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THE PUZZLING SMC5/6 COMPLEX

PIECING TOGETHER FUNCTIONS IN SEGREGATION AND REPAIR

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The cover shows a still image of live cells merged with EGFP-tubulin and a td-Tomato fluorescent region 35 kb from centromere of chromosome V.

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The Puzzling Smc5/6 Complex

Piecing Together Functions in Segregation and Repair

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Public defense of thesis for doctoral degree at the Andreas Vesalius lecture hall.
Berzelius väg 3, Karolinska Institutet Solna.
Friday the 29th of May at 9.00 am.

Dedicated to the loving women in my life.

In memory of my mother, for all your sacrifices made in the past.

To my wife, for your care and support at the present.

For my daughter and our bright future.

Thank you for helping me to reach this moment.

ABSTRACT

Genome stability is essential in order for cells to survive. During their life cycle, parental cells must divide and properly allocate their genetic material to daughter cells. To ensure correct distribution, multiple proteins are involved in replicating and segregating the genome. Even under unchallenged conditions, replication and segregation errors can occur frequently but are in most cases efficiently repaired. Replication, segregation and repair are all fundamental processes that are under tight control. Failure to correctly execute these processes leads to genomic instability, which can drive tumor development. The structural maintenance of chromosomes 5/6 (Smc5/6) protein complex is involved in all of these fundamental processes and is therefore considered to be an important guardian of genome stability. However, the mechanism of Smc5/6 function in these processes remains to be determined. The diversity of Smc5/6 functions in chromosome replication, segregation and repair has puzzled researchers since the discovery of the complex in the 1990's. The aim of this thesis was to explore novel functions of the Smc5/6 complex in DNA repair and chromosome segregation in the budding yeast *Saccharomyces cerevisiae*.

We first studied the Smc5/6 complex role in DNA repair, the results of which are presented in **paper I**. We drew the conclusion that modification of the Smc5/6 subunit Mms21 by phosphorylation was needed for its full SUMO ligase activity. Phosphorylation of Mms21 was dependent on the Mec1 kinase, a checkpoint protein sensor that phosphorylate substrates in response to DNA damage. Two targets of Mms21 SUMOylation were investigated, and both were SUMOylated at a reduced level without Mms21 phosphorylation. In the presence of DNA damage, Mms21 phosphorylation was important for maintaining genome stability.

In **paper II**, we focused on chromosomal association of the Smc5/6 complex. Smc5/6 associated to DNA in a cohesion-dependent manner during S-phase, followed replication fork progression, and accumulated in the G2/M-phase. Moreover, Smc5/6 binding along chromosome arms increased in the absence of Topoisomerase II (Top2) activity, the lack of which is known to accumulate sister chromatid intertwinings (SCIs). The level of Smc5/6 enrichment predicted the missegregation pattern of Top2 mutants, suggesting that Smc5/6 binds SCIs. In addition, we observed that Smc5/6 promoted segregation of chromosomes that accumulated SCIs in the absence of functional Top2. This led to the conclusion that the Smc5/6 complex is likely to bind SCIs and to facilitate their resolution.

In **paper III,** we further investigated the role of Smc5/6 in chromosome segregation. This study revealed that Smc5/6 mutants activated the spindle assembly checkpoint (SAC) in a Mad2-dependent pathway. Together with earlier and present investigations in our lab, we speculate that Smc5/6 mutants delay replication of especially longer chromosomes, which hinders proper kinetochore-microtubule attachment. This in turn activates the SAC and halts the cell cycle.

These results will add more pieces to the puzzle to build a more comprehensive picture of Smc5/6 function.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following scientific papers.
They will be referenced in the text by their Roman numerals.

I. **Kristian K. Carlborg**, Takaharu Kanno, Sidney D. Carter and Camilla Sjögren

"Mec1-dependent phosphorylation of Mms21 modulates its SUMO ligase activity"

DNA Repair (2015) doi: 10.1016/j.dnarep.2015.01.006

II. Kristian Jeppsson, **Kristian K. Carlborg**, Ryuichiro Nakato, Davide G. Berta, Ingrid Lilienthal, Takaharu Kanno, Arne Lindqvist, Maartje C. Brink, Nico P. Dantuma, Yuki Katou, Katsuhiko Shirahige and Camilla Sjögren

"The chromosomal association of the Smc5/6 complex depends on cohesion and predicts the level of sister chromatid entanglement"

PLoS Genetics (2014) doi: 10.1371/journal.pgen.1004680

III. **Kristian K. Carlborg**, Sidney D. Carter and Camilla Sjögren

"Mutants of the Smc5/6 complex trigger a Mad2-dependent checkpoint arrest"

Manuscript

CONTENTS

LIST OF SELECTED ABBREVIATIONS

CONCEPTS

PTMs	Post-translational modifications
SUMO	Small ubiquitin-like modifier
MMS	Methyl methanesulfonate
WB	Western blotting
DSB	Double-strand break
SCIs	Sister chromatid intertwinings
ChIP	Chromatin immunoprecipitation
ts	Temperature sensitive
WT	Wild-type
SAC	Spindle assembly checkpoint
CEN	Centromere
LCI	Live cell imaging

PROTEINS

Smc1	Stability of minichromosomes 1
Smc2-6	Structural maintenance of chromosomes 2-6
Nse1-6	Non-Smc element 1-6
Mms21	Methyl methanesulfonate sensitivity 21
PIKK	Phosphatidylinositol 3-kinase-related kinase
Mec1	Mitotic entry checkpoint 1
Tel1	Telomere maintenance 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
Top2	Topoisomerase II
Scc1, 3	Sister chromatid cohesion 1, 3
Mad2	Mitosis arrest deficient 2

Chapter 1
MOLECULES OF LIFE, THE DNA

All forms of life are made up of cells, from small unicellular organisms like yeasts to larger multicellular organisms like humans. Cells come in a great variety of different sizes and have many various properties. The common denominator for them all is their ability to divide and to forward their genetic material, encoded by the deoxyribonucleotide acid (DNA) molecule. The DNA is a long double-stranded molecule and its backbone is made up by two chains of alternating sugar and phosphate groups. Attached to each sugar residue is one of four different bases: adenine (A), cytosine (C), guanine (G) and thymine (T). These four bases are able to interact with one specific partner on the other strand, A with T, and G with C. The paired bases form double-strand stretches that run antiparallel to one another and twist around its own axis, to give the DNA its characteristic double helical structure (Watson and Crick, 1953).

Every human cell contains almost 2 meters of DNA in its nucleus, which is equivalent of a chain with several millions of paired bases. This is an incredible feat, considering that the average nucleus diameter is no more than a couple of micrometers. DNA compaction is accomplished by an elaborate packing process where the DNA is tightly organized into structures called chromosomes **(Figure 1)**. Some organisms have a single chromosome that constitutes their entire genetic material, such as bacteria. Other organisms have several different chromosomes present in one copy; these are known as haploid organisms. Other species such as the entire animal phylum have two copies of each chromosome, they are known as diploid organisms.

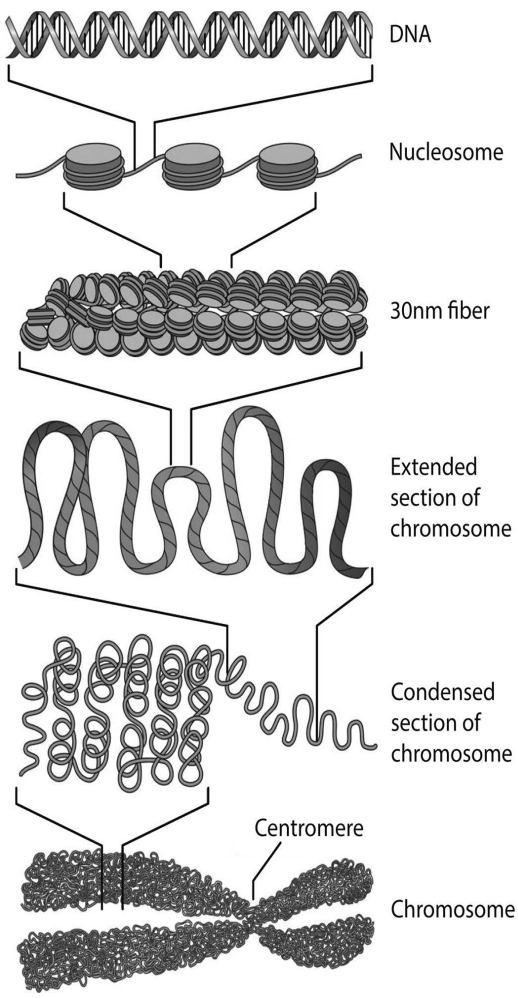

Figure 1. DNA compaction into chromosomes (Jansen and Verstrepen, 2011).

As cells grow and divide, daughter cells must be able to inherit the same genetic material as the mother cell. This means that it is necessary for the chromosomes to be copied and correctly distributed between their progeny. In order to support cellular viability, all these

processes must be fulfilled during a typical cell cycle **(Figure 2)**. The cell cycle starts with a growth phase called gap 1 (G1), which is followed by the synthesis (S) phase. In S-phase, chromosomes are duplicated and form sister chromatids in a process termed replication. Once the chromosomes have been replicated the cells go through a secondary gap phase (G2). The final cell cycle phase is called mitosis (M). At this stage the sister chromatids are separated to opposite poles within the cell, which is then cleaved in two parts in a process called cytokinesis. Thereby, two genetically identical copies of the original cell are created. The process of separating the duplicated chromosomes in two daughter cells is called chromosome segregation. Once segregation and cytokinesis is completed the cell cycle starts over again.

The results in this thesis are based on experiments performed with the budding yeast *Saccharomyces cerevisiae* as a model organism, which is further described in chapter 5. The budding yeast nomenclature of gene and protein names is used in the following chapters unless otherwise stated.

Replication

It is a challenging task to access the highly packed DNA-strands prior to entering S-phase. During this time the DNA must be unwound and the double-stranded DNA (dsDNA) must temporarily become single-stranded (ssDNA) in order for replication to take place. Once all

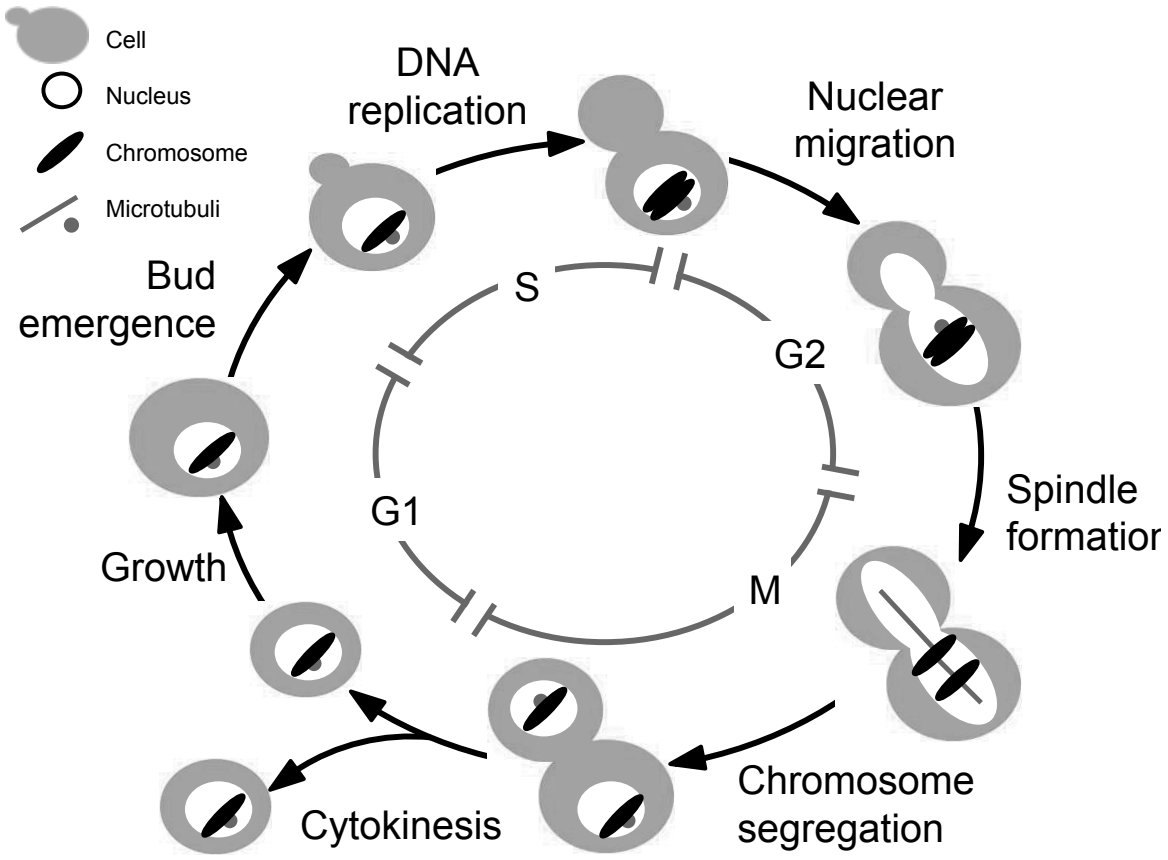

Figure 2. Cell cycle of the budding yeast *S. cerevisiae*.

chromosomes have been replicated, cells can proceed in the cell cycle.

DNA replication is initiated at distinct sites on chromosomes called replication origins. Bacterial chromosomes often have a single origin while chromosomes of most other species have many. Origins are generally spaced several thousands of base pairs apart in an uneven pattern, which typically contain specific nucleotide sequences that are rich in A and T bases. The reason for this is thought to be because A and T are bound together by only two hydrogen bonds and thus easier to break in comparison to G and C which interacts via three hydrogen bonds. The sequence of replication origins is identified by the origin recognition complex (ORC), which is required for initiation of replication (Romanowski et al., 1996, Grallert and Nurse, 1996).

