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STRUCTURAL MAINTENANCE OF CHROMOSOMES –

A complex tale of genomic integrity

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ABSTRACT

Genomic integrity is an absolute requirement for cell survival. Programmed events such as genome rearrangements and DNA replication can cause lesions in the DNA, as can exogenous agents such as radiation and chemicals. One of the most austere types of lesion is DNA double strand breaks (DBSs). In *Saccharomyces cerevisiae*, budding yeast, they are preferentially repaired by the homologous recombination (HR) pathway using a homologous DNA sequence as template.

The Structural maintenance of chromosomes (SMC) family proteins are essential for cell viability and have functions in chromosome condensation, segregation and in DNA repair by HR. The cohesin complex is important for cohesion and correct segregation of sister chromatids. The Smc5/6 complex functions late in the HR process and it has another function, not yet entirely elucidated, that makes the complex essential.

We have investigated the chromosomal localization of the Smc5/6 complex and found that the complex associates with specific sites along the chromosome arms in a chromosome length-dependent manner. This association is dependent on the cohesin loading protein Scc2. The complex also localizes to chromosomal regions surrounding a DNA DSB in the G2/M phase. Localization to DSBs is dependent on the damage-sensing HR protein Mre11, but not on Scc2. Smc6 mutants exhibit a delay in chromosome segregation and a closer investigation suggests that this delay is caused by persisting replication forks.

The length-dependent distribution of the Smc5/6 complex on chromosomes was found to reflect a function of the complex that is independent of its function in HR. A possible explanation for this length-dependency is the accumulation of replication-induced topological structures on longer chromosomes due to their inability to swivel off the torsional stress. A circular short chromosome is therefore expected to generate more unresolved topological structures than a linear version of the same chromosome. Smc5/6 complex components showed an increase in binding regions on a circular chromosome compared to the linear version. Deletion of Top1, a protein required for release of replication-induced torsional tension in DNA, also shows a similar chromosome length-specific phenotype as the Smc5/6 complex components, indicating that topology is the inherent cause of the Smc5/6 complex association with chromosomes.

The main function of the cohesin complex is linking the sister chromatids from S phase until the metaphase-to-anaphase transition. To investigate a role for cohesin in DSB repair, we examined its localization in response to a site-specific DSB. Cohesin is normally loaded onto DNA in late G1/early S phase, but when a DNA break has been induced in G2/M, cohesin localizes to the break area in a Scc2-dependent manner. In addition, we have demonstrated that cohesin recruited in response to DSBs in G2/M phase can mediate cohesion, supporting the idea that cohesin and sister chromatid cohesion have a role in DNA repair.

The damage-induced cohesion can be distinguished from cohesion formed during replication and the regulation and function of this damage-induced cohesion was found to be dependent on Mre11 and the Tel1 and Mec1 kinases. The HR protein Rad52 protein was not required, showing that contrary to S phase-established cohesion, formation of damage-induced cohesion in G2/M phase is independent of DNA synthesis.
A single DNA DSB is enough to generate cohesion throughout the entire genome. Mec1, Scc2, Smc6 and the establishment of cohesion protein Eco1 are also required for genome-wide cohesion after DSB induction, and the damage-induced cohesion is required for DNA repair.

LIST OF PUBLICATIONS

This thesis is based on the following articles and manuscript. They will be referred to in the text by their roman numerals.

I. **Hanna Betts Lindroos***, Lena Ström*, Takehiko Itoh, Yuki Katou, Katsuhiko Shirahige and Camilla Sjögren
Chromosomal association of the Smc5/6 complex reveals that it functions in differently regulated pathways.
Molecular Cell (2006) 22:755-767

II. **Hanna Betts Lindroos***, Andreas Kegel*, Xiaolan Zhao, Katsuhiko Shirahige and Camilla Sjögren
The Smc5/6 complex acts together with topoisomerase I in the resolution of chromosome length-dependent stress.
Manuscript

III. Lena Ström, **Hanna Betts Lindroos**, Katsuhiko Shirahige and Camilla Sjögren
Postreplicative recruitment of cohesin to double-strand breaks is required for DNA repair.
Molecular cell (2004) 16:1-20

IV. Lena Ström, Charlotte Karlsson, **Hanna Betts Lindroos**, Sara Wedahl, Yuki Katou, Katsuhiko Shirahige and Camilla Sjögren
Postreplicative formation of cohesion is required for repair and induced by a single DNA break.
Science (2007) 313:242-245

* these authors contributed equally to this work.

ABBREVIATIONS

ARS	autonomously replicating sequence
ChIP	chromatin immunoprecipitation
DSB	double strand break
HR	homologous recombination
NHEJ	non-homologous end-joining
ORC	origin recognition complex
PCNA	proliferating cell nuclear antigen
PFGE	pulsed field gel electrophoresis
rDNA	ribosomal DNA
RFB	replication fork barrier
ssDNA	single stranded DNA
SUMO	small ubiquitin-like modifier
ts	temperature sensitive
wt	wild type

Proteins

Cdc6	cell division cycle
Cdt1	cdc10-dependent transcript
Chk1	checkpoint kinase
Ddc2	DNA dependent checkpoint
Dun1	DNA damage uninducible
Eco1	establishment of cohesion
Esc2,4	establishes silent chromatin
Esp1	extra spindle pole bodies
MCM	minichromosome maintenance
Mec1	mitosis entry checkpoint
Mrc1	mediator of the replication checkpoint
Mre11	meiotic recombination
Nse1-6	non-SMC element
Pds1	precocious dissociation of sisters
Rad9, 50 etc.	radiation sensitive
RPA	replication protein A
Scc1-4	sister chromatid cohesion
Smc1-6	structural maintenance of chromosomes
Tel1	telomere maintenance
Top1,2	topoisomerase
Xrs2	X-ray sensitive

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1. INTRODUCTION

Survival of an organism – no matter how few or how many cells it contains – depends on its ability to accurately transfer an identical copy of the complete genetic material to its daughter cells. To achieve this, the entire genome must first be faithfully replicated and then distributed with profound precision to the two cells. The replication process itself is fraught with danger, and spontaneously occurring DNA breaks as well as those induced by exogenous agents must therefore be mended promptly to prevent genome instability. Central players in these processes are the structural maintenance of chromosomes (SMC) proteins. They are essential proteins, conserved throughout evolution and required for diverse actions such as chromosome condensation, cohesion and segregation, as well as for DNA repair by homologous recombination. All eukaryotic SMC proteins form heterodimers which associate with additional protein subunits to form large complexes. Smc1 and Smc3 together with two additional subunits form the cohesin complex involved in sister chromatid cohesion and DNA repair. Smc2 and Smc4 along with three other subunits make up the condensin complex required for proper chromosome condensation and segregation. Lastly, the Smc5/6 complex, consisting of Smc5, Smc6 and six non-SMC subunits, functions in DNA repair and checkpoint response (Losada and Hirano 2005).

The aim of this thesis was to elucidate more about the function(s) of the cohesin and the Smc5/6 complex and for this we used the budding yeast *Saccharomyces cerevisiae* as a model organism.

2. YEAST AS A MODEL ORGANISM

Yeast is in many respects an ideal model organism. It is a unicellular eukaryote with many of the properties for which bacteria are appreciated as a model, such as rapid growth and a well-defined genetic system. Being a eukaryote organism, many insights into cellular processes in yeast can provide insight into functions in mammalian cells, without having to deal with the complexity of multi-cellular organisms. It's also non-pathogenic and requires virtually no precautions for handling.

Saccharomyces cerevisiae propagates by budding off a daughter cell (*Fig. 1*), as opposed to *Schizosaccharomyces pombe* that divides by fission, hence the common names budding and fission yeast respectively. Budding yeast propagate in both haploid (vegetative) and diploid (sexual) states. The haploid forms have either one of two separate sexes, a or α, that can mate and form diploid cells. When reproducing sexually, four haploid gametes are formed that cling together in a spore-sack, called an

ascus, allowing for study of the progeny and its genetics. The haploid genome is made up of 16 chromosomes ranging between 200 and 2200 kilobases (kb) in size, with a total of 14000 kb. The entire genomic sequence was presented in 1996 and was the first complete eukaryotic genome sequenced.

Budding yeast has a highly efficient DNA recombination system that has enabled development of gene cloning and genetic engineering techniques. Transformed cells use homologous recombination to integrate the DNA into the genome with remarkable efficiency. This makes gene deletions, gene substitutions, epitope tagging and modification of genes using plasmid- or cloned sequences relatively straightforward, the expected total time for production of a gene knock-out is approximately two weeks! (Sherman 1991; Oliver 1999)

Saccharomyces cerevisiae is our model organism of choice and all information in this thesis such as schematic models, gene names and cellular processes will refer to budding yeast if not otherwise specified.

3. THE CELL CYCLE

Cells beget more cells by duplicating their content and dividing it up in two parts. This event is known as a cell cycle and it is essential to all living organisms. The cell cycle is divided into four phases (*Fig. 1*). The replication of the genetic material occurs during the synthesis (S) phase and the segregation of the compacted chromosomes in the mitosis (M) phase. The remaining two phases are known as gap (G) phases, G1 and G2 during which the cells grow, mature and prepare for the S and M phases respectively (Hartwell and Weinert 1989). The Gap phases will not be further discussed here.

