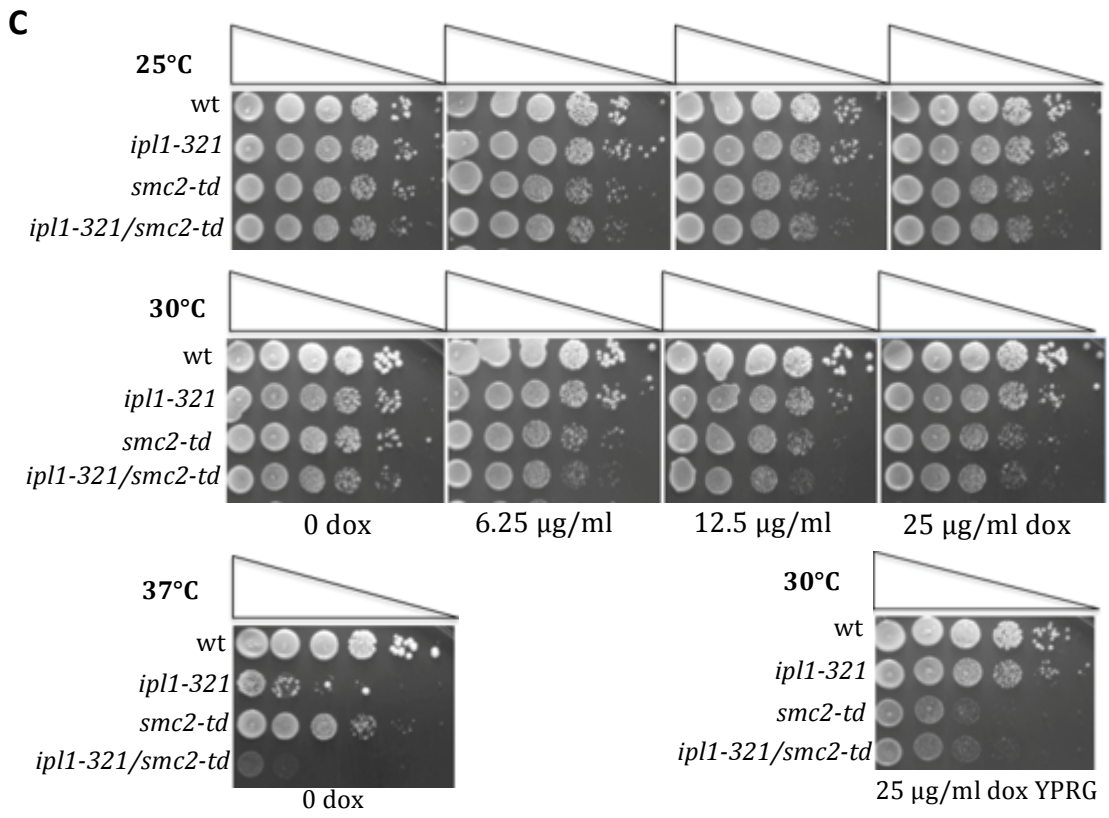


Figure 5.9 continued.

C) Spot test Spot test showing the cell viability of wild-type, *ipl1-321* (360), *smc2-td* (278) *and ipl1-321/smc2-td* (358) strains under various growth conditions. 10-fold serial dilutions of exponentially growing cells were plated onto YPD solid media with no doxycycline, 6.25 µg/ml, 12.5 µg/ml or 25 µg/ml doxycycline, or YPRG with 25 µg/ml doxycycline and incubated at either 25°C, 30°C or 37°C for up to 48 hours.

5.1.5 Cdc7/DDK (Dbf4-dependent kinase) is not required for the mitotic enrichment of condensin on chromatin

Ipl1 is important for condensin enrichment but not thought to directly phosphorylate the complex. We've also demonstrated that Cdc5, the proposed intermediary of Ipl1 activity, is not necessary for condensin enrichment. Perhaps then, there is another kinase acting downstream of Ipl1 that is important for the phosphorylation of condensin non-SMC subunits, and its enrichment on chromatin. If another kinase is able to phosphorylate the non-SMC subunits, this could explain why the proposed Cdc5 phosphorylation sites, but not the Cdc5 kinase, are required for condensin

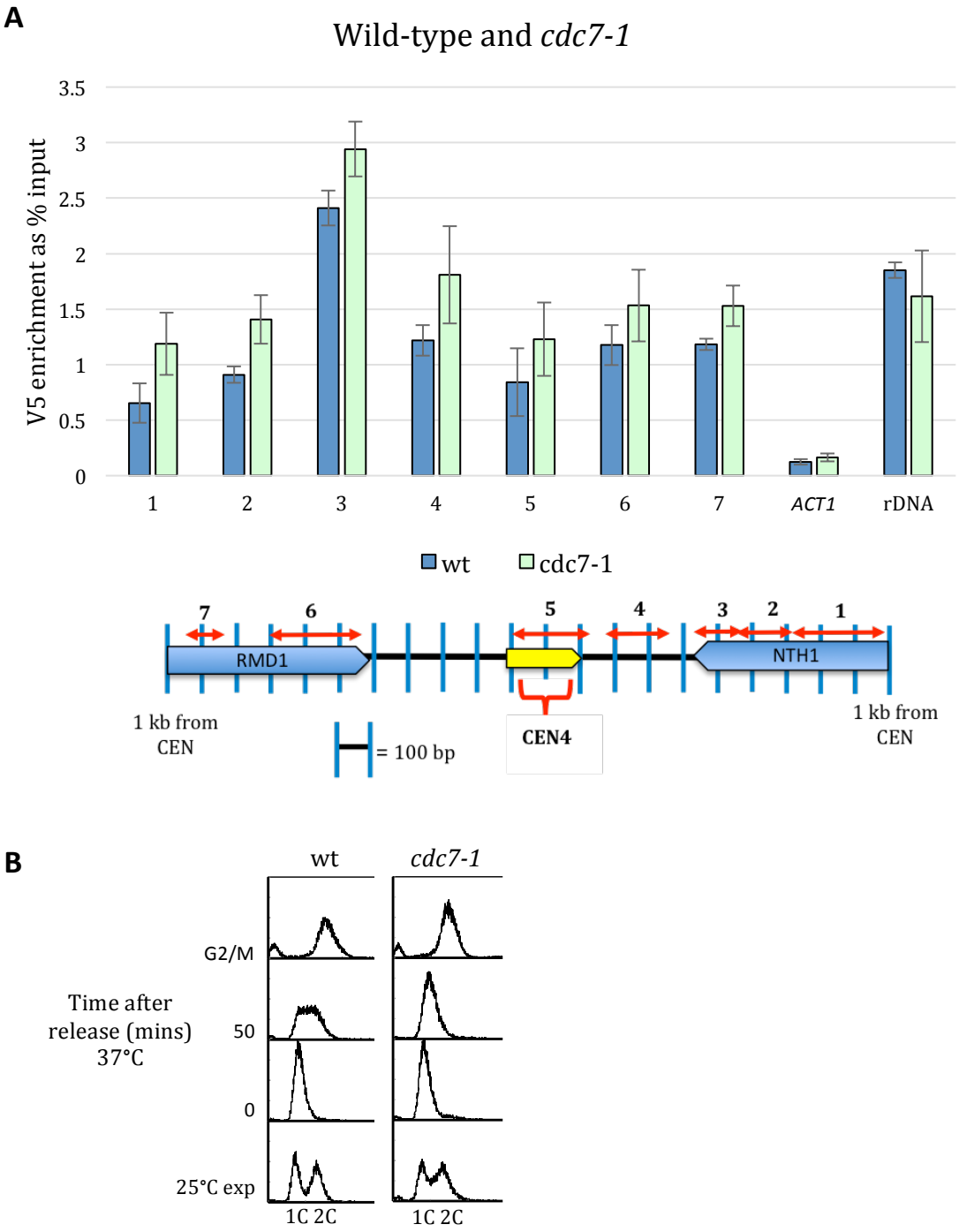


Figure 5.10 Cdc7 is not required for the mitotic enrichment of condensin on chromatin.

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were not processed according to the standard block and release protocol. Cells are arrested in G1 using alpha factor mating pheromone then released into and a subsequent G2/M block using nocodazole, restrictive conditions were implemented once budding was apparent in c*dc7-1*, ~ 60 mins after G1 release. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *cdc7-1* (1072) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

enrichment on chromatin. In meiosis there is some evidence of functional crossover between Cdc5 and another kinase known as Cdc7/DDK, in the recruitment of the monopolin complex to kinetochores (Matos *et al.* 2008). With this in mind, we speculated that perhaps there is a similar functional crossover with the regulation of condensin, in mitosis. To test a possible role of Cdc7 in the chromatin enrichment of condensin during mitosis, we used ChIP to assess the enrichment levels of condensin in the *cdc7-1* mutant. Cdc7 is important for initiation of replication, and the inactivation of the kinase results in a G1 arrest (Donaldson *et al.* 1998). Therefore, we had to alter the standardised cell culture protocol (2.5.7.1) for this experiment. Exponentially growing cells were blocked in G1 using alpha factor mating pheromone, then released into a subsequent G2/M arrest using nocodazole. Cells were subjected to restrictive conditions once S-phase was underway (as assessed by the presence of budding in *cdc7-1* cells). Figure 5.10 shows that there was no significant difference between levels of condensin enrichment in *cdc7-1* and wild type cells. Cdc7 is therefore not a requirement for condensin enrichment during mitosis.

5.1.6 Mutations in *SMC4* phosphorylation sites reduce condensin association with chromatin

It has been shown that Smc4 is targeted by CDK1 and the phosphorylation of this subunit is crucial in condensin function (Robellet *et al.* 2015). However, it is not known how the phosphorylation of Smc4 affects condensin association with chromatin. Therefore, next we wanted to investigate the role of phosphorylation of the Smc4 subunit in the mitotic enrichment of condensin. To do so, we used an Smc4 phosphorylation site mutant allele *smc4-10A* (figure 5.11A - generated and previously characterised by (Robellet *et al.* 2015). I demonstrated the ablation of phosphorylation site serine 4 by western blot using a phospho-specific antibody (specified in 2.6.2.1) and provided by (Robellet *et al.* 2015). The serine 4 phosphorylation of Smc4 detectable in the wild-type strain is not present in *smc4-10A* (figure 5.11B).

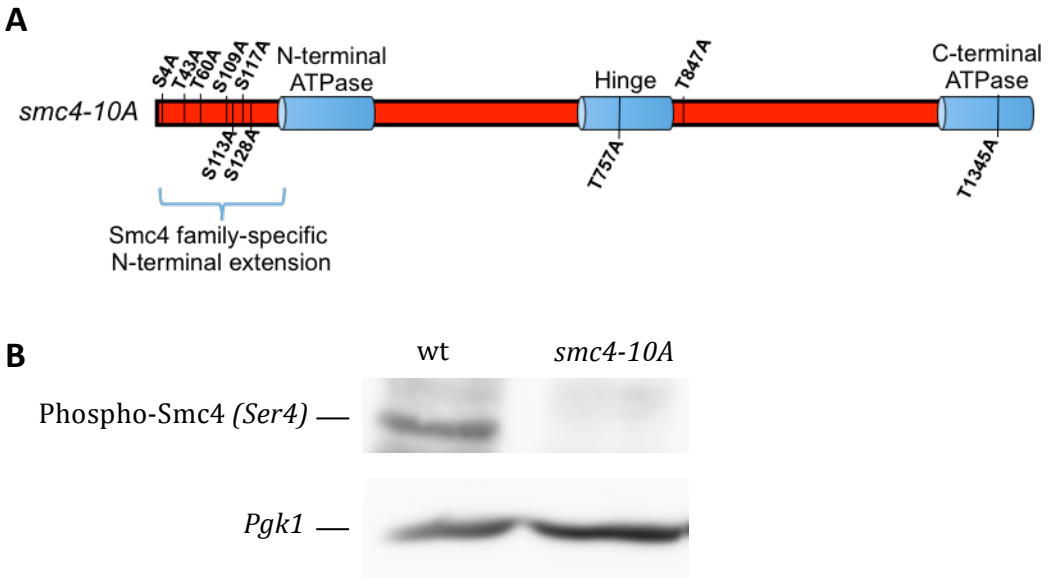


Figure 5.11 Characterisation of *smc4-10A* phosphorylation site mutant

A) Schematic representation of the *smc4-10A* mutant adapted from a figure in (Robellet *et al.* 2015). **B)** Western blot analysis demonstrates the loss of phosphorylation at serine 4 in the *smc4-10A* mutant. Wt (tet-degron wt 708) and *smc4-10A* (1070) cells were synchronised in G2/M using nocodazole. Samples were taken and prepared for western blot analysis using whole-cell TCA extraction (detailed in 2.6.1). Western blot was undertaken as detailed in 2.6.2 and samples were run on a 6% polyacrylamide gel.

I then conducted a ChIP analysis of *smc4-10A* to assess whether the mutation of phosphorylation sites on Smc4 affected condensin association with chromatin. The ChIP analysis (figure 5.12A) showed that the loss of Smc4 phosphorylation sites resulted in a loss of condensin enrichment on chromatin at all loci investigated, with the exception of the negative control actin locus. Therefore, Smc4 phosphorylation sites are required for the mitotic enrichment of condensin on chromatin.

