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THE SMC5/6 COMPLEX: THE MYSTERIOUS GUARDIAN OF GENOME STABILITY

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The Smc5/6 complex: the mysterious guardian of genome stability

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother

ABSTRACT

An intricate network of proteins ensures the faithful transmission of genetic information through cell generations. The Structural Maintenance of Chromosomes (SMC) protein complex family plays a pivotal role in maintaining genome stability. Initially, the three eukaryotic SMC complexes, cohesin, condensin and Smc5/6 complex (Smc5/6) were identified for their functions in chromosome cohesion, condensation and recombination. Later, it was shown that SMC complexes also control replication and transcription. Another important group of proteins involved in the maintenance of genome stability are the topoisomerases. These enzymes control DNA topology to ensure faithful replication, transcription and chromosome segregation.

Defects in processes that control genome maintenance lead to cell death and chromosomal aberrations, including aneuploidy and translocations, which are hallmarks of cancer cells. Therefore, it is essential to reveal the details of how genome stability is maintained in order to fully comprehend the underlying causes of tumor development.

The aim of the projects described in this thesis was to increase the knowledge of Smc5/6, which is the least characterized of the three SMC complexes. Using the budding yeast *Saccharomyces cerevisiae* as model organism, our work shed light on the involvement of Smc5/6 in chromosome replication, segregation and, possibly, transcription. Moreover, *in vitro* analysis of purified Smc5/6 revealed new details of how the complex interacts with DNA.

In **Paper I**, it was demonstrated that Smc5/6 accumulates onto chromosomes after sister chromatids are tethered by cohesin. Smc5/6 is also shown to facilitate segregation of short entangled chromosomes. Our data suggest that the chromosomal association of Smc5/6 occurs at sites where sister chromatids are entangled, and that entanglement is proportional to the level of superhelical stress.

In **Paper II**, it was shown that Smc5/6 can both bind directly to, and topologically entrap DNA molecules *in vitro*. It was also demonstrated that Smc5/6 topologically entraps more than one DNA molecule at the time, stimulating their catenation by topoisomerase 2, which interacts with the complex.

In **Paper III**, it was shown that Smc5/6 is recruited to the intergenic region between two highly transcribed genes. This suggests that Smc5/6 accumulates in regions of high transcription-induced superhelical stress.

LIST OF SCIENTIFIC PAPERS

I. Jeppsson K, Carlborg KK, Nakato R, **Berta DG**, Lilienthal I, Kanno T, Lindqvist A, Brink MC, Dantuma NP, Katou Y, Shirahige K, Sjögren C.
The chromosomal association of the Smc5/6 complex depends on cohesion and predicts the level of sister chromatid entanglement.
PLoS Genet. 2014, 10(10):e1004680.

II. Kanno T, **Berta DG**, Sjögren C.
The Smc5/6 complex is an ATP-dependent intermolecular DNA linker.
Cell Rep. 2015, 12(9):1471-82.

III. **Berta DG**, Jeppsson K, Sjögren C.
Transcription-induced superhelical stress recruits Smc5/6 complex onto chromosomes between convergently oriented genes.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ALT	Alternative lengthening of telomeres
APB	ALT-associated promyelocytic leukemia nuclear bodies
ARS	Autonomously replicating sequences
ATP	Adenosine triphosphate
Bp, kb	Base pairs, kilobase pairs
Cdc6, 20, 45	Cell division cycle 6, 20, 45
CDK	Cyclin-dependent kinase
ChIP	Chromatin immuno precipitation
Ctd1	Chromatin licensing and DNA replication factor 1
Ctf19	Chromosome transmission fidelity 19
DDK	Dbf4-dependent kinase
DSB	Double strand break
Eco1, ESCO1, 2	Establishment of cohesion 1, 2
GFP	Green fluorescent protein
GINS	Go-ichi-ni-san (Sld5, Psf1, Psf2, and Psf3)
HRP	Horseradish peroxidase
Mcm2-7	Mini-chromosome maintenance
Mms21	Methyl methane sulfonate sensitivity 21
Nse 1-6	Non-SMC element 1-6
ORC	Origin recognition complex
Pds5	Precocious dissociation of sisters 5

qPCR	Quantitative polymerase chain reaction
Rad	Radiation sensitive
rDNA	Ribosomal DNA
Scc1-4	Sister chromatid cohesion 1-4
SCI	Sister chromatid intertwining
SDS	Sodium dodecyl sulfate
SMC	Structural Maintenance of Chromosomes
SUMO	Small Ubiquitin-like Modifier
Top1, 2, 3	Topoisomerase 1, 2, 3
tRNA	Transfer RNA
WAPL	Wings apart-like protein

1. INTRODUCTION

The maintenance of genome stability is crucial for the proper transmission of genetic information from mother to daughter cells. This is guaranteed by the activity of a broad network of proteins, which ensures faithful DNA replication, chromosome segregation and DNA repair. This thesis focuses on the so-called Structural Maintenance of Chromosomes (SMC) complexes, known to regulate all three processes.

1.1. DNA REPLICATION

DNA is composed by two non-covalently bound single strands of deoxyribonucleotides polymers. The structure of the nucleotide building blocks determines the polarity of the DNA strands, and the double helix is composed by two 5' to 3' anti-parallel nucleotide strands. DNA replication is semi-conservative and occurs only in the 5'-3' direction (Hirt, 1966). Due to this, the so-called leading strand is synthesized as a unique molecule, while the lagging strand is generated in a stepwise manner forming so-called Okazaki fragments, which are subsequently ligated together (Okazaki et al., 1967; Sinha et al., 1980). Replication begins at multiple origin sites, where two replisomes are assembled onto DNA. These molecular machines, which contain all the proteins required to ensure DNA synthesis, progress in opposite directions, creating a bubble of replicated DNA in between (Fang et al., 1999; Umek and Kowalski, 1988). At both ends of the replication bubble, parental DNA is unwound to allow DNA synthesis, producing a Y-shaped structure called replication fork. After completion of replication all chromosomes have been duplicated into two identical DNA molecules, called sister chromatids.

1.1.1. Replication initiation

In eukaryotes, the origin recognition complex (ORC) binds to replication origins (Bell and Stillman, 1992), which in budding yeast are defined by conserved sequences called autonomously replicating sequences (ARSs) (Palzkill and Newlon, 1988). In the budding yeast genome, ~400 ARSs have been identified, and they can be divided into early- and late-firing origins depending on when in S-phase they are activated (Raghuraman et al., 2001; Wyrick et al., 2001; Yabuki et al., 2002). ORC and Cdc6, together with Cdt1, recruit two copies of the hexameric helicase Mcm2-7, building the pre-replication complex (Bell and Stillman, 1992; Cocker et al., 1996; Donovan et al., 1997; Remus et al., 2009; Tanaka and Diffley, 2002). This

is the first step of replication and it occurs between late mitosis and G1-phase (Dahmann et al., 1995; Detweiler and Li, 1998; Piatti et al., 1996). Prior to initiation of replication, other factors are assembled to the pre-replication complex, such as Cdc45 and the GINS complex, creating the pre-initiation complex (Hopwood and Dalton, 1996; Kanemaki et al., 2003; Takayama et al., 2003). Replication is finally activated through CDK- (Cycling-dependent kinase) and DDK- (Dbf4-dependent kinase) dependent phosphorylation of subunits belonging to the pre-initiation complex (Bousset and Diffley, 1998; Cheng et al., 1999; Donaldson et al., 1998; Mendenhall and Hodge, 1998; Perez-Arnaiz et al., 2016).

1.1.2. Replication termination

While replication initiation has been well characterized, much less is known about termination process. Differently from replication origins, termination sites have not been associated to specific DNA sequences, and their location is determined by activity of flanking origins (Hawkins et al., 2013; McGuffee et al., 2013). However, there are genomic regions where replication termination occurs at specific sites. These regions are characterized by replication fork pausing elements and include centromeres, rDNA and tRNA genes (Brewer and Fangman, 1988; Deshpande and Newlon, 1996; Fachinetti et al., 2010; Greenfeder and Newlon, 1992).

1.2. CHROMOSOME SEGREGATION

Segregation of sister chromatids occurs during anaphase. From S-phase until segregation, the chromatids are held together by sister chromatid cohesion. This process is mediated by the protein complex cohesin, and it will be presented in detail in the chapter 1.4.2. Sister chromatid segregation depends on the attachment of each chromatid to the microtubules of the mitotic spindle. Microtubules are connected to a protein structure called kinetochore located in the centromeric region of each chromatid. In preparation for anaphase, each kinetochore of a chromatid pair is attached to opposite poles of the mitotic spindle. The pulling force of the spindle will be opposed by sister chromatid cohesion, thereby generating tension. This leads to alignment of the two sister chromatids in the middle of the mitotic cell (Ng et al., 2009; Tanaka et al., 2000). The tension is also one of the signals sensed by the spindle assembly checkpoint, which surveils that each kinetochore pair is properly attached to the spindle apparatus. In budding yeast, microtubules are attached to kinetochores all through the cell cycle, except for a short time during S-phase when the centromere is replicated (Kitamura et al., 2007). At

anaphase onset, the anaphase-promoting complex is activated by its regulator Cdc20 and induces degradation of the protein securin (Ciosk et al., 1998; Cohen-Fix et al., 1996; Sethi et al., 1991; Shirayama et al., 1998). Securin has, until then, prevented the protease separase to cleave one of the subunits of the cohesin complex. Thus, securin degradation triggers the removal of cohesin from sister chromatids, thereby allowing their separation into the nascent daughter cells (Uhlmann et al., 1999).

1.3. DNA TOPOLOGY

The two DNA strands are wrapped around each other, forming a right-handed double helix. This feature is called "twist". In its relaxed state, the double helix completes a full turn every 10.5 base pairs (bp). Processes such as DNA replication and transcription constantly alter this conformation. The field that focuses on the structural changes of DNA is called the study of DNA topology. To envisage the topological properties of DNA, one can imagine two ropes wrapped around each other, and fixed at one end. This represents the DNA molecule. If the two strands are separated from each other at the free ends, as displayed in Figure 1, the twist will be confined to a shorter region, leading to over-winding of the double helix. Excessive over-winding leads the DNA to fold on itself, generating supercoils (Figure 1).