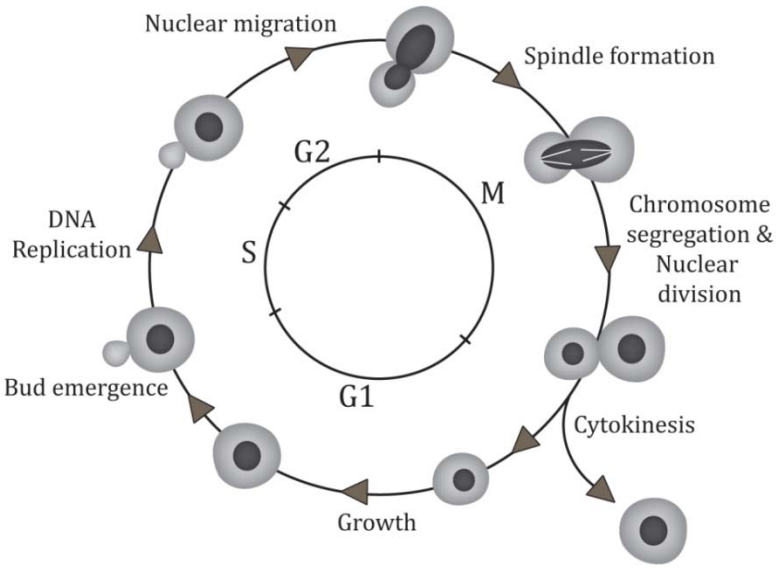

Fig. 1. **The budding yeast cell cycle.**

3.1 DNA Synthesis

The initiation of replication occurs at several sites along the chromosomes. These sites are referred to as origins of replication, and the DNA that is replicated from one origin is known as a replicon. Replication initiates at the origins and proceeds outwards in both directions and eventually meets and fuses with the neighbouring replicons (*Fig. 2*) (Kelly and Brown 2000; Sclafani and Holzen 2007).

The budding yeast origins are often referred to as autonomously replicating sequences (ARSs). Unlike most organisms, budding yeast contains a specific ARS sequence, a 17 base-pair A-T rich consensus region. ARSs are located every 40-150 kb (Branzei and Foiani 2007) on the 16 chromosomes of budding yeast and during one S phase most are used to initiate replication, even though some are decidedly more active than others. Not all ARSs start replication at the same time, the temporal distribution of the firing of origins is determined in the G1 phase of the cell cycle (Sclafani and Holzen 2007).

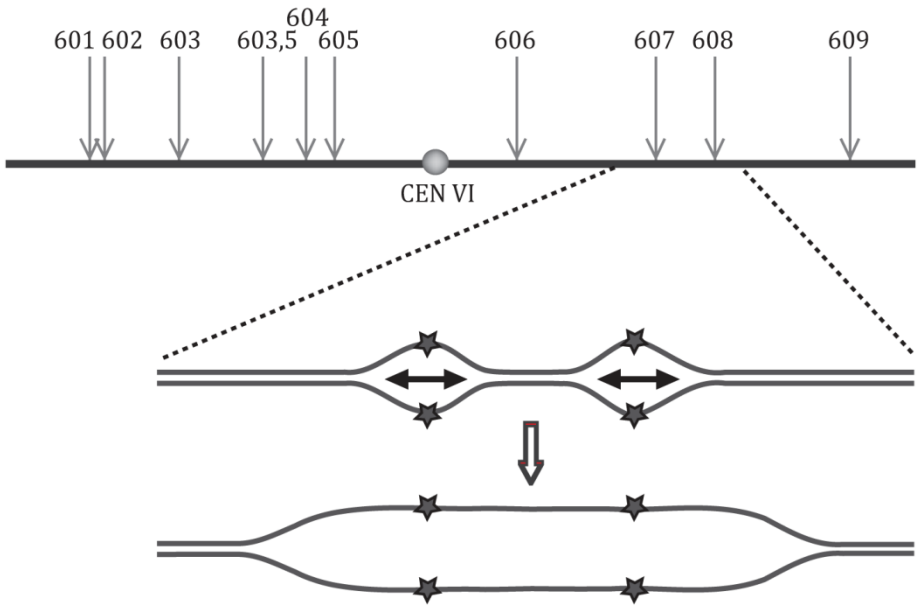

Fig. 2. **Replication origins on chr.VI and a close-up of two fusing replicons.** The thin, grey arrows indicate the positions of the origins and the double-headed arrows show the direction of replication fork progression. The stars denote the origins.

Initiation of replication is a two-step process that begins in G1 with the origin recognition complex (ORC) binding to the origin (*Fig. 3*). The ORC serves as a landing platform for a complex of MCM helicases that is loaded by the cofactors Cdc6 and Cdt1. The role of the MCM complex is to unwind the DNA double–helix, producing single-stranded (ss)DNA (Sclafani and Holzen 2007; Forsburg 2008).

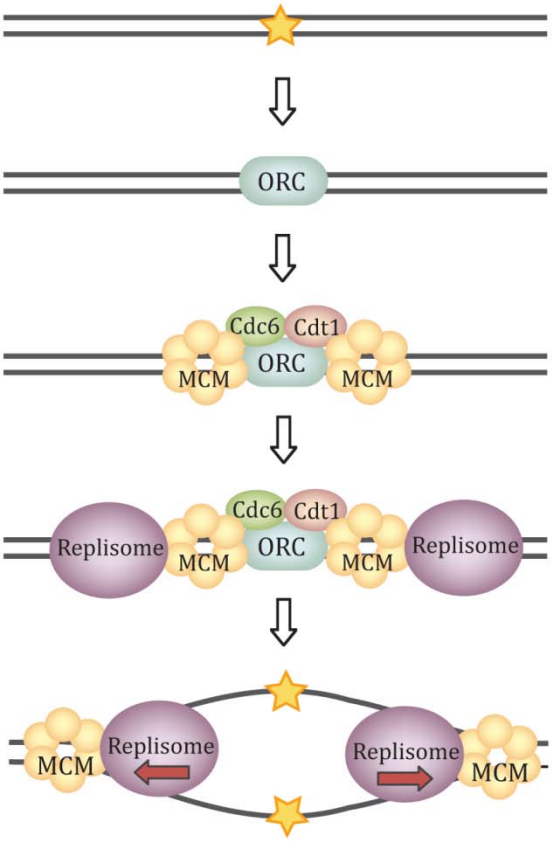

Fig. 3. **The steps in replication initiation and the proteins required for this process.** The stars denote the origin.

The second step in replication initiation is activation of the origin through creation of a replication fork, which is the junction where double stranded DNA becomes ssDNA as a result of helicase activity. This requires several additional cofactors that are recruited by the ORC and they activate the MCM complex and load the replisome (Kelly and Brown 2000). DNA polymerases ε and δ, replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) make up the replisome that copies the exposed ssDNA (Branzei and Foiani 2007). PCNA is a ring-shaped replication factor that clamps the replicative polymerases ε and δ to DNA, and RFC is the loader of the PCNA clamp (Moldovan, Pfander et al. 2007).

The replisome and the MCM complex move with the replication fork as it progresses along the DNA with newly synthesized DNA trailing in its wake. Occasionally the moving fork encounters damage to the DNA template, highly transcribed genes e.g. transfer RNA (tRNA) genes, or a replication fork barrier (RFB), a site where the proteins bind tightly to DNA. These events hinder the progression of the replication fork causing it to stall (Labib and Hodgson 2007). Stalled forks activate a checkpoint in the cell that causes a new subset of proteins bind to the fork to stabilize the ssDNA and prevent the fork from collapsing (see also chapter 5.2). When a replication fork encounters a fork moving in the opposite direction replication terminates and the replisome is disassembled. When all chromosomal DNA is replicated, the cell contains two identical copies of each chromosome, known as sister chromatids. To prevent premature and faulty separation of the chromatids they need to be identified by the cell as sisters immediately when they are formed. This is achieved by the protein complex cohesin that holds the sister chromatids together until it is time to separate them in mitosis (Hagstrom and Meyer 2003; Skibbens, Maradeo et al. 2007).

3.1.1 Topoisomerases

When the replication fork moves forward, torsional stress accumulates as a result of separation of the two strands in the DNA helix. Ahead of the progressing fork the stress is transformed into positive supercoils, and behind the fork sister chromatid intertwinings are generated. These structures must be resolved for the progression of replication and for correct chromosome segregation in mitosis. This task is performed by enzymes known as topoisomerases, of which there are two types; those that cleave one DNA strand are defined as type I, and those that cleave both strands are known as type II (Champoux 2001).

The role of the type I topoisomerase Top1 is to relax supercoils that build up ahead of replication and transcription by nicking one DNA strand to release tension by rotation of the loose strand around the intact strand, and then re-ligating the two strands ends (*Fig. 4*). Top1 association with DNA is normally very transient and difficult to detect. Treatment with the camptothecin type of anti-cancer drugs traps Top1 at DNA as it is performing the nick. When the replication fork collides with a trapped Top1 molecule, replication is inhibited (Pommier, Barcelo et al. 2006). Treatment with this type of drug will also lead to an accumulation of supercoils ahead of the replication fork. This, too, will hinder progression of the replication fork (Koster, Palle et al. 2007).

Top2 is a type II topoisomerase that catalyzes an ATP-dependent movement of one double-helix through another. It is required for resolution of chromatid intertwinings known as DNA catenations that are

a by-product of replication. Top2 activity is therefore indispensable for correct chromosome segregation (Wang 2002) (Diaz-Martinez, Gimenez-Abian et al. 2008).