The ORC is used as an anchor for the MCM helicase, which is loaded by specific factors (Tanaka et al., 1997, Maiorano et al., 2000, Devault et al., 2002). The MCM helicase is composed of several subunits and its main function is to unwind the DNA double helix so that the single strands become accessible. This action is not activated until the ORC also recruits polymerase δ, polymerase ε and replication factor C (RFC), which in turn is responsible for loading the proliferating cell nuclear antigen (PCNA) onto DNA (reviewed in Baker and Bell, 1998). The PCNA is associated to the MCM helicase, with the main purpose to act as a platform that loads the polymerases δ and ε onto DNA. The protein complex with MCM helicases, PCNA and DNA polymerases are referred to as the replication fork. As the helicases unwind the DNA helix and thereby exposing the single strands, the PCNA acts as a scaffold that follows behind and holds the DNA polymerases δ and ε that perform the actual copying of the single strands (Moldovan et al., 2007).

When attempting to copy the entire genome, the replication fork encounters many problems that might cause it to stall. Naturally, replication forks moving in opposite directions can cause fork stalling. Presence of DNA damage during replication can be another reason. In both cases, stalled forks activate an intra S-phase checkpoint that halts the entire cell cycle in order to assess the problem (Tercero et al., 2003). The role of checkpoints for cell cycle progression is further described in chapter 3.

Once replication has finished it is crucial that the products of replication, known as sister chromatids, are held together until segregation in mitosis. Otherwise sister chromatids are separated prematurely, which cause failure to evenly distribute the genetic material. The sister chromatids are held together by cohesin (Michaelis et al., 1997, Guacci et al., 1997), a protein complex made up of four subunits. It is proposed that cohesin encircles both sister chromatids and thereby physically holds them together (Haering et al., 2008). The cohesin complex will be further described in more detail in chapter 4.

Segregation

Following replication, the cells go through a second preparation phase before entering mitosis. At mitosis, the genetic material of the two replicated chromatids must be equally

divided between the mother and daughter cells in order to sustain viability. This process is called chromosome segregation.

Mitosis is split into several phases: prophase, metaphase, anaphase and telophase. As cells enter mitosis in prophase, chromosomes get compacted by the protein complex condensin. Animal chromosomes become highly condensed and are fully visible under a microscope. The chromosomes are also held together by cohesin along their entire length. In budding yeast, all cohesin stays on chromosomes until anaphase onset. In humans, cohesin is removed along the arms of the chromosomes during prophase, leaving only a cohesed region where the sister chromatids meet. This region is called the centromere, attached to it are up to 80 proteins, among them the so-called centromere proteins (CENP) (Balczon and Brinkley, 1987, Cheeseman and Desai, 2008).

CENP and other proteins together form the kinetochore structure that serves as a platform for microtubule attachment. In budding yeast, microtubules are attached to the kinetochore already during replication. In human cells, this occurs at the end of prophase and beginning of metaphase. Microtubules are highly dynamic tubes of protein that emerges from the centrosome, an organelle that is associated to the nuclear membrane. The equivalent organelle in budding yeast is the spindle pole body. In mitosis the centrosome has been copied and is located at opposite poles of the cell. Chromosomes have two kinetochores, one on each sister chromatid. Microtubules emerging from one centrosome attach to the kinetochore of one sister chromatid while microtubules emerging from the centrosome at the opposite pole attach to the homologous chromatid. This is called bipolar attachment and is a requirement for mitosis to proceed. At the end of metaphase, chromosomes are subjected to tension that is created by the pulling forces of attached microtubules directed towards each pole. These pulling forces are opposed by the cohesed sister chromatids. The tug of war between kinetochore microtubules pulling sister chromatids apart and cohesin holding them together aligns the chromosomes to the middle of the cell. This is referred to as the metaphase plate.

In anaphase, the sister chromatids are released from cohesin entrapment and pulled to opposite cell poles. Synchronously the cell itself is also elongated by polar microtubules, these are attached to both centrosomes at opposite poles and not to kinetochores. In budding yeast, the daughter cell emerges as a bud from the mother cell and is not elongated by polar microtubules. In telophase, the sister chromatids migrate to opposite ends of the cell together with an accompanying centrosome. Mitosis is rounded off by cytokinesis, the process where the elongated cell or budneck is cleaved in the middle, which thereby finalizes the process of creating two genetically identical daughter cells.

Topology

When the replication machinery needs to access single strands the DNA helix is unwound by helicases. The two strands of the DNA helix can be considered as two long ropes that are twisted around each other. The replication process can be illustrated by trying to pull apart the

intertwined ropes. As a result the ropes will coil up if they are long enough and can therefore only be separated to a certain extent. To further complicate things, the ropes might get entangled in themselves causing knotting and catenation. Catenation is a state where the two ropes cannot be completely separated without cutting one. DNA coiling, knotting and catenation are referred to as topological stress and are discussed under the topic of DNA Topology **(Figure 3)**.

The nature of the intertwined strands of the DNA helix causes topological problems. During the process of replication, chromosomes become overwound due to the opening of the DNA helix. This in turn causes topological problems by accumulation of supercoils further ahead on the DNA molecule. These problems must be resolved in order for replication to finish and to allow correct segregation of chromosomes. To counteract buildup of topological problems, specialized proteins called topoisomerases aid the progression of the replication fork and facilitate segregation. Topoisomerases possess enzymatic activity, which enables them to cut the DNA in order to remove accumulated tension (Champoux, 2001).

There are two classes of topoisomerases. The first class is the type I topoisomerases, consisting of Top1 and Top3 in budding yeast, which act on DNA by cutting one of the strands (DNA nicking). The strand that has been cut can then rotate in the appropriate direction around the uncut strand. In this way

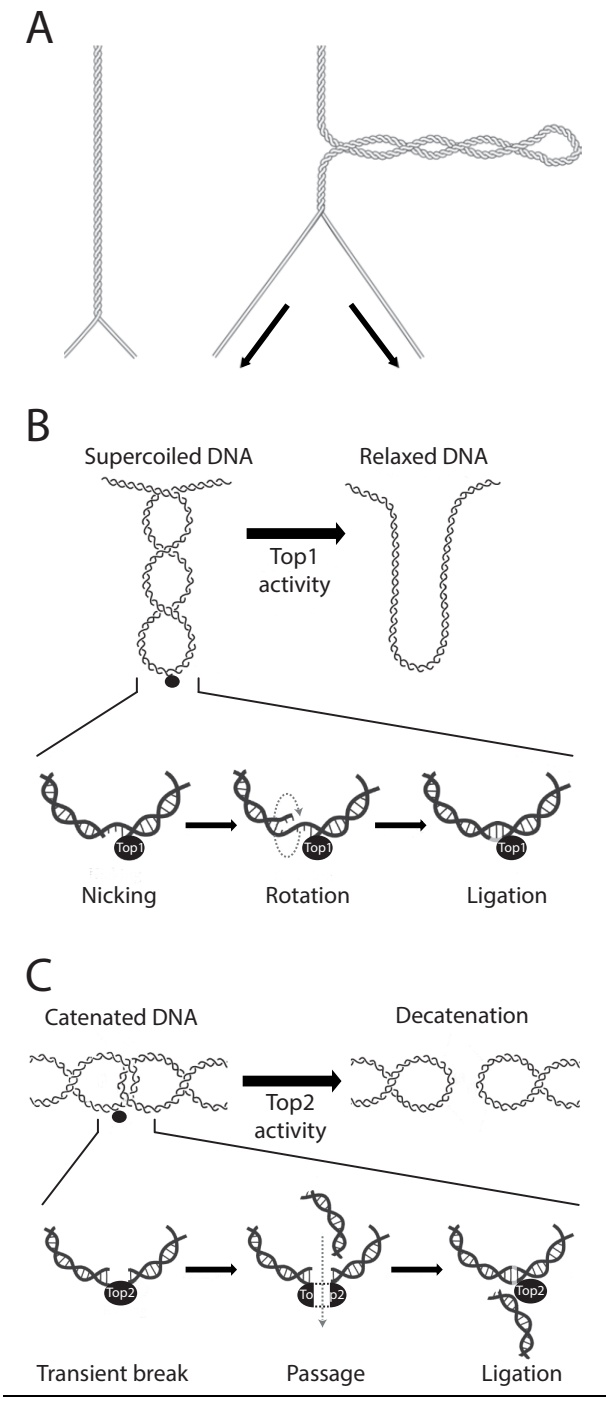

Figure 3. DNA topology. **(A)** Accumulation of DNA supercoiling illustrated by two intertwined ropes that are being pulled (Carter and Sjogren, 2012). **(B)** Topoisomerase I resolve DNA supercoiling by nicking one DNA strand, which allows rotation around its axis (Adapted from Pommier, 2006). **(C)** Topoisomerase II resolves catenated DNA by introducing a transient break, which allows passage of one part of the DNA molecule through another.

overwound DNA, caused by replication fork progression, can be relaxed from having too many or too few twists in the DNA helix. Once relaxation has been achieved, type I topoisomerases can re-ligate the cut DNA strand. Inhibition of type I topoisomerases, by the drug Camptothecin (CPT), have been shown to stall replication fork progression and to induce DNA damage. Since you can inhibit cell growth in this manner, type I topoisomerases are therefore used as targets for anti-cancer drugs (Pommier et al., 2003).

The second class of topoisomerases is the type II topoisomerases, Top2 in budding yeast. This class operates on dsDNA by cleaving both strands and thereby enabling the movement of another intact DNA molecule through it. This process is adenosine triphosphate (ATP) dependent. Top2 activity is needed for resolving DNA catenations, two interlocked DNA molecules that are remnants of replication. There are also studies showing that type I and type II topoisomerases work together to remove supercoils in order to maintain replication fork integrity (Bermejo et al., 2007). Without the action of type II topoisomerases, severe segregation problems are induced during mitosis with chromosome breakage as a consequence. This has lethal outcome for the cell (DiNardo et al., 1984, Holm et al., 1989).

In **paper II**, we further investigated the role of Top2 in chromosome segregation. The aim was to link accumulation of topological structures to the function of the Smc5/6 complex, the main protein of interest in this thesis. This connection is further discussed in chapter 6.

Chapter 2
ALTERING PROTEIN FUNCTION

The composition of base pairs lead to a great variation of DNA sequences, some of which encode a protein. These sequences are called genes and are used as template to build proteins in the processes of transcription and translation. Every gene encodes a protein and different proteins have diverse functions in regulating cellular processes. Proteins can be modified by binding chemical groups to them, which can change the properties of the original protein. These modifications are known as post-translational modifications (PTMs) and are widely studied for their role in altering protein function. There are several PTMs that can change protein function in various cellular processes. In this chapter, the focus will be on two types of PTMs, phosphorylation and SUMOylation of proteins.

Phosphorylation

Phosphorylation is an important regulatory mechanism for proteins and exists in both eukaryotes and prokaryotes. The phosphorylation process is often reversible, kinases covalently attach one or more phosphate groups (PO_4^{3-}) onto its substrates, and phosphatases can then dephosphorylate them. Serine, threonine and tyrosine residues are usually the targets of phosphorylation in eukaryotes. These amino acids have a hydroxy group (-OH) onto which phosphate groups are transferred from a phosphate donor (usually ATP), in a reaction catalyzed by protein kinases (Deutscher and Saier, 1983). Kinase activity is usually triggered through phosphorylation of activating its domains, which occurs either by autophosphorylation or by other kinases (Johnson and Lewis, 2001). Protein kinases not only recognize the target residue, but also flanking consensus motifs (Pawson and Nash, 2003). Once phosphorylated, target proteins undergo conformational changes that alter their function. The addition of a phosphate group can change parts of the modified protein from hydrophobic to hydrophilic, thereby inducing a conformational change (Polyansky and Zagrovic, 2012). This has been observed for many proteins using X-ray crystallography. In addition, computational methods have also predicted conformational changes of proteins induced by phosphorylation, which is important to understand the mechanism of PTMs (Groban et al., 2006).

The role of protein phosphorylation in cell cycle progression has been extensively studied. Numerous proteins involved in the transition between cell cycle stages are known to be phosphorylated in unchallenged cells. Depending on the protein, phosphorylation can either induce or inhibit cell cycle progression. In the presence of DNA damage, phosphorylation also plays key roles in modifying proteins to halt the cell cycle by inhibiting degradation of downstream proteins. The importance of phosphorylation in these pathways is discussed in chapter 3.

Sumoylation

Small ubiquitin-like modifier (SUMO) is another type of PTM that alters protein function. SUMO is a small protein, with a molecular weight of around 11 kilodalton (kDa). There are

four distinct SUMO-proteins in humans, while the *SMT3* gene encodes the single SUMO in *S. cerevisiae* (Johnson et al., 1997). SUMO is conjugated to its targets via a three-step pathway that is dependent on ATP hydrolysis. First, SUMO binds to the E1 activating enzyme heterodimer Aos1-Uba2 (Johnson et al., 1997). Second, SUMO is transferred to the E2 conjugating enzyme Ubc9 (Johnson and Blobel, 1997). Lastly, SUMO is ligated to its targets by the three E3 ligases Siz1, Siz2 and Mms21 (Zhao and Blobel, 2005, Johnson and Gupta, 2001). SUMO E3 ligases are characterized by the SP-RING domain, which is essential for interaction to Ubc9 and for ligation to target proteins (Hochstrasser, 2001). SUMO is conjugated to a lysine residue on the target protein in a specific consensus site, ΨKxE, where Ψ is a large hydrophobic residue, K the lysine, x any amino acid and E glutamic acid. (Mahajan et al., 1998, Matunis et al., 1998, Kamitani et al., 1998, Sternsdorf et al., 1999, Desterro et al., 1998, Muller et al., 2000). However, SUMO conjugation to lysine in non-consensus sites has also been reported. Addition of SUMO to protein targets is a reversible process. Two isopeptidases, Ulp1 and Ulp2 cleave SUMO at the C-terminal to remove it from the substrates (Li and Hochstrasser, 1999, Li and Hochstrasser, 2000).

The SUMO cascade is essential in most organisms, and similarly to phosphorylation, SUMOylation has diverse functions in many cellular processes. SUMOylation of target proteins is proposed to alter protein-protein interactions in three different ways, based on studies of their structure in primarily human cells (reviewed in Geiss-Friedlander and Melchior, 2007). Conjugation of SUMO to targets can prevent interaction to other proteins by masking existing binding sites on the substrate. SUMO itself can add supplementary binding sites to increase interaction. In addition, SUMOylation is proposed to subject proteins to conformational changes that can either reveal or hide existing binding sites.