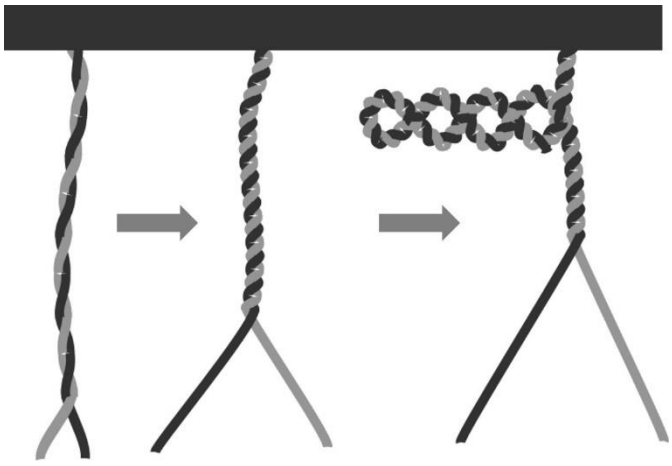

Figure 1. Increase in twist determines supercoiling. Separation of the free ends of a pair of ropes twisted around each other first increases the number of twists per unit length. At a certain level of twists per unit, the ropes fold on themselves creating supercoils.

Supercoil accumulation generates topological, also called superhelical, stress. Supercoils can assume either plectonemic or toroidal conformation. Plectonemic supercoils are positive if over-winding has a left-handed conformation, negative if right-handed. Toroidal supercoils are positive if right-handed, negative if left-handed (Figure 2). The handedness of a supercoil indicates the direction in which the overlying double helix must be rotated to be aligned to the

underlying double helix. Clockwise direction characterizes a left-handed molecule, counter-clockwise a right-handed molecule.

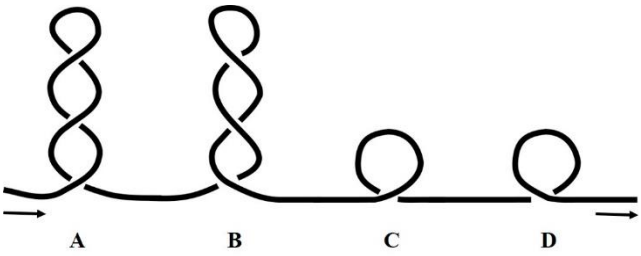

Figure 2. Supercoil conformation. (A) Right-handed negative plectonemic supercoils; (B) Left-handed positive plectonemic supercoils; (C) Left-handed negative toroidal supercoils; (D) Right-handed positive toroidal supercoils.

Topological structures can be generated not only within a single DNA molecule, but also between two molecules. The most well known structures of this kind are sister chromatid entanglements, also called sister chromatid intertwinings (SCIs).

1.3.1. Topoisomerases

Enzymes responsible of resolving supercoils and SCIs are called topoisomerases. These enzymes relax DNA through transesterification reactions, during which the DNA backbone is cleaved, and a covalently bound DNA-enzyme intermediate is generated.

Topoisomerases are classified in type I if they create a single strand break in the double helix, or type II if they create double strand breaks. Type I topoisomerases are divided into subtypes IA and IB. Topoisomerases IA covalently bind to the 5' end of the induced DNA break and catalyze one passage of the noncovalently bound 3' end around the intact strand before resealing the gap. Through this mechanism, they relax negative supercoils with an efficiency that is proportional to the level of supercoiling. Topoisomerases IB bind the 3' end of the break, leaving the 5' end free to rotate several times around the intact strand before resealing. Through this mechanism of "DNA rotation", they relax both positive and negative supercoils. Type II topoisomerases act as dimers and generate a transient double strand break in the DNA molecule, through which another double helix passes before resealing. This reaction is dependent on ATP. Type II topoisomerases can resolve both supercoils within a single DNA molecule, and intertwinings between two different DNA helices (Wang, 2002).

In budding yeast, three topoisomerases have been identified: Topoisomerase 1 (Top1) (type IB), Topoisomerase 2 (Top2) (type II) and Topoisomerase 3 (Top3) (Type IA). Top1 is non-essential in *S. cerevisiae* (Thrash et al., 1985), while Top2 is required for viability and *top2* mutants die at the time of mitosis, because of defects in chromosome segregation (DiNardo et

al., 1984; Holm et al., 1985). Top3 is non-essential, but *top3* mutants display slow growth and aberrant recombination (Bailis et al., 1992). This phenotype can be rescued by depletion of the helicase Sgs1 (Gangloff et al., 1994).

1.3.2. Topology and replication

During replication, positive supercoils accumulate ahead of the replication fork in response to the unwinding of the double helix. In budding yeast, Top1 and Top2 act redundantly to resolve this topological stress, and in the absence of both topoisomerases replication is blocked (Brill et al., 1987). Replication termination is executed by Top2 (Fachinetti et al., 2010). Top3, together with Sgs1, cooperates redundantly with Top2 to promote replication termination only at the rDNA locus, which is the genomic region where ribosomal RNA is transcribed (Mundbjerg et al., 2015). Absence of Top1 alone does not inhibit replication (Thrash et al., 1985), although replication delay of long chromosomes has been reported in budding yeast (Kegel et al., 2011). This suggests that absence of Top1 leads to superhelical stress that slows down replication, and that this stress can be passively resolved on short chromosomes. The same replication delay of long chromosomes was observed in *top3* mutants (Kegel et al., 2011), but the exact function exerted by Top3 remains unknown.

Topoisomerase-dependent resolution of superhelical stress during replication is supplemented by rotation of the replication fork. Fork rotation reduces the accumulation of positive supercoils ahead of it, but instead leads to the formation of entanglements between the newly replicated sister chromatids (Figure 3) (Champoux and Been, 1980).

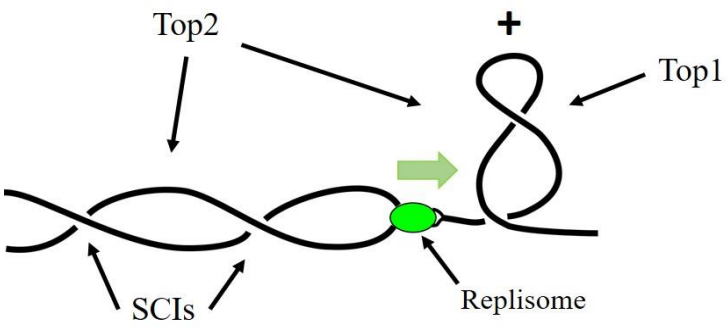

Figure 3. DNA topology during replication. Positive supercoils (+) accumulate ahead of the replisome. Fork rotation reduces supercoil accumulation and generates right-handed sister chromatid intertwinings (SCIs) behind the fork. Top1 resolves positive supercoils ahead of the fork; Top2 resolves both supercoils and SCIs.

When entanglements are formed during plasmid replication, they are called precatenanes because they generate catenated plasmids at the end of replication if left unresolved. Catenanes on linear chromosomes are called sister chromatid intertwinings, in short SCIs. The essential

function for type two topoisomerases (Top2 in budding yeast) is to resolve SCIs, which is crucial for proper chromosome segregation (Buchenau et al., 1993; Downes et al., 1991; Giménez-Abiàn et al., 1995; Shamu and Murray, 1992).

1.3.3. Topology and transcription

Transcription requires DNA unwinding to allow the production of the mRNA molecule by the RNA polymerase. Transcription elongation generates positive supercoils ahead of the polymerase, and negative behind, according to the "twin supercoiled domain" model (Figure 4) (Liu and Wang, 1987).

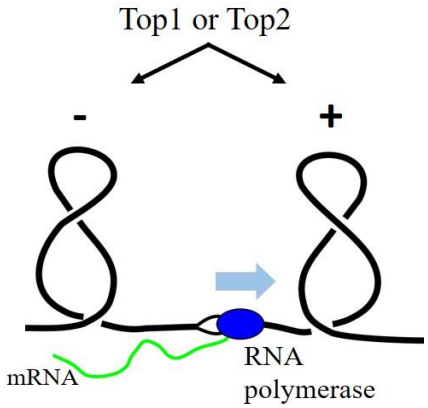

Figure 4. DNA topology during transcription. DNA unwinding during transcription generates positive supercoils (+) ahead of the RNA polymerase and negative supercoils (-) behind. Top1 and Top2 resolve both positive and negative supercoils.

Analysis in budding yeast shows that topological stress negatively affects transcription if genes are located more than 100 kb away from telomeres (Joshi et al., 2010). This suggests that superhelical stress can be resolved by rotation of chromosome ends, which is in line with the length-dependent delay of replication in *top1* mutants (Kegel et al., 2011). Top1 and Top2 resolve superhelical stress during transcription (Wang, 2002), although none of the two topoisomerases is crucial for RNA polymerase II-dependent transcription, which is only reduced by one third in *top1top2* double mutants (Brill et al., 1987). Due to this, it has been suggested that superhelical stress also can be reduced by the merge of positive and negative supercoils, which then cancel each other (Stupina and Wang, 2004). Although Top1 and Top2 can resolve both positive and negative supercoils, it is now clear that the two topoisomerases do not necessarily act redundantly, but have specific functions. In particular, several studies indicate that Top1 acts preferably on negative supercoils, while Top2 on positive ones (Brill and Sternglanz, 1988; French et al., 2011; Joshi et al., 2012; Mondal and Parvin, 2001). Moreover, Top2, but not Top1, is required to ensure proper transcription of genes longer than 3 kb in budding yeast (Joshi et al., 2012).

Related to this thesis, it is important to highlight the regulation of topological stress at intergenic sites between two genes that are transcribed in convergent orientation. Top2, but not Top1, is required to maintain genome stability between convergently-oriented genes, and *top2* mutants display increased gross chromosomal rearrangements at these sites (Pannunzio and Lieber, 2016). In contrast, another study showed that Top1 is the major responsible of removing supercoils between two convergently-oriented genes on a plasmid, while Top2 plays just a marginal role in supercoils relaxation (García-Rubio and Aguilera, 2012). Despite these conflicting results, it is clear that topoisomerases function at these sites is essential, even though the details are not fully elucidated.

1.4. THE SMC PROTEIN FAMILY

The SMC protein complex family plays a central role in maintenance of genome stability. Six SMC proteins have been identified in eukaryotes (named Smc1-6). These proteins have a similar structure and are found to be conserved through evolution (Jones and Sgouros, 2001). Each SMC protein is composed by five domains. A central hinge domain is connected to the N- and C- globular ends by two coiled-coil regions (Figure 5A). The N- and C-termini contain nucleotide-binding motifs (Niki et al., 1991). SMC proteins fold at the hinge domain, creating an anti-parallel coiled-coil region that brings the two globular ends in close proximity to each other (Melby et al., 1998). The association of the N- and C-termini domains creates an ATP-binding cassette (ABC) with ATPase activity (Hirano et al., 2001; Lowe et al., 2001). SMC proteins form heterodimers, and the hinge domain is the dimerization interface. The Smc1 and Smc3 proteins are part of the cohesin complex (Losada et al., 1998; Michaelis et al., 1997), Smc2 and Smc4 constitute the core of condensin (Hirano et al., 1997) and Smc5 and Smc6 associate to generate the homonymous complex Smc5/6 (Fousteri and Lehmann, 2000; Taylor et al., 2001) (Figure 5B). Cohesin and Smc5/6 were discovered in yeast and condensin was identified in *Xenopus* extracts (Hirano and Mitchison, 1994; Lehmann et al., 1995; Strunnikov et al., 1993). The focus of this thesis is on cohesin and Smc5/6.