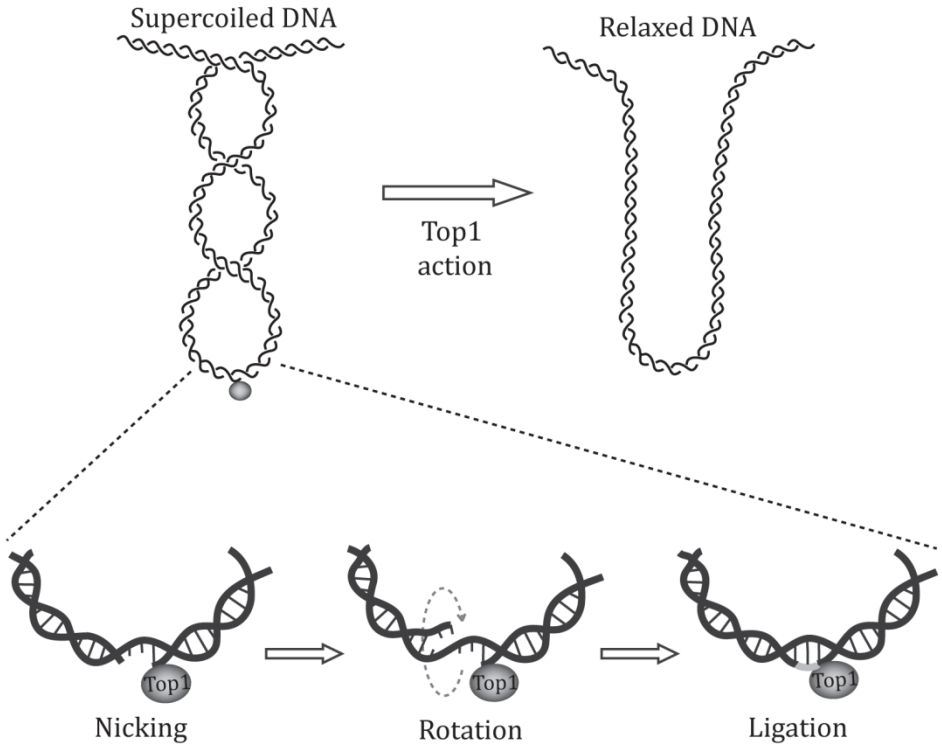

Fig. 4. **Top1 function in resolving replication-induced supercoils.** (Adapted from (Pommier 2006))

3.2 Mitosis

The M phase is a complex process requiring a multitude of proteins. Briefly it can be divided into six stages – prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis that occur sequentially.
In prophase condensation of the chromosomes is initialized, a process requiring the condensin SMC protein complex. The mitotic spindle is an assembly of microtubules that forms between opposite poles of the cell. In prometaphase and metaphase microtubules from the spindle poles will attach to the protein structure at the centromeric region known as the kinetochore. A tug of war commences, resulting in alignment of the fully condensed chromosomes at the bud neck. The connection between the sister chromatids is lost at anaphase onset, leaving sisters free to segregate to opposite poles of the cell. As cells progress to telophase the chromosomes have reached opposite the poles of the cell and decondense. Cytokinesis then takes place, during which the cytoplasm of the cells divide to generate two genetically identical daughter cells (Alberts 2002) (Hagstrom and Meyer 2003).

4. REPAIR OF DNA DOUBLE-STRAND BREAKS

Upholding genomic integrity is crucial for cell cycle progression. DNA damage can severely compromise chromosomal stability if left unrepaired. Double strand breaks (DSBs) are perhaps the most harmful type of damage to a cell, and must therefore be recognized immediately and efficiently mended. DSBs are produced by a multitude of naturally occurring cellular processes such as replication fork collapse and programmed genome rearrangements, as well as by external DNA damaging agents such as ionizing radiation and chemicals (Shrivastav, De Haro et al. 2008).

4.1 HR vs NHEJ

Eukaryotes have two alternative methods for repair of DSBs. Yeast cells preferentially utilize a method for repair known as homologous recombination (HR) that employs a homologous DNA sequence as template for repair, thereby ensuring a high degree of fidelity (Krogh and Symington 2004). The alternative method for repair of DSBs is non-homologous end joining (NHEJ), which is used extensively by higher eukaryotes. This mechanism is able to join DNA ends with little or no homology, but can lead to a substantial loss of genetic material in the process (Aylon and Kupiec 2004).

The balance between the two repair methods shifts during the budding yeast cell cycle, from NHEJ being the preferred method for repair in G1 phase – to HR as the cells progress through S toward G2/M phase where a sister chromatid is available as template. NHEJ is still active in S and G2 phase, but to a much lower extent (Shrivastav, De Haro et al. 2008).

Cell type also matters in the choice between HR and NHEJ. In haploid cells HR is only efficient after DNA replication when a sister chromatid has formed. In diploid cells a homologous template is always available and therefore HR is preferred over NHEJ. It has even been shown that non-homologous repair pathways are repressed in diploid cells (Aylon and Kupiec 2004).

4.2 Homologous recombination

A DSB in the genome is identified by the MRX (Mre11-Rad50-Xrs2) complex (Fig. 5). Mre11 has intrinsic nuclease activity and aids in the next step of the recombination process; resectioning of the DNA ends in the 5’ to 3’ direction, thereby creating 3’ ssDNA tails. The resected ends are then coated with RPA, possibly to remove secondary structures from the ssDNA (Krogh and Symington 2004). Rad52-dependent deposition of Rad51 on the RPA-coated DNA displaces the RPA and converts the

ssDNA into a nucleofilament with the ability to interact with another DNA molecule and initiate strand exchange. Rad51 continues to accumulate on the ssDNA until a homologous region has been located in the template. The nucelofilament then associates with the intact donor template, a process known as strand invasion that is facilitated by several other Rad proteins. At this time Rad51 is removed from the filament, and the invading ssDNA strand acts as a primer for DNA synthesis. (Aylon and Kupiec 2004).

After elongation of the resected DNA, the second end of the DSB is captured and acts as primer for synthesis of the other template strand. All strands are then ligated, generating a double Holliday junction intermediate that must be resolved for accurate segregation of the DNA molecules, a process that requires type I topoisomerases (Krogh and Symington 2004).

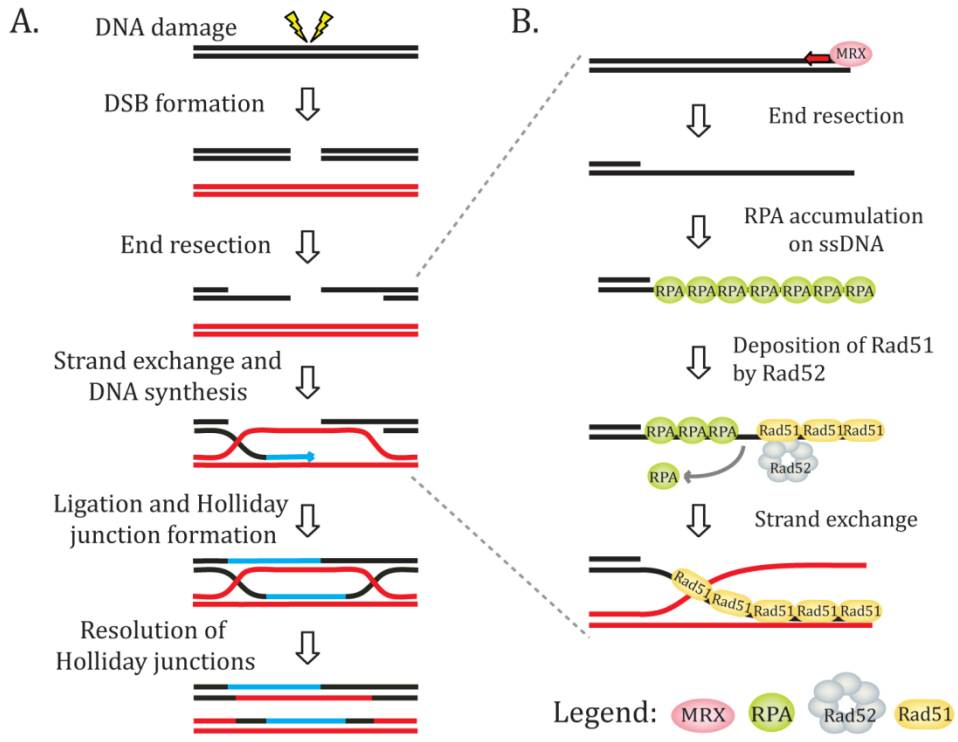

Fig. 5. **Homologous recombination. A.** Schematic overview of the sequential steps in HR. **B.** Detailed view of nucleofilament formation and the proteins required for this process. See text for details (A. Based on (Krogh and Symington 2004; San Filippo, Sung et al. 2008))

5. CHECKPOINTS

For maintaining genomic stability and preventing the catastrophic effects of a DSB, cells have developed surveillance mechanisms known as checkpoints that monitor the state of the chromosomes. Damage to the DNA or perturbation of the replication process is sensed by the cell and

signals are sent to the cell cycle machinery that in response halts or slows down the cell cycle to allow repair before replication proceeds or cells engage in mitosis (Carr 2002; Kolodner, Putnam et al. 2002).

The checkpoint proteins function in signalling cascades that interact to form large networks. Recruitment of checkpoint proteins to sites of DNA damage occurs via ssDNA intermediates created by repair complexes that signals to activate the checkpoint machinery (Branzei and Foiani 2008).

5.1 Checkpoint response to DSBs

The earliest known sensor of a DNA DSB in budding yeast is, as previously noted, the MRX complex (*Fig. 5*). It binds to the DNA ends and tethers them to each other through Rad50 and Mre11 aids in resection of the DNA which is the initial step in repair. The MRX complex then recruits the Tel1 kinase to the damaged area (*Fig. 6*) (Lisby and Rothstein 2005). The ssDNA generated by resection of DNA at the break site recruits the central damage checkpoint component Mec1 and its regulator Ddc2 to the site of damage (Yeung and Durocher 2008).
Downstream in the checkpoint network is Rad9, which is phosphorylated in response to DNA damage in a Tel1/Mec1-dependent manner. It interacts with the Rad53 kinase and promotes Rad53 autophosphorylation, thereby activating its kinase activity (Fu, Pastushok et al. 2008) (Branzei and Foiani 2006). The Rad9/Rad53 complex in turn phosphorylates Dun1 which regulates the transcriptional response to DNA damage. Another kinase, Chk1, is also activated by Mec1 in a Rad9-dependent manner. Among Chk1's numerous activities is the phosphorylation of Pds1 which prevents its degradation and thereby inhibits the metaphase-anaphase transition (see also chapter 7.1.3) (Jessberger 2002) (DeMase, Zeng et al. 2005).