We wanted to see whether the loss of condensin enrichment would generate a growth phenotype. The original characterisation of the mutant showed the *smc4-10A* mutant to have growth defects at 37°C (Robellet *et al.* 2015), however we were unable to replicate this finding. A spot test to assess the temperature sensitivity of the mutant (figure 5.12C) showed no obvious growth defect at 25°C or 37°C suggesting that these phosphorylation sites are not vital for condensin function even when pushed to higher temperatures.

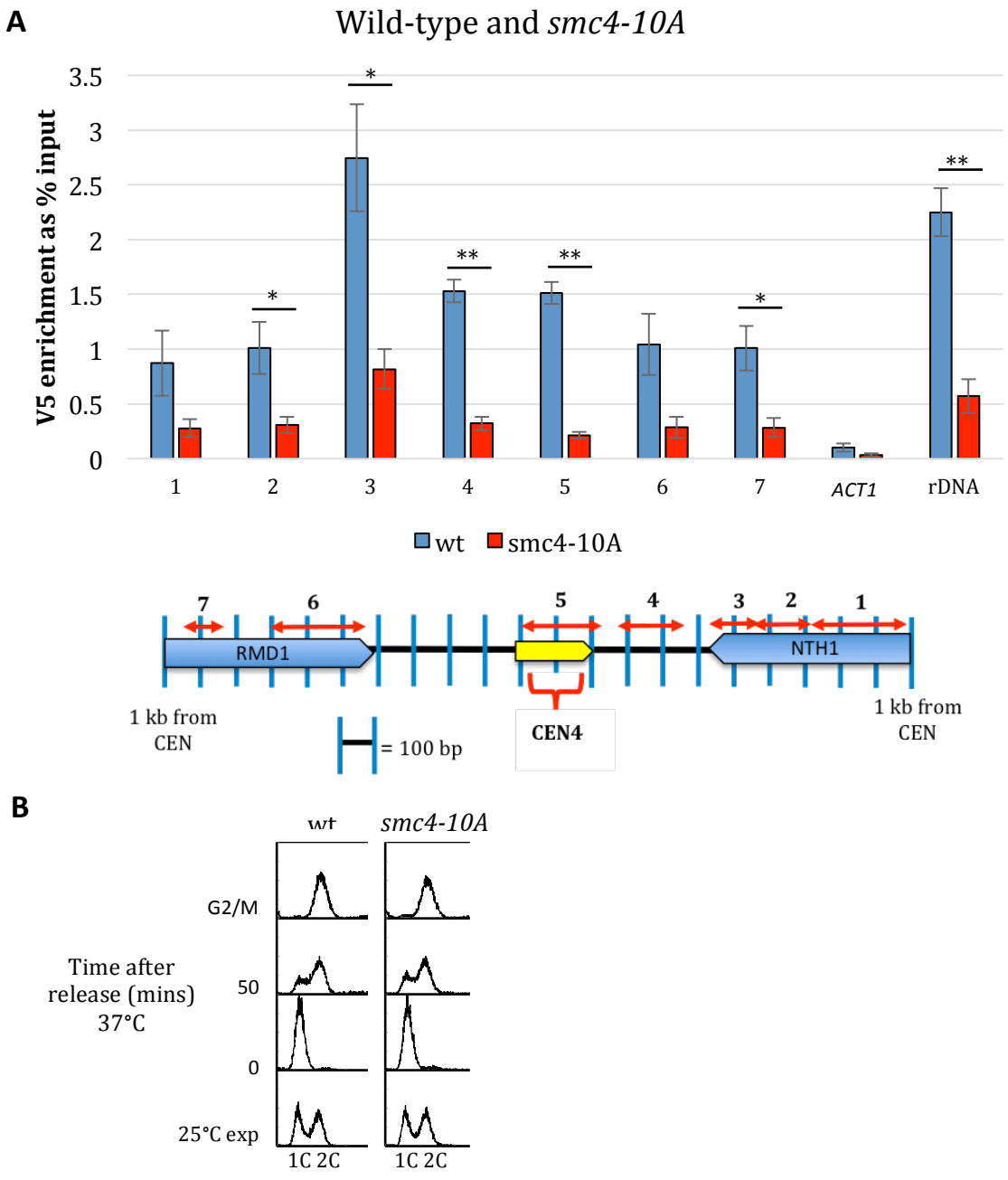


Figure 5.12 Mutations in Smc4 phosphorylation sites significantly reduce condensin association with chromatin

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 **B)** Representative FACS data showing the progression of wild-type (tet-degron wt 1291) and *smc4-10A* (1109) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.(figure continued on following page)

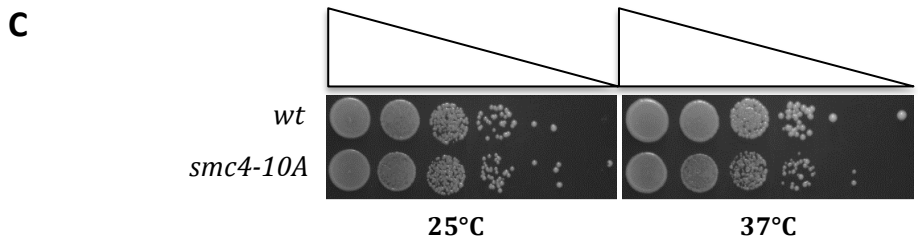


Figure 5.12 continued

C) Spot test showing the cell viability of phosphorylation site mutant *smc4-10A* (1070) compared to wt (708). 10-fold serial dilutions of exponentially growing cells were plated onto YPD solid media and incubated at 25°C or 37°C for up to 48 hours.

Smc4 proteins have an N-terminal extension not present in other SMC proteins. As evident in figure 5.11A the majority of Smc4 phosphorylation sites reside in the Smc4 N-terminal extension. To further probe the role of smc4 phosphorylation sites, I used an Smc4 N-terminal delete mutant (constructed by S. Schalbetter) to see whether removal of the N-terminal region containing most of the phosphorylation sites would have a similar effect on condensin enrichment as the mutation of the sites themselves. The deletion of the Smc4 N-terminal extension is lethal (Robellet *et al.* 2015). Therefore, the mutated version of the Smc4 gene (with the N-terminus deleted up to and including Arg153) was placed ectopically, under the control of a *GAL* promoter in an *smc4-td* tetracycline-repressible Smc4 degron mutant strain. In the resulting yeast strain (*smc4_n_termΔ*) endogenous *SMC4* can be conditionally depleted (as detailed in 3.1.4), and the mutated Smc4 with the deleted N-terminus can be conditionally expressed.

To characterise the growth phenotype of *smc4_n_termΔ,* I conducted spot tests (figure 5.13A) in which I compared its growth to the *Smc4 o/e* (over expression) strain. *Smc4 o/e* contains the *smc4-td* degron and ectopically expresses wild type Smc4 under the control of a *GAL* promoter. I also included the *smc4-td* mutant strain that allows conditional depletion of endogenous *SMC4* but does not ectopically express any variant of Smc4. On YPD at both 25°C and 37°C all three yeast strains have similar growth phenotypes. Under conditions that facilitate the conditional depletion of endogenous Smc4, and conditional expression of the endogenous *SMC4 or* N-terminus deleted Smc4 (figure 5.11A panel 3), the *smc4-td* and *smc4_n_termΔ* mutants were

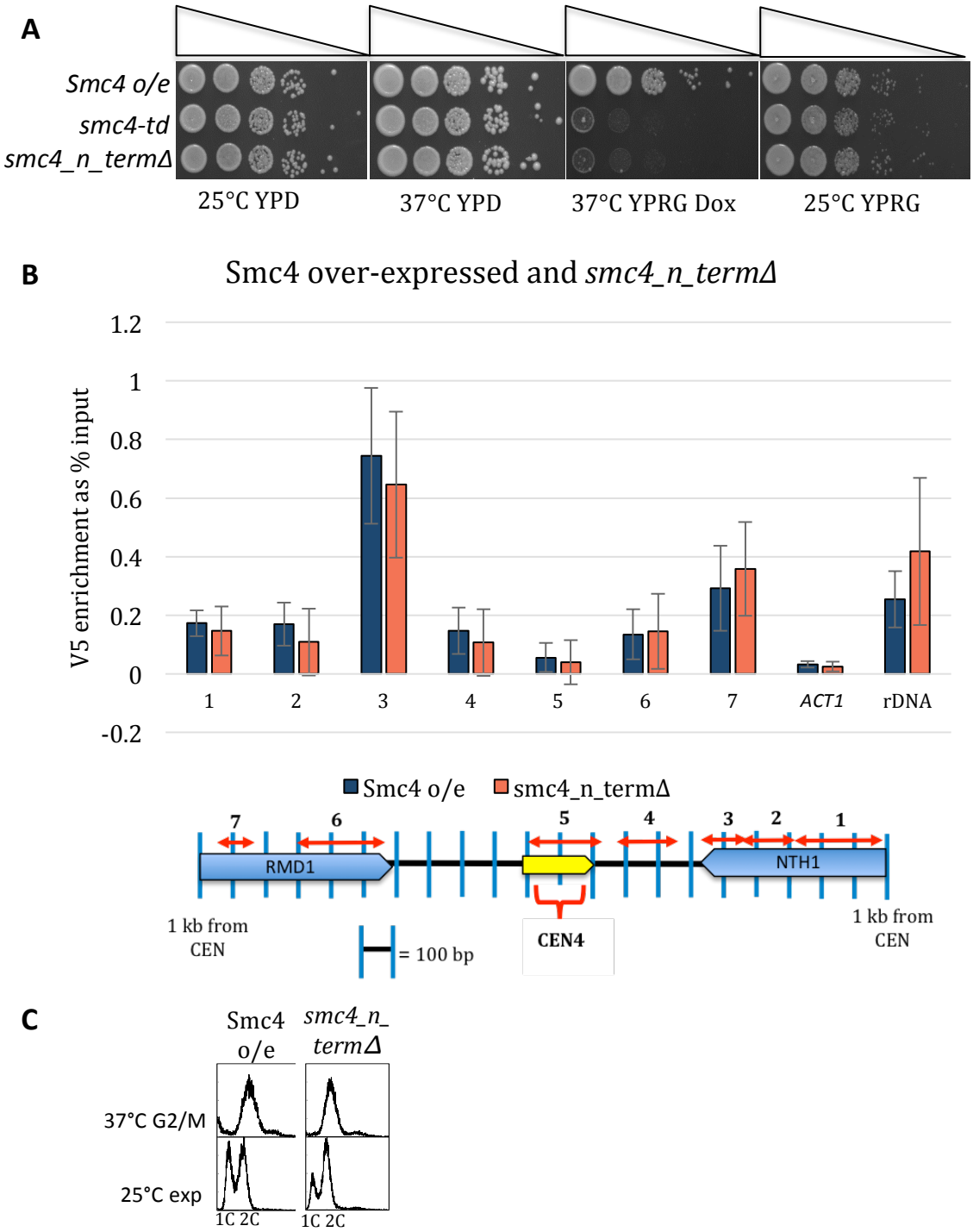


Figure 5.13 Conditional expression of *smc4_n_termΔ* does not affect condensin enrichment on chromatin

A) Spot test showing the growth phenotype of *Smc4 o/e* (1112), *smc4-td* (1111) and *smc4_n_termΔ (1113).* 10-fold serial dilutions of exponentially growing cells were plated onto YPD or YPRG solid media with and without 25 µg/ml doxycycline and incubated at 25°C or 37°C for up to 48 hours. **B)** Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Exponentially growing cells were arrested in G2/M using nocodazole and subjected to restrictive conditions for 1.5 hours. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. **C)** Representative FACS data showing *Smc4 o/e* (1112) and *smc4_n_termΔ (1113) as* 'exp' = exponentially growing cells and at to the nocodazole block the G2/M time point taken just prior to cell fixation.

inviable whilst the *Smc4 o/e* was still growing almost as well as in wild-type conditions. This shows that as expected, over-expression of wild-type *SMC4* but not N-terminus deleted Smc4 rescues the conditional depletion of Smc4. I also tested whether the over expression of the N-terminal delete allele of Smc4 was dominant negative by using conditions that facilitate the over expression of the ectopic Smc4 but not the depletion of endogenous Smc4 (right panel). The spot test revealed that the over expression of Smc4 N-terminal delete mutant had a similar growth phenotype to *Smc4 o/e* and is therefore not dominant negative. Next we analysed the N-terminal delete mutant alongside *Smc4 o/e* by ChIP. The *smc4_n_termΔ* mutant strain had a G1 release defect and so after several attempts at processing these yeast strains using the standardised cell culture protocol (see 2.5.7.1), we had to alter the cell culture treatment. Exponentially growing cells were therefore treated with nocodazole and once synchronised in a G2/M arrest, were placed in restrictive conditions for 1.5 hours before being fixed with formaldehyde and processed for analysis by ChIP. The ChIP data (figure 5.13B) showed no significant difference between the *Smc4 o/e* and *smc4_n_termΔ* mutant, suggesting that the loss of this n terminal domain is not vital for condensin enrichment. However, because we could not perform the standardised cell culture protocol we would need to confirm the depletion of endogenous Smc4 by western blot to verify this finding.

