Imperial College London

Doctoral Thesis

Functional Analysis of the Condensin Complex during the Metaphase to Anaphase Transition of Budding Yeast

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in the

Cell Cycle Group
Institute of Clinical Science

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Declaration of Authorship

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Abstract

Functional Analysis of the Condensin Complex during the Metaphase to Anaphase Transition of Budding Yeast

by Joanne Caroline Leonard

Cell Cycle Group
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The precise division of DNA into two daughter nuclei is a critical event in every cell cycle. The pairing of sister chromatids in order to allow accurate segregation, is achieved by two primary mechanisms – the SMC complex cohesin binds as replication ends and holds sisters together with protein-mediated links; and the process of replication intertwines sister chromatids in a way that results in DNA-DNA mediated links called catenations. At the point of segregation, it is crucial that cohesin is destroyed and all catenations are resolved.

Topoisomerase 2 (Top2) is the primary actor in S. cerevisiae able to resolve DNA intertwinings. This enzyme appears to maintain an equilibrium level of catenations between S phase and mitosis and it has been shown to be capable of both introducing and removing catenations by its strand passage mechanism. At the metaphase to anaphase transition, a shift to positive supercoiling may help to direct the action of topoisomerase enzymes towards full decatenation at the crucial moment of DNA segregation. Further, it has been shown that condensin, another SMC complex, has a pivotal role in this supercoiling transition.

Here, work is presented to link this change in supercoiling to physical tension across sister chromatids aligned on the metaphase plate. The regulation of this transition requires Cdc5 kinase, Ipl1 kinase and acts independently of the Spindle Assembly Checkpoint. Furthermore, the supercoiling transition is linked to a global enrichment of Smc2 across the entire length of chromosomes as the spindles form and anaphase progresses. This enrichment is matched by Top2 binding and this Top2 enrichment depends directly on the action of Smc2. It is hypothesised that this recruitment of Top2 is dependent on catenations being revealed by the supercoiling action of Smc2. If Top2 is not active, then Smc2 continues to bind globally to DNA and further increase the level of positive supercoiling, suggesting that the complex is able to sense the presence of remaining catenations. Finally, a secondary last minute catenane resolution pathway may have been identified that occurs after the supercoiling transition and is regulated by the Anaphase Promoting Complex.
Acknowledgements

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>1D/2D</td>
<td>1 dimensional/2 dimensional</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenylyl-imidodiphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
</tr>
<tr>
<td>CatA</td>
<td>Catenated dimer, both relaxed</td>
</tr>
<tr>
<td>CatB</td>
<td>Catenated dimer, one relaxed, one supercoiled</td>
</tr>
<tr>
<td>CatC</td>
<td>Catenated dimer, both negatively supercoiled</td>
</tr>
<tr>
<td>CatC*</td>
<td>Catenated dimer, both positively supercoiled</td>
</tr>
<tr>
<td>CCCm</td>
<td>Closed circular supercoiled monomer</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CEN</td>
<td>Centromere</td>
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<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<td>ChIP paired with full genome sequencing</td>
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<td>Chromosome</td>
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<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
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<td>Cyclin-dependent kinase inhibitors</td>
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<td>Crosslinking Kinetics</td>
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<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
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<td>CPC</td>
<td>Chromosomal Passenger Complex</td>
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<td>Dbf4-Cdc7 dependent kinase</td>
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<tr>
<td>DSB</td>
<td>Double strand break</td>
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<tr>
<td>DAPI</td>
<td>4’6-diamidino-2-phenylindole</td>
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<td>DDR</td>
<td>DNA damage response</td>
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<tr>
<td>DHFR</td>
<td>Dihydrofolate reductase gene</td>
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<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FEAR</td>
<td>Cdc Fourteen Early Anaphase Release</td>
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<td>Mating type a/α</td>
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<td>MEN</td>
<td>Mitotic exit network</td>
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<tr>
<td>MMS</td>
<td>Methyl methane sulfonate</td>
</tr>
<tr>
<td>NAT</td>
<td>Nourseothricin</td>
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<tr>
<td>OCm</td>
<td>Open circular relaxed monomer</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PBS-T</td>
<td>Phosphate buffered saline - Tween®</td>
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<td>PCR</td>
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<td>PEG</td>
<td>Polyethylene glycol</td>
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<td>Phenylmethanesulfonylfluoride</td>
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<td>Protein Phosphatase 2A</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real time PCR (quantitative PCR)</td>
</tr>
<tr>
<td>rcf</td>
<td>Relative centrifugal force</td>
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<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>rpm</td>
<td>Rotations per minute</td>
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<tr>
<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
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<tr>
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<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
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<tr>
<td>SMC</td>
<td>Structural Maintenance of Chromosomes</td>
</tr>
<tr>
<td>SPB</td>
<td>Spindle Pole Body</td>
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</table>
Abbreviations

SSC  Saline sodium citrate
ssDNA  Single stranded DNA
TBE  Tris/Borate/EDTA buffer
TCA  Trichloroacetic acid
TEMED  Tetramethylethlenediamine
tRNA  Transfer RNA
UV  Ultraviolet
YP  Yeast Extract Peptone
YPD  YP 2% glucose
Chapter 1

Introduction

1.1 The *Saccharomyces cerevisiae* Cell Cycle

The process by which cells multiply is commonly referred to as the cell cycle, consisting of interphase (G₁ (Gap1), S (synthesis), G₂ (Gap2)) and mitosis (M). The G₁ phase typically consists of a pause in the progression of the cell cycle to allow synthesis of cellular components and for the cell to either enter the cell cycle, attempt mating, or enter quiescence (G₀). If the cells do begin division, they enter S phase and begin to replicate their DNA and form kinetochores at the centromeres of newly replicated DNA. G₂ phase follows, which is a second growth phase in preparation for division - this is very short in *S. cerevisiae*. Finally, M phase is the process of cell division which in turn can be subdivided into five stages: prophase where the chromosomes condense and centrioles separate to form a spindle; metaphase as the spindles form fully and the chromosomes align on the metaphase plate; anaphase as the chromosomes begin to separate due to depolymerisation of the microtubules from the kinetochore-associated end (see Section 1.4); telophase where two distinct nuclei are formed; and finally cytokinesis in which the cytoplasm is divided and the membrane closes around two separate cells.

There are many forms of the cell cycle. The process explained above is mitosis - a form that gives rise to two genetically identical daughter cells (asexual division) and can happen with cells in both haploid and diploid forms to maintain the chromosome number. Meiosis is a highly specialised form of cell division which results in four haploid cells, each with half the chromosome number of the mother cell. These haploid daughter cells can go on to function as gametes or spores and participate in sexual reproduction. The differences between the two types of cell division occur largely in M phase which, in mitosis, consists of a single segregation event to divide the sister chromatids. In meiosis
the first segregation event separates homologous chromosomes, and then a second division occurs immediately afterwards to separate sister chromatids, ultimately producing four haploid cells.

In *S. cerevisiae* both haploid and diploid forms of the organism can multiply via rounds of mitosis. Alternatively, the decision can be made to move between haploid and diploid forms - either via mating (haploid to diploid) or sporulation (producing a tetrad of 4 haploid cells from a diploid mother) as shown in Figure 1.1. In haploid form, the yeast cell is of either a or α mating type and each will respond to the mating pheromone of the other type, exiting the cell cycle during G₁ phase in order to mate. Furthermore, *S. cerevisiae* contains an endonuclease which enables it to switch mating type. This ensures that mating can always take place, when favoured, as a single population can ensure a mix of mating types. Yeast strains used in laboratories have often been modified to remove this endonuclease which allows the maintenance of a genetically identical haploid population, forcing the yeast to remain as either a or α mating type and therefore unable to mate. Using haploid yeast cells makes genetic manipulation that much easier as there is only one copy of the genome and the mating pheromone can be used as a tool for cell cycle arrest. However, the existence of a diploid form is also useful for more complex studies especially of multiple or essential genes.

**Figure 1.1:** The *S. cerevisiae* cell cycle. Cartoon shows movement between the haploid and diploid form of *S. cerevisiae* and the cycling in either form. It also shows the exit points that can be taken to G₀, a quiescent state where the cells do not cycle (shown in red) (de Virgilio, 2014)
1.1.1 Cell cycle control

Movement through the cell cycle and distinction between cell cycle stages is controlled by cyclins and cyclin-dependent kinases (Cdk). A Cdk is inactive until bound by a partner cyclin and this cyclin targets the kinase activity of a Cdk to a specific set of proteins. The phosphorylation of different targets by Cdk can activate or inactivate a protein, or target it for degradation, any of which can alter transcription levels of various other genes. In \textit{S. cerevisiae} the single Cdk (Cdk1/Cdc28) acts throughout the cell cycle, in cooperation with nine different cyclins, see Figure 1.2. In vertebrate cells, there are multiple Cdks but Cdk1 alone is still capable of controlling the whole cell cycle in vertebrates, although Cdk2, 4 and 6 are still required for development of specialised tissues (Enserink, 2010). The mechanism of Cdk governed cell cycle control is very well conserved and vertebrate Cdk1 can substitute for \textit{S. cerevisiae} Cdc28.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.2.png}
\caption{The cyclin-cdk partners that control progression through the cell cycle in \textit{S. cerevisiae} (Bloom, 2007). This shows the timing of cyclin involvement in progressing the \textit{S. cerevisiae} cell cycle, where they promote or restrict activities to different timings within the cycle. Note that there is only one Cdk, Cdc28, which is the kinase in all partnerships, shown in grey.}
\label{fig:cycle}
\end{figure}

There are nine cyclins in \textit{S. cerevisiae} and their levels increase and decrease with specific timing during the cell cycle. In G\textsubscript{1} Cdk activity is low due to the absence of all cyclins and the presence of cyclin dependent kinase inhibitors (CKIs) (Enserink, 2010). As the cell progresses through G\textsubscript{1}, cyclin Cln3 levels increase (Baroni \textit{et al.}, 1994). Cln3 pairs with Cdc28, enabling it to phosphorylate Whi5, releasing the inhibitory interaction
between Whi5 and a transcription factor complex, the SBF. This leads to increased transcription of many genes required during G₁ which include Cln1 and Cln2, cyclins which then further activate Cdc28, acting as a positive feedback loop.

The Cln-Cdc28 complexes also phosphorylate Sic1, a CKI. This phosphorylation results in the degradation of Sic1, allowing transcription of Clb5 and Clb6 which pair with Cdc28 to stimulate DNA replication (Epstein and Cross, 1992, Feldman et al., 1997, Nash et al., 2001, Schwob and Nasmyth, 1993, Schwob et al., 1994). Importantly, the Clb-Cdc28 complexes also phosphorylate the SBF transcription factor complex (which has been activated in G₁ by Cln-Cdc28 removing its inhibitor) and this phosphorylation acts to downregulate the G₁ transcriptional program leading to the reduction of G₁ cyclins, promoting unidirectionality of the cell cycle (Enserink, 2010). While Cdk activity is low in G₁, the pre-replicative complex is loaded onto DNA replication origins and subsequent phosphorylation by Clb-Cdc28 triggers replication. This two-step process helps to ensure a single replication event per origin. This mechanism is accompanied by phosphorylation of Cdc6 by Clb-Cdc28, a protein which is required for initiation of replication and is targeted for degration by this phosphorylation (Enserink, 2010).

Clb3 and (slightly delayed) Clb4 are then expressed as the cells go into S phase, and later in S phase Clb1 and Clb2 are also expressed. Clb3/4 are largely responsible for the movement across the S/G₂ boundary and all four cyclins are present in the G₂/M transition. All cyclins are degraded with the exit from mitosis (Grandin and Reed, 1993). Each of these mitotic cyclins interacts with Cdc28 and will induce specific events, either by subcellular localisation or target selection for Cdc28 (Bailly et al., 2003). These complexes are involved in triggering spindle assembly and organisation; chromosome condensation; Golgi fragmentation and in regulating the anaphase promoting complex (APC) (Miele, 2004, Nigg, 2001).

The APC is an E3 ubiquitin ligase (King et al., 1995) which cooperates with its regulatory subunits (Cdc20 at anaphase onset, Cdh1 later in mitotic exit) to trigger cascades of protein degradation. Ubiquitination occurs on lysine residues and acts as a degradation signal, resulting in degradation by the proteasome (King et al., 1995). The APC is also involved in exit from mitosis (with Cdh1 replacing the Cdc20 regulatory subunit), as it degrades the cyclins and causes the release of Cdc14 (part of the FEAR pathway (Cdc fourteen early anaphase release)). This also results in the activation of the mitotic
exit network (MEN) together with the FEAR pathway and together these guide the cell back to G$_1$. This process includes dephosphorylation by Cdc14 of many Cdc28 targets, ending mitotic specific activities (McCollum and Gould, 2001, Stegmeier et al., 2002).

1.1.2 Checkpoints

Progression between the different stages of the cell cycle is controlled by various ‘checkpoints’ which can be seen as either ‘stop’ or ‘go’ points. These either rely on the presence of positive signals (such as enough nutrients to replicate DNA) or the absence of negative signals (such as DNA damage). These checkpoints arrest the cell cycle to allow time to resolve any problems, such as waiting until replication has finished before progressing out of S phase or until there are enough nutrients to divide. Problems with checkpoints often lead to incorrect genomic segregation and aneuploidy - a common feature of cancerous cells. Checkpoints also act as a ‘point of no return’ as the cycle cannot be reversed, and once a cell has passed one checkpoint, it will continue until the following one. The checkpoints in *S. cerevisiae* are summarised in Figure 1.3.

At the G$_1$/S phase transition, there is a checkpoint called the restriction point or START which was the first checkpoint to be identified (Hartwell, 1974). START encompasses a choice to enter the cell cycle or retreat to G$_0$ and depends on nutrient availability and the presence of mating pheromones in the environment, but also on internal signals such as cell size, growth rate, cell stress and DNA damage (Figure 1.3).

The next major checkpoint is the DNA damage checkpoint, or G$_2$/M transition, which ensures full replication of the DNA and an absence of the DNA damage response before allowing the cells to continue towards mitosis. There may also be a DNA replication checkpoint, or intra-S checkpoint, which can delay the firing of late replication origins and stabilise stalled replication forks, slowing replication in response to DNA damage (Fanning et al., 2006, Marini et al., 2006). This has been investigated extensively through the use of DNA-damaging agents, e.g. methyl methane sulfonate (MMS) or ultra-violet (UV) radiation. It seems the major signal for the detection of DNA damage is single stranded DNA (ssDNA) which is quickly bound by a three protein complex, RPA (Replication Protein A) (Fanning et al., 2006). This sets in motion the DNA damage response (DDR) which includes halting the cell cycle (Fanning et al., 2006, Marini et al., 2006).
The final major checkpoint is the spindle assembly checkpoint (SAC) which is capable of delaying anaphase until centromeric kinetochores attach to microtubules in a tension-generating way, i.e. one from either spindle pole body (SPB) (Rieder et al., 1994). The SAC is automatically active as the spindles are attaching and produces an inhibitory signal to prevent anaphase. When full bipolar attachment is achieved, the SAC is satisfied and the sister chromatids are pulled apart (see Section 1.4). If unattached chromosomes are artificially destroyed in mammalian cells then this is sufficient to down regulate the checkpoint and allow progression into anaphase (Rieder et al., 1995). Treatment with some drugs, such as nocodazole, will cause depolymerisation of the microtubules and therefore will artificially trigger this checkpoint (Foley and Kapoor, 2013, Rieder, 1992). Indeed the cell cycle can be artificially stopped at multiple points by depleting certain proteins, or by adding exogenous chemicals including those that cause DNA damage. This is a useful tool for examining cellular processes.

Figure 1.3: Cell cycle checkpoints. A cartoon showing the progression through the S. cerevisiae cell cycle with the four major checkpoints marked (red rectangles). The diagram also shows the two points where cells can exit the cell cycle to a resting state (during G₁ and G₂) and the main protein complexes driving progression through the cell cycle.
1.1.3 Pairing of sister chromatids

The cell cycle revolves around the accurate replication and division of the DNA to give each daughter cell a single complete copy of the genetic material. The equal segregation of DNA relies on knowing the identity of sister chromatids (in the case of haploid *S. cerevisiae*). Cells physically pair nascent chromatids during their replication in S phase, maintaining these links until chromosomes are aligned on the metaphase plate ready for segregation (Aragón, 2011).

Initial work done by Sundin and Varshavsky (1980) showed that replication of a circular genome (in this case the genome of the animal virus SV40) produced dimers which were catenated, i.e. the double helices themselves were intertwined. These DNA-mediated links are thought to be introduced by replication of the genome due to the double helical structure of DNA (explained further in Section 1.3.2.1). Work by Holm *et al.* (1989) showed that genetically inactivating an enzyme from the topoisomerase family (Topo II, see Section 1.3.4) prevented the separation of replicated chromatids in anaphase, instead producing elongated strings of DNA between the separating nuclei. These DNA linkages can also be added to sister chromatids due to certain mechanisms of DNA damage repair, e.g. homologous recombination will invade the sister chromatid to copy a missing or mismatched base and can result in a link that must be resolved by topoisomerase enzymes (Downes and Johnson, 1988).

However, a further mechanism of connecting the sisters was suggested by Koshland and Hartwell (1987) as they were able to observe small plasmids which remained paired in metaphase with no DNA-mediated links, suggesting that protein structures must also be involved. Genetic screens in several labs then led to the identification of a family of proteins called Structural Maintenance of Chromosomes (SMC) which consists of cohesin (SMC1 and SMC3 – identified by these studies), condensin (SMC2 and SMC4) and the SMC5/6 complex (Fousteri and Lehmann, 2000, Guacci *et al.*, 1997, Michaelis *et al.*, 1997, Strunnikov *et al.*, 1995, Strunnikov, 1993).

More recently, Farcas *et al.* (2011) carried out similar experiments looking at the maintenance of DNA-mediated linkages as cells reach metaphase. They used larger plasmids – 26 kb instead of 14 kb - and this time saw significant levels of catenations were still maintained as cells reached metaphase in up to 50% of the plasmid population, contrary
to the previous work in smaller plasmids (Koshland and Hartwell, 1987). They also found that the level of catenations correlated with increasing plasmid size and could detect the presence of intertwining in short linear chromosomes. Taken together, these data suggest that full linear chromosomes are likely to have a high level of catenations remaining at metaphase and that these may play an additional role alongside the protein-mediated linkages, dependent upon cohesin, for maintaining cohesion of sister chromatids (Aragón, 2011).

### 1.2 SMC proteins

SMC proteins are conserved in all organisms including eukaryotes, bacteria and archaea (Hirano, 1999). While bacteria have a single SMC protein (which forms multiple complexes) (Hirano and Hirano, 1998), eukaryotes have the three SMC complexes referred to above: cohesin, condensin and the SMC5/6 complex (see Figure 1.4). Although they were originally assigned simple and separate roles – sister chromatid cohesion, chromosome condensation and DNA damage response – over time the picture has become more complicated with all three complexes being assigned multiple roles in all realms of DNA structure, repair and manipulation (De Piccoli et al., 2009, Hudson et al., 2009, Jeppsson et al., 2014).

![Figure 1.4: The three S. cerevisiae SMC complexes.](image)

Figure 1.4: The three S. cerevisiae SMC complexes. Cartoons showing the SMC complexes; cohesin, condensin and the Smc5/6 complex, and their non-SMC subunits, with the currently assumed structure shown. Cohesin (left hand side) is made up of Smc1 and Smc3 and the non-SMC components Scc1, Scc3 and Pds5. Condensin (central) contains Smc2/4, Brn1, Ycs4 and Ycs5 (Ycg1). Finally, the Smc5/6 complex (right hand side) is made up of Smc5/6 and Nse1-6 (De Piccoli et al., 2009)
SMC proteins have a shared architecture - globular N- and C-terminal domains linked by two long coiled-coil sections, themselves interrupted by a ‘hinge’ region (Haering et al., 2002, Melby et al., 1998). This hinge folds to allow the formation of an antiparallel coiled-coil which brings the globular N- and C-terminal regions into contact, allowing the Walker A and Walker B motifs in these domains to bind and hydrolyse ATP (Haering et al., 2002, Melby et al., 1998). ATP hydrolysis is required for cohesin to stably bind to chromosomes and DNA binding also stimulates ATP hydrolysis in condensin and the bacterial Smc-ScpAB complex (Hirano and Hirano, 2006). In each complex, the two SMC proteins form a heterodimer via their hinge regions (Hirano, 2002), and the non-SMC proteins bind to this dimer. Some of these (the kleisins) help to form a ring-like structure by binding across the globular heads of the SMC dimer (Schleiffer et al., 2003) and others bind elsewhere on the complex.

Structural studies have shown that the coiled coils of all three complexes appear to exist in either V-shaped, O-shaped or rod-like conformations (Anderson et al., 2002, Fuentes-Perez et al., 2012, Haering et al., 2002, Matoba et al., 2005, Melby et al., 1998). Primarily, the condensin SMC dimer was seen as a rod and Smc1-Smc3 from cohesin was seen as a V-shaped molecule by electron microscopy (Anderson et al., 2002). However, it is not clear whether all complexes are capable of all conformations, if these conformations reflect complex-specific forms, or if they are due to experimental conditions (Soh et al., 2014). Most recently a paper by Soh et al. (2014) showed bacterial and eukaryotic condensins forming rod-shaped holocomplexes with rigid and juxtaposed coiled coils which change conformation upon binding of DNA and ATP. This model suggests that condensin complexes bind ATP and alter the conformation of their coiled coils in order to reveal a binding site for DNA on the hinge region (Soh et al., 2014).

### 1.2.1 Condensin

Condensin is an SMC complex with five subunits: Smc2 and 4 and three non-SMC proteins Brn1, Ycs4 and Ycg1 (in S. cerevisiae (Freeman et al., 2000)). Condensin is conserved from bacteria to humans (Hirano, 2012) and shows a rod-shaped structure in vitro (Anderson et al., 2002). A more recent paper by Soh et al. (2014) suggests that the ‘rods’ seen by electron microscopy reflect the unbound form of condensin and that binding, first to ATP and then to DNA, produces an entirely different conformation.
Enzymatic cleavage of condensin causes its dissociation from DNA (Cuylen and Haering, 2011). Thus, it has been suggested that condensin may form a ring to entrap DNA, as cohesin does (Cuylen and Haering, 2011). Combining the two studies, it seems likely that condensin is also interacting directly with DNA, as well as any topological entrapment.

Condensin has been shown to have roles in the condensation of chromosomes (Hirano et al., 1997) and in repair, recombination and segregation (Hirano, 2005). Condensation of chromosomes is essential for correct segregation and is particularly needed at the rDNA locus where it regulates recombination events, genetic stability and can cause rDNA condensation during interphase in response to nutrient starvation (Freeman et al., 2000, Hirano et al., 1997, Tsang et al., 2007a,b).

Brn1 is a member of the kleisin family (Schleiffer et al., 2003) and interacts with Smc2 and Smc4 through its N- and C-terminal parts, respectively. Ycs4 and Ycg1 (also called Ycs5) bind to the condensin complex through binding to Brn1 and are both involved in the regulation of condensin activity. All three non-SMC subunits are essential in vivo, however the condensin Smc2-4 dimer has been shown to have DNA binding and supercoiling activity in vitro without its non-SMC subunits (see Section 1.3.2) (Akai et al., 2011, Hirano and Mitchison, 1994, Stray et al., 2005). Higher eukaryotes have two isoforms of condensin, I and II, which differ in their accessory subunits and this results in different localisation patterns, activities and mutant phenotypes (Ono and Fang, 2004). Depletion of condensin I, which only has access to the DNA during mitosis, results in a swollen chromosome shape (see Figure 1.5b and f); whereas the loss of condensin II, which is constantly present in the nucleus, gives curly chromosomes (see Figure 1.5c and g). Depletion of both complexes gives cloud-like uncompacted chromosomes, as shown in Figure 1.5d and h for depletion of a non-SMC protein from both complexes simultaneously, and the final two panels show depletion of an SMC subunit which also prevents activity from either complex (Ono et al., 2003).

Condensin is present at similar protein levels throughout the cell cycle (Takemoto et al., 2004). However, it is phosphorylated on all subunits by different kinases at different points in the cell cycle, leading to different levels of activity (Bazile et al., 2010). Cdk-mediated phosphorylation of Smc4 is required to prime condensin to be hyperphosphorylated by Cdc5 (St-Pierre et al., 2009). Cdc5 is a Polo-like kinase that has been shown to have phosphorylation targets on all three regulatory subunits of condensin (Brn1,
Figure 1.5: **Condensin loss alters chromosome conformation.** Depletion of a non-SMC protein from either condensin I or condensin II produces differently shaped chromosomes; depletion of a subunit from both produces further loss of condensation, and loss of an SMC protein results in a cloud of DNA (Ono et al., 2003). Top panels show DAPI-stained DNA and lower panels show images stained with anti-CAP-E antibody, showing the location of condensin (I and II). Abbreviations: G-dep: Depletion of CAP-G, homologous to Ycg1 in condensin I; G2-dep: Depletion of CAP-G2, homologous to Ycg1 in condensin II; E-dep: Depletion of CAP-E, homologous to Smc2 Ycg1 and Ycs4). This phosphorylation is key for full activation of condensin (St-Pierre et al., 2009). Additionally, Ipl1 is an aurora B kinase and has multiple targets which include Brn1 and Ycg1 to promote and maintain chromosome condensation (St-Pierre et al., 2009, Vas and Clarke, 2008).

Human condensin I is also phosphorylated prior to mitosis by Casein kinase 2 (CK2). CK2 phosphorylation is inhibitory to condensin function and must be removed as the cells go into mitosis (Takemoto et al., 2006). A diagram of the suggested phosphorylation sites in the non-SMC subunits of human and *S. cerevisiae* condensin is shown in Figure 1.6 (Bazile et al., 2010). Mutants expressing forms of condensin subunits which cannot be fully phosphorylated show condensation defects (St-Pierre et al., 2009).

Condensin exists as two pools in the cell, a chromatin bound fraction and a soluble fraction and is one of the most abundant components of mitotic chromosomes (Takemoto et al., 2004). Binding patterns for condensin have been proposed to various levels of detail. ChIP-chip has shown a largely unchanging distribution through the cell cycle, with roughly one peak every 10 kb and increased binding at rDNA, tRNAs and telomeres at all times, and a mitosis specific increase at centromeres (D’Ambrosio and Schmidt, 2008). More recent studies have suggested a presence of condensin at actively transcribed
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Figure 1.6: **Phosphorylation sites of condensin subunits** Schematic showing the identified phosphorylation sites of the condensin non-SMC subunits in *S. cerevisiae* and vertebrates (Bazile et al., 2010). A shows Ycg1 from *S. cerevisiae* and its homolog CapG from *Homo sapiens*, B shows Ycs4 and its homolog CapD2 and C shows the kleisin Brn1 and its homolog CapH. This shows the tendency for clustering of phosphorylation sites into specific regions, and the wealth of sites on the kleisin.

genes, with varying placements and explanations. Kim et al. (2013) have reported an enrichment of condensin I in mammalian cells at promoter sequences, as well as centromeres and telomeres, and Kruesi et al. (2013) report that recruitment of condensin to promoters in X-linked genes is required for dosage compensation in *Caenorhabditis elegans*. Bernard and Vanoosthuyse (2015) have reported that although some transcription machinery has been shown to facilitate the binding of condensin (Piazza et al., 2013), in the telomeres it is necessary to remove Pol II before condensin can bind, and the binding to the rDNA requires the inactivation of Pol I (Bernard and Vanoosthuyse, 2015). This work suggests that highly transcribed regions during interphase are altered in a way that is able to recruit condensin in mitosis (Bernard and Vanoosthuyse, 2015).
As such, it seems that condensin may have a role to play at transcription sites, but perhaps is not linked to active transcription. In *Schizosaccharomyces pombe* it was shown that condensin and RPA (single strand binding protein) compete for highly transcribed regions and that condensin can displace RPA and reanneal ssDNA (single stranded DNA) *in vitro* (Akai et al., 2011). Therefore, perhaps the link to transcription is either as a marker for mitosis loading, or in helping to maintain the integrity of the DNA double helix.

### 1.2.2 Condensation and mitotic chromosomes

Chromosomes change dramatically upon entry into mitosis. During interphase the DNA exists in a diffuse form in the nucleus (Figure 1.7a) which, upon entry into mitosis, condenses into structures that can be visualised individually in many model organisms and mammalian cells (Figure 1.7b-f). *S. cerevisiae* cells do not compact their DNA to this extent but condensation does occur (Guacci et al., 1997). Condensation of chromosomes begins in G₂ and persists through mitosis, aiding with segregation of DNA. A lack of condensation can result in DNA stretching between the cells at the moment of cytokinesis, resulting in the ‘cut’ phenotype after which the non-SMC subunits of condensin are named in *S. pombe* (Hirano et al., 1986).

Condensed chromosomes are formed through the looping and folding of the DNA, in a manner not specified by sequence (Strukov and Belmont, 2009) and this process is continuously ongoing until the cells exit mitosis. If mitotic progression is delayed by the use of spindle poisons, then condensation of chromosomes continues (Rieder, 1992). Condensation appears to be dependent on condensin (Freeman et al., 2000, Vagnarelli et al., 2006) and separate from DNA cohesion. Furthermore, condensin has been shown to play a vital role in the metaphase to anaphase transition in *S. cerevisiae* mitosis (Baxter et al., 2011) and to have additional roles in meiosis, particularly in the removal of cohesin in anaphase I (Yu and Koshland, 2005).

As this condensation has a significant effect on other cellular processes which require access to the DNA, such as transcription, it must be well regulated. In *S. pombe*, condensin is only transported into the nucleus at the onset of mitosis (Sutani et al., 1999), and in vertebrates condensin I also becomes nuclear only at mitosis (Ono and Fang, 2004). In *S. cerevisiae*, condensin is present in the nucleus throughout the cell
Figure 1.7: Chromosome condensation through the cell cycle. Microscopy images showing the nucleus at different stages in the cell cycle, demonstrating the condensation and segregation of chromosomes (Department of Engineering Science, 2014). Taken from 3D time-lapse image sequences of human epithelial adenocarcinoma (HeLa) cells tagged with a red chromatin marker, H2B-mCherry, acquired with a 60× microscope objective.

cycle so regulation occurs through phosphorylation. As mentioned previously, it seems condensin is negatively phosphorylated by CK2 (at least in HeLa cells) and that removal of this phosphorylation plus phosphorylation by Cdc2 is key to convert condensin into an active complex (Kimura, 1998, Takemoto et al., 2006). Further phosphorylations by Cdc5 and Ipl1 are required for full activation and the maintenance of chromosome condensation in S. cerevisiae (St-Pierre et al., 2009, Vas and Clarke, 2008).

Many other proteins and protein modifications are involved in chromosome condensation. Furthermore, DNA-DNA interactions and intertwines are of great importance to the structure of condensed chromosomes (Poirier et al., 2000). These DNA-based connections are introduced into DNA by the combined actions of the condensin complex, the cohesin complex and topoisomerase enzymes. Topoisomerase enzymes are able to break and religate DNA strands, and as such are powerful tools for controlling these interactions (Liu et al., 1980, Rattner et al., 1996). Indeed, topoisomerases are required to maintain condensation as the chromosomes must be able to tolerate pulling forces without breaking the DNA. As such Top2 is required throughout mitosis to remove and
1.3 DNA Structure, Compaction and Supercoiling

1.3.1 DNA compaction in vivo

DNA must be highly packaged in order to fit inside the nucleus of a cell. This is a significant feat as the DNA is orders of magnitude longer than the cell (entirely without compaction) and it is heavily negatively charged, see Figure 1.8 for a cartoon representation of the stages of DNA compaction in vivo.

In eukaryotic cells, the initial level of compaction is via the formation of nucleosomes. This is achieved by the wrapping of approximately 147 base pairs of DNA around a protein octamer made up of two copies each of the histones H2A, H2B, H3 and H4 (Bentley et al., 1984, Richmond and Davey, 2003, Richmond et al., 1984). This stage of compaction is commonly referred to as ‘beads on a string’ due to its appearance by electron microscopy, or the 10 nm fiber due to its size (Luger et al., 1997). By wrapping the DNA around the protein octamer, the DNA is twisted in a way that alters the turn of the double helix resulting in a slightly more relaxed twist, as the twist is counter to the natural turn of the DNA (see below). Furthermore, as histones are positively charged, this process neutralises the negative charge of DNA (Fussner et al., 2011) and the N-terminal tails of the histone proteins can be modified to help regulate DNA-nucleosome interactions. This can alter the compaction or activation state of that stretch of DNA (Xu et al., 2009). A further compaction to 30 nm fibers is also suggested, helped by histone H1 (Fussner et al., 2011).

Finally, as cells enter into mitosis, additional compaction is necessary for accurate segregation. This process requires many different proteins including cohesin and condensin complexes, Top2 and a number of signalling molecules (Earnshaw et al., 1985, Hirano and Mitchison, 1994, Lewis et al., 1982).
Figure 1.8: DNA compaction levels The levels of compaction hypothesized to exist in DNA in vivo (MechanoBio, 2014). Wrapping of DNA around nucleosomes achieves an initial 6-fold compaction level (1 - 2). Further chromatin condensation into 30 nm fibers (i.e. zigzag or solenoid) is suggested by in vitro data and is yet to be proved or discredited to exist in vivo (3). During interphase (4), chromatin is folded into 300-700 nm domains, which together comprise a chromosome territory. The structure of territories remains the matter of debate and was proposed to exist in the form of solenoid, or zigzag, or nucleosomes, or a hybrid of those. A final level of compaction, mediated by condensin, is added as cells go into metaphase (7).

1.3.2 DNA supercoiling

The double helical structure of DNA was first described by Watson and Crick (1953). They briefly noted the struggles that a double helix would pose if access was required to the bases within. Relaxed DNA generally completes one turn around the double helix every 10.4 bases (Griffith, 1978, Wang et al., 1979). If force is applied to the DNA helix where it is unable to freely rotate (as is true in vivo due to protein structures creating topological domains within chromosomes (Worcel and Burgi, 1972)) this will change the number of base pairs per turn, and this in turn will change the stability of the helix, see Figure 1.9.

The helix is wound in a right handed manner, i.e. if viewed down the axis of DNA it turns clockwise away from the viewpoint (Figure 1.9A). If rotational force is applied in the direction of this left handed turn, then the DNA helix will 'overwind', increasing its
stability by increasing the energy required to access the bases within. If force is applied in the opposite direction then this will ‘underwind’ the helix and open its conformation slightly (Bates and Maxwell, 1993). The overwound form is referred to as positive supercoiling, and the underwound, negative. This can be represented by the Linking Number (Lk) of a DNA molecule, representing the number of times and with which handedness, the two backbones are intertwined, see Figure 1.9 (Bates and Maxwell, 1993). A relaxed DNA molecule thus has a linking number of 0.