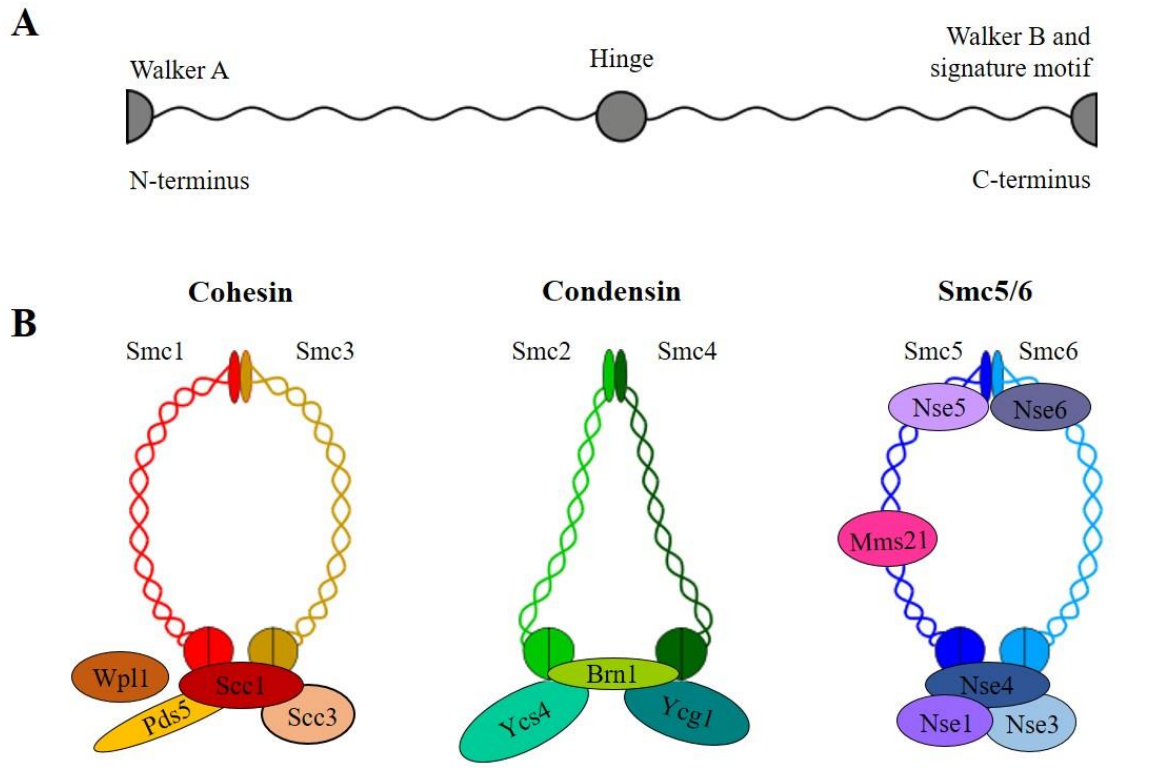

Figure 5. Structure and composition of SMC complexes. (**A**) Structure of an unfolded SMC protein. The protein folds at the hinge domain, bringing the N- and C-termini in close proximity. Vicinity of Walker A, Walker B and signature motif generates an ATP binding site within the globular head. (**B**) Structure and composition of the three SMC protein complexes in *S. cerevisiae*.

1.4.1. ABC ATPase domain

The ATP binding cassette is characterized by two domains that are called the RecA-like domain and the helical domain. Within these two domains, three regions are highly conserved, and they have been named Walker A, B and C, to honor the scientist who discovered them (Walker et al., 1982). Walker A and Walker B belong to the RecA-like domain, while Walker C is also known as signature motif and belongs to the helical domain (Higgins et al., 1986; Hung et al., 1998; Mimura et al., 1991). Several studies on ABC-like proteins (ABC transporters and SMC proteins) showed that the Walker A motif is essential for ATP binding, while the signature motif is required to promote ATP hydrolysis. Walker B motif is required for both processes, since mutations in this region stabilize ATP binding and delays its hydrolysis (Cobbe and Heck, 2006; Fetsch and Davidson, 2002; Hirano and Hirano, 2004; Hirano et al., 2001; Mimura et al., 1991; Schmees et al., 1999; Shyamala et al., 1991). In SMC proteins, Walker A is found at the N-terminus, Walker B and the signature motif at the C-terminus (Figure 5A) (Niki et al., 1991).

A close relative of the SMC family, Rad50, possesses ABC-like ATPase activity, and ATP binding determines dimerization of Rad50, with two nucleotides sandwiched between the dimer (Hopfner et al., 2000). A similar process has been observed for SMC proteins, as evidences suggest that two ATP molecules bind to opposing Walker A and signature motif of the SMC dimer to assemble the complex (Lammens et al., 2004). Mechanism of ATPase-mediated DNA binding has been thoroughly investigated for cohesin (as it will be discussed in paragraph 1.4.2.2.), while very little is known about Smc5/6. **Paper II** in this thesis shed light on this process.

1.4.2. Cohesin

The cohesin complex was first identified in budding yeast thanks to a genetic screen aimed to identify genes that allowed sister chromatids to separate in the absence of functional anaphase promoting complex (Guacci et al., 1997; Michaelis et al., 1997).

1.4.2.1. Structure of cohesin

Cohesin is constituted by the two SMC proteins Smc1 and Smc3, whose ATPase heads are bridged by the kleisin subunit Scc1, forming a ring-like structure (Gruber et al., 2003). The C-terminus of Scc1 associates with the head domain of Smc1, while the N-terminus binds to the head domain of Smc3 (Haering et al., 2002). Scc3 is an additional subunit of the cohesin complex, and promotes establishment and maintenance of sister chromatid cohesion (Roig et al., 2014; Toth et al., 1999). Cohesin interacts with several other proteins that regulate cohesion function. In particular, Pds5 and Wpl1 form a dimer that interacts with cohesin subunit Scc1 (Hartman et al., 2000a; Kueng et al., 2006; Panizza et al., 2000). The main functions of these two proteins are to promote maintenance of cohesion between sister chromatids (mediated by Pds5) and cohesin release from chromosomes (mediated by both Pds5 and Wpl1) (Murayama and Uhlmann, 2015; Panizza et al., 2000; Rowland et al., 2009; Stead et al., 2003; Sutani et al., 2009; Vaur et al., 2012).

1.4.2.2. Cohesin's DNA binding mechanism

Cohesin loading onto chromosomes is dependent on the loading factor Scc2-Scc4 and occurs before DNA replication (Ciosk et al., 2000). After loading, cohesin binding to chromosomes is unstable due to destabilization triggered by Wpl1 (Sutani et al., 2009; Vaur et al., 2012). In

budding yeast, acetylation of Smc3 on two conserved lysines performed by the Eco1 acetyltransferase inhibits Wpl1 activity and leads to the establishment of sister chromatid cohesion (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009; Sutani et al., 2009; Unal et al., 2008; Zhang et al., 2008a). In vertebrates, establishment of cohesion is achieved not only through Smc3 acetylation, but it requires also recruitment of sororin to counteract Wapl (the human homolog of budding yeast Wpl1) (Nishiyama et al., 2010; Rankin et al., 2005). It is important to note that acetylation of Smc3 only leads to establishment of cohesion during DNA replication (Lengronne et al., 2006). A large number of investigations have been performed to reveal how cohesin binds to DNA in order to perform its function. It has been shown that cohesin can topologically entrap DNA molecules (Ivanov and Nasmyth, 2005; Murayama and Uhlmann, 2014). Initially, *in vivo* work suggests that the so-called DNA "entry gate" is located at the hinge between Smc1 and Smc3 (Gruber et al., 2006). Recently, however, *in vitro* analysis of the complex suggests that the gate is found at the Smc3-Scc1 interface (Murayama and Uhlmann, 2015).

ATP plays a pivotal role in cohesin loading. Not only ATP binding is required for cohesin complex assembly, but ATP hydrolysis is also needed to allow cohesion establishment (Arumugam et al., 2003; Weitzer et al., 2003). The central role for ATP hydrolysis (at the head domains), has been taken as an argument against the localization of the DNA entry gate at the hinge (Murayama and Uhlmann, 2014). However, the SMC-protein related Rad50 homodimer undergoes large conformational change upon ATP hydrolysis that also affects the coiled coil domains (Lim et al., 2011; Williams et al., 2011). This could also be the case for cohesin, and thereby ATP hydrolysis at the head could lead to opening of the ring at the hinge.

In budding yeast, removal of cohesin during mitosis occurs through cleavage of the kleisin subunit Scc1 at the anaphase onset (Uhlmann et al., 1999). In vertebrates, this process occurs through two different mechanisms. Centromeric cohesin is removed by cleavage of the kleisin subunit at the anaphase onset (Waizenegger et al., 2000). Before that, the so-called prophase pathway is responsible of cohesin removal from chromosome arms by non-proteolytic opening of the Smc3-Scc1 interface (Buheitel and Stemmann, 2013; Gandhi et al., 2006; Kueng et al., 2006; Sumara et al., 2000). In budding yeast, there are no intact cohesin rings after anaphase, due to destruction of all Scc1, while the cohesin rings removed by the prophase pathway are still intact in vertebrates. This explains why reloading of cohesin occurs already during telophase in vertebrates, while in budding yeast cohesin loading occurs in late G1-phase because Scc1 needs to be synthesized *de novo* (Gerlich et al., 2006; Michaelis et al., 1997; Uhlmann and Nasmyth, 1998).

Even though cohesion is solely dissolved through Scc1 cleavage at anaphase in budding yeast, non-acetylated cohesin complexes are constantly removed by a pathway regulated by Wpl1, Pds5, Scc3 and cohesin ATPase activity (Chan et al., 2013; Elbatsh et al., 2016; Rowland et al., 2009). Experiments performed on fission yeast suggest that cohesin release from DNA without Scc1 cleavage occurs in two different steps. First, ATP hydrolysis triggers opening of the Smc1-Smc3 heads. Subsequently, Wapl (the fission yeast homolog of Wpl1) facilitates opening of the Smc3-Scc1 interface, so-called "exit gate", to free the DNA (Beckouet et al., 2016; Chan et al., 2012; Murayama and Uhlmann, 2015).

It is still debated how cohesin holds two sister chromatids together. One of the two most common models, called "handcuff model", suggests that two cohesin complexes embrace one sister chromatid each, and interact to create cohesion (Huang et al., 2005). The other, called "one-ring embrace model", proposes that one cohesin ring is able to hold two sister chromatids (Haering et al., 2002).