The Mec1 and Tel1 kinases are also responsible for the phosphorylation of histone 2A, which in its phosphorylated form is referred to as γH2A. This occurs within minutes of break formation and stretches for several hundred base-pairs around the break site. DSBs always trigger γH2A formation, regardless of the origin of the damage. The exact function of γH2A is not yet mapped out, but it is known to promote the recruitment of repair factors to the DNA region immediately adjoining a break (Fillingham, Keogh et al. 2006).

5.2 Replication checkpoint

As already mentioned, the replication fork progression along template DNA is slowed down or stalled at several sites in the genome. If the stalled fork is not stabilized, the replisome dissociates from DNA and the fork collapses, creating a DSB. Cells then have to activate other

mechanisms to protect the fork from being processed incorrectly (Branzei and Foiani 2007).

The exposure of ssDNA at a stalled or collapsed fork promotes checkpoint activation. A certain amount of ssDNA must be generated to reach the threshold level for checkpoint activation. Similar to initial events in the DNA damage checkpoint, the exposed ssDNA recruits Mec1/Ddc2 (*Fig. 6*) (Branzei and Foiani 2005), but in response to a stalled fork Mec1 phosphorylates Mrc1, a protein involved in both checkpoint response and replication fork progression (Katou, Kanoh et al. 2003; Osborn and Elledge 2003), rather than Rad9. Phosphorylation of Mrc1 is suggested to stop the MCM complex from unwinding the DNA helix and aid in replication fork restart once the replication block has been removed (Nedelcheva, Roguev et al. 2005). Subsequent phosphorylation of the Rad53 kinase by Mrc1 is a requirement for stabilization of the replisome at the fork and full checkpoint response (Osborn and Elledge 2003).

In response to fork stalling the checkpoint activates three pathways: The first prevents firing of late replicating origins to inhibit replication of flawed DNA, the second stabilizes the replisome at the fork to prevent fork collapse and the third suppresses the activity of recombination enzymes at the stalled fork. (Branzei and Foiani 2005)

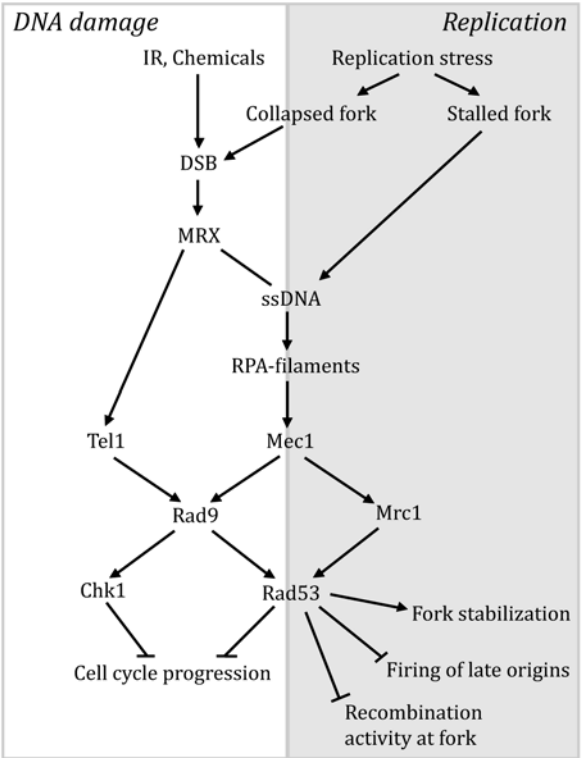

Fig. 6. **The network of checkpoint events and proteins required for the replication checkpoint and the checkpoint response to DSBs**.

6. POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications of proteins provide opportunities for additional function(s) of the protein in question. They are fundamental for regulation and stability of checkpoint components and may affect the recruitment of repair proteins to DNA lesions (Branzei and Foiani 2008). Several of the SMC proteins and their associated subunits are post-translationally modified and/or contain enzymatic activity required for the protein modifications (Zhao and Blobel 2005). Post-translational modifications such as phosphorylation, glycosylation and methylation are perhaps the most familiar ones, but in some cases the modification can be a protein in itself.

The most commonly known protein modifier is ubiquitin, first identified for its role in protein degradagtion by the proteasome. In three enzymatically catalyzed steps ubiquitin is added to the protein targeted for degradation. First ubiquitin is activated for transfer by an E1 activating enzyme. The activated form is then transferred to an E2 conjugating enzyme and finally an E3 ligase transfers ubiquitin to the substrate protein. The process is the repeated until the target protein is tagged with a chain of ubiquitin molecules that signals to the proteasome that degradation is required. Ubiquitin has several additional roles in the cell not involving its degradation function, e.g. in DNA repair and stress response (Kroetz 2005) (Hanna and Finley 2007).

More recently another post-translational protein modifier was identified and named SUMO (small ubiquitin-like modifier.) Unlike ubiquitylation, modification by sumoylation does not lead to degradation of the target protein. Among the many substrates for sumoylation are proteins involved in signalling pathways, transcription and regulation of sub-cellular localization (Lee and O'Connell 2006). Like ubiquitylation, sumoylation in budding yeast requires E1, E2 and E3 enzymes. There are three known E3 ligases, of which the Smc5/6 complex subunit Nse2 is one (Watts 2007). Sumoylation is a reversible process that can be revoked by a protease. It is also an essential process in yeast as demonstrated by mis-segregation phenotypes of conditional SUMO mutants (Kroetz 2005; Watts 2007). Several proteins involved in DNA repair are sumoylated or associate with SUMO e.g. Rad51 and Rad52, PCNA and the topoisomerases Top1 and Top2 (Lee and O'Connell 2006).

7. SMC PROTEIN COMPLEXES

The SMC proteins are highly conserved proteins that, as the name implies, are required for the organization of chromosomes. They have important functions in chromosome segregation, gene regulation and

recombinational DNA repair. The proteins in the SMC family are large proteins with a distinct and unique architecture. The globular heads of the proteins are connected by long helices; coiled-coils, to a “hinge” domain that allows the helices to fold back on themselves, creating an ATP-binding pocket between the globular heads. All SMC proteins form dimers which associate with additional protein subunits to form large complexes. The two proteins in the dimer associate through the hinge domains giving the whole molecule a U- or V-shape (*Fig. 7A*).

Eukaryotes contain six SMC family members, Smc1-6, that form heterodimers, each with a specific binding partner (*Fig. 7B-D*). The cohesin complex is required for connecting the sister chromatids as they form during S-phase and for correct segregation of the sisters in mitosis. It consists of the Smc1/Smc3 heterodimer and two additional subunits, Scc1 and Scc3. The Smc2/Smc4 dimer constitutes the core of the condensin complex that promotes chromosome condensation and segregation. The third and last of the eukaryotic SMC complexes is the Smc5/6 complex. It was the last of SMC protein complexes to be characterized and less is known about this complex than the other two. It functions in homologous recombination and checkpoint maintenance and consists of six non-SMC subunits in addition to Smc5 and Smc6 (Losada and Hirano 2005).

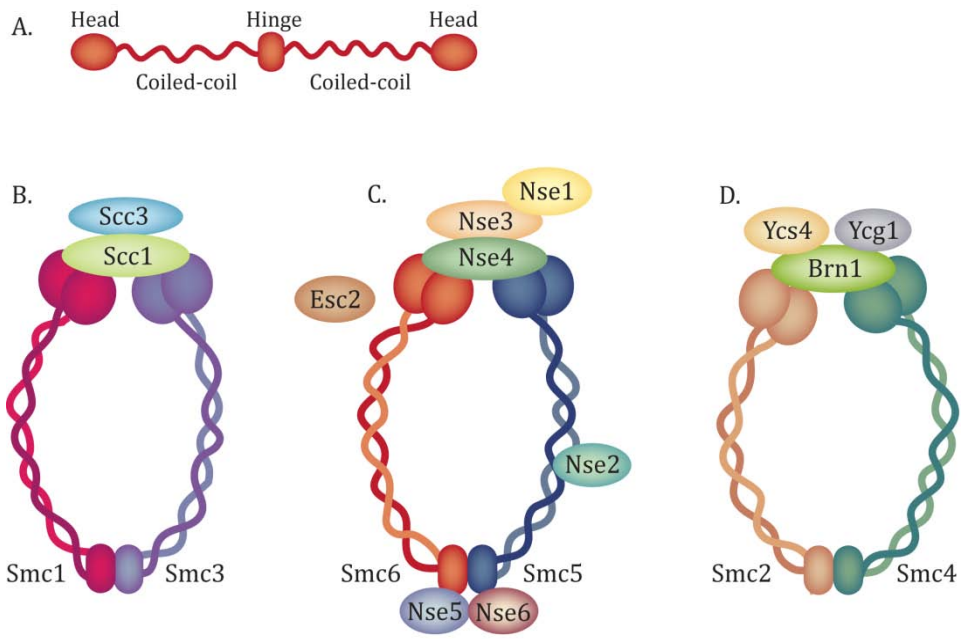

Fig. 7. **SMC proteins. A.** SMC protein structure. Schematic illustrations of the three SMC protein complexes. **B.** Cohesin, **C.** The Smc5/6 complex, **D.** Condensin (D from (Ghosh, Hajra et al. 2006))

7.1 Cohesin

The cohesin complex is the most investigated of the three eukaryotic SMC complexes. The main function of cohesin is connecting the sister chromatids as soon as they are formed during S phase. If they fail in this the chromatids will separate prematurely, leading to chromosome missegregation, most often with fatal consequences. Cohesins also have important functions in DNA repair by homologous recombination. As long as the sisters are cohesed a template for DSB repair is always close by if DNA breaks should occur (Sjogren and Nasmyth 2001; Haering and Nasmyth 2003). Smc1 and Smc3 make up the core of the cohesin complex. Bridging the gap between the globular heads of the SMC proteins is the non-Smc subunit Scc1, thus giving the molecule a ring-shape. Connected to the complex through binding to Scc1 is also another non-Smc subunit; Scc3 (*Fig. 7B*) (Haering and Nasmyth 2003)

7.1.1 Cohesin loading and localization on chromosomes

A substantial amount of cohesin is located at the centromeric regions. In addition, distinct sites called cohesin-associated regions (CARs) are evenly spread out along the chromosome arms, with an average distance of 10 kb between sites. Some cohesin is also found on the telomeres in budding yeast. The arm binding sites are located in intergenic regions, specifically regions of converging transcription (Glynn, Megee et al. 2004).