**Figure 1.9: DNA winding and supercoiling** A) Cartoon showing handedness of the helix, and the addition of tension resulting in over- or underwinding. B) Cartoon demonstrating how DNA which is not free to rotate will become supercoiled. C) A cartoon demonstrating the supercoiling that is induced if a double helix is unwound from one end, as would happen in replication. D) Electron microscopy of plasmids with different linking numbers, and therefore assuming different supercoiling forms. Left hand image shows a plasmid where Lk=0, increasing through the images to the right (University of Maine, 2014).

Over- or underwinding introduces tension into the helix, and while a small change can be accommodated by spreading through surrounding regions of the DNA, a large amount of strain results in a higher level structure, a superhelical coil, where the axis of the helix twists to relieve the torsional stress (Marko and Siggia, 1995). Supercoiling of a DNA molecule alters its progression when separated by gel electrophoresis and this can
be used to identify the supercoiled state of a DNA molecule (Figure 1.9D). However, the extraction must be gentle as nicking either strand will allow rotation and therefore release any tension, returning the molecule to a relaxed state (Shure and Vinograd, 1976, Thorne, 1966). Certain molecules are capable of inserting themselves into the double helix in a way that introduces strain into the DNA double helix, which changes the structure of the molecule, leading to an apparent change in supercoiling (although this cannot change the Linking number). The most commonly used of these molecules are ethidium bromide and chloroquine. These can be added during separation of DNA by gel electrophoresis and aid in differentiating between differently supercoiled forms of DNA molecules (Shure et al., 1977). Both of these molecules insert into the DNA molecule in a manner that, in a negatively supercoiled DNA molecule, will add strain and move the apparent supercoiling level towards that of a relaxed molecule. If these are used at higher and higher concentrations, the DNA molecules will begin to run as if positively supercoiled (Shure et al., 1977).

For most of the cell cycle, in most organisms, DNA is maintained in a slightly underwound, negatively supercoiled form. As cells go into mitosis, it is known that the chromosomes condense through the formation of inter-DNA linkages mediated by condensin. Condensin has also been shown to be able to induce positive supercoiling into DNA in vitro and to act in conjunction with topoisomerases to induce a major supercoiling shift at the metaphase to anaphase transition (Bazett-Jones et al., 2002, Cuylen and Haering, 2011, Kimura and Hirano, 1997, St-Pierre et al., 2009, Stray et al., 2005).

1.3.2.1 Supercoiling in vivo

Regions of the genome which are being actively transcribed or replicated are likely to be negatively supercoiled so that the helicase, which is unwinding the helix to allow the transcriptional machinery to access the DNA bases, requires less energy to progress through the gene. Replication is a global process and the entire genome must be unwound in order for each base to be copied. This will result in the introduction of positive supercoiling – overwinding – ahead of the replication fork and negative supercoiling in the region just replicated, see Figure 1.10 (Ishimi and Matsumoto, 1993, Kegel et al., 2011, Wang, 2002). The positive supercoiling tension ahead of the fork can be released by topoisomerases (see below) provided there is space for them to access the DNA
(Wang, 2002). Indeed their action is required otherwise the energy needed for the fork to progress would be too great (Fierro-Fernández, 2007, Nitiss, 2009).

Figure 1.10: Supercoiling and its relief during DNA replication A cartoon showing the helical stress induced by a progressing replication fork and the sites of action of topoisomerases (McClendon et al., 2005). A) shows progression of a replication fork along DNA and the relief of tension by topoisomerases ahead and behind the fork. B) shows two converging forks preventing access for topoisomerases to the DNA, and C) shows the suggested swivelling of the forks that results in precatenanes, or twists in the newly produced sister chromatids. D) shows fully catenated sister chromatids, and through the action of Top2 their resolution to produce decatenated, but cohesed, sister chromatids in E). Small and large blue hexagons represent Top1 and Top2 respectively, the 6 part grey ring is the helicase and yellow circles represent the polymerases progressing in the wake of the helicase. The red rings around DNA show the addition of cohesin to the newly replicated DNA.

However, as forks converge, the space between them becomes limited and it is hypothesised that at some point topoisomerases cannot access the positively supercoiled region. At this point, it is suggested, the whole replication fork could swivel on its axis, transferring the positive supercoiling ahead of the fork to behind the fork, where the two nascent sister chromatids are already formed (Postow et al., 2001). This process would wrap the sisters in a way that, after the completion of replication, can only be reversed.
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by breaking one of the DNA strands and religating in a new configuration (also carried out by topoisomerases) (Kegel et al., 2011, Nitiss, 2009, Wang, 2002).

Condensin has been shown to be capable of introducing positive supercoiling in vitro (Bazett-Jones et al., 2002, Kimura and Hirano, 1997). Bazett-Jones et al. (2002) visualised condensin supercoiling naked DNA in vitro and one condensin molecule, without topoisomerases, can introduce two superhelical twists into a plasmid, see Figure 1.11. Although this would not go far to compact a whole chromosome, the presence of many other DNA features in vivo - histones, cohesin, topoisomerases - would be able to amplify this activity, for example using topoisomerases to cut and religate the DNA at one of these supercoils so that the twist is maintained as condensin dissociates. Indeed Kimura and Hirano (1997) show the introduction of positive supercoils into plasmids by condensin in conjunction with bacterial Topo I. Given the requirement of Top2 and condensin for chromosome compaction, they may cooperate to introduce the necessary loops that condense chromosomes ready for mitosis.

**Figure 1.11:** Condensin is capable of introducing supercoiling into DNA
A single molecule of condensin is capable of supercoiling a plasmid of naked DNA in vitro. The four images in A) represent a single condensin molecule with a naked DNA plasmid and ATP, B) is the same conditions but with AMP-PNP and no ATP, and D) represents a nicked plasmid, (Bazett-Jones et al., 2002). Supercoiling can be seen in the images where condensin is fully activated due to the presence of ATP and DNA (A), but fails to occur when the ATP is replaced by the inactive AMP-PNP (an analogue of ATP which cannot be metabolised) or where the plasmid was nicked and so cannot become supercoiled. The white spot is where the condensin complex has bound DNA, and the path of the DNA can be clearly seen.
1.3.3 Catenanes

Catenanes are two molecules of DNA which are linked in such a way that resolution requires the breaking of the backbones of one of the DNA molecules (Marini and Miller, 1980). The number of links or crosses by which the two molecules are intertwined are referred to as nodes. Catenanes were first observed in vivo in 1967 in human cells (Hudson and Vinograd, 1967) and are formed in every cell cycle. As referred to above, they were originally thought to be the only mechanism by which sister chromatids were held together after S phase (Holm et al., 1989). Although protein complexes have subsequently been identified, catenations persist until metaphase and there is some suggestion that protein complexes aid in the creation of catenations by maintaining the proximity of the sister chromatids (Haering et al., 2008, Losada and Hirano, 2001).

The number of nodes (crossing points) in a catenated molecule will alter its rigidity and as such, the molecules can be resolved differently by two dimensional (2D) gel electrophoresis (Martínez-Robles et al., 2009). Each DNA molecule in the catenane can exist in a supercoiled or relaxed state as discussed above for monomers, and this will further change its migration through a gel, see Figure 1.12 (Martínez-Robles et al., 2009).

![Figure 1.12: Cartoon showing the possible catenated states of two plasmids](image)

These cartoons show plasmids that are intertwined by either one, two or zero nodes (central, right and left hand images respectively). Top2 (orange hexagon) is known to decatenate plasmids, therefore moving the molecule from higher numbers of nodes to lower catenation states. Top2 has also been shown to be able to introduce catenations, both in vitro and in vivo.

It has been shown that catenations can be added enzymatically in vitro (Martin and Wang, 1970). Unpublished data (Aragón lab) has now shown that this can happen in metaphase in S. cerevisiae in vivo and that the likelihood of catenation depends upon the proximity of DNA molecules, maintained by cohesin. The enzymes which control
these processes are topoisomerases which are capable of breaking either one or both backbones of a DNA molecule.

1.3.4 Topoisomerases

Topoisomerases are enzymes capable of altering DNA structures by breaking DNA backbones. They act by breaking one strand of DNA and passing another through that gap, before resealing the break, and this alters the linking number, or catenation number, depending on the identity of the DNA molecules. They are found in all organisms and at least one must be functional for a cell to be viable as they are essential for untangling and decatenating DNA after replication in order to segregate DNA to new cells in mitosis (Spell and Holm, 1994). They are also essential for replication and transcription, to allow localised unwinding of the duplex, lowering the energy requirement for accessing the DNA bases (Wu et al., 1988).

There are two major classes of topoisomerases, type I and type II, classified by whether they break one or both backbones of a DNA double helix. However, those from different organisms can have different specificities within these classes, e.g. E. coli type I topoisomerase can only act on negatively supercoiled DNA whereas others are able to relax both positive and negative supercoiling to a lower linking number (Champoux and Dulbecco, 1972, Wang, 1971).

S. cerevisiae has three topoisomerases, one type II (Top2) and two type I (Top1 and Top3). Top3 typically acts in a complex with two other proteins Sgs1, a helicase, and Rmi1, and these are reportedly involved in altering supercoiling or catenation state (in cooperation with RPA) (Cejka et al., 2012, Chang et al., 2005, Gangloff et al., 1994, Mankouri and Hickson, 2007, Mullen et al., 2005). Figure 1.13 shows the different forms a plasmid can take, and the roles of topoisomerases in moving between these forms.

1.3.4.1 Type I Topoisomerases

Type I topoisomerases are capable of breaking a single DNA strand and can therefore alter supercoiling but not catenation levels. There are many different type I topoisomerases, distinguished by different modes of action and substrate preference. These fall into two main categories - type IA and type IB which have surprisingly different protein
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Figure 1.13: Differently catenated and supercoiled forms of a plasmid DNA can become supercoiled or catenated in vivo. Cartoons demonstrate the pathways between these forms for a circular plasmid. The processes involved in changing between the forms and the topoisomerase enzymes which help to carry out these changes are also marked, Top1 shown as a blue oval and Top2 as an orange hexagon. Supercoiling is generally only added as an indirect consequence of other DNA processes such as transcription or translation, and catenation by replication. However, it is possible for these to be introduced by topoisomerases during certain stages of the cell cycle.

structures resulting in different mechanisms of interaction with DNA. Type I topoisomerases of both classes act on DNA to reduce its linking number and move the DNA towards a more relaxed state, with the exception of Reverse Gyrase. This unusual type IA topoisomerase can actively introduce positive supercoils into DNA and is found in thermophile archaea whose DNA is positively supercoiled in vivo, in a manner thought to help protect the fragile bases against the extreme temperatures that they inhabit (Bates and Maxwell, 1993).

Type IA topoisomerases break a single strand of a DNA duplex, and pass the other strand of the same duplex through the break, towards a cavity within the enzyme, before resealing the original duplex. This will alter the supercoiling of the molecule by one linking number and this cavity is well conserved in this class of topoisomerases (Bates and Maxwell, 1993).

Type IB enzymes cleave a single strand in the same way but the 5’-OH end of the cleaved strand is released and allowed to rotate once, before religation, also relaxing the structure by one linking number. These enzymes lack the internal cavity seen in type IA topoisomerases (Bates and Maxwell, 1993). Both classes, therefore, act to reduce the
linking number of a DNA molecule by one, and can act upon both positive and negative supercoiling to move the DNA molecule towards a more relaxed state.

1.3.4.2 Type II Topoisomerases

Type II topoisomerases are different in that they cleave both strands of a DNA duplex, creating a double strand break (DSB) in the DNA helix, pass another DNA duplex through the break and then religate the break. This action can occur on two DNA strands from the same molecule, in which case the action results in a change of two in the linking number of the DNA (relaxing its level of supercoiling). Alternatively, it can act on strands from two different DNA molecules to alter their catenation state, reducing or increasing the number of links between two DNA molecules (Martin and Wang, 1970).

In order to safely introduce a DSB into DNA, type II topoisomerases act as a dimer where each subunit is responsible for breaking one strand - this broken strand is referred to as the gate segment (G-segment). The break is created by a tyrosyl residue in the active site of the enzyme, resulting in a phospho-tyrosine bond between the enzyme and the DNA, ensuring the enzyme keeps hold of the broken DNA strands which could be very dangerous to the cell (Nitiss, 2009). The enzyme then passes a different DNA section, called the transfer or T-segment, through the DSB and reseals the G-segment (Bates and Maxwell, 1993). The T-segment can be from the same DNA molecule or from another DNA molecule, as explained above.

Type II topoisomerases can be further subdivided into types IIA and IIB. The enzymes are made up of a dimer, with each monomer containing A and B subunits - A is responsible for binding the G segment and distinguishes the two subclasses of type II topoisomerases, while the B subunit is very similar across the two classes suggesting a single common strand passage mechanism (Berger, 1998). The A subunit differs in molecular structure, and this affects how it binds to the G-segment with type IIA enzymes forming a four base pair staggered overhang and type IIB forming a two base pair staggered overhang (Buhler et al., 2001). Type IIA topoisomerases also bend the DNA strand up to 150° prior to cleavage and this is an essential part of their catalytic cycle (Lee et al., 2013). The B subunit is well conserved across type II topoisomerases and also MutL proteins (a bacterial DNA repair enzyme). This subunit contains the ATP
binding domain, which in turn is responsible for the dimerisation of the enzymes and large conformational changes responsible for the strand passage mechanism (Corbett and Berger, 2003).

Type IIA topoisomerases include bacterial DNA gyrase, topoisomerase IV and eukaryotic Top2 (Nitiss, 2009), type IIB includes TopoIV from plants and Spo11 homologues which act in meiotic recombination (Lichten, 2001). Almost all of these enzymes act to relax positively or negatively supercoiled DNA. DNA gyrase is unique as its action introduces negative supercoiling to DNA (Berger, 1998). *S. cerevisiae* Top2 is a type IIA topoisomerase and the enzymatic cycle is shown in Figure 1.14. The crystal structure has also been solved for a large internal fragment by Berger *et al.* (1996).

Although a weak consensus sequence has been suggested for sites of Top2 action, it seems to be largely independent of the DNA sequence (Capranico and Binaschi, 1998) and more recent studies have suggested that the specificity could actually depend on local structure of the DNA molecule (Vélez-Cruz *et al.*, 2005). However, neither local structure nor sequence would allow the enzyme to know the ‘identity’ of the strands it is acting on, whether they come from the same or different DNA molecules, nor the global topological state of the DNA. As such, the enzyme cannot know the consequences of its action and it has been suggested that the catenation level must lie at an equilibrium with Top2 acting on any two DNA strands which lie in close enough proximity whether that action will result in the addition or resolution of catenations. Although it is considered favourable for cells to maintain topological links between sister chromatids from S phase to mitosis, it is essential that these can be removed fully prior to going through anaphase as any that remain as the sisters are separated will result in DNA bridges and incorrect segregation.

### 1.3.5 Topoisomerases in replication

It is essential that all cells retain the function of at least one topoisomerase enzyme. The supercoiling state of DNA is a variable thing and certain processes require changes to be made in the supercoiling or catenation state of the DNA. For example, condensation of chromosomes is thought to at least partly rely on DNA-mediated links introduced and maintained by type II topoisomerases and transcription requires a negatively supercoiled substrate to reduce the energy requirement of accessing the DNA bases. If a cell is
Figure 1.14: The enzymatic mechanism of *S. cerevisiae* Top2. Cartoon representation of the catalytic cycle of Top2 (Schmidt et al., 2012). (1) Homodimer at the beginning of cycle. One monomer is shaded beige and the other is coloured to show the ATPase domain (yellow), the conserved acidic triad as part of the Toprim domain (red) and the core of the enzyme (blue), as indicated in the key. The enzyme binds and bends a Gate-segment (G-segment, shown in black). (2) The binding of two ATP molecules (black stars) promotes capture of the Transfer-segment (T-segment, shown in green). (3) Hydrolysis of one ATP to ADP (black circle) leads to G-segment cleavage and T-segment transport through the break. Following strand transport, the ATPase domains swivel about each other to impede backward translocation of the T-segment. (4) G-segment ends are brought back together for religation. ATPase domains have wrapped around each other to prevent backwards motion of the T-segment, as shown by the red stars. (5) T-segment escape, followed by ADP release, resets the enzyme (Schmidt et al., 2012).

Lacking type II topoisomerases then it will produce anaphase bridges during segregation - remaining DNA strands which are linked by intertwines cannot be resolved in the absence of Top2 and so the DNA stretches between the two separating nuclei. Eventually these strands will either be ripped by spindle forces or cut by cytokinesis, either of which will result in uneven segregation of DNA.

Topoisomerases are particularly essential during replication as this involves the unwinding of entire chromosomes and resolution of linkages and supercoiling within the DNA, see Figure 1.10. As explained in Section 1.3.2.1, the progression of a replication fork through the DNA must be accompanied by the action of topoisomerases. The energy
required to unwind the helix as the forks move through the chromosome would eventually cause the fork to halt (Fierro-Fernández, 2007), and so topoisomerases must relax the over- and underwinding either side of the fork, keeping the energy requirements low enough for replication to continue. This process becomes harder as forks converge and fork swiveling has been suggested to relieve some of this tension although termination of replication is not well understood.

Either by fork swiveling or by allowing the supercoiling tension to spread along the DNA molecule (Witz and Stasiak, 2010), the newly replicated segments of DNA are likely to become wrapped around each other to form pre-catenanes which require strand breakage to be resolved and will become catenations if they are not resolved before the end of replication (Wang, 2002). It is estimated that these are formed so often by the process of unwinding the entire genome that Top2’s action must be required roughly every 10-20 bases (Hardy et al., 2004). Type I and II topoisomerases are acting throughout replication and continue to be needed through mitosis (Nakazawa et al., 2011). Cells can complete replication with only type I or type II topoisomerases, but without type II the cell is unable to resolve the intertwining of catenanes. Even in wild type cells, with Top2 fully active, some catenanes persist until metaphase (Baxter et al., 2011) and these must be resolved prior to segregation to ensure accurate partition of the DNA to daughter cells.

1.4 Segregation of sister chromatids at anaphase

The actions of both condensin and topoisomerases are required throughout mitosis, right through to the end of anaphase, to ensure that DNA remains compact and untangled as it is segregated to daughter nuclei. Furthermore, the actions which culminate in the segregation of the DNA - cleavage of the protein-mediated links, generation of tension across the mitotic spindles - must be tightly regulated to only occur once full replication, condensation and decatenation have taken place. Errors during this process will lead to genome instability.

At the onset of mitosis, sister chromatids which have been paired since S phase by a combination of topological and protein links align on the metaphase plate in the centre of the cell. At the centromere of each chromatid, a large protein complex forms, called
a kinetochore, which forms an attachment between the DNA and mitotic spindles (a single spindle in the case of *S. cerevisiae*). These spindles are microtubule structures that extend from either end of the cell to create a link between the DNA and the spindle pole body (SPB). Once attached, the spindles are able to generate tension via the actions of motor proteins (see Section 1.4.5) and this is the force that pulls sister chromatids apart to the two daughter nuclei at the onset of anaphase.

**1.4.1 Centromeres and Kinetochores**

*S. cerevisiae* has a point centromere which is defined by a single nucleosome containing a histone-H3 variant called Cse4 (Fitzgerald-Hayes *et al.*, 1982), bound at an 80 base pair conserved sequence, but the proteinaceous kinetochore which forms on this site is far from simple. Sequences surrounding the central nucleosome are also key for defining the centromeric, and pericentromeric, region and are well conserved. The kinetochore is known to comprise more than 100 proteins (Cheeseman and Desai, 2008) and can broadly be grouped into proteins that interface with centromeric DNA (inner kinetochore), those that interact with spindle microtubules (outer kinetochore) and those in between (central kinetochore), see Figure 1.15 (Cheeseman *et al.*, 2002).

The inner kinetochore consists of the centromeric nucleosome Cse4, the CBF3 complex which binds just outside the Cse4-containing nucleosome, the Ctf19 complex and a Mif2 homodimer, which together make up the CCAN (Centromere-Associated Network of Proteins) and remains bound to centromeres throughout the cell cycle (Cheeseman and Desai, 2008, Cieśliński and Ries, 2014, Ortiz *et al.*, 1999). Even small sequence changes in this centromere defining region of *S. cerevisiae* is enough to prevent CBF3 binding and this leads to a segregation failure (Cleveland *et al.*, 2003). All of these proteins are essential for kinetochore formation and function (Goh and Kilmartin, 1993, Meluh and Koshland, 1995, Niedenthal *et al.*, 1993, Ortiz *et al.*, 1999, Sorger *et al.*, 1994).

The central kinetochore consists mainly of the MIND complex (Mtw1, Dsn1, Nsl1 and Nuf1) (Hornung *et al.*, 2011, Pinsky *et al.*, 2003, Westermann and Weber, 2003) which is recruited by Ndc10 (a subunit of the CBF3 complex) and Cse4 (Pinsky *et al.*, 2003), and this combines with the Spc105 complex and Ndc80 of the outer kinetochore to form the KMN network, able to bind to microtubules (Cheeseman *et al.*, 2006, Nekrasov *et al.*, 2003, Pinsky *et al.*, 2006).
The outer kinetochore then is the DNA binding portion of the KMN network, consisting of the Ndc80 and Dam1 complexes (Cieśliński and Ries, 2014, DeLuca et al., 2006, Janke et al., 2001). The Ndc80 complex binds directly to the inner kinetochore and the microtubules and is regulated via phosphorylation by the Aurora B-like kinase Ipl1 (Akiyoshi et al., 2010, Cheeseman et al., 2006, Petrovic et al., 2010, Yamagishi et al., 2014). The Dam1 complex is made up of 10 subunits and by electron microscopy it appears to form a ring around the microtubules, thought to be important in coupling the movement of chromosomes to the depolymerisation of microtubules (Westermann et al., 2005) by interacting directly with the Ndc80 complex and encircling the microtubule (Maure et al., 2011, Yamagishi et al., 2014).

There are also accessory proteins, not required for the integrity of the kinetochore but which aid in the segregation of DNA, which associate with the kinetochore or with the plus ends of microtubules. These include the kinesin-like motor proteins, Kip1, Kip3, Cin8 and Kar3 (Pagliuca et al., 2009, Tanaka et al., 2005, Tytell and Sorger, 2006), Bik1 which is an end-on microtubule plus-end tracking protein (He et al., 2001), Stu1 and Stu2 which seem to play a major role in microtubule dynamics and regulatory proteins including the spindle assembly checkpoint proteins Mps1, Mad1, Mad2, Bub1 and Bub3 (Biggins, 2013, Cheeseman et al., 2001, He et al., 2001, Janke et al., 2002, Lin et al., 2001, Middleton, 1994).

The kinetochore is a flexible structure. When attached to microtubules, the protein contacts within the kinetochore can be altered by the tension put on the structure by dynamic microtubule binding (Maresca and Salmon, 2009). An intact kinetochore is essential for correct spindle attachment and to enable the Spindle Assembly Checkpoint (SAC) to function correctly (Janke et al., 2001). Yeast kinetochores form on the new sister chromatid centromeres during S phase and remain near spindle poles until the formation of spindles and segregation during mitosis.

Unlike in mammalian cells in which around 48 microtubules bind per kinetochore, only one microtubule attaches to each kinetochore in *S. cerevisiae*. This attachment can happen to a microtubule which has originated from either SPB and as such both sisters could become primed to travel to the same pole but must be corrected until each pair is attached in tension-generating pairs (Anderson et al., 2009, Tanaka et al., 2013, Yeh et al., 2008). As such, the cell has ‘tension sensing’ mechanisms which ensure that each
sister of a pair is attached to a microtubule from either pole, see Section 1.4.5. These mechanisms signal through the SAC to prevent progression into anaphase in the case of either incorrect binding, errors in kinetochore formation or incorrectly formed spindles, as explained below.

### 1.4.2 Spindle Assembly Checkpoint

The machinery of the SAC is made up of a number of proteins which can form complexes in multiple combinations. Key players Mad1, 2 and 3, Bub1 and 3 and Mps1 are all needed to arrest the cell cycle and were originally identified by mass screening (Hoyt et al., 1991, Li and Murray, 1991). MPS1 is the only essential gene from this group and encodes a kinase which plays a role in other cellular processes (Jones et al., 2001, Winey et al., 1991). The remaining checkpoint genes are not essential but have defects in segregation when the system is stressed, i.e. with microtubule poisons (Biggins, 2013,

The key step which enables this checkpoint to halt progression through the cell cycle is the sequestering of Cdc20 (Fang et al., 1998, Hwang et al., 1998, Kim et al., 1998, Li and Nicklas, 1997). Cdc20 is the regulatory subunit of the Anaphase Promoting Complex (APC), and is sequestered in various complexes with SAC subunits, predominantly a mitotic checkpoint complex (MCC) of Cdc20, Mad2, Mad3 and Bub3 whilst the spindles are forming and attaching to kinetochores (Brady and Hardwick, 2000, Hardwick et al., 2000, Sudakin et al., 2001, Tang et al., 2001). This MCC complex prevents Cdc20 from binding to the APC, delaying its activation and preventing the movement of the cell into anaphase (Amon, 1999). Without components of this checkpoint, e.g. Mad2, the cells are unable to activate the SAC and so the APC may become active in the absence of accurate spindle formation or attachment (Biggins, 2013, Li and Murray, 1991).

The sequestration of Cdc20 by the MCC is also directly related to the binding of spindles to kinetochores. Mps1 (the kinase) recruits checkpoint components to kinetochores (Hardwick et al., 1996, Heinrich et al., 2012, Yamagishi et al., 2014) via interaction with Ndc80 (Biggins, 2013, Kemmler et al., 2009). Mps1 then phosphorylates the Spc105 complex of the kinetochore and this recruits Bub1 and Bub3 which then recruit Mad1 and Mad2 (Gillett et al., 2004, Heinrich et al., 2012, Kiyomitsu et al., 2011, 2007, Krenn et al., 2012). The binding of Mad2 to kinetochores in association with Mad1 converts Mad2 into a “closed” form (De Antoni et al., 2005, Luo et al., 2000, 2002, Sironi et al., 2002). Cdc20 has a similar Mad2 binding domain to Mad1, and so the conversion of Mad2 to its closed form enhances its ability to bind Cdc20 and inhibit the APC (Biggins, 2013, Luo et al., 2000, 2002, Mapelli et al., 2007).

When tension is generated at kinetochores, Mad1 and Mad2 proteins no longer bind (Gillett et al., 2004) and satisfaction of the checkpoint is also thought to depend on de-phosphorylation by both Cdc14 and protein phosphatase 1 (PP1) and possibly the degradation of these subunits (Mirchenko and Uhlmann, 2010, Pinsky et al., 2009, Vanoosthuyse and Hardwick, 2009). Once Mad2 is lost from attached kinetochores, it reverts to its open conformation and loses its interaction with Cdc20, allowing activation of the APC (De Antoni et al., 2005, London et al., 2012, Luo et al., 2000, Sironi et al., 2002). Components of the SAC are then degraded preventing its reactivation as the

The sensing of microtubule binding and tension generation is thought to happen through changes in the availability of substrates for certain kinases due to the stretching of kinetochores in response to the tension. Ipl1 (Aurora B kinase) is known to localise to the inner centromere during metaphase and phosphorylate Dam1 and Ndc10 of the kinetochore in a manner which disrupts their binding to microtubules (Biggins, 2013, Gestaut et al., 2008, Sarangapani et al., 2013). This is also thought to be the mechanism for satisfaction of the checkpoint response, allowing the action of phosphatases, as mentioned above, to dominate and dismantle the checkpoint.

Upon binding of two microtubules from opposite poles to each of a pair of sister chromatids, the pulling forces across the pair will stretch the kinetochore and pull the substrates of Ipl1 out of reach. Ipl1 can therefore no longer phosphorylate these targets and the interaction will be stabilised (Tanaka et al., 2005). Elegant support for this theory was provided by Liu et al. (2009) where they fused biosensors able to detect Ipl1 phosphorylation activity with proteins recruited either to the centromere or the kinetochore. This showed that when kinetochores came under tension, only the biosensor at the centromere remained phosphorylated, whereas when tension was lost, both biosensors showed Ipl1 activity (Liu et al., 2009).

Furthermore, Ipl1 mutants are unable to detach from the spindle pole, and show segregation defects, as well as monopolar attachments of paired sister chromatids (Biggins et al., 1999, He et al., 2001, Tanaka et al., 2002). Ipl1 forms part of the chromosomal passenger complex (CPC) along with Sli15 (INCENP), Bir1 (Survivin) and Nbl1 (Borealin), and its inner centromere localisation lasts until anaphase when it relocates to the spindles. This is crucial as anaphase will release the tension on the kinetochores (due to loss of the forces holding sisters together) and this would put Ipl1 back in reach of its targets, meaning the interactions would be destabilised (Carmena et al., 2012, Mirchenko and Uhlmann, 2010, Oliveira et al., 2010, Parry et al., 2003). This localisation pattern, along with a tension-dependent drop in the phosphorylation level of its substrates and defects caused by ectopic localisation of Ipl1, support a central role in tension sensing for Ipl1, see Figure 1.16 (Keating et al., 2009, Liu and Lampson, 2009, Sandall et al., 2006, Shimogawa et al., 2009, Tanaka et al., 2002, Welburn et al., 2010).
Debate remains over whether the SAC detects the presence of tension generating connections at each kinetochore, or if the presence of an unattached kinetochore is sufficient to sustain its activation. Artificially obliterating unattached chromosomes has resulted in the inactivation of the SAC (Rieder et al., 1995) and in mantid spermatocytes applying mechanical tension to unattached chromosomes was also able to allow cells to move into anaphase (Li and Nicklas, 1997). However, the story may be different in *S. cerevisiae* as at least some studies have shown that a lack of tension failed to activate the SAC but that loss of kinetochore components could (Guacci et al., 1997, Tavormina and Burke, 1998).

**Figure 1.16:** A model for the biorientation mechanism for microtubule-kinetochore attachments
Cartoons depicting a model for the mechanism by which tension across kinetochores is sensed in *S. cerevisiae* (Tanaka et al., 2005). On the left, attachment of spindles from the same pole to both sisters shows incorrect attachment (green ball and rods show the spindle pole body and microtubules) and this means no tension is created across the kinetochores (shown as orange balls). As such, Ipl1 (localised to the inner kinetochore, red oblongs) is in contact with its substrates within the kinetochore in its relaxed state. As tension is created on the right, resisted by the cohesin complexes (pale circle), the structure of the kinetochore is altered and Ipl1 can no longer phosphorylate its substrates on the outer kinetochores, stabilising the attachment.

### 1.4.3 The pericentromeric region

The pericentromeric region in almost all organisms (except *S. cerevisiae*) is a heterochromatic region defined by di- and trimethylation of histone H3 on lysine 9 to which the protein HP1 is recruited (Marston, 2015). Heterochromatic or not, the pericentromeric
region is essential in all organisms for accurate segregation of chromosomes (Gartenberg, 2009, Tanaka et al., 2002) and attracts high levels of cohesin, shugoshins (see below), Ipl1, condensin and the kinetochore proteins.

Several of the kinetochore subunits, including the Ctf19 subcomplex and Dbf4-Cdc7 kinase (DDK), are crucial for establishment of the pericentromeric region, and its enrichment in the complexes mentioned above (Natsume et al., 2013). These proteins recruit Scc2/4 to the centromere and this enables loading of the cohesin complex resulting in a higher level of cohesin at the centromeric region when compared to the chromosome arms (Fernius et al., 2013). DDK, the Dbf4-dependent kinase, seems to be heavily involved in several of these processes. It is recruited to heterochromatin in fission yeast (S. pombe) and by kinetochore components in S. cerevisiae (Bailis et al., 2003, Natsume et al., 2013) and is essential to the loading of cohesin and to triggering the early firing of replication origins in these regions (Natsume et al., 2013, Tanaka et al., 2013).

In S. cerevisiae where the centromeres are defined by just a single Cse4-containing nucleosome, transferring the centromere consensus sequence to another site within the chromosome is sufficient to establish a fully functional centromere and pericentromeric environment, including the early replication of these regions which appears crucial for proper kinetochore assembly (Hill and Bloom, 1989, Pohl et al., 2012, Tanaka et al., 1999, 2013).

The centromere is key for the creation of a functional kinetochore and also for the assembly of a pericentromeric region and recruitment of pericentromeric factors which themselves help to support kinetochore function and ensure accurate chromosome segregation.

1.4.4 Shugoshin

Shugoshins were first identified for their role in centromeric cohesion protection during meiosis (Kitajima et al., 2004, Marston and Amon, 2004) where the removal of cohesin during meiosis is a multi-step process. During metaphase I, cohesin is lost from the arms via cleavage by separase (Nasmyth, 2001, Page and Hawley, 2003, Petronczki et al., 2003). This cleavage is activated by phosphorylation of the Rec8 kleisin subunit of
coheshin. Shugoshin prevents this by recruiting protein phosphatase 2A to the centromere, this phosphatase removes the phosphorylation from Rec8 and cohesin at the centromere is not cleaved (Clift and Marston, 2011, Hamant et al., 2005, Moore et al., 1998). The centromeric localisation of shugoshin is dependent on the centromere, many components of the kinetochore, Bub1 kinase and the enriched cohesion of the pericentromeric region (Kerrebrock et al., 1995, Kiburz et al., 2005, Vaur et al., 2005).

A similar set of events occurs during mammalian mitosis where cohesin is removed in a two-step pathway as well (McGuinness et al., 2005, Waizenegger et al., 2000). During prophase, a protein called sororin which associates with cohesin prior to mitosis and aids in maintaining DNA cohesion (Rankin et al., 2005) is phosphorylated and this causes its dissociation from cohesin which has also been phosphorylated on its Scc3 subunit and these phosphorylations result in the loss of cohesin from chromosomes (Waizenegger et al., 2000). Again, due to the presence of shugoshin at centromeres and its recruitment of PP2A, sororin at the centromere is dephosphorylated and cohesion is maintained until separase cleavage during anaphase (Nishiyama et al., 2010, Uhlmann et al., 2000, Waizenegger et al., 2000).

However, the distribution of shugoshin in S. cerevisiae mitosis was also found to be centromeric and not to affect cohesion (Kiburz et al., 2005). Instead, it plays a role in the correct biorientation of sister chromatids both in mitotic metaphase and metaphase II of meiosis (Kawahima et al., 2007, Kiburz et al., 2008, Vanoosthuyse et al., 2007). Sgo1 is the only shugoshin in S. cerevisiae but it is not an essential gene. However, cells lacking Sgo1 struggle to recover from mitotic spindle damage, for example treating cells with the microtubule-depolymerising drug nocodazole results in missegregation of chromosomes (Indjeian et al., 2005).