1.4.2.3. Cohesin's chromosomal localization

The chromosomal binding pattern of cohesin was first determined in budding yeast. In unchallenged cells, the complex was shown to be highly enriched at centromeres, pericentromeric area and was also found in AT-rich regions every ~11 kb along chromosome arms (Megee et al., 1999; Tanaka et al., 1999). Subsequent genome-wide analysis demonstrated that cohesin was present at intergenic regions between convergently oriented genes along chromosome arms (Lengronne et al., 2004). Interestingly, the binding pattern of the cohesin's loading factor Scc2-Scc4 did not overlap with cohesin, and was proposed to correlate with transcription, suggesting that cohesin could be pushed to the intergenic regions by transcription machineries (Lengronne et al., 2004). However, Scc2 and cohesin colocalize at centromeres, where the recruitment of the cohesin loader is dependent on Ctf19 (Fernius et al., 2013). This indicates yet another difference in the regulation of centromeric and arm cohesin/cohesion.

1.4.2.4. Cohesin's functions

Cohesin's main function is to promote cohesion between the newly replicated sister chromatids (Michaelis et al., 1997). Cohesin promotes cohesion both in a direct way, by holding sister chromatids together (Gligoris et al., 2014; Haering et al., 2008), and indirect, by protecting

them from Top2-mediated resolution of SCIs (Farcas et al., 2011). In addition to this, cohesin and its loader have been implicated in transcriptional regulation, although how this role is performed is still unclear (Kagey et al., 2010; Zuin et al., 2014). Moreover, it has been demonstrated that cohesin promotes condensation of the rDNA region (Guacci et al., 1997). In further support for a role of cohesin in condensation, depletion of Wapl, which leads to stabilization of cohesin on chromosomes, promotes condensation of interphase chromosomes (Tedeschi et al., 2013).

1.4.3. The Smc5/6 complex

Among the three members of the SMC family, Smc5/6 is the least characterized. Smc6 was first discovered in fission yeast *Schizosaccharomyces pombe* (*S. pombe*) and initially named Rad18, and it was shown to promote repair of DNA damage caused by UV-irradiation (Lehmann et al., 1995).

1.4.3.1. Structure of Smc5/6

The *S. cerevisiae* Smc5/6 complex consists of Smc5, Smc6 and six additional subunits, named Non SMC Elements (NSE) (Nse1, Nse2/Mms21, Nse3-6) (Palecek et al., 2006; Zhao and Blobel, 2005). Smc5 and Smc6 dimerize through their hinge domains, and a subcomplex composed by Nse1, Nse3 and the Scc1-like subunit Nse4 bridges Smc5 and Smc6 head domains (Palecek et al., 2006). Nse2/Mms21 is a small ubiquitin-like modifier (SUMO) E3 ligase and associates to the middle of the coiled coil domain of Smc5 (Duan et al., 2009a). Nse5 and Nse6 subunits form a subcomplex that has been reported to bind to the hinge region of Smc5/6 in *S. cerevisiae*, and the head domains in *S. pombe* (Duan et al., 2009b; Palecek et al., 2006; Pebernard et al., 2006). All the subunits are essential in budding yeast, while in *S. pombe* Nse5 and Nse6 are nonessential (Pebernard et al., 2006).

1.4.3.2. Smc5/6 DNA binding mechanism and chromosomal localization

Smc5/6 binding mechanism onto DNA was explained for the first time in **Paper II**. Smc5/6 requires ATP to bind directly to DNA and ATP hydrolysis for DNA topological entrapment *in vitro*. This suggests a two-step mechanism that requires first direct interaction of the complex with DNA and then ATP hydrolysis allows topological entrapment. It was also shown that

Smc5/6 can topologically entrap more than one DNA molecule at the time, suggesting that the complex can entrap sister chromatids *in vivo* (Kanno et al., 2015).

Upon DNA damage, Smc5/6 is recruited to double strand breaks (DSBs) in G2/M phase, and to collapsed replication forks during S-phase (Ampatzidou et al., 2006; Lindroos et al., 2006; De Piccoli et al., 2006; Potts et al., 2006).

How Smc5/6 localization is regulated in unchallenged cells is not fully elucidated. In budding yeast, Smc5/6 was found to localize at telomeres and rDNA, suggesting a specific function at repetitive DNA regions (Torres-Rosell et al., 2005). Moreover, the complex colocalizes with cohesin at centromeres in G2/M-phase, and its enrichment is positively correlated to chromosome length (Kegel et al., 2011; Lindroos et al., 2006). Inactivation of Top2 increases frequency of Smc5/6 binding, suggesting that Smc5/6 localization is triggered by SCIs (Kegel et al., 2011). Initially, chromatin immuno-precipitation experiments followed by microarray hybridization (ChIP-on-chip) showed that in the absence of the cohesin loader Scc2, but not in the absence of functional cohesin, Smc5/6 chromosome recruitment is significantly decreased (Lindroos et al., 2006). Instead, in **Paper I** it is demonstrated that cohesin, as well as establishment of sister chromatid cohesion, are required for Smc5/6 localization to chromosomes (Jeppsson et al., 2014). This discrepancy is probably due to the lower sensitivity of microarray hybridization compared to sequencing and quantitative PCR (Ho et al., 2011), which were used by Jeppsson et al., (2014). In budding yeast Smc5/6 maximum enrichment is reached in G2/M (Lindroos et al., 2006; Pebernard et al., 2008). In human cells, Smc5/6 binds to chromosomes during interphase, and dissociates from mitotic chromosomes (Gallego-Paez et al., 2014). Finally, the unloading mechanism is currently unknown. **Paper I** provides several new insights about Smc5/6 chromosomal localization.

1.4.3.3. Smc5/6 functions in DNA repair

Cells without fully functional Smc5/6 are hypersensitive to DNA damaging agents and to replication fork blocking agents (Ampatzidou et al., 2006; Branzei et al., 2006; Lehmann et al., 1995) and display gross chromosomal rearrangements (Hwang et al., 2008). This phenotype is due to defects in resolution of homologous recombination intermediates, and is conserved from yeasts to vertebrates (De Piccoli et al., 2006; Potts et al., 2006; Stephan et al., 2011). In line with this function, Smc5/6 is recruited at stalled replication forks (Ampatzidou et al., 2006; Branzei et al., 2006; Irmisch et al., 2009; Lindroos et al., 2006). Moreover, Smc5/6 promotes

repair of double strand breaks by regulating a recombination function of cohesin (De Piccoli et al., 2006; Potts et al., 2006; Ström et al., 2007).

1.4.3.4. Smc5/6 functions in unchallenged cells

While Smc5/6 role in DNA damage conditions has been studied intensively, its essential role in unchallenged conditions remains elusive. Budding yeast and human cells lacking a functional Smc5/6 complex display missegregation during mitosis, aberrant chromosome structures and incomplete replication at both repetitive and unique DNA regions (Gallego-Paez et al., 2014; Lindroos et al., 2006; Torres-Rosell et al., 2005, 2007; Yong-Gonzales et al., 2012). These phenotypes appear to be connected to a role of Smc5/6 in the resolution of replication-induced superhelical stress, which is discussed in the following paragraph. In addition, Smc5/6 protects chromosomal fragile sites, *i.e.:* natural pausing sites, from recombination-mediated fragility, and promotes resolution of recombination intermediates that are formed in response to endogenous replication stress (Menolfi et al., 2015).

Moreover, recent analysis suggest a transcriptional function for Smc5/6. Analysis of hepatitis B virus provided results indicating that Smc5/6 binds to episomal DNA and acts as transcriptional repressor when a gene is present in a plasmid, but not when it is integrated in the host genome (Decorsiere et al., 2016; Domingues et al., 2015; Murphy et al., 2016). Since episomal DNA is expected to be under higher superhelical stress than chromosomal DNA, this suggests that Smc5/6 also has a transcriptional function connected to the topology of DNA. The results presented in **Paper III** provide further evidence that this indeed could be the case.

1.4.3.5. Interplay between Smc5/6 and superhelical stress

In the absence of functional Smc5/6 during S-phase, replication delay is observed on long chromosomes in budding yeast (Kegel et al., 2011). This delay was observed also in the absence of Top1, suggesting a role for Smc5/6 in reducing superhelical stress. In unchallenged cells, Smc5/6 accumulates in the pericentromeric area, and the level of enrichment positively correlates to chromosome length. Lack of functional Top2 during replication, which leads to an increase in the number of SCIs, triggers the recruitment of Smc5/6 to chromosome arms. Reactivation of Top2 in G2/M-phase, which removes additional SCIs, also restores wild-type Smc5/6 binding. Altogether, this suggests that the presence of SCIs triggers Smc5/6 recruitment onto DNA (Jeppsson et al., 2014; Kegel et al., 2011). Moreover, the length-

dependent binding pattern suggests that Smc5/6 recruitment is regulated also by replication-induced superhelical stress. Based on these observations, Kegel et al., proposed a role for Smc5/6 in replication fork rotation (Kegel et al., 2011). The model is that Smc5/6 sequesters SCIs generated behind the replication fork, thereby facilitating fork rotation and reducing superhelical stress ahead of the fork (Kegel et al., 2011). In **Paper I**, it is shown that Smc5/6 chromosome binding increases with the level of missegregation in the absence of functional Top2 (Jeppsson et al., 2014). This suggests that Smc5/6 acts as a marker of structures that hamper sister chromatid segregation, most likely SCIs (Jeppsson et al., 2014).

2. METODOLOGIES

2.1. MODEL ORGANISM

In all the three papers presented in this thesis, budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) was used as model organism. *S. cerevisiae,* commonly known as the baker's yeast, is an eukaryote unicellular organism that provides several advantages in cell biology research: it has a very short life cycle in optimal conditions (around 90 minutes), its genome is completely sequenced (Dujon, 1996; Goffeau et al., 1996), it is easy to manipulate, economically advantageous and easy to culture.

The entire *S. cerevisiae* genome is approximately 12,000 kb, organized into 16 linear chromosomes. Around 6000 open reading frames (ORFs) have been identified, and most of the genome is composed by coding DNA. Gene density is ~50-fold higher than in human genome, with one gene every ~2 kb, and an ORF measures 1450 bp in average. Divergent genes are spaced by 618 bp in average, while by only 326 bp if convergently oriented. Finally, only 4% of genes contain introns (Dujon, 1996; Goffeau et al., 1996).

Despite the differences between *S. cerevisiae* and higher eukaryotic genome organizations, several studies performed on budding yeast allowed to identify proteins and understand processes that are highly conserved through evolution. For example, cohesin was discovered and further characterized in budding yeast (Guacci et al., 1997; Michaelis et al., 1997) and most functions of cohesin were later shown to be conserved in higher eukaryotes.