SUMOylation of targets can also be enhanced by phosphorylation of proteins in the SUMO cascade. In **paper I**, we investigated phosphorylation of the SUMO E3 ligase Mms21 with the aim to understand the consequences for substrate SUMOylation in genome stability.

Chapter 3
CHECKPOINTS, CONTROLLING THE CELL CYCLE

Every minute, cells in our body manage to duplicate and successfully forward their genetic information to the next generation. However, damage to DNA can be the source of complications for completing the cell cycle. These rare events can be caused by endogenous factors, such as products of cellular metabolism that can be harmful for the genome. Exogenous factors can also contribute to DNA damage, like toxic chemicals and irradiation from the sun. All organisms are exposed to these hazards and it is therefore essential that the genome is protected against it.

Any type of DNA damage requires that cell cycle progression halts or slows down in order to assess the problem and to allow repair. If damage is not taken care of, potential mutations and chromosomal aberrations could be inherited or cause cell death. To cope with this problem, cells have developed safety mechanisms that control the state of chromosomes at specific cell cycle stages. These are called checkpoints and are responsible for halting cell cycle progression in order to assess the presence of DNA damage and to ensure alignment of chromosomes to the metaphase plate. The DNA damage checkpoint and the spindle assembly checkpoint (SAC) will be presented in this chapter.

DNA damage checkpoint

All forms of DNA damage, as well as incomplete replication, triggers checkpoint activation. The DNA damage checkpoint is conserved from yeasts to humans and is responsible for mediating cell cycle arrests in either G1, intra-S or in the G2/M phase (Siede et al., 1994, Paulovich and Hartwell, 1995, Weinert and Hartwell, 1988). The main goal for the DNA damage checkpoint is to recruit appropriate repair factors and prevent the cell cycle to proceed (Harper and Elledge, 2007).

Central to activating the DNA damage checkpoint is the initiation of a protein phosphorylation cascade mediated by the Tel1 and Mec1 kinases in *S. cerevisiae.* Tel1 and Mec1 belong to the phosphatidyl inositol 3-kinase-related kinases (PIKK) family and are homologs of mammalian ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR) respectively. Upon DNA damage, one of the first protein complexes to localize at the site of double-strand breaks (DSBs) is the MRX complex, consisting of Mre11, Rad50 and Xrs2 in *S. cerevisiae* (Lisby et al., 2004). The MRX complex is essential in mediating DSB repair via the non-homologous end-joining and homologous recombination pathways (D'Amours and Jackson, 2002). Tel1 is recruited to these sites by the MRX complex through its association to the C-terminal domain of the Xrs2 subunit (Nakada et al., 2003, Falck et al., 2005, Lee and Paull, 2005, You et al., 2005). Kinase activity of Tel1 is proposed to be triggered by its association to DSBs, but also to be stimulated by MRX (Fukunaga et al., 2011, Bakkenist and Kastan, 2003). In addition to DNA damage sensing, the MRX complex also aids end resection, which exposes ssDNA overhangs at the broken ends of DSBs (Bonetti et al., 2010). These overhangs are then coated with RPA, which

recruits Mec1 in a process that is dependent on the Mec1 interacting protein Ddc2 (Dubrana et al., 2007, Paciotti et al., 2000). Activation of Mec1 kinase activity in *S. cerevisiae* is however not stimulated by DSB association, but rather through interaction with the Ddc1-Rad17-Mec3 protein heterotrimer, also known as the 9-1-1 complex that is independently recruited to ssDNA (Majka et al., 2006). Recruitment of Dpb11 to DNA damage via the Ddc1 subunit of the 9-1-1 complex has been shown to further stimulate Mec1 activation (Navadgi-Patil and Burgers, 2008, Pfander and Diffley, 2011). Ddc1 is phosphorylated by Mec1 upon their interaction and this has been suggested to be crucial for Dpb11 function, thereby creating a positive feedback loop that further stimulates Mec1 activation (Pfander and Diffley, 2011). Once Tel1 and Mec1 become active, they phosphorylate numerous downstream mediators that are involved in checkpoint signaling (**Figure 4**).

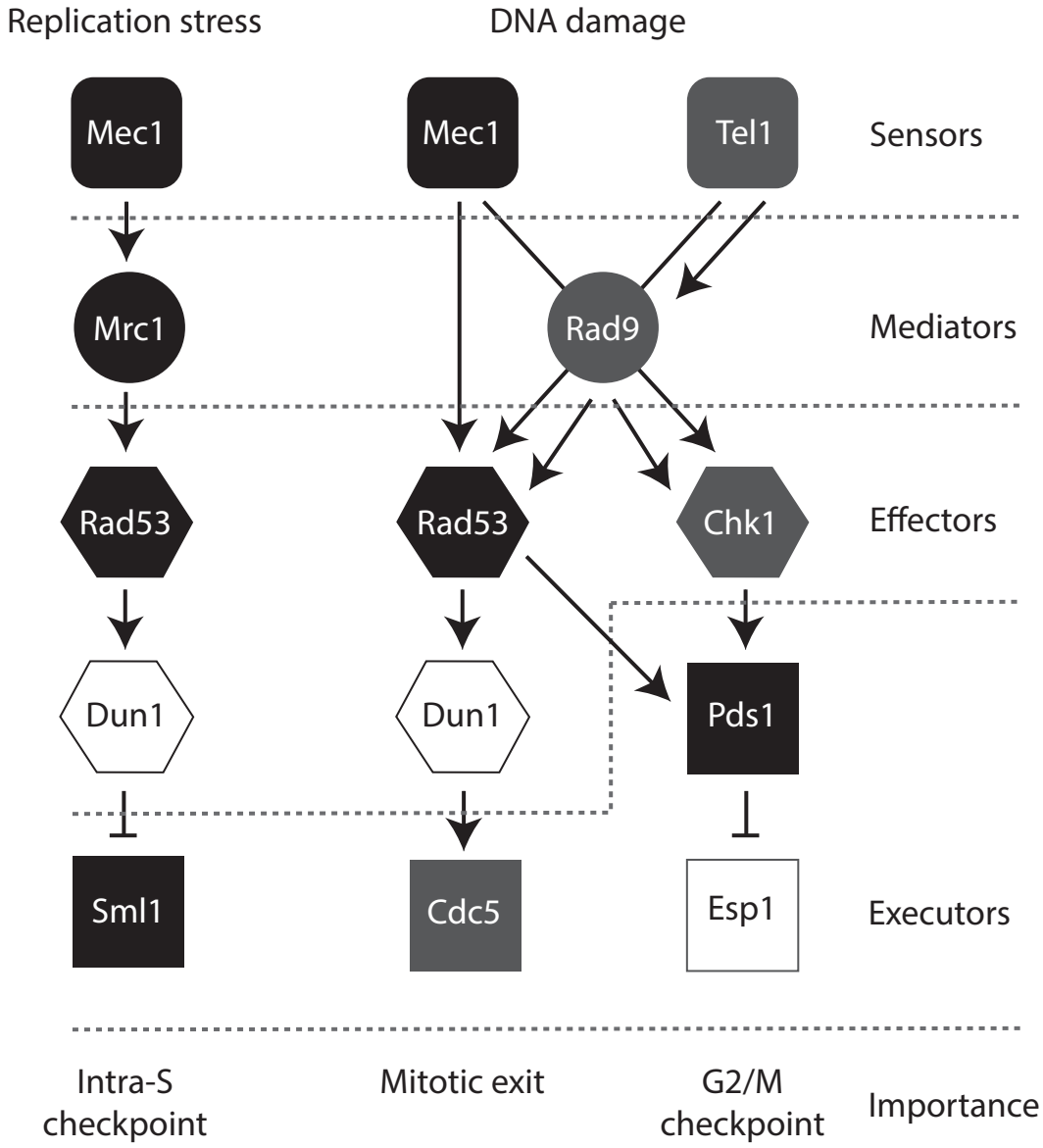

Figure 4. The Mec1 and Tel1 phosphorylation cascade.

In response to DSBs, both Mec1 and Tel1 phosphorylate histone H2A, which is a hallmark of DSB presence (Downs et al., 2000, Shroff et al., 2004). H2A phosphorylation and H3 methylation by the Dot1 methylase, promote recruitment of the mediator kinase Rad9 to sites of damage (Hammet et al., 2007, Huyen et al., 2004). Important for checkpoint-mediated arrest in the G2-phase, Rad9 can also be recruited to DSBs in a histone-independent pathway, which requires the interaction between Ddc1 and Dpb11 (Puddu et al., 2008). At the site of damage, Rad9 becomes phosphorylated by Mec1 and Tel1 (Emili, 1998). Rad9 phosphorylation is important as it acts as a docking site for binding the effector kinase Rad53 (Schwartz et al., 2002, Sweeney et al., 2005, Sanchez et al., 1996). Binding of Rad53 to phosphorylated Rad9 triggers Rad53 autophosphorylation, and brings it in close proximity for additional Mec1-dependent phosphorylation (Gilbert et al., 2001, Sweeney et al., 2005, Stern et al., 1991, Sun et al., 1996). In addition, Rad9 also facilitates the Mec1-dependent phosphorylation of a second effector kinase, Chk1 (Sanchez et al., 1999, Blankley and Lydall, 2004). Once the effector kinases Rad53 and Chk1 become active by Mec1- and Tel1-dependent phosphorylation, they arrest the cell cycle at specific stages by targeting key regulators.

For G1 checkpoint activation, active Rad53 directly phosphorylates the regulatory subunit of the Swi4/6-dependent cell cycle box binding factor (SBF) transcription factor, which inhibits the transcription of G1/S cyclins Cln1 and Cln2 (Sidorova and Breeden, 2003, Sidorova and Breeden, 1997). The reduced activity of cyclins was shown to inhibit the degradation of the cyclin inhibitor Sic1, an event that is required for the G1 to S transition (Schwob et al., 1994, Verma et al., 1997). Phosphorylation of Chk1 has also been shown to be important for G1 arrest, but it remains unknown how this directly influences the checkpoint (Grenon et al., 2001).

In *S. cerevisiae*, the intra-S checkpoint depends solely on Mec1 and Rad53 activation. The intra-S checkpoint mediates cell cycle arrest in response to both DNA damage and stalled replication, the latter known as the replication checkpoint, by two separate pathways. In response to replication fork stalling, Mec1 is proposed to be recruited at these sites through interaction with RPA coated ssDNA, and then to promote phosphorylation of the replication machinery protein Mrc1 (Osborn and Elledge, 2003). Mrc1 is another mediator that upon phosphorylation promotes the downstream phosphorylation of the effector kinase Rad53 (Osborn and Elledge, 2003). Mrc1-dependent Rad53 activation leads to cell cycle arrest by inhibiting firing of late replication origins, and by promoting fork stability (Santocanale and Diffley, 1998, Shirahige et al., 1998). The replication checkpoint involves regulation of several other factors that inhibits S-phase progression, including replisome components, cell-cycle dependent kinases (CDKs) and transcription factors (reviewed in Finn et al., 2012). The replication checkpoint seems to be activated independently of DNA damage and does not require activity of the Rad9 mediator. Rad9 instead specifically facilitates intra-S checkpoint arrest in response to DNA damage through Rad53 activation. Replication stress also induces the Rad53-dependent phosphorylation of another effector kinase, Dun1 (Gardner et al., 1999, Zhao and Rothstein, 2002). A downstream target of Dun1 is the ribonucleotide reductase

inhibitor Sml1, which control the rate of DNA synthesis by negative regulation of dNTP pools (Zhao and Rothstein, 2002).

The G2/M arrest (G2 arrest in mammalian cells) in response to DNA damage is a prominent checkpoint in eukaryotes. In the fission yeast *Schizosaccharomyces pombe* and human cell lines, the G2 checkpoint is regulated by inhibition of cyclin-dependent kinase 1 (Cdk1) activity by phosphorylation of a conserved tyrosine residue (Krek and Nigg, 1991, Gould and Nurse, 1989, Lee et al., 1994, Parker et al., 1992). This does not seem to be the case in *S. cerevisiae*, where G2/M arrest is instead achieved by inhibiting the metaphase to anaphase transition. Both Chk1 and Rad53 activation contribute to G2/M checkpoint arrest by phosphorylation of Pds1, an anaphase inhibitor (Sanchez et al., 1999, Wang et al., 2001). It is possible that phosphorylated Pds1 cannot be degraded, which prevents anaphase entry. This pathway will be described further in the spindle assembly checkpoint section. In addition, Rad53 has also been shown to prevent mitotic exit by maintaining high CDK activity and by phosphorylation of Cdc5 via the effector kinase Dun1 (Gardner et al., 1999). Cdc5 phosphorylation is thought to prevent mitotic cyclin degradation, which inhibits mitotic exit (Sanchez et al., 1999).

In **paper I**, we found an additional target of Mec1: the Mms21 subunit of the Smc5/6 complex. The function of Mms21 phosphorylation is discussed in chapter 6.

Spindle assembly checkpoint

Apart from ensuring correct repair of damaged DNA, cells must also segregate chromosomes accurately to avoid genome instability. To certify that the segregation process is executed properly, all chromatids must establish bipolar attachment by microtubule binding to the kinetochore. Bipolar attachment of all chromosomes ensures that tension is created and enables entry into mitosis. This process is monitored by the spindle assembly checkpoint (SAC), which delays cell cycle progression in the metaphase to anaphase transition when active.