Sgo1’s localisation to the centromere in mitosis depends on Bub1 phosphorylation of H2A (a histone) at the pericentromere and centromere (Boyarchuk et al., 2007, Huang et al., 2007, Kawashima et al., 2007, Yamagishi, 2010). Bub1 is part of the kinetochore, and upon tension generation across sister chromatids, Bub1 can no longer phosphorylate H2A and this causes the loss of Sgo1, aided by dephosphorylation by Sgo1’s PP2A recruitment (Boyarchuk et al., 2007, Eshleman and Morgan, 2014, Haase et al., 2012, Kawashima et al., 2007, Nerusheva et al., 2014, Tanno et al., 2010).
Sgo1 is in fact essential to recruit condensin to the pericentromere, which helps to aid in the initial biasing of kinetochore capture towards spindles from opposite poles and for the recruitment and maintenance of the CPC, including Ipl1, at incorrect microtubule-kinetochore attachments (Peplowska et al., 2014, Rattani et al., 2013, Rivera et al., 2012, Verzijlbergen et al., 2014). By dissociating from the centromere in response to tension, Sgo1 concomitantly removes its associated phosphatase, PP2A-Rts1, followed by the loss of Ipl1 and condensin (Nerusheva et al., 2014, Rattani et al., 2013).

1.4.5 Biorientation and segregation

Kinetochore attachment and tension sensing are complicated events controlled by phosphorylation of many substrates which leads to the localisation or loss of several different proteins at the centromere and pericentromere, including Ipl1 and Sgo1 (Haase et al., 2012, Parry et al., 2003, Peplowska et al., 2014, Tanaka et al., 2005).

When tension is sensed at every sister kinetochore pair, cohesin is cleaved, the DNA links are resolved and the sisters are pulled to the two new nuclei. This process occurs through the de-polymerisation of the microtubules from the kinetochore-associated end, aided by motor proteins (Desai and Mitchison, 1997). Microtubules are made up of heterodimers of α- and β-tubulin and are polar with the minus end oriented towards the spindle pole and the plus end aiming for kinetochores (Desai and Mitchison, 1997).

The microtubules can be extended or disassembled from either end, however extension and disassembly is much more dynamic at the plus end. There is an intrinsic GTPase activity of tubulin dimers and its action causes spontaneous depolymerisation of microtubules from the plus end unless a tubulin-GTP ‘plus end cap’ is present (Duro, 2015). Kinesin-5 motors (Cin8 and Kip1 in S. cerevisiae) act in the assembly of the mitotic spindle, maintenance of it prior to the anaphase transition and anaphase spindle elongation (Gerson-Gurwitz et al., 2009). Cin8 and Kip1 localise to the spindle midzone, and they are suggested to act by crosslinking and sliding interpolar microtubules, i.e. in the nucleus (Gerson-Gurwitz et al., 2009).

Dynein (Dyn1) is a minus end directed motor protein located at the cell cortex in the bud. This seems to act by applying pulling forces to the spindle via cytoplasmic MTs and is essential for mitotic spindle positioning in S. cerevisiae and other eukaryotes.
Together, these three motor proteins Cin8, Kip1 and Dyn1 provide the forces to separate the sister chromatids along spindles after cohesin cleavage, see Figure 1.17.

**Figure 1.17: Suggested mechanisms of microtubule force generation.** A) Microtubules (grey beaded rods) could be attached to proteins such as Ndc80 (orange rods) by random binding events and as these proteins rebind further along the microtubule, the microtubule depolymerises behind their binding location, biasing the movement towards tension generation. B) The DASH/Dam1 rings (purple rings) could potentially encircle the microtubules in such a way that the depolymerisation would carry the kinetochore along the microtubule. C) Fibrillar proteins (red rods) could attach to the peeling microtubules in such a way to generate force. D) The microtubule could polymerise against a rigid barrier to generate force but these forces are not thought to be significant in kinetochore motility (Bloom and Joglekar, 2010).

### 1.4.6 Cohesion dissolution

For the segregation of sister chromatids to occur in anaphase, cohesin-mediated links must be removed. In *S. cerevisiae* this occurs as cells enter anaphase and is controlled by the APC, as mentioned above. Once Cdc20 is released from Mad2 following the achievement of biorientation of sister chromatids coming under tension from the spindles, the APC is activated and targets multiple proteins for degradation, including a protein called Pds1 (securin) (Cohen-Fix *et al.* 1996, Zou *et al.*, 1999). The degradation of Pds1 results in the activation of Esp1 (separase), a protease that cleaves the kleisin subunit of cohesin, Scc1, releasing the sisters, see Figure 1.18 (Ciosk *et al.*, 1998, Uhlmann
et al., 2000, Waizenegger et al., 2002). The relationship between separase and securin is slightly more complex though, as securin also plays a role in the full activation of separase (possibly via targeting it to the chromosomes (Ciosk et al., 1998, Jäger et al., 2001, Siomos et al., 2001)). This relationship was elegantly demonstrated by loss of both securin alleles or a single separase allele, neither of which caused lethality but loss of a single separase allele in a securin null mutant was inviable (Wirth et al., 2006).

Separase null mutants are thought to be viable due to the need for phosphorylation of Scc1 by Cdc5 (Polo-like kinase) to target the cleavage action of separase (Alexandru et al., 2001, 1999, Yamamoto et al., 1996) and further levels of regulation for this cleavage are enforced by Sgo1, as it recruits a phosphatase (PP2A-Cdc55) which counters the Cdc5 phosphorylation and inhibits separase action (Clift et al., 2009, Yaakov et al., 2012). Full segregation also requires the degradation of the C-terminal fragment of Scc1 (Rao et al., 2001) suggesting cleavage is only the first step of the process.

![Cohesin removal at the metaphase to anaphase transition.](image)

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In vertebrates, cohesin is removed in two steps - the prophase pathway, in prophase, and Scc1 cleavage as the cells move into anaphase. The prophase pathway removes cohesin without cleavage by separase but instead depends on Wap1, Cdc5, Aurora B and condensin and potentially separates cohesin at the Smc3-Scc1 interface (Buheitel and Stemmann, 2013, Haarhuis et al., 2013, Waizenegger et al., 2000). The prophase pathway removes approximately 90% of cohesin and that which remains is localised to the centromeric and pericentromeric regions (protected by shugoshin). This remaining
cohesin is cleaved as in *S. cerevisiae* by separase at the metaphase to anaphase transition. In *S. cerevisiae*, the prophase pathway is not present and all cohesin is removed upon activation of separase as the cells progress to anaphase.

### 1.4.7 Catenation resolution

As mentioned earlier, replication introduces an enormous amount of topological strain into the DNA and although most of this is resolved by topoisomerases during S phase, a number of catenations between sister chromatids persist until anaphase (Baxter *et al.*, 2011). A recent paper (Farcas *et al.*, 2011), showed that in all likelihood full-size chromosomes will have a significant number of catenations that persist into mitosis and all of these must be removed before segregation, in order to prevent chromosome missegregation, see Figure 1.19.

![Figure 1.19: Complete catenation removal prior to anaphase is essential](image)

The removal of cohesin at the metaphase to anaphase transition is not sufficient to allow accurate segregation of the genome. If the DNA-mediated links are not resolved then this will lead to chromosome bridges as the sister chromatids are pulled apart. The two sister chromatids are represented as black and grey threads, held together by red and blue cohesin complexes and put under tension by binding to the microtubules in yellow. The right hand image shows the catenanes exposed as cohesin is removed and spindle tension dominates.

As the topoisomerase enzymes cannot sense DNA ‘identity’, it is likely that other mechanisms are needed to promote full decatenation. It was suggested that the bend introduced by type IIA topoisomerases into DNA could orient the enzyme (supercoiling crosses are thought to have different geometries) but this is not supported experimentally
(Hardin et al., 2011, Klenin et al., 2002, Stuchinskaya et al., 2009, Vologodskii et al., 2001). Alternatively, topoisomerases could detect catenation states in local topological domains (estimated to be 20 kb in *E. coli*) (Hardy et al., 2004), that spindle forces propagate along chromosome arms to expose catenations or that the enzymes simply reduce catenations past the equilibrium level by the use of ATP as an energy source (Rybenkov, 1997).

It was recently shown that a dramatic change in the catenation level occurs at the moment of spindle formation and bipolar attachment of the sister chromatids, i.e. just prior to anaphase, and this is accompanied by a change to positive supercoiling (Baxter et al., 2011). Catenations are present in a nocodazole-mediated arrest, without spindles, but are removed as the cells progress to a Cdc20-mediated cell cycle arrest (which acts by depleting Cdc20, the regulatory subunit required for the APC to trigger anaphase) (Baxter et al., 2011).

It is thought this could present catenations in a geometry that favours decatenation by Top2 (Figure 1.20) similarly to the original hypothesis involving spindle forces. However, this mechanism implies a global topological change and this could be more effective for removal of catenations distal to the centromeres. Furthermore, extensive supercoiling would pull freshly decatenated strands away from each other, potentially reducing the chance of erroneous re-catenation.

**Figure 1.20:** A positive supercoiling transition may help expose catenations. A change in the supercoiling of the DNA (represented as red and blue double helices from separate DNA molecules) at the moment of spindle attachment could help to expose any remaining catenations in a way that makes them more obvious for Top2 (green dimer) to decatenate. On the left, multiple locations on the DNA appear to be intertwined, while on the right the supercoiling of the DNA has resolved two of these an exposes the single tru catenation.
1.5 Project Introduction

The change in supercoiling identified at the metaphase to anaphase transition (Baxter et al., 2011) is an important step in understanding the mechanisms used by the cell to ensure the final resolution of catenations prior to the moment of segregation. Baxter et al. (2011) showed that Smc2 is required for the transition to a positively supercoiled state, implicating the condensin complex, and subsequent work has also highlighted Cdc5 (Polo-like kinase) as essential for activating the supercoiling of the transition. This could be acting via its phosphorylation of condensin as this has been reported to increase condensin’s positive supercoiling activity in vitro (St-Pierre et al., 2009).

The first objective of this project was to understand the mechanisms which allow activation and progression of the supercoiling transition. It is unknown how a tension sensing mechanism triggers the supercoiling transition. It may be regulated via the SAC, the action of kinases involved in tension sensing or physical tension itself. Is the effect an all-or-nothing commitment and could it be reversed or prolonged? Finally, was it a local or global phenomenon and could we directly link it to catenation resolution?

Another aim of the project was to investigate the mechanism by which condensin was involved in the transition. Is the condensin complex the primary enactor of the supercoiling transition? Does this involve an enzymatic activity of condensin, as seen in vitro, and does it make use of topoisomerases? How is this change in activity regulated and is it linked to altered localisation of condensin on the DNA?

Finally, could the role of topoisomerases in this supercoiling transition be further elucidated. If the function of the supercoiling transition is to highlight catenations for resolution by Top2, could we also see a recruitment of Top2 to chromosomes? Furthermore, is it possible to visualise a localisation of Top1 or Top3 in this process, presumably responsible for the topological shift visualised by Baxter et al. (2011) in the absence of Top2?
Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Media

Yeast extract peptone (YP) and synthetic media lacking various amino acids were made according to standard recipes. 2% agar was added for solid media to make plates. Antibiotics used for selection included 100 $\mu$g/ml nourseothricin (NAT selection), 300 $\mu$g/ml hygromycin B (HPH selection) and 200 $\mu$g/ml geneticin (KAN selection). To analyse some mutations, benomyl was used on plates at 10 $\mu$g/ml from a stock dissolved in dimethyl sulfoxide (DMSO).

Glucose, galactose, and raffinose have all been used as a carbon source, all at a concentration of 2%. These were prepared as separate stocks, filter sterilized and then added to autoclaved media.

2.1.2 Cell cycle synchronisation

Alpha-factor was added to liquid MATa cultures to a final concentration of 50 ng/ml for bar1Δ strains and 5 $\mu$g/ml for BAR1+ strains. Secondary doses of half the amount were added every 90 minutes to maintain the arrest. Cultures were monitored by microscopy until around 95% of cells had arrested in G1 (typically 2-3 hours). For release, cells were spun down at 4,000 rpm for 2 minutes and washed with 0.5 volumes of equivalent media containing 0.1 mg/ml pronase (Sigma). This wash was repeated twice more and then the pellet resuspended in fresh media, also containing 0.1 mg/ml pronase.

To arrest in G2/M, nocodazole was used to a final concentration of 15 $\mu$g/ml (stock was 1.5 mg/ml in DMSO) and then half doses added every 90 minutes to maintain the
arrest. To release from the arrest, cells were pelleted and washed as for α-factor release but in media lacking pronase.

For the inactivation of temperature sensitive proteins, the culture was shifted for at least one hour to 37°C to ensure full inactivation. If the mutation is intended to induce a cell cycle arrest then the temperature shift was maintained until the cells, as monitored by microscopy, were arrested.

For proteins expressed under the galactose-inducible promoter, cells were grown in 2% raffinose for 24 hours prior to the experiment and then 2% galactose added to begin induction.

To induce the destruction of a protein tagged with a doxycycline-induced degron, doxycycline was added to 50 µg/ml which represses the promoter, galactose added to 2% to induce expression of the ubiquitin-ligase Ubr1 and the culture shifted to 37°C. The temperature shift induces a conformational change, exposing a lysine which is ubiquitinated by the overexpressed Ubr1, triggering degradation of the protein and the promoter is shut down so no new protein is produced. This is explained further in Chapter 3.

2.1.3 Growth assay

To assess the viability of certain mutants, 10-fold dilutions of cells from an optical density of 1 were made in water and then spotted (2 µl) onto a plate of appropriate media, incubated for 3 – 5 days and the growth analysed.

2.2 Strain creation

2.2.1 Competent cells

Based on Knop et al. (1999), cells were harvested and washed with 0.5 volumes of sterile distilled water and then with 1 ml of Sorbitol buffer (100 mM LiOAc, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1 M sorbitol; filter sterilized). Cells were resuspended in 360 µl of SORB per 50 ml of original culture and carrier DNA (salmon sperm DNA) was added to 1 mg/ml concentration. Cells were aliquoted and stored at -80°C until required for transformation.
2.2.2 Transformation

Based on Knop et al. (1999), approximately 0.2 µg of circular plasmid DNA, or 1 µg of linearized plasmid or PCR product were added to 30 µl of competent cells. 6 volumes of polyethylene glycol (PEG; 100 mM LiOAc, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 40% (w/v) PEG-3350, filter sterilized) were added and this mixture incubated for 30 - 60 minutes at room temperature. DMSO was then added to 10% of final volume and this mixture heat shocked at 42°C for 15 minutes. The cells were spun at 10,000 rpm for 2 minutes and then either resuspended in sterile distilled water and plated immediately for amino acid drop-out selection or resuspended in YPD and allowed to grow for approximately 3 hours before being plated for antibiotic selection.

2.2.3 Integration by Homologous Recombination

Chromosomal replacement of genes, deletion of genes or insertion of epitope tags was carried out by PCR and homologous recombination (Janke et al., 2004). Plasmids were used that contained a selection marker and, where appropriate, an epitope tag. These were amplified using primers with sequence tails designed to enable integration into the genome by homologous recombination. Positive transformants were then tested by colony PCR, Western blot, growth tests or microscopy.

2.2.4 Restriction digest of DNA

Restriction enzymes (New England Biolabs) allowed site-specific cleavage of DNA. 5 – 10 units of enzyme were used for each 1 µg of DNA to be digested. The mixture was then incubated at the recommended temperature for 1 - 12 hours.

2.3 Cell Analysis Techniques

2.3.1 Fluorescence-Activated Cell Sorting (FACS)

Samples were fixed in 70% ethanol and stored at 4°C for a minimum of one hour. Cells were spun down at 10,000 rpm for 2 minutes, resuspended in 250 µl saline-sodium citrate
solution (SSC) (150 mM NaCl, 15 mM trisodium citrate, pH 7) with 25 µg RNase A (Qiagen) and incubated overnight at 37°C. 1.5 µl Proteinase K were added with 50 µl SSC and the mixture vortexed before incubating for one hour at 50°C. Samples were sonicated for 4 rounds of 30 seconds on low power (30 seconds off in between) at 4°C. After, 250 µl SSC were added and 15 µl of propidium iodide (final concentration 1 µg/ml). This was incubated for one hour at room temperature and then 200 µl of each sample diluted in 1 ml of SSC for FACS analysis using FACScan cytometer and CellQuest Pro software (both Becton Dickinson).

### 2.3.2 Microscopy

Cells were spun down from liquid culture and frozen at -80°C or used immediately. The pellets were washed in 1% Triton® X-100 for 2 minutes and then resuspended in distilled water. To visualize the nuclei of intact cells, a final concentration of 25 ng/ml of 4’,6-diamidino-2-phenylindole (DAPI)/Antifade (Millipore) was used in water. For fluorescence microscopy, a series of z-focal plane images were taken for each sample on a Leica IRB microscope using a Hamamatsu D742-95 digital camera and OpenLab software (Improvision). A controlled light source (Polychrome IV (Photonics)) with a Xenon lamp or an ultraviolet mercury lamp (Leica) was used. Images taken in different z-axis planes were flattened into a 2D projection and processed with OpenLab.

### 2.4 Protein Analysis Techniques

#### 2.4.1 TCA protein extraction

Trichloroacetic acid (TCA) extraction extracts proteins closely bound to chromatin more efficiently and therefore it was commonly used when looking at the proteins of cohesin or condensin complexes. Cells were collected by centrifugation at 4,000 rpm for 2 minutes and washed with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) (PBS). The PBS was removed and the pellets frozen at -80°C. From here on, all work was done on ice with pre-chilled solutions and machines. Cells were resuspended in 250 µl of 20% TCA and glass beads added, before breaking in a FastPrep® machine for 2 20 sec cycles at a power setting of 5.5 (FastPrep® FP120,
BIO 101). The tubes were then pierced with a hot needle, placed into a new eppendorf and spun at 2,000 rpm for 2 minutes to collect the lysate. The glass beads were washed with 300 µl of 5% TCA and this was spun down again to collect the wash together with the first lysate. This lysate was mixed with 700 µl of 5% TCA and then spun at 14,000 rpm for 10 mins and the pellets were washed with 750 µl of 100% ethanol. The pellet was resuspended in an appropriate volume of 1 M Tris pH 8 and 2 volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.03% bromophenol blue). This mixture was then boiled for 10 minutes at 95°C, centrifuged for 5 minutes at 15,000 rpm and the supernatant was taken for analysis by SDS-PAGE.

2.4.2 RIPA protein extraction

Radioimmunoprecipitation assay (RIPA) extraction for protein analysis was carried out on cells collected by centrifugation at 4,000 rpm for 2 minutes and washed with PBS. The PBS was removed and the pellets frozen at –80°C. From here on, all work was done on ice with pre-chilled solutions and machines. Cells were resuspended in 15 µl of RIPA buffer (25 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) and glass beads added, before breaking in a FastPrep® machine for 2 20 sec cycles at a power setting on 5.5 (FastPrep® FP120, BIO 101). 150 µl of RIPA were added to each tube and then the tubes pierced with a hot needle, placed into a new eppendorf and spun down at 2,000 rpm for 2 minutes to collect the lysate. The eppendorf containing the lysate was then spun down at 14,000 rpm for 10 mins and the supernatant taken to a new tube. The level of protein was assessed with Pierce® bicinchoninic acid (BCA) protein assay (Thermo Scientific) and then the samples diluted to equalise the protein concentrations in each sample. More commonly, samples were made comparable by taking equal volumes of cells during the experiment. The correct volume of the samples was taken, adjusted with water where necessary, and loading buffer added (4x Lithium dodecyl sulfate (LDS) sample buffer + 8% β-mercaptoethanol). These samples were then boiled for 5 minutes and loaded onto SDS-PAGE gels for analysis (see below).
2.4.3 Alkaline Protein Extraction

Post-alkaline protein extraction was performed for rapid protein extraction (Kushnirov, 2000) by taking cells from an agar plate and resuspending them in 100 µl of sterile distilled water. 100 µl of 0.2 M NaOH was then added, and incubated for 5 minutes at room temperature. Afterwards, cells were pelleted at 13,000 rpm for 30 seconds, resuspended in 25 µl of 1 M Tris pH 8 and 50 µl SDS-PAGE loading buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.03% bromophenol blue) and boiled at 95°C for 3 minutes. Samples were then spun down at 12,000 rpm for 2 minutes. Finally, the supernatant was loaded onto a SDS-PAGE gel and analysed by Western blot (see below).

2.4.4 SDS-PAGE

The Bio-Rad Mini-PROTEAN® 3 system or the Invitrogen NuPAGE® system was used for SDS-PAGE. The Bio-Rad system used 8-10% acrylamide gels (Resolving gel: 26.7 – 33.4% Protogel Acrylamide solution (30% acrylamide (acrylamide:bisacrylamide 37.5:1), National Diagnostics), 26% ProtoGel Resolving Buffer (final concentrations: 0.375 M Tris-HCl, 0.1% SDS, pH 8.8), 0.1% ammonium persulphate (APS), 0.1% N,N,N1,N1 tetramethylethylethylenediamine (TEMED; National Diagnostics) and Stacking gel: 13% ProtoGel Acrylamide solution (as above), 25% ProtoGel Stacking Buffer (final concentrations: 0.125 M Tris-HCl, 0.1% SDS, pH 6.8), 0.1% APS, 0.1% TEMED). Gels were run at 140 V for 30 minutes and then 200 V for the desired time in Tris-glycine-SDS running buffer (0.025 M Tris base, 0.192 M glycine, 1% SDS, National Diagnostics).

For the Invitrogen NuPAGE® system, pre-cast 4-12% Bis-Tris or 3-8% Tris-Acetate NuPAGE® gels were run in NuPAGE® Bis-Tris or Tris-Acetate buffer respectively (Bis-Tris: 50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3 and Tris-Acetate: 50 mM Tricine, 50 mM Tris base, 0.1% SDS, pH 8.24) at 200 V according to manufacturer’s instructions.
2.4.5 Western Blot

SDS-PAGE gels were transferred to polyvinylidene fluoride transfer membrane (Hybond-P, Amersham Biosciences) in the appropriate system – Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell or for Invitrogen gels the XCell SureLock® Mini Cell Transfer module. The Bio-Rad system used Tris-Glycine blotting buffer (0.025 M Tris base, 0.192 glycine, pH 8.4, National Diagnostics) with 20% methanol and the NuPAGE system used NuPAGE transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2), also with 20% methanol. Transfers were run at 250 mA for NuPAGE and 280 mA for Bio-Rad systems, both for 1 hour.

Membranes were blocked in 5% skimmed milk powder, in PBS with 0.1% Tween® 20 (PBS-T) for a minimum of 45 minutes at room temperature then incubated overnight at 4°C in 5% milk in PBS-T with appropriate primary antibody. These were then washed three times with PBS-T and then incubated in 5% milk in PBS-T with the appropriate secondary antibody, conjugated to IgG Horseradish Peroxidase for 90 minutes at room temperature. These were then washed again three times in PBS-T and then twice in PBS. Membranes were then incubated with Enhanced Chemiluminescence (ECL) Plus/Prime Western Blotting Detection System (GE Healthcare) followed by exposure to ECL Hyperfilm (GE Healthcare).

2.4.6 Immunoprecipitation and Co-Immunoprecipitation

Co-immunoprecipitation experiments were performed based on Arumugam et al. (2004) with minor modifications: 100 OD$_{600}$ of cells were harvested at 4,000 rpm for 2 minutes, washed once with ice-chilled water, and the cell pellet frozen at -80°C. Cells were resuspended in 200 µl of pre-chilled lysis buffer (50 mM HEPES, 150 mM KCl, 1.5 mM MgCl$_2$, 0.5 mM DTT, 0.5% Triton® X-100, pH 7.5) supplemented with Complete protease inhibitor cocktail tablets (without EDTA, from Roche), and an equal volume of glass beads was added. Cells were then broken by one 45 seconds cycle at power 5.5 in a FastPrep® machine (FastPrep® FP120, BIO 101). Tubes were pierced with a hot needle and the cell lysate spun down into new eppendorfs at 2,000 rpm for 2 minutes. The cell lysate was then clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C.
and protein concentration was determined using a Micro DC protein assay (from Bio-Rad). 50 µl of protein G Dynabeads (Invitrogen) coupled to either anti-Myc or anti-HA antibody (30 min at room temperature) were added to the lysate and the suspension was incubated for 2 hours at 4°C. After protein binding, beads were washed with wash buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, pH 7.5) 5 times and then eluted in 40 µl of eluting buffer (10% sacarose, 0.025% bromophenol blue, 0.05% sodium azide, 4% SDS, 250 mM Tris-HCl pH 6.8) supplemented with 4% β-mercaptoethanol at 37°C for 5 minutes. This was then boiled at 95°C for 2 minutes, loaded onto SDS-PAGE gels and subsequently analysed by Western blot (see above).

2.5 DNA Analysis Techniques

2.5.1 DNA extraction

For DNA extraction, samples were spun down at 4,000 rpm for 2 mins, flash frozen on dry ice and stored at -80°C. The pellet was resuspended in 400 µl of DNA extraction buffer (1% SDS, 100 mM NaCl, 50 mM Tris-HCl pH 8, 10 mM EDTA), 40 units of lyticase (Sigma L2524) were added and the tube inverted repeatedly to mix. This was incubated for 10 minutes at 37°C and then 500 µl of phenol/chloroform/iso-amylalcohol (25:24:1) were added and rotated on a wheel for 20-40 minutes at room temperature. The phenol emulsion was transferred to a phase lock tube (5 prime) and spun down at 12,000 rcf for 5 minutes. The upper aqueous phase was taken to a fresh tube and 2 volumes of cold ethanol were added. The samples were spun down at 15,000 rpm for 30 minutes at 4°C, washed with 1 ml 70% ethanol and spun down again. The pellet was air-dried for a minimum of 1 hour. The pellet was then resuspended in 150 µl sterile water, slowly and without vortexing to try to prevent damage to the DNA. It is possible to pass the DNA twice through the phase lock tubes to increase the purity, before precipitating with ethanol.

2.5.2 Simple DNA gel electrophoresis

DNA samples in loading buffer (Promega) were run on 0.8% agarose gels with 0.5 µg/ml ethidium bromide at 95 V in 1x Tris-Borate-EDTA (TBE) buffer (45 mM Tris-borate, 1
mM EDTA, pH 8). DNA can then be visualized using a UV transilluminator (324 nm).

### 2.5.3 One dimensional gel electrophoresis

To analyse DNA extracts to look at catenanes during the transition, by Neutral 1D gel electrophoresis. 3.2 g of Mega Sieve agarose were dissolved in 400 ml 0.5 x TBE buffer (22.5 mM Tris-borate, 0.5 mM EDTA, pH 8) (0.8% agarose). This was heated to dissolve the agarose but not allowed to boil to prevent loss of volume or damage to the polymerised agarose chains. This was then left to cool to 60°C before pouring to set. Usually 25 µl of sample was loaded with 12.5 µl of loading buffer, along with 15 µl of 1 kb DNA ladder (Promega) and run at 50 V for 24 hours at 4°C. The gel is then cut to remove the ladder, which is incubated for a minimum of 90 minutes in water with ethidium bromide and photographed under UV light. The remaining gel, containing the experimental samples, was analysed by Southern blot (see below).

### 2.5.4 Two dimensional gel electrophoresis with ethidium bromide

To analyse DNA extracts to look at catenanes during the transition, Neutral-Neutral 2D gel electrophoresis with ethidium bromide was used. The first dimension was run in 0.4% agarose (1.4 g of Mega Sieve agarose in 350 ml 1x TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8)). Up to 35 µl of sample was loaded with 8 µl of 6x loading buffer (Promega) (either as is, or digested with NbBsm1 for 1 hour at 65°C to nick all catenanes to become CatA). 15 µl of 1 kb DNA ladder (Promega) and a plasmid from *E. coli* were also run on the gel, to be able to monitor the running in the first dimension. This first dimension was run at 30 V for 28 hours at room temperature in 1x TBE buffer. The ladder and guide plasmid were stained with ethidium bromide and visualised using a UV transilluminator (324 nm). The body of the gel was then cut to individual sample lanes and to where the fastest migrating form of the guide plasmid had run and this fragment was reset such that the first dimension ran left to right and new agarose poured around this fragment. This second gel was 1% agarose (4 g of Mega Sieve agarose in 400 ml 1x TBE buffer) and ethidium bromide was added to 0.3 µg/ml. The second dimension gel was run, in 1x TBE also containing 0.3 µg/ml ethidium bromide, for 15 hours at 135 V at 4°C.
2.5.5 Two dimensional gel electrophoresis with chloroquine

To analyse DNA extracts by monomer supercoiling state, Neutral-Neutral 2D gel electrophoresis was used with chloroquine as an intercalating agent. The first dimension was run in 0.4% agarose (1.4 g of Mega Sieve agarose in 350 ml 1x TBE buffer) with 0.5 µg/ml chloroquine. Up to 35 µl of sample was loaded with 8 µl of 6x loading buffer (Promega), along with 15 µl of 1 kb DNA ladder (Promega) and a guide plasmid from *E. coli* and run at 30 V for 40 hours at room temperature in 1x TBE buffer with 0.5 µg/ml chloroquine. The ladder and guide plasmid were stained with ethidium bromide and visualised using a UV transilluminator (324 nm). The body of the gel was then cut to individual sample lanes and the portion containing 2kb - 12 kb, as judged by the guide plasmid, was reset such that the first dimension ran left to right. This was set into a gel containing 1.2% agarose (4.8 g of Mega Sieve agarose in 400 ml 1x TBE buffer) and 1 µg/ml chloroquine. This second dimension gel was run, in 1x TBE also containing 1 µg/ml chloroquine, for 10 hours at 125 V at 4°C.

2.5.6 Southern Blot

After DNA gel electrophoresis the gels were briefly washed in water, then incubated in depurinating solution (0.125 M HCl) for 10 minutes, washed again with water for 10 minutes and then incubated with denaturing solution (0.4 M NaOH, 1 M NaCl) for 30 minutes, and finally neutralising buffer (1.5 M NaCl, 0.5 M Tris-HCl, adjusted to pH 7.5), for 30 minutes. Hybond-N membrane (Amersham Biosciences) was cut to size, wet in water and then incubated in 20x SSC for at least 20 minutes.

DNA was then transferred to the membrane using 20x SSC buffer and blotting paper and paper towels to draw the liquid through. This set up was left for a minimum of 10 hours (overnight) and then the membrane was transferred to UV Stratalinker and auto-crosslinked (1200 J/cm2) DNA side up and uncovered. Following crosslinking, the membrane was washed in 5x SSC until hybridization.

The membrane was placed into a glass tube and incubated with 50 ml of blocking solution (0.1% SDS, 5% Dextrane sulphate, 5% Blocking liquid (GE), dissolved in 5x SSC) rolling at 65°C for a minimum of 2 hours. The probe was denatured by boiling at 95°C for 30 minutes and then left on ice to cool. This denatured probe was added
in place of the blocking solution and left rolling at 65°C overnight. The membrane was then washed twice in primary washing buffer (1x SSC, 0.1% SDS) for 15 minutes and then twice in secondary washing buffer (0.5x SSC, 0.1% SDS) for 10 minutes, all also at 65°C. The membrane was rinsed at room temperature in 50 ml of AB buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 250 ml of 1% milk powder in AB buffer at room temperature for 2 hours. Following blocking, the membrane was rinsed again in 50 ml AB buffer and then incubated in 250 ml of 0.5% milk in AB buffer with 1:250,000 dilution of Anti-Fluorescein-AP Fab fragments (Roche) for 1 hour. Finally the membranes are washed three times in AB buffer with 0.2% Tween 20 (Sigma) for 10 minutes at room temperature and then incubated with CDP-Star® Detection reagent (GE) for 10 minutes at room temperature, followed by exposure to ECL Hyperfilm (GE Healthcare).

### 2.5.7 Chromatin Immunoprecipitation (ChIP)

The ChIP analysis protocol was based on Nelson et al. (2006) but adapted for use with *S. cerevisiae* and further for this thesis. 100 ml of culture at an OD$_{600}$ of 1 were fixed with formaldehyde (final concentration of 1.42%) for 15 minutes at 25°C, and quenched with glycine (final concentration 125 mM) for 5 minutes before the cells were harvested by centrifugation at 4,000 rpm for 2 minutes. The pellet was washed in PBS and transferred to a screw cap tube and frozen on dry ice. The pellets were stored at -80°C.

Pellets were resuspended in 100 µl of IP buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, NP-40 (0.5% v/v), Triton® X-100 (1% v/v)) containing phenylmethane-sulfonyl fluoride (PMSF, final concentration 1 mM) and Complete protease inhibitor cocktail (without EDTA, from Roche), and 500 µl glass beads were added. Cells were broken by five 20 sec cycles, power setting 5.5 in a FastPrep® FP120 (BIO 101) machine with 1 min on ice after each second cycle. 300 µl IP buffer, containing PMSF and protease inhibitors, were then added, the tubes vortexed thoroughly and then pierced with a hot needle and placed into new eppendorfs and spun (2,000 rpm for 2 min) to collect the lysate. The cell lysate was spun down for 10 minutes at 13,000 rpm at 4°C. This pellet (containing the nuclei) was resuspended in 1 ml IP buffer containing PMSF and protease inhibitors, and this solution was sonicated for 1 hour (30 sec on, 30 sec off)
at high power at 4°C (Diagenode Bioruptor). Post-sonication, samples were spun down again for 10 minutes at 13,000 rpm.

100 µl of sonicated chromatin were taken as ‘input’ and the remaining volume was divided between tubes for incubation with antibody.

Sodium acetate (NaOAc) was added to a final concentration of 0.3 M and 2.5 volumes of cold ethanol were added to the input DNA and mixed. The mixture was then spun down at 15,000 rpm for 30 minutes, and the supernatant removed. The pellet was air dried, and then 250 µl of 10% (w/v) Chelex 100® suspension were added and the pellet fully resuspended before boiling for 30 minutes. After boiling, the tubes were spun down at 6,000 rpm and the supernatant was cleaned using the PCR purification kit (Qiagen) according to the manufacturer’s instructions. Finally, DNA was eluted in 250 µl of sterile distilled water.

The main IP sample was incubated with the appropriate amount of antibody for 30 minutes in an ultrasonic water bath at 4°C. After sonication, it was spun down at 13,000 rpm for 5 min, and the supernatant was added to a 100 µl slurry of 50:50 Protein A and Protein G beads (Roche), which had been equilibrated in IP buffer. The sample and beads were incubated for 2 hours at 4°C and then the beads washed 4 times in IP buffer with inhibitors and twice more in IP buffer without inhibitors. Washes were done by spinning down at 6,000 rpm for 1 minute, allowing another minute for the beads to settle, aspirating the supernatant and then adding 1.5 ml of washing buffer and inverting the tubes 30 times. After the final wash, 250 µl of 10% (w/v) Chelex 100® were added and samples boiled for 30 minutes. After a final spin at 6,000 rpm for 1 minute, the supernatant was transferred to a new tube and stored, along with the input DNA at 4°C for short periods or -20°C for up to several months.