2.2. CHROMATIN IMMUNO-PRECIPITATION (CHIP)

Chromatin immuno-precipitation (ChIP) is a technique that allows the identification of genomic loci where a specific protein associates to, which is essential to understand the chromatin-related function(s) of the protein. This technique was employed in all the three papers presented in the thesis. The first step of ChIP is to grow cells under desired conditions. Then, cells are collected and treated with formaldehyde to crosslink proteins bound to DNA. Cell lysis is then followed by chromatin shearing by sonication, which generates DNA fragments of approximately 400-500 bp. Immuno-precipitation of the protein and associated DNA fragments is then performed using a specific antibody, most often directed against a C-terminal epitope tag, the sequence of which has been inserted into genomic version of the

corresponding gene. It has first to be tested that the epitope-tag does not interfere with protein function. Crosslinks are then reversed and DNA is purified (Figure 6) (Katou et al., 2006).

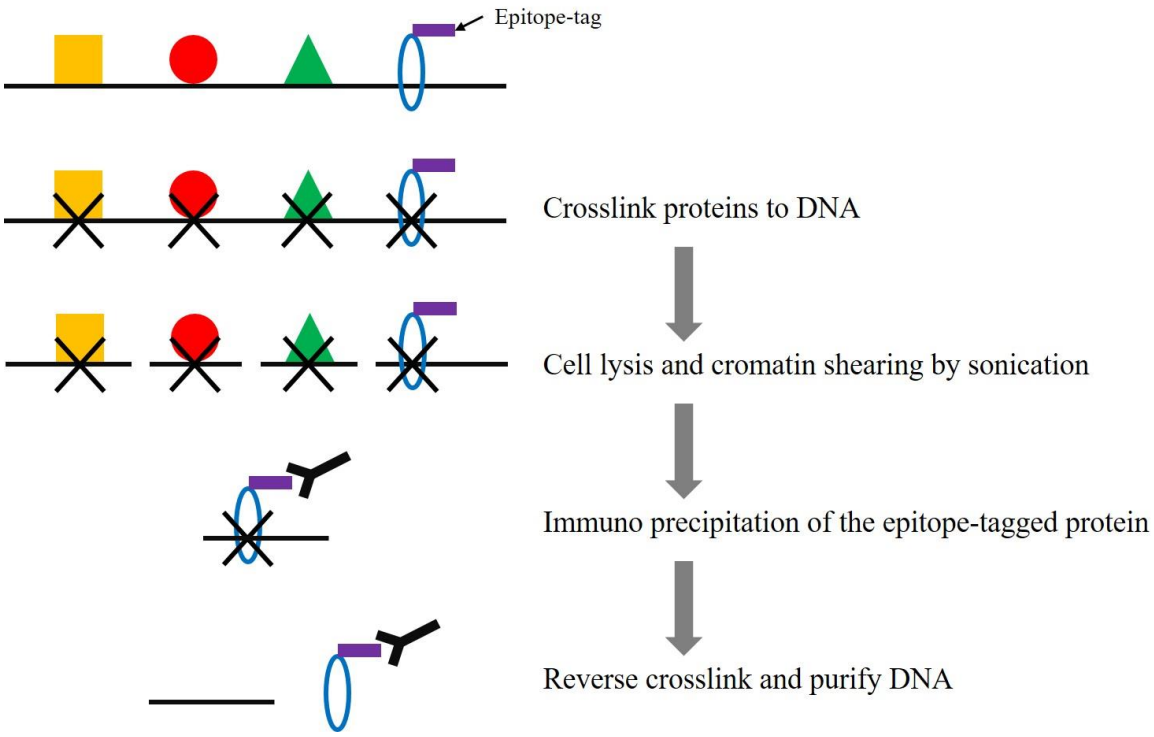

Figure 6. Schematic representation of Chromatin Immuno Precipitation (ChIP). The protein of interest is tagged with an epitope (violet rectangle) recognized by a specific antibody.

The amount of purified DNA is finally quantified and compared to the level of DNA present in the whole cell extract (input fraction). This can be done by using microarrays (ChIP-on-chip), sequencing (ChIP-seq) or quantitative PCR (ChIP-qPCR). ChIP-on-chip and ChIP-seq provide an analysis of the protein-binding pattern in the whole genome. ChIP-seq provides better resolution than ChIP-on-chip and better signal/noise ratio. ChIP-qPCR is, on the other hand, fully quantitative, and provides information about a specific locus (Ho et al., 2011). It is important to notice that ChIP is a technique performed on cell populations. Therefore, it does not provide information to understand if a lower signal at a specific site as compared to others is due to fewer cells with bound protein, or generally weaker binding of the protein.

As indicated above, one advantage of using budding yeast is the ease by which a protein can be marked with a specific epitope, recognized by a highly specific antibody. With the use of tagged proteins, two negative controls can be added to the analysis. One is a strain lacking any epitope-tag, the other a strain in which another nuclear protein that does not bind to DNA is tagged. Another advantage of using the same epitope-tag and antibody is that it facilitates comparison between experiments on different proteins (but tagged with the same epitope).

2.3. RNA EXTRACTION

To measure the expression level of *MCR1* and *DBR1* genes in **Paper III**, RNA was first extracted and purified. The protocol used to extract RNA is called acid guanidinium thiocyanate-phenol-chloroform extraction, and was first published by Chomczynski and Sacchi (1987). Briefly, cells are homogenized and the addition of phenol-chloroform allows separation of an upper aqueous phase from a lower organic phase. Nucleic acids are found in the upper phase together with chloroform, while proteins in the lower together with phenol. RNA precipitation occurs through addition of isopropanol to the upper phase. Following wash with ethanol and resuspension in DNase/RNase-free water, the sample is treated with DNase, and RNA is subsequently reverse transcribed into cDNA. Finally, qPCR is performed targeting the genes of interest. Comparison of expression levels of different genes, or of a single gene in different conditions, is performed through normalization using a reference gene, normally a housekeeping gene, as internal control. In **Paper III**, actin was used as reference gene.

2.4. ADDITIONAL TECHNIQUES

In **Paper I** and **Paper III**, DNA replication was monitored through two-dimensional gel electrophoresis. This technique allows detection of DNA structures, such as recombination and replication intermediates, at specific loci in the genome. This technique is based on the fact that linear and branched DNA can be separated during electrophoresis (Bell and Byers, 1983). DNA is digested with restriction enzyme in order to obtain the sequence of interest in the middle of a fragments of 4-6 kb. In the first dimension, the digested DNA is separated by gel electrophoresis according to the size, using low voltage and agarose concentration. Sample lanes are excised and, in the second dimension, gel electrophoresis is performed in a 90 degrees angle as compared to the first dimension. In the second dimension, through the addition of ethidium bromide and the use of higher voltage and agarose concentration it is possible to determine separation according to the structure of DNA. The fragment of interest is finally detected through Southern blot using a radiolabeled probe (Friedman and Brewer, 1995). The structures that can be visualized through two-dimensional gel electrophoresis include replication intermediates on linear chromosomes (Brewer and Fangman, 1987) (**Paper I** and **Paper III**), replication termination (Fachinetti et al., 2010), recombination intermediates (Branzei et al., 2006) and hemicatenane formation (Lopes et al., 2003).

In **Paper I**, DNA catenation of a reporter plasmid in the absence or presence of functional Top2 was detected using one-dimensional gel electrophoresis and Southern blot. Since the SCIs on

linear chromosomes are unstable structures that are dissolved after restriction cleavage, they cannot be detected by two-dimensional gel electrophoresis.

In **Paper I**, live cell imaging was performed to monitor chromosome segregation. Through a fluorescent marker integrated in a locus in the genome, it is possible to follow separation of sister chromatids, and subsequent segregation into the mother and daughter cells. To do so, multiple tetracycline operators were integrated at a chosen locus in the genome of a strain expressing tetracycline repressors tagged with the fluorescent marker td-Tomato. Tetracycline repressors bind to operators with high specificity. To monitor spindle elongation, tubulin was tagged with another fluorescent marker, GFP. Using a computer software, which automatically detected when the mitotic spindle reached a certain level of elongation, the timing of sister chromatid separation could be determined. Subsequently, the moment of sister chromatid segregation into the mother and daughter was timed in relation to separation.

In **Paper I** and **Paper II**, Western blot (Burnette, 1981; Laemmli, 1970; Towbin et al., 1979) was performed to detect expression level of specific proteins. Several protocols are available to extract proteins and the choice of protocol is dependent on the purpose of the study. In **Paper I**, the level of cohesin subunit Scc1 was measured in cells arrested in telophase or prometaphase in different conditions. Proteins were extracted by glass beads disruption (Dunn and Wobbe, 2001). A modification of the protocol was addition of benzonase to remove nucleic acids, since cohesin is a DNA-binding protein. In **Paper I** and **Paper II**, the phosphorylated isoform of Rad53 was measured. Phosphorylation of Rad53 represents a marker of DNA damage checkpoint activation. Here, protein extraction was performed through trichloroacetic acid (TCA)-precipitation, which is a rapid way to extract and inactivate proteins thereby maintaining the phosphorylated state of the protein (Wright et al., 1989). In **Paper I** and **Paper II**, Scc1 and phosphorylated Rad53 were detected by chemiluminescent detection. A secondary antibody conjugated to horseradish peroxidase (HRP) is allowed to interact with the specific primary antibody. In presence of chemiluminescent substrate, HRP oxidizes the substrates, which can be detected by a charge-coupled device (CCD) camera. Although Western blot is not a quantitative technique, it is often used to quantify the expression level of a protein. When comparison between samples is required, measurement of expression level of a housekeeping gene provides information about amount of sample loaded (Scc1 in **Paper I**).

3. RESULTS AND DISCUSSION

The subject of the three papers presented in the thesis is the Smc5/6 protein complex. Findings discussed below concern the chromosomal binding features of the complex, DNA binding mechanism and evidences that transcription can drive Smc5/6 localization between highly-expressed convergently-oriented genes.

3.1. PAPER I

The purpose of this study was to better understand how chromosomal localization of Smc5/6 is regulated. It was previously demonstrated that Smc5/6 localizes onto chromosomes as a consequence of DNA replication, and that the binding level increases in the absence of functional Top2 (Kegel et al., 2011; Lindroos et al., 2006). This suggested that Smc5/6 chromosomal association might be driven by SCIs, implying that proximity between sister chromatids should be required. Previous ChIP-on-chip analysis did indeed reveal that Smc5/6 binding was reduced in the absence of functional Scc2, the cohesin loading factor (Lindroos et al., 2006). However, the same study was not able to provide a clear answer concerning the requirement of cohesin itself, which seemed to affect Smc5/6 localization, but not its association. This suggested that proximity of sister chromatids is not required for Smc5/6 binding to DNA, arguing against a role of SCIs in Smc5/6 localization.