A protein complex consisting of Scc2 and Scc4 is required for loading of cohesin onto the DNA, an event that occurs already in late G1 phase. Both Scc2 and Scc4 are essential in budding yeast. Scc2 has orthologs in most eukaryotes whereas Scc4 is much less conserved (Losada and Hirano 2005), but functional orthologs have recently been discovered in other species (Bernard, Drogat et al. 2006; Seitan, Banks et al. 2006; Watrin, Schleiffer et al. 2006).

Somewhat surprisingly the Scc2/4 cohesin loader and the cohesin complex show different binding patterns on chromosomes (Uhlmann and Nasmyth 1998; Ciosk, Shirayama et al. 2000), but cohesin association in an *scc2-4* mutant shows a distribution similar to that of wt Scc2/4. When binding is dissected in wild type (wt) cells after release from G1 arrest, this shows that the first sign of cohesin is detected at Scc2/4 binding sites. After 30 minutes cohesin has relocated to other binding sites. A possible explanation for this behaviour of cohesin is that the complex is loaded at Scc2/4 complex binding sites, and from there it re-distributes across the genome to its more permanent binding sites at the site of converging transcription (Lengronne, Katou et al. 2004).

7.1.2 Establishment of cohesion

Loading of cohesin on chromosomes and establishment of cohesion are two separate processes. The loading normally occurs in late G1 phase. Establishment of cohesion however, occurs during S phase in undamaged cells (Uhlmann and Nasmyth 1998), and is dependent on the protein Eco1, an essential, replication fork-associated acetyltransferase (Ben-Shahar, Heeger et al. 2008). It is not a subunit of the cohesin complex and is not required for cohesin assembly, chromosomal association/ dissociation, or for maintenance of cohesion in G2/M (Toth, Ciosk et al. 1999). Eco1 has been shown to interact with PCNA; an interaction which is required for Eco1 function in sister chromatid cohesion (Moldovan, Pfander et al. 2006). Establishment of cohesion during S phase has been linked to replication. Recent work has shown that Eco1 acetylates Smc3 during S phase, and that this modification is essential for cell viability (Ben-Shahar, Heeger et al. 2008; Unal, Heidinger-Pauli et al. 2008).

7.1.3 Cohesin in chromosome segregation

Cohesion between the sister chromatids is maintained from S phase until the metaphase-to-anaphase transition. Dissolution of cohesin cannot take place until all chromosomes are aligned and bi-oriented at the bud neck Segregation of the chromosomes to opposite spindle poles is initiated when the protease Esp1, also known as separase, cleaves the cohesin subunit Scc1. Cleavage of Scc1 is irreversible and therefore separase is kept inactive until the right moment by its association with Pds1 (securin). Ubiquitination of Pds1 by the anaphase promoting complex (APC), targets it for degradation which is effectuated by the proteasome at anaphase onset (Haering and Nasmyth 2003; Sun and Fasullo 2007). To avoid degradation of cohesin when chromosomes are damaged, Pds1 is phosphorylated in a Chk1-dependent manner, thereby preventing ubiquitination and the subsequent degradation (Sun and Fasullo 2007).

Cohesins are not the only players in cohesion. Even if cohesin is not functional, the pairing of the sister chromatids is never fully dissolved. Therefore other factors must also influence cohesion. Condensin and ORCs have been shown to mediate cohesion, as have DNA catenations (Onn, Heidinger-Pauli et al. 2008).

7.1.4 Cohesin in DNA damage repair

Cohesin subunits as well as the proteins required for loading and for establishment of cohesion are all indispensable for repair of damaged DNA. The distinct cohesin binding pattern changes in the area surrounding a DNA DSB. Cohesin subunits appear in large amounts in the area close to the break and up to 100 kb around the lesion as

determined by chromatin immunoprecipitation (ChIP) and ChIP analysed on a micro array, so called ChIP on chip (Strom, Lindroos et al. 2004; Unal, Arbel-Eden et al. 2004). The new distribution of cohesin at the break site is dependent on the loading factor Scc2, the DNA damage checkpoint kinases Mec1, Tel1, Rad53 and the damage sensor Mre11 (Unal, Arbel-Eden et al. 2004).

7.2 The Smc5/6 complex

The Smc5 and Smc6 proteins are the most distantly related SMC proteins, and also the least investigated of the three eukaryotic dimers. The complex has at least two functions, one in DNA repair by homologous recombination and another, essential function that is still under investigation.

7.2.1 Composition of the Smc5/6 complex

The complex consists of eight essential subunits; Smc5, Smc6 and six non-SMC elements, Nse1-6 (*Fig. 7C*) (Verkade, Bugg et al. 1999; Fousteri and Lehmann 2000; Fujioka, Kimata et al. 2002; Hazbun, Malmstrom et al. 2003). Components of the complex have intrinsic activities that are likely involved in the complex's function. Nse1 contains a RING motif that is commonly found in E3 ubiquitin ligases and Nse2 is an E3 SUMO ligase required for sumoylation of several proteins, among them Smc5 (Zhao and Blobel 2005). Nse3 contains a MAGE domain often found in mammalian proteins expressed in tumours (Pebernard, Wohlschlegel et al. 2006). Nse1, 2 and 3 are also important in meiosis, possibly for homologous recombination after initiation of meiosis-specific DSBs (Pebernard, McDonald et al. 2004). Nse4 is orthologous to the cohesin subunit Scc1, binding the globular heads of Smc5 and Smc6. In fission yeast, Nse5 and Nse6 were the last subunits to be identified. Their sequences are very divergent from those of the *S. cerevisiae* orthologues, and unlike in budding yeast, gene deletions of *NSE5* and *NSE6* are viable in fission yeast (Pebernard, Wohlschlegel et al. 2006).

The Nse's assemble into smaller sub-complexes that associate with the Smc5/6 core at specific sites. Nse1, 3 and 4 forms one such sub-complex and it associates with the heads of the SMC proteins. Nse2 binds to the coiled-coil region of Smc5 (Sergeant, Taylor et al. 2005; Palecek, Vidot et al. 2006). The Nse5-6 sub-complex associates with the hinge regions of Smc5 and Smc6 in budding yeast (Duan, Yang et al. 2009), but appears to bind to the coiled-coil regions in fission yeast (Palecek, Vidot et al. 2006).

Rad60 in *S. pombe* has also been shown to interact with the Smc5/6 complex. Rad60 is an essential protein involved in HR, that is

phosphorylated in response to hydroxyurea (HU)-induced replication stress in a Cds1(*S.c.* Rad53)-dependent manner (Miyabe, Morishita et al. 2006). Esc2, the budding yeast homologue of Rad60, together with the Smc5/6 complex has recently been implicated in prevention of accumulation of recombination structures at damaged replication forks (Sollier, Driscoll et al. 2009).

7.2.2 Functions of the Smc5/6 complex

In DNA repair

Smc6 was the first of the complex's subunits to be identified. The temperature sensitive *smc6-X* mutant was found in a screen for radiation sensitive proteins in *S. pombe*. The mutation is located in the hinge region, next to the coiled-coil region and renders the cells sensitive to ionizing radiation and to exposure to ultraviolet light. The mutant was shown to be epistatic with *RHP51*, the fission yeast homologue of *RAD51* required for homologous recombination in DSB repair. Yeast cells are viable in the absence of a functional Rad51 pathway, suggesting that the repair function of the Smc5/6 complex is not what makes it essential (Lehmann, Walicka et al. 1995).

Mutants of all the subunits have since been generated and they are all sensitive to DNA damaging agents and defective in recombinational repair as shown by epistatis analysis with Rad51, Rad52 and other proteins required for HR (McDonald, Pavlova et al. 2003; Harvey, Sheedy et al. 2004; Morikawa, Morishita et al. 2004; Onoda, Takeda et al. 2004) . Mutants of the Smc5/6 complex components are able to initiate checkpoint arrest after DNA damage but are unable to maintain it, an inability that leads to aberrant mitosis resulting in fragmented chromosomes (Verkade, Bugg et al. 1999; Ampatzidou, Irmisch et al. 2006; Pebernard, Wohlschlegel et al. 2006). Taken together, this indicates that the complex exerts its function at a later stage in HR, after strand invasion which is the step that requires Rad51 and Rad52.