5.2 Discussion

Phosphorylation has long been implicated in facilitating condensin's association with chromatin. Early in *vitro* work showed that adding a broad-range kinase inhibitor to *Xenopus* mitotic egg extract completely blocked the chromosomal targeting of condensin (Hirano *et al.* 1997). One model proposed that the phosphorylation of Histone H3 at serine 10 (which is highly correlated with mitotic chromosome condensation (Wei *et al.* 1999)), acted to recruit condensin to chromatin in *S. pombe* (Petersen and Hagan 2003). However, no phosphorylation-dependent interaction was evident between condensin and histone H3 in *Xenopus* extracts (Kimura and Hirano

2000). Another model proposes Aurora B-dependent phosphorylation of condensin CAP-H directs condensin loading onto histone H2A and H2AZ in *S. pombe (Tada et al. 2011).* However, recent studies suggest that condensin preferentially binds nucleosome-free DNA *in vivo* (Sutani *et al.* 2015, Toselli-Mollereau *et al.* 2016).

In this chapter we have explored the effects of perturbing condensin phosphorylation sites and mitotic kinases on condensin association with chromatin. Although the mechanism by which phosphorylation facilitates condensin's association with chromatin remains unclear, we have demonstrated that the direct phosphorylation of condensin subunits is required. In line with the notion that phosphorylation is an important regulator of condensin function (as discussed in 1.4.4.2), the mutation of phosphorylation sites in the non-SMC subunits and in Smc4 lead to a significant loss of condensin association with chromatin in mitosis (figures 5.2-5.5 and 5.12). Interestingly, the loss of phosphorylation sites on Brn1 and Ycg1 appears to cause a greater loss of condensin enrichment than that of the Ycs4 mutant. Suggesting that perhaps the phosphorylation of Ycs4 is less important for the association of condensin with chromatin.

5.2.1 Ipl1 but not Cdc5 is required for condensin association with chromatin in pre-anaphase mitosis

The current model of phosphorylation and activation of condensin in *S. cerevisiae* is that CDK1 phosphorylation of Smc4 is the primary activation event, followed by hyper-activation of the complex via direct phosphorylation by Cdc5 downstream of Ipl1 in late mitosis (anaphase). In contrast to this model, I have demonstrated using ChIP analysis that Ipl1 but not Cdc5 is required for condensin enrichment on chromatin (figures 5.7-5.9). This is unexpected considering that we have demonstrated that the loss of the putative Cdc5 phosphorylation sites on non-SMC subunits causes a significant loss of condensin enrichment. Furthermore, the *cdc5-99* allele used in this study, has been previously demonstrated to cause a considerable reduction in the levels of phosphorylation of the non-SMC condensin subunits in mitosis (St-Pierre *et al.*

2009). One explanation for this may be that the point at which we probe for condensin enrichment is just prior to the metaphase-anaphase transition, and Cdc5 may only be important for condensin enrichment once anaphase commences (Figure 5.14, Model 1). Potentially, we are looking at a point in mitosis that is too early to detect the effects of perturbing Cdc5 on condensin enrichment with chromatin. However, we also demonstrated that Ipl1 is required for condensin enrichment at this point of mitotic arrest (figure 5.9). Previously it has been argued that the role of Ipl1/Aurora B kinase in condensin activity was limited to anaphase in budding yeast (Lavoie *et al.* 2004) similar to as in humans (Mora-Bermudez *et al.* 2007). It therefore seems unlikely that we would see a loss of condensin enrichment for Ipl1 (supposedly working upstream of Cdc5) but not Cdc5 if the current model were correct. In model 1 we propose that Ipl1 may work independently of Cdc5 in metaphase.

Since Ipl1 does not directly phosphorylate condensin subunits *in vitro* (St-Pierre *et al.* 2009) the current data suggests that Ipl1 is not acting directly on condensin. Therefore, alternatively, in *S. cerevisiae* another kinase may work alongside Cdc5 in the phosphorylation of condensin downstream of Ipl1, leading to its association with chromatin (Figure 5.14, Model 2). We tested to see whether Cdc7 had a role in condensin enrichment with chromatin (figure 5.10), however we saw no significant change in condensin association in the *cdc7-1* mutant allele. However, if Cdc5 were working alongside another kinase in a redundant manner to promote condensin association with chromatin it may be necessary to perturb both kinases to see an effect on the enrichment of the complex. If we had more time we would have liked to complete ChIP analysis of a *cdc5-99/cdc7-1* double mutant to see if this had an effect on condensin enrichment in mitosis. If there was still no change in condensin enrichment values in the *cdc5-99/cdc7-1* double mutant, we could probe other kinases linked to condensin function (see 1.4.4.2). Another alternative kinase that could act downstream of Ipl1 is Mps1. A recent study in *C. elegans* revealed that PLK1 functionally substitutes for Mps1 in SAC checkpoint initiation (Espeut *et al.* 2015). However, we did not have sufficient time to investigate this possibility.

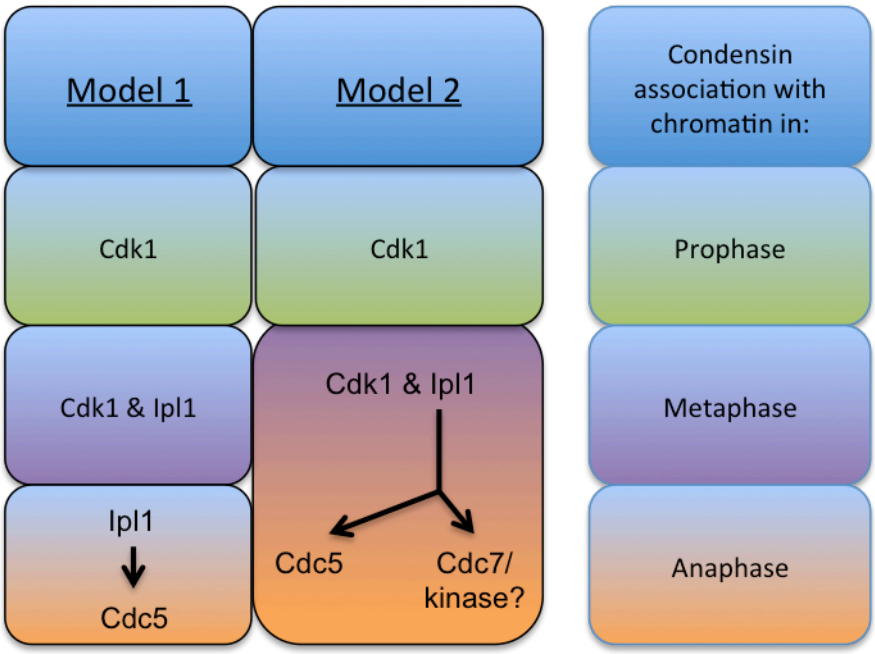


Figure 5.14 Potential models of how mitotic kinases participate in facilitating condensin association with chromatin in *S. cerevisiae* mitosis.

In model 1 Cdk1 phosphorylation of Smc4 is important for condensin association with chromatin up to and including metaphase (after which Cdk1 is degraded). Ipl1 is directly important for condensin enrichment in metaphase and in anaphase indirectly via Cdc5. In model 2 Cdk1 phosphorylation of Smc4 is important for condensin association with chromatin up to and including metaphase. Throughout metaphase and anaphase Ipl1 acts via Cdc5 and another kinase (potentially Cdc7) to facilitate condensin enrichment on chromatin.

5.2.2 Ipl1 important for condensin enrichment at the pericentromere but not rDNA in pre-anaphase mitosis

We have demonstrated for the first time in *S. cerevisiae,* not only that Ipl1/Aurora B is required for condensin association with chromatin, but also that it has a pre-anaphase role in condensin regulation in this organism. As discussed in 1.4.4.2 Aurora B kinase has been shown to be important for condensin loading from pre-metaphase to anaphase, in several other organisms including *D. melanogaster* (Giet and Glover 2001), *C. elegans* (Collette *et al.* 2011), human cells (Lipp *et al.* 2007) and *Xenopus* egg extracts (Takemoto *et al.* 2007). In *S. pombe* Ark1/Aurora B kinase is required for the stable association of condensin with chromosomes (particularly centromeres and

rDNA) right up until telophase (Nakazawa *et al.* 2008). In contrast up until now there had been no evidence that that condensin association with chromatin is regulated by Ipl1/Aurora kinase in *S. cerevisiae*. Furthermore, the role of Ipl1 in condensin regulation was thought to be limited to late mitosis (from the metaphase-anaphase transition onwards) (Lavoie *et al.* 2004). These findings suggest the regulation of condensin may be more similar between *S. cerevisiae* and other organisms than previously thought.

Our data shows that in the metaphase arrest, Ipl1 is important for condensin enrichment at the pericentromere but not the rDNA or directly on the centromere (figure 5.9). Ipl1 function in *S. cerevisiae* has been shown to be important for rDNA condensation post-anaphase (Lavoie *et al.* 2004). This finding suggests that there may be differences in the role of Ipl1 in the regulation of condensin pre- and post-anaphase. Aurora B kinase as part of the CPC is re-localised in anaphase to the spindle mid-zone, which could account for this difference (Kitagawa and Lee 2015). It would be interesting to analyse the effect of perturbing Ipl1 function on condensin enrichment in an anaphase mitotic arrest.

5.2.3 The mutation of *SMC4* phosphorylation sites, but not the loss of the N-terminus of *SMC4*

Work in this chapter (figure 5.12) shows that the mutation of *SMC4* phosphorylation sites causes a loss of condensin enrichment in mitosis. However, a mutant in which the N-terminal region of Smc4 (containing seven out of the ten putative Cdk1 phosphorylation sites), does not have an effect on condensin enrichment. As mentioned in 5.1.6, we have used an Smc4 over-expression and depletion tool to test for the effect of removing the N-terminus. We have not yet verified the depletion of Smc4 in the cell culture protocol used. Once verified however, this data would indicate that the presence of non-functional phosphorylation sites is more detrimental to condensin enrichment that the absence of the N-terminus of *SMC4*. One explanation for this could be that the three functional phosphorylation sites present in the

smc4_n_termΔ are sufficient for facilitating condensin association with chromatin. Alternatively, it is possible that the N-terminal extension of Smc4 promotes disassociation of the condensin complex from chromatin unless phosphorylated. Therefore, having the N-terminus present but unable to be phosphorylate would promote dissociation of the condensin complex from chromatin, whereas removal of the N-terminus entirely would in theory, not effect condensin chromatin association as seen by ChIP. To distinguish between these possibilities, it would be interesting to see whether mutating the three remaining phosphorylation sites in the *smc4_n_termΔ* mutant is sufficient to reduce chromatin enrichment.

5.2.4 Discrepancies between chromatin enrichment and the functionality of the condensin complex

An interesting observation is that despite condensin phosphorylation sites being so important for the association of the complex with chromatin, mutations of the phosphorylation sites on individual subunits do not have a significant impact on condensin function (as assessed by cell viability and FACS analysis – figures 5.6 and 5.12). The original study of the non-SMC phosphorylation site mutants demonstrated that although singularly the mutants do not obviously affect condensin function, in combination they do. This suggests that phosphorylation sites of the non-SMC subunits are redundant in some way. This concept fits in with the partial loss of viability seen for the *ycs4-543/ycg1-521* double mutant (figure 5.6A), (to be discussed further in chapter 7). Similarly, despite the loss of condensin enrichment in the *ipl1-321* mutant allele, there was no evidence of a genetic interaction between Ipl1 and condensin in figure 5.9C. Together with the discrepancies seen for the chromatin enrichment and functionality of condensin in the phosphorylation site mutants, this finding suggests that that only a small amount of condensin is required for viability. However, the spot test probing for genetic interaction by partially perturbing essential genes is a crude method, and we should be cautious in our interpretation.

6 Investigating the role of condensin ATPase activity in the association of the complex with chromatin

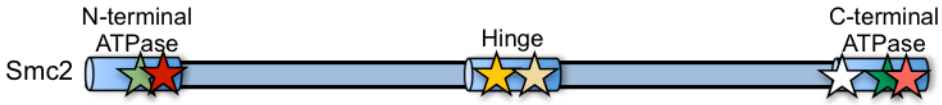
6.1 Results

6.1.1 Introduction

As discussed in 1.4.4.4, SMC proteins are ATPase enzymes that harness energy from catalysing the hydrolysis of ATP. The ATPase cycle of SMC protein complexes has been shown to be important for their *in vitro* and *in vivo* activity and association with chromatin (Arumugam *et al.* 2003, Stray and Lindsley 2003, Weitzer *et al.* 2003, Strick *et al.* 2004, Hudson *et al.* 2008, Ladurner *et al.* 2014, Murayama and Uhlmann 2014, Kanno *et al.* 2015, Kinoshita *et al.* 2015). Early investigation into the role of SMC ATPase activity was conducted using *in vitro* analysis of bacterial SMC complexes. Most likely due to the speed of the ATPase cycle of SMC complexes, there was some difficulty in analysing the effects of ATP on DNA-binding by SMC proteins (discussed in (Hirano and Hirano 2006). In order to circumvent this problem, 'transition-state' mutations were introduced in order to perturb the ATPase cycle of SMC proteins at the point of ATP-binding and ATP-hydrolysis (Hirano and Hirano 1998, Hirano *et al.* 2001, Hirano and Hirano 2004, Hirano and Hirano 2006). Following the *in vitro* work in bacteria, similar mutations have been used to investigate the role of eukaryotic SMC ATPase activity *in vitro* (Weitzer *et al.* 2003, Haering *et al.* 2004, Arumugam *et al.* 2006) and *in vivo (Arumugam et al. 2003, Haering et al. 2004, Hudson et al. 2008, Kanno et al. 2015)*.