The SAC is largely controlled by the ubiquitin ligase anaphase promoting complex or cyclosome (APC/C), which mediates protein degradation by attaching the small ubiquitin protein onto its targets. APC/C activity is regulated through the accessory protein Cdc20, which binds both to APC/C and to substrates targeted for degradation (Shirayama et al., 1998, Shirayama et al., 1999, Luo et al., 2000). Anaphase onset is promoted via the APC/C-Cdc20 interaction, while unattached kinetochores to inhibit this association, resulting in SAC activation. Unattached kinetochores recruit Mad1, which in turn binds Mad2 (Chen et al., 1998). Mad1 and Mad2 are both essential proteins that are required for SAC activation in humans, budding yeast and many other eukaryotes (Li and Murray, 1991, Hoyt et al., 1991, Winey and Huneycutt, 2002). According to the "template model" **(Figure 5)**, Mad1 recruits Mad2 to the kinetochore and it is only thereafter that Mad2 gains the ability to also bind Cdc20 directly (Li et al., 1997, Fang et al., 1998, Luo et al., 2000). This observation, together with studies on the tertiary Mad2 structure suggested that Mad2 existed in two conformations

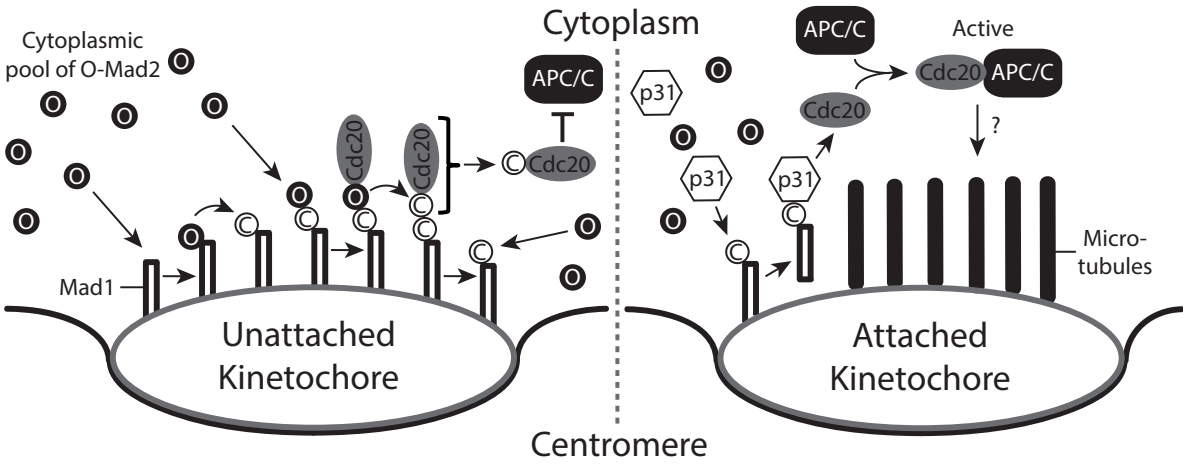

Figure 5. The "template model", describing the function of Mad2 in the SAC.

(Sironi et al., 2002). The proposed model postulates that there is a cytoplasmic pool of Mad2 in the open conformation (O-Mad2) that gets recruited by Mad1 to unattached kinetochores. The interaction to Mad1 triggers a conformational change of O-Mad2 to the closed state (C-Mad2). Additional O-Mad2 is then recruited to the Mad1-C-Mad2 complex, thereby forming C-Mad2-O-Mad2 dimers bound to Mad1 (Sironi et al., 2002, Luo et al., 2002). O-Mad2 association to the Mad1-C-Mad2 complex was found to be crucial for its ability to bind Cdc20, thereby inhibiting its further interaction to APC/C (Luo et al., 2002, DeAntoni et al., 2005). When O-Mad2-Cdc20 interaction is established, O-Mad2 undergoes a conformational change to the C-Mad2 state, which causes the "new" C-Mad2-Cdc20 complex to dissociate Mad1-C-Mad2 at the kinetochore (DeAntoni et al., 2005). This also makes the kinetochore-bound Mad1-C-Mad2 receptive to new O-Mad2 binding, which maintains APC/C-Cdc20 inhibition. The "template model" is further supported by experiments utilizing a C-terminally truncated version of Mad2 (*mad2ΔC*). This mutant is locked in the O-Mad2 conformation, it only forms Mad2 monomers and can neither bind to C-Mad2-Mad1 complexes or Cdc20 (DeAntoni et al., 2005). Once all kinetochores have attached to microtubules, the checkpoint antagonist p31^{comet} becomes active and competes with O-Mad2 for binding Mad1-C-Mad2 at the kinetochore (Westhorpe et al., 2011). O-Mad2 then loses the capability of trapping Cdc20, which is free to associate to APC/C.

Attachment of all kinetochores to microtubules is not enough to satisfy the SAC. In addition, chromosomes must establish bipolar attachment. This enables a buildup of tension since the protein complex cohesin holds sister chromatids together. Cohesin is responsible for holding sister chromatids together from time of replication until anaphase onset (Michaelis et al., 1997). Tension stabilizes the bipolar attachments, which prevents chromatid missegregation to the same daughter cell (Swedlow, 2013). When the criteria for kinetochore and bipolar attachment are fulfilled, the SAC becomes inactivated and sister chromatids are released from their cohesin-mediated entrapment to allow chromosome segregation. The segregation process is initiated by the degradation of Pds1 (also known as securin) and the cell cycle

regulating protein cyclin B. Pds1 and cyclin B are both substrates of APC/C-Cdc20, which upon Mad2 inhibition at anaphase onset stimulates their degradation (Uhlmann et al., 1999). Pds1 is normally phosphorylated by Chk1 in unchallenged cells, and additionally phosphorylated by Rad53 in response to DNA damage, which is thought to prevent Pds1 degradation by APC/C (Sanchez et al., 1999, Wang et al., 2001). Pds1 is an anaphase inhibitor, which acts to keep the protease Esp1 (also known as separase) inactive. This is achieved through a stable interaction with Esp1 (Haering and Nasmyth, 2003, Sun and Fasullo, 2007, Oliveira et al., 2010). Phosphorylation of Pds1 is thought to further induce its stable interaction to Esp1 (Sun and Fasullo, 2007). When the damage has been repaired, Pds1 and cyclin B are degraded, which results in Esp1 activation and cleavage of the cohesin subunit Scc1 (Uhlmann et al., 1999, Cohen-Fix et al., 1996, Shirayama et al., 1999). This causes the dissolution of cohesion and enables sister chromatid segregation to mother and daughter cells.

In **paper III**, we investigated the role of the Smc5/6 complex in chromosome segregation. We found that non-functional Smc5/6 protein prolonged activation of the SAC and that the additional deletion of *MAD2* allowed passage through mitosis.

Chapter 4
STRUCTURAL MAINTENANCE OF CHROMOSOMES

There are many different proteins involved in maintaining genome stability. The structural maintenance of chromosomes (Smc) family of protein complexes has several essential roles required for replication, segregation and repair, which are all fundamental processes in preserving genome stability. In all eukaryotic organisms there are six Smc proteins that form the backbone of three evolutionary conserved protein complexes: cohesin, condensin and the unnamed Smc5/6 complex.

Apart from having overlapping roles in several cellular processes, these complexes also share a common structure. In eukaryotes, the different Smc proteins associate to each other in pairs, thus forming a heterodimer that constitutes the core of each complex. The Smc proteins are long helical proteins that form coiled-coil domains, with a globular N- and C-terminus at each end. The coiled-coil domain is folded in the middle, thereby creating a "hinge" domain at one end, which in turn brings the N- and C-termini together at the other end. The two pairs of Smc proteins in each complex interact with each other via their "hinge" domains while their N- and C-termini ends form ATPase active globular heads, which are bridged by additional proteins **(Figure 6).** In this chapter, the focus will be on roles of the cohesin and Smc5/6 complexes in genome integrity.

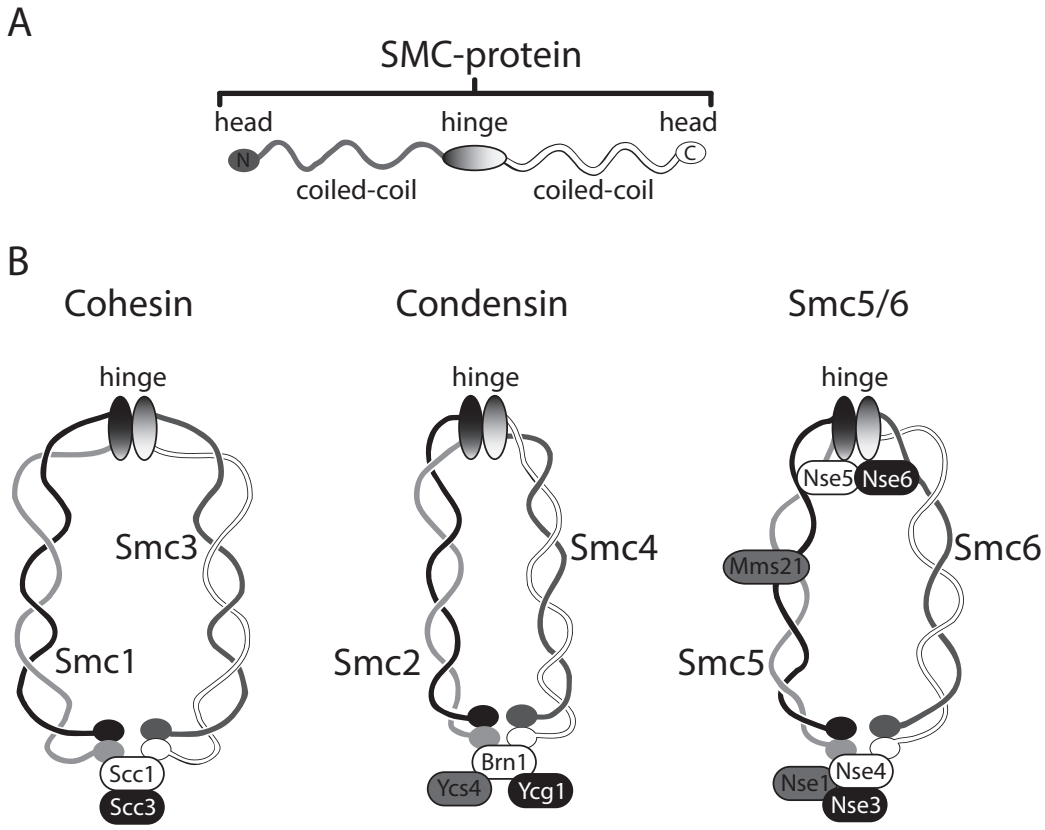

Figure 6. The Smc complexes. **(A)** Architecture of Smc protein domains. **(B)** Composition of subunits that make up cohesin, condensin and the Smc5/6 complex.

Cohesin

The cohesin complex was the first protein in the SMC family to be discovered in mid 1990's by the Nasmyth and Koshland research groups (Strunnikov et al., 1993, Michaelis et al., 1997). Due to this, cohesin has been the most extensively studied out of the three eukaryotic Smc complexes. Smc1 and Smc3 compose the core of the cohesin complex. The globular heads of Smc1 and Smc3 are bridged by the sister chromatid cohesion 1 (Scc1) protein, which in turn is associated to Scc3 (Toth et al., 1999, Gruber et al., 2003). The cohesin complex forms a ring-shaped structure that is enclosed by the binding of Scc1 to the Smc1/Smc3 heterodimer, therefore Scc1 is also referred to as the kleisin subunit of cohesin.

Sister chromatid cohesion

The first annotated function of cohesin was to keep the newly synthesized sister chromatids together after replication in S-phase until they are segregated during the following mitosis (Michaelis et al., 1997). This is called sister chromatid cohesion. Lack of cohesion leads to premature separation of the sister chromatids and as a result, the chromosomes often missegregate which have catastrophic consequences for cell survival. Keeping the sister chromatids cohesed is not only important for chromosome segregation but also for DNA repair. In the presence of a DSB, cohesin was shown to be recruited to sites of damage (Strom et al., 2004). New establishment of cohesion is required for post-replicative repair, where the undamaged sister chromatid serves as template for recombinational repair (Sjogren and Nasmyth, 2001).

Cohesin localization to chromatin

Important information on the function of cohesin comes from studying its chromosomal association. In budding yeast, cohesin is loaded onto chromosomes in late the G1-phase at centromeres and are also found on chromosome arms at an average distance of 10 kilobasepairs (kb) apart, known as cohesin attachment regions (CARs). It was suggested that cohesin is translocated to these sites by the replication fork after initially being associated to centromeres and regions of highly transcribed genes (Lengronne et al., 2004, Hu et al., 2011). Studies have revealed that the CAR sites are specifically regions of convergent transcription (Glynn et al., 2004, Lengronne et al., 2004). Some cohesin binding can also be seen on telomeres.

Cell cycle of cohesion

It has been hypothesized that cohesin encircles both DNA double strands in order to keep the sister chromatids together until their release at anaphase to ensure proper segregation (Haering et al., 2008). Cohesion is not only dependent on association of cohesin to chromosomes but also on establishment, which takes place during S-phase (Uhlmann and Nasmyth, 1998). In budding yeast, cohesin is loaded onto chromatin in the G1-phase by the Scc2/4 complex but is not yet able to form cohesion (Ciosk et al., 2000). Cohesin is also associated to a protein called Wapl (also known as Rad61), which has been shown to

destabilize cohesin binding to chromosomes in both budding yeast and human cells (Lopez-Serra et al., 2013, Gandhi et al., 2006, Kueng et al., 2006). This makes the cohesin-chromosome interaction highly dynamic. During S-phase, cohesion is established through the acetylation of the Smc3 subunit of cohesin by the Eco1 acetyltransferase (Unal et al., 2008, Rolef Ben-Shahar et al., 2008). Eco1 is essential for budding yeast viability and Eco1 ts mutants display severe of cohesion defects. Deleting Wapl makes Eco1 dispensable for cell survival (Rolef Ben-Shahar et al., 2008, Sutani et al., 2009, Rowland et al., 2009). This might indicate that removing the destabilizing effect on cohesin might enable cohesion establishment without Eco1 activity. However, the role of Wapl in cohesion is more complex since Wapl mutants has minor cohesion defects (Sutani et al., 2009). In addition, it is under debate whether deleting Wapl rescues the cohesion defect of Eco1 mutants (Guacci et al., 2015, Guacci and Koshland, 2012, Rowland et al., 2009, Rolef Ben-Shahar et al., 2008). In human cells, Eco1-dependent acetylation of Smc3 during S-phase was shown to dissociate Wapl from cohesin, thereby making cohesion more stable (Nishiyama et al., 2010). The same model has been proposed in budding yeast, but Wapl displacement has not been shown directly (Rowland et al., 2009). In addition to establishment of cohesion, association of Pds5 to cohesin was required for sustaining cohesion during the G2-phase and mitosis (Hartman et al., 2000, Panizza et al., 2000). Following entry into mitosis, Pds5 becomes SUMOylated and dissociates from cohesin (Panizza et al., 2000, Stead et al., 2003).