### 2.6 RNA extraction

Samples of approximately 25 ml of culture at an OD$_{600}$ of 0.4 were taken and spun down at 4,000 rpm for 2 minutes, washed with PBS and transferred to sterile screw cap tubes. These were then spun down for 2 minutes at 10,000 rpm and flash frozen on dry ice before storing at -80°C. For RNA extraction, a Qiagen kit was used, and all buffers were provided by Qiagen. The samples were defrosted on ice and 100 µl of RLT buffer
containing 1% β-mercaptoethanol was added, along with glass beads. The cells were broken in a FastPrep machine for 3 20 sec cycles at a power setting of 6.0 (FastPrep® FP120, BIO 101) with 2 minutes on ice in between. 500 µl of RLT buffer containing 1% β-mercaptoethanol were then added and the samples vortexed. The bottom of the tube was then pierced and the lysate collected in a new tube by spinning at 2,000 rpm for 2 minutes, then the new eppendorfs were spun down at 15,000 rpm for 5 minutes. The supernatant was collected into a new eppendorf and 1 volume of 70% ethanol was added (570 µl) to precipitate the RNA (and DNA). It was then added to the extraction column, spun down for 15 seconds at 12,000 rpm and the eluent discarded. The column was washed once with 350 µl of RW1 and then DNase was added in RDD buffer directly to the column, incubated at room temperature for 30 minutes before washing the column once more with 350 µl RW1 and then twice with 500 µl RPE buffer. The eluent was continually discarded and the empty column spun down a final time after all washes. The RNA was then eluted to a new sterile tube by adding 50 µl RNase free water, spinning this through the column and readding the eluent to the top of the column for a second pass. The column work must be done at room temperature but as soon as the RNA is collected all further work must be done on ice. A small amount of this extraction was run on a 0.8% agarose gel to assess the quality of the RNA extracted.

2.7 Polymerase Chain Reaction (PCR)

2.7.1 Standard PCR

1 µl of DNA was made up to a 25 µl reaction mixture, containing 0.3 µM primers, 300 µM dNTPs, 1 µl GoTaq Flexi DNA polymerase (Promega) and 20% GoTaq Buffer (Promega).

Table 2.1: PCR conditions for Standard PCR

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Repeat Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>5 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>95</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>30 sec</td>
<td>30 cycles</td>
</tr>
<tr>
<td>72</td>
<td>1 min/kb</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>
For longer fragments of DNA, or difficult amplifications, the Expand High Fidelity System (Roche), with 0.3 \( \mu \text{M} \) of each primer and 300 \( \mu \text{M} \) dNTPs, was used.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Repeat Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>3 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>94</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>30 sec</td>
<td>10 cycles</td>
</tr>
<tr>
<td>72</td>
<td>1 min/kb</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>30 sec</td>
<td>20 cycles</td>
</tr>
<tr>
<td>72</td>
<td>1 min/kb</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

2.7.2 Colony PCR

For colony PCR, a small amount of yeast was resuspended in 10 \( \mu \text{l} \) sterile water and boiled for 10 min. It was cooled on ice and 15 \( \mu \text{l} \) of PCR reaction mix were added, made with 0.3 \( \mu \text{M} \) primers and 300 \( \mu \text{M} \) dNTPs, 1\( \mu \text{l} \) of GoTaq Flexi DNA polymerase and 5 \( \mu \text{l} \) of buffer (Promega). Finally, the PCR was run with the same conditions as shown in Table 2.1.

2.7.3 Retrotranscription

7 \( \mu \text{l} \) of RNase free water and 2 \( \mu \text{l} \) of the RNA sample were added to each tube and incubated at 65°C for 5 minutes and cooled on ice. Two ‘master mixes’ were made for each reaction, the first containing 1 \( \mu \text{l} \) of random hexamers and 2 \( \mu \text{l} \) of dNTP mix per sample; the second containing 4 \( \mu \text{l} \) cDNA buffer, 1 \( \mu \text{l} \) 0.1 M DTT, 1 \( \mu \text{l} \) RNase OUT, 1 \( \mu \text{l} \) RNase free water and 1 \( \mu \text{l} \) Thermoscript (polymerase) per sample and kept on ice. 3 \( \mu \text{l} \) of mix 1 and 8 \( \mu \text{l} \) of mix 2 were added to each tube and incubated as shown in Table 2.3 and then returned to ice for PCR. All solutions used are from Invitrogen.
Table 2.3: PCR conditions for reverse transcription

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Repeat Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>60 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td></td>
</tr>
</tbody>
</table>

2.7.4 Real time PCR (qPCR)

For each primer pair to be used, 7 µl of a primer mix (final concentration of 0.78 µM of each primer in nuclease free water), either 2 µl of 1:50 dilution of cDNA or 3 µl of DNA from ChIP and 10 µl of Sensimix were added per well (Bioline). The plate was sealed and spun briefly to 1,500 rpm. Plates were then run in a CFX96 qPCR machine (Bio-Rad) as shown in Table 2.4.

Table 2.4: PCR conditions for real time PCR

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Repeat Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>2 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>95</td>
<td>30 sec</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>30 sec</td>
<td>45 cycles</td>
</tr>
<tr>
<td>72</td>
<td>30 sec</td>
<td>(minimum)</td>
</tr>
</tbody>
</table>

Melt Curve 70 °C - 95 °C
Chapter 3

Regulation of the positive supercoiling transition

Objective

- To identify the factors required for the positive supercoiling change observed at the metaphase to anaphase transition in *S. cerevisiae*

Experimental approach

The protocol used to observe the supercoiling transition was previously optimised by other lab members (Baxter *et al.*, 2011). Cells pass through a single cell cycle in the absence of Top2 (*S. cerevisiae*’s only type II topoisomerase) and Cdc20 (the regulatory subunit of the APC required for progression into anaphase). Cells are released from a G₁ arrest to an arrest in G₂/M caused by the lack of Cdc20. In the absence of Top2, replication proceeds as normal because Top1 can relieve the superhelical tension in front of replication forks, but catenations cannot be resolved. In order to achieve this, strains were constructed which used a temperature sensitive degron (Figure 3.1).

Temperature sensitive degron

In order to create a strain where the protein of interest can be degraded at will, a heat-inducible degron construct is added to the N-terminal of the gene to be targeted. This consists of the TetO promoter which replaces the endogenous promoter, and a ‘degron module’ which will be expressed fused to the N-terminal of the protein. This is a Ubiquitin moiety, followed by an Arginine residue (which destabilises the protein due
Figure 3.1: **The temperature inducible degron construct** Top panel shows the genetic construct made with the TetO promoter followed by the Ub-Arg-DHFRts-Myc-degron module upstream of the gene of interest. Bottom panel shows the production of stable protein and the transition, at non-permissive temperature, to an unstable protein which is then targeted for degradation. Diagram shows the ORF of the target gene (blue) with an N-terminal Myc tag (red) and the temperature-sensitive DHFR (green) under the control of the TetO promoter (orange). The Arginine (R) which speeds up degradation by the N-end rule pathway is shown in yellow, and the lysine (K) which receives the ubiquitination is shown in purple. Abbreviations: Ub - ubiquitin, DHFRts - temperature sensitive dihydrofolate reductase gene, TetO - Tet operator.

This protein, along with its new N-terminal degron module, is stable and behaves like wild type at the permissive temperature as the DHFR conformation obscures both the N-terminal arginine and any lysines that could be suitable for ubiquitination, both of which destabilise a protein. Therefore, at 25°C the cells grow as wild type.

A copy of Ubr1, an E3 ubiquitin ligase involved in the N-end rule pathway, is also added to the strain under a galactose-inducible promoter. Therefore, this is not expressed provided cells are grown in media with glucose or raffinose as the sole carbon source.

Three steps are required to degrade the protein in vivo. Firstly, the addition of galactose to the medium (in the absence of glucose) results in the overexpression of Ubr1. Secondly, addition of the drug doxycycline to the medium suppresses transcription of
the target protein from the TetO promoter. Finally, the culture is shifted to 37°C which results in a conformational change in the DHFRts at the N-terminal end of the target protein, revealing suitable ubiquitination targets and the arginine residue. Together, this results in no new protein being created while the existing protein pool is degraded via ubiquitination by Ubr1 and degradation by the N-end rule pathway (Dohmen and Varshavsky, 2005, Gossen and Bujardt, 1992).

**Supercoiling transition protocol**

In these experiments, the degron system is used to target Top2 and Cdc20, as explained above. Cells pass through a single cell cycle, released from G1 to G2/M, without Top2 and this produces catenated DNA (Baxter et al., 2011).

A small circular centromeric plasmid was used in order to be able to visualise the topological changes occurring to DNA in these cells. In the case of genomic DNA, topological domains are maintained by proteinaceous structures, cytoskeletal elements and other DNA binding proteins (Worcel and Burgi, 1972). During extraction, these proteins are removed from DNA, and as such the topological state of the genomic DNA is lost. Because a plasmid is a closed piece of DNA, the removal of all proteins cannot alter its supercoiling state and this can therefore be visualised by neutral gel electrophoresis (Baxter et al., 2011). The plasmid used was a yeast centromeric plasmid (pRS316 or pRS314) so that its segregation dynamics would mimic the endogenous chromosomes as closely as possible.

Figure 3.2 shows the protocol used to visualise this topological transition. Cells are arrested in G1 and then released into the cell cycle with the degron activated. Cells were cultured in YP media with raffinose as the sole carbon source, at 25°C, until they were in exponential growth phase, thus ensuring no degron activation. Once growing exponentially, the mating pheromone α-factor was added to the culture which causes a cell cycle arrest in G1 as the cells halt the cell cycle to attempt mating. Once cells are in G1, the degron is triggered by addition of galactose, doxycycline and moving the cultures to the non-permissive temperature, 37°C, see Figure 3.2. Once the degrons have removed all target protein, cells are released from the G1 arrest. This is achieved by
washing in media without α-factor and adding pronase (an enzyme which degrades α-factor). Cells are released in two conditions, one which progresses to the cell cycle arrest obtained due to the lack of Cdc20, and one which arrests in G2/M due to the addition of nocodazole to the media which depolymerises the spindle and causes activation of the SAC. As such, the culture blocked in Cdc20-mediated arrest will have undergone spindle formation, tension generation and the supercoiling transition whereas the nocodazole arrested culture will not - both situations are visualised by neutral gel electrophoresis and Southern blotting, see Figure 3.3.

This system allows the characterisation of the positive supercoiling transition regulation. By creating strains which have the top2-td, cdc20-td pRS316 background but which are also lacking other proteins, we can test genetic requirements for the supercoiling transition. For example, previous work identified Smc2 as being necessary by use of a temperature-sensitive allele, smc2-8, which cannot enact the supercoiling transition (Figure 3.7).
3.1 Previous work

The protocol described above was used to show the original transition, see Figure 3.3. The left hand gel is showing a release through the cell cycle from G_1 in the presence of a wild type Top2 and although higher molecular weight bands are seen, they disappear quickly. The central gel shows a cell cycle in the absence of Top2 from a G_1 arrest to an arrest caused by nocodazole addition. Nocodazole prevents spindle formation and activates the SAC. Here, dimers form due to catenations which cannot be resolved in the absence of Top2 and run as higher molecular weight species. Characterisation showed these to be dimers where both plasmids are negatively supercoiled, see Section 3.1.1, referred to as CatC. The final gel on the right shows passage through the cell cycle, in the absence of Top2, to an arrest due to the absence of Cdc20. In this gel, the dimer appears but then the band reduces in intensity and a ‘smear’ appears at the same height as the monomer band - this corresponds to a positively supercoiled species, again see Section 3.1.1 below, (Baxter et al., 2011).

3.1.1 Characterisation of the supercoiled species

Plasmid dimers can exist in three states, CatA, CatB and CatC. The cartoon in Figure 3.4 shows these three forms, distinguished by the supercoiling state of each plasmid - CatA consists of two relaxed plasmids, CatB has one relaxed plasmid and one supercoiled, CatC is two negatively supercoiled plasmids and CatC* has both plasmids positively supercoiled. Nicking one or both plasmids will create CatB or CatA from CatC. The different forms of the plasmid can be visualised by two dimensional gel electrophoresis (Baxter et al., 2011, Martín-Parras, 1998)

To assign identities to the bands visualised by 1D gel electrophoresis, 2D gel electrophoresis is necessary. Catenation and supercoiling states of DNA can be observed by running DNA through agarose gels of different percentages and with different intercalating agents. In order to investigate the change that was occurring as cells reach metaphase, a different approach was used where the cells were allowed to pass through S phase in the presence of wild type Top2 which means that the cells reach G_2/M with monomer plasmids instead of catenanes.
Chapter 3. Supercoiling mechanism

Figure 3.3: An example of the positive supercoiling transition protocol. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G1 and, if necessary, the degrons triggered and the cells released into the cell cycle. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blots. A probe to the plasmid was used so only the different forms of the plasmid are seen. The left hand gel shows release with functional Top2 - higher molecular weight bands appear and are then rapidly resolved into monomers. The central gel shows arrest in the absence of Top2 and the presence of nocodazole. The centromeric plasmids cannot be resolved and so a dimer band appears and persists in the arrest. The right hand gel shows release with functional Top2 - higher molecular weight bands appear and then quickly resolve into monomers. The centromeric plasmids cannot be resolved and so a dimer band appears and persists in the release.

Legend:

- Ocm - open circular monomer (relaxed)
- CatC - catenanes where both plasmids are negatively supercoiled
- CatC* - catenanes where both plasmids are positively supercoiled
- CCCm - supercoiled closed circular monomers
Chapter 3. **Supercoiling mechanism**

**Figure 3.4:** *Forms taken by catenated plasmids.* The cartoons show the different species that have been identified in the supercoiling transition protocol. The monomer can be either supercoiled or relaxed, and the dimer can exist in three states with either both relaxed or supercoiled, or one of each. Abbreviations: Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled), CatA - catenane where both plasmids are relaxed, CatB - catenane where one plasmid is supercoiled and the other relaxed, CatC/CatC* - catenane where both plasmids are negatively/positively supercoiled respectively.

Once cells are arrested in G$_2$/M by addition of nocodazole, the cell is then depleted of Cdc20 alone or Cdc20 and Top2. The removal of Cdc20 will arrest cells at the metaphase to anaphase transition, and the removal of Top2 allows the preservation of topological states by slowing the cell’s ability to resolve and relax DNA.

In order to look at the topological state of the monomers, it is necessary to run the DNA in two dimensions using the intercalating agent chloroquine at two different concentrations, along with changing the agarose concentration and the voltage. The first dimension (low agarose, low voltage) separates the molecules predominantly by size and the second dimension (high agarose, high voltage) separates DNA molecules predominantly by shape. This means the monomers are resolved according to their supercoiling state as the supercoiling of a plasmid alters its shape in a way that changes its migration through the gel.

Visualisation of the monomer plasmids during the release from a nocodazole-mediated
arrest to a Cdc20-mediated arrest in the absence of Top2 shows a change in the distribution of these supercoiled monomers (Baxter et al., 2011), Figure 3.5. This demonstrates the new species seen by one dimensional separation of the catenanes is representative of a shift to positive supercoiling at the moment of spindle formation.

\[\text{Figure 3.5: 2D gel electrophoresis demonstrates the topological shift.} \ (\text{Baxter et al., 2011})\] The cartoon shows how the movement of the monomers changes based on topological state, and explains the running conditions. The gels show a release from nocodazole to a Cdc20-mediated arrest with or without Top2 (top and bottom panels respectively). The cells are arrested in nocodazole, and then the degron is triggered removing Top2 and Cdc20 protein. A sample is taken and then the arrest is released by washing out the nocodazole. Samples were taken every 20 minutes and processed for 2D gel electrophoresis in the conditions shown in the cartoon. Note that, without Top2, an accumulation of positively supercoiled species occurs, highlighted by the red box. Gels taken from Baxter et al. (2011).

3.1.2 Supercoiling transition \textit{in vivo}

The accumulation of positively supercoiled species can only be visualised in the absence of functional Top2. This suggests that Top2 is usually acting in the cells to release this tension as quickly as it is introduced. Therefore, in wild type cells it is likely that this transition is transient. Furthermore, this technique is examining a small plasmid and in full length chromosomes it may be that the shift towards positive supercoiling is happening on a domain-by-domain basis and so will not be seen as a full global shift to a positively supercoiled state but as a transient movement towards positive supercoiling in individual topological domains at different times.
To determine if this topological shift is indeed capable of aiding the resolution of catenanes, an experiment was carried out where recombinant Topo II was presented with different substrates in vitro - either a plasmid pool taken from nocodazole arrested cells, prior to the transition, or taken from Cdc20 arrested cells, after the supercoiling transition has occurred. Shown in Figure 3.6 is the topological state of the plasmid dimers upon addition of increasing amounts of purified Topo II in vitro.

**Figure 3.6:** Topo II preferentially decatenates positively supercoiled substrates. *In vitro* assessment of decatenation and topological relaxation of plasmid populations taken from either nocodazole or Cdc20 arrested cells. These reactions were carried out *in vitro* with differing amounts of recombinant Topo II, before neutral gel electrophoresis (Baxter *et al.*, 2011). The plasmids from nocodazole arrested cells shows Topo II concomitantly decatenates and relaxes the supercoils in the plasmids. Conversely, when Topo II is added to the samples from a Cdc20-mediated arrest, the catenations are almost immediately resolved, and the supercoiling relaxed with higher levels of enzyme. Gels taken from Baxter *et al.* (2011). Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled), Lk - linking number.

The left hand of the image shows the action of Topo II on negatively supercoiled dimers, i.e. a population taken from nocodazole arrested cells. Here, Topo II first relaxes the supercoils, and subsequently decatenates the plasmids. The right hand side panel shows that when plasmids are taken from a Cdc20-mediated arrest, a positively supercoiled
substrate, the catenations are resolved almost immediately, followed by relaxation of the supercoiling at higher levels of Topo II. It takes a 10-fold lower concentration of Topo II to fully decatenate the substrate plasmids when they are taken from a Cdc20 arrested population instead of a nocodazole arrested population (Baxter et al., 2011). These results indicate that Top2 has a bias towards decatenation when acting on positively supercoiled dimers and this would link the supercoiling transition to resolution of catenations at the metaphase to anaphase transition, i.e. when under spindle tension.

3.1.3 Known players in the transition

Finally, the Baxter et al. (2011) paper showed that Smc2 is required for the transition to occur. Subsequent work in the lab also identified the need for a functional cohesin complex (Scc1) and for Cdc5, the polo-like kinase, as can be seen in Figure 3.7. Smc2 was tested for involvement due to the evidence showing that condensin is capable of inducing positive supercoiling in vitro (Bazett-Jones et al., 2002, Kimura and Hirano, 1997). This was tested by a temperature-sensitive mutant of Smc2, smc2-8, which was triggered alongside the temperature sensitive degrons in the experimental protocol. With this additional mutation, the cells failed to go through the transition in the Cdc20-mediated arrest, shown in Figure 3.7. This indicates that Smc2 is required for the transition to occur (Baxter et al., 2011).

The next candidate tested was the SMC complex cohesin, due to its central role in the cohesion of sister chromatids. The loss of cohesin, and therefore cohesion, is likely to mean that bipolar tension cannot be generated during spindle formation and attachment as there is nothing to counter the spindle pulling forces. As such, if the transition is dependent on tension, then it may not happen in a cohesin mutant. In this strain, the kleisin subunit of cohesin, Scc1, was replaced with a temperature-sensitive allele scc1-73 which will render the protein non-functional at the non-permissive temperature used to trigger the degrons. In Figure 3.7, the CatC species persists in the third panel showing the supercoiling transition in the absence of functional Scc1. From this, it can be concluded that functional cohesin is indeed required for the transition to occur.

Finally, the supercoiling activity of condensin is not fully active unless it is phosphorylated by Cdc28 and Cdc5 (Bazett-Jones et al., 2002, Kimura and Hirano, 1997). Both
Figure 3.7: Condensin, cohesin and Cdc5 are required for the positively supercoiled transition. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G1, the degrons triggered and cells released into the cell cycle. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. The left hand panel shows the background strain enacting the transition as it reaches a Cdc20-mediated arrest. There is a reduction in the CatC band, and a smear appears behind the monomer band, representing CatC*. The next three panels show three separate strains going through the same protocol - strains containing a temperature-sensitive allele of either Smc2 (left hand), Scc1 (middle) or Cdc5 (right hand). None of these show the shift to positive supercoiling, the CatC species persists in all three experiments. This suggests all three proteins are individually required for the transition. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled).
of these kinases are highly active with many targets but due to the link between Cdc5 phosphorylation and condensin’s supercoiling activity, Cdc5 was chosen for analysis. The endogenous copy of Cdc5 was mutated to a temperature-sensitive allele, \textit{cdc5-1}. The right hand panel of Figure 3.7 demonstrates a persistence of the negatively supercoiled CatC species and the absence of the transition in this \textit{cdc5-1} strain. However, it cannot be known whether the removal of Cdc5 prevented the transition due to a direct loss of phosphorylation of condensin or a more indirect path.

Nevertheless, these experiments allow the conclusion that the positive supercoiling transition requires functional condensin and cohesin, and the kinase activity of Cdc5, as well as spindle formation. Given cohesin is, so far, known only to topologically link sections of DNA, and condensin has been shown to be capable of supercoiling DNA \textit{in vitro}, condensin was thought likely to be the prime actor in this process. Tension must exist for the transition to be triggered, explaining the requirement for Scc1, and phosphorylations are key in the activation of this topological change.

The following sections demonstrate the investigation of multiple other proteins as potential regulators of the supercoiling transition. The proteins tested included further investigation of the timing of transition activation, the requirement of condensin and the kinases involved.
3.2 Determining the role of condensin subunit Brn1

Given the requirement for Smc2, and the knowledge that all five subunits of condensin are essential for the viability of *S. cerevisiae*, it seemed likely that the need for Smc2 was representative of a requirement for the condensin complex as a whole. To test this, we looked at Brn1. Brn1 is the kleisin subunit of condensin, known to be phosphorylated by both Cdc5 and Ipl1 (Aurora B) and essential for chromosome condensation in *S. cerevisiae* (Lavoie et al., 2000, Ouspenski et al., 2000). A temperature-sensitive allele was used, *brn1-60*, which was integrated in place of the endogenous *BRN1* gene. The mutant used was completely inviable at 37°C suggesting the mutation was successful in eliminating the function of Brn1 (Figure 3.8).

![Figure 3.8: The role of Brn1 in the supercoiling transition.](image)

Growth assay shows that the *brn1-60* mutation renders the cells inviable at the non-permissive temperature. FACS profiles show the two cultures pass through the cell cycle with similar kinetics. Southern blots showing a centromeric plasmid, *pRS316*, during passage through the cell cycle. Cells were arrested in G1, the degrons triggered and then released into the cell cycle, with nocodazole added to one culture. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. The strain used contained a *brn1-60* temperature-sensitive allele. The CatC species fades over time, as in a wild type transition, and there is a smear that is slightly weaker than in a normal transition. The transition seems largely unaffected by the loss of Brn1 function. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled).
Figure 3.8 shows the supercoiling transition protocol carried out with this strain. As can be seen, it behaves almost entirely as the ‘wild type’ (in this case wild type refers to the background top2-td, cdc20-td, pRS316) in both the nocodazole block and the Cdc20-mediated arrest. The transition is perhaps weaker than normally seen in the wild type but it is definitely able to trigger the change in topology. This would suggest that the inactivation of Brn1 has some effect but does not entirely prevent the transition, meaning Brn1 functionality is not required.

This was unexpected as all in vivo functions of condensin so far reported have been shown to require all five subunits. It is possible that we have identified a function for Smc2, or the Smc2/4 dimer, separate from the remaining subunits. Alternatively, it could be that the temperature sensitive form of the protein is still present and this may be sufficient to allow the complex to carry out its function for the duration of the experiment, even at the non-permissive temperature.

To test this, a Western blot was used to see whether the temperature-sensitive allele remained stable at non-permissive temperature. Figure 3.9B shows that the protein is indeed stable, and its levels increase as the cells are released from an α-factor mediated arrest and go through a single cycle at 37°C.

Therefore, if the temperature-sensitive protein is not degraded, does it maintain its interaction with the condensin complex? To investigate this, a co-Immunoprecipitation was carried out, pulling down Smc2-6HA with anti-HA antibody conjugated to Protein G Dynabeads, and a Western blot was used to look for the Brn1-60 protein, or wild type Brn1, both tagged with nine Myc epitopes (in different strains). Figure 3.9C shows that there is a significant drop in the quantity of Brn1 interacting with Smc2 at 37°C (highlighted by the blue box) but that the interaction is not entirely lost. Indeed, when compared to the amount of Smc2-6HA pulled down in the two conditions (red box) then the difference in protein ratios (Smc2:Brn1-60) is approximately equivalent.

As such, it seems that the temperature sensitive form of the protein is stable and remains at least partly associated with the condensin complex at the non-permissive temperature. This may be enough for the complex to retain some functionality and perhaps the lethality is conveyed via other processes which require a different function of the Brn1 protein, or due to a loss of activity over multiple cell cycles.
3.3 Activation of condensin via phosphorylation

Condensin is present throughout the cell cycle, and is phosphorylated by multiple kinases at multiple sites on all subunits. As such the confinement of this positive supercoiling activity to such a specific point in the cell cycle seems likely to be controlled by some of these phosphorylations.

Previous work in the lab used the overexpression of phospho-mimic forms of the non-SMC subunits of condensin (versions of the proteins under the galactose-inducible promoter with all the key phosphorylation sites mutated to aspartate, an amino acid which has been shown to mimic phosphorylated residues) to see if the transition could be forced in a nocodazole-arrested culture. However, this had no success. Additionally, an Smc4 protein mutated to mimic Cdc28 phosphorylation (a key first step in condensin’s activation) and a phospho-null mutant of the same residues (to alanine, an unphosphorylatable amino acid) also showed almost wild type behaviour - potentially a slight delay was seen
but they still transitioned successfully (work carried out by another lab member, data not shown).

These forms of the proteins could not replace the endogenous genes as cells containing phospho-mutants of the condensin subunits are inviable. However, it is unlikely that the endogenous copies were enough to counter an otherwise mutant phenotype as the mutant proteins were being expressed to a much higher level than the endogenous wild type protein. Therefore, it may be a more subtle mixture of phosphorylations occurring with different timings to trigger the transition.

3.3.1 Overexpression of Cdc5

As mentioned above, removing the Cdc5 kinase was sufficient to prevent the transition from occurring. However, this kinase has very many targets involved in mitosis and so this could be a highly non-specific and indirect effect. The idea that the removal of Cdc5 prevents the transition directly by failing to phosphorylate condensin is suggested by the fact that Cdc5 has been shown to phosphorylate all three non-SMC subunits of the condensin complex in anaphase and, in vitro, these phosphorylations trigger a positive supercoiling activity in condensin (St-Pierre et al., 2009).

If preventing these phosphorylations could prevent the transition, then could inducing them early force the cells to transition prematurely? To answer this question, a copy of the S. cerevisiae CDC5 gene was inserted under the control of the galactose promoter, on a centromeric plasmid pRS414. This will express Cdc5 when galactose is added to the culture, in this experiment this will happen as the degrons are triggered. Cells pass through one cell cycle in the presence of higher than normal Cdc5 protein levels but as shown in Figure 3.10 this was not enough to trigger the transition in cells blocked with nocodazole.

Simply overexpressing Cdc5 is therefore not enough to force the transition in the absence of spindle tension. Under normal circumstances, Cdc5 is present and active prior to the transition. Cdc5 requires phosphorylation by Cdc28 to become an active kinase (Mortensen et al., 2005) so perhaps the overexpressed Cdc5 cannot be phosphorylated fast enough to activate it all. Alternatively a change in localisation of Cdc5 or targetting of its activity, which occurs upon spindle formation, is required to activate this process.
Figure 3.10: **Cdc5 overexpression alone cannot trigger the supercoiling transition.** Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G₁, the degrons triggered and the cells released into the cell cycle, with nocodazole added to one culture. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. Cdc5 is under the galactose inducible promoter and as such would be expressed alongside the degron machinery. FACS profiles show the two cultures pass through the cell cycle with similar kinetics. This strain behaves as wild type during the transition, and does not transition prematurely in the nocodazole-mediated arrest. Therefore, Cdc5 overexpression cannot force the transition. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled).

### 3.3.2 Elimination of Ipl1

Another likely candidate for the activating phosphorylation of condensin (potentially alongside Cdc5) is Ipl1, the *S. cerevisiae* homologue of Aurora B. Ipl1 is known to phosphorylate the non-SMC condensin subunits, particularly Brn1, and is required until telophase for accurate DNA segregation (Nakazawa et al., 2011). Additionally, phospho-null mutants of Cnd2, the *S. pombe* homologue of Brn1, result in problems during anaphase (Nakazawa et al., 2011). To assess the role of Ipl1 in the positive supercoiling transition, a temperature-sensitive allele of Ipl1 was introduced, *ipl1-321*, which has a point mutation in the catalytic domain, D283N (Biggins et al., 1999). This was integrated into the endogenous *IPL1* site in a strain which is *top2-td, cdc20-td, pRS414* (the plasmid was changed due to a conflict of selection for the *ipl1-321* allele). Again,
these mutants were selected for by temperature sensitivity and then the supercoiling transformation protocol was carried out with the resultant clone, see Figure 3.11.

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**Figure 3.11: Loss of Ipl1 may affect the supercoiling transition.** Growth assay to evaluate the viability of a strain carrying wild type *IPL1* or the *ipl1-321* allele at permissive and non-permissive temperatures. The *ipl1-321* strain is completely dead at 37°C. Southern blots showing a centromeric plasmid, pRS414, on passage through the cell cycle. Cells were arrested in G₁, the degrons triggered and the cells released into the cell cycle, with nocodazole added to one culture. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. The temperature sensitivity of *ipl1-321* will mean function is lost as the degrons are triggered. FACS profiles show the two cultures pass through the cell cycle with similar kinetics. The CatC signal fades over time, and a smear does appear but to a lesser extent than the wild type transition, meaning the loss of Ipl1 prevents a full transition. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled).

Similar to the result from the *brn1-60* strain, the result was not a conclusive outcome. However, here it suggests that Ipl1 is required for full activation of the supercoiling transition. Although the CatC species does become fainter over time, and there is some appearance of the positively supercoiled CatC* species, it is not a complete transition as observed in wild type conditions. It seems cells cannot fully enact the supercoiling transition with the loss of Ipl1 phosphorylation activity. This could mean that the phosphorylations carried out by Ipl1 include targets (possibly condensin) which help to upregulate the transition, but are not essential to the process. Alternatively, this partial transition could be linked to Ipl1’s role in error correction during bipolar spindle
attachment (see Section 1.4.5). If the spindles cannot be accurately attached in a tension generating manner, perhaps the transition cannot be fully activated.

3.4 The Spindle Assembly Checkpoint does not regulate the transition directly

Although the moment of spindle attachment seems like a single event, several processes occur between the nocodazole-mediated arrest and the Cdc20-mediated arrest, including the formation of spindles, attachment to the kinetochores, tension generation and stabilisation, and deactivation of the Spindle Assembly Checkpoint (SAC), as well as the positive supercoiling transition. To narrow down the key processes in the activation of the transition, we tried to ascertain whether the SAC was involved.

The SAC will be active in the nocodazole-mediated arrest as the spindles cannot form correctly. In the Cdc20-mediated arrest, spindles form correctly and attach to kinetochores meaning the SAC will be satisfied, although segregation is prevented due to the absence of Cdc20. The protein Mad2 is a key component of the *S. cerevisiae* SAC and is necessary to activate the checkpoint (Musacchio and Salmon, 2007). Mad2 is not an essential protein and so to investigate the necessity of the SAC in preventing the transition, the gene was deleted in the background *top2-td cdc20-td pRS316* strain. Successful transformants could be checked by increased sensitivity to the drug benomyl (Figure 3.12). Without the ability to activate the SAC, the cells do not cope as well with the presence of low levels of this microtubule poison as they cannot delay segregation to wait for correct spindle formation. The supercoiling transition protocol was then carried out with this strain.

Without Mad2, the cells are unable to activate the SAC in response to the addition of nocodazole. Figure 3.12 shows a result identical to the wild type background strain. The transition occurs as wild type in the Cdc20-mediated arrest, but still does not occur in the culture where nocodazole has been added even though the SAC will not be active.

However, the primary mechanism by which the SAC prevents progression to anaphase is via the sequestration of Cdc20 by Mad2. In this strain, the absence of Mad2 would mean that this sequestering of Cdc20 cannot occur, but the fact that the degrons have
Figure 3.12: SAC activation is not essential in preventing the transition
Growth assay to evaluate the viability of a strain carrying wild type MAD2 or mad2∆
growing on YPD with no benomyl or 10 µg/ml benomyl. The mad2∆ strain copes
much less well with the mild spindle poison compared to the wild type strain. Southern
blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells
were arrested in G1, the degrons triggered and the cells released into the cell cycle, with
nocodazole added to one culture. Samples were taken every 20 minutes and processed
for DNA extraction before running in one dimensional neutral gel electrophoresis for
Southern blotting. A probe to the plasmid was used so only the different forms of the
plasmid are seen. The deletion of the MAD2 gene results in cells that cannot activate
the SAC in response to the addition of nocodazole. FACS profiles show the two cultures
pass through the cell cycle with similar kinetics. However, the nocodazole-arrested
sample is still unable to undergo the positive supercoiling transition. Abbreviations:
CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane
where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed),
CCCm - closed circular monomer (supercoiled), YPD - yeast peptone with glucose.

been triggered would also mean that there was no Cdc20 protein present in the cells.
This means that there is no release of Cdc20 to activate the APC and so, effectively,
the checkpoint is still active. Therefore, a mad2∆ top2-td pRS316 strain was created,
notably lacking the degron tag at the CDC20 locus. Here, upon addition of nocodazole
the Cdc20 cannot be sequestered by Mad2 and so activates the APC despite the lack of
spindles.

Figure 3.13 shows the positive supercoiling protocol carried out on this mad2∆ top2-
td pRS316 strain, where the cells are released from α-factor. The right hand panel
shows the progression through the cell cycle and activation of the transition as cell
reach mitosis. On the left hand side, we see the same experiment but with the addition
of nocodazole, preventing the formation of spindles as the cells reach mitosis. This
Figure 3.13: Lack of SAC activation seems to cause topological change in the presence of nocodazole. Growth assay to evaluate the viability of a strain carrying wild type MAD2 or mad2Δ growing on YPD with no benomyl or 10 µg/ml benomyl. The mad2Δ strain copes much less well with the mild spindle poison compared to the wild type strain. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G1, the degrons triggered and the cells released into the cell cycle, with nocodazole added to one culture. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. The deletion of the MAD2 gene results in cells that cannot activate the SAC in response to the addition of nocodazole. FACS profiles show the two cultures pass through the cell cycle with similar kinetics. Here, the nocodazole containing sample is altered from other nocodazole-arrested cultures, perhaps an inability to activate the SAC means some part of the transition occurs. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled), YPD - yeast peptone with glucose.

shows an apparent lack of the positive supercoiling transition, but the catenanes show a different profile of bands than normally seen in a nocodazole-mediated arrest (Figure 3.3).