In replication – at stalled and collapsed replication forks

Several lines of evidence imply that the Smc5/6 complex functions at replication forks. The first indication came from Verkade et al showing that the *smc6-74* mutation in fission yeast can be suppressed by overexpression of Brc1 (Verkade, Bugg et al. 1999), whose budding yeast homologue Esc4 has been implicated in stabilization of replication forks (Chin, Bashkirov et al. 2006). Disturbed DNA replication and/or DNA damage triggers phosphorylation, and thereby activation, of the protein kinase Rad53. As previously noted, the effect is cell cycle arrest, stabilization of the replication fork and prevention of late origin firing. In fission yeast *rad62* (*S.c. NSE4*) and budding yeast *smc5* temperature

sensitive mutant cells at restrictive temperature, Rad53 is activated (Hu, Liao et al. 2005) (Cost and Cozzarelli 2006), indicating that the DNA has been damaged or the DNA replication has been affected by the absence of functional Smc5/6 complex proteins. In *S. pombe, cds1* mutant cells treated with HU, stalled replication forks collapse. The processing of these collapsed forks is defective in smc6 mutants and leads to accumulation of X-shaped recombination intermediates between sister chromatids that fail to be recognized by the intra-S-checkpoint in Smc6 mutant cells, resulting in aberrant mitosis (Ampatzidou, Irmisch et al. 2006). X-shaped intermediates also accumulate at damaged forks in *nse2* sumoylation defective mutants in a Rad51-dependent manner, suggesting that Nse2-dependent sumoylation has a role in inhibiting the formation of this type of recombination intermediates (Branzei, Sollier et al. 2006). In addition, several proteins known to be involved in the recovery from replication block are synthetically lethal with mutants of *rad62* (Morikawa, Morishita et al. 2004) which indicates that the complex is involved in regulating the stability and/or processing of stalled and damaged forks.

In ribosomal DNA and nucleolar maintenance

The ribosomal DNA (rDNA) region has proved to be very interesting from a Smc5/6 point of view. Situated on chrXII in budding yeast, it is a heterochromatin region consisting of approximately 100-150 tandem repeats copies of a 9.1 kb unit. Each repeat contains three ribosomal RNA (rRNA) genes, RFBs and a non-transcribed spacer containing an ARS. The number of rDNA repeats varies, and its regulation depends on proteins required for replication fork blocking at RFBs, and on proteins suppressing recombination within the rDNA (Kim, Ishikawa et al. 2006).

When transcription and replication progress in opposite directions the encounter often lead to collapse of the replication fork and ensuing processing of the generated DSB. The rDNA repeat is equipped with RFBs to prevent this type of collision. The role of RFBs is to ensure that replication goes in the same direction as transcription and this is achieved by stalling of the fork progressing in the wrong way (Kobayashi, Heck et al. 1998). In line with a function of the Smc5/6 complex in maintenance of stalled and damaged replication forks, more X-shaped intermediates are formed in the rDNA region in Smc6-deficient cells than in the wt cells. In the absence of the central HR protein Rad52, the levels of aberrant recombination structures decrease. Lack of Rad52 partially restores viability in *smc6* ts mutants, supporting the idea that the Smc5/6 complex is involved in the processing of recombination intermediates. In addition, chromosome segregation is defective in the chromosomal region distal to the rDNA in Smc6-deficient cells, further reinforcing the idea of

a role for the complex at compromised replication forks (Torres-Rosell, Machin et al. 2005).

The Smc5/6 complex has been shown to accumulate on rDNA in budding yeast and the entire region locates to the crescent-shaped nuclear compartment known as the nucleolus (Torres-Rosell, Sunjevaric et al. 2007), where rRNA is synthesised and processed, and where ribosomes are assembled (Irmisch, Ampatzidou et al. 2009). The Smc5/6 complex seems to have a function in nucleolar maintenance, as indicated by nucleolus segregation and organization defects seen in *nse1* mutants (Pebernard, Perry et al. 2008) and a *nse2* RING-mutant in which the sumoylation function is disabled (Zhao and Blobel 2005). This, too, is consistent with a role in the prevention of recombination intermediate formation.

8. PRESENT INVESTIGATIONS

The aim of this study was to elucidate the functions of the two SMC protein complexes cohesin and the Smc5/6-containing complex.

With regards to the Smc5/6 complex, we have investigated the binding of the complex to chromosomes: its regulation, timing and pattern, in unchallenged cells and in cells with a site-specific, endonuclease-induced DSB. Utilizing a short circular chromosome and a fragmented long chromosome, we have investigated a chromosome length-specific role of the complex. We discovered that this function of the complex is due to the formation of torsional stress generated by the replication process that in the absence of Smc5/6 is not resolved, leading to the formation of branched structures during replication and segregation problems. We have also shown that the complex is required for DNA DSB repair.

Much more was already known about the cohesin complex, so the prime goal has been to investigate the requirement of cohesin in DNA repair. Cohesin was found to localize to the area around a DNA DSB, and was found to mediate cohesion. The damaged-induced cohesion was found to be a prerequisite for DNA repair and was analyzed with respect to other necessary proteins. One single DSB in the whole genome was found to be sufficient for induction of damage-induced cohesion genome-wide.

8.1 Paper I

The main method utilized in this publication is the ChIP on chip method in which immunoprecipitated DNA is purified, amplified and hybridized to oligo probes on a microarray (Affymetrix) (*Fig. 8*). The binding is analyzed by software provided by the manufacturer and modified by our collaborators in Japan.

Using this technique we identified the binding sites for the Smc5/6 complex components on budding yeast chromosomes in the different phases of the cell cycle. We found that while no binding is seen in G1 phase, replicated regions of the genome and early replicating origins (ARSs) show binding in early S phase and onwards. The binding to the early ARSs however, is only seen after an extended treatment with HU, conditions also known to accumulate proteins required for stabilization of the replication fork. Shorter exposures to HU do not convey Smc6 binding at the ARSs, but the fork maintenance proteins still accumulate at the site. This argues that Smc6 is not associated with stalled replication forks but rather with forks that have collapsed due to the prolonged HU arrest.

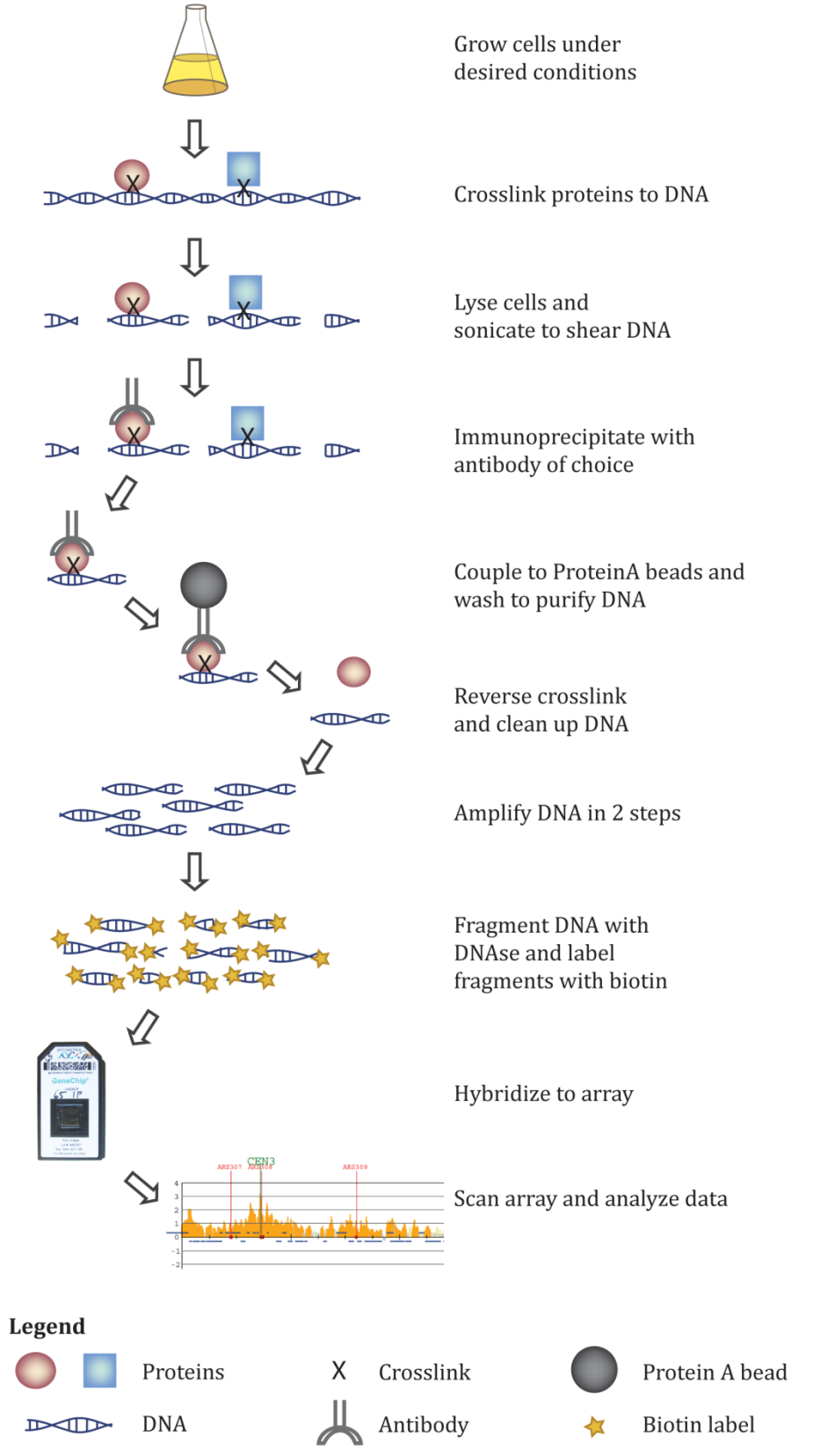

Fig. 8. **The ChIP-on-chip method** (adapted from Affymetrix® Chromatin Immunoprecipitation assay protocol)

As previously noted, one of the proteins required for stabilization of the stalled replication forks, is the Rad53 kinase. It functions in the replication checkpoint and in cells lacking functional Rad53 stalled replication forks collapse, creating DSBs. During these circumstances, Smc6 does indeed localize to the early replicating origins already after one hour of HU-treatment, supporting the idea that the complex binds to collapsed forks, rather than the stable stalled fork.