In **Paper I,** we exploited the higher sensitivity of ChIP-seq as compared to ChIP-on-chip, to clarify the role of cohesion in Smc5/6 binding. ChIP-seq and ChIP-qPCR analysis revealed that in the absence of functional Scc1 or Scc2 during S-phase, Smc5/6 binding was decreased to a similar extent, in contrast to what observed by Lindroos et al., (2006). This result indicated that the role of Scc2 in loading of Smc5/6 is not direct, but exerted through cohesin. The finding that cohesin is required for Smc5/6 binding onto chromosomes was confirmed by experiments performed in the absence of Eco1 or Pds5. The former is required for establishment of sister chromatid cohesion, the latter for its maintenance (Hartman et al., 2000b; Panizza et al., 2000; Skibbens et al., 1999), and Smc5/6 binding to DNA was significantly reduced in the absence of any of the two proteins. These results indicate that cohesin loading onto chromosomes is not sufficient to induce Smc5/6 recruitment, but cohesion establishment has to occur. In line with this assumption, Smc5/6 displayed a wild type DNA binding pattern in *wpl1eco1* double mutant, in which Eco1 becomes dispensable for cohesion formation (Rolef Ben-Shahar et al., 2008; Unal et al., 2008). Together, these results indicate that Smc5/6 binding onto DNA requires proximity of sister chromatids.

By using a no-tag control, high sensitivity of ChIP-seq allowed the identification of several false positive binding sites. This showed that Smc5/6 localization in wild-type background displayed specific binding sites at centromeres and between convergently oriented genes in the pericentromeric areas, and that Smc5/6 colocalizes with cohesin at these sites. No or low binding of Smc5/6 was detected in G1-phase, and the highest level was reached in G2/M-phase, before disappearing during anaphase. Because Smc5/6 is detected at stalled replication forks in early S-phase (Bustard et al., 2012), the idea that the complex could travel together with the fork was tested. After nucleotide depletion during S-phase, which leads to a replication block, Smc5/6 binding pattern differed from G2/M-phase, and the complex was specifically found in replicated regions of the genome.

Earlier work showed that Smc5/6 accumulation positively correlates with chromosome length, suggesting that Smc5/6 recruitment depends on replication-induced superhelical stress (Kegel et al., 2011). It has been proposed that, in budding yeast, chromosome ends can swivel off the superhelical tension (Joshi et al., 2010). Based on this observation and the new results showing that Smc5/6 specifically accumulates in the pericentromeric areas, it was investigated if the centromere–telomeres distance was correlated to Smc5/6 enrichment in a 100 kb region spanning around the centromere. This showed that the correlation increased when Smc5/6 accumulation was compared to the length of the shortest chromosome arm, as compared to full chromosome length. This result suggests that superhelical stress in the pericentromeric area is proportional to the distance between centromere and the closest telomere. We speculate that the longer this distance is, the more fork rotation might occur to reduce positive supercoils ahead of the replication fork in the pericentromeric area. This would generate more SCIs that are bound by Smc5/6.

ChIP-seq and ChIP-qPCR confirmed that inactivation of Top2 during replication triggers Smc5/6 recruitment along chromosome arms, as it was shown by Kegel et al., (2011). This new analysis also revealed that Smc5/6 binding is not altered around centromeres in *top2* mutants and that the new binding sites along chromosome arms are located between convergently oriented genes. These sites are also occupied by cohesin, and Smc5/6 binding sites triggered by Top2 inactivation were also shown to be cohesin–dependent. Cohesin binding pattern was, however, not affect by Top2 inactivation. Because Top2 resolves supercoils during both transcription and replication, it was investigated if inhibition of Top2 specifically in G1- or G2/M-phase was able to trigger Smc5/6 accumulation along chromosome arms. ChIP-seq revealed that Smc5/6 accumulation occurs only after Top2 inhibition during S-phase,

suggesting that Smc5/6 enrichment is correlated to a specific function of Top2 during replication.

Since Smc5/6 accumulates at sites of double strand breaks (DSBs) and plays a role in homologous recombination (Lindroos et al., 2006; De Piccoli et al., 2006), it was examined if recruitment of the complex after Top2 inactivation was due to double strand breaks at cohesin sites. To this purpose, Smc5/6 binding was measured in *top2* background in the absence of Mre11, which recruits Smc5/6 at DSB sites (Lindroos et al., 2006), and in the absence of Rad52, which is required for homologous recombination. None of these mutants affected Smc5/6 accumulation along chromosome arms. This, together with the finding that DNA damage checkpoint is not activated after S-phase in the absence of Top2, indicated that Smc5/6 binding on chromosome arms in *top2* mutant is not related to DSBs or homologous recombination.

Because Smc5/6 localizes at stalled replication forks (Bustard et al., 2012) and Top2 is required to promote replication termination (Fachinetti et al., 2010), it was investigated if in the absence of functional Top2 during S-phase, Smc5/6 was recruited to stalled forks and/or termination sites. ChIP-on-chip of polymerase ε did not detect any replisome on DNA in G2/M-phase when Top2 was inactivated during S-phase, and increasing fork stalling by deletion of the helicase Rrm3 (Ivessa et al., 2003) did not result in Smc5/6 accumulation onto chromosomes. Finally, no sign of replication fork stalling in G2/M-phase after Top2 inhibition during S-phase could be detected using two-dimensional gel electrophoresis.

Excluding a correlation with DSBs, homologous recombination and replication intermediates, it was examined if structures that accumulate as consequence of Top2 inhibition during replication could be resolved by restoration of Top2 function in G2/M-phase. Top2 reactivation in G2/M-phase led to the restoration of wild type binding pattern of Smc5/6 and Smc5/6 binding was not reduced after a prolonged arrest in G2/M-phase with inactivated Top2. This suggested that Top2 could resolve the structures created in its absence during replication in G2/M-phase. It was also demonstrated that restoration of Top2 function specifically in G2/M-phase could resolve SCIs between reporter plasmids, in line with requirement of Top2 during mitosis to promote segregation (Holm et al., 1985). Together this supports the notion that Smc5/6 indicates the presence of SCIs on replicated chromosomes.

To further challenge this idea, the level of missegregation in *top2* mutants was investigated. Top2 was inhibited from either G1-phase until G2/M-arrest, or when cells were arrested in G2/M-phase. In the first condition, Smc5/6 binding sites along chromosome arms was

observed, in the second condition Smc5/6 displayed wild-type binding pattern. It was demonstrated that missegregation level positively correlates with Smc5/6 accumulation after Top2 inhibition from G1-phase. Moreover, chromosomes displayed a higher level of missegregation when Top2 was inhibited from G1-phase than only in G2/M-phase. It is conceivable that the structures accumulating after Top2 inhibition are indeed SCIs, but an essay that could provide direct evidence of their presence still needs to be established.

In order to understand which function Smc5/6 could exert at SCIs, it was tested if inactivation of Smc5/6 affected missegregation in *top2* background. The experiment was performed on chromosome 1, which is short and unaffected by Top2 inactivation. It was observed that concomitant inactivation of Smc5/6 and Top2 during replication leads to a three-fold higher level of missegregation compared to a *top2* single mutant. Since the *S. pombe top2smc6* double mutant fails to remove a subset of cohesin from chromosomes in a separase-independent pathway (Outwin et al., 2009), it was investigated if missegregation was due to defects in removal of cohesin. However, no difference in overall cohesin, or cohesin bound to DNA in telophase-arrested cells, could be observed in *top2smc6* mutant compared to wild-type cells. This indicated that Smc5/6 plays a role in segregation of short chromosomes, likely promoting Top2-independent SCIs resolution. It can, however, not be excluded that the complex has a role in preventing accumulation of other linkages between entangled sister chromatids.

In conclusion, **Paper I** suggests that Smc5/6 is a marker of sister chromatid entanglements, which accumulate in response to replication-induced superhelical stress. This is in line with the observation that cohesin protects SCIs from Top2-mediated resolution (Farcas et al., 2011) and that Smc5/6 facilitates segregation of entangled sister chromatids.

3.2. PAPER II

The purpose of this study was to characterize Smc5/6 binding mechanism to DNA. It has been shown that cohesin and condensin association to DNA is controlled by ATP binding and hydrolysis (Arumugam et al., 2003; Hudson et al., 2008; Soh et al., 2015; Weitzer et al., 2003). Cohesin can function both as intra-and inter-molecular DNA linker (Fudenberg et al., 2016; Guacci et al., 1997; Hadjur et al., 2009; Haering et al., 2008), while condensin functions as intra-molecular linker only (Haeusler et al., 2008; Kimura et al., 1999). Before this study, it was unknown if Smc5/6 could act as DNA linker, and how its association to DNA is regulated.

In the first set of experiments, plasmid relaxation was observed after incubation of negatively supercoiled plasmid with purified Smc5/6. This raised the possibility that the complex either possesses topoisomerase-like activity or that there was a topoisomerase in the purified Smc5/6 fraction. Further experiments revealed that plasmid relaxation occurred independently of plasmid sequence and size. Relaxation of positive supercoils excluded that Smc5/6 could relax negative supercoils by introducing positive ones through a condensin-like activity (Kimura and Hirano, 1997). In addition to this, plasmid catenation was also observed following plasmid relaxation, 30 minutes after incubation start, indicating presence also of a Top2-like activity. Presence or absence of ATP or ATPγS, which is a non-hydrolysable ATP analog, revealed that plasmid relaxation was independent of ATP, while catenation required ATP hydrolysis. To finally determine if the topoisomerase-like activity was intrinsic of Smc5/6 or was due to contamination, the complex was purified from cells depleted of Top1, Top2 or Top3. Results indicated that sub-stoichiometric amounts of Top1 and Top2 co-purified with Smc5/6. In particular, it was demonstrated that while Top1 was a contaminant of the purification process, Top2 interacts with Smc5/6. Co-immunoprecipitation experiment confirmed that Top2 directly binds to Smc5/6 *in vivo*. It was also shown that plasmid relaxation occurred due to the contamination by Top1, while Top2 caused catenation of relaxed plasmids.

To understand if Smc5/6 played a role in Top2-dependent catenation, it was investigated if catenation activity was affected by the presence of Smc5/6. Addition of Smc5/6 purified from *top2* mutants decreased the amount of human recombinant TOP2α required to promote catenation of relaxed plasmid. These results indicated that Smc5/6 directly promotes Top2-dependent catenation. To further elucidate this novel Smc5/6 function, it was examined if ATP binding and/or hydrolysis were required for catenation. The assay required first incubation of relaxed plasmid with Smc5/6 purified from *top2* mutants, and subsequent addition of human recombinant TOP2α. Both steps were performed in presence or absence of ATP and/or ATPγS, in several combinations. Catenation formation occurred only when ATP was supplied during the first incubation of Smc5/6 with plasmid, indicating that catenation is promoted by ATPase activity of Smc5/6.