To investigate what had caused this altered running of the DNA, and if it is related to the positive supercoiling transition, two dimensional gels were run using ethidium bromide as an intercalating agent, to assess the DNA species present. Figure 3.14 shows this process carried out on the 0, 20 and 40 min samples from the mad2Δ top2-td pRS316 culture released to nocodazole containing media (left hand panel, Figure 3.13).
Chapter 3. *Supercoiling mechanism*

The DNA is first separated through a low percentage agarose gel in neutral conditions, and then through a second dimension in the presence of ethidium bromide and a higher density of agarose to separate the DNA according to both size and shape, detecting catenation and supercoiling changes (Martínez-Robles et al., 2009). The bands appearing as the cells pass through S phase are indeed catenanes, as would be expected in the absence of Top2. However, this does not explain the altered appearance of the catenane band when compared to a strain arrested in nocodazole with wild type Mad2 present.

**Figure 3.14**: Identification of DNA species present on passage through S phase without functional Mad2. 2D gel electrophoresis with ethidium bromide to show a centromeric plasmid, *pRS316*, on passage through the cell cycle. Cells were arrested in G1, the degrons triggered and the cells released into the cell cycle, with nocodazole added to the medium. Shown here is G1 (A), 20 minutes (B) and 40 minutes (C) post release from the same experiment as shown in left hand panel of Figure 3.13. Samples were run without ethidium bromide through a low agarose density in the first dimension and then with 0.3 μg/ml ethidium bromide and higher agarose density in the second dimension. A probe to the plasmid was used so only the different forms of the plasmid are seen, and a cartoon is shown in D) depicting the identity of the DNA species seen. A) shows mostly monomer species present, B) shows the first signs of dimers, and C) shows a high level of catenanes, particularly CatCs. Abbreviations: CatA - catenane where both plasmids are relaxed, CatB - catenane where one plasmid is supercoiled and the other relaxed, CatC - catenane where both plasmids are negatively supercoiled, Ocm - open circular monomer (relaxed), Ocd - open circular dimer, Lm - linear monomer, Ld - linear dimer.

To fully understand the identity of this expanded catenane band, samples with and without functional Mad2 in a nocodazole-mediated arrest were nicked with the NbBsm1 enzyme. This relaxes all catenanes to CatA species as nicking releases the supercoiling tension from the DNA molecules by allowing rotation. The DNA is then run under the same conditions as Figure 3.14.
In this situation, it is possible to examine the catenane population of the cell and determine the number of intertwines, or nodes, by which each pair of plasmids are linked, as shown in the cartoon (Figure 3.15C)). The other two panels of Figure 3.15 show strains lacking Top2 with either wild type Mad2 (left hand panel) or mad2Δ which were synchronised in G1 and released to progress through S phase in the absence of functional Top2 - producing catenated dimers - to an arrest in nocodazole.

**Figure 3.15:** Lack of Mad2 results in a change in catenane distribution in the presence of nocodazole. 2D gel electrophoresis with ethidium bromide to show a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G1, the degrons triggered and the cells released into the cell cycle, with nocodazole added to the medium. Shown here are samples taken once cells reached G2/M in the presence of nocodazole, 60 minutes after release from G1 with (A) functional Mad2 and (B) no Mad2. These samples were nicked with NbBsm1 enzyme to produce relaxed monomers and this allows the number of catenated nodes in the plasmids to be identified, see (C) a cartoon of this distribution. Samples were run without ethidium bromide through a low agarose density in the first dimension and then with 0.3 µg/ml ethidium bromide and higher agarose density in the second dimension. A probe to the plasmid was used so only the different forms of the plasmid are seen. The distribution of catenanes alters between the two samples. On average, there are fewer nodes in the catenanes in the cells lacking Mad2 than in those with wild type Mad2. Abbreviations: CatA - catenane where both plasmids are relaxed, Ocm - open circular monomer (relaxed), Od - open dimer.

The lack of Mad2 has altered the distribution of catenated nodes in the plasmid population. With wild type Mad2, the densest part of the arc shows that the most common number of intertwines between plasmids is between 8 and 14 nodes, while in the mad2Δ strain this is reduced, with between 1 and 11 nodes being most common (Figure 3.13).
This may suggest that a decatenation activity has been triggered without Mad2 and some resolution of the intertwines is occurring.

Importantly, these cells have no active Top2 as the degrons were triggered before S phase, and so it is likely another topoisomerase is involved. Top3 has been shown to be able to decatenate DNA in vitro as part of the complex Top3-Sgs1-Rmi1 (Cejka et al., 2012). This may be demonstrating an additional catenane resolution pathway which can be activated as the cells go into anaphase to ensure full resolution of all catenanes before chromosome segregation. This redundancy emphasises the importance of full catenation resolution at this point in the cell cycle, in order to allow accurate segregation of the DNA.

This also explains the altered distribution seen by 1D gel electrophoresis, as under wild type conditions if the film is overexposed catenanes can be seen running the full length of the gel behind the labelled CatC and monomer bands (for an example see Figure 3.10) with the densest portion being labelled as CatC presumably representing those linked by 8-14 nodes. The stronger, thicker band seen in the mad2Δ cells is therefore actually demonstrating a partial decatenation of the plasmids, producing a condensation of the catenane smear to approximately 1 cm long instead of 5 cm as usually seen in wild type cells. This makes it more visible, appearing as the expanded ‘doublet’, see Figure 3.13.

Finally, although the 1D gel did not show signs of the positive supercoiling transition occurring, this partial resolution of catenanes could have been masking a change in supercoiling. Therefore an experiment was carried out where the mad2Δ top2-td pRS316 strain was delayed in G2/M by addition of nocodazole, having passed through S phase with a functional Top2 to produce monomers instead of catenanes. A sample was taken as the degron was triggered to remove Top2 and then 60 and 90 minutes later, while nocodazole was maintained in the media. This will allow us to see if the supercoiling transition is able to be activated in a situation where there is no spindle formation, but also no Mad2 to activate the SAC.

Figure 3.16 shows that while the cells were held in the presence of nocodazole, no change in supercoiling state occurs. From the 1D we know that if the cells are allowed to form functional spindles then the transition does occur in this strain. Therefore it is clear that the supercoiling transition requires functional spindles to occur and that the activation of the transition is not dependent upon satisfaction of the SAC but spindles themselves.
Figure 3.16: Lack of SAC activation does not cause a supercoiling change without functional spindles. 2D gel electrophoresis in the presence of chloroquine showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were synchronised in G\textsubscript{1}, released through S phase, and then the degron of Top2 triggered. A sample was taken at this point, when arriving in G\textsubscript{2}/M in the presence of nocodazole, and then 60 and 90 minutes later. The samples were run in the 1st dimension with low levels of both chloroquine and agarose, and then in a second dimension with higher levels of both. This separates the monomer plasmids according to their supercoiled state. A probe to the plasmid was used so only the different forms of the plasmid are seen. The deletion of the MAD2 gene means that the cells cannot activate the SAC in response to the addition of nocodazole. However, the transition still does not occur in the absence of spindles.

3.5 Tension generation is essential

Based on this evidence, the inactivation of the SAC is not the key step in activating the positive supercoiling transition. Therefore, it is likely that either the attachment of the spindles, or the tension generated by correct attachments, is the instigating event for the supercoiling transition. \textit{S. cerevisiae} has four motor proteins, and it can lose two of these and still be viable, while loss of the third is lethal and prevents tension generation through spindles (Dyn1, Kip1 and Cin8 - Kar3 cannot generate spindle tension alone) (Gerson-Gurwitz \textit{et al.}, 2009). As such, a strain was created in the \textit{top2-td cdc20-td pRS316} background where two motor proteins \textit{DYN1} and \textit{KIP1} were deleted and the third motor protein Cin8 was replaced by a temperature-sensitive allele, \textit{cin8-3}. The addition of the \textit{cin8-3} allele allowed selection by viability at 37°C and total removal of motor protein function. The supercoiling transition protocol was then carried out with this strain.

Figure 3.17 shows that this strain does not undergo the transition in the Cdc20-mediated arrest. The CatC species persist and there is no significant appearance of CatC*.
suggests that the ability to generate tension through the spindles is necessary for the transition to occur and that attachment of the spindles alone is not enough.

**Figure 3.17:** Inability to generate tension through spindles means cells are unable to trigger the supercoiling transition. Growth assay to evaluate the viability of a strain carrying wild type motor proteins, dyn1Δ, dyn1Δ kip1Δ or dyn1Δ kip1Δ cin8-3 temperature-sensitive allele at permissive and non-permissive temperatures. The strains are viable until the addition of the cin8-3 allele and then the strain is completely dead at 37°C. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle, with nocodazole added to one culture. Cells were arrested in G1, the degrons triggered and the cells released into the cell cycle. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. FACS profiles show the two cultures pass through the cell cycle with similar kinetics. Cells lacking all three motor proteins are unable to generate tension along the microtubules of the spindle, and the cells which reach the Cdc20-mediated arrest do not undergo the positive supercoiling transition and instead maintain the CatC signal, as in the nocodazole arrested cells. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled).

### 3.6 Determining the role of Shugoshin

Recent papers are suggesting an increasingly complex and central role for shugoshin in the centromeric assembly of proteins, and particularly in pericentromeric recruitment of condensin (Peplowska *et al.*, 2014). To investigate if Sgo1 (*S. cerevisiae* shugoshin) plays a role in the coordination of the supercoiling transition, the *SGO1* gene was deleted. The supercoiling transition protocol was carried out and is shown in Figure 3.18.
Figure 3.18: **Loss of Sgo1 does not prevent the positive supercoiling transition** Growth assay to evaluate the viability of a strain carrying wild type SGO1 or sgo1Δ growing on YPD with no benomyl or 10 µg/ml benomyl. The sgo1Δ strain copes much less well with the mild spindle poison compared to the wild type strain. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G₁, the degrons triggered and the cells released into the cell cycle, with nocodazole added to one culture. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. The strain used here is lacking SGO1. FACS profiles show the two cultures pass through the cell cycle with similar kinetics, albeit not fully reaching the G₂/M arrest. Deletion of SGO1 is not enough to prevent the transition from occurring, although it appears weaker than in a ‘wild type’ strain and possibly delayed. The FACS also shows an altered profile but mainly in the quality of the G₂/M arrest and not the speed with which the cells progress through the cell cycle. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled), YPD - yeast peptone with glucose.

As can be seen, the transition does occur in a strain without Sgo1 as the cells arrive at a Cdc20-mediated arrest. Therefore, Sgo1 is not essential for the positive supercoiling transition to occur. However, the timing of the transition is slightly delayed as the shift normally occurs at 80 minutes in a reproducible manner, and here there are no CatC* species detectable until 100 minutes. Furthermore, it does not look like a complete transition, with some remaining negatively supercoiled dimers, CatC, even at 140 minutes. The FACS profile shows that these cells do not appear entirely as ‘wild type’ as they go through the cell cycle but the progression through S phase is similar to that of the normal experiment.

Sgo1 has been shown to be responsible for the recruitment and maintenance of condensin
at the centromeric and pericentromeric region (Nerusheva et al., 2014, Peplowska et al., 2014, Verzijlbergen et al., 2014). Perhaps, without Sgo1, condensin is not bound to chromosomes at the moment of the transition and so it takes longer to recruit condensin to the DNA to enact the change. However, this recruitment must be one of several redundant mechanisms given that the topological change does occur in the absence of Sgo1. It could be that the release of condensin from the centromere as tension is created (Nerusheva et al., 2014, Peplowska et al., 2014, Verzijlbergen et al., 2014) allows it to enact the supercoiling change, and in fact tethering of Sgo1 at the centromeres could have a negative impact on this process, if condensin needs to move away from centromeres for this action. Further information is needed on the mediation of condensin recruitment and release during the transition.

3.7 Examining the role of a histone chaperone

The wrapping of DNA around histone octamers to form nucleosomes introduces negative supercoiling into the DNA, see Section 1.3.1. As such, it is likely that the change to positive supercoiling will require the displacement or alteration of histones. Nap1 is a small histone chaperone protein which helps to alter the conformation of histone molecules, primarily through displacement of H2A/H2B, in a manner thought to help promoter activation and transcription elongation (Peterson et al., 2007). This change in histone conformation has been shown to be capable of affecting the supercoiling of DNA, and allowing it to accommodate positive supercoiling more easily. As such it was possible that Nap1 could be important in the switch to positive supercoiling and that without it, perhaps the topological transition could not be enforced. Nap1 was deleted and the positive supercoiling transition carried out with this strain. Figure 3.19 shows that the loss of Nap1 does not affect the supercoiling transition, suggesting this protein is not required for the topological changes taking place.

The positively supercoiled species, CatC*, that are produced by the supercoiling transition fade over time as a Cdc20-mediated arrest is maintained. If Nap1 plays a role in altering histone conformations to accommodate positive supercoiling then it seemed possible that an overexpression of Nap1 could mean that the topological shift would persist longer in a Cdc20-mediated arrest. As such, an extra copy of Nap1 under the
Figure 3.19: Nap1 is not required for the positive supercoiling transition. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle, with nocodazole added to one culture. Cells were arrested in G1, the degrons triggered and the cells released into the cell cycle. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid was used so only the different forms of the plasmid are seen. FACS profiles show the two cultures pass through the cell cycle with similar kinetics. Deletion of the histone chaperone Nap1 does not seem to affect the positive supercoiling transition. Abbreviations: CatC - catenane where both plasmids are negatively supercoiled, CatC* - catenane where both plasmids are positively supercoiled, Ocm - open circular monomer (relaxed), CCCm - closed circular monomer (supercoiled).

galactose-inducible promoter was added to the strain and this would be triggered alongside the degrons. To look at the persistence of the supercoiled species, samples were taken for the 2D monomer analysis. This allows cells to pass through a synchronised S phase in the presence of functional Top2 before arresting in nocodazole, triggering the degrons and releasing to a Cdc20-mediated arrest in the absence of Top2. The transition should occur as the cells form and attach spindles, but perhaps overexpression of Nap1 would be sufficient to create longer lived positively supercoiled species.

Figure 3.20 shows that the transition occurs as expected, with the appearance of the positively supercoiled species in the 20 min sample, in the pGAL1-NAP1 strain as in the wild type experiment. However, these species fade and are gone by 60 minutes in the mutant, slightly earlier even than the wild type strain. Therefore perhaps the
overexpression of Nap1 is allowing more rapid resolution of the supercoiling changes but it is certainly not forcing the DNA towards a positively supercoiled state.

![Overexpression of Nap1](image.png)

**Figure 3.20:** Overexpression of Nap1 does not help the longevity of the positively supercoiled species. 2D supercoiling transition protocol in the presence of overexpression of Nap1. Cells were arrested in nocodazole, the degrons were triggered (removing Top2, Cdc20 and overexpressing Nap1) before release to Cdc20-mediated arrest and samples taken every 20 minutes. Samples were processed for DNA extraction before running in two dimensional gel electrophoresis in the presence of chloroquine according to the conditions explained in Figure 3.5. The top panels show this result in the presence of excessive Nap1, while the bottom panels show the wild type experiment and a cartoon explaining the forms separated by 2D gel electrophoresis (Baxter et al., 2011). The positively supercoiled species do not persist any longer with the overexpression of Nap1 than in the wild type strain, suggesting this protein is not enacting positive supercoiling or its persistence in the DNA.

Therefore, it seems that neither the deletion of NAP1, nor its overexpression, significantly influence the transition and so Nap1 is unlikely to be involved in enacting the topological changes. However, this certainly does not rule out a role for some histone remodelers or chaperones in the physical process of accommodating this transition towards positive supercoiling of the DNA. Further work looking for other candidates and examining their involvement would definitely be interesting.
Chapter 3. Supercoiling mechanism

3.8 Summary

A change in supercoiling towards a more positive state was found to occur at the point of spindle formation during the metaphase to anaphase transition. This was found to depend on spindles, the condensin complex (Smc2), the cohesin complex (Scc1) and the Polo kinase (Cdc5). It was hypothesised that spindle tension across sister chromatid kinetochores, resisted by cohesin complexes, triggered the hyperactivation of condensin’s DNA overwinding activity, directly or indirectly via phosphorylation by Cdc5. This was supported by the demonstration that significant changes occur on DNA at the metaphase to anaphase transition (resolution of catenations, higher compaction of DNA and induction of supercoiling (Baxter et al., 2011, Lavoie et al., 2002, Nakazawa et al., 2011, Oliveira et al., 2010, Uhlmann et al., 2000, Vas and Clarke, 2008)), condensin can positively supercoil DNA in vitro (Bazett-Jones et al., 2002, Kimura and Hirano, 1997, Stray et al., 2005) and that phosphorylation of condensin by Cdc5 hyperactivates it in vitro, and occurs in vivo (Bazile et al., 2010, St-Pierre et al., 2009).

Here, the transition was shown to depend directly on spindle tension (Figure 3.17), as well as formation, and not on the satisfaction or dismantling of the SAC (Figures 3.13 and 3.16). Condensin recruitment by Sgo1 is not required to enact the supercoiling transition (Figure 3.18). It is possible that the release of condensin from centromeres in response to tension (Sgo1 dependent) is important, but that its initial recruitment is not (for the transition to occur).

Furthermore, work here has shown that although loss of Smc2 prevents the supercoiling transition from occurring, the temperature-sensitive allele of Brn1, brn1-60, did not prevent the transition from occurring (Figure 3.8). However, the protein remained associated with the complex and was not degraded (Figure 3.9) so it seems that the mutation here was not sufficient to eliminate the topological change.

Loss of the Ipl1 complex appears to reduce the supercoiling transition, but not eliminate it (Figure 3.11). This suggests its phosphorylation of condensin may only provide an enhancement of its supercoiling activity, or that it is not related to the topological transition. Unfortunately, overexpression of Cdc5 was not sufficient to produce the supercoiling change without spindle tension (Figure 3.10) and this may be due to additional modifications needed, either on Cdc5 or condensin itself.
Chapter 4

Genome wide ChIP-seq of Smc2

Objective

- To examine the localisation pattern of condensin in more detail during the super-coiling transition at the progression from metaphase to anaphase

Experimental approach

Given the essential role condensin appears to play in the observed positive supercoiling transition, it seemed interesting to look at condensin’s localisation during that time. Published studies have shown condensin to be localised to centromeres during mitosis and rDNA throughout the cell cycle (D’Ambrosio and Schmidt, 2008, Wang et al., 2005). These studies have been carried out in a G\textsubscript{1} arrest and a G\textsubscript{2}/M arrest enforced by nocodazole, and investigated by ChIP-chip. As such the picture of condensin localisation is limited to only single moments in the cell cycle. The supercoiling transition occurs on a very short time scale in the aftermath of spindle formation, and this time period has not been covered by ChIP. As such, it seemed there were gaps in this knowledge which could be filled in by the use of ChIP-seq at the time of the positive supercoiling transition.

ChIP-seq

The ChIP-seq experiments were carried out in the Shirahige lab at the University of Tokyo. The protocols used for the ChIP-seq were theirs, but the experimental protocol was as outlined in Figure 4.1. The strain used had the promoter of the CDC20 gene replaced with a promoter responsible for one of the methionine synthesis genes; as such
if the cells are cultured in a media lacking the amino acid methionine then they grow as wild type. However, if they are grown in media with methionine present (such as YPD) then the gene under that control would be repressed. Therefore, addition of methionine to the media would cause a cell cycle arrest by the lack of Cdc20 protein as the promoter is shut down.

Cells were cultured overnight in media without methionine, and then arrested at four points in the cell cycle where samples were taken. The first was a $G_1$ arrest under the influence of the mating pheromone $\alpha$-factor. Cells were then resuspended into media which contained methionine but no $\alpha$-factor which means the promoter expressing $CDC20$ will shut down, and nocodazole was added to the media to force cells to arrest due to no functioning spindles and activation of the SAC. At this point, the second sample was taken and the culture was washed to release cells from the nocodazole-mediated arrest meaning spindles reform, the SAC is satisfied but cells arrest due to a lack of Cdc20, meaning that the APC cannot be activated to progress the cells into anaphase. Here, the third sample was taken and then nocodazole was added back into the culture and time allowed for dissolution of the spindles for sample four, see Figure 4.1.

**Figure 4.1:** Experimental protocol for ChIP-seq experiments. Cartoon representation of the cytological state of the yeast during the protocol, showing exponential at the beginning, $\alpha$-factor arrested cells with mating shmoos and the dumbbells of a $G_2/M$ arrest, with and without spindles. ChIP sample time points are indicated.
The samples taken were 100 ml samples and fixed for 30 minutes with formaldehyde at room temperature before being processed for ChIP-seq. The protocol for the ChIP is outlined in Section 2.5.7 and Appendix C and the protein being pulled down was Smc2 with nine Myc epitopes attached to the C-terminus. The full ChIP-seq maps for each sample are included in Appendix C.

4.1 Smc2 binds at the rDNA throughout the cell cycle

In order to check that the ChIPs had successfully pulled down Smc2-9Myc, it seemed wise to check that we had reproduced previously published results - an enrichment of condensin at the rDNA locus throughout the cell cycle (D’Ambrosio and Schmidt, 2008, Freeman et al., 2000). Figure 4.2 shows a portion of the rDNA locus in the four samples taken for ChIP-seq - G₁ arrested by mating pheromone, G₂/M arrested by nocodazole addition and SAC activation, a G₂/M arrest mediated by the repression of Cdc20 through methionine addition, and the readdition of nocodazole into the Cdc20-mediated arrest. The previously reported enrichment in the rDNA is seen in all four samples (Figure 4.2). Therefore, the ChIP-seq has been successful at pulling down Smc2 and identifying areas of enrichment.

![Figure 4.2: ChIP-seq enrichment of the rDNA locus at the four time points.](image)
ChIP-seq carried out according to the protocols of the Shirahige lab. All four cell cycle samples showed enrichment across the rDNA locus, as has been previously published. Graphs show the enrichment level in the IP sample divided by the input value, therefore giving the fold increase in Smc2 binding at each location.
Although it is definitely true that the rDNA is enriched for Smc2-9Myc in all four samples, the level of enrichment varies slightly between the different cell cycle points, suggesting it may vary through the cell cycle.

### 4.2 Condensin localisation at centromeres is mitosis specific

Based on published Smc2 binding data, the sample arrested in G\(_1\) was expected to show low level centromeric binding of condensin (D’Ambrosio and Schmidt, 2008, Wang et al., 2005) with a higher enrichment going into mitosis. Here, this ChIP-seq shows no condensin localisation to the centromeres in G\(_1\), as shown for the first eight centromeres in Figure 4.3 (the ChIP was successful due to rDNA binding, full maps are in Appendix C). Condensin was then seen to be highly enriched in the mitosis sample (peaks were seen in all three samples taken in G\(_2/M\)). This also confirms previous reports of condensin binding to centromeres during mitosis but shows a greater distinction here between levels in G\(_1\) and G\(_2/M\) than was published (D’Ambrosio and Schmidt, 2008).

![Figure 4.3: Condensin localises to centromeres only in mitosis](image)

**Figure 4.3:** Condensin localises to centromeres only in mitosis. Panels show the centromeric region of Chromosomes I - VIII. Top panels show a G\(_1\) sample obtained through blocking with α-factor and the bottom panel shows a nocodazole-mediated G\(_2/M\) arrest. The lower panels show a high level of Smc2 enrichment in mitosis, and the top panels show an absence of binding in G\(_1\). Graphs show the fold enrichment of Smc2 binding in the IP sample over input.

### 4.3 Spindle-dependent localisation

The focus of this ChIP-seq was to look at the localisation of condensin at the moment of the positive supercoiling transition, the change between the nocodazole-mediated arrest,
with no spindles, and the Cdc20-mediated arrest, with spindles, where the DNA would be in the positively supercoiled, ‘transitioned’ state.

I also took a third sample where nocodazole was readded because the readdition of nocodazole to a culture which had already progressed to a Cdc20-mediated arrest would cause the spindles to re-depolymerise and it would be possible to see if the transition was reversible. If the transition is reversible it would be interesting to see if the localisation of condensin also returned to the pre-transition state. Figure 4.4 shows the centromeres of Chromosomes I - VIII in the three G₂/M arrest samples that were analysed.

Figure 4.4 shows, in the top panels, that in the initial nocodazole-mediated arrest there are high levels of enrichment of Smc2-9Myc across the centromere of each chromosome and, slightly lower, extending across the pericentromeric region. The full maps of the ChIP-seq show a general lack of Smc2 enrichment away from the centromeres. The middle panels show that, having washed out the nocodazole and allowed the cells to form spindles and progress to a Cdc20-mediated arrest, these peaks were lower, but similarly localised. This suggests that the localisation of condensin to the centromeres is at least partly linked to the pre- and post-transition state of the chromatin, and the formation of spindles.

Finally, after readdition of nocodazole and dissolution of spindles, shown in the bottom panels, these peaks returned to their original level showing that the localisation of Smc2 to centromeres can be reinstated in the case that spindle tension is lost after progression to a Cdc20-mediated arrest.

**Figure 4.4:** Condensin enrichment at centromeres is affected by spindles. Panels show the centromeric region of chromosomes I - VIII. Top panels show the original nocodazole block, the middle panel the Cdc20-mediated arrest and the bottom panel shows the readdition of nocodazole. The degree of enrichment of Smc2-9Myc at the centromeres alters reversibly with and without spindle formation. Graphs show the fold enrichment of Smc2 binding in the IP sample over input.
A previous ChIP had been carried out in similar conditions (nocodazole-arrested and Cdc20-depleted) by a previous lab member. However, the strains used in these data above were done in the presence of functional Top2, whereas the previous ChIP used a top2-4 mutant as was used to visualise the topological transition. In the first ChIP, binding was seen at the centromere in the nocodazole-mediated arrest followed by a complete loss of this enrichment when the cells were allowed to reach a Cdc20-mediated arrest. This suggests the change in condensin binding may be more pronounced in the absence of Top2, and accompanies the stronger topological change seen in the absence of Top2. Alternatively it could represent only a partial transition occurring here, potentially due to an incomplete removal of the nocodazole.

4.4 The topological transition is also reversible

Here, we have shown that condensin localisation changes are reversible as spindles are formed and lost. Therefore, it seemed interesting to examine the reversibility of the topological transition in the same way. The positively supercoiled species that appear as cells reach a Cdc20-mediated arrest do not persist for extended amounts of time (Figure 3.5). Therefore, to examine the reversibility of the topological change, nocodazole had to be readded as soon as possible after the topological transition had taken place, and a second culture was maintained in a Cdc20-mediated arrest for comparison.

The positive supercoiling transition protocol was used from Chapter 3 (Figure 3.2), except both cultures were allowed to progress to a Cdc20-mediated arrest. At 100 minutes, the transition should have occurred in the cultures and the catenanes should be in a positively supercoiled state, CatC*. At this point, nocodazole was added to one culture and this should re-depolymerise the spindles, as for the ChIP, potentially removing the activating signal for the transition. If the reversal of the Smc2 localisation pattern was representative of a reversal in topology, then the CatC species (the negatively supercoiled form, pre-transition) should reappear.

Figure 4.5 shows the two cultures passing through the cell cycle in the absence of Top2 and Cdc20. At 80 minutes, the CatC species fades, and the CatC* appears, showing that the transition has occurred. After the readdition of nocodazole at 100 minutes, the spindles would dissolve and the SAC be reactivated. As can be seen in the red box, the
CatC band reappears after this time. This suggests that the topological transition can also be reversed upon dissolution of spindles and loss of spindle tension.

**Figure 4.5:** Positive supercoiling transition is reversible. Southern blots showing a centromeric plasmid, pRS316, on passage through the cell cycle. Cells were arrested in G₁, the degrons triggered and the cells released into the cell cycle. Samples were taken every 20 minutes and processed for DNA extraction before running in one dimensional neutral gel electrophoresis for Southern blotting. A probe to the plasmid highlights the multiple forms of the plasmid present in each sample, seen. Nocodazole was added to one culture at 100 minutes, marked by a star. The red box highlights the reappearance of the CatC band in the culture with added nocodazole. Abbreviations: CatC - catenanes where both plasmids are negatively supercoiled, CatC* - catenanes where both plasmids are positively supercoiled, Ocm - open circular monomers (relaxed), CCCm - closed circular monomers (supercoiled).

Combined with the ChIP-seq results showing the reversibility of the condensin localisation to the centromere, this may suggest that upon tension generation, condensin alters its binding and changes the topological state of the chromosomes. If the spindle tension is lost then the condensin is recruited back to the centromeres and the chromosomes return to the negatively supercoiled state.

### 4.5 Unexpected G₁ peaks

Interestingly, there were very clear and highly enriched peaks showing condensin binding at distinct sites in the G₁ arrested sample. Based on previous work, it was expected that the G₁ arrested ChIP-seq would show very little condensin enrichment, with localised
peaks potentially linked to tRNA genes (D’Ambrosio and Schmidt, 2008, Freeman et al., 2000). rDNA and telomere enrichment was seen as predicted in all samples. However, more extensive peaks were seen in the G₁ arrest, not only at tRNAs but at some Pol II transcribed genes along the chromosome arms, Figure 4.6.

These peaks covered the entire open reading frame (ORF) of the genes they occupied, and these genes included about 40 α-factor response genes, 10 stress response genes and multiple housekeeping genes, some of which showed binding in the G₂/M arrests as well. This suggested a possible link between active transcription and condensin localisation.

Figure 4.6 shows a selection of these peaks from the ChIP-seq which are aligned with the gene layout and their occupancy in a nocodazole-mediated arrest. Note that the enrichment of condensin appears to occupy the full length of the genes, mapping exactly to the ORF. The genes which are only expressed in response to α-factor show no enrichment in the G₂/M arrest but some genes do, those which may also be transcribed in that arrest, including stress response or cellular maintenance genes. This certainly required further investigation.

**Figure 4.6: Condensin localisation across active ORFs in G₁.** Example peaks seen in a G₁ arrested sample at actively transcribed genes which fall into three categories, mating specific, stress response or housekeeping. Top panels show the chromosome map, central panels show the G₁ sample and the bottom panel shows a nocodazole-mediated G₂/M arrest. The mating specific genes show high levels of enrichment in G₁ and none in G₂/M whereas some of the stress response and housekeeping genes do show enrichment, to lower levels, in the G₂/M samples. Graphs show fold enrichment of Smc2 binding in the IP sample over input.
4.6 Summary

Previous reports of the binding patterns of condensin have suggested a binding at telomeres, tRNA and the rDNA throughout the cell cycle and an increase at centromeres in mitosis (D’Ambrosio and Schmidt, 2008, Lavoie et al., 2002, Verzijlbergen et al., 2014, Wang et al., 2006).

Here, the ChIP-seq carried out using Smc2-9myc also showed a binding at the telomeres and rDNA throughout the cell cycle and saw a binding to approximately 50% of tRNAs (Figure 4.2). This analysis also showed a complete absence of Smc2 at centromeres in G$_1$ and an increase in G$_2$/M across centromeres and pericentromeric regions (Figures 4.3) and this binding in mitosis was seen to respond reversibly to the presence of spindles (Figure 4.4).

This reversibility in the localisation of condensin in response to spindles was reflected by the reversibility of the topological supercoiling change (Figure 4.5) suggesting that the change in localisation could be related to the enacting of the positive supercoiling transition. No binding outside of the centromere was seen but the supercoiling transition is a transient and rapid change, and it may be that the timing of samples taken here were too slow to catch any binding associated with the change.

Finally, unexpected peaks were seen in the G$_1$ arrest at the ORFs of various genes thought to be active during this cell cycle arrest (Figure 4.6). This required further investigation to verify them, particularly given an absence of an untagged or no antibody control.
Chapter 5

Technical pitfalls of ChIP

Objectives

- To confirm the genome wide condensin ChIP-seq results using qPCR
- To confirm condensin binding to transcribed ORFs in G₁
- To assess ChIP protocol for potential artifacts

Experimental approach

As mentioned in the previous chapter, the Smc2 ChIP-seq of a G₁ arrested sample showed a number of highly enriched regions which precisely matched the ORF of various genes. Further inspection of these genes showed that they fell largely into three categories of genes, which could all be expected to be actively transcribed in the G₁ arrest - mating or G₁ specific, stress response and cellular maintenance genes.

Although a role for condensin in active transcription has not been reported, the level of enrichment at these peaks suggested it was worth further investigation. The first steps were to confirm the ChIP-seq enrichment, and check that these genes were indeed actively transcribed.

For confirmation of the ChIP-seq profile, strains in which the protein of interest was tagged with Myc or HA epitopes were used and the enrichment at specific locations was tested by real time PCR (qPCR) to see if the result could be recapitulated.

For assessment of transcription levels, the RNA was extracted and converted to cDNA by reverse transcription. The relative amount of any given sequence was then tested by qPCR normalised to a control region.
Transcription

In order to look at the transcription level of certain genes, the relative abundance of the RNA transcript level was analysed. Firstly, the total genetic material of the cell is extracted which purifies the DNA, as well as mRNA, tRNA and rRNA. This purified genetic material is treated with DNase I in order to leave just the RNA and due to the ease of degradation of RNA it is essential to check the quality of the RNA extraction by gel electrophoresis at this stage (see Figure 5.1B).

Provided the RNA is still intact, the purified RNA is used as a substrate for a PCR reaction using a viral reverse transcriptase enzyme, in order to produce the cDNA from each sample. The RNA is then removed by RNase treatment.

Finally, the cDNA is used as the substrate for qPCR to evaluate the level of a transcript in the sample. For analysis of each gene, three pairs of primers were used through the open reading frame and one in the promoter region, see Figure 5.1. The signal was normalised by dividing the qPCR signal at the target region by a qPCR primer pair in the constitutively transcribed gene, TUB1, which is representative of the total cDNA present. This means the value given ultimately represents the relative abundance of the RNA of interest in each sample. This should be representative of the transcription levels of that sequence, although it can also be affected by the half-life of the mRNA produced.

ChIP analysis

To confirm the peaks seen in the genome wide ChIP-seq results, the ChIP was repeated with strains where the target protein was tagged with either Myc or HA epitopes.

Briefly, as the samples are taken, the DNA is crosslinked to any proteins bound to it using formaldehyde. Formaldehyde can crosslink anything which is within close enough proximity, including DNA-protein interactions but also protein-protein interactions. As such, it is important that the length of crosslinking treatment, the concentration of formaldehyde and the temperature are all controlled. The crosslinking process is stopped by quenching with glycine.
Cells are broken and DNA (and bound proteins) purified by centrifugation. This is then sonicated to produce fragments of DNA approximately 200-500 bases long, as optimised by reversing the cross linking and running on a simple agarose gel with ethidium bromide staining. The length of these fragments is important for specificity of the ChIP enrichment as it limits how long a stretch of DNA is pulled down by a bound protein.

The sonicated chromatin can then be separated from remaining insoluble material by centrifugation and incubated with the appropriate antibody. This will pull down the target protein and so enrich the DNA for sequences bound by that protein. This mixture is then incubated with agarose beads to pull down the antibody (and the target protein-DNA complexes). Finally these beads are washed to remove any unbound genetic material.