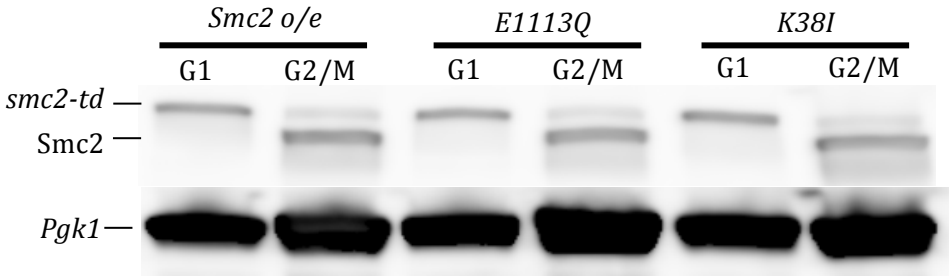
The role of condensin's ATPase activity has been investigated in vertebrate cells. Fluorescence microscopy was used to analyse the effects of perturbing different stages of the ATPase cycle (Hudson *et al.* 2008). The study indicated that ATP-binding but not ATP-hydrolysis is required for condensin association with chromatin. This finding is in

A



Mutation	*SMC2* domain	Predicted Effect
K38I	Walker A	No ATP-binding
D1112A	Walker B	No ATP-Binding
E1113Q	Walker B	Slows ATP-hydrolysis
R58A	R-Loop	No DNA-stimulated ATP-hydrolysis
S1085R	Signature Motif	No ATP-dependent dimerisation
L567K	Hinge	Disruption of Smc2/Smc4 interaction
L665R	Hinge	Disruption of Smc2/Smc4 interaction

B



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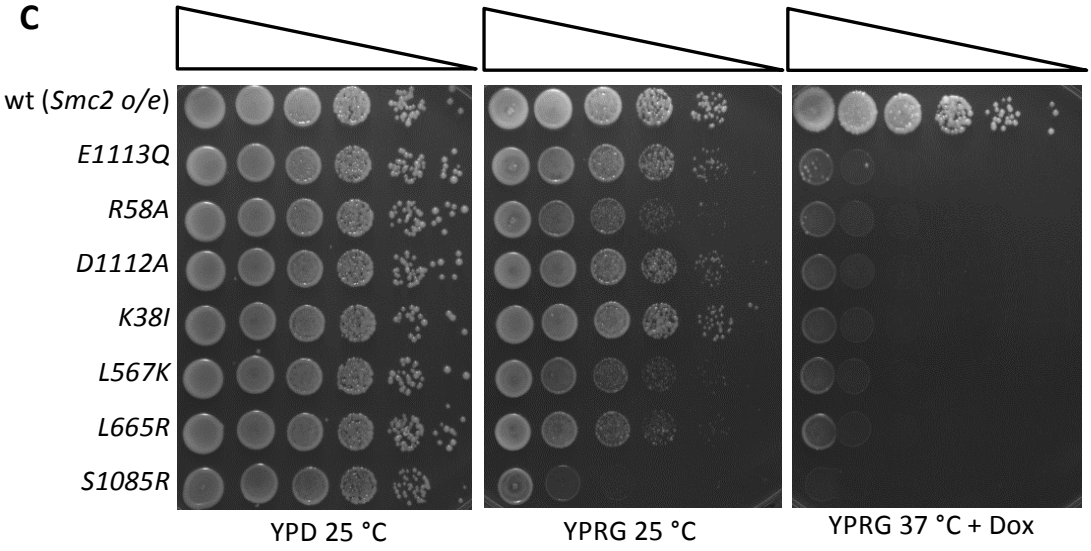

Figure 6.1 Characterising *SMC2* ATPase mutants

A) At the top, a schematic showing the approximate locations of the *SMC2* mutations. Below is a table detailing the location of the mutations and predicted effects of the *SMC2* mutants used in this chapter. **B)** Western blot analysis demonstrating the degradation of (HA-tagged) *smc2-td* and the expression of either wild-type or mutated (HA-tagged) *SMC2 (E1113Q or K38I) in G2/M*. Cells (817, 820, 821) were synchronised in G1 using alpha factor mating pheromone and subjected to restrictive conditions (detailed in 2.5.7) and released into a G2/M arrest using nocodazole. Samples were taken in G1 in which smc2-td is not depleted and in G2 in which smc2-td is depleted and either WT or mutant Smc2 is expressed. Samples were then prepared for western blot analysis using whole-cell TCA extraction (detailed in 2.6.1). Western blot was undertaken as detailed in 2.6.2 and samples were run on a 6% polyacrylamide gel. Pgk1 used as a loading control. **C)** Spot test showing the growth phenotypes of the *SMC2* mutants. 10-fold serial dilutions of exponentially growing cells (from top to bottom 817, 820, 824, 819, 821, 822, 823, 825) were plated onto YPD, YPRG and YPRG with 25 µg/ml doxycycline and incubated at 25°C or 37°C for up to 48 hours.

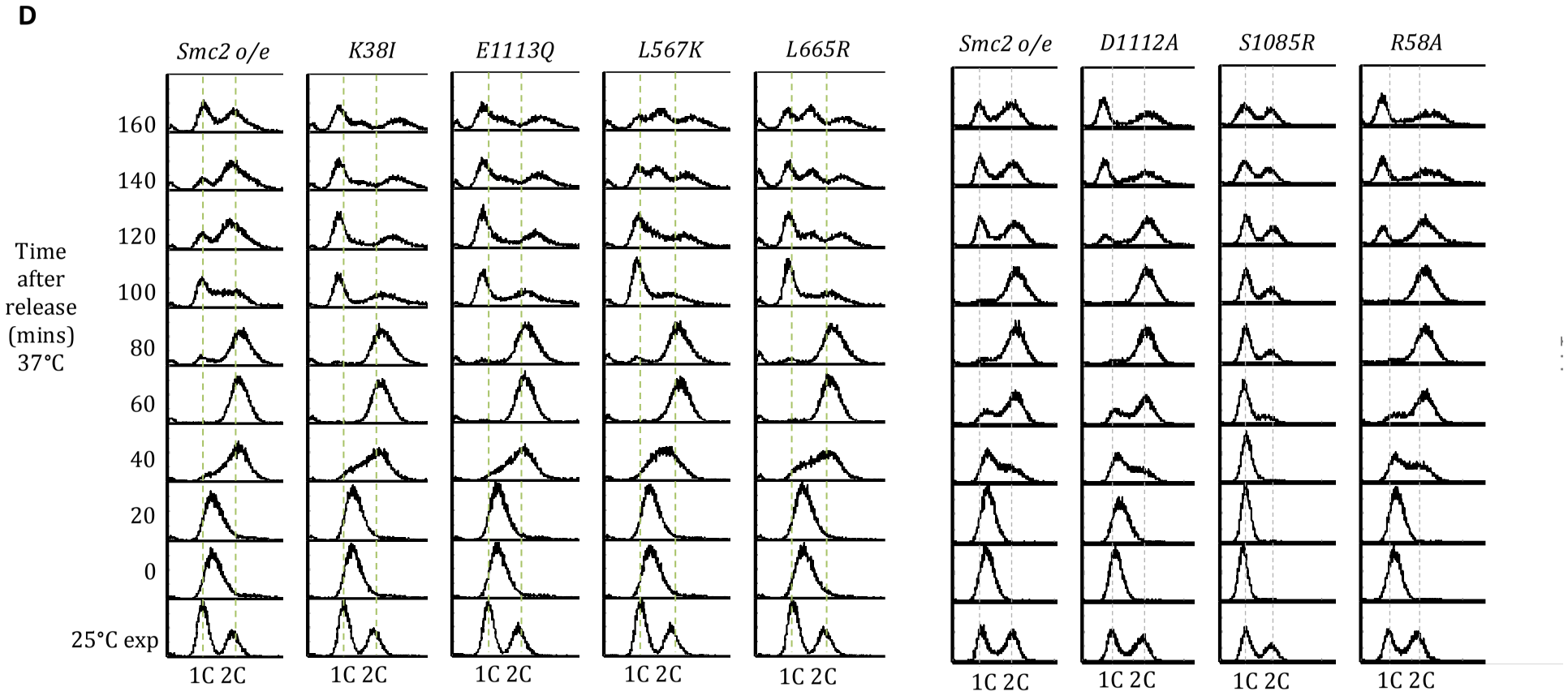

Figure 6.1 continued - Characterising *SMC2* mutants.

D) FACS data showing the progression of *SMC2* mutants (from left to right: 817, 821, 820, 822, 823 – 817, 819, 825, 824) through the cell cycle. Exponentially growing cells were arrested in G1 using alpha factor mating pheromone and subjected to restrictive conditions (the addition of 2% galactose, 25 µg/ml and doxycycline at 37°C) before being released from G1 to progress through the cell cycle. Cell samples for FACS analysis were taken just before release (0 min) and every 20 minutes after for 160 minutes)

contrast to what has been shown for the cohesin and Smc5/6 complex, for which ATP-hydrolysis is required for stable binding of the complex with chromatin (Arumugam *et al.* 2003, Weitzer *et al.* 2003, Kanno *et al.* 2015). We used ATPase mutants to gain further insight into the role of condensin's ATPase cycle in chromatin association in *S. cerevisiae.*

6.1.2 Characterising *SMC2* enzymatic mutants

To begin investigating the effects of condensin's ATPase cycle on the chromatin enrichment of the complex during mitosis, a series of enzymatic mutants that perturb the ATPase cycle in several different ways, was generated in the Baxter lab (designed by T. Oliver and constructed by M. Yu). These included ATP-binding mutants (*K38I* and *D1112A*), ATP-hydrolysis mutants (*E1113Q* and *R58A*), a head-destabilisation mutant (*S1085R*) and two hinge mutants (*L567K* and *L665R*) (see figure 6.1A). The ATP binding, ATP-hydrolysis and head-destabilisation mutants are modelled on the series of SMC mutations from bacteria (Hirano and Hirano 1998, Hirano *et al.* 2001, Hirano and Hirano 2004, Hirano and Hirano 2006) and cohesin (Arumugam *et al.* 2003, Weitzer *et al.* 2003, Haering *et al.* 2004, Arumugam *et al.* 2006), and are similar to those used in the study of condensin ATPase activity in vertebrate cells (Hudson *et al.* 2008). The *S1085R* mutant is modelled on the BsSMC *S1090R* which binds ATP but is unable to promote the engagement of the two catalytic head domains (Hirano *et al.* 2001). The hinge mutants are designed to disrupt the interaction between Smc2 and Smc4 at the apex of the SMC complex. They were based on the cohesin hinge mutants described by (Mishra *et al.* 2010, Hu *et al.* 2011).

Due to the lethality of these mutant alleles, they were placed ectopically in *smc2-td* strains under the control of a *GAL* promoter. This system allowed the conditional depletion of endogenous *SMC2* and conditional expression of the *SMC2* mutant allele as demonstrated by western blot in figure 6.1B. Although I only tested *E1113Q* and *K38I* as shown in figure 6.1B, M. Yu investigated *SMC2* expression levels of all the condensin enzymatic mutants used in this chapter, and found them to be consistent. To characterise these *SMC2* mutants, I first conducted a spot test to investigate their

growth phenotypes. In figure 6.1C (left panel) we can see that all of the mutants have a similar growth phenotype to the *Smc2 o/e* strain when grown in permissive conditions (YPD 25°C). The lethality of the *SMC2* mutations was confirmed using conditions that facilitate depletion of endogenous SMC2, and over expression of wild-type *SMC2* or the *SMC2* enzymatic mutant allele (right panel). We can see that whilst the *Smc2 o/e* (over expression) strain was viable, every *SMC2* mutant was inviable under these conditions (YPRG 37°C with doxycycline). I also tested whether the mutant alleles were dominant negative by using conditions (YPRG 25°C) which would lead to their over expression without the depletion of endogenous Smc2 (middle panel). The spot test revealed that the ATP-binding mutants *D1112A* and *K38I* and also ATP-hydrolysis mutant *E1113Q* did not appear to be effected by over expression of the mutant allele in the presence of endogenous Smc2. The cells containing hinge mutants *L567K* and *L665R,* as well as ATP-hydrolysis mutant *R58A* grew at least 10-fold less than the wild-type. The *S1085R* head-engagement mutant however, shows significant growth defect when expressed alongside endogenous Smc2. Therefore, there appear to be no dominant negative effects of the *D1112A*, *K38I* and *E1113Q* mutant alleles, and a weak dominant negative effect from *R58A*, *L567K* and *L665R*, but quite a severe dominant negative effect of the *S1085R* mutant allele.