The most prominent association of the Smc5/6 complex is seen in the G2/M phase. The complex localizes to all centromeres, and to distinct sites on chromosome arms, similar to what was previously shown for the cohesin complex (Glynn, Megee et al. 2004). A closer look at the binding sites of the two complexes show that the Smc5/6 binding sites overlap with cohesin sites to 63,7%. However, while the arm binding sites of cohesin are evenly spaced on all chromosomes, the frequency of Smc6 arm binding sites increases with increasing chromosome length. This size-dependent interaction likely reflects a function of the complex, and this was further investigated in paper II.

Others have shown that the Smc5/6 complex is required for DNA repair by homologous recombination (Onoda, Takeda et al. 2004; Cost and Cozzarelli 2006). Many proteins, cohesin included, that are required for repair localize to the region of the chromosome that surrounds the site of DNA damage (Lisby, Antunez de Mayolo et al. 2003; Lisby, Barlow et al. 2004). This prompted us to investigate the Smc6 localization to a site-specific DSB caused by an inducible endonuclease. Smc6 localizes to the area immediately surrounding the break and the first signs of binding appear 30 minutes after initiation of break induction. The amount of Smc6 bound to the area of damage increases with time and reaches maximum levels after 70 minutes. The striking similarities between the cohesin and the Smc5/6 complex further inspired us to investigate the break association of Smc5/6 complex components in the absence of factors required for cohesin association to damaged DNA. The DNA damage sensor Mre11 is required, but the Mec1 and Rad53 checkpoint kinases are not. Intriguingly, the Scc2 protein is not required for break association of Smc6, but it is required for Smc6 distribution on chromosomes in unchallenged cells. Together this information illuminates the differences between two otherwise similar complexes. It also shows that the Smc5/6 complex functions in at least two separate pathways: one Scc2-dependent pathway which is active in unchallenged cells and one Mre11-dependent that govern the association of the complex to sites of DNA damage. Cell cycle analysis of *smc6* temperature sensitive (ts) mutants indicates that the complex exerts its function in late G1 or S phase. A chromatid separation assay shows a delay in segregation, but no replication delays are visible by FACS. This indicates that the complex is required for completion of replication and was further analyzed by PFGE of *smc6* mutants during a

synchronous cell cycle. In this assay only fully replicated chromosomes are able to enter the gel while chromosomes containing any type of branched molecules remain in the well. The results from this analysis support the idea that the mutant has problems in completing replication leading to the conclusion that persisting replication forks are what prevent chromosomes from entering the gel (see also paper II).

Conclusions from Paper I:
* The Smc5/6 complex associates with duplicated regions of the genome and with collapsed replication forks.
* In G2/M phase, the complex binds to all centromeres and to specific sites along the chromosome arms with a frequency that increases with chromosome length.
* Smc5/6 binds to the DNA surrounding a DSB in an Mre11-dependent but Scc2-independent manner.
* The complex is required for completion of replication.

8.2 Paper II

To address a plausible chromsome length-dependent function of the Smc5/6 complex, ChIP on Chip (see paper I) was used to study the binding pattern of the Smc5/6 complex components on chromosomes that had been artificially shortened. In this strain chromosome IV was divided close to the centromere so that each of the new chromosomes contained one of the arms of chromosome IV. The amount of Smc6 binding on the arms decreased to levels matching those on natural chromosomes of similar sizes. The two chromosome parts have the same sequence as the original long chromosome, demonstrating that it is the length of the chromosomes, not the sequence that determines Smc5/6 binding.

PFGE analysis of BrdU-labeled chromosomes from a temperature-sensitive *smc6* and a sumolyation-deficient *nse2* mutant at different time points after replication illustrate that the relative amount of DNA that enters the gel is significantly less from a long chromosome than from a short chromosome. This indicates that the complex is required for resolving structures that inhibit entry of the DNA into the gel that occur on long but not on short chromosomes and supports our presumption from paper I: that the complex has a length-dependent function.

In paper I we demonstrated that Scc2 is required for loading of the Smc5/6 complex onto chromosomes. Using the PFGE technique we show that Scc2 also has a length-dependent function. To rule out that aberrant recombination structures are what prevent chromosomes in the *smc6* mutant from entering the gel, *RAD51* was deleted in a *smc6* mutant background. The double mutant has the same phenotype as the single

smc6 mutant, suggesting that the chromosome length-dependent function is separate from its function in HR.

A possible explanation for the chromosome length-dependency of the Smc5/6 complex is the accumulation of unresolved topological structures on longer chromosomes due to their inability to physically swivel off the torsional stress. Circularization of a short chromosome is therefore expected to generate increased amounts of unresolved topological structures compared to a linear version of the same chromosome. ChIP on chip examination of the localization of Smc5/6 complex components to a circular chromosome vs. a linear chromosome showed a dramatic increase in binding regions not evident for either the cohesin or the condensin complexes. This argues that the requirement in resolving topological stress is unique for the Smc5/6 complex. Lack of Top1, a protein required for release of replication-induced torsional tension in DNA, also shows a similar chromosome length-specific phenotype as the Smc5/6 complex components when analyzed by PFGE, indicating that replication-induced topological stress is indeed the inherent cause of the Smc5/6 complex association with chromosomes.

Conclusions from Paper II:
* Chromosome length, not DNA sequence, determines frequency of Smc5/6 interaction sites.
* The complex has a length-dependent function.
* Accumulation of topological stress is what recruits the Smc5/6 complex to chromosomes

8.3 Paper III

It was previously believed that sister chromatid cohesion could only be established during S-phase in connection with replication. In this publication we show that cohesin is recruited to a site-specific DSB induced in G2/M phase. Furthermore, that DNA damage can lead to the formation of cohesion after completion of S phase.

When cohesin function is impaired, DNA repair and recombination are also affected. This implies that sister chromatid cohesion formed during S phase is required for repair (Sjogren and Nasmyth 2001). Other discoveries augment this implication, for example, interaction of human cohesin with Rad50, a member of the DNA damage-sensing and repair complex MRN (Mre11, Rad50, Nbs1(*S.c.* Xrs2))(van den Bosch, Bree et al. 2003). Cohesin has also been shown to accumulate at laser-induced DNA damage in a Rad50-dependent fashion (Kim, Krasieva et al. 2002). If budding yeast cohesin is also involved in DNA damage repair, we would expect to find it located in the immediate surroundings of a DNA break. This was investigated by ChIP of the cohesin subunits Smc1 and

Scc1 in the area surrounding a site-specific DSB. Analysis showed an accumulation of cohesin subunits Scc1 and Smc1 around the break as far as 1 kb upstream and 7 kb downstream of the break, whereas no accumulation was evident in the control samples. DSB repair is most efficient in the G2/M phase of the cell cycle, when cells can use the homologous chromosome as repair template. If cohesin is involved in DSB repair we would expect to find them around the break site at this stage of the cell cycle. ChIP of G2/M arrested cells showed and accumulation of Scc1 and Smc1 around the break site, implying a role for cohesin in DNA repair.

Cohesin loading in late G1 and S phase requires the loading complex consisting of Scc2 and Scc4. They are, however, not required for maintenance of normal cohesion at later stages. It is therefore possible to study if they have a role in loading of cohesin to DSBs in the G2/M phase. In wt cells Scc1 still accumulates at the DSB, but not in *scc2* mutant strains, showing that functional Scc2 is required for loading of cohesin subunits in G2/M phase. Is the cohesin loading complex also required for DSB repair in G2/M? Irradiation with γ –rays to induce DSBs in wt, *scc2* or *scc4* mutant cells, and subsequent DNA repair analysis by PFGE of samples from a recovery period after irradiation showed that wt cells were proficient at DNA repair, whereas the *scc2/scc4* mutant cells failed to repair the damaged DNA. We therefore conclude that the cohesin loading complex Scc2/Scc4 is required for DNA DSB repair in G2/M.

Because cohesins are located to the area around the break site in a Scc2/Scc4-dependent manner, and DNA repair is most efficient in G2/M, we wanted to investigate if the cohesin recruited in response to DNA damage also mediates cohesion, thereby facilitating DNA repair. An elaborate system was set up in which only cohesins recruited in response to damage are present on chromosomes (*Fig. 9*). Ts *smc1* mutant cells expressing the wt *SMC1* gene from a galactose(GAL)-inducible promoter and also containing the tet-operon/repressor system (see below) were arrested at permissive temperature in G2/M phase in galactose-free media. Here, the ts version of *smc1* was expressed during G1 and S phase and under such conditions was able to mediate cohesion. When the cells had reached G2 the culture was split in half and wt *SMC1* expression was induced in one half by addition of galactose. After an additional hour cultures were split again, and half of each was treated with γ–rays. After irradiation cells were permitted a recovery period during which cohesin were allowed to load at irradiation-induced DSBs. In the galactose-treated cells both wt and mutated versions of *SMC1* were expressed, but in cells grown in galactose-free medium only the ts *smc1* was expressed. After the recovery period, the temperature was raised to destroy mutant *smc1* function. In doing so, all the mutant cohesins loaded, both at S-phase and at G2/M, are rendered non-functional. Samples for scoring of sister

chromatid separation were continually withdrawn from irradiation treatment and onwards. Sister chromatid separation was then scored using a system in which the URA3 locus, close to the centromere on chr.V, is labelled with tetracycline (Tet) operators in a cell expressing Tet repressors fused to GFP. The repressors are visible as fluorescent green dots: one dot is seen if sister chromatids are held close together or two dots when the sisters have separated (Straight, Belmont et al. 1996; Michaelis, Ciosk et al. 1997).