The beads are then boiled in the presence of Chelex® (from Bio-Rad) which reverses the crosslinking reaction and the DNA is released into solution, meaning it can be separated once more from insoluble material by centrifugation (here leaving behind protein, agarose beads, antibody and Chelex®). This purified DNA should have a higher proportion of any sequences which were bound by the protein of interest, and this enrichment is tested by comparing to an aliquot which is taken prior to antibody treatment and as such should represent the natural abundance of the sequences (referred to as the input). These two samples, the antibody-treated and the input sample are used as the substrates for qPCR reactions using primer pairs to a chosen sequence of DNA. Any increase or decrease of signal in the antibody-treated sample compared to the input should be due to the presence, or absence, of the target protein at that location in the genome.

For all details of the primers used for qPCR in this study, refer to Appendix B for sequences and representative melt curves.
5.1 Condensin-enriched genes in G\textsubscript{1} are actively transcribed

The genes which showed condensin enrichment in G\textsubscript{1} are reported to be active in this cell cycle phase. In order to confirm active transcription at genes of interest, the RNA transcript levels were measured in cells arrested in G\textsubscript{1} by addition of the mating pheromone \(\alpha\)-factor.

Two target genes were chosen for assessment which showed very clear condensin enrichment, to differing degrees, in the genome wide ChIP-seq (see Figure 4.6). Over 6-fold enrichment was seen at FIG2 which is a G\textsubscript{1} specific gene that responds to the presence of \(\alpha\)-factor and encodes a cell wall adhesin. FAS2 is a non-G\textsubscript{1} specific gene, but it is most likely transcribed nonetheless as it is a cellular maintenance gene encoding a fatty acid synthetase, and showed over 3-fold enrichment over the background condensin binding. Finally the silenced mating locus, HMR, was used as a negative control as it showed no condensin enrichment in either G\textsubscript{1} or G\textsubscript{2}/M and should not be actively transcribed.

Figure 5.1 shows the transcription levels of these three target genes, normalised to TUB1, in an \(\alpha\)-factor mediated G\textsubscript{1} arrest. Cells were arrested by addition of the mating pheromone and samples taken for analysis of transcript levels by RNA extraction and reverse transcription, followed by qPCR.

Figure 5.1 shows that FIG2 is highly transcribed, and FAS2 transcripts are present at approximately 2.5% of the level of FIG2. However, both are actively transcribed by comparison with HMR transcripts which are barely detectable.

Furthermore, a pattern can be seen that the primer pair closest to the 5’ end of the gene is the most abundant, with the level reducing through the gene body, as not every transcript which is initiated will run the whole length of the gene (Sims et al., 2004).

These data appear to show a link between the level of transcription at a locus and the enrichment of condensin as seen by ChIP-seq, at least in this G\textsubscript{1} sample and in the three genes tested.
5.2 Replicating the ChIP-seq results in G₁ by manual ChIP

It was important to verify the results seen in the genome wide ChIP-seq, and particularly the unexpected enrichment in G₁. In order to do this, a strain was used with 3 HA epitopes attached to the C terminal end of the SMC2 gene. The cells were cultured and then arrested either in G₁ by addition of α-factor, or in G₂/M by the addition of nocodazole. Once the cells were blocked, formaldehyde was added to a final concentration of 1.42% for 15 minutes and then quenched with glycine.
These samples were processed for ChIP, resulting in an input sample which was not subject to the IP process, a ‘no antibody’ sample which is subject to the IP but with no antibody present and a full IP with an anti-HA antibody. The input sample shows the un-enriched signal each loci gives by qPCR, the ‘no antibody’ sample shows the enrichment caused by the beads and the IP process, and the ‘antibody’ sample shows the specific enrichment caused by the tagged protein’s presence at certain sequences. Therefore, each sample is controlled for the absolute amount of DNA by the input sample (this also helps to control for other problems such as the high copy number of rDNA), controlled for background enrichment caused by the IP by the ‘no antibody’ sample, and finally it is normalised to the HMR locus which is not bound by condensin and as such acts as a negative control for the qPCR.

Figure 5.2 shows the outcome of this ChIP analysis. It is plotted to show the level of qPCR signal in a Smc2-3HA ChIP divided by the ‘no antibody’ ChIP to show a fold-level enrichment which should be dependent on the presence of the antibody, and as such, its target Smc2-3HA. The samples were tested across three loci, FAS2 and FIG2 which were enriched on the genome wide ChIP-seq and tested for transcription in the previous section and CDC15 which is inactive and unenriched in G1. The primers were located throughout the ORF of each gene, and one outside, as shown in Figure 5.1. The level of enrichment at FAS2 and CDC15 is approximately the same in both arrests, although slightly higher in CDC15 in a nocodazole-mediated block, and vice versa for FAS2. However, FIG2 shows a large enrichment for Smc2-3HA, specifically in the α-factor blocked sample, exactly when its transcription is highly induced.

This confirmed an increase in ChIP signal during an α-factor arrest over that in the nocodazole-mediated result, as seen in the G1 genome wide ChIP-seq at the FIG2 locus. The genome wide ChIP-seq also showed a peak at the FAS2 location in G1, albeit much lower than the enrichment in FIG2. Indeed, the level of enrichment at FIG2 on the ChIP-seq exceeded the 6-fold level, and here it is only 4-fold higher than background enrichment, potentially reflecting a lower level of signal achieved by the manual ChIP. This could explain why no enrichment is seen at the FAS2 locus, where it was on the ChIP-seq analysis (Figure 4.6), perhaps due to lower levels of crosslinking used here.
Figure 5.2: Manual ChIP of suspected G\textsubscript{1} enrichment loci ChIP-qPCR of three gene loci pulling down Smc2-3HA in an α-factor mediated arrest, red bars, and a nocodazole-mediated arrest, blue bars across the promoter and ORF of three genes, FAS2, FIG2 and CDC15. The primer pairs were located through the gene, identically to Figure 5.1. Cultures were arrested in either G\textsubscript{1} or G\textsubscript{2}/M and crosslinked for 15 minutes with 1.42\% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR. Each sample was divided by a ‘no antibody’ value to give a fold increase which is normalised to an HMR qPCR signal and then plotted. This is showing the signal from a single experiment. The ChIP shows an increased binding of Smc2 at FIG2 specifically in G\textsubscript{1}, and very little enrichment at FAS2 or CDC15. Abbreviation: ORF - open reading frame

5.3 Further investigation of the FIG2 locus

Given we can recapitulate the binding of Smc2 to the FIG2 gene manually, is the localisation limited as specifically to the ORF as seen in the ChIP-seq? To test this, primers were designed for qPCR at 1, 4 and 8 kilobases distal from either end of the gene.

Samples were taken, one in an asynchronous culture and one arrested in G\textsubscript{1} by addition of α-factor. The transcription of FIG2 was checked, confirming a high level of expression only in the G\textsubscript{1} arrest (Figure 5.3A), and ChIP was performed using an anti-HA antibody to enrich the Smc2-3HA protein (Figure 5.3B). The same specificity is shown for the enrichment of Smc2 - enrichment within the gene and not outside the ORF. Additionally, the ChIP enrichment followed the same pattern as the RNA transcript level with a higher level of enrichment at the 5’ end of the gene, see Figure 5.3.

Due to the small numbers involved in these calculations, it is more accurate to subtract the ‘no antibody’ enrichment rather than to divide as the numbers are so small that
dividing will amplify any error within the numbers disproportionately. As such, the axes in Figure 5.3 represents the PCR signal remaining when the ‘no antibody’ sample is removed after both have been normalised to the input and represents a percentage increase over the ‘no antibody’ sample, and not a fold increase as before.

Also shown in Figure 5.3 are ChIPs carried out in the same conditions targeting other proteins. Figure 5.3C) shows ChIP using the same anti-HA antibody but in a strain where the tag is attached to one of the non-SMC subunits of condensin, Brn1, instead of Smc2, and D) shows a direct ChIP using an antibody which recognises all three subunits of the single strand DNA binding complex RPA (made up of Rfa1-3 subunits in *S. cerevisiae*).

All three were analysed with a mixture of Protein A and G agarose beads to ensure background binding effects were comparable. RPA was targeted as *S. pombe* condensin has been shown to compete with RPA for single stranded DNA in vitro in order to reanneal the DNA to a double stranded structure (opposed by RPA binding single stranded DNA (Akai *et al.*, 2011)) and it seemed possible that this competition could be occurring at a site of active, rapid transcription such as those detected by this ChIP-seq.

All three ChIP profiles show similar binding patterns - virtually no binding in an asynchronous culture and specific enrichment across the ORF when the gene is activated by addition of α-factor. In particular, enrichment is higher at the 5’ end of the gene and remains low outside of the ORF. One thing to note is the scale of each ChIP graph. The enrichment levels for the condensin subunits are very low, especially when compared to the RPA signal. This suggests that the RPA interaction at this site is likely to be far stronger than that of condensin. It also raised questions about the specificity of this signal.
Figure 5.3: Manual ChIP of expanded Fig2 locus. Blue bars show asynchronous samples in all four graphs and red bars show an α-factor mediated G₁ arrest. The primer pairs were located as shown in the cartoon in the bottom panel. Graph A) shows the transcription level of Fig2 in asynchronous and G₁ arrested samples. RNA was extracted, reverse transcribed to cDNA and this tested by qPCR, normalised to Tub1. The graph shows the fold increase in transcripts over Tub1 levels in each sample. Primers are located in the promoter and ORF of Fig2. Transcription is occurring in the G₁ arrested sample and not in an asynchronous culture. Graphs B) - D) show ChIP from the same asynchronous and G₁ arrested conditions, crosslinked for 15 minutes with 1.42% formaldehyde. ChIP was performed with the appropriate antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR. Each sample was normalised to the respective input signal and the ‘no antibody’ sample was subtracted. Further, each sample is then normalised to an HMR qPCR signal. Graphs show the relative signal retrieved compared to the input, where the signal from the IP carried out with no antibody is subtracted from the signal with an antibody used in the processing. All three show similar enrichment across the ORF, higher at the 5’ end, only when the gene is active in G₁.

5.4 Specificity of the enrichment

5.4.1 Testing the role of the antibody in G₁ peaks

A paper published by Teytelman et al. (2013) showed that ChIP as a technique can be susceptible to artificial enrichment signals when a gene is highly transcribed. The pattern of enrichment seen in this study at the highly transcribed genes in G₁ was not only linked to transcriptional activity but the profile of the enrichment maps well to the
transcriptional profile of the gene, i.e. higher levels at the 5’ end, and this is replicated exactly in all three ChIP profiles. As such, it seemed possible that these data could in fact be a result of this artificial effect, and not true enrichment of condensin.

Initially, this similarity in ChIP profile was taken to imply that the enrichment, although very small, was real and distinct from background noise. However, in light of this paper, the fact that all three profiles mapped so neatly to the level of transcription suddenly seemed highly suspect. Furthermore, to be able to see this enrichment for the condensin subunits had taken a large amount of optimisation, a large cell volume, and even then the level of the enrichment was very small.

In order to test if this enrichment was an artefact of ChIP at highly transcribed regions or a physiological binding site of condensin, a ChIP was performed with a strain where Smc2 was tagged with 9 Myc epitopes using both an anti-HA and an anti-Myc antibody. As such, each sample was processed to give an input sample, a ‘no antibody’ sample, an anti-RPA sample, an anti-HA sample and an anti-Myc sample. In theory, if the condensin enrichment was real, the anti-Myc sample would produce peaks over and above those in the anti-HA ChIP as this would be pulling down Smc2-9Myc and enriching for sequences bound by Smc2. Similarly, if the RPA binding was real, this level of enrichment would be higher than the target-less anti-HA antibody. If the level of enrichment seen is simply a reaction to the transcription at that site, then the anti-HA ChIP should give a similar level of enrichment, despite not targetting a specific protein.

As can be seen in Figure 5.4, analysing a ChIP of Smc2-9Myc using both anti-Myc and anti-HA antibodies gives almost identical enrichment profiles across the FIG2 locus. Once more, the enrichment is only present inside the transcribed ORF and only in the α-factor arrested sample. However, the targetless anti-HA ChIP indicates that this enrichment seems to correlate with increased transcription at that location and not be linked to the presence of condensin at this site.

Furthermore, both of these signals have been normalised by the ‘no antibody’ processed sample and so this enrichment is specifically due to the presence of an antibody, but not the presence of a target. It could be that active transcription leads to the localisation of so many proteins that there is an increase in targets for non-specific binding of the antibody and this helps to produce the false positive enrichment signal. Alternatively, it may be an alteration in the structure of the DNA region which increases its ‘ChIPability’,
but this must depend on the presence of an antibody, as it is an enrichment seen on top of the background ‘no antibody’ signal.

Significantly, the anti-RPA ChIP shows a far higher level of enrichment, well above the ‘targetless’ sample, approximately 20-fold higher. This suggests that this represents a real enrichment dependent on the specificity of the antibody, and therefore the target, whereas the Smc2 and Brn1 ChIPs do not.

**Figure 5.4:** Enrichment specificity by ChIP with multiple antibodies at *FIG2*. Graphs A) - C) show ChIP from the same asynchronous and G₁ arrested conditions, crosslinked for 15 minutes with 1.42% formaldehyde. ChIP was performed with the indicated antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR. Each sample was normalised to its corresponding input signal and the ‘no antibody’ sample was subtracted. Further, each sample is then normalised to an HMR qPCR signal. Graphs therefore show the percentage of input recovered in the IP sample with an antibody, minus that retrieved using no antibody in the preparation. The primer pairs were located as shown in the cartoon at the bottom of each graph, outside the gene (grey bar), in the promoter (green bar) and within the gene body (orange bar). Blue bars show asynchronous samples in all three graphs and red bars show an α-factor mediated G₁ arrest. A) shows an anti-Myc ChIP targeting Smc2-9Myc, B) shows an anti-HA ChIP which has no specific target and C) shows an anti-RPA ChIP. Note that the anti-HA and anti-Myc ChIP are identical and the non-specific signal seen in anti-HA ChIP is still G₁ specific. The RPA ChIP shows a much higher enrichment profile.

### 5.4.2 Validating the enrichment of condensin seen in mitosis

Given the signal of condensin enrichment across the *FIG2* locus could be replicated with an untagged strain, it seemed probably that this was a false positive peak. Alternatively,
it could be that the enrichment seen on the genome wide ChIP-seq was representative of condensin binding, but the conditions used in this manual protocol could not efficiently bring down the condensin subunits and so had not successfully replicated the enrichment (over the low level artificial enrichment seen due to active transcription at those loci).

To test this possibility, the same protocol was used to try to reproduce the well documented presence of condensin at the centromeres in mitosis (D’Ambrosio and Schmidt, 2008, Nerusheva et al., 2014, Verzijlbergen et al., 2014). As before, samples were blocked in G$_1$ by addition of α-factor and in mitosis by addition of nocodazole. These samples were then processed for ChIP analysis in the same way and this time qPCR was carried out targeting both the FIG2 region but also the centromere of Chromosome IV.

Figure 5.5 shows a ChIP analysis done using an anti-Myc antibody in two strains - one where Smc2 is tagged with 9 Myc epitopes and should be brought down during the ChIP, and one where there is no target for the Myc antibody. Samples were taken in an α-factor mediated arrest and a nocodazole-mediated arrest, crosslinked and processed for ChIP. Both were processed for an input sample, a ‘no antibody’ sample and an ‘anti-Myc’ sample. In the nocodazole-mediated arrest and only when Smc2 is tagged with 9 Myc epitopes, the qPCR at the centromere of Chromosome IV does indeed show significant enrichment. By contrast, the FIG2 gene shows only a tiny enrichment over the ‘targetless’ strain.

As such, the ChIP protocol used here is indeed able to detect enrichment of condensin at the centromere in a manner dependent on a tagged Smc2 protein and so the failure of the ChIP can be ruled out. It seems likely, therefore, that there is no real enrichment at the FIG2 gene but instead this signal is demonstrating an artificially increased ‘ChIP-ability’ of highly transcribed regions, at least partly dependent on the presence of an antibody but not of a target for that antibody.

The full ChIP-seq maps included in Appendix C show that there are small peaks occurring throughout the chromosomes, and these appeared particularly high in the G$_1$ arrested sample. It is important to bear in mind that ChIP is prone to these false positives and to use the most stringent controls possible, here found to be the use of an untagged strain rather than a sample processed in the absence of antibody.
Figure 5.5: Condensin enrichment verified manually at centromeres

Graphs A and B show an anti-Myc ChIP in a strain with a tagged Smc2-9Myc (green bars) and one which is untagged (purple bars) with samples taken from A) an α-factor arrested G₁ sample and B) a nocodazole arrested G₂/M sample. The samples were crosslinked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-Myc antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR. Each sample was normalised to its corresponding input signal and the ‘no antibody’ sample was subtracted. Further, each sample is then normalised to a HMR qPCR signal. Therefore the graph shows the percentage of input recovered in the IP with antibody over and above that retrieved when processed without antibody. The primer pairs were located along the FIG2 promoter and ORF, and around CenIV as shown in the cartoon at the bottom of each graph. Note that the difference between an Smc2-targeted ChIP and a targetless ChIP is significant at the centromere in mitosis, and minimal at FIG2 in an α-factor arrested sample.

5.5 Protocol validation

Given this experience, it seemed important to be sure that future ChIP results represented physiologically real enrichments. The elimination of background is always important, but similarly to a transcriptionally active region where the high number of proteins may provide a multitude of false binding targets for antibodies, the centromere is also bound by many proteins, including the kinetochore, and this is especially true during mitosis. As such, if the antibodies are pulling down anything in an unspecific manner, it seems this effect could be amplified in a region which is bound by so many proteins. Verification of the controls to remove unspecific background enrichment is therefore very important.

Therefore, instead of using a tagged strain and processing a ‘no antibody’ sample, experiments were carried out with two strains, a tagged and untagged strain and both were processed with the appropriate antibody. This appears to give a more stringent control of the background as this removes any additional binding that may be caused by the
presence of an antibody and leaves only that being caused by the tagged target protein. The ChIP signal for both is normalised to their respective input sample and then the signal from the untagged strain (not specific to the target protein) is subtracted from the tagged strain to leave behind the enrichment caused by the presence of the tag, and therefore the target protein.

It was also decided to no longer normalise the qPCR by a ‘negative control’, previously the *HMR* locus, as later ChIPs were trying to identify global changes in enrichment levels which could be lost if normalised this way (see Figure 5.6). To counter the removal of this control - which is intended to help normalise any variation between individual qPCR runs - the PCRs were run with all samples analysed simultaneously for each primer site. In this way, variation between plates would be between the primer sites instead of between samples, as the variable of interest was the change over time at each primer site. Finally, a 6-HA tag or 9-Myc tag were seen to be the most reliable in allowing specific enrichment to be separated from background and as such these were used for all future ChIPs.

Figure 5.6 shows a ChIP carried out using this system of controls and shows that a difference between the tagged and untagged strains is only seen at the centromere in mitosis and the rDNA in both *G_1* and *G_2/M* (higher in mitosis). The ‘enrichment’ seen at the *HMR* and a site which is on the arm of chromosome IV (100 kb away from the centromere) is seen in both the untagged and tagged strains and so is not specific to the presence of Smc2. It is important to note that this non-specific enrichment is at the level seen for the suspected *G_1* peaks. As such, subtracting the untagged strain from the tagged is able to eliminate non-specific enrichment. This protocol was used for all subsequent ChIPs.
Chapter 5. Technical pitfalls

Figure 5.6: Verification of controls for condensin ChIP Graph showing ChIP in both a tagged Smc2-6HA (Green bars) and untagged (Purple bars) strain from A) α-factor arrested G₁ sample and B) nocodazole arrested G₂/M sample. The samples were crosslinked for 15 minutes with 1.42% formaldehyde. ChIP was performed with an anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR. Each sample was normalised to its corresponding input sample only - not to a ‘no antibody’ or to HMR. As such, the graphs show the percentage of input signal retrieved by IP treatment for both tagged and untagged strains. The primer pairs were located at the centromere of Chr IV (CEN IV), 100 kilobases away from Cen IV, at the HMR locus and in the rDNA. Error bars represent the range from two biological replicates for the tagged strain. * p=0.0658, ** p=0.0261.. Note that the difference between an Smc2-targeted ChIP and a targetless ChIP is significant at the centromere in mitosis and the rDNA in both (albeit a larger enrichment in mitosis).

5.6 Summary

Attempts to replicate the peaks seen at active gene ORFs by ChIP-seq (Figure 4.6) were ultimately undermined by the inclusion of a no tag control (Figure 5.6). This is in agreement with published result showing that the inclusion of an antibody in the processing of the sample for IP produced additional background when compared to completing the IP with no antibody present, even without a target for that antibody (Teytelman et al., 2013).

For future ChIPs, a no tag control was always used. Furthermore, the normalisation to Tub1 was no longer carried out. The less normalisation being added to the data, the more realistic any changes seen are likely to be.
Chapter 6

Condensin binds globally to DNA during the transition and recruits Top2

Objectives

- To investigate the localisation of condensin during the supercoiling transition
- To investigate the factors involved in the regulation of condensin localisation
- To confirm the timescale of the transition
- To assess the role for topoisomerases in the transition

Experimental approach

The genome wide ChIP-seq showed that the localisation of condensin during mitosis alters in a manner dependent on the formation of spindles at the metaphase to anaphase transition. However, the samples used for ChIP-seq were taken over a long time scale - cells were allowed over an hour between the nocodazole- and Cdc20-mediated arrests to ensure all cells had reached the intended arrested state.

Topological studies regarding the transition have shown it to be a more rapid and transient event. Therefore, if the changes in localisation were linked to the transient changes in topology, it seemed possible that these samples could have missed important changes. As such, I decided to assess the localisation of condensin on a shorter time scale during the positive supercoiling transition by manual ChIP.
Furthermore, it seemed interesting to investigate the localisation of condensin during the progression through anaphase, i.e. during the physical separation of sister chromatids. To study this, in place of the cdc20-td mutant, a cell cycle block was introduced which used a temperature sensitive form of Cdc15, cdc15-2, which means that when the cells are moved to the non-permissive temperature (37°C) the Cdc15 protein becomes non-functional. Cdc15 is a kinase involved in the MEN pathway and as such the cells cannot progress through telophase and instead arrest with two separated nuclear masses inside one cell. Alternatively, no cell cycle block was used at all, simply releasing the cells from G₂/M by removing the nocodazole.

6.1 Timescale of transition

6.1.1 Condensin localisation at the centromere

As mentioned in the introduction, the positive supercoiling transition appears to be a transient event and when the cells are held in the Cdc20-mediated arrest, the positively supercoiled form of the dimer fades over time (Baxter et al., 2011). Furthermore, here and in the genome wide ChIP-seq, the ChIP is being carried out with wild type Top2 present which will mean even more transient topological changes are occurring. The samples which were taken for analysis by genome wide ChIP-seq were taken a significant amount of time after the removal of nocodazole. This was due to the reported lack of synchrony in a release from nocodazole (Cooper et al., 2006), and it seemed better to ensure all cells in the culture had reached a Cdc20-mediated arrest. However, allowing this much time to pass most likely meant that any activity linked to the triggering of the supercoiling transition may have been missed, although the positively supercoiled species do persist up to 60-80 minutes (in the absence of Top2).

As shown in Chapter 4, the genome wide ChIP-seq showed condensin binding to the centromeres in a nocodazole-mediated arrest, the level of this enrichment reduced in the Cdc20-mediated arrest, and returned to the same level when nocodazole was added back into the cell culture medium (Figure 4.4). However, in the process of manually validating this result, it became obvious that the timing with which samples were taken during the release from a nocodazole-mediated arrest was vital to the levels of condensin.
enrichment seen. As such, it seemed wise to take systematic samples during the release from a nocodazole-mediated arrest to build a clearer picture.

Figure 6.1 shows a ChIP carried out in a strain tagged at SMC2 with 9 Myc epitopes and where the CDC20 gene is under the control of a methionine promoter, the same strain as was used for ChIP-seq. The culture was blocked in α-factor, then released into media with methionine and nocodazole present. As such the cells arrested without Cdc20, but also without spindles in a pre-supercoiling transition state. A sample was taken in the nocodazole-mediated arrest, the drug washed out and then samples taken at short time intervals. The centromere shows an enrichment of condensin in the nocodazole-mediated arrest and this enrichment increases immediately upon washing out the drug and allowing the formation of spindles. This effect seems to be reduced even as close as the outer centromere, and condensin binding fades at all locations as the Cdc20-mediated arrest is maintained.

**Figure 6.1:** Enrichment of condensin at the centromere of Chromosome IV during formation of spindles. ChIP of Smc2-9Myc through release from nocodazole-mediated arrest. Cells (both tagged and untagged strains) were blocked in α-factor, released into media with methionine and nocodazole. A sample was taken once cells reached a nocodazole-mediated arrest, the drug washed out and then samples taken through the release to a Cdc20-mediated arrest. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-Myc antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR at CEN IV and a negative control at the HMR locus. Each sample was normalised to an input sample and an untagged strain, the mean and range of technical replicates is plotted, with the pattern verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05, ** represents a p value of less than 0.01, as calculated using the data from one biological replicate. FACS image shows the arrests at each time point where samples were taken. Note a high level of enrichment across the centromere in the nocodazole-mediated arrest which then increases immediately upon release, and reduces gradually, while HMR remains low throughout.
Given the changes in Smc2 localisation occur immediately upon release into media lacking nocodazole, does the topological change occur on the same time scale? To investigate this, the top2-td, cdc20-td, pRS316 strain used to assess the positive supercoiling transition was used to carry out the 2D monomer supercoiling assay with samples taken to match the timing of the manual ChIP samples. Figure 6.2 shows that the positively supercoiled species appear at the very first time point, coinciding with the initial peak in condensin enrichment at the centromere.

**Figure 6.2: Supercoiling transition occurs rapidly as spindles form**

A) 2D monomer supercoiling protocol was carried out in a top2-td cdc20-td pRS316 strain. Cells are synchronised in G1 and then released to pass through S phase with functional Top2, arresting due to the presence of nocodazole. The culture is then shifted to a non-permissive temperature to remove the function of Top2 and Cdc20 and then released from the nocodazole-mediated arrest and samples taken every 10 minutes. The DNA is extracted and run in agarose gel electrophoresis firstly in a low agarose concentration with 0.5 µg/ml chloroquine, and then in a higher percentage gel with 1 µg/ml chloroquine to separate the monomer form of the plasmid by its supercoiling state. B) shows a cartoon representation of the supercoiling topoisomers as separated by this process. C) The graph shows the intensity of the positively supercoiled species, with the level seen in nocodazole normalised to zero. Note the absence of positive supercoiling in the sample taken in the nocodazole arrested culture but that this topological change occurs immediately upon nocodazole removal. Abbreviations: NZ - nocodazole-mediated arrest.

The ‘Cdc20 sample’ analysed by genome wide ChIP-seq most closely matches the 70 minute sample in timing (taken at 75 minutes). Both samples show a reduction in Smc2 enrichment when compared to the sample taken in the nocodazole-mediated arrest. However, the ChIP-seq profile therefore missed the increase in enrichment which can be seen here happening as soon as the spindles are allowed to form. As such, it seems
that the centromere is bound by condensin as the cells go into mitosis, but upon spindle formation and tension generation, more condensin is recruited to the centromere before this reduces over time as the cells are held in a Cdc20-mediated arrest.

6.1.2 Condensin localisation away from the centromere

From these data, it is clear that the widely spaced samples taken for ChIP-seq missed some rapid changes in condensin localisation during the positive supercoiling transition. Could condensin levels be changing elsewhere on the chromosomes away from the centromeres, on a timescale not detected by my ChIP-seq experiment?

The positive supercoiling transition is a topological change happening across the whole genome. The mechanism of condensin’s involvement is unknown - it is possible that condensin bound at the centromere is capable of instigating a change in topology across the full length of chromosomes, or it may be that condensin localises away from the centromere to enact this change. Again, although the genome wide ChIP-seq in the Cdc20-mediated arrest sample did not detect any localisation of condensin away from the centromeres, the samples used for analysis may have missed what could be a transient change, as is seen here for the centromere enrichment during spindle formation. In the scenario that condensin is binding away from centromeres to cause the positive supercoiling transition, is it sliding away from the centromere or acting at random along the full length of the chromosome arms upon activation?

The manual ChIP was repeated in the same strains and conditions but the qPCR analysis was carried out with primers designed 10 kb, 50 kb and 100 kb away from the centromere along Chromosome IV as well as to the centromere and outer centromere as before. The primer locations were chosen at random, but all were placed away from transcribed regions. The results are shown in Figure 6.3.

Once again, Smc2 enrichment is seen at the centromere in a nocodazole-mediated arrest, and this is absent elsewhere along the chromosome arms. This signal at the centromere increases immediately nocodazole wash out and this is also confined to the centromere. However, as the cells are allowed to progress further into the Cdc20-mediated arrest, the binding at the centromere decreases and the binding of condensin along the arms
Figure 6.3: Localisation of condensin along Chromosome IV during formation of spindles ChIP of Smc2-9Myc through the positive supercoiling transition. Cells (both tagged and untagged strains) were blocked in α-factor, released into media with methionine and nocodazole. A sample was taken once cells reached a nocodazole-mediated arrest, the drug washed out and then samples taken through the release to a Cdc20-mediated arrest. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-Myc antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR at CEN4 and at sites spaced along the long arm of Chr IV, as shown in the cartoon. Each sample was normalised to an input sample and an untagged strain, pattern verified by biological replicates, n=2. Statistics could not be done with this data, as it represents a single repeat. FACS image shows the arrests at each time point. Graph A) shows this data plotted without further normalisation, B) shows the same data normalised by the nocodazole signal at each primer pair. Note the enrichment at the centromere in nocodazole increases immediately upon release as before, and then fades. Elsewhere on the chromosome arms, condensin enrichment increases at 40 minutes, showing peaks away from the centromere. Normalisation to the nocodazole signal shows the increase in condensin binding is similar at the centromere and along the arms.

Increases in the 20 min sample and becomes positive enrichment at 40 minutes. This increase in enrichment is also transient, reducing by 70 min.

If these same data were replotted by normalising to the signal in the nocodazole sample at that location, then the increase in Smc2 enrichment that occurs after the release appears to be of approximately the same magnitude, at the centromeres first and then later along the arms. It seems that the distance from the centromere is not affecting this enrichment as the condensin binding here appears simultaneously at the three sites away from the centromere.
6.1.3 Anaphase progression

These more detailed timecourses show Smc2 localising to the centromeres in a nocodazole-mediated arrest and that this localisation increases upon spindle formation, firstly at the centromeres and then subsequently along the arms, before reducing. These experiments are strictly limited in the timeframe that they are examining, i.e. the formation of spindles, tension generation and satisfaction of the SAC, and do not, as the natural timeline would, move rapidly through these tasks and progress through anaphase.

Even on this timeline the localisation of condensin is shortlived, so if the cells are allowed to progress through metaphase and anaphase, presumably as the supercoiling transition is resolved, it seems likely condensin localisation would also alter further. A strain was created with the endogenous promoter at the \( \text{CDC20} \) locus and instead a temperature sensitive form of Cdc15, \( \text{cdc15-2} \), was added. This means when the cells are moved to a non-permissive temperature, the Cdc15 protein becomes non-functional and the MEN pathway cannot be activated. Cells arrest with two separated nuclear masses in one cell. This strain was also tagged at \( \text{SMC2} \) with 6 HA epitopes and experiments carried out with a tagged and untagged strain. The cultures were blocked in nocodazole and then released at \( 37^\circ \text{C} \), meaning that the cells will form spindles, generate tension, inactivate the SAC, activate the APC, cleave cohesin and segregate the DNA. The localisation of Smc2-6HA through all of this was measured at 10 minute intervals.

Microscopy, shown in Figure 6.4B, demonstrates that 40 minutes is sufficient time for cells to reach a Cdc15-mediated arrest, having passed through spindle formation, tension and anaphase. As such, these timings were used for future experiments looking at protein localisation during the supercoiling transition.

Graph 6.4A shows the ChIP of Smc2-6HA during this progression through anaphase. The primers used here were localised to suggested cohesin binding sites, and those not linked to cohesin enrichment, along Chromosome IV. A paper by Renshaw \textit{et al.} (2010) showed a process of chromosome stretching and recoiling during anaphase which was dependent on remaining cohesin at cohesin binding sites and also on condensin. As such, it seemed plausible that condensin was being recruited to these remaining cohesin complexes and could even have been acting in concert with them to remove remaining catenations.
Chapter 6. Condensin and Top2

Figure 6.4: Localisation of condensin during progression through anaphase

ChIP of Smc2-6HA through the positive supercoiling transition. Cells (both tagged and untagged strains) were blocked by addition of nocodazole then released at 37°C to progress through anaphase to a Cdc15-mediated arrest, with samples taken every 10 min. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR at CEN4 and at cohesin and non-cohesin binding sites along the long arm of Chr IV, as shown in the cartoon. Each sample was normalised to an input sample and an untagged strain, pattern verified by two biological replicates. Statistics could not be done with this data, as it represents a single repeat. Graph B) shows the progression of the release, measured by the presence of two nuclei by microscopy. Note the enrichment at the centromere in nocodazole increases upon release as before. An enrichment in Smc2 binding is seen away from the centromere later in the release. Abbreviations: Coh - cohesin, NCoh - non-cohesin, NZ - nocodazole-mediated arrest.

The pattern of enrichment seen here is similar to that seen in cells progressing between nocodazole-mediated arrest and Cdc20-mediated arrest. Smc2 enrichment is seen at the centromere in nocodazole and increases immediately upon release from the arrest. Shortly after that, the level of Smc2 enrichment increases along the full length of Chromosome IV, away from the arms. By 40 minutes, condensin binds solely at the centromere once again, well before the 70 min sample examined in the Cdc20-mediated arrest.

Interestingly, the cohesin binding site which is closest to the centromere shows an increase in Smc2 binding in the first release sample, as the centromeric region does, but all other
sites show an enrichment later in the release (30 min). The association of a particular site with cohesin binding does not appear to affect the degree of Smc2 binding, nor the timing, and neither does distance from the centromere.

However, the extended release here (nocodazole-Cdc15) gives a more narrowly defined enrichment of Smc2 on chromosome arms, falling away in almost all samples by 40 minutes when compared to the nocodazole-Cdc20 release. By microscopy, most cells have reached a two-nuclei state by 40 minutes and so have finished their progression through anaphase. Perhaps, therefore, condensin localisation to chromosomes is limited to progression through anaphase and segregation of the DNA and by a Cdc15-mediated arrest, its enrichment to chromosomes is lost.

If this is true, is the topological change also lost by the Cdc15-mediated arrest? The monomer supercoiling protocol was carried out in a top2-td cdc15-2 pRS316 strain, seen in Figure 6.5, and indeed the positively supercoiled species grows strongest at 20 and 30 minutes and is almost gone by 50 minutes. This suggests that this localisation of Smc2 may well be linked to the topological change seen in the positive supercoiling transition, and both are resolved by a Cdc15-mediated arrest.