Because Smc5/6 promoted catenation specifically of relaxed plasmids, binding affinity of the complex for positively and negatively supercoiled, relaxed and linear plasmids was measured. To prevent topoisomerases activity, Smc5/6 was purified from *top1* cells, and binding affinity was measured before catenation took place. This experiment demonstrated that Smc5/6 could bind to DNA independently of DNA supercoiling state, sequence and plasmid size.

These results prompted us to investigate if Smc5/6 binding onto DNA occurred through direct interaction and/or topological entrapment. To discriminate between the two possibilities, the Smc5/6-DNA mixture was eluted through washes in presence of high salt concentration or Sodium dodecyl sulfate (SDS). High salt condition disrupts all electrostatic interactions, preserving only topological binding, while SDS treatment dissolves topological entrapment of DNA. This demonstrated that Smc5/6 topologically entraps circular plasmids, while direct interaction was observed on linear plasmids, indicating that the complex interacts with DNA through both binding mechanisms. To understand the role of ATP in Smc5/6 binding to DNA, the reaction was performed in the absence of ATP or in presence of ATPγS. This provided results indicating that ATP hydrolysis, but not binding, was required for topological entrapment. To explain this contradictory result, it was hypothesized that Smc5/6 was purified in ATP-bound state, thereby allowing a first round of DNA binding in the absence of ATP in the reaction. To test this, a Smc5/6 complex containing a *SMC6* mutant allele in Walker A motif (*SMC6K115E*), which is predicted to abolish ATP binding, was analyzed (Arumugam et al., 2003; Fousteri and Lehmann, 2000). This mutation decreased ATPase activity compared to wild-type Smc5/6 complex, despite the presence of a functional ATP-binding site on Smc5. Additional experiments using this mutant confirmed that ATP binding is required for efficient association of the complex onto DNA *in vitro*. This result was further strengthen by ChIP-qPCR experiment on Smc6K115E mutant cells, which revealed that the mutant complex displayed low level of chromosomal binding *in vivo*. Finally, ATP binding to the complex was shown to be required for efficient stimulation of TOP2α-dependent catenation. Together, these results indicate that functional ATP binding site of Smc6 is required for both direct binding to, and topological entrapment of DNA, and for the complex to bridge two DNA molecules and promote catenation.

In the last set of experiments, it was investigated if ATP binding to Smc6 was required for cell growth. This was achieved by inducing overexpression of Smc6K115E in cells also expressing wild-type Smc6. In this way, it was conceivable that cells would accumulate mutated version of the complex. Smc6K115E overexpression, but not of the wild type protein, inhibited cell growth, indicating that ATP binding to Smc6 indeed is required for proper cell growth. The inhibition was likely due to the accumulation of DNA damage, since phosphorylation of Rad53 was observed 24 hours after Smc6K115E overexpression. These results indicate that in the absence of ATP-bound Smc6, cells undergo DNA damage, checkpoint activation and cell death. *smc6* mutants have been shown to enter anaphase without completing replication (Torres-Rosell et al., 2007) and eventual chromosome breakage during segregation activates DNA damage checkpoint (Torres-Rosell et al., 2005). This could explain chromosome

aberrations, DNA damage checkpoint activation and cell death observed in Smc6K115E mutants.

This study shed light on the crucial roles of ATP binding and hydrolysis in Smc5/6 association to DNA. In particular, ATP binding is required for direct interaction with DNA, and ATP hydrolysis is needed for topological entrapment of DNA and to promote Top2-dependent catenation activity. This suggests a mechanism for Smc5/6 binding to DNA that in a first step requires direct ATP-dependent interaction, and subsequently ATP hydrolysis allows topological entrapment. The ability of Smc5/6 to promote Top2-dependent catenation suggests that the complex can tether two DNA molecules together. A low level of DNA catenation was detected when the complex was mixed with monomeric plasmids. This suggests that the complex acts as inter-molecular DNA linker like cohesin, rather than working intra-molecularly like condensin. The mechanism by which Smc5/6 can entrap DNA remains, however, elusive. To reveal this, detailed structural analysis of the complex is required.

The biological reason for the ability of Smc5/6 to promote Top2-dependent catenation *in vitro* remains unanswered. Both Smc5/6 and Top2 localize at replication forks (Bermejo et al., 2007; Jeppsson et al., 2014) and the catenation activity observed *in vitro* could therefore indicate that Smc5/6 promotes Top2-dependent SCIs formation. On the other hand, absence of Smc5/6 or Top2 leads to unresolved linkages between sister chromatids, indicating a role in SCIs resolution (Gallego-Paez et al., 2014; Wang et al., 2008). To fit these observations in the model proposed by Kegel et al., (2011) and Jeppsson et al., (2014) the following can be envisaged. Smc5/6 first promotes SCIs formation by stimulating Top2-dependent catenation behind the replication machinery. This facilitates fork rotation, and thus reducing the level of replication-induced supercoils. Finally, prior to anaphase, both Smc5/6 and Top2 are needed to remove all SCIs to allow proper sister chromatid segregation.

3.3. PAPER III

This study had the purpose to further investigate the hypothesis that Smc5/6 is recruited at SCIs in response to superhelical stress during replication, presented in **Paper I**. It has been shown that positive supercoils are generated ahead of transcription machineries as a consequence of DNA unwinding (Liu and Wang, 1987). Superhelical stress also accumulates on plasmids in response to strong convergent transcription (García-Rubio and Aguilera, 2012). Aiming for a direct evidence that superhelical stress drives Smc5/6 localization onto chromosomes, we measured by ChIP-seq and ChIP-qPCR if Smc5/6 accumulated at a site where superhelical

stress was specifically increased. To do so, wild-type promoters of two convergently-oriented genes were replaced by strong constitutive ones. Before replacement of the promoters, it was ensured that cohesin, but not Smc5/6, was enriched at the intergenic region between the two chosen genes, *MCR1* and *DBR1*. ChIP-qPCR analysis showed that Smc5/6 accumulates specifically at the intergenic region when the two genes are overexpressed.

Two-dimensional gel electrophoresis was used to inquire if the increase of transcription altered replication fork progression. It was shown that replication fork is paused during mid-S-phase when *MCR1* and *DBR1* are overexpressed. Since Smc5/6 localizes at stalled replication forks (Bustard et al., 2012), it was possible that Smc5/6 accumulation at the intergenic region could be dependent on the fork pausing event. To challenge this idea, Smc5/6 binding level was measured in the absence of the pausing factor Tof1, which promotes replication fork pausing (Mohanty et al., 2006). This showed that Smc5/6 binding was unchanged in the absence of Tof1. To further test if Smc5/6 enrichment and replication fork pausing are unrelated events, replication pattern and Smc5/6 binding were measured when *MCR1* and *DBR1* were overexpressed one at the time. The results showed that *DBR1* overexpression is sufficient to pause the replication fork, while Smc5/6 accumulation requires convergent transcription. This confirmed that Smc5/6 accumulation was not dependent on replication fork pausing. It also showed that replication fork pausing was due to head-on collision between the replication and transcription machineries progressing in opposite directions, as it was previously demonstrated on plasmid (Prado and Aguilera, 2005).

Because Smc5/6 has been implicated in preventing accumulation of recombination intermediates generated during template-switching of post-replicative repair (Choi et al., 2010), it was investigated if the complex was recruited also in the absence of Mms2, which is an ubiquitin conjugating-like enzyme required for this pathway (Broomfield et al., 1998). ChIP-qPCR analysis revealed that Smc5/6 binding remains unaltered in *mms2Δ* cells, indicating that template switching is not triggering Smc5/6 accumulation at the intergenic region.

In **Paper I** it is shown that Smc5/6 recruitment onto chromosomes requires cohesion and increases along chromosome arms after Top2 inhibition during replication (Jeppsson et al., 2014). Therefore, it was decided to characterize Smc5/6 enrichment between *MCR1* and *DBR1*, investigating how the binding is affected in the absence of functional cohesin or Top2. Surprisingly, Smc5/6 recruitment at the intergenic region is independent of cohesin and is markedly reduced after Top2 inactivation during S-phase, indicating a different regulation of Smc5/6 localization as compared to the replication-induced binding. Reduced levels of cohesin at the site of convergent transcription were also detected when *MCR1* and *DBR1* were

overexpressed, which contrasts with the hypothesis that cohesin is pushed by transcription to the intergenic region between convergently oriented genes (Lengronne et al., 2004).

These results indicate that Smc5/6 recruitment between *MCR1* and *DBR1* does not follow any feature of the known replication-induced DNA binding. Therefore, we decided to elucidate if Smc5/6 recruitment was dependent on superhelical stress generated by transcription alone. Because Smc5/6 binding triggered by replication reaches a peak in G2/M-phase and is not detected in G1 (Jeppsson et al., 2014), an inducible system was used to create high convergent transcription specifically in G1-, S- or G2/M-phase. It was observed that Smc5/6 is recruited at the intergenic region during all cell cycle phases tested, suggesting that high convergent transcription is able to directly recruit Smc5/6. It was however noticed that when strong convergent transcription was active during replication, higher Smc5/6 enrichment was observed, suggesting that replication still contributes partially to Smc5/6 recruitment at the site of convergent transcription.

These results open several speculative scenarios concerning the role of Smc5/6 at the site of high convergent transcription. Recently it was demonstrated that Smc5/6 binds to episomal DNA in cohesin-independent way and acts as transcriptional repressor only when the investigated gene is present in the plasmid DNA. The complex had no effect on transcription of the gene when it was integrated into chromosomal DNA (Decorsiere et al., 2016; Murphy et al., 2016). Possibly, superhelical stress accumulates to a higher extent on closed circular plasmids than on linear chromosomes, which could explain why Smc5/6 specifically acts on episomal DNA. Strong convergent transcription between *MCR1* and *DBR1* could generate an exceptionally high accumulation of superhelical stress, which normally is not found on the linear chromosomes of budding yeast. At this site, Smc5/6 could downregulate transcription to prevent excessive accumulation of positive supercoils in the intergenic region. Top2, which is required to maintain genome stability between convergently-oriented genes (Pannunzio and Lieber, 2016) and physically interact with Smc5/6 (**Paper II**), could play a role in recruiting Smc5/6 at this site. To challenge this hypothesis, *MCR1* and *DBR1* transcription levels need to be measured after Smc5/6 inactivation. A technique able to measure the level of supercoiling on linear chromosomes is also needed to inquire if Smc5/6 is recruited at sites characterized by exceptionally high superhelical stress.

This project suggests that Smc5/6 recruitment could be triggered by transcription-induced superhelical stress. The chromosome structure recognized by the complex remains unknown. It is conceivable that positive supercoils formed ahead of the transcription machinery create a structure in the intergenic region that resembles SCIs, i.e. a right-handed DNA crossing.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

4.1. SUMMARY OF THE FINDINGS

This thesis increases the understanding of the DNA binding mechanism of Smc5/6, the control of Smc5/6 chromosomal association and the role of superhelical stress in this process.