As described in chapter 3, the segregation process is initiated by cleavage of the cohesin ring to release sister chromatid cohesion. Two proteins, Esp1 and Pds1, are central players in this release. Esp1 is a protease, which in its active form in mitosis cleaves the Scc1 subunit of cohesin (Uhlmann et al., 1999). Esp1 is however kept in an inactive state by being bound to Pds1. In addition, cyclin B stimulates the Chk1-dependent phosphorylation of Pds1 to further prevent its premature degradation (Sun and Fasullo, 2007). Once the requirements to silence the SAC are met, APC/C-dependent ubiquitination targets Pds1 and cyclin B for degradation, which enables chromosome segregation (Haering and Nasmyth, 2003).

Cohesin in DNA repair

Keeping the replicated sister chromatids cohesed is not only essential for chromosome segregation. Cohesion provides a second copy of genetic information in close proximity in case of damage to chromosomes. This is essential when both strands of the DNA helix are damaged and the only way to restore the chromosome to its original state is to access the identical sister chromatid. In addition to cohesion formed during S-phase, damage-induced cohesion was shown to be distinct from S-phase cohesion in response to DNA damage. A single DSB leads to post-replicative recruitment of cohesin, which induced the rearrangement of cohesin association to chromosomes so that it bound to the surrounding region of the break (Unal et al., 2004, Strom et al., 2004). This pathway is dependent on the cohesin loader Scc2, DNA damage checkpoint proteins Mec1, Tel1 and Rad53 as well as DNA damage sensor Mre11 (Strom et al., 2004).

Cohesin in SAC

In budding yeast, non-functional cohesin does not activate the SAC despite the lack of tension between sister chromatids (Michaelis et al., 1997). In contrast, human cells activate the SAC in the absence of cohesin (Hoque and Ishikawa, 2002, Tachibana-Konwalski et al., 2013). The simplest explanation for these interspecies differences would be that cohesin-dependent tension between sister chromatids is not required in budding yeast. This assumption is supported by studies on kinetochore attachment to microtubules, which in budding yeast are attached throughout the cell cycle, except for a short period during centromere replication in early S-phase (Kitamura et al., 2007). In human cells, attachment of microtubules to the kinetochore is initiated after disassembly of the nuclear envelope during mitosis (Rieder, 1990). Therefore, budding yeast chromosomes might come under tension without a functional cohesin complex during replication, due to unreplicated regions along chromosome arms. It is possible that the tension requirement might be fulfilled already in S-phase. Human cells must however establish tension post S-phase and is thereby fully dependent on a functional cohesin complex to create tension

In addition, requirement of cohesin in human cells has been suggested for efficient production of the mitotic checkpoint complex (MCC) (Tachibana-Konwalski et al., 2013). This complex is important to inhibit APC/C activation, which promotes anaphase onset. Cohesin is therefore proposed to play additional roles in SAC activation at least in human cells.

The Smc5/6 complex

The least understood member of the SMC family is the Smc5/6 complex. The *RAD18* gene was the first subunit of the Smc5/6 complex to be discovered in a screen for radiation sensitive mutants in *S.pombe* (Lehmann et al., 1995). Although Smc5/6 function has been linked to various cellular processes, its main role remains elusive.

Subunits and architecture of the Smc5/6 complex

The Smc5/6 complex contains the Smc5 and Smc6 proteins that make up the core of the complex. There are additionally six non-Smc element (Nse) proteins that bind to the core at different positions. These are Nse1, Mms21 and Nse3-6. Several of these subunits have distinct domains that are likely to contribute to the Smc5/6 complex function (Verkade et al., 1999, Fujioka et al., 2002, Hazbun et al., 2003, Pebernard et al., 2004). Nse1 contains a RING motif that is commonly found in ubiquitin E3 ligases (Fujioka et al., 2002) but no ubiquitylation activity has yet been reported. Mms21, also known as Nse2, has been identified as a SUMO E3 ligase (Zhao and Blobel, 2005) and some of the known substrates for Mms21 are Smc1, Smc3, Smc5, Smc6 as well as Mms21 itself (Zhao and Blobel, 2005, Potts and Yu, 2005, Takahashi et al., 2008, Albuquerque et al., 2013). Nse3 is the only gene in yeast that contains a MAGE homology domain, which is found in a large family of mammalian proteins that are highly expressed in tumors (Pebernard et al., 2004, Guerineau et al., 2012). The function of the MAGE domain remains unclear. Nse4 is the kleisin subunit of

the complex and the ortholog of Scc1. It is also related to the Eid family of transcription repressors. It bridges the N- and C-terminal of Smc5 and Smc6 and has been shown to be essential for the DNA repair function of the Smc5/6 complex (Hu et al., 2005).

Smc5 and Smc6, as well as Nse1, Mms21 and Nse3-4 share well conserved homologous sequences between species in eukaryotes. Nse5 and Nse6 however are not as conserved and orthologs in humans have not been found. Both subunits have also been shown not to be essential for viability in fission yeast (Pebernard et al., 2006).

Binding of the various Nse proteins to the Smc5/6 core structure at different sites make up the complete octameric protein complex. Mms21 is bound to the coiled-coil region of Smc5 (Sergeant et al., 2005, Duan et al., 2009a, Duan et al., 2011). Nse1, Nse3 and Nse4 make up a subcomplex that binds to the globular heads of Smc5-Smc6, where Nse4 directly associates to Smc5 and Smc6. Nse1 binds to Nse3, which in turn binds to Nse4. It has been suggested that MAGE domains enhances ubiquitin ligase activity, which could explain the interaction of Nse1 and Nse3 (Doyle et al., 2010). There is some debate of where Nse5 and Nse6 bind to the rest of the Smc5/6 complex. In fission yeast, Nse5 and Nse6 form a secondary bridge between Smc5 and Smc6 globular heads (Pebernard et al., 2006). In budding yeast, Nse5 and Nse6 associate to the hinge domain of Smc5-Smc6 (Duan et al., 2009b).

Smc5/6 localization to chromatin

In budding yeast, Smc5/6 binding in the G1-phase is not prominent. Instead the complex seems to localize to chromosomes during S-phase in a cohesion-dependent manner (Jeppsson et al., 2014). It associates primarily to centromeres but also subtelomeric and arm regions, a process that initially was thought to be dependent on the Scc2/4 loading factor (Lindroos et al., 2006). Moreover, Smc5/6 associates to replication forks and accumulates in G2/M at cohesin sites (Jeppsson et al., 2014). After Top2 inactivation, Smc5/6 is additionally found to accumulate along chromosome arms (Kegel et al., 2011). Interestingly, the same study showed that the Smc5/6 complex binds to longer chromosomes with higher frequency as compared to shorter ones (Kegel et al., 2011). These results suggest that Smc5/6 function is executed during replication and is involved in the resolution of topological structures. The Smc5/6 complex is not present on chromosomes in telophase, as shown by the binding pattern in Cdc15 mutant-mediated arrest (Jeppsson et al., 2014). How the dissociation of the complex from chromosomes is regulated remains unknown.

Smc5/6 in DNA repair

As mentioned before, Smc6 was first discovered in a screen for radiation sensitive mutants in fission yeast, where it rescued the sensitivity of *rad18-X* mutants (Lehmann et al., 1995). It is maybe because of this that most research on the complex has involved its function in the DNA repair process. This does however not exclude that the main function of the Smc5/6 complex might be coupled to other processes. Characterization of the Smc5/6 complex showed that various subunit mutants were defective in DNA repair (Andrews et al., 2005,

Pebernard et al., 2006, Santa Maria et al., 2007, Pebernard et al., 2008). Smc5/6 has been shown to directly target DNA double strand breaks (DSBs), through its assembly around a single DSB. This assembly was Mre11 dependent but Mec1- and Rad53-independent (Lindroos et al., 2006). The Smc5/6 complex also has roles in checkpoint signaling, as Smc6 mutants were shown to decrease checkpoint response via Mec1-dependent phosphorylation of Rad53 in presence of damage and replication stress (Chen et al., 2013). Smc6 has also been shown to be required for G2/M checkpoint response in fission yeast (Verkade et al., 1999). Furthermore, the SUMO-ligase activity of Mms21 is required for proper DNA damage repair (Andrews et al., 2005, Potts and Yu, 2005) and also for damage-induced cohesion possibly through its SUMOylation of Smc1 and Smc3 (Takahashi et al., 2008, McAleenan et al., 2012). The DNA damage repair function of the Smc5/6 complex is likely to operate through the homologous recombination (HR) pathway, since Smc6, Nse1 and Mms21 are shown to be epistatic with the key regulator of HR, Rad51 (Lehmann et al., 1995).

Smc5/6 associates to damaged replication forks

There is substantial evidence showing that the Smc5/6 complex is involved in the rescue of stalled and collapsed replication forks. Smc6 temperature sensitive (ts) mutants accumulate aberrant chromosome structures in response to collapsed replication forks by HU which are efficiently removed once the complex is re-activated (Ampatzidou et al., 2006). This was further supported by the finding that the Smc5/6 complex is required for dissolving chromosome junctions or ongoing replication, so called sister chromatid linkages, after MMS induction (Bermudez-Lopez et al., 2010). SUMO-ligase activity of Mms21 is also needed to prevent accumulation of replication intermediates, so-called X-molecules, at damaged replication forks (Branzei et al., 2006, Sollier et al., 2009). Investigation of Smc6 localization shows it has binding affinity to stalled and collapsed replication forks induced by HU treatment, which further supports a role for the complex at perturbed forks (Lindroos et al., 2006, Bustard et al., 2012). Smc6 and Mms21 function have been linked to the Mph1 helicase, since the accumulation of aberrant recombination intermediates are suppressed by inactivation of Mph1 in Smc6 and Mms21 mutants (Chen et al., 2009). In addition, Mph1 activity also induces the DNA damage checkpoint signaling via Rad53 phosphorylation in Smc6 mutants, indicating a connection between replication stress and the DNA damage response (Chen et al., 2013).

Smc5/6 in rDNA and nucleolar maintenance

The Smc5/6 complex also functions in ribosomal DNA (rDNA) and nucleolar maintenance. The transcription of rDNA takes part in the nucleolus, a small compartment within the cell nucleus. The rDNA is a large heterochromatic region of tandem repeats located on chromosome XII in budding yeast. Because of substantial repetitive copies of rDNA genes, there is high activity of transcription and replication machineries that travel in opposite directions. To counteract this problem the *S. cerevisiae* genome contains replication fork barrier (RFB) sites. These are 100 basepair DNA sequences located at the 3' end of rDNA genes. At RFBs, the Fob1 protein binds tightly to DNA to hinder replication fork collapse

and can also aid termination of replication (Kobayashi, 2003). In Smc5 and Smc6 mutants, X-shaped intermediates accumulate that prevents correct segregation of chromosomes at the rDNA region locus. As a consequence, rDNA fragmentation is observed (Torres-Rosell et al., 2005b). Smc5/6 mutants also exhibit replication delays at rDNA sites contributing to the failure in chromosome segregation (Torres-Rosell et al., 2007a). In addition, Smc5 and Smc6 mutants trigger the accumulation Ddc1 foci in the nucleolus following mitosis, an indication of DNA damage (Torres-Rosell et al., 2005b). Inactivation of Smc5 or Mms21 SUMO-ligase activity renders in fragmentation and irregular shaping of the nucleolus (Torres-Rosell et al., 2005b, Zhao and Blobel, 2005, Torres-Rosell et al., 2005a). These findings support a role of the Smc5/6 complex in nucleolar maintenance. This notion is further supported by the fact that Smc6 preserves rDNA repetitiveness, and also Mre11, by controlling HR. Uncontrolled HR can cause various DNA alterations, including loss of repetitive sequence. Smc6 acts in this pathway by excluding one of the central proteins in HR, the Rad52 protein, from the nucleolus (Torres-Rosell et al., 2007b). The hypothesis is that Smc5/6 decreases the level of recombination, thereby preventing genome instability at the rDNA locus.

Smc5/6 and its role in topology

Recently a new role for the Smc5/6 complex in DNA topology has been identified. As stated before, DNA must be opened up for the replication machinery to access the two single strands. This causes topological stress in the form of supercoils ahead of the advancing fork, when the parental double helix becomes overwound. Topoisomerases can to some extent solve this problem by relieving the topological stress ahead of the fork. Our group found that the Smc5/6 complex might aid the resolution of such topological structures by rotation of the replication fork. This has however the consequence that sister chromatid intertwinings (SCIs) form behind the fork (Kegel et al., 2011). This model was based on the findings that Smc6 mutants delays replication of long chromosomes specifically, Smc6 binding frequency increases with chromosome length and that Smc6 reduces the level of intertwined sister plasmids in the absence of Top2 function. (Kegel et al., 2011). Additionally, Smc6 binding increased along chromosome arms in the absence of functional Top2 activity, known to increase the level of SCIs (Kegel et al., 2011, Jeppsson et al., 2014). Accumulation of SCIs in the absence of Top2 function has long been known to inhibit chromosome segregation (DiNardo et al., 1984, Holm et al., 1989). In **paper II**, we analyzed segregation of long and short chromosomes in Top2 mutant cells and confirmed that longer chromosomes, and especially arm regions, failed to segregate. Short chromosomes however segregated fairly well without Top2 activity. Removing both Smc6 and Top2 function caused even the short chromosomes to missegregate. These results further support a role for the Smc5/6 complex to aid the resolution of SCIs. Resolving these structures is crucial for the segregation process and genome stability.

Chaper 5
STUDYING CELLULAR PROCESSES

S. cerevisiae is a haploid unicellular eukaryote, the genome of which has been completely sequenced. It contains 5885 protein-encoding genes that are distributed across 16 chromosomes, with the combined length of 12,068 kb (Goffeau et al., 1996). This means that there is a gene approximately every 2 kb, which makes the *S.cerevisiae* genome very compact. In comparison, the human genome encodes a gene every 30 kb.