A common phenotype of condensin mutants is the miss-segregation of chromosomes leading to aneuploidy. To see whether this is evident in the *SMC2* mutant alleles I used FACS analysis to see the cellular DNA content of populations of cells expressing either wild-type *SMC2 or* an *SMC2* mutant allele (figure 6.1D). Exponentially growing cells were arrested in G1 using alpha factor mating pheromone, subjected to restrictive conditions, and released synchronously to progress through the cell cycle (still under restrictive conditions). Cell samples were taken every 20 minutes for 160 minutes after release, and processed for analysis by FACS. Due to experimental constraints, I analysed the SMC mutants in two separate batches, on the left I compared *K38I E1113Q*, *L567K* and *L665R* with *Smc2 o/e.* On the right I compared *D1112A, S1085R* and *R58A* with *Smc2 o/e.* Miss-segregation and aneuploidy can be identified by peaks

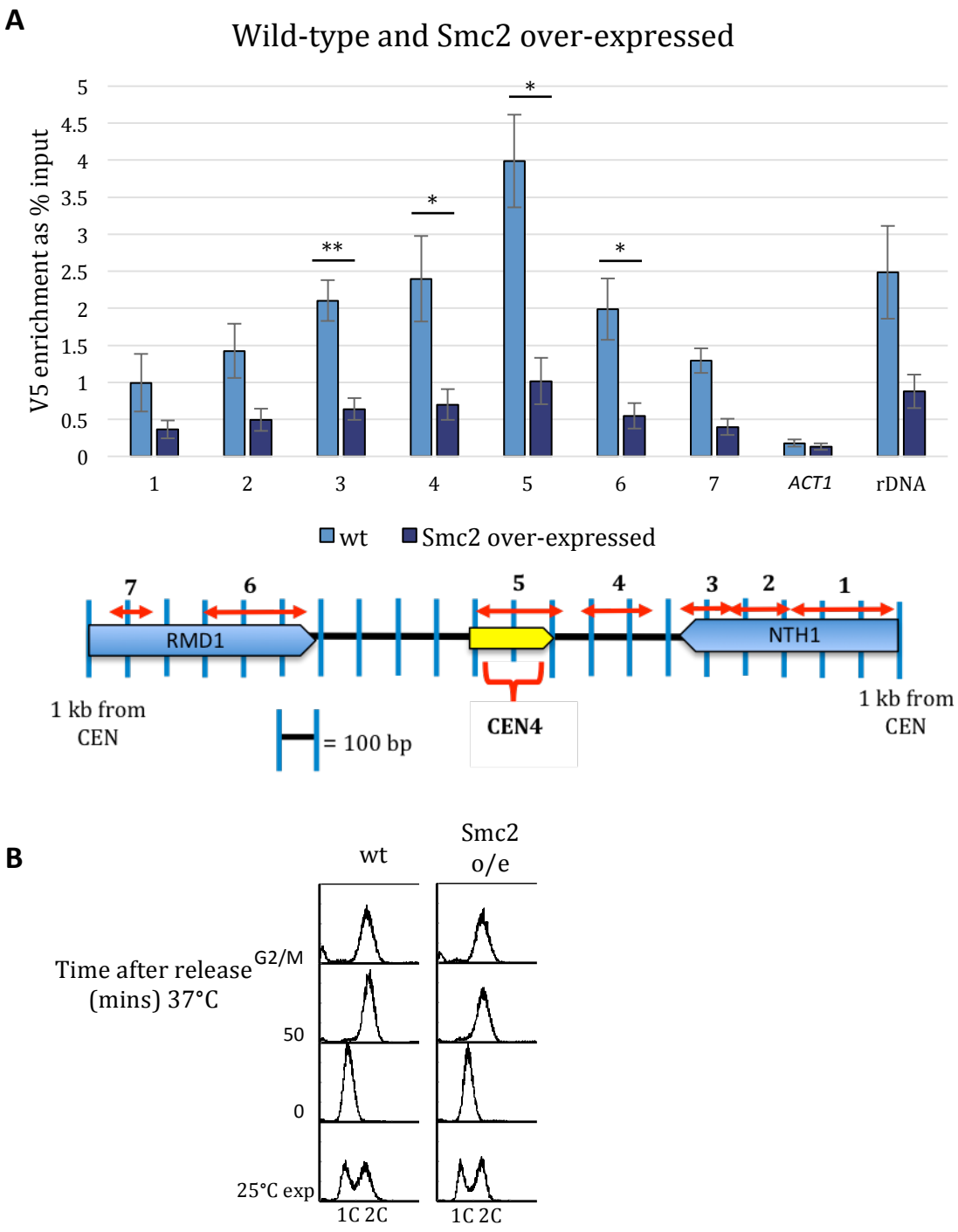

Figure 6.2 Over expression of Smc2 causes a loss of condensin enrichment on chromatin

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of wild-type (tet-degron wt 708) and *Smc2 o/e* (817) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

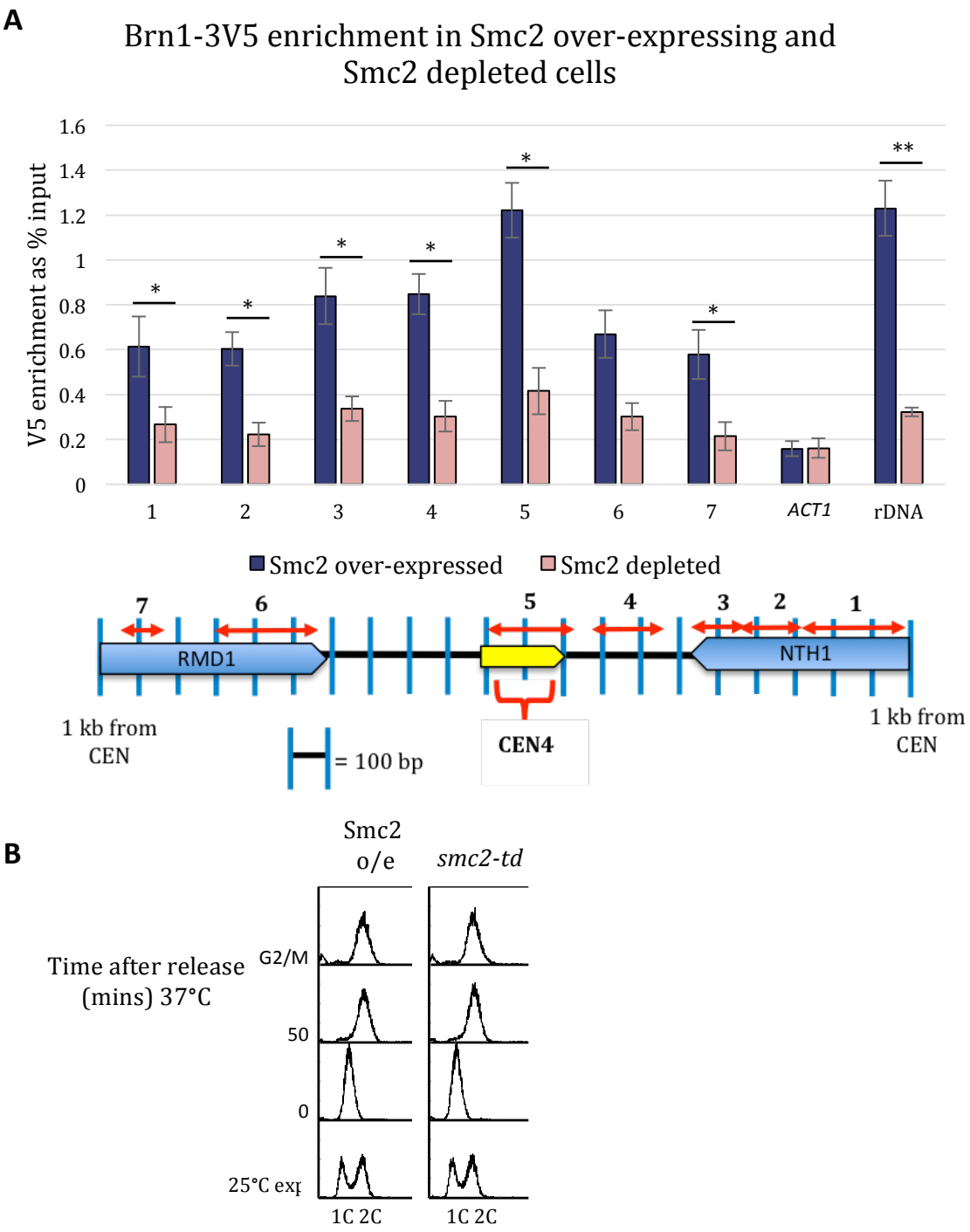

Figure 6.3 The depletion of Smc2 generates a significant loss of Brn1-3V5 enrichment compared with Smc2 over expression

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of *Smc2 o/e* (817) and *smc2-td* (818) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding

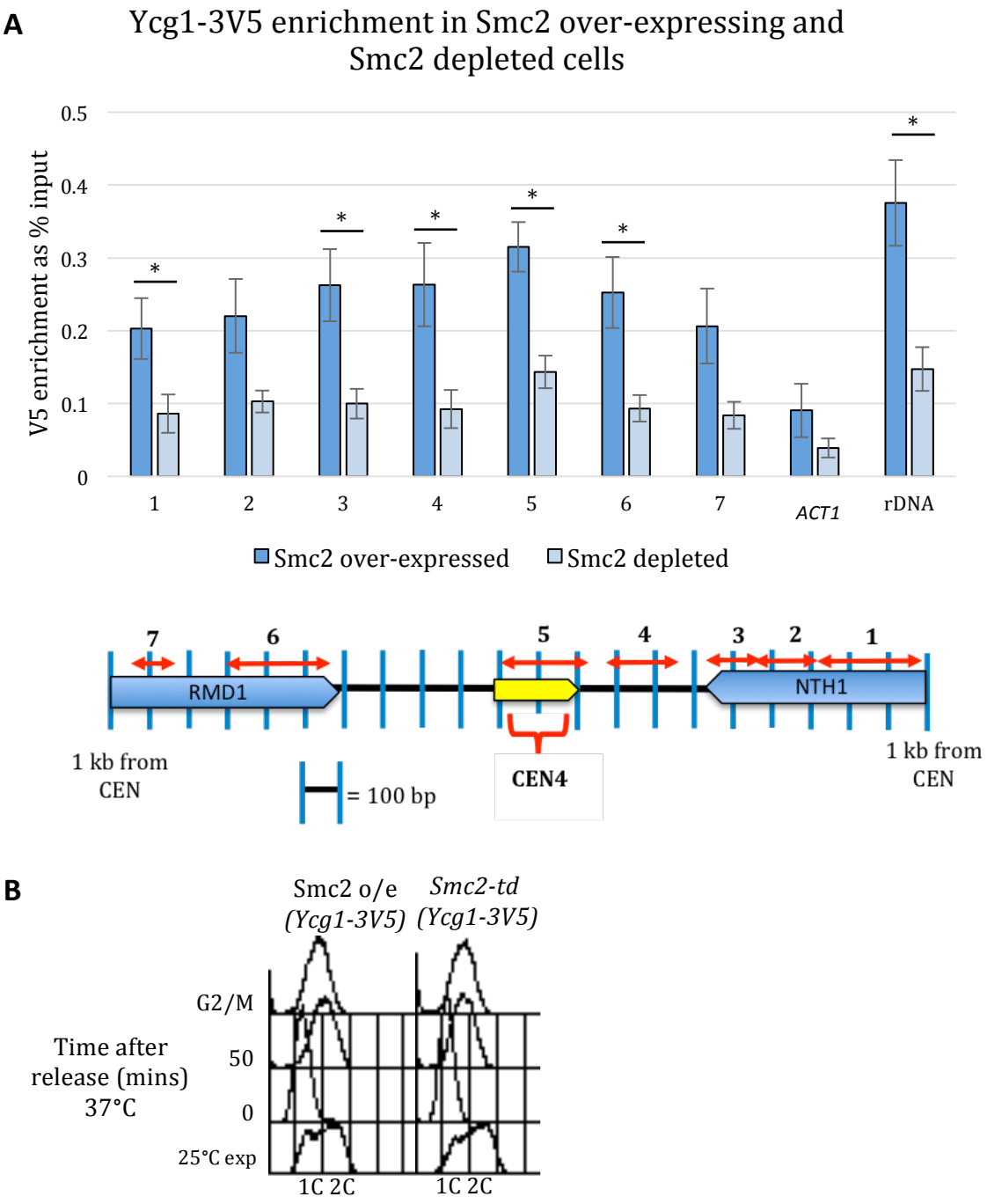

Figure 6.4 The depletion of Smc2 generates a significant loss of Ycg1-3V5 enrichment compared with Smc2 over expression

A) Histogram showing levels of Ycg1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01. B**)** Representative FACS data showing the progression of *Smc2 o/e* (1101) and *smc2-td* (1102) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

that fall outside of the two grey dotted lines, as these represent the 1C and 2C (complement) peaks of DNA. In the left panel we can see that all of the mutants exit G1 along with *Smc2 o/e* at around 40 minutes. Following mitosis between 80 and 100 minutes, we can see for all of the mutants tested (not *Smc2 o/e*), an accumulation of DNA content above and below the 1C and 2C marker lines. Therefore, the *K38I*, *E1113Q*, *L567K* and *L665R SMC2* mutants all cause chromosomal miss-segregation. In the right panel we can see that *Smc2 o/e*, *D1112A* and *R58A* begin to exit G1 at 40 minutes. *D1112A* and *R58A* go on to exhibit miss-segregation of chromosomes with cells having less than 1 and more than 2 complements of DNA. The *S1085R* mutant cells however showed a severe delay in leaving G1, with only a small percentage of cells exiting by 60 minutes a population of cells appears to remain arrested in G1 throughout. Therefore, all of the SMC2 ATPase and Hinge mutants lead to the miss-segregation of chromosomes, with the exception of *S1085R, which* generated a population of G1-arrested cells. Since earlier in the study we have shown that mitotic entry is required for condensin enrichment by ChIP, we did not carry out ChIP analysis of the *S1085R*.