**Figure 6.5: Supercoiling transition is resolved as cells reach a Cdc15-mediated arrest.** A) 2D monomer supercoiling protocol was carried out in a top2-td cdc15-2 pRS316 strain. Cells pass through a synchronised S phase with functional Top2, are arrested by addition of nocodazole, shifted to a non-permissive temperature to remove the function of Top2 and Cdc15 and then released from the arrest and sampled every 10 minutes. The DNA is extracted and separated in a low agarose concentration with 0.5 µg/ml chloroquine, and then in a higher percentage gel with 1 µg/ml chloroquine to separate the monomer form of the plasmid by its supercoiling state. B) shows higher exposures of the positively supercoiled species and C) shows a quantification of the same portion of the gel. The supercoiling change occurs by 10 mins and has been mostly lost by 50 min, suggesting it is a state lost by the end of anaphase.
Finally, the experiment was repeated in strains which lack any mutations affecting the cell cycle, simply with an Smc2-6HA tag and processed as above, to be sure that this was not an artefact of these arrests. Here, binding was examined along Chromosome XII, so as to include the rDNA locus, and all primers were designed away from sites of known cohesin binding or transcription, see Figure 6.6. Here again, the pattern is replicated - Smc2 is enriched at the centromere and rDNA in the nocodazole-mediated arrest, and this binding increases rapidly upon release. Interesting to note that the rDNA matches the centromere in this pattern of enrichment. At 50 minutes, there is then an increase in Smc2 binding across the whole chromosome, including all sites along the arms. The timing is delayed compared to the previous experiment, likely due to a slower release from nocodazole. This demonstrates that the binding seen is not an artefact dependent on artificial cell cycle arrest but occurs during a wild type mitosis.

**Figure 6.6: Localisation of condensin during progression into the next cell cycle**

A) ChIP of Smc2-6HA through the positive supercoiling transition. Cells (both tagged and untagged strains) were blocked by addition of nocodazole then released at 30°C to progress through anaphase, with samples taken every 10 min. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR at CEN4 and along Chr XII, as shown in the cartoon. Each sample was normalised to an input sample and an untagged strain, mean and range of two technical repeats is plotted and the pattern verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05, ** represents a p value of less than 0.01, as calculated using the data from one biological replicate.  

B) shows the progression of anaphase as measured by microscopy. Condensin is further enriched at the centromere upon release and, later, condensin enrichment is seen along the arms. The rDNA locus shows a similar binding pattern of condensin as the centromere. 

Abbreviations: NZ - nocodazole-mediated arrest.
6.2 Preventing the supercoiling transition alters the binding pattern of condensin subunit Smc2

These ChIPs were intended to examine the localisation of Smc2 during the supercoiling transition known to depend on its functionality. The enrichment pattern of Smc2 demonstrates an increase in binding during the formation of the spindles, which correlates well with the timing of this transition. Furthermore, the global binding pattern identified may suggest a role for condensin which requires localisation along the full length of the DNA. However, condensin has well reported roles during mitosis in chromosome condensation and compaction which are crucial for accurate segregation, but unrelated to the transition.

To try to further solidify a connection between the topological change and this localisation pattern of Smc2, we used a mutant which has been shown to fail to go through the transition, the Polo-like kinase Cdc5 (see Section 3.1.3). Using strains which contain cdc5-1, a temperature sensitive allele of the kinase, one tagged with Smc2-6HA and one untagged, the same protocol was carried out by arresting the cells in G2/M with nocodazole and then switching the cultures to the non-permissive temperature (37 °C) before taking a sample and releasing the cells to go through anaphase in the absence of functional Cdc5.

Figure 6.7 shows that the loss of Cdc5 does not affect Smc2 binding to centromeres and the rDNA locus in the nocodazole-mediated arrest. However, there is no significant binding of Smc2 at the centromeres, or globally, as the spindles form. The loss of Cdc5 activity has prevented the enrichment of Smc2, both at centromeres and globally, during progression through anaphase. This is particularly striking as loss of Cdc5 prevents the supercoiling transition but not condensation of chromosomes, and here it does not alter centromeric condensin binding in the nocodazole-mediated arrest but it does abolish the enrichment pattern during the time of the supercoiling transition. This supports the idea that this global recruitment of condensin is linked to enacting the topological changes involved in the transition.
Figure 6.7: Localisation of condensin in the absence of functional Cdc5. ChIP of Smc2-6HA through the positive supercoiling transition in the absence of functional Cdc5 kinase shows altered localisation. A) shows cdc5-1 cells fail to produce the positively supercoiled CatC* species as they reach mitosis. B) shows ChIP analysis of Smc2-6HA as cells are released from a nocodazole-mediated arrest without functional Cdc5. Cells with the cdc5-1 allele (tagged and untagged) were blocked by addition of nocodazole then shifted to 37°C to disrupt Cdc5 function, sampled and released into the cell cycle, with samples taken every 10 min. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR along Chr XII with Cen4, as shown in the cartoon. Each sample was normalised to an input sample and an untagged strain, mean and range of technical replicates are plotted and the pattern was verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05, ** represents a p value of less than 0.01, as calculated using the data from one biological replicate. C) shows progression through the cell cycle as monitored by microscopy. Enrichment of Smc2 is seen at the centromere and rDNA in the nocodazole arrested sample, but no subsequent global increase in enrichment is seen during the release.

6.3 Timescale of Smc2 loading

This rapidly changing localisation pattern of condensin during mitosis is contrary to the original view that it was, more or less, a structural protein. However, evidence that condensin is capable of enzymatic interactions with DNA is accumulating and this would correlate well with the idea that it is central to the positive supercoiling transition (Baxter et al., 2011, Bazett-Jones et al., 2002, Hirano et al., 1997, Losada and Hirano, 2001). But does this mean that the binding of condensin is highly changeable
throughout the cell cycle, or is this specific to the period around the transition? At what point is it loaded onto the DNA and is this more stable than the binding during mitosis?

To investigate the localisation of condensin over the length of the chromosome during progression through a normal cell cycle, samples were taken every 30 minutes as cells were released from a synchronised G\textsubscript{1} to a nocodazole-mediated arrest. This was done with an untagged strain and a strain tagged at SMC2 with 6 HA epitopes, and qPCR analysis carried out along Chromosome XII including the rDNA.

Figure 6.8 shows these data plotted at the different loci as the cells progress through the cell cycle. In G\textsubscript{1}, there is a high level of Smc2 enrichment at the rDNA locus which drops as the cells go into S phase (at 60-90 min, confirmed by FACS). There is little binding elsewhere on the chromosome, including the centromere, during this time but as the cells exit S phase, Smc2 enrichment increases once more on the rDNA and also at the centromere and pericentromere.

![Figure 6.8: Localisation of condensin during the cell cycle](image)

**Figure 6.8:** Localisation of condensin during the cell cycle ChIP of Smc2-6HA through the cell cycle released from an α-factor arrest. Cells (both tagged and untagged) were blocked by addition of α-factor then released to a nocodazole-mediated block with samples taken every 30 minutes. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR. Each sample was normalised to an input sample and an untagged strain. qPCR was carried out at the Cen IV and at sites spread along chromosome XII including the rDNA. FACS image shows the progress through the cycle where each ChIP sample was taken. The graph is plotted at the different chromosome loci over time, including the rDNA (orange) and centromere (green). Note the condensin localisation is high in α-factor only at the rDNA, even this drops as the cells go through S phase and then peaks in the rDNA and the centromere as the cells move towards a nocodazole block.
This study did not detect any global changes similar to those seen during the release of cells from a nocodazole-mediated arrest. It is interesting to note that there appears to be a removal of Smc2 from the DNA, including even the rDNA, as the cells pass through S phase and this could be examined further. In particular, this could be simply an artefact from the ChIPping of DNA going through S phase, care must be taken to verify the physiological relevance of the apparent effect. Additionally, the rapid binding changes were not seen until examined on a 10 minute timescale during the topological transition, and with these samples taken 30 minutes apart it is possible that we are missing interesting changes. It could be interesting to examine this further but it seems likely that the global binding of Smc2 seen during mitosis is unique.

6.4 Kinetics of Smc2 binding during the positive supercoiling transition

The protocol for ChIP requires the crosslinking of proteins and DNA which are in close proximity by the use of formaldehyde. This is a balancing act between preserving true interactions and unwittingly crosslinking proteins which are not interacting in vivo but get caught by over-crosslinking. The crosslinking reaction takes approximately 5 seconds, and in almost all ChIPs produced here the cells were crosslinked for 15 minutes (the ChIP-seq used 30 minutes of crosslinking). This should allow true interactions to be maintained and detected via ChIP without too much unphysiological crosslinking, while using the untagged strain as a control should allow the removal of this background enrichment.

A technique called crosslinking kinetic analysis (CLK) was used by Viswanathan et al. (2014) which utilises very short crosslinking times to study the binding dynamics of a specific protein to DNA. Given that a link can be formed in only 5 seconds in the presence of formaldehyde, a protein which is firmly bound to DNA would be crosslinked immediately upon addition of formaldehyde to the culture. If the crosslinking reaction is allowed to continue then it will catch other protein molecules which bind to DNA in that time. The longer the formaldehyde is present, the more proteins will become crosslinked. This is true of different proteins, but also the number of molecules of a given protein which is crosslinked to a given binding site, and this also depends strongly on
the concentration of that protein. If a given protein has rapid on-off binding dynamics then a small increase in crosslinking time will capture more molecules of that protein and produce a larger increase in signal than a protein which is recruited slowly meaning no extra protein is bound during the additional crosslinking time as no new molecules are recruited to the binding site, see Figure 6.9.

By crosslinking all samples for 15 minutes, this information is lost. The level of signal seen in these conditions could represent a protein where 30 molecules bind in the first minute of crosslinking or over the course of the full 15 minutes. The single data point
retrieved by traditional ChIP would be indistinguishable between the two scenarios. However, these different binding dynamics can provide information about the nature of the interaction between protein and DNA.

During the progression from metaphase to anaphase, the condensin complex alters its localisation pattern on DNA, suggesting a change in its activation state. The concentration of the protein remains constant through the cell cycle, as shown previously and indeed by subsequent IP ((Freeman et al., 2000) and Figure 6.12). Previous ChIPs using a 15 minutes crosslinking period have shown changes in condensin enrichment but cannot detect any changes in recruitment - it may interact with DNA in a different manner and this change could be detected by CLK analysis (Viswanathan et al., 2014).

Figure 6.9 shows samples taken before and after the formation of spindles being crosslinked for very short periods of time. This was normalised by 30 minutes crosslinking to concentrate on the rate of change in signal and not the ultimate degree of Smc2 binding. The initial part of the curve reflects the binding dynamics and the steeper curve of the 'post-release' sample suggests more rapid recruitment of Smc2-6HA to the centromere when the positive supercoiling transition is induced. This may suggest a more active form of the condensin complex, and a different recruitment or binding mechanism.

Figure 6.10: Binding dynamics of Smc2-6HA as detected by CLK. ChIP of Smc2-6HA pre- and post-transition with varied crosslinking times. Cells (both tagged and untagged) were blocked by addition of nocodazole, samples taken and then released and a further set of samples taken. The samples were cross-linked for between 5 seconds and 30 minutes with 1.42% formaldehyde and quenched with glycine added to 280 mM. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR at Cen 4. Each sample was normalised to an input sample and an untagged strain, and to the signal generated by 30 minutes crosslinking, mean and the range of two biological replicated is plotted. The Smc2 enrichment increases more rapidly in the samples taken after formation of spindles.
6.5 Localisation of non-SMC condensin subunit Brn1 during the positive supercoiling transition

The condensin complex is made up of the heterodimer Smc2-Smc4 and three non-SMC subunits Ycs4, Ycg1 and the kleisin, Brn1 (see Section 1.2.1 (Freeman et al., 2000)). Given these ChIPs have been carried out using a tagged Smc2, it was interesting to assess whether the same pattern was seen for another subunit, in this case the kleisin Brn1. This was tagged and analysed by ChIP in the same way as Smc2. Unlike cohesin, there is little evidence of the condensin complex being cleaved or dismantled during the cell cycle and so it was expected that the ChIP analysis would be similar for the two subunits.

However, the binding pattern of Brn1, shown in Figure 6.11, does not mirror that of Smc2. The ChIP analysis shows binding of Brn1 to the chromosomes at the centromere and at the rDNA locus, as previously reported (D’Ambrosio and Schmidt, 2008), but there is no enrichment as anaphase progresses. The amount of enrichment varies but not in a definable pattern before it is lost, presumably as the cells begin to exit mitosis.

![Figure 6.11: Localisation of Brn1 during progression through anaphase](image)

**Figure 6.11:** Localisation of Brn1 during progression through anaphase ChIP of Brn1-6HA through the positive supercoiling transition. Cells (tagged and untagged) were blocked by nocodazole addition then sampled and released, sampling every 10 min. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR along Chr XII with Cen IV, as shown in the cartoon. Each sample was normalised to an input sample and an untagged strain, mean and range of technical repeats is plotted, with the pattern verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05, ** represents a p value of less than 0.01, as calculated from one biological replicate. Enrichment is seen at the centromere and the rDNA but not globally during the release through anaphase.
In Section 3.2, a strain carrying a temperature sensitive form of Brn1, brn1-60, was tested for its ability to carry out the positive supercoiling transition. Again here, it was assumed that the dependency on Smc2 indicated a requirement for the entire condensin complex. However, this was not the case either and the brn1-60 strain completed the shift to positive supercoiling almost entirely as wild type strains, see Figure 3.8.

The combined fact that Brn1 is not required for the topological transition to occur and that the localisation pattern is also different during this stage of the cell cycle suggest that the full condensin complex is not involved in the supercoiling transition. The different localisation pattern - shared in nocodazole, different upon release into anaphase - could reflect a different composition of the complex, perhaps lacking the Brn1 subunit. Alternatively, it could reflect a different mechanism of binding to the DNA, i.e. the interaction of the complex with DNA post-release is predominantly via the Smc2/4 dimer and so the Brn1-DNA interaction is too weak or distant to be detected by ChIP.

If the composition of the complex does change here then this should be detectable by Co-IP. A Co-IP as in Section 3.2 was used to look at the complex pre- and post-transition. Protein G beads associated with anti-Myc antibody were used to pull down Brn1-9Myc and then Western blots were used to look for the quantity of Smc2 which interacts with Brn1 to show if the complex remained intact, and also to look at the Brn1 subunit which was brought down by the IP to see if there was any detectable difference post-transition.

Figure 6.12 shows that the strength of the Smc2 signal that is interacting with Brn1-9Myc is the same in the pre- and post- transition samples (top right panels, red arrow), as is the form and quantity of Brn1 brought down by the IP (left hand panels, green arrows). This suggests that no change in the composition of the condensin complex or modification of Brn1 occurs during spindle formation.

As such, perhaps the noted differences in Brn1 ChIP enrichment and Smc2 enrichment is not due to a break down of the complex but an alteration in the manner in which condensin interacts with DNA in its transition-activating role, and this renders Brn1-DNA interactions harder to detect. The fact that a brn1-60 strain can still carry out the transition may demonstrate no active role for the Brn1 subunit in the enzymatic process of the topological change. However, the fact that the Brn1-60 protein remains part of the complex perhaps allows Smc2/4 to carry out their supercoiling role. In
Figure 6.12: Co-IP of Brn1-9Myc and Smc2-6HA during the formation of spindles

Co-IP pulling down Brn1-9Myc and looking at both Brn1 and Smc2 by Western blot. Cells with no tags, Brn1-9Myc or both Brn1-9Myc and Smc2-6HA tagged were blocked in nocodazole, a sample taken and then released into anaphase and another sample taken. The proteins were extracted and incubated with Protein G beads coupled with an anti-Myc antibody. The input and the IP samples were then run on a Western blot to detect both Brn1 (by anti-Myc antibody) and Smc2 (by anti-HA antibody). Left hand panels show the anti-Myc Western blot and the green arrows highlight Brn1; Right hand panels show the anti-HA Western blot and the red arrows highlight Smc2. Top panels show a low exposure and lower panels show a long exposure. There is no difference in the quantity or form of Brn1 or Smc2 before and after formation of spindles.

vitro experiments which showed the ability of condensin to induce positive supercoiling required only the Smc2-Smc4 dimer to do so (Stray et al., 2005). As such, the evidence here suggests that the complex remains intact but Brn1 does not play an active role in the topological transition. It will be important to check the localisation patterns of the other condensin subunits, and the integrity of the complex, to check which of those can be seen to localise globally.

6.6 Localisation of Top2 during the supercoiling transition

6.6.1 Localisation of Top2 during spindle formation

Topoisomerase 2 is a crucial actor in altering the supercoiling and catenation state of DNA through its ability to break both of the sugar-phosphate backbones of a DNA double helix and pass another through the gap (see Section 1.3.4, (Liu et al., 1980)). The topological studies of the positive supercoiling transition have made use of strains lacking Top2 in order to be able to visualise the supercoiling change. The fact that the
removal of Top2 affects supercoiling means it must be involved in the process in vivo. Additionally, if the physiological role of the transition is linked to catenane resolution, then Top2 must also be involved in this process too.

It therefore seemed interesting to check if it could be seen to localise with condensin during this time in the cell cycle. A strain was tagged at TOP2 with 6 HA epitopes in a cdc15-2 background. This strain was blocked by addition of nocodazole and then released at 37°C to a Cdc15-mediated arrest. Previous results have suggested that Top2 localises to chromosomes in mitosis and specifically to the centromere in metaphase (Baldwin et al., 2009).

Figure 6.13 shows Top2 localising to the centromere and rDNA at low levels in a nocodazole-mediated arrest and then an increase in enrichment along the arms of Chr XII as the cells arrest in anaphase, with timing matching the enrichment of Smc2 as seen by ChIP. This supports the idea that Smc2’s action may recruit Top2 to act upon DNA.

**Figure 6.13:** Localisation of Top2 during the release from nocodazole to a Cdc15-mediated arrest ChIP of Top2-6HA through release from nocodazole. Cells (both tagged and untagged) were blocked by addition of nocodazole then sampled and released into the cell cycle, with samples taken every 10 min. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR along Chr XII with Cen4. Each sample was normalised to an input sample and an untagged strain, mean and range of technical repeats is plotted, with the pattern verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05, ** represents a p value of less than 0.01, as calculated from one biological replicate. B) shows the progression through anaphase as monitored by microscopy. Note Top2 localises predominantly to the rDNA in the nocodazole-mediated arrest and then binds along Chr XII as cells arrest in anaphase with two statistically significant increases.
6.6.2 Dependency of Top2 localisation on Smc2

The enrichment of Top2 across the arms of Chromosome XII with similar timing to Smc2 during the formation of spindles lends further weight to the hypothesis of Top2 involvement in the topological change, most likely in resolving both the topological strain and the catenations exposed in the process.

However, the segregation of DNA that is also occurring at this point in the cell cycle could be responsible for this recruitment of Top2, rather than being dependent on the positive supercoiling transition. If it is an active recruitment due to the action of Smc2, then it should depend on the presence of functional Smc2.

To look at this, strains, tagged at TOP2 and untagged, were transformed with a temperate sensitive allele of Smc2, smc2-8. These strains were arrested in nocodazole, moved to 37°C to eliminate Smc2 function and then released to progress through anaphase. Samples were taken every 10 minutes and processed for ChIP analysis, shown in Figure 6.14. The analysis of Top2 binding in wild type circumstances (Figure 6.13) was also carried out at 37°C so the two ChIPs are comparable.

There is a higher level of Top2 enrichment across the chromosome in the nocodazole-mediated arrest, which may be due to the uncompacted form chromosomes would take in the absence of condensin function (Section 1.2.1). This level then drops away in two statistically significant drops, one on release from the nocodazole-mediated arrest and one when the cells exit anaphase (as monitored by microscopy, see Figure 6.14).

Therefore the global enrichment of Top2 during spindle formation is largely dependent on the function of Smc2. Without functional Smc2, Top2 is bound during early mitosis but as spindles form on release from the nocodazole-mediated arrest Top2 is mostly released from chromosomes. Subsequently, as the cells exit anaphase and enter the next cell cycle there is another drop in Top2 bound to DNA.

This experiment also suggests that perhaps in the absence of functional Smc2, more Top2 is recruited to the rDNA, potentially due to a higher number of substrates due to loss of compaction of this DNA. The loss of the global enrichment as the cells progress through anaphase adds weight to the idea that the action of Smc2 during this time may be able to recruit Top2 to the DNA for catenane resolution.
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Figure 6.14: Localisation of Top2 during the release from nocodazole without Smc2. ChIP of Top2-6HA through release from nocodazole without functional Smc2. Cells (both tagged and untagged) were blocked by addition of nocodazole, moved to 37°C to inactivate Smc2 and then sampled and released into the cell cycle, with samples taken every 10 min. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR along Chr XII with Cen4. Each sample was normalised to an input sample and an untagged strain, mean and range of technical repeats is plotted, with the pattern verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05. B) shows the progression through anaphase as monitored by microscopy. Top2 localisation is higher in the nocodazole arrested sample but then drops away in two statistically significant steps, one immediately upon release and one as the cells exit anaphase.

6.6.3 Dependency of Smc2 localisation on Top2

Therefore, if Top2 is recruited via the action of Smc2 in order to deal with the topological changes or aid in the resolution of catenations, would the localisation of Smc2 alter if Top2 could not be recruited? To test this, the localisation of Smc2 during release from a nocodazole-mediated arrest was assessed in the absence of functional Top2. Strains, tagged and untagged, with a temperature-sensitive allele of Top2, top2-4, were arrested by addition of nocodazole, shifted to 37°C and released in the absence of functional Top2.

The loss of Top2 appears to alter the localisation of Smc2 during this release from nocodazole. The pattern of initial increase at the centromere and subsequent enrichment along the arms is lost. Instead Smc2 binds everywhere immediately upon release and this remains until the cells exit anaphase (as seen by microscopy Figure 6.15B). Perhaps, when it is not possible for Smc2 to successfully recruit a functioning Top2 enzyme by its actions, the recruitment of Smc2 to the chromosomes persists.
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6.7 Localisation of Smc2 during the release from nocodazole without functional Top2

Figure 6.15: Localisation of Smc2 during the release from nocodazole without functional Top2. Cells (both tagged and untagged) were arrested by addition of nocodazole and then released at 37°C and sampled every 10 minutes. The samples were cross-linked for 15 minutes with 1.42% formaldehyde. ChIP was performed with anti-HA antibody and a mix of Protein A and G agarose beads, and purified DNA was used for qPCR along Chr XII with Cen4. Each sample was normalised to an input sample and an untagged strain, mean and range of technical repeats is plotted, with the pattern verified by two biological replicates. * indicates the change is significant with a p value of less than 0.05, ** represents a p value of less than 0.01, as calculated from one biological replicate. B) shows the progression through anaphase as monitored by microscopy. Note that the Smc2 enrichment increases globally upon release from nocodazole-mediated arrest and persists for longer than seen with functional Top2, then drops as cells exit anaphase.

As such, it seems that in the absence of functional Top2, Smc2 enrichment levels are higher and that this binding persists for longer. This correlates well with the fact that the positively supercoiled species are only detectable by gel electrophoresis without functional Top2 (Section 3.1.1). If indeed in the absence of Top2, Smc2 continues to overwind the DNA to a greater degree, this would imply that Smc2 is aware of the action of Top2 having occurred or not, via a mechanism which would require further investigation.

6.7 Localisation of Top1 during the positive supercoiling transition

The positively supercoiled DNA seen during the transition by gel electrophoresis in the absence of Top2 means that Top1 and Top3 must be involved. For the change in supercoiling to be visualised by gel electrophoresis, the DNA linking number must be altered (an action carried out by topoisomerases) as all proteins are lost during the
extraction (Figures 3.3 and 3.5). If Top1 is involved, is it possible to also see Top1 being recruited by the topological shift?

The localisation pattern seen in Figure 6.16 does not closely resemble that of Top2 or Smc2. Instead, Top1 is enriched in the nocodazole-mediated arrest and the first couple of time points of the release before fading as the cells exit mitosis. This enrichment is greater in the rDNA where the requirement of topoisomerases for accurate segregation has been previously reported (Baldwin et al., 2009, Holm et al., 1989, Nitiss, 2009).

As such, in a wild type situation it seems unlikely that Top1 is being recruited via the same mechanisms responsible for the recruitment of Smc2 and Top2. It may be that Top1 is involved in the topological changes required for the accurate segregation of DNA and indeed in the switch to positive supercoiling. However, the pattern of enrichment is different from that seen for Top2, perhaps due to the different roles these topoisomerases are playing - topological changes versus catenane resolution. It could be that the recruitment of Top1 when the function of Top2 is compromised would be very different, or that Top3 is also involved.
6.8 Summary

Previous work has shown the localisation of condensin to the centromeres during mitosis (D’Ambrosio and Schmidt, 2008, Wang et al., 2005) and there has been suggestion of its loss from centromeres with a diffuse relocalisation during anaphase (Nerusheva et al., 2014, Verzijlbergen et al., 2014).

ChIPs carried out here, with the use of an untagged strain to control artefacts, show a localisation of condensin to centromeres in a nocodazole-mediated arrest. As the drug is washed out, this level of condensin enrichment increases and then drops away at the centromere (Figure 6.1) - as would be expected from work showing tension causes the loss of Sgo1 from centromeres, and this allows the dissociation of condensin (Nerusheva et al., 2014, Verzijlbergen et al., 2014).

Subsequent analysis along the arms of Chr IV and XII showed enrichment as cells formed spindles which was limited to the timescale of sister chromatid segregation (Figure 6.3, 6.4 and 6.6). Furthermore, I have shown a localisation of Top2 with similar timing as the cells progress through anaphase (Figure 6.13) and that this is dependent on functional Smc2 (Figure 6.14).

The recruitment of Smc2 appears to be well coordinated with the previously reported positive supercoiling transition (Baxter et al., 2011) as the timing of spindles formation, switch to positive supercoiling (Figure 6.2), and enrichment along the chromosome arms matches well (Figure 6.3). Further support comes from the loss of the kinase Cdc5, which does not transition to positive supercoiling, and does not show the same enrichment pattern of Smc2 binding (Figure 6.7).

Finally, ChIP of Brn1 shows a different localisation pattern than that of Smc2 (Figure 6.11) despite the complex remaining intact (Figure 6.12) and this will require further work to clarify why the two components of the complex are apparently differently localised.
Chapter 7

Discussion

With each cell cycle, the entire genome must be faithfully replicated and segregated to the daughter cells in order to maintain genome stability. The process of replication pairs sister chromatids by DNA-mediated linkages which require the breaking of DNA backbones to resolve the link, and protein-mediated linkages by the cohesin complex which must be cleaved to release the DNA. DNA-mediated links can be added via sister chromatid intertwines which result from the process of replication or recombination intermediates which occur as part of various DNA damage repair pathways. These links are thought to benefit the cell as they maintain the identity of copied DNA as sister chromatids. However, at the moment of DNA segregation it is essential that no DNA linkages persist, as cleavage of cohesin allows the spindle pulling forces to dominate and tangled DNA could be stretched and broken as the sisters are pulled apart by the spindles and separated by cytokinesis.

Topoisomerase 2 is able to safely resolve these catenations in *S. cerevisiae* in vivo (Liu *et al.*, 1980). However, Top2 is also capable of introducing de novo catenations when two DNA strands are in close enough proximity (unpublished data, Aragón lab), presumably by acting ‘blindly’ on overlying DNA. All catenations must be removed prior to DNA segregation in anaphase, requiring the actions of Top2 to be exclusively decatenating.

Previous work (Baxter *et al.*, 2011) showed a positive supercoiling change occurs as mitotic spindles form and that this biases the action of Topo II towards decatenation (Figure 3.5 and 3.6). This change can only be visualised in the absence of functional Top2, most likely because the speed of action of Top2 resolves these topological changes to rapidly to be extracted, and that this change is brief and localised in full length chromosomes.
Previous examination of this transition showed it depended on mitotic spindles, Scc1, Smc2 and Cdc5 (Figure 3.7). From this, a model was built suggesting that cohesin is required to hold sister chromatids together as the spindles form and bind to kinetochores, creating tension across the centromeres (Michaelis et al., 1997). This activates Cdc5 which in turn, directly or indirectly, activates the supercoiling activity of the condensin complex (Alexandru et al., 2001, Bazett-Jones et al., 2002, Kimura and Hirano, 1997, St-Pierre et al., 2009, Stray et al., 2005). The aim of this work was to clarify the regulation of the supercoiling transition and investigate the role of condensin in enacting this transition.

7.1 Regulation of the positive supercoiling transition

The positive supercoiling transition occurs between the block caused by the addition of nocodazole and the absence of Cdc20 protein, distinguished by spindle formation and SAC satisfaction. As part of this study, mutants lacking various genes were created in order to assess which of these multiple processes is involved in the positive supercoiling transition also known to be activated between the two arrests.

7.2 Tension generation or checkpoint inactivation?

To distinguish whether it was the attachment of spindles or the generation of tension through those spindles which triggered the positive supercoiling transition, a mutant lacking three motor proteins was used (Figure 3.17). This failed to transition when allowed to progress to a Cdc20-mediated arrest which demonstrates that it is the generation of physical tension across kinetochores which is essential for activating the transition and not simply attachment between the spindle and the kinetochore.

Cells do not progress through the cell cycle until all sister chromatid pairs are attached to spindles in tension-generating combinations, and this is sensed through the Spindle Assembly Checkpoint. If tension is required to trigger the supercoiling transition, could elimination of this checkpoint bypass this requirement? Deletion of the MAD2 gene eliminates the cell’s ability to trigger the SAC, but this loss does not allow a premature activation of the supercoiling change in a nocodazole-mediated arrest (Figure 3.13). A
change in the form of the DNA is seen in response to the loss of this checkpoint, but further characterisation showed that this was an alteration in the form of the catenanes (Figure 3.15) and that the supercoiling transition was not activated as no change is seen in the supercoiling of the DNA (Figure 3.16).

These two mutants together suggest that the initiation of the positive supercoiling transition is linked to the physical generation of tension, separate from checkpoint activity.

7.3 Condensin’s involvement

Cells lacking functional Smc2, a subunit of the condensin complex, are unable to activate the positive supercoiling transition (Figure 3.7). This suggests a central role for the condensin complex in the supercoiling change, a finding which fits well with its identified ability to induce positive supercoiling into DNA (Bazett-Jones et al., 2002, Kimura and Hirano, 1997). Additionally, the transition to positive supercoiling is an actively maintained, reversible state which also supports the theory of an enzymatically maintained topological change, briefly introduced to aid in catenation resolution (Figure 4.5).

There is currently little evidence that the condensin complex is dismantled or degraded in the process of the cell cycle and so it was expected that the requirement for Smc2 would extend to Brn1, a non-SMC subunit of the complex. However, the inactivation of the non-SMC Brn1 subunit had little effect on the supercoiling transition (Figure 3.8). However, the temperature-sensitive allele used does not dissociate from the complex and is not degraded, so it may be that this allele still allows the complex to complete some functions and its lethality may not be through a first cell cycle arrest (Figure 3.9).

To complicate this question, whilst Smc2 appears to have a dynamic localisation pattern during the transition, Brn1 does not (Figures 6.3 and 6.11). This may indicate that Brn1 is in fact disassociating from the complex at this time in the cell cycle but a Co-IP strongly suggests that no change in the complex’s composition occurs (Figure 6.12). Together these results suggest Brn1 may play a limited role in the enzymatic process of supercoiling the DNA, but perhaps plays a regulatory role.
7.4 Phosphorylation of condensin

Condensin is multiply phosphorylated by Cdc28, CK2, Ipl1 and Cdc5 and these phosphorylations regulate condensin’s localisation and activity. CK2 phosphorylation is thought to inhibit the complex (shown in HeLa cells), and Cdc5 hyperactivates its supercoiling ability (Nakazawa et al., 2011, Ono and Fang, 2004, Piazza et al., 2013, St-Pierre et al., 2009, Takemoto et al., 2006). Eliminating the activity of Cdc5 is enough to prevent the transition, but overexpression could not initiate a premature transition in a nocodazole arrested culture (Figures 3.7 and 3.10). Previous work has shown that overexpression of phosphomimic forms of the condensin non-SMC subunits could not force the transition and expressing a phospho-null allele of brn1 could not prevent it (data not shown).

Ipl1 is crucial to sensing spindle tension and is known to phosphorylate condensin subunits (Liu and Lampson, 2009, Peplowska et al., 2014, Vas et al., 2007). Eliminating the function of Ipl1 significantly reduces the efficiency of the positive supercoiling transition but does not prevent it. This may be through the loss of its phosphorylation of Brn1, or due to an increase in incorrect spindle attachments in this mutant (Figure 3.11).

It is possible that the overexpressed Cdc5 was not fully activated, as it requires phosphorylation itself by Cdc28 (Mortensen et al., 2005) for full activity, or that Cdc5 phosphorylation must happen together with phosphorylation by Cdc28 to activate condensin (reported by St-Pierre et al. (2009)). The Cdc28-dependent phosphorylations are required prior to the addition of Cdc5-dependent phosphorylations, and so are also likely to be a key step in the activation of the transition.

Alternatively, a combination of these phosphorylations and re-localisation of the complex may be required for the supercoiling transition. Future work could look into the phosphorylation states of the condensin subunits in a pre- and post-transition state.

Therefore, the formation and attachment of spindles, and the generation of force through them, activates the supercoiling transition in a distinct pathway from the Spindle Assembly Checkpoint, potentially involving Ipl1 phosphorylation. This is communicated to condensin, either directly or indirectly, and most likely via phosphorylation by Cdc5 or Ipl1. Condensin can then induce a positive supercoiling transition, as it has been demonstrated to do in vitro.
Figure 7.1: Regulation of the positive supercoiling transition The positive supercoiling transition has been shown to be activated as the spindles bind to the kinetochores in tension-generating pairs - one from either pole. This physical tension is sensed by the cells in a pathway separate from the Spindle Assembly Checkpoint, and most likely mediated via Cdc5 (yellow box) and Ipl1 (blue box) kinases to activate condensin’s ability to supercoil DNA. Condensin (Orangy/yellow cartoon) then acts to induce a transient change in the supercoiling of the DNA in such a way that attracts the decatenating action of Top2 (green cartoon).

7.5 Condensin localisation

7.5.1 Shugoshin is not controlling recruitment for the supercoiling transition

The shugoshin protein, Sgo1, has been shown to be crucial for the recruitment of condensin and Ipl1 to centromeres (Peplowska et al., 2014, Verzijlbergen et al., 2014). However, elimination of shugoshin does not prevent the positive supercoiling transition from occurring (Figure 3.18). This actually matches well with the reported loss of condensin from centromeres in response to tension (Nerusheva et al., 2014, Peplowska et al., 2014, Verzijlbergen et al., 2014). It would be interesting to try eliminating Sgo1 in a motor protein mutant - perhaps without the maintenance of condensin at centromeres, the transition could be activated without spindle tension.