S. cerevisiae is an excellent model organism for research due to the evolutionary conservation of the Smc5/6 complex. Other main advantages of using *S. cerevisiae* is its rapid cell cycle, which takes approximately 2 hours to complete when grown under ideal conditions. In addition, several techniques have been developed over the years that allow genome manipulation by deleting, replacing, tagging or mutating genes of interest. The study of such manipulations can be used to investigate the function of genes in various cellular processes. There are established methods for arresting *S. cerevisiae* at specific cell cycle stages, switching on or off gene expression and releasing cells back into a cycling state. This allows for complete control of the experimental setup.

Below, I will describe the main technique used to study cellular processes in each of the three scientific papers.

Western blotting

In **paper I**, we used western blotting (WB) analysis to study protein expression and presence of post-translational modifications (PTMs) in various Mms21 mutants. The WB technique is a two-part procedure, where proteins are first separated based on their molecular mass and then detected by antibody recognition of specific proteins or protein tags. Analysis using the same protein tag on different proteins requires one primary antibody. In budding yeast, this is advantageous over using several primary antibodies that may differ in substrate specificity. The main drawback is the need to carry out careful analysis to verify that the tagged protein is fully functional.

In our investigations, we C-terminally tagged variants of the Mms21 protein with 3xHA6xHis and separated them using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The negatively charged SDS cover the proteins loaded on the gel, which thereby move along the electric gradient through the gel and towards the positive electrode. In order to make proteins accessible for detection, the blotting procedure transfers proteins from the gel onto a membrane made of polyvinylidene difluoride (PVDF). At this step, a tag-specific primary antibody can recognize the tagged proteins. A horseradish peroxidase-linked secondary antibody is then allowed to bind the primary antibody. The tagged proteins are detected by applying a chemiluminescent agent to the membrane, which is cleaved by the horseradish peroxidase in a reaction that produces luminescence. The emitted light can then be visualized with a CCD camera.

Proteins that are modified by phosphorylation or SUMOylation have greater molecular mass than the WT protein. Phosphorylated proteins also bind less SDS making them more positively charged (Lee, 2013). Modified proteins will therefore move slower through the polyacrylamide gel mesh. This difference in mobility is visualized as a slower migrating form of the analyzed protein. WB is a qualitative method that we have used to detect presence of modified Mms21 protein and how the modification changes in various mutant backgrounds.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) combined with DNA sequencing (seq), hybridization to microarrays (on-chip) or quantitative polymerase chain reaction (qPCR) was the basis for **paper II**. These methods are widely used to study where proteins bind to the genome, which can give important clues on protein function. ChIP-seq and ChIP-on-chip analyses are based on large cell populations that offer an overview of genome-wide binding. ChIP-seq data returns higher spatial resolution of binding sites as compared to ChIP-on-chip, this because hybridization to microarrays is more unspecific than DNA sequencing (Ho et al., 2011). ChIP-qPCR on the other hand is a quantitative assay, which provides information of protein binding at a specific locus.

ChIP was performed as previously described (Katou et al., 2006) **(Figure 7)**. We have used cells in which the protein of interest was tagged C-terminally with a 3xFLAG6xHis construct. These cells were grown under desired conditions, harvested and then treated with formaldehyde. The formaldehyde treatment cross-links proteins bound to chromosomes. The protein-bound DNA is then sheared by sonication. This input fraction is next enriched by immunoprecipitation, which is performed using an antibody with specificity to the protein tag. The cross-linked DNA is then eluted, referred to as the ChIP fraction, and the DNA-protein cross-links are reversed. The DNA is then purified and amplified by PCR. For analysis, the amount of DNA for each specific locus in the ChIP fraction is compared relative to the DNA in the input fraction.

Using these methods, we investigated the chromosomal association of the Smc5/6 complex. We performed experiments using a variety of conditions to determine the requirement of Smc5/6 binding. Using this approach, we studied how the Smc5/6 binding pattern changed in the absence of functional proteins required for sister chromatid cohesion, and in addition how binding was influenced in the absence of Top2 activity. The overall aim was to further investigate Smc5/6 function during the cell cycle and to unravel its possible connection to SCIs as proposed in a previous study (Kegel et al., 2011).

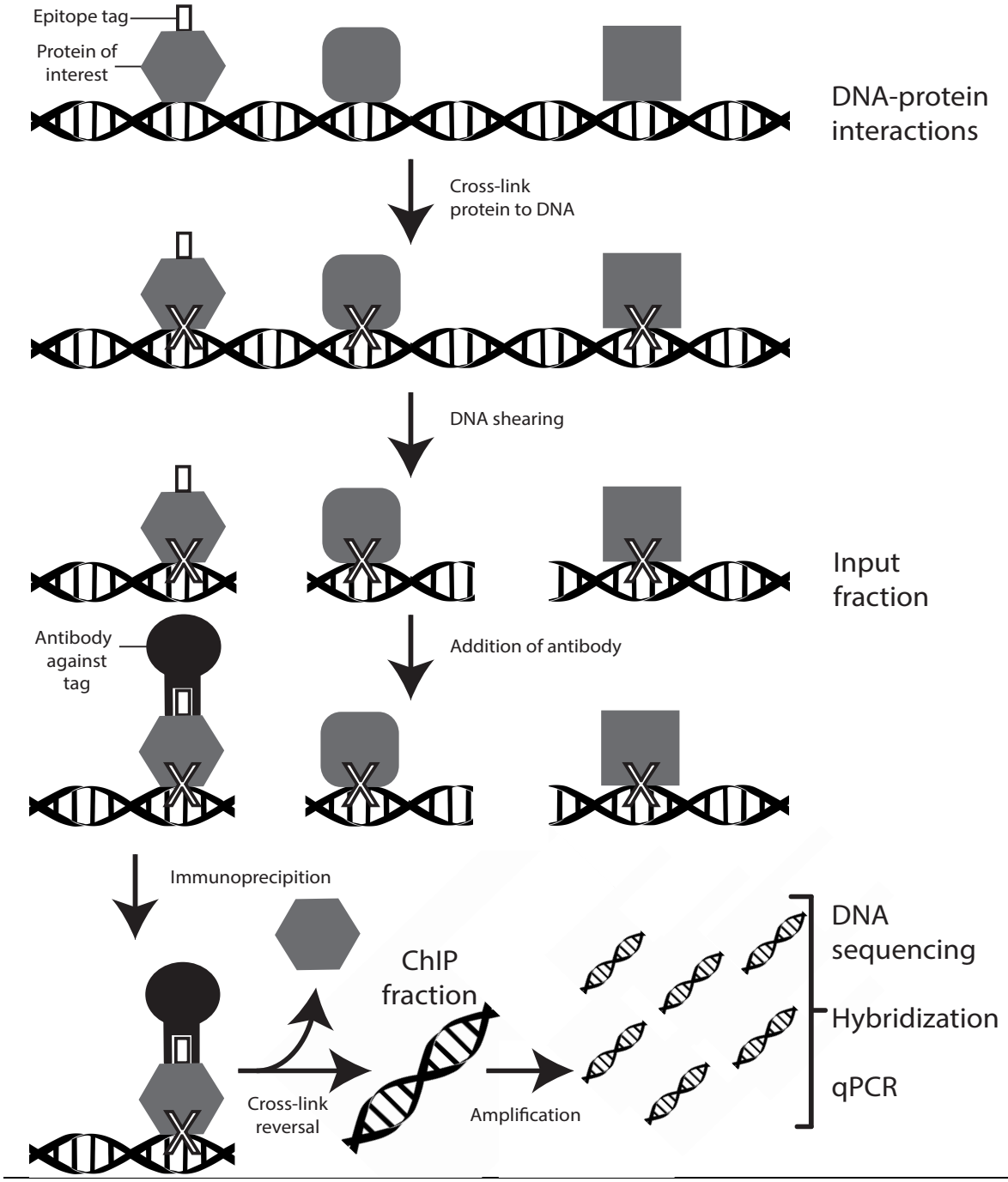

FIGURE 7. The ChIP workflow.

Live cell imaging

An important tool to visualize chromosomes and analyzing their segregation during mitosis is by live cell imaging (LCI) **(Figure 8)**. This technique was used in **paper II** and **paper III**. As the name suggests, LCI takes advantage of studying individual living cells instead of using population-based analysis. Additionally, LCI is independent of cell fixation that might affect analysis. Using time-lapse microscopy, images can be taken very frequently in an automated process. We wanted to analyze segregation in Smc5/6 mutants of a long, intermediate and short chromosome. This was based on observations that the Smc5/6 complex binds with

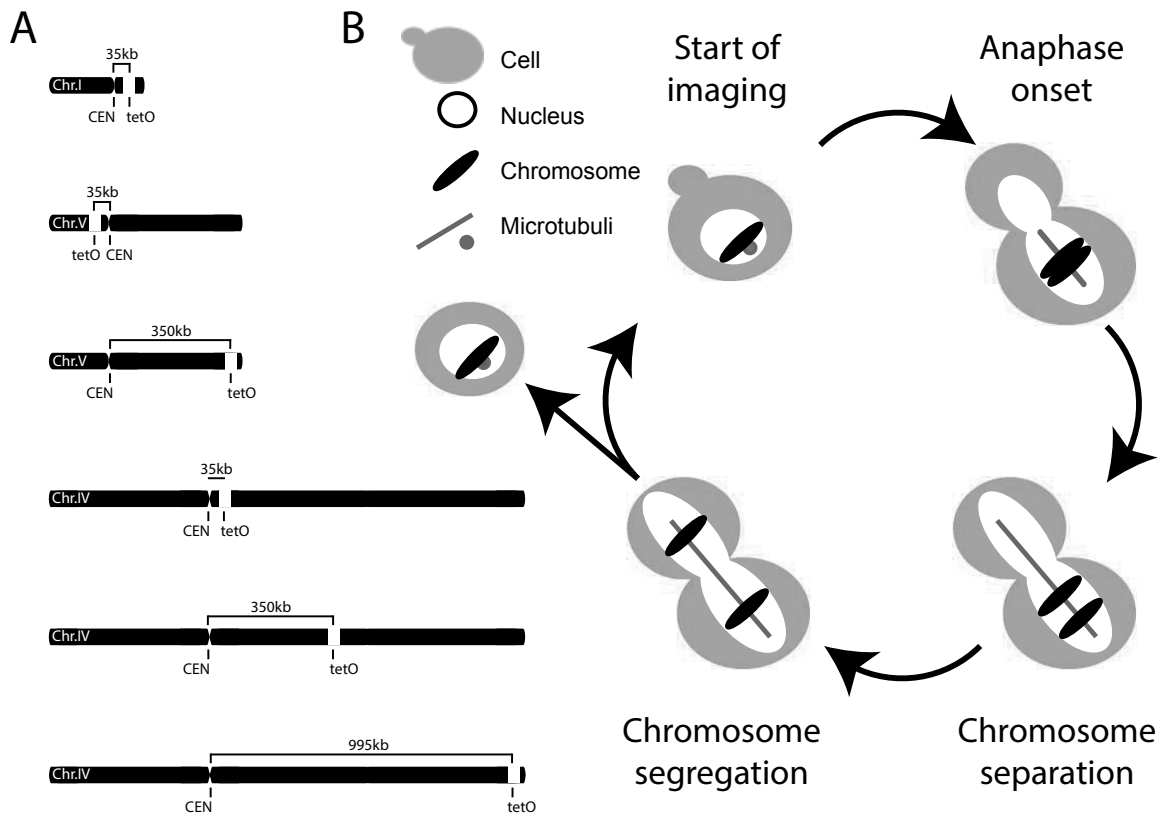

Figure 8. Strains and experimental setup for LCI. **(A)** Positions of tetracycline operons integrations on a short, intermediate and long chromosome. **(B)** Cells with tetracycline repressors fused with fluorescent td-Tomato, which bind to the tetracycline operons, and EGFP-tubulin were used. Time-lapse microscopy enabled analysis of chromosome separation and segregation in reference to anaphase onset, determined by EGFP-tubulin length.

higher frequency to longer chromosomes and that mutants delay replication of long chromosomes specifically (Kegel et al., 2011).

To visualize chromosomes, we first integrated multiple tetracycline operon (tetO) repeats in the genome 35 kb from centromere of chromosome VI (long), chromosome V (intermediate) and chromosome I (short). TetO repeats were also integrated 350 kb from centromere of chromosome IV and V, and 995kb from centromere of chromosome IV. Next, a tetracycline repressor-tandem dimeric Tomato (tetR-tdTomato) fusion was inserted at a different genomic locus. The use of the tetO/tetR system was previously described (Michaelis et al., 1997). TetR binds to tetO at the specific integrated position, and the fused tdTomato protein emits red fluorescence at this site upon excitation by light of 554 nm in wavelength. This allows visualization of chromosomes at the specific position as a bright red dot. In order to score chromosome separation and subsequent segregation in relation to anaphase onset, we tagged tubulin with an enhanced green fluorescent protein (EGFP). Anaphase onset was determined empirically by measuring the length of the EGFP-tubulin structure when sister chromatids of wild-type (WT) cells separated at the centromere.

With this setup, living cells were imaged during mitosis upon release from G1-phase arrest. Cells were excited with red and green light every 30 seconds, and the emitted light allowed us to follow chromosome and tubulin dynamics with high temporal resolution. We used this technique to analyze chromosome separation and segregation in detail. The aim was to understand how segregation is affected in the absence of a functional Smc5/6 complex and Top2 activity.

Chapter 6
PRESENT INVESTIGATIONS

Paper I

Aim: Very little is known about post-translational regulation of the Smc5/6 complex. The impact of such modifications on the molecular function of the complex is even less studied. We therefore initiated this investigation with the aim to reveal when and how the Smc5/6 complex was modified. Once identified, we wanted to determine the functional relevance of the modifications.