6.1.3 Validating the *SMC2* over-expression system

Since we were using an over-expression system, we carried out several control experiments to validate the system before we began investigating the impact of the *SMC2* mutations on condensin enrichment. To begin, we tested whether the over-expression of Smc2 resulted in a change in condensin enrichment on chromatin. To test this, we conducted a ChIP experiment comparing the levels of condensin enrichment in the wild-type (tet-degron wt) with that the *Smc2 o/e* (over-expression) cells (Figure 6.2). Surprisingly we found that the over-expression of Smc2 actually resulted in a significant loss of condensin enrichment. We speculated that the over-expression of one condensin subunit might generate a population of partially incomplete complexes that are unable to associate with chromatin efficiently.

To continue with the over-expression system after this finding, we needed to ensure that the system is sensitive to a loss of condensin enrichment caused by factors other than the over-expression of Smc2. To test this, we conducted a ChIP experiment comparing levels of condensin enrichment (Brn1-3V5) in cells over-expressing Smc2 (*Smc2 o/e*) with the levels of enrichment in cells depleted of Smc2 (*smc2-td*). Figure 6.3 shows that there is a significant loss of condensin enrichment upon depletion of Smc2, when compared with the enrichment levels in cells over-expressing Smc2. Due to other areas of investigation, we had strains available in which we could over-express Smc2 and deplete Smc2, in cells in which the Ycg1 rather than Brn1 subunit was tagged with a -3V5 moiety. To further validate the Smc2 over-expression system we conducted a second ChIP experiment comparing levels of condensin enrichment (Ycg1-3V5) in cells over-expressing Smc2 (*Smc2 o/e*) with the levels of enrichment in cells depleted of Smc2 (*smc2-td*). Figure 6.4 shows that when we immuno-precipitated Ycg1-3V5 we again observed a significant loss of condensin enrichment upon depletion of Smc2 when compared to over-expression of Smc2.

Despite the finding that over-expression of Smc2 results in a loss of condensin enrichment when compared with wild-type (tet-degron wt) cells, the system is still sensitive to depletion of condensin. Therefore, the over-expression system is appropriate for use in investigating the effects of *SMC2* ATPase mutants on condensin association with chromatin.

6.1.4 ATP-binding but not ATP-hydrolysis is required for condensin association with chromatin

To investigate whether perturbing condensin ATPase activity affects the complex's association with chromatin, we conducted a series of ChIP experiments comparing the levels of condensin enrichment in the ATP-binding (*K38I* and *D1112A*) and ATP-hydrolysis (*E1113Q* and *R58A*) with the *Smc2 o/e* strain. Ideally we would have liked to test each of the *SMC2* mutants in parallel with each other, however due to experimental restrictions I conducted the cell culture and ChIP experiments in smaller

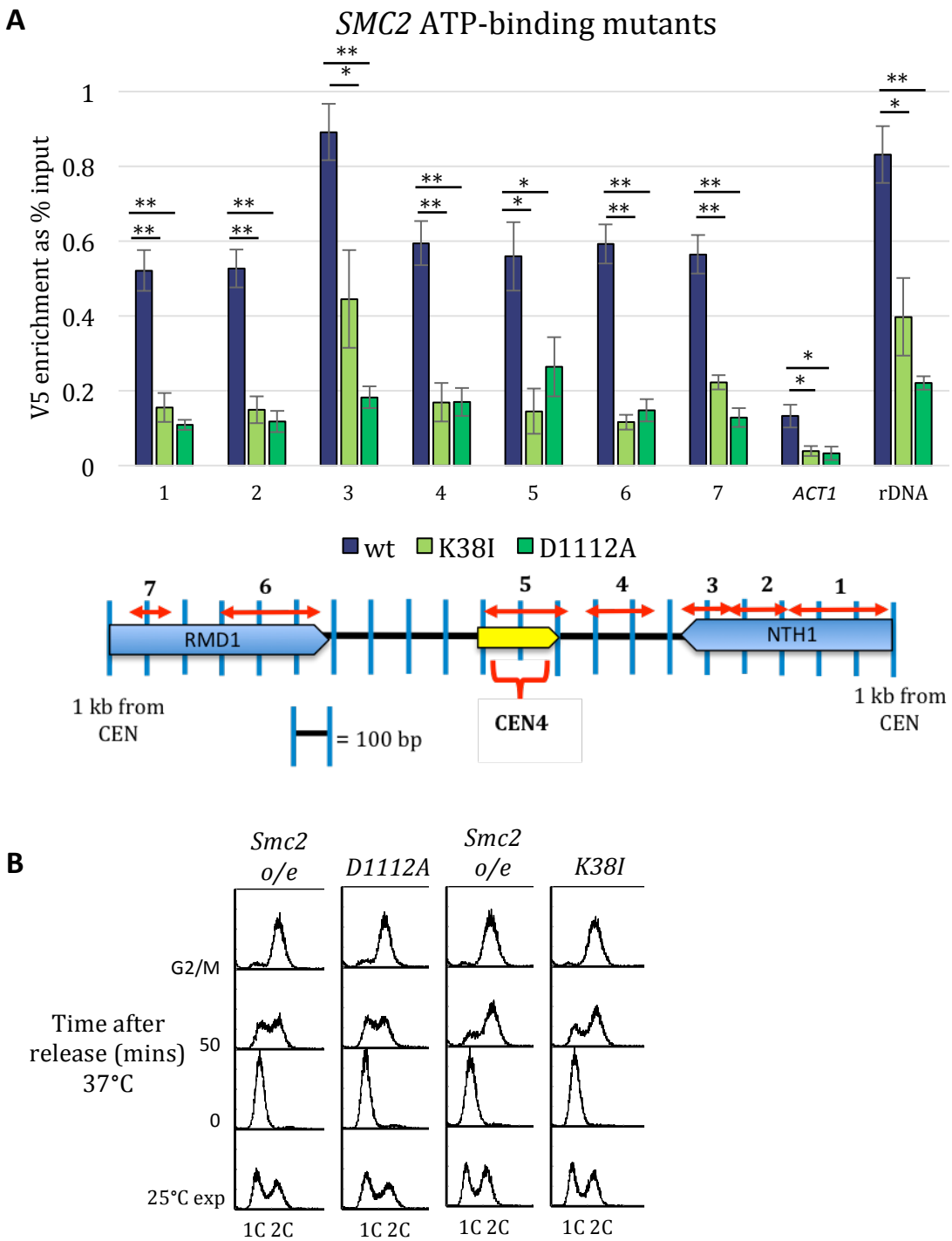

Figure 6.5 ATP-binding is required for condensin association with chromatin

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of *Smc2 o/e* (817), *K38I* (821) and *D1112A* (819) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

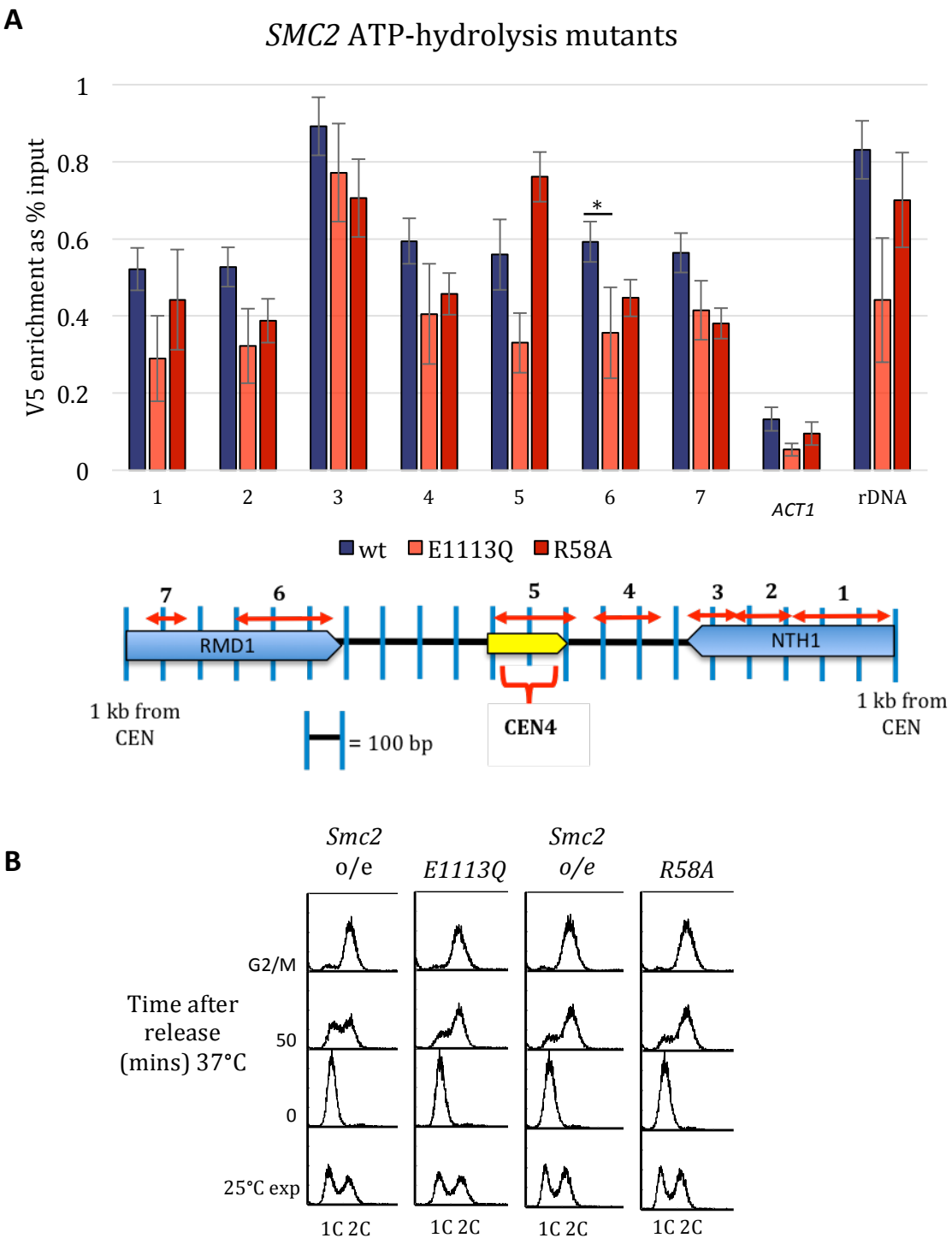

Figure 6.6 ATP-hydrolysis is not required for condensin association with chromatin

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of *Smc2 o/e* (817), *E1113Q* (820) and *R58A* (824) through the G1 block and release protocol 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

batches. Between experiments, the enrichment values obtained for the *Smc2 o/e* were very consistent. For comparative purposes we compare each *SMC2* mutant to the average of *Smc2 o/e* from all experiments conducted (9 overall). Cells were processed using the standardised alpha-factor block and release to G2/M protocol detailed in 2.5.7.1. Figure 6.5 shows the data obtained from ChIP experiments investigating condensin enrichment on chromatin in ATP-binding mutants *K38I* and *D1112A*. For each ATP-binding mutant we observed a significant loss of condensin enrichment on chromatin, at each locus investigated (including the actin gene locus). Therefore, the process of ATP-binding is required for association of condensin with chromatin. The data obtained from ChIP analysis of ATP-hydrolysis mutants *E1113Q* and *R58A* (Figure 6.6) however has a much less significant effect on condensin enrichment. For *E1113Q* we only observed a statistically significant reduction in condensin enrichment at one pericentromeric loci (6) when compared with *Smc2 o/e*. For the *R58A* mutant we see no significant difference between enrichment levels when compared with *Smc2 o/e.* From these findings we conclude that ATP-binding but not ATP-hydrolysis is required for condensin association with chromatin.