7.5.2 Smc2 binds globally during the activation of the transition

To further investigate the role of condensin in the transition, ChIP was used to assess its localisation during this period of the cell cycle. By ChIP-seq, it was verified that condensin is bound at centromeres in metaphase, and that spindle tension causes the
loss of this enrichment (Figure 4.4, D’Ambrosio and Schmidt (2008), Nerusheva et al. (2014), Peplowska et al. (2014), Wang et al. (2005)).

By manual ChIP, this was again verified, and the binding of Brn1 to centromeres was also seen (Figures 6.1 and 6.11, D’Ambrosio and Schmidt (2008), Wang et al. (2005). Using narrower sampling timescale however, allowed the observation of an immediate increase in Smc2 enrichment at centromeres as nocodazole is removed, and then an enrichment across chromosome arms as the cells progress towards anaphase (Figure 6.6). This localisation is partly maintained in a Cdc20-mediated arrest but more transient when the cells are released to a Cdc15-mediated arrest or with no further cell cycle block (Figure 6.3 vs 6.4 vs 6.6). The binding is significant enough to be detected by 10 minute time points but it is nevertheless localised to a very specific moment in the cell cycle during the metaphase to anaphase transition.

The various primers used in the manual ChIPs do not detect any subdivision in the binding of condensin along chromosome arms - not by distance from the centromere and cohesin binding sites do not seem to have a higher or more prolonged interaction with condensin (Figure 6.3, 6.4 and 6.6). This may reflect the true situation and condensin is binding globally, or it may be due to a lack of sophistication in ChIP paired with qPCR and averaged over a population. It would be interesting to look into this, using genome wide ChIP-seq or using samples taken closer together at the moment of enrichment to see if a difference could be detected. From these data shown here, it can be concluded that the condensin recruitment to chromosome arms is a non-specific global phenomenon.

7.5.3 Localisation pattern of Brn1

Interestingly, the same pattern was not observed for the Brn1 subunit, this appears to bind centromeres and rDNA but does not relocalise during anaphase progression (Figure 6.11), despite no evidence of complex separation (Figure 6.12). It is hard to explain these conflicting results. It may be that the manner in which the condensin complex is interacting with the DNA when instigating the supercoiling change is different from the binding to the centromere and is a process which provides detectable interaction with the Smc2 subunit but not Brn1. Alternatively, there may be changes in the complex which are not detected by the methods used here, or the protocol for ChIP that was used must be optimised differently for the Brn1 subunit. This should be investigated further,
perhaps using longer crosslinking times as a first step to try to detect Brn1 along the chromosome arms.

### 7.5.4 Kinetics of Smc2 recruitment

A CLK analysis was also employed (crosslinking kinetics) which makes use of short crosslinking times to investigate whether there is a change in the binding kinetics of Smc2 as spindles form and the cells initiate the topological change (Figure 6.9, Viswanathan et al. (2014)). A change can be seen at the centromere between the nocodazole- and Cdc20-mediated arrests as the rate at which the ChIP signal increases is more rapid once the spindles have formed - suggesting a more active recruitment of Smc2 to the DNA (Figure 6.10). This fits well with the data shown here suggesting condensin is activated by spindles, altering the manner in which the complex interacts with DNA. Given the differences we see between the ChIP profile of Smc2 and Brn1, it could be interesting to look at Brn1 using the same technique - perhaps its binding activity would not be altered in the same way.

### 7.6 Connecting the localisation to the topological change

A cdc5-1 strain is unable to generate positive supercoiling in a Cdc20-mediated arrest, potentially due to loss of condensin phosphorylation by Cdc5, shown to activate its supercoiling activity (Figure 3.7, St-Pierre et al. (2009)). Strong evidence linking the topological changes to this localisation of condensin is therefore provided by the loss of this global Smc2 localisation pattern when cells are released from nocodazole in the absence of functional Cdc5 (making use of the same temperature sensitive allele, cdc5-1 (Figure 6.7)).

The current model for this transition suggests its purpose is to recruit Top2 to resolve catenations. Top2 is also likely to be involved in the supercoiling changes as the positively supercoiled species can only be visualised when Top2 is inactivated (Figure 3.5). A link between the supercoiling transition and the action of Top2 would be strengthened if its interaction with DNA could be visualised. A ChIP of Top2-6HA in a nocodazole-mediated arrest showed low level enrichment along the arms of Chromosome XII with higher levels at the centromere and the rDNA (higher in the rDNA). As the cells are
released from the nocodazole block, a higher degree of Top2 binding is seen at 20 and 30 minutes and then a significant peak is seen at 50 minutes after removal of the drug (Figure 6.13), coinciding with Smc2 enrichment in an open release (Figure 6.6).

To investigate the dependence of Top2 enrichment on condensin supercoiling, an smc2-8 top2-6HA strain was used and showed that this anaphase-specific enrichment of Top2 was lost in the absence of Smc2 function (Figure 6.14). However, Smc2 binding still occurred in a top2-4 smc2-6HA strain (Figure 6.15), suggesting Smc2 binds first and its action or presence recruits Top2.

The increased Top2 enrichment in the nocodazole-mediated arrest could reflect the loss of chromosome condensation (smc2-8 mutant), which would be particularly apparent in the rDNA, and could provide more substrate for the action of Top2.

Topologically, the positive supercoiling transition is stronger and longer-lived when the function of Top2 is compromised and the extended binding of Smc2 matches this finding well (Figure 6.15). Perhaps, when Top2 cannot be recruited, Smc2 continues to bind globally and further overwind the DNA in order to compact it and to promote Top2’s decatenation activity. How Smc2 is detecting the action of Top2 requires further investigation.

Finally, for the topological change to happen in a Top2 mutant, Top1 or Top3 must be involved. As an initial foray into this subject, Top1 localisation was analysed by ChIP in the same manner as Smc2 and Top2. However, the pattern is distinct from that of Top2 and Smc2 (Figure 6.16) as Top1 is bound to DNA in the initial time points, particularly at the rDNA, and then lost as the cells exit anaphase. This demonstrates that Top1 is actively interacting with DNA during anaphase but suggests a different mechanism of recruitment. It may be interesting to look at the localisation of Top1 in a top2-4 mutant where perhaps more of the topological challenges of the supercoiling transition would have to be dealt with by Top1.

7.7 Mechanism of the transition

The long linear chromosomes of S. cerevisiae and other organisms are made up of topological domains defined by protein structures (Worcel and Burgi, 1972) which alter their
supercoiling state independently. Additionally, in cells with functional Top2 this change in supercoiling is transient, presumably due to the efficiency of Top2 in resolving the topological changes. The transition is therefore likely to be occurring in individual topological domains rather than globally, and transient rather than sustained.

The dependence of the transition on Smc2 and the pattern of Smc2 enrichment during this time may reflect the direct induction of the supercoiling transition by condensin’s global binding to DNA. Furthermore, the simultaneous binding across chromosome arms may demonstrate that condensin, once activated, binds and acts at random across the whole genome (Figure 7.2). This also hints that the regulation of the supercoiling transition is at least partly dependent on the release of condensin from the centromeres and its ability to localise along the arms, perhaps controlled via Cdc5 as the enrichment pattern is lost in the absence of functional Cdc5.

Condensin then appears to recruit Top2 to the DNA, as a significant enrichment is seen with similar timing to Smc2, and this is abolished if Smc2 function is compromised. It also seems that if the function of Top2 is lost then the localisation of Smc2 is extended. This suggests that Smc2 is able to detect failed recruitment of Top2, or the absence of its activity, and condensin continues to induce positive supercoiling.

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**Figure 7.2: Localisation of condensin and Top2 during the positive supercoiling transition** The positive supercoiling transition has been shown to happen as the spindles bind to the kinetochores in tension-generating pairs (green dashed lines). In these conditions, condensin (orange cartoon) is enriched at the centromere, dependent on Sgo1 (purple trapeziums), before being released as tension is generated by motor proteins (red rectangles) via phosphorylation by Cdc5 (yellow square) and possibly Ipl1 (blue square). Condensin then appears to bind randomly along the full length of chromosomes, overwind the DNA and recruit the action of Top2 (green cartoon) to resolve catenations.
7.8 An additional Mad2-regulated catenation resolution strategy?

The elimination of MAD2 leaves cells unable to activate the Spindle Assembly Checkpoint and this appears to result in a shift in the catenation distribution towards resolution in a culture delayed in G$_2$/M by addition of nocodazole (Figure 3.15). This is not regulated via a change in supercoiling and does not require Top2 (Figure 3.16). It may be that this is operating through Top3 which has recently been shown to have an in vitro decatenation activity as part of the Sgs1-Top3-Rmi1 complex in conjunction with RPA (Cejka et al., 2012). As examined here, this change occurred in the absence of Mad2 and the presence of Cdc20 which suggests activation through the APC.

It would be interesting to investigate if this altered catenane distribution could be seen in cells allowed to progress to a Cdc15-mediated arrest, in the presence of functional Mad2 but still without functional Top2, as this is a more ‘wild type’ situation than eliminating a checkpoint.

7.9 ChIP controls

An essential optimisation step for ChIP is having the correct controls in place to eliminate non-specific enrichment. The enrichment detected is affected by many things (including the quantity of DNA, the crosslinking process and the extraction) and originally an ‘input’ sample and a ‘no antibody’ sample were used to try to eliminate differences between samples due to factors other than specific protein binding.

The ChIP-seq detected very strong enrichment over the open reading frame of certain genes in the G$_1$ arrest (Figure 4.6). Manual ChIP against Brn1, Smc2 and the RPA complex seemed to confirm the existence of this enrichment (Figure 5.3) but this low level enrichment could be replicated simply by the presence of an antibody, with no need for a target protein (Figure 5.4). If this background was eliminated then it negated the Smc2 and Brn1 peaks, but the RPA remained significant.
Susceptibility to antibody-dependent background seems particularly high at highly transcribed regions. This may be due to crosslinking chain reactions and non-specific antibody targets in these protein-rich areas, also likely to be true at the centromere.

It was concluded that the most stringent control was to process strains with and without an epitope tag on the target protein, adding antibody to the IP of both samples (Figure 5.6). This means any difference which is seen can be attributed to the presence of the target protein and its tag.

The full ChIP-seq maps shown in Appendix C show a significant number of seemingly random peaks along the chromosome arms in all four samples. Many of these are likely to be linked to this artificial enrichment seen at actively transcribed regions, emphasising the importance of using untagged strains as a control.

7.10 Condensin binding at active genes

Previous ChIPs of condensin had suggested a binding to all tRNA sites (D’Ambrosio and Schmidt, 2008) and that this may play a role in their clustering in the nucleolus and elimination of RNA pol II transcription in that region (Haeusler et al., 2008, Thompson et al., 2003, Wang and Strunnikov, 2008). Further links to transcription, both recruitment to regions of active transcription and recruitment in order to repress transcription have also been reported (Clemente-Blanco et al., 2009, Freeman et al., 2000, Iwasaki et al., 2010, Johzuka et al., 2006, Tanaka et al., 2012, Wang et al., 2006). Therefore, it is likely that condensin recruitment is linked to the regulation of transcription - perhaps negatively interacting with Pol II and aiding in the activation of Pol III and Pol I, but the picture is still murky.

In the ChIP-seq carried out for this thesis, these G1 peaks appeared significant but showed very low enrichment when replicated manually which was further undermined when using an untagged strain as a control (something not done for the ChIP-seq) (Figures 4.6 and 5.4). As mentioned above, several papers have suggested a genuine enrichment of condensin at actively transcribed genes and loss of Ycs4, a non-SMC subunit of condensin, is defective in silencing the mating loci (Bhalla et al., 2002), a phenotype which has also been observed in smc2-8 cells (unpublished results, Aragón lab).
The low level of enrichment seen here could therefore be indicative of real condensin binding but it is not high enough above background to be considered significant. This may be due to a lower level of formaldehyde crosslinking, or the cell cycle stage in which this effect was looked for. A more complex role for condensin in the organisation of chromosomes throughout the cell cycle seems likely and will require further work.

7.11 Concluding remarks

This thesis aimed to investigate the mechanism underlying a change in the supercoiling occurring as \textit{S. cerevisiae} cells progress from metaphase to anaphase. By using a combination of topological investigations and ChIP localisation studies, a model has been built which places Smc2 of the condensin complex at the centre of the topological change and strongly supports the idea that this is a global interaction involving direct global binding of condensin to DNA. Although it has previously been suggested that condensin plays an extended role in mitosis, with specific anaphase roles (Cuylen \textit{et al.}, 2013, Lavoie \textit{et al.}, 2004, Machín \textit{et al.}, 2004, Renshaw \textit{et al.}, 2010, St-Pierre \textit{et al.}, 2009) the regulation and importance of this have remained unclear.

These data show specific recruitment of Top2 by Smc2 (Figures 6.13 and 6.14). Condensin has been shown to be necessary for the recruitment of TopoIIα to mitotic chromosomes in chicken DT40 cell lines (Samejima \textit{et al.}, 2012) and to be needed for the decatenation of minichromosomes in yeast (Charbin \textit{et al.}, 2013). If condensin is able to promote overwinding and compaction of the DNA to recruit Top2, and Top2 is able to respond to this activity, both by relaxing condensin-dependent overwinding and removing exposed catenations (Baxter and Aragón, 2012) then this suggests an elegant model for the restructuring of chromosomes at the metaphase to anaphase transition.

Recent work with mammalian mitotic chromosomes in cells lacking either condensin or TopoIIα function showed opposing activities for the two proteins - problems caused by condensin loss were partially rescued if TopoIIα was also mutated (Samejima \textit{et al.}, 2012). Furthermore, the stabilisation of condensin binding seen in the absence of Top2, and the increase in supercoiling that this creates, also support an antagonistic model (Figures 6.14 and 3.5).
The CLK analysis of Smc2 binding at CEN 4 pre- and post-spindles suggests a two-fold increase in the binding rate of condensin upon spindle formation (Figure 6.10) and this matches well with a highly dynamic FRAP pattern seen for condensin I in mammalian cells (Gerlich et al., 2006), and suggests the mechanism behind the sudden increase in its activity - resulting in the overwinding being visible by gel electrophoresis (Figure 6.2).

Further work may help to solidify the links between the stages of this process - identifying the phosphorylation sites of condensin which control its activation, looking for physical interaction between Smc2 and Top2, and clarifying whether the entire condensin complex is involved. However, this study has highlighted a more dynamic role for condensin in the restructuring of chromosomes as cells begin to segregate their DNA and has shown global condensin binding, recruitment of Top2 and a cooperation between the two proteins is key in genome stability.
Appendix A

List of strains

These tables contain the strains used in this study. Those used for ChIP were created in the background As499 specifically for this study. The cdc5-1 and cdc15-2 backgrounds were created previously in the lab and adapted for use here. Those used in the topological experiments were created for Baxter et al. (2011) in the W303-1 background and further modified as required.

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<th>Key mutations</th>
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**Figure A.1:** List of *S. cerevisiae* strains used in this study.
### Appendix A. List of strains

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<tr>
<td>CCG10429</td>
<td>Mata; leu2-3,112; ura3-52; his3-D200; trp1-D63; ade2-1; lys2-801; bar1; pep4::His3; smc2-9myc (hyg)</td>
<td>smc2-9myc</td>
<td>As499</td>
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<tr>
<td>CCG10490</td>
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<td>pMET-cdc20, smc2-9myc</td>
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<td>CCG11726</td>
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<td>pMET-cdc20, smc2-6HA</td>
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</tr>
<tr>
<td>CCG11139</td>
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<td>top2-4</td>
<td>As499</td>
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<td>CCG12260</td>
<td>Mata; leu2-3,112; ura3-52; his3-D200; trp1-D63; ade2-1; lys2-801; bar1; pep4::His3; top2-4 (ade2); smc2-6HA (hyg)</td>
<td>top2-4, smc2-6HA</td>
<td>As499</td>
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<td>CCG907</td>
<td>Mata; bar1D::leu2-3,112; ura3-52; his3-D200; trp1-D63; ade2-1; lys2-801; pep4; brn1-9myc (trp)</td>
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<td>CCG12361</td>
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<td>brn1-60-9myc</td>
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<td>CCG12259</td>
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<td>smc2-6HA, brn1-60-9myc</td>
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<td>CCG12263</td>
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<td>brn1-9myc, smc2-6HA</td>
<td>As499</td>
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List of *S. cerevisiae* strains used in this study.
<table>
<thead>
<tr>
<th>Strain number</th>
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<th>Key mutations</th>
<th>Background</th>
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<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (His3); leu2-3::CM244 (CMVp-tetR’-SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-TA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker</td>
<td>top2-td</td>
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<td>top2-td, pRS316</td>
<td>W303-1a</td>
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<td>CCG9121</td>
<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (His3); leu2-3::CM244 (CMVp-tetR’-SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-TA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; cdc15-2; pRS316 (ura3)</td>
<td>top2-td, cdc15-2, pRS316</td>
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<tr>
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<td>top2-td, cdc20-td, pRS316</td>
<td>W303-1a</td>
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<tr>
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<tr>
<td>CCG9723</td>
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<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (His3); leu2-3::CM244 (CMVp-tetR’-SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-TA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; cdc20-td CDC20 5' upstream -100 to -1 replaced with KanMX-TA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; ipl1-231 (ura); pRS414 (trp)</td>
<td>top2-td, cdc20-td, pRS414, ipl1-231</td>
<td>W303-1a</td>
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## Appendix A. List of strains

<table>
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<tr>
<th>Strain number</th>
<th>Genotype</th>
<th>Key mutations</th>
<th>Background</th>
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<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL10-Ubiqitin-M-LacI fragment-myc-UBR1 (His3); leu2-3::pCM244 (CMVp-tetR'::SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; mad2Δ:: NAT; pRS316 (URA3)</td>
<td>top2td, cdc20-td, pRS316, mad2Δ</td>
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<td>CCG10013</td>
<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL10-Ubiqitin-M-LacI fragment-myc-UBR1 (His3); leu2-3::pCM244 (CMVp-tetR'::SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; cdc20-td CDC20 5' upstream -100 to -1 replaced with KanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; dyn1Δ:: NAT; kip1Δ::Hsg; cyn8-3 (trp); pRS316 (URA3)</td>
<td>top2td, cdc20-td, pRS316, dyn1Δ, kip1Δ, cyn8-3</td>
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<tr>
<td>CCG10327</td>
<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL10-Ubiqitin-M-LacI fragment-myc-UBR1 (His3); leu2-3::pCM244 (CMVp-tetR'::SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; cdc20-td CDC20 5' upstream -100 to -1 replaced with KanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; nap1Δ:: NAT; pRS316 (URA3)</td>
<td>top2td, cdc20-td, pRS316, nap1Δ</td>
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<td>CCG10388</td>
<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL10-Ubiqitin-M-LacI fragment-myc-UBR1 (His3); leu2-3::pCM244 (CMVp-tetR'::SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; pGAL-nap1 (ade); pRS316 (URA3)</td>
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<td>W303-1a</td>
</tr>
<tr>
<td>CCG11724</td>
<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL10-Ubiqitin-M-LacI fragment-myc-UBR1 (His3); leu2-3::pCM244 (CMVp-tetR'::SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; cdc20-td CDC20 5' upstream -100 to -1 replaced with KanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; sgo1Δ::HYG; pRS316 (URA3)</td>
<td>top2td, cdc20-td, pRS316, sgo1Δ</td>
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<tr>
<td>CCG11932</td>
<td>W303-1a; Mat a; ade2-1; his3-11; leu2-3; trp1-1; ura3-1; can1-100; UBR1::GAL10-Ubiqitin-M-LacI fragment-myc-UBR1 (His3); leu2-3::pCM244 (CMVp-tetR'::SSN6 LEU2) x3; top2td TOP2 5' upstream -100 to -1 replaced with kanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; cdc20-td CDC20 5' upstream -100 to -1 replaced with KanMX-tTA (tetR-VP16)-tetO2 - Ub - DHFRts - myc - linker; ipl1-321 (ura); sgo1Δ:: NAT; pRS314</td>
<td>top2td, cdc20-td, pRS314, ipl1-3231, sgo1Δ</td>
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<tr>
<td>CCG12364</td>
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<td>top2td, pRS316, mad2Δ</td>
<td>W303-1a</td>
</tr>
</tbody>
</table>

List of *S. cerevisiae* strains used in this study.
Appendix B

List of qPCR primers

This appendix contains the sequences and melt curves for all primers used in this study for qPCR. Primers were designed with a melting temperature as close to 59.4°C as possible and in pairs which would amplify a region of 150 bp (or as close as possible) to try to minimise differences in the qPCR signal caused by primer design. Primers which gave unusual melt curves - signifying non-specific binding or dimer formation - were discarded. Where a number is given (e.g. Fig2 +400), this signifies the number of bases between the primer and the start of the gene (i.e. 400 bp downstream of Fig2). For the primers described by a single number (e.g 0.02), this is the coordinate of Chromosome XII where it amplifies.

<table>
<thead>
<tr>
<th>Collection Number</th>
<th>Target</th>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
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<tbody>
<tr>
<td>2676 2677</td>
<td>rDNA-5-1</td>
<td>GGAAGCGGAAAATACGGAAAC</td>
<td>TCTGAAGCGTGATTCCGTCAC</td>
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<tr>
<td>3372 3373</td>
<td>Fas2 -983</td>
<td>TGGATTCACAATCAAGGAGG</td>
<td>TGCTTTGAGAAGATCTGCTG</td>
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<td>3374 3375</td>
<td>Fas2 +139</td>
<td>ATCGGTCTCCTCACAATTTG</td>
<td>CTTGAGCATCCTCGGAATAGC</td>
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<tr>
<td>3376 3377</td>
<td>Fas2 +2164</td>
<td>ACGCCAAATATGGTGCTAAGG</td>
<td>TCCAATCTAAACCAACATTC</td>
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<td>3378 3379</td>
<td>Fas2 +4736</td>
<td>TCTTGACTGCTCATCCAAAGG</td>
<td>TGCTCAAAGATCTATCCACG</td>
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<tr>
<td>3380 3381</td>
<td>Fig2 -796</td>
<td>GAGCAACTCAGAGAAAAAGC</td>
<td>CCAGTCATCGTGACAAATTC</td>
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<tr>
<td>3382 3383</td>
<td>Fig2 +179</td>
<td>CCTCTTATCGTATGTCAGC</td>
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<tr>
<td>3384 3385</td>
<td>Fig2 +1987</td>
<td>TCACTTTAGAGGCTCTACG</td>
<td>GTCACACTAGAAGCTACATG</td>
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<tr>
<td>3386 3387</td>
<td>Fig2 +4125</td>
<td>ACAACCAACTACTCCATCCAGC</td>
<td>TAAAGCAGATGGAGAGTAC</td>
</tr>
<tr>
<td>3388 3389</td>
<td>Tub1 +145</td>
<td>GTCAAGCTGGTGTGAGTTG</td>
<td>AAAAGTGGAGAAACCTCTTC</td>
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<td>3396 3397</td>
<td>HMR +798</td>
<td>TCAAGAGTGTCGGTAATTCG</td>
<td>ATACCAAGGGGCTAGAGAATC</td>
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<td>3416 3417</td>
<td>HPT1 +51</td>
<td>ATGTCAGATCTCGGAGAG</td>
<td>AAATCTGATGGTGGCACGC</td>
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</table>

Figure B.1: List of qPCR primers used in this study.
### List of qPCR primers

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<thead>
<tr>
<th>Collection Number</th>
<th>Target</th>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
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<tbody>
<tr>
<td>3428, 3429</td>
<td>Cdc15</td>
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<td>CGGTAATATTTCAGCAGCCC</td>
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<tr>
<td>3430, 3431</td>
<td>Cdc15</td>
<td>AATTATGAGTGTCTGTCGCG</td>
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<td>Cdc15</td>
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<td>3434, 3435</td>
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<td>GGTCTGTACGAAAAGTCTCC</td>
<td>GCTTCAACTCGCATAGAATTGC</td>
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<td>CEN4</td>
<td>GCGCAAAGCTTGGCAAAAGGTCCATG</td>
<td>GAATCTTATTTTGCCGCTCCTAGGCTA</td>
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<tr>
<td>3492, 3493</td>
<td>Act1</td>
<td>GGTATTGTTTTGGATTCCGTTGA</td>
<td>TTGCAATTTCTTGTGCAAGTCCA</td>
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<tr>
<td>3500, 3501</td>
<td>Fig2</td>
<td>AACGCCCTAGATAGTGACC</td>
<td>CGGCGCAATTTTTTCCATCAC</td>
</tr>
<tr>
<td>3502, 3503</td>
<td>Fig2</td>
<td>AACAAAGACAATCCACAAGTCG</td>
<td>TCAGGTTGCTTTTGTATCCAGC</td>
</tr>
<tr>
<td>3504, 3505</td>
<td>Fig2</td>
<td>CTCCTCCTCAAGAAATCAG</td>
<td>ATCCGCTTCAATGTCATACC</td>
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<tr>
<td>3506, 3507</td>
<td>Fig2</td>
<td>AATGAAGCCATAAAGCCTCCG</td>
<td>TTGTTAGGCTTACCAGATCAG</td>
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<td>Fig2</td>
<td>AAACAGGGAAGAAAGGCTTGG</td>
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<td>3510, 3511</td>
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<td>3512, 3513</td>
<td>CEN4 1</td>
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<td>CCGCAACTACATTCTGGGGAATTTG</td>
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<tr>
<td>3514, 3515</td>
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<td>TCAGTAGTGCCCTTTAAGTCAG</td>
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<tr>
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<tr>
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<td>3522, 3523</td>
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<td>3779, 3780</td>
<td>Cen4</td>
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<td>3781, 3882</td>
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<td>Coh-1</td>
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<td>Coh-2</td>
<td>TCACATAGCTGGTCTCAG</td>
<td>TTGTACTGGAAAACCTCAGAGC</td>
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</tbody>
</table>

List of qPCR primers used in this study.
Appendix B. **List of qPCR primers**

<table>
<thead>
<tr>
<th>Collection Number</th>
<th>Target</th>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
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<tbody>
<tr>
<td>3890  3891</td>
<td>Ncoh-1 (Chr IV)</td>
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<td>CAATACCTCATTGCACCTGGC</td>
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<td>Ncoh-2 (Chr IV)</td>
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<td>3894  3895</td>
<td>Ncoh-3 (Chr IV)</td>
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<td>TGACATGTCACTGGCCACCTTC</td>
</tr>
<tr>
<td>3896  3897</td>
<td>Coh-3 (Chr IV)</td>
<td>CCCCCCCCCAGTAGCATAATAC</td>
<td>CGAAAATTTTTCCTCCATCCC</td>
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<tr>
<td>3898  3899</td>
<td>Ncoh-4 (Chr IV)</td>
<td>CTTTGGAATGCAAGAAGACCCG</td>
<td>CAAGTTGGGGCTTGTGAATGG</td>
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<tr>
<td>3963  3964</td>
<td>0.02 (Chr XII)</td>
<td>AGTTGCTAACGAAAGGATGG</td>
<td>TTGGTACAAGGCCTTGTCCC</td>
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<tr>
<td>3967  3968</td>
<td>0.22 (Chr XII)</td>
<td>GAAAGAAATGATTCTGCAGC</td>
<td>TACTGATGGAATCGTTGTCCG</td>
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<tr>
<td>3971  3972</td>
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<td>GTCGGGAGTTCCATTCTATCT</td>
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<tr>
<td>3975  3976</td>
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<td>TGAAAAATGGCGCTCAGAAAAG</td>
</tr>
<tr>
<td>3979  3980</td>
<td>0.51 (Chr XII)</td>
<td>GCTCAGTTAAGAAACTTGACCG</td>
<td>TTTCAGGGAAGCCAGAAACAC</td>
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<tr>
<td>3981  3982</td>
<td>0.58 (Chr XII)</td>
<td>TTTGCCTCCCATATCTCGTG</td>
<td>ATTACTACCCTCAACAGGGG</td>
</tr>
</tbody>
</table>

List of qPCR primers used in this study.
Appendix B. List of qPCR primers

Figure B.2: Melt curves for qPCR primers used in this study.
Representative melt curves from at least two experiments for primers used in this study.

Melt curves for qPCR primers used in this study.
Appendix C

ChIP-seq maps

This appendix contains the full ChIP-seq maps for the ChIP carried out in the Shirahige Lab at the University of Tokyo. Briefly, samples were taken at the four time points and cells were crosslinked for 30 minutes with formaldehyde. Protein G magnetic beads crosslinked to anti-Myc antibody (9E11 from abcam) were used to enrich the target protein and this was normalised to an ‘input’ sample which is not processed as the IP.

The statistics representing the efficiency of the ChIP-seq are shown in Figure C.1. ChIP-seq maps are plotted as a fold-enrichment over the input sample and are mapped as individual chromosomes (Figures C.2 - C.5). The first sample was arrested in G\textsubscript{1} (Figure C.2), the second was taken when cells reached a nocodazole-mediated arrest (Figure C.3), the third was released from nocodazole and allowed to progress to an arrest due to loss of Cdc20 (Figure C.4) and the final sample represents readdition of nocodazole to Cdc20-arrested cells (Figure C.5). See Chapters 3 and 4 for more details.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of reads</th>
<th>Unique reads (Percentage of genome)</th>
<th>Unmapped reads (Percentage of genome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChIP - Smc2-9myc G1</td>
<td>7506990</td>
<td>5793585 (77.18%)</td>
<td>1713405 (22.82%)</td>
</tr>
<tr>
<td>ChIP - Smc2-9myc Nocodazole</td>
<td>6884668</td>
<td>5321853 (77.30%)</td>
<td>1562815 (22.70%)</td>
</tr>
<tr>
<td>ChIP - Smc2-9myc Cdc20</td>
<td>7237486</td>
<td>5010437 (69.23%)</td>
<td>2227049 (30.77%)</td>
</tr>
<tr>
<td>ChIP - Smc2-9myc Readdition of Nocodazole</td>
<td>7189607</td>
<td>5223503 (72.65%)</td>
<td>1966104 (27.35%)</td>
</tr>
<tr>
<td>Input - Smc2-9myc G1</td>
<td>6332371</td>
<td>5288141 (83.51%)</td>
<td>1044230 (16.49%)</td>
</tr>
<tr>
<td>Input - Smc2-9myc Nocodazole</td>
<td>7571361</td>
<td>6271990 (82.84%)</td>
<td>1299371 (17.16%)</td>
</tr>
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Figure C.1: Efficiency of Smc2-9myc ChIP-seq. Statistics showing the number of reads from each ChIP sample (both the antibody enriched sample and the input sample). The number of reads which were then mapped to a unique region of the genome, and those which could not be mapped are also shown.
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G1
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G\textsubscript{1}
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G1
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G1
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁

chrVII_2

genes

Smc2_G1

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁

genes

Smc2_G1
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁

chrIX

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁

chrX_1
genesis

Figures C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G\textsubscript{1}
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁

chrXIII_2

genes

Smc2_G1

0.0 3.0 6.0
0.600M 0.610M 0.620M 0.630M 0.640M 0.650M 0.660M 0.670M 0.680M 0.690M

genes

Smc2_G1

0.0 3.0 6.0
0.700M 0.710M 0.720M 0.730M 0.740M 0.750M 0.760M 0.770M 0.780M 0.790M

genes

Smc2_G1

0.0 3.0 6.0
0.800M 0.810M 0.820M 0.830M 0.840M 0.850M 0.860M 0.870M 0.880M 0.890M

genes

Smc2_G1

0.0 3.0 6.0
0.900M 0.910M 0.920M

Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G1

chrXV_1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1
Appendix C. ChIP-seq maps

Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁

chrXVI_1

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes

Smc2_G1

genes
Figure C.2: Smc2-9myc ChIP-seq map of cells arrested in G₁.

chrXVI_2

genes

Data not shown.
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole

[Diagram of Smc2-9myc ChIP-seq map]
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Appendix C. ChIP-seq maps

Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole

chrIII

genes

Smc2_pNZ

0.100M 0.110M 0.120M 0.130M 0.140M 0.150M 0.160M 0.170M 0.180M 0.190M

0.0 0.3 0.6

genes

Smc2_pNZ

0.100M 0.110M 0.120M 0.130M 0.140M 0.150M 0.160M 0.170M 0.180M 0.190M

0.0 0.3 0.6

genes

Smc2_pNZ

0.100M 0.110M 0.120M 0.130M 0.140M 0.150M 0.160M 0.170M 0.180M 0.190M

0.0 0.3 0.6

genes

Smc2_pNZ

0.100M 0.110M 0.120M 0.130M 0.140M 0.150M 0.160M 0.170M 0.180M 0.190M

0.0 0.3 0.6

Appendix C. ChIP-seq maps

216
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Appendix C. ChIP-seq maps

Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole

chrVII_1

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole

chrVII_2

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Appendix C. ChIP-seq maps

Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Appendix C. ChIP-seq maps

Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Appendix C. ChIP-seq maps

Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole

chrXII_2

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Appendix C. ChIP-seq maps

231
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole

chrXV_2

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ

genes

Smc2_pNZ
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.3: Smc2-9myc ChIP-seq map of cells arrested by addition of nocodazole
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Appendix C. ChIP-seq maps

Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20.

chrII_1

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

Appendix C. ChIP-seq maps
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chrV_1

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Appendix C. ChIP-seq maps

Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chrVI

genes

Smc2_pnNZ

0.0 0.10M 0.12M 0.14M 0.16M 0.18M 0.20M 0.22M 0.24M 0.26M 0.28M 0.30M

0.0 0.10M 0.12M 0.14M 0.16M 0.18M 0.20M 0.22M 0.24M 0.26M 0.28M 0.30M

0.0 0.10M 0.12M 0.14M 0.16M 0.18M 0.20M 0.22M 0.24M 0.26M 0.28M 0.30M

genes

Smc2_pnNZ

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0

0.0

3.0

6.0
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chrIX

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chrX_1

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

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Smc2_pnNZ

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Smc2_pnNZ

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Smc2_pnNZ

genes

Smc2_pnNZ
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chrXI_2

genes

Smc2_pnNZ
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20.
Appendix C. ChIP-seq maps

Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Appendix C. ChIP-seq maps

Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chrXIV_2

genes

Smc2_pnNZ

genes

Smc2_pnNZ
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20

chr XV_2

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ

genes

Smc2_pnNZ
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
Figure C.4: Smc2-9myc ChIP-seq map of cells arrested by depletion of Cdc20
**Figure C.5:** Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved

Appendix C. ChIP-seq maps
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

Appendix C. ChIP-seq maps
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Appendix C. ChIP-seq maps

Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

Figure showing ChIP-seq maps for Smc2-9myc in cells arrested by nocodazole readdition.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

chrXII_2

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Appendix C. ChIP-seq maps

Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

chrXIII_2

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

Smc2_pnpNZ
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Appendix C. ChIP-seq maps

Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

chrXV_1

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.
Appendix C. ChIP-seq maps

Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

chrXVI_1

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes
Figure C.5: Smc2-9myc ChIP-seq map of cells arrested by readdition of nocodazole, after Cdc20-mediated arrest achieved.

chrXVI_2

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

genes

Smc2_pnpNZ

Appendix C. ChIP-seq maps
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