Summary (Figure 9): In **paper I**, we first purified the Smc5/6 complex and then used mass-spectrometry to get an overview of what subunits were modified. We found that all of the Smc5/6 subunits, except Smc6, were phosphorylated. We focused on the Mms21 subunit of the Smc5/6 complex, which is a SUMO E3 ligase. We detected phosphorylation of three serines at the N-terminal in unchallenged cells and of two serines at the C-terminal after exposure to ionizing irradiation. Interestingly, the two C-terminal serines were also part of an S/T-Q motif, which is a consensus site for Mec1 and Tel1 kinases. The three N-terminal serines were lacking obvious candidate kinases, therefore we decided to focus on the Mms21 C-terminal serines. By WB analysis, a slower migrating form of Mms21 was detected in unchallenged cells, which accumulated in the presence of DNA damage induced by MMS. MMS is an alkylating agent that methylates the DNA and induces replication fork stalling (Lundin et al., 2005). The results displayed by WB were consistent with the presence of modified Mms21 obtained by mass-spectrometry in the presence of damage. The modification accumulated during S-and G2-phases, which correlates with proposed functions of the Smc5/6 complex. The upshifted form of Mms21 was abolished when treated with phosphatases, which confirmed the presence of phosphorylated residues. Additionally, the Mec1 kinase was shown to be mainly responsible for Mms21 modification, which likely

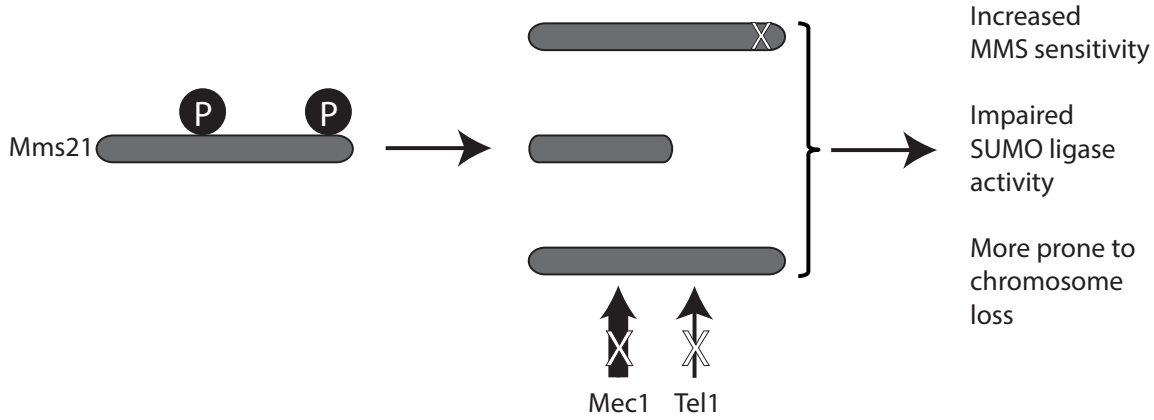

Figure 9. Summary of Paper I. Mms21 phosphorylation (P) was abolished following non-phosphorylatable substitutions of C-terminal serines, removal of the entire Mms21 C-terminus or inactivation of Mec1. Lack of Mms21 phosphorylation reduced its SUMO ligase activity, increased sensitivity/rate of chromosome loss in presence of DNA damage.

phosphorylated the C-terminal serines, as they were part of Mec1/Tel1 consensus site. Cells with C-terminal mutations of Mms21, *mms21-S260AS261A* and *mms21-S260IS261I*, were not phosphorylated and were also sensitive to MMS exposure. We next investigated the functional relevance for this C-terminal phosphorylation and found that phosphoablative Mms21 mutants had reduced SUMO ligase activity. Two Mms21 substrates, Smc1 and Smc6, were not fully SUMOylated when the C-terminal serines were mutated. We observed that the reduction of Mms21 SUMO activity was important for genome integrity, as phosphoablative Mms21 cells lost chromosomes more frequently under damaging conditions. We therefore proposed that Mec1-dependent C-terminal phosphorylation of Mms21 promotes genome integrity.

Perspectives: This was the first time that the Smc5/6 complex was shown to be under the control of the DNA damage checkpoint sensor Mec1. However, the exact mechanism by which Mec1 mediates genome stability through Smc5/6 remains to be determined. Our conclusion is that Mec1 phosphorylates Mms21, predominantly at the C-terminal sites. The importance for the N-terminal phosphorylation sites remains to be investigated. A feature of the phosphoablative Mms21 mutant was that it could SUMOylate its substrates, but to a much reduced level than in wild-type cells. In unchallenged cells the SUMO activity of the phosphoablative Mms21 mutant was very similar to a catalytic dead Mms21 mutant. In the presence of DNA damage, the phosphoablative Mms21 mutant displayed reduced SUMO ligase activity and cells were more prone to chromosome loss. The mechanism by which phosphorylation control the SUMO ligase activity could be explained by the Mms21 crystal structure. Based on this structure (Duan et al., 2009a), the C-terminal lies relatively free in space, which could make it particularly susceptible to post-translational modifications. Perhaps C-terminal phosphorylation of Mms21 triggers a conformational change of the protein that in turn makes the SUMO ligase catalytic site more active. To test this hypothesis, it would be interesting to investigate if the *mms21-S260AS261A* mutant changes the structure of the Mms21.

Since Mec1 phosphorylates Mms21, it is likely that the SUMO ligase function of Mms21 is a mediator in the DNA damage response. In addition, the Smc family of proteins is primarily SUMOylated by Mms21, while other proteins are mainly SUMOylated by the two other SUMO E3 ligases (Albuquerque et al., 2013). Therefore reduced SUMOylation levels of cohesin, condensin or the Smc5/6 complex in combination with their respective roles in the DNA damage response could explain the chromosome loss phenotype observed in the phosphoablative Mms21 mutants.

In regards to cohesin function, SUMO catalytic dead Mms21 mutants were important for establishing cohesion in response to damage (McAleenan et al., 2012). However, the phosphoablative Mms21 mutant formed normal damage-induced cohesion. This suggests that reduced SUMO ligase activity is sufficient for proper cohesin function under damaging conditions. The reason for more frequent chromosome loss events in the Mms21 phosphoablative mutant is therefore likely not in the cohesin pathway.

In phosphoablative and SUMO catalytic dead Mms21 mutants, we observed a similar reduction of SUMOylated Smc6. As shown before by 2D-gel analysis, SUMO catalytic dead Mms21 mutants accumulated recombination intermediates at blocked replication forks (Branzei et al., 2006). These mutants have also been shown to delay replication of longer chromosomes in unchallenged cells (Kegel et al., 2011). As chromosome loss events are triggered by DNA damage and faulty replication (Klein, 2001), it is possible that the replicative function of the Smc5/6 complex could play a key role for preventing chromosome loss. To further understand the role of Mms21 phosphorylation, analysis of the phosphoablative mutant for timely replication of long chromosomes and presence of recombination intermediates will be valuable.

Paper II

Aim: Previous studies in our research group showed that binding of the Smc5/6 complex to chromosomes accumulated in the absence of Top2 activity (Kegel et al., 2011). In addition, fewer so-called SCIs accumulated on a reporter plasmid in cells lacking combined Smc5/6 and Top2 functions, as compared to single Top2 mutants. This indicated that the Smc5/6 complex associates to SCIs or other structures that accumulate in the absence of Top2. Such association would however require the close proximity of the sister chromatids to one another. Therefore, chromosomal association of the Smc5/6 complex should decrease when cohesion is disrupted. According to an earlier investigation using ChIP-on-chip analysis, Smc5/6 binding to chromosomes was shown to be dependent on the cohesin loader Scc2. In the absence of cohesin, the chromosomal binding pattern of Smc5/6 changed but the complex still bound to chromosomes (Lindroos et al., 2006). The differences of Smc5/6 binding in Scc2 and cohesin mutants were difficult to reconcile since cohesin is absent from chromosomes in an Scc2 mutant. We therefore investigated the chromosomal binding pattern of the Smc5/6 complex using next generation ChIP-sequencing with the aim to further elucidate the accumulation of Smc5/6 binding in Top2 mutants.

Summary (Figure 10): In **paper II**, we first revisited the chromosomal association of the Smc5/6 complex in a cohesin mutant background. In contrast to previous results (Lindroos et al., 2006), Smc5/6 binding was reduced in the absence of Scc1 when using the ChIP-sequencing technique. The difference is likely due to that ChIP-on-chip is a less accurate method. The result obtained by ChIP-seq was confirmed by ChIP-qPCR and by immunofluorescence microscopy of chromosome spreads. Chromosomal binding of Smc5/6 was also dependent on the close proximity of sister chromatids, as shown by the absence of Smc5/6 on chromosomes in other cohesion-disrupting mutants; Scc2, Pds5 and Eco1. Smc5/6 binding accumulated on chromosome arms in the absence of Top2 activity, as shown previously (Kegel et al., 2011). In addition, Smc5/6 binding was shown to be independent of recombination, DNA breaks, and replication fork stalling. Instead, all our results indicated that the chromosomal binding of the Smc5/6 complex is due to a structure that is removed by Top2 activity. Additionally, this structure was most likely formed during replication and required cohesed chromosomes. A plausible explanation would be that Smc5/6 binds to SCIs.

To test this, we further examined segregation of chromosomes in the absence of Top2. Shorter chromosomes were able to segregate almost like wild-type but longer chromosomes often missegregated, which is in line with previous studies (Holm et al., 1989). These results show that chromosomes in Top2 mutant cells segregated as expected when SCIs were allowed to accumulate. Since the Smc5/6 complex assumedly accumulates at regions were SCIs are found, it would be predicted to perform a function there. We therefore investigated segregation of a short chromosome, which in the absence of Top2 function segregated fairly well. In the additional absence of functional Smc5/6, the short chromosome missegregated three times more frequently. This suggests that the Smc5/6 not only binds to these topological structures but also facilitates their resolution in order for chromosomes to segregate.

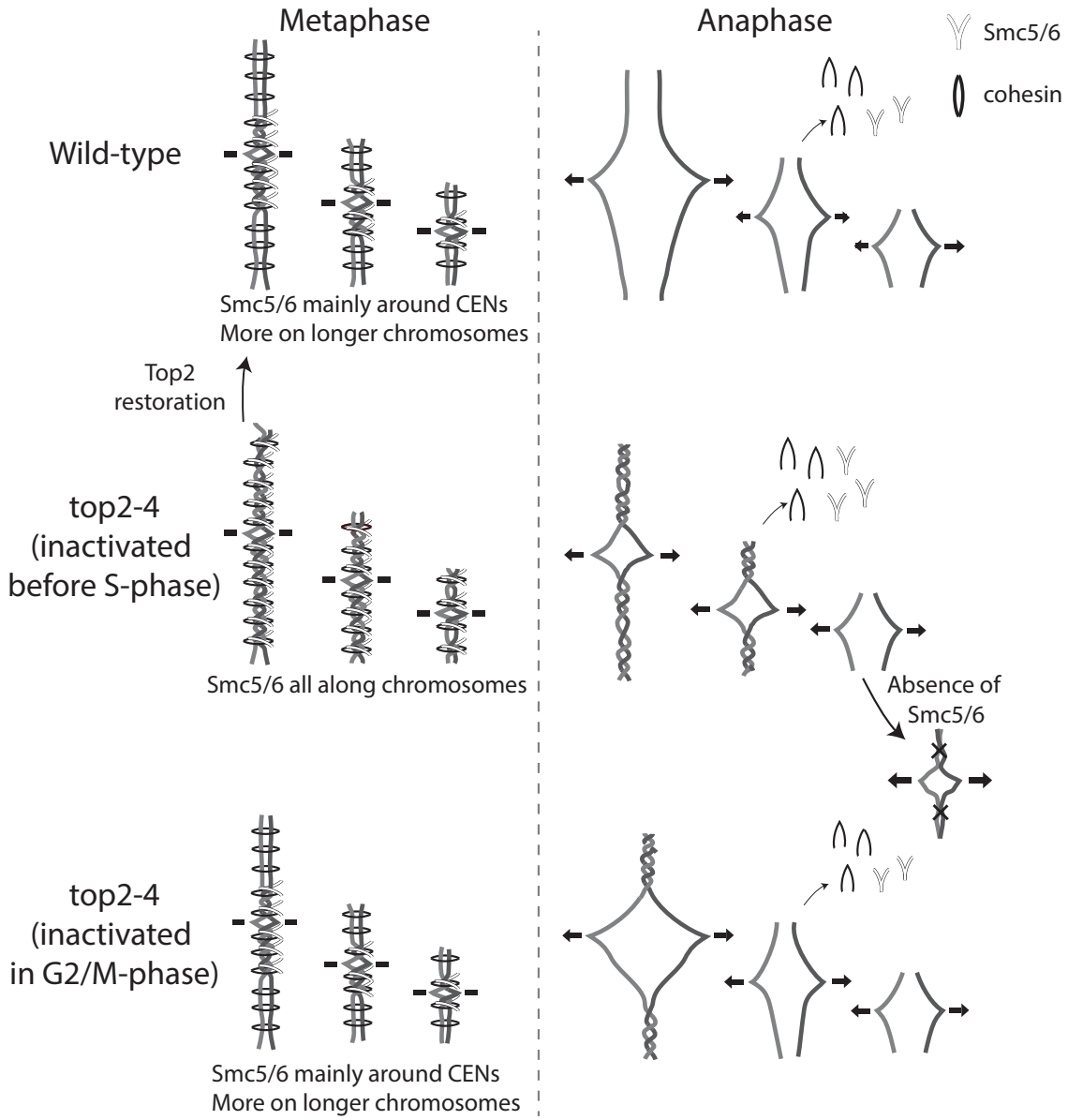

Figure 10. Summary of Paper II. Model describing Smc5/6 and cohesin binding in metaphase and the correlation to chromosome segregation in anaphase. The Smc5/6 complex most likely binds SCIs and, at least in the absence of functional Top2, facilitates their resolution.