6.1.5 Mutations in the SMC2 hinge region result in an increase in condensin enrichment on chromatin in mitosis

Next, we wanted to investigate the role of the hinge interface between Smc2 and Smc4 in the association of condensin with chromatin. The hinge region of condensin complexes isolated from *S. pombe* and vertebrates has been shown to have DNA-binding activity (Yoshimura *et al.* 2002, Griese *et al.* 2010), and the mutation of the condensin hinge region in *S. pombe* (*cut14-Y1*) causes a loss of DNA-binding and annealing activity (Akai *et al.* 2011). In order to investigate the role of the *S. cerevisiae* hinge region in the association of condensin with chromatin, we conducted ChIP analysis to compare the condensin enrichment levels of hinge mutants *L567K* and *L665R* with those of *smc2 o/e* (Figure 6.7). Remarkably, we found that the condensin enrichment in both hinge mutants was significantly higher than that seen in the cells

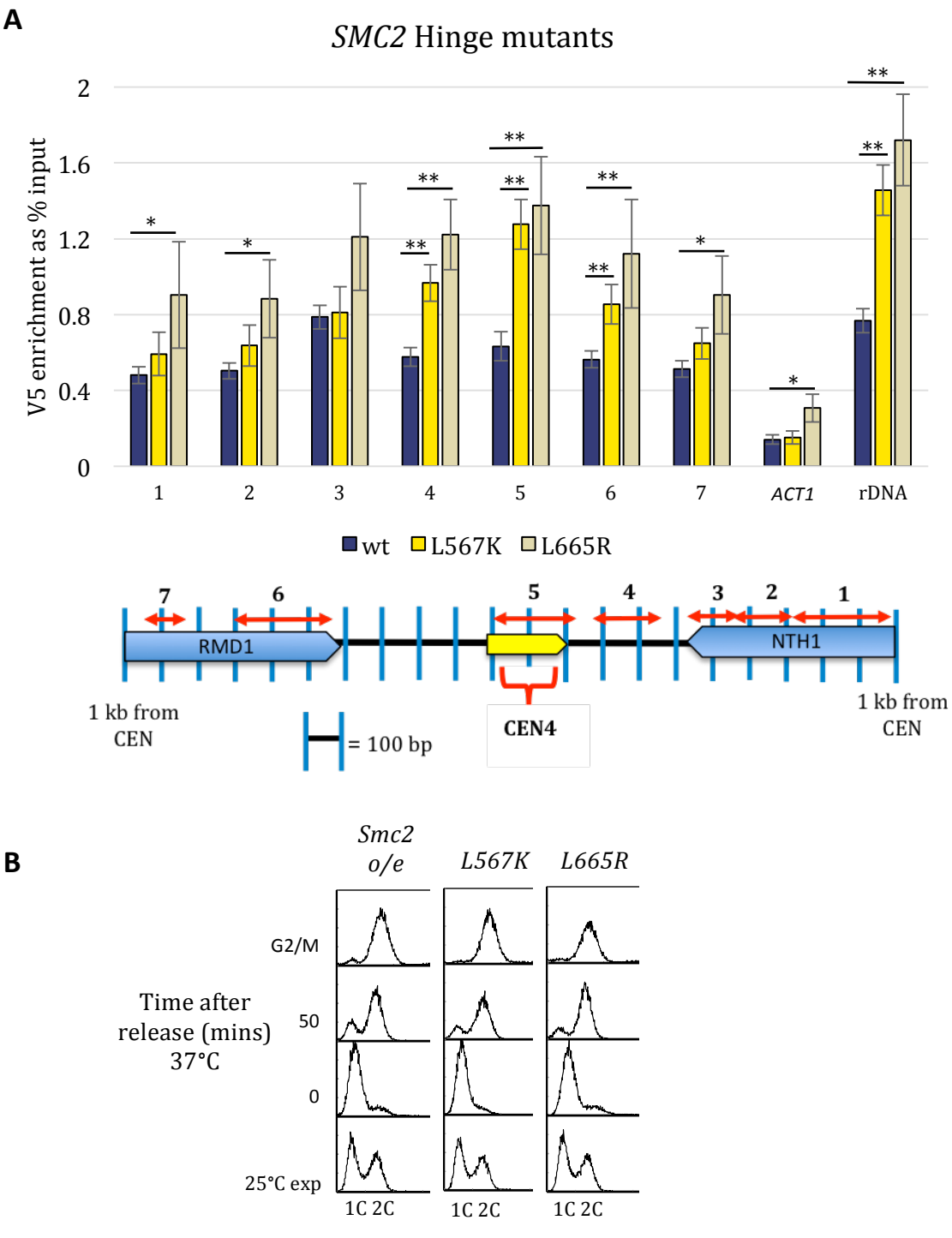

Figure 6.7 Mutations in condensin hinge region cause a significant increase in condensin enrichment on chromatin

A) Histogram showing levels of Brn1-3V5 enrichment at specified loci surrounding *CEN4* (depicted in cartoon for reference), at the actin gene and the rDNA. Cells were processed according to the standard block and release protocol in which cells are arrested in G1 using alpha factor mating pheromone, switched to restrictive conditions and released into a G2/M block using nocodazole. Cell samples were then processed according to the ChIP protocol detailed in materials and methods. Each sample was normalised to its corresponding input signal. Error bars represent SEM from three separate ChIP experiments. Stars represent statistical difference as calculated by a two-tailed homoscedastic T test. * = P <0.05 ** = P <0.01 .**B)** Representative FACS data showing the progression of *Smc2 o/e* (817), *L567K* (822) and *L665R* (823) through the G1 block and release protocol, 'exp' = exponentially growing population. '0 mins' time point was taken just prior to release from G1 block. The G2/M time point taken when cells reach G2/M as assessed by budding index.

expressing wild-type *SMC2* cells (*Smc2 o/e*). The significant increase in condensin enrichment is evident at all loci except for primer region 3 in the *L665R* mutant. For *L567K,* the increase in enrichment is limited to the *CEN4*, the two primer regions closest to the centromere (4,6) and the rDNA. Therefore, the disruption of the hinge region in *S. cerevisiae* condensin causes an increase in condensin association with condensin as seen by ChIP. This finding suggests that a full-functioning hinge is not required for condensin association with chromatin, but may be involved in the disassociation of the complex from chromatin.

6.2 Discussion

6.2.1 The role of ATP-binding and ATP-hydrolysis in condensin association with chromatin in mitosis

In this chapter, we have characterised a set of *SMC2* mutations that perturb the enzymatic cycle of condensin in *S. cerevisiae.* This work represents, to our knowledge, the first investigation into the effects of perturbing the ATPase cycle on condensin association with chromatin in *S. cerevisiae*. Our work shows that ATP-binding but not hydrolysis, is required for the association of condensin with mitotic chromosomes. In cells expressing *SMC2* mutations predicted to prevent ATP-binding (*K38I* and *D1112A*), condensin association with chromatin was significantly decreased. In contrast, in cells expressing *SMC2* ATP-hydrolysis mutants predicted to block ATP-hydrolysis (*R58A*) or slow the hydrolysis step (*E1113Q*), condensin associated with chromatin at levels comparable to wild-type. These findings are in agreement with *in vitro* work investigating *Bacillus subtillus* BsSMC, in which the ATP-binding mutant (*K37I*) does not exhibit ATP-stimulated DNA binding, but the ATP-hydrolysis mutant does (Hirano and Hirano 2004). Furthermore our results are consistent with a study in chicken DT40 cells, in which the binding of condensin to chromosomes in analogous ATP-binding (*K38I* and *D1113A*) and ATP-hydrolysis (*E1114Q*) mutants was analysed using fluorescent microscopy techniques *in vivo (Hudson et al. 2008)*. The study

demonstrated that ATP-binding but not ATP-hydrolysis is required for the association of condensin with mitotic chromosomes in vertebrate cells.

In the *in vitro* analysis of BsSMC mutants, it was shown that like the ATP-binding mutant (*K37I*), the head-engagement mutant (*S1090R,* analogous to the *S1085R* characterised in figure 6.1), did not exhibit ATP-stimulated DNA-binding (Hirano and Hirano 2004). Unfortunately, we were unable to investigate by ChIP whether this was the case for *S1085R* in *S. cerevisiae* due to a population of cells failing to exit a G1 arrest when expressing that mutation (figure 6.1D). Since the *S1090R* can bind ATP but fails to bring the ATPase heads together, this finding suggests that it is not the binding of ATP *per se* that facilitates condensin loading to chromatin, but the conformational change brought about by the ATP-binding (Lammens *et al.* 2004, Hirano 2005). Studies using purified human SMC2 demonstrated a conformational shift of the protein in the presence of ATP, which lead to the hypothesis that ATP-binding may open the hinge region to facilitate chromatin association (Onn *et al.* 2007). There has also been evidence that the loading of cohesin onto chromosomes involves the transient opening of its SMC hinge (Gruber *et al.* 2006).

6.2.2 The role of the hinge region in condensin association with chromatin

We have demonstrated that mutations of the hinge region of Smc2 (predicted to disrupt the Smc2/Smc4 interface, but not prevent complex formation) result in an increase in condensin association with chromatin in mitosis. In theory, this increase in enrichment of condensin could be down to an increase in loading of condensin, or a decrease in the disassociation of the complex. One model predicts that the ATP-driven conformational change of the condensin complex, causes the opening of the hinge to facilitate condensin loading (Onn *et al.* 2007). In line with this model, the disruption of the hinge might by-pass that ATP-dependent conformational change, to allow constitutive chromatin association of condensin, without the rate-limiting step of binding and hydrolysing ATP. However, it is difficult to conceive that this would result in stable binding, because if the open hinge allowed the entry of chromatin; it would

surely just as likely allow the exit of it. Alternatively, if a fully functional hinge region is required for the disassociation of condensin from chromatin, then mutations in this region may cause an accumulation of the complex on chromatin.

The hinge mutations that were used in this chapter were modelled on cohesin hinge mutations (*L665R* based on *SMC1 M665R* and *L567K* based on *SMC3 M557K)*. *L665R* and *L567K* are predicted to disrupt hydrophobic interfaces 1 and 2 respectively. These cohesin mutants (*M665R* and *M557K*) caused a partial reduction in cohesin binding, or did not have any significant effect on the association of cohesin with chromatin, as measured by immunofluorescence and ChIP (Mishra *et al.* 2010, Hu *et al.* 2011). Interestingly, FRAP (fluorescence recovery after photo-bleaching) revealed that the *M665R and M577K* cohesin mutations cause an increased recovery compared to wild-type suggesting more rapid turnover. These findings are not consistent with the data we obtained from the condensin hinge mutants *L567K* and *L665R,* suggesting either the mutations are not perturbing the hinge region of the two complexes in the same way, or that the hinge is important for different steps of chromatin association in condensin and cohesin. Indeed, we must be cautious in making comparisons between cohesin and condensin because their loading mechanism appears to be subtly different. In contrast to our finding that ATP-hydrolysis is not required for condensin loading, in ATP-hydrolysis mutants of either *SMC1* or *SMC3*, the cohesin complex fails to bind to chromosome arms (Arumugam *et al.* 2003). Similarly recent studies of Smc5/6 revealed that the stable binding of the complex to chromatin also requires ATP-hydrolysis (Kanno *et al.* 2015).

6.2.3 Potential model for how the ATPase cycle of Condensin facilitates condensin associates with and disassociates from chromatin.

On the premise of the current understanding of SMC proteins and data presented in this chapter, we put forward a possible model for the role of the ATPase cycle in condensin association with chromatin (figure 6.9). The condensin complex has been shown to bind DNA in the absence of ATP (Kimura and Hirano 1997, Kimura *et al.*

1999). However, stable loading of the complex requires the ATP-binding step of the condensin ATPase cycle (figure 6.5) (Hudson *et al.* 2008). How might the ATP-independent transient DNA-interactions be stabilised by the ATP-binding of the complex? ATPase head domains of SMC subunits are thought to engage upon nucleotide binding, and to disengage upon subsequent ATP-hydrolysis (Lammens *et al.* 2004, Hirano 2005). The process of ATP-binding is thought to generate a conformational change of the complex from a rod-shaped structure to a more open ring-shaped conformation (Soh *et al.* 2015). This conformational change may facilitate the opening of the condensin complex to allow topological entrapment of chromatin, or it may reveal DNA-binding sites, which are not accessible prior to ATP-binding.

Although the hydrolysis of ATP is not required for stable binding of the complex, it is an essential component of condensin activity. The disengagement of condensin heads may facilitate further conformational changes required for theoretical condensin functions, such as loop extrusion (Alipour and Marko 2012, Burmann and Gruber 2015). Alternatively, the ATP-hydrolysis step may be vital to allow a further DNA-binding event, in which a second DNA duplex may become stably bound to the complex. This could be important for the formation of long-range intrachromosomal interactions. Indeed, one model predicts that condensin compacts chromatin by the sequential entrapment of two DNA helices by a single condensin ring (Cuylen *et al.* 2011). The role of ATP-hydrolysis in condensin function remains to be clarified, and further work to understand how condensin compacts chromatin will be crucial for this.

Our data indicates that a mutation of the hinge region causes an accumulation of condensin on chromatin (figure 6.7). Following the required ATPase cycles, it is possible that the hinge region is required for subsequent disassociation of the condensin complex. This may occur via a transient opening of the hinge region, a process linked with complex loading for cohesin (Gruber *et al.* 2006, Murayama and Uhlmann 2014). To investigate whether the hinge really is required for condensin disassociation from chromatin, it would be interesting to observe the dynamics of condensin complexes containing *L567K* and *L665R* mutants using FRAP. In future experiments, it would also be interesting to compare the effect of hinge perturbation

on condensin I and condensin II in vertebrate cells since FRAP experiments have shown condensin I to have a much more dynamic association with mitotic chromosomes (Gerlich *et al.* 2006, Oliveira *et al.* 2007).