Our data suggest that Smc5/6 is recruited at sites of entanglement between sister chromatids after cohesion has been established (**Paper I**). Smc5/6 binds to DNA through both direct interaction and topological entrapment (**Paper II**). This suggests a two-step mechanism of Smc5/6 loading, which involves direct electrostatic interaction of the complex with DNA, and subsequent ATP hydrolysis, which leads to topological entrapment (**Paper II**). We demonstrated that Smc5/6 is able to entrap two DNA molecules and promote Top2-mediated catenation *in vitro* (**Paper II**). This function could reflect the ability of the complex to bind inter-molecularly the two sister chromatids and promote Top2-dependent SCIs formation *in vivo*. In **Paper III**, we demonstrated that Smc5/6 is recruited at sites of high convergent transcription, possibly in response to high superhelical stress that accumulates between the two genes.

Altogether, these results indicate a complex role of Smc5/6 in the regulation of genome stability. In the following section, future investigations, open questions and the importance of SMC complexes in maintenance of genome stability are discussed.

4.2. SMC5/6 AND COHESION

In **Paper I,** it was shown that Smc5/6 is found at cohesin-binding sites in the pericentromeric areas and that chromosomal association occurs after cohesion has been established. This indicates that cohesin binding to chromosomes in late G1-phase is not sufficient to recruit Smc5/6, but Smc3 acetylation by Eco1 is also required. This opens for the possibility that acetylated Smc3 recruits Smc5/6 to chromosomes and, to determine this, ChIP analysis of acetylated Smc3 should be performed. Although the wild-type binding pattern of Smc5/6 in *wpl1eco1* mutant argues against the acetylation-mediated recruitment model, sister chromatid cohesion as such appears to be an essential determinant for Smc5/6 chromosomal association (**Paper I**). If true, cohesion establishment occurs only in the pericentromeric areas in wild-type cells, and Top2 inhibition promotes cohesion along chromosome arms. Alternatively, Smc5/6 is found only at cohesin sites where also SCIs are present.

In line with Smc5/6 recruitment after S-phase, it has been shown that cohesion establishment occurs during replication (Uhlmann and Nasmyth, 1998), and Eco1 is associated to the replication fork (Lengronne et al., 2006). In human cells in which acetylation of cohesin is inhibited, the speed of the replication fork is significantly decreased (Terret et al., 2009). It is intriguing to speculate that this effect is due to the absence of Smc5/6 on chromosomes, which could lead to excessive accumulation of replication-induced superhelical stress (**Paper I**; Kegel et al., 2011).

4.3. SCI: INTERPLAY BETWEEN SMC5/6, COHESIN, TOP2

SCIs formation occurs during replication as consequence of replication fork rotation (Champoux and Bean, 1980), and Top2 is essential for the resolution of entanglements (Baxter et al., 2011; Holm et al., 1985). It has also been shown that sister chromatid cohesion is required for SCIs maintenance, but not for their formation (Farcas et al., 2011). Another study reports that proximity of sister chromatids promotes Top2-mediated SCIs formation in prometaphase-arrested cells (Sen et al., 2016). It remains unclear if cohesin contributes to Top2-dependent catenation by maintaining cohesion, if it protects SCIs from Top2-mediated resolution, or both. Even though it has been shown that cohesin can maintain cohesion in the absence of SCIs (Farcas et al., 2011), it is still possible that cohesin-dependent SCIs stabilization/formation contribute to the stability of sister chromatid cohesion.

In **Paper II**, it is shown that Smc5/6 promotes Top2-dependent catenation *in vitro* (Kanno et al., 2015), arguing that Smc5/6 could play a similar role *in vivo*. This is further supported by the observation that a *smc6-56* mutant decreases the level of plasmid catenation after Top2 inhibition (Kegel et al., 2011). However, a *smc6-9* mutant did not lead to a similar decrease in the level of SCIs (Farcas et al., 2011). This discrepancy could be due to the use of different *smc6* alleles, which display different level of Smc5/6 inhibition. In addition to a role in promoting SCI formation/stability, it has also been shown that the enrichment of Smc5/6 on chromosomes correlates with the level of sister chromatid missegregation in *top2* mutants (**Paper I**). This raises the possibility that Smc5/6 not only facilitates SCIs formation during replication, but also promotes their resolution in a Top2-independent manner. To investigate further SCIs dynamics and positioning, a technique able to visualize these structures has to be developed.

4.4. SMC5/6 AND TRANSCRIPTION REGULATION

In **Paper III,** we show that strong convergent transcription recruits Smc5/6 onto chromosomes, and we speculate that transcription-induced superhelical stress could be the underlying reason for this. Possibly, Smc5/6 could negatively regulate transcription to prevent excessive accumulation of superhelical stress at these sites. Since superhelical tension on linear chromosomes cannot be measured directly, the following experiments could be performed to challenge this idea. The convergent transcription model system with promoters of different strength, genes of different length or at different distances to telomeres could provide further indications that Smc5/6 recruitment is proportional to the increase of superhelical tension. Since supercoil accumulation is expected to be proportional to transcription levels, promoters of different strength would proportionally affect Smc5/6 binding. Longer genes (more than 3 kb) have been shown to trigger higher levels of positive supercoils during transcription (Joshi et al., 2012). Finally, since chromosomal ends can rotate to dissipate superhelical tension (Joshi et al., 2010), placing the convergent transcription site in the proximity of a telomere would lead to a decrease in Smc5/6 accumulation.

Another interesting aspect of Smc5/6 recruitment in response to transcription-induced superhelical stress is the decrease in binding of the complex after inhibition of Top2 during the preceding replication (**Paper III**). This result is in striking contrast to the increase of Smc5/6 binding along chromosome arms after Top2 inactivation during S-phase (**Paper I**). Since Smc5/6 and Top2 physically interact (**Paper II**), and Top2 is required to maintain genome stability between convergently oriented genes (Pannunzio and Lieber, 2016), it is possible that Top2 recruits Smc5/6 (or vice versa) at sites of transcription-dependent superhelical tension to downregulate transcription, preventing chromosomal instability. The following experiments are required to validate this model. Top2 localization at sites of strong convergent transcription must be analyzed. Furthermore, gene expression level after Smc5/6 inactivation, Smc5/6 binding after Top2 inactivation in G1- or G2/M-phase, and Smc5/6 binding after re-activation of Top2 function have to be determined.

4.5. SMC COMPLEXES IN DISEASE

Because of the crucial role in maintaining genome stability, deregulation of the SMC complexes can lead to cancer and developmental diseases. Mutation in the cohesin loader NIPBL (human homolog of budding yeast Scc2) is responsible of Cornelia de Lange syndrome, which is characterized by short stature, moderate or severe intellectual disability, distinct facial

features and abnormalities in upper limbs (Krantz et al., 2004; Tonkin et al., 2004). Mutations in cohesin subunits SMC1A and SMC3 are involved in chromosome instability in colorectal cancer (Barber et al., 2008) and changes of RAD21 expression level (human homolog of budding yeast Scc1) have been linked to breast cancer (Atienza et al., 2005; Yun et al., 2016). Cohesin-interacting proteins are also overexpressed in several kinds of cancer: WAPL in cervical cancer (Oikawa et al., 2004), separase in breast cancer (Zhang et al., 2008b), osteosarcoma ad prostate cancer (Meyer et al., 2009), ESCO2 in melanoma (Ryu et al., 2007) and Securin in pituitary tumor (Zou et al., 1999).

Smc5/6 has been involved in Alternative Lengthening of Telomeres (ALT) pathway in cancer cells, which occurs through homologous recombination. Shortly, in cancer cells that elongate telomeres through ALT, Smc5/6 associates to the so-called ALT-associated promyelocytic leukemia nuclear bodies (APBs). APBs are responsible for ALT-mediated telomere lengthening and Mms21-dependent SUMOylation of APBs subunits is crucial for both APBs formation and activation (Chung et al., 2011; Potts and Yu, 2007). Mutation of the hSMC5/6 subunit NSMCE3 (homolog of Nse3) has been linked to an autosomal recessive chromosome breakage syndrome characterized by immunodeficiency and pulmonary disease (van der Crabben et al., 2016). Finally, mutations in NSMCE2 (homolog of Nse2/Mms21) have been identified in primordial dwarfism and insulin resistance (Payne et al., 2014) and mutations in SMC5 have been discovered in human brain metastasis (Saunus et al., 2015).

4.6. AN OPEN ENDING

Despite the increasing interest to reveal the role of Smc5/6 in unchallenged cells, the understanding of this complex is still at its infancy. Initially, it was discovered that Smc5/6 could prevent the deleterious effects of homologous recombination, because inhibition of this pathway improved viability of *smc6* mutants in budding yeast (Torres-Rosell et al., 2005). Even if an interesting observation, this could not be the pivotal role of Smc5/6 because homologous recombination is not essential in this yeast (Paques and Haber, 1999). It was also observed that Smc5/6 promotes segregation of the rDNA region (Torres-Rosell et al., 2005), but *smc6* mutant viability was not improved by rDNA relocalization to a multicopy plasmid from the endogenous location on chromosome 12 (Torres-Rosell et al., 2005). Not even the replication delay of long chromosomes observed in *smc6* mutant reflects the pivotal role of Smc5/6, since the same delay was observed in *top1* and *mms21-CH* mutants, which are viable (Kegel et al., 2011).

A possible way to investigate Smc5/6 essential function is to identify genes that suppress lethality of Smc5/6 mutants. For example, recently it was discovered that deletion of the helicase Mph1 can partially suppress defects of Smc5/6 mutants, including accumulation of recombination intermediates (Chen et al., 2009). *In vitro*, Mph1 is involved in replication fork regression and Smc5/6 counteracts this function (Xue et al., 2014). Possibly, cells undergo aberrant fork regression, which generates toxic DNA structures in the absence of Smc5/6, and Mph1 deletion abolishes the formation of these structures (Chen et al., 2009).

The new information concerning Smc5/6 chromosomal localization presented in this thesis (**Paper I**) also provide insights into the function of the complex. Identification of Smc5/6 binding sites gives the possibility to identify novel proteins that colocalize with Smc5/6 and/or affect its chromosomal association, or are regulated by the complex. The discovery that Smc5/6 topologically entraps more than one DNA molecule and promotes Top2-mediated catenation (**Paper II**) suggests that the complex can entrap sister chromatids and promote SCIs formation. This function needs to be confirmed on linear chromosomes *in vivo*, but again, this requires a technique that allows SCIs visualization. Finally, we propose that Smc5/6 localization can be driven by strong convergent transcription on chromosomes (**Paper III**), which might open a new field for investigations on Smc5/6-mediated transcription regulation.

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