Perspectives: Accumulation of Smc5/6 binding to chromosomes in the absence of Top2 activity was not due to presence of DNA breaks, recombination structures or stalled replication forks. The association of the Smc5/6 complex to chromosomes was instead dependent on cohesion between sister chromatids. In addition, the binding accumulated on chromosome arms in the absence of Top2 activity during S-phase. Restoration of Top2 function after replication removed Smc5/6 from chromosomes and also reduced the level of missegregation. These results suggest that Smc5/6 senses topological structures and a likely scenario is that the complex binds to SCIs. This is further supported by the correlation of Smc5/6 binding to the degree of chromosome missegregation in the absence of Top2 function. When Top2 was inactivated from the G1-phase, chromosome missegregation at telomere and arm regions was the predominant phenotype. Top2 inactivation after replication did not influence Smc5/6 binding, it did however lead to improved segregation of an intermediate chromosome. Arm regions of a long chromosome also segregated to a higher degree while the telomeric regions still missegregated. These results suggest that topological structures accumulate in the absence of Top2 function during S-phase and cause chromosome missegregation. Short chromosomes as well as telomeric regions on intermediate chromosomes are able segregate fairly well in the absence of Top2 during G2/M. This is in line with the idea that the pulling forces of the mitotic spindle can push SCIs from centromeres towards chromosome ends. The SCIs might therefore swivel off shorter chromosomes arms but not on longer ones. This concept can be illustrated by wrapping two ropes around each other and then trying to pull them apart **(Figure 4)**. The longer the ropes are, the more likely they are to "intertwine" and thereby preventing their separation.

The main argument against this hypothesis is the lack of direct evidence that the chromosome regions bound by Smc5/6 actually are SCIs. As SCIs were not observed directly by any assay used, we instead tried to eliminate other possibilities. One possibility to detect SCIs directly would be to excise the bound Smc5/6 sites and to perform 2D-gel analysis of these fragments. However, these topological structures must be "locked" in the same state as they appear on chromosomes. This might prove to be a difficult task since excising these Smc5/6 binding sites may release SCIs that have accumulated.

Another possibility is that Smc5/6 binds indirectly to SCIs, possibly via cohesin. Smc5/6 binding was dependent on cohesin in our investigation. In addition, Smc5/6 binding overlapped with that of cohesin. A previous study linked cohesin to SCIs, where it was speculated to protect SCIs from resolution by Top2 (Farcas et al., 2011). It was proposed that SCIs are important for mediating cohesion and maybe the role of Smc5/6 is to further protect these sites from premature resolution. This scenario could explain why a short chromosome missegregated more frequently in the absence of both Smc5/6 and Top2 function, in comparison to each single mutant. This suggests that Smc5/6 is needed for resolution of SCIs independently of Top2 function after completion of replication. However, absence of functional Smc5/6 has been shown to accumulate recombination intermediates at centromeric regions (Yong-Gonzales et al., 2012). To distinguish between the roles of SCIs and

recombination structures in chromosome segregation, Rad52 Smc5/6 Top2 triple mutants could be investigated.

Paper III

Aim: The Smc5/6 complex has been linked to the process of replication. The complex was found to associate to the replication fork and bind with higher frequency to longer chromosomes in unchallenged cells (Kegel et al., 2011, Jeppsson et al., 2014). In addition, Smc5/6 ts mutants delayed replication on long chromosomes, but not short ones (Kegel et al., 2011). Perhaps the replicative function of the Smc5/6 complex could also control chromosome segregation events in mitosis. The relatively unknown function for the Smc5/6 complex in segregation prompted us to initiate this study. Our aim was to investigate if segregation was delayed on longer chromosomes in Smc5/6 mutants.

Summary (Figure 11): We utilized live cell imaging to study the segregation process in **paper III**. To visualize the chromosomes, different positions of a long, intermediate and short chromosome were targeted with a fluorescent marker. Chromosome separation and segregation was scored in relation to tubulin elongation, a marker for anaphase onset. To our

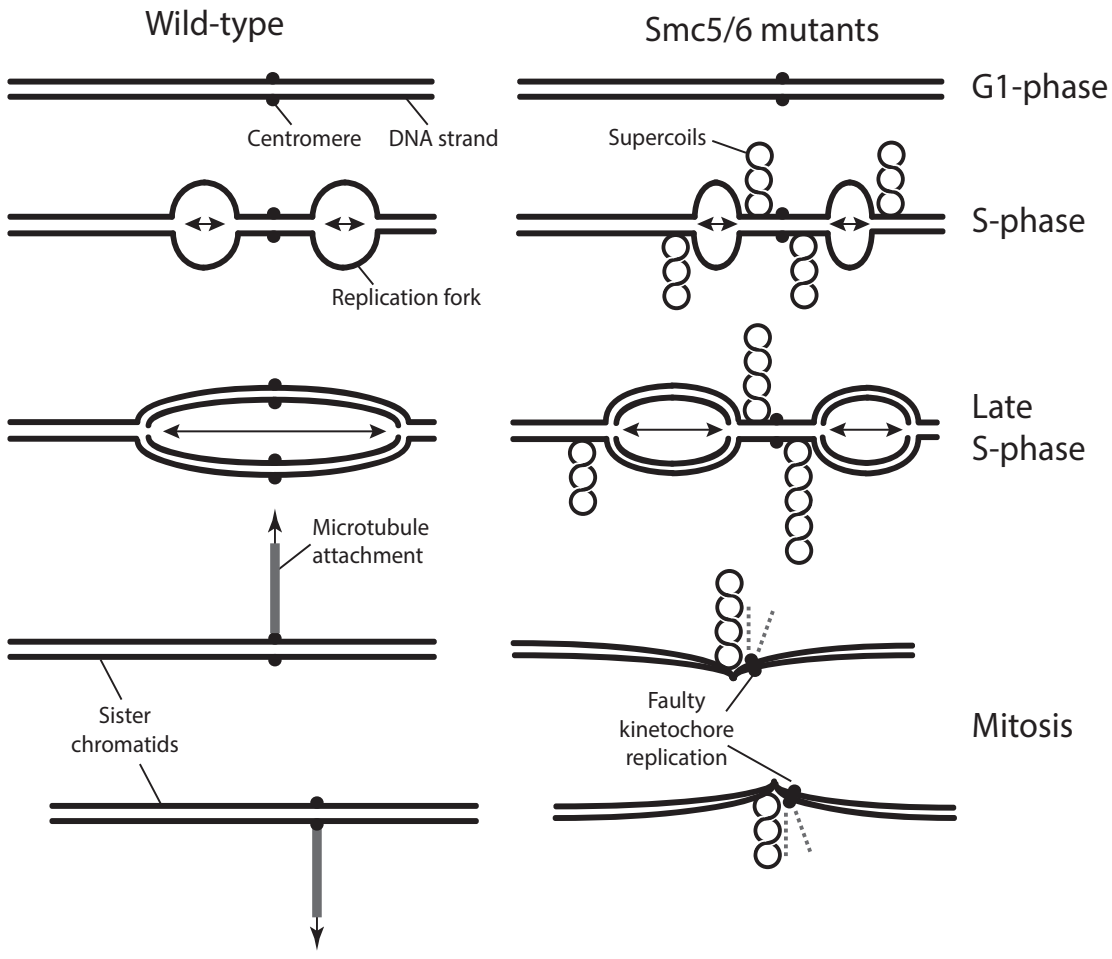

Figure 11. Summary of Paper III. In Smc5/6 mutants, delay of replication might cause faulty kinetochore attachment to microtubules, which triggers the SAC.

surprise, we did not observe a chromosome length-dependent missegregation in Smc5/6 ts mutants. At all chromosomal positions investigated, the same low percentage of Smc5/6 mutant cells missegregated their chromosomes. Instead, a larger population of cells was arrested in the first mitosis upon release from G1-phase with non-functional Smc5/6. The behavior of tubulin in these cells suggested that the SAC was active. This notion was confirmed by the observation that the Smc6 ts mutant segregated similarly to WT cells after disruption of the SAC by deleting the checkpoint protein Mad2. To further validate these results, other ts mutants and an Nse4 null allele were analyzed. While the same phenotype was observed, the number of checkpoint arrested cells varied in the ts and null mutants. To understand this variability, we analyzed the Nse4 null allele at high (35°C) and low temperatures (25°C). The reason for this was the observations that the Smc5/6 complex delayed replication on longer chromosomes, and that this delay might be due to a feature of replication that occurs more frequently at high temperature. Therefore, the need of the Smc5/6 complex might be greater at high as compared to low temperature. We observed that more Nse4 null mutant cells were checkpoint arrested when grown at high temperature compared to growth at low temperature (25°C). At both conditions, the null mutant went through mitosis like WT cells after disruption of the SAC by deleting *MAD2*. This suggests that replication in Smc5/6 mutants is more perturbed at high temperature, which in turn correlates with the amount of checkpoint arrested cells. Together with a study showing that the Smc5/6 complex also localizes to spindles at the kinetochore (Yong-Gonzales et al., 2012), we propose that the replicative function of Smc5/6 promotes proper kinetochore-microtubule attachment and segregation of chromosomes.

Perspectives: We observed a varying degree of checkpoint arrested cells of different Smc5/6 ts mutants. In addition, some of these mutants grew slower than WT even at the permissive temperature (25°). Therefore, ts mutants might not be optimal to use for studies of Smc5/6 function. The use of the Nse4 null mutant is temperature independent. In these cells, few were checkpoint arrested at low temperature. At high temperature on the other hand, more Nse4 null mutant cells were arrested. Together with the known role of the Smc5/6 complex for completion of replication on longer chromosomes, this indicates that Smc5/6 function is needed to overcome a replication related problem. It might be that DNA supercoiling accumulates in the absence of functional Smc5/6, and even more so at elevated temperatures. This is supported by the observation that Smc5/6 is needed to deal with replication-induced supercoiling (Jeppsson et al., 2014). Furthermore, unpublished results from our lab suggest that the Smc5/6 complex also has a role in handling trancription-induced supercoiling (Jeppsson and Sjögren, unpublished). Therefore, increasing temperature would more likely to induce supercoiling in Smc5/6 mutants as a result of higher speed of the replication and transcription machineries. Consequently, longer chromosomes should be more prone to trigger the checkpoint in Smc5/6 mutants, while disrupting the SAC in these cells would cause long chromosomes to missegregate more often. We have analyzed segregation on the arm of the long chromosome IV in an Smc6 ts mutant after disrupting the SAC and these cells seldom missegregated chromosomes. Therefore, segregation analysis of the telomere on

chromosome IV could be used to test this hypothesis. Other possible experiments would include investigating segregation on long linear chromosome fusions or circularized chromosomes (Kegel et al., 2011, Titos et al., 2014).

So far, we have only investigated checkpoint arrest in the first mitosis following repression of mutants. Since only segregation in ts mutants has been analyzed, the next step would be to introduce the lac-operon/lac-repressor (LacO/LacR) system into the Nse4 null mutant background. This analysis will also reveal the reason why depletion of Nse4 and other conditional Smc5/6 null mutants (Carlborg, Carter and Sjögren, unpublished) arrest in the second mitosis when grown at 25°C. It is plausible that if topological problems are allowed to accumulate over an entire cell cycle, a more pronounced checkpoint arrest might be observed during the second mitosis. Additionally, it would be interesting to test the Nse4 null mutant in replication and repair assays to confirm and refine a general Smc5/6 phenotype. In the future, careful analysis of Nse4 and other conditional null alleles of Smc5/6 will establish if this is correct.

In addition to Smc5/6 function, SUMO ligase activity of Mms21 was also reported to equally contribute to replication of long chromosomes and to spindle elongation (Kegel et al., 2011, Yong-Gonzales et al., 2012). In our assay, we did not detect any checkpoint arrested cells when SUMO ligase activity was compromised. This could be explained by the setup of our analysis. Yong-Gonzales et al. looked at tubulin morphology 75 minutes post G1-release while we performed a time-course experiment with the final timepoint being at 115 minutes post G1-release. Perhaps compromised SUMO ligase activity delays anaphase onset but cells are able to silence the checkpoint earlier than the ts and null mutants. Since we used spindle elongation as a marker for anaphase onset, we were only interested in whether the spindle elongated or not. Re-analysis of the timepoint when spindle elongation occurs in relation to when cells were G1-released would answer if SAC is prolonged in SUMO ligase mutants. Finally, Mms21 SUMO ligase activity was also shown to be important for kinetochore localization to spindles (Yong-Gonzales et al., 2012). It would of interest to investigate if Smc5/6 ts and null mutants also disrupt kinetochore localization to spindles and if Mad2 localizes to these sites, an indication of unattached kinetochores. Based on the phenotypes of the Smc5/6 complex in replication, kinetochore localization to spindles and SAC, an interesting model would be that the replicative defects in Smc5/6 mutants delay centromere replication, and especially longer chromosomes would therefore have faulty kinetochore attachment. This in turn would lead to persistent SAC activation.

Chapter 7
CONCLUDING REMARKS

The Smc5/6 complex is an evolutionary conserved protein. We have studied Smc5/6 post-translational modifications coupled to DNA repair, Smc5/6 chromosomal association, and also its functions in segregation in the budding yeast *S. cerevisiae*. It is likely that the role of Smc5/6 in segregation and repair are connected to the complex' replicative function. Replication, segregation and repair are all fundamental cellular processes that are essential for maintaining genome stability. Since genome instability is tightly connected to human disease, studying the conserved Smc5/6 complex is not only important from a basic research point of view, but also relevant from a medical perspective. There is accumulating evidence that defective replication, segregation and/or repair can drive tumor development. The exploration of the molecular mechanisms behind these processes are therefore thought to lead to the identification of novel cancer drug targets (Rajagopalan and Lengauer, 2004).

We have shown that mutants of the Smc5/6 complex are more prone to chromosome loss events under damaging conditions, which cause genomic instability. In addition, we find that Smc5/6 is likely to bind SCIs and aid their resolution prior to mitosis. Although SCIs might play important roles to facilitate sister chromatid cohesion and DNA replication, they must be removed prior to anaphase onset in order for chromosomes to segregate properly. If SCIs are left unresolved, there is an elevated risk of chromosome missegregation with resulting aneuploidy in the next generation of cells. Chromosomal aberrations and aneuploidy can cause developmental disorders and are often found in tumor cells. This is yet another reason that makes the Smc5/6 complex an important protein to study in the quest to understand cancer development and other syndromes.

The general aim of our research is to unravel the molecular mechanisms of the Smc5/6 complex in replication, segregation and repair. These processes together protect the genome against chromosomal abnormalities. Since the Smc5/6 complex has established roles in all of these processes, it is important to understand how these functions are linked. With more knowledge of the molecular mechanisms for maintaining genome stability, more insights will be gained that will help to understand cancer development and the cause of developmental disorders. With the present investigations, I hope to have contributed with some pieces that will solve the puzzle of Smc5/6 function.

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