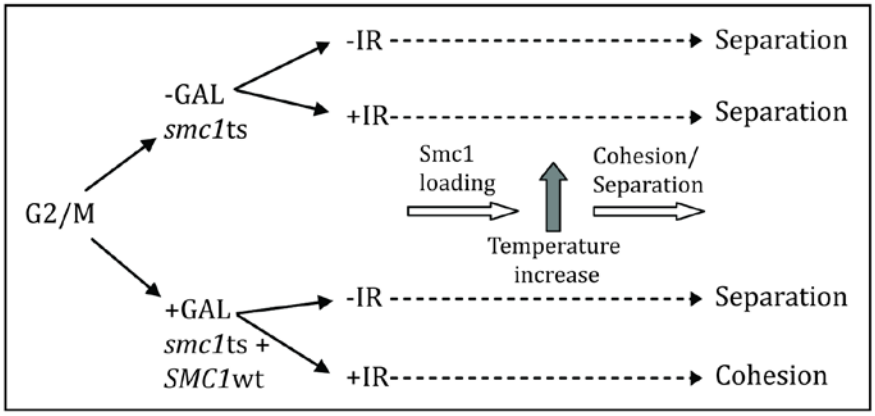

Fig. 9. **The experimental setup for investigation of cohesin recruited in response to DNA damage** (adapted from paper III).

Before irradiation and during the recovery period the sister chromatids remained cohesed in all cultures. After the temperature increase sisters separated in both cultures expressing ts *smc1* and in the non-irradiated cells expressing wt *SMC1*. However, in the irradiated cultures expressing wt *SMC1* the sisters remained close. This suggested that cohesin recruited to DSBs in G2/M phase can mediate cohesion, supporting the idea that cohesion triggered in response to DNA damage has a function in DNA repair.

Conclusions from Paper III:
* Cohesin accumulates at a DNA DSB in G2/M in a Scc2-dependent manner.
* Cohesin recruited to a DSB can mediate cohesion.
* Scc2/4 is required for repair of DNA DSBs in G2/M**.**

8.4 Paper IV

Cohesion established during replication is required for repair of DNA DSBs in the subsequent G2/M phase in budding yeast. The cohesin complex is recruited to the site of damage independently of replication and can generate cohesion under these conditions (paper III (Strom,

Lindroos et al. 2004). We have investigated this damage-induced cohesion with regards to its formation, regulation and possible requirement for DSB repair.

Damage-induced cohesion can be distinguished from cohesion formed during replication through the previously described system in which cells with a temperature sensitive version of Smc1 are arrested in G2/M at permissive temperature. In response to DSBs created by γ-irradiation, cohesion is established through expression of galactose-inducible wt *SMC1* and is visualized by sister chromatid separation of chr.V in the tet-repressor-GFP/tet operator system (see paper III). Several proteins central to the DNA damage response were investigated with respect to the formation of damage-induced cohesion. Both Mre11 and γH2A are required for recruitment of cohesin to damaged DNA. Consequently, formation of damage-induced cohesion is defective in cells lacking functional Mre11 or in cells expressing a non-phosphorylatable version of H2A. The phosphorylation of H2A is performed by the Tel1 and Mec1 kinases, and in the absence of these proteins damage-induced cohesion is defective. Tel1 and Mec1 also activate the downstream transducer Rad9 through phosphorylation, but the absence of Rad9 has no effect on the generation of cohesion induced by DNA damage. *rad52* deletion mutants were also analyzed for formation of damage-induced cohesion. The absence of the central HR protein Rad52 had no effect on cohesion, implying that contrary to S phase-established cohesion, formation of damage-induced cohesion is independent of DNA synthesis.

The γ-irradiation dose applied to cells generates approximately one DSB per chr. V and since cohesion is determined on this chromosome only in these experiments, we questioned whether as little as one DSB per cell is enough to trigger cohesion. To investigate this, cells expressing an uncleaveable version of Scc1 were used. When Scc1 can no longer be cleaved by separase, chromatid separation at anaphase is blocked. Expression of uncleavable Scc1 in the absence of DNA damage does not affect separation of chr.V, but induction of an endonuclease generating a site-specific DSB on chr.III prevented sister chromatid separation of chr.V, indicating that a single DSB is enough to activate damage-induced cohesion throughout the genome. The regulation of the genome-wide cohesion was investigated using the ts *smc1*/gal-inducible, wt *SMC1* system as previously described, but with the addition of the inducible endonuclease. These experiments demonstrate that the genome-wide cohesion triggered by one single DSB is dependent on Mec1 and partly on Tel1 and phosphorylated H2A.

Other proteins required for the regulation of cohesin were also examined for their role in genome-wide cohesion. The results indicate the requirement of the cohesin loading protein Scc2, the Smc5/6 complex component Smc6 and the cohesion establishment factor Eco1. The

requirement for Eco1 in G2/M-established cohesion shows that it functions outside S phase and suggests that Eco1 is reactivated to form cohesion under these conditions. The *eco1* mutant does not influence localization of cohesin to chromosomes, and the mutant could thus be used to determine that damage-induced cohesion is required for DNA repair.

All in all our research shows that newly established cohesion is generated on all chromosomes, even undamaged, in response to one single DSB on chromosome III and that this is dependent mainly on the Mec1 kinase, but independent of DNA synthesis.

Conclusions Paper IV:
* Damage-induced cohesion is dependent on Mre11, γH2A, Tel1 and Mec1, but not on Rad9 or Rad52.
* One single DSB is enough to trigger damage-induced cohesion genome-wide.
* Genome-wide cohesion is dependent on Scc2, Smc6 and Eco1.

9. PERSPECTIVES AND CONCLUDING REMARKS

Size matters. At least when it concerns Smc5/6 localization on chromosomes: the longer the chromosome, the more Smc5/6 binding sites per kb. This likely reflects a role of the complex in resolving torsional stress induced by the replication process that is independent of the previously reported function in repair by homologous recombination. A function in topology has already been described for an SMC protein complex, namely condensin. But rather than relieving them, like the Smc5/6 complex seems to do, the complex induces positive supercoils into DNA (Stray, Crisona et al. 2005).

Topology may also be what recruits the Smc5/6 complex and condensin to tRNA genes. The condensin complex was recently shown to localize to tRNA genes and other RNA pol III transcribed genes in the budding and fission yeast genomes (D'Ambrosio, Schmidt et al. 2008).
In fission yeast, the Smc5/6 complex has been shown to localize to transcriptionally active tRNA genes (Pebernard, Schaffer et al. 2008). Although not thoroughly analyzed, our results indicate that this is the case for budding yeast too, especially in the G2/M phase of the cell cycle. The tRNA genes are highly transcribed, and the association of the Smc5/6 complex may reflect its proposed function in the removal of torsional stress that is generated when the transcription machinery is performing its task, an issue that calls for detailed investigation.

Like the Smc5/6 complex and cohesin, the condensin complex has been shown to localize to intergenic regions, but unlike that of the other

two complexes, condensin binding in this regions seems independent of ORF orientation (D'Ambrosio, Schmidt et al. 2008). Correct function of the three eukaryotic SMC complexes requires the Scc2/Scc4 complex, first identified as a cohesin loader (Ciosk, Shirayama et al. 2000) . The cohesin and condensin complexes are loaded onto DNA but require an activating event to become functional. For the cohesin complex this task is performed in S phase by the Eco1 protein (Toth, Ciosk et al. 1999). What activates condensin is not yet known, but the fact that it is loaded onto chromosomes in G1 phase and does not start condensing the DNA until M phase implies that such a mechanism must exist (Wang, Eyre et al. 2005). This feature has not yet been described for the Smc5/6 complex, but as the apparent similarities of the complexes in chromosomal localization and loading suggest similar modes of activation, this may be interesting to investigate.

The significance of the SMC protein complexes in cell viability is clear from the numerous and diverse roles they perform during the cell cycle. In higher eukaryotes, the cohesin and Smc5/6 complexes have been implicated in different genetic disorders. The Smc5/6 complex has a role in telomere maintenance in cancer cells. The telomeres in somatic cells are gradually shortened, a piece at the time during each cell division, until nothing is left, at which point the cell undergo apoptosis. Cancer cells typically become immortal by upregulating the enzymes that lengthen telomeres, thereby inhibiting senescence. Certain types of cancers, for instance several sarcomas and Li-Fraumeni syndrome tumors, employ a HR-based method for telomere maintenance, known as alternative lengthening of telomeres (ALT). The Smc5/6 complex is required for preservation of the telomere length in ALT cells and this is dependent on sumoylation of telomere-binding proteins by MMS21(*S.c.* Nse2) (Potts and Yu 2007). Mutations in the cohesin complex components Smc1, Smc3 or the NIPBL, the human homologue of *S.c.* Scc2, can cause Cornelia de Lange syndrome, characterized by craniofacial and upper limb abnormalities, growth and mental retardation. Individuals with mutations in SCC2 exhibit the most striking phenotypes. Roberts syndrome, another cohesin-related disorder is distinguished by growth deficiency, cleft lip and mental retardation. This disorder is associated with mutations in ESCO1, homologous to *S.c.* Eco1 (McNairn and Gerton 2008). It is clear that the SMC protein complexes play key roles in genomic integrity and human disorders, confirming that further studies of these complexes are relevant and crucial.

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