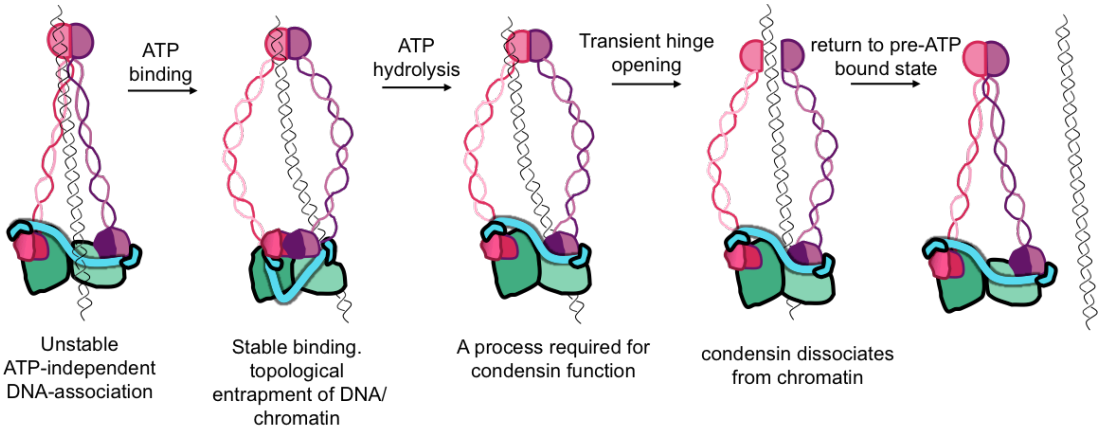

Figure 6.8 A hypothetical model for the role of the ATPase cycle in condensin association with and disassociation from chromatin

The condensin complex can bind DNA in the absence of ATP (Kimura and Hirano 1997, Kimura *et al.* 1999). However ATP-binding is required for stable loading of the complex (figure 6.5) (Hudson *et al.* 2008). The binding of ATP is thought to generate a conformational change of the complex from a rod-shaped structure to a more open ring-shaped conformation (Soh *et al.* 2015). This conformational change may potentially bring about stable loading by an opening of the complex to allow topological entrapment of double stranded DNA and/or by revealing sites for direct binding of chromatin to the heat proteins and hinge domain regions. ATP-hydrolysis is not required for stable association of the complex with chromatin (figure 6.6) (Hudson *et al.* 2008) but is vital for condensin function. Upon completion of the essential condensin function (perhaps after multiple rounds of ATPase activity), transient opening of the hinge-region may allow condensin disassociation from chromatin.

7 Final discussion

7.1.1 Factors affecting condensin enrichment with chromatin

For the stable association of the condensin complex with chromatin, factors are required first for its recruitment to local areas of enrichment, and then to facilitate the stable binding of the complex. For SMC complexes stable binding is thought to be achieved via the topological entrapment of chromatin (Uhlmann 2016). In this study we have demonstrated that condensin enrichment at mitotic centromeres is dependent on fully functioning inner and outer kinetochore components Ndc10 and Ndc80 (figures 4.3-4.4), and on the phosphorylation of condensin subunits (figures 5.2-5.5 and 5.12). So far, we cannot determine whether the kinetochore factors and the phosphorylation of condensin subunits work to recruit condensin, or to promote stable binding of the complex to the chromatin, or both. However, due to the centromere-specific location of kinetochore factors, it is most likely that their role in condensin association with chromatin is in *cis*-recruitment rather than establishing stable binding of the complex.

We have also shown that the mutation of condensin phosphorylation sites results in a loss of condensin enrichment, not only at the centromere and pericentromeric regions but also the rDNA. It seems that phosphorylation is required for condensin association with chromatin globally. However, the disruption of Ipl1 function results in the loss of condensin enrichment at the peri-centromere but not directly on the centromere or at the rDNA. Therefore, the phosphorylation of condensin may also contribute to site-specific association of the complex. By what mechanism does phosphorylation promote condensin association with chromatin? Potentially, phosphorylation of condensin subunits may stabilise the complex. We have demonstrated *in vivo* that the perturbation of one condensin subunit reduces the chromatin binding of other subunits. Indeed, depletion of Smc2 or Ycg1 (figures 3.3, 3.5), or the mutation of phosphorylation-sites on any single subunit resulted in a significant loss of Brn1 enrichment. *In vitro* data suggests that non-SMC proteins are able to associate with

DNA independently of the Smc2/4 heterodimer (Piazza *et al.* 2014) and *vice versa (Sakai et al. 2003, Stray and Lindsley 2003, Stray et al. 2005).* In contrast, our findings support the notion that the complex as a whole is required for condensin enrichment on chromatin *in vivo.* The hypothetical stabilisation of the condensin complex by phosphorylation could facilitate condensin enrichment by physically strengthening the pentameric structure in a way that promotes its recruitment to chromatin, or that would enhance stable binding by topological entrapment.

Alternatively, the phosphorylation of condensin subunits may facilitate the association of the complex with chromatin by promoting its enzymatic activity. In the final chapter of this study we demonstrated that the ATPase cycle of the condensin complex is very important for its enrichment, in a global manner. In our proposed model, ATP-binding is a necessary step in the association of condensin with chromatin. The phosphorylation of condensin subunits could promote the binding of ATP and therefore the association of the complex with chromatin in this manner. While we have shown that they do affect chromatin enrichment, at this stage we can only speculate as to how phosphorylation of condensin, and kinetochore factors do so. Further work is required to shed more light on the mechanisms that lead to the stable association of the condensin complex with chromatin.

7.1.2 The disparity between the enrichment of condensin on chromatin and the functionality of the complex.

Several times in this study we have demonstrated that the perturbation of a factor may result in a significant loss of condensin enrichment, without having a major impact on cell viability or chromosome segregation. We have shown that while the phosphorylation-site mutants of individual condensin non-SMC subunits (*brn1-570*, *ycg1-521*, *ycs4-543*) and Smc4 (*smc4-10A*) all generated a significant loss of condensin enrichment, none of them displayed any obvious growth or chromosome miss-segregation phenotypes as assessed by spot tests and FACS analysis. It could be contested that our analysis isn't sensitive enough to detect subtle chromosome miss-

segregation phenotypes; FACS analysis is a blunt tool for detecting chromosome segregation defects, and also we only have investigated the impact of these mutants over a short space of time (160 minutes from G1 release). However, our results are in agreement with the characterisation of the non-SMC phosphorylation-site mutants in the study in which they were generated (St-Pierre *et al.* 2009). The authors found that the mutation of phosphorylation sites in single subunits did not generate any growth phenotypes or any significant problems in rDNA condensation and segregation (St-Pierre *et al.* 2009). Colony sectoring investigations could explore whether these single subunit phosphorylation site mutants lead to miss-segregation in a more sensitive and long-term manner.

Without further investigation we cannot rule out the possibility that condensin is still present at these regions we probed, but at a level undetectable by ChIP. However, our finding that mutations generating a significant loss of condensin enrichment do not necessarily cause viability or chromosome segregation defects, suggests that only a relatively low level of condensin association with chromatin is required for its function in mitosis.

Another way in which this study highlights the distinction between condensin enrichment and condensin function, is by demonstrating that mutants affecting condensin function do not necessarily cause a reduction in condensin enrichment. In Chapter 6 we showed that the ATP-hydrolysis (*R58A* and *E1113Q*) and hinge mutants (*L567K* and *L665R*), which are incompatible with cell viability and in which miss-segregation of chromosomes was detectable by FACS, did not significantly impact condensin enrichment, and significantly increased it respectively. These findings underline the fact that the stable association of condensin with chromatin is not sufficient for its function. Therefore, we predict that the essential activity of condensin occurs following stable chromatin binding.

7.1.3 Role of condensin at the centromere pre-anaphase

What is the function of condensin at the pre-anaphase centromere/pericentromere? As discussed in 1.4.2.2, condensin has been shown to be important for generating centromeric tension and elasticity important for the segregation of sister chromatids in metazoan cells (Oliveira *et al.* 2005) (Gerlich *et al.* 2006) (Ribeiro *et al.* 2009). In *S. cerevisiae,* condensin has also been shown to be important in maintaining centromere structure (Yong-Gonzalez *et al.* 2007). To protect against aneuploidy, systems are in place to ensure that chromosomes attach to spindles from opposite poles in mitosis (biorientation). Two branches of the spindle assembly check-point (SAC) prevent the onset of anaphase in the absence of biorientation. The first branch is satisfied when kinetochore-microtubule (MT) connections have been made. The second branch of the SAC is not satisfied by the attachment of microtubules to kinetochores, but requires the generation of tension from correct biorientation before the onset of anaphase is permitted. The tension-sensing pathway allows for error-correction of imperfect kinetochore-MT attachments. Aurora B/Ipl1 is a key component of the tension-sensing and error correction pathway, and is thought to promote turnover of incorrect attachments by phosphorylating kinetochore components at centromeres lacking tension (Tanaka *et al.* 2002). Subsequent tension-induced dephosphorylation then triggers SAC silencing and anaphase progression (Jin and Wang 2013) (Yamagishi *et al.* 2014).

Perturbation of the condensin complex can result in the loss of tension-sensing and error correction of kinetochore-MT interactions (Yong-Gonzalez *et al.* 2007). During the course of this study, the Marston group (Verzijlbergen *et al.* 2014) investigated the role of condensin in the biorientation of sister-kinetochores at the centromere in *S. cerevisiae.* They showed that shugoshin (Sgo1) acts to maintain Ipl1 localisation and recruit condensin to centromeric regions. They go on to demonstrate that Sgo1 and condensin are both important for imposing a bias on sister kinetochores to biorient. The authors propose that condensin organises pericentromeric chromatin to generate a structural rigidity that imposes a back-to-back geometry of sister-chromatin kinetochores, favouring biorientation. Once amphitelic kinetochore-MT have been

established, Sgo1 and condensin dissociate from the centromeric region and anaphase can progress (Nerusheva *et al.* 2014).

We have shown that kinetochore factors are also important for condensin recruitment to the centromere in *S. cerevisiae*. Whether this is alongside or upstream/downstream of Sgo1 recruitment remains to be seen. We have demonstrated that significantly reduced levels of condensin enrichment at the centromere sites pre-anaphase, is sufficient for cell viability without inducing aneuploidy, bringing into question the importance of condensin's role in biorientation. Either the role of condensin here is not essential for biorientation, or only a small amount of condensin is sufficient for its function, in which case it appears that excessive copies of the condensin complex are recruited here to ensure its success. Further studies are required to address whether biorientation is delayed but not prevented in some of the viable condensin mutants in which condensin enrichment is inhibited.

7.1.4 Condensin as a therapeutic target

The findings in this thesis contribute to the understanding of factors regulating the association of condensin with chromatin. Crucially, the data suggests that relatively low level of condensin association with chromatin is required for its function in mitosis. What may be the clinical relevance of such information? Condensin has been identified as a potential cancer therapeutic target by multiple research groups (Dávalos *et al.* 2012, Shiheido *et al.* 2012). Broadly speaking, there are two ways in which condensin could contribute to cancer progression. Firstly, chromosomal aberration and genomic instability are hallmarks of cancer, and the loss of condensin function can lead to miss-segregation and aneuploidy. Several different loss-of-function mutations in hCAP-E (SMC2) and hCAP-C (SMC4), as well as chromosome segregation defects, were identified in leukemia-lymphoma cell lines (Ham *et al.* 2007). A recent study has identified that loss of even one copy of the tumour suppressor gene RB1 (retinoblastoma) reduces the recruitment of condensin (II) to pericentromeres, causing defective chromosome segregation in mitosis (Coschi *et al.* 2014).

Secondly, cancer cells can up-regulate cellular mechanisms to supress the cellular toxicity of DNA-replication stress concomitant with aneuploidy and oncogenic activation. These adaptations include hyper-activation of genome stability mechanisms such as components of DNA damage repair pathways. Cancer cells can become dependent on the hyper-activation of such genome-stability mechanisms, a process known as “oncogene addiction” (Weinstein and Joe 2006). Condensin is key in maintaining genomic instability and also has a role in protecting the genome from exogenous DNA-damage (Aono *et al.* 2002, Heale *et al.* 2006, Akai *et al.* 2011). Condensin has been shown to be up regulated in several cancers including human intestinal and colorectal tumour cells (Dávalos *et al.* 2012). Furthermore, a derivitive of the kinase inhibitor aniloquinazoline has been shown to bind hCAP-G2 and inhibit the proliferation of a panel of human cancer cell lines by inhibiting normal segregation of chromosomes (Shiheido *et al.* 2012). We have shown that some condensin mutants that cause a significant loss of chromatin enrichment, do not generate obvious phenotypes to suggest a loss of condensin function. Therefore In tackling condensin as a therapeutic target, we would propose the most effective route would be to focus on inhibiting the enzymatic activity of the complex rather than recruitment mechanisms.

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