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FUNCTIONAL ANALYSIS OF SMC5/6 COMPLEX IN HUMAN CELLS

by
LINA MARCELA GALLEGO PAEZ

Dissertation
Presented to the International Graduate School
of Bioscience and Biotechnology
Tokyo Institute of Technology

For the Degree of
DOCTOR OF PHILOSOPHY
Tokyo Institute of Technology
Yokohama, Japan
January, 2014

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To my beloved dad, who always supported me in this hard journey far from home. He dreamed this moment, and I'm sure he is watching it from somewhere.

FUNCTIONAL ANALYSIS OF SMC5/6 COMPLEX IN HUMAN CELLS

LINA MARCELA GALLEGO-PAEZ, PhD.

The Tokyo Institute of Technology, 2014
KATSUHIKO SHIRAHIGE, Ph.D.

The structural maintenance of chromosomes (SMC) proteins constitute the core of several highly conserved protein complexes with critical roles in chromosome architecture and organization during the cell cycle and are essential elements for the preservation of genomic stability. In eukaryotes, the Smc1/3 heterodimer belongs to the cohesin complex, which ensures the pairing of sister chromatids until they segregate during anaphase, whereas condensin complexes containing the Smc2/4 heterodimer participate in the regulation of mitotic chromosome condensation together with the activity of topoisomerase IIα. A third complex, consisting of Smc5/6 heterodimers and several non-SMC elements (Nse1–6) is less well characterized. Some of these Nse proteins provide relevant enzymatic activity such as Nse1, which contains a RING finger domain typical of ubiquitin ligases, and Mms21/Nse2, which has SUMO ligase activity. In budding yeast, all components of the Smc5/6 complex are essential for cell viability, and epistasis analysis with the Rad51 protein indicates that the Smc5/6 complex participates in several aspects of double-strand break repair by the homologous recombination pathway.

These functions have been corroborated in other species. However, relatively little is known about the involvement of the complex in general chromatin organization in the absence of DNA damage. A recent study highlighted the important contribution of the

Smc5/6 complex, specifically of the Mms21 SUMO ligase activity, to the resolution of a variety of DNA-mediated linkages arising not only in the context of DNA repair but also during DNA replication. Gross chromosome missegregation can occur if those abnormal linked structures are not efficiently removed prior to anaphase. Regions of DNA containing repetitive sequences, such as ribosomal DNA loci and telomeres, have consistently been shown to become highly unstable in yeast Smc5/6 mutants as a consequence of incomplete replication and the persistence of DNA linkages that ultimately result in chromosome breakage.

Interestingly, chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis in Saccharomyces cerevisiae revealed that the frequency of chromosomal association sites of the Smc5/6 complex increases in response to increased superhelical tension caused by chromosome lengthening, chromosome circularization, or inactivation of topoisomerase IIα (topo IIα). This has been proposed to reflect the requirement of Smc5/6 in preventing the accumulation of positive supercoiling ahead of the replication machinery by promoting replication fork rotation on a chromosome scale at sites of sister chromatid intertwining. Although this model implies the possible roles of Smc5/6 function in chromosome maintenance in yeast, it remains to be determined to what extent the complex exerts these structural roles in higher eukaryotes, which contain longer and more-complex chromatin.

Here we analyzed the cell cycle profile of the human Smc5/6 complex and its contribution to chromosome structure under physiological unperturbed conditions. Smc5 and Smc6 associate with chromatin during interphase and largely dissociate during mitosis, in a fashion similar to that of the human cohesin complex. Interestingly, depletion of Smc5 and Smc6 results in aberrant mitotic chromosomal structures and impaired chromosomal axial localization of topo IIα and the condensin Smc2.

Moreover, a detailed analysis of chromosome structure during interphase by premature chromosome condensation (PCC) assay revealed that a perturbed progression of replication after Smc5/6 depletion interferes with the timing of topo IIα binding and re-localization onto chromosomes before cells enter mitosis, suggesting an essential role for the Smc5/6 complex in linking DNA replication with higher-order chromatin structure.

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Lina Marcela Gallego-Paez, Hiroshi Tanaka, Masashige Bando, Motoko Takahashi, Naohito Nozaki, Ryuichiro Nakato, Katsuhiko Shirahige, and Toru Hirota. (2014). Smc5/6-mediated regulation of replication progression contributes to chromosome assembly during mitosis in human cells. Mol. Biol. Cell 25(2), 302-317.

CHAPTER I: INTRODUCTION

Structural Maintenance of Chromosome Proteins

Accurate segregation of genetic material to the daughter cells during mitosis is mainly dependent on the proper structural conformation of chromosomes. This attribute is the consequence of a tightly regulated series of processes during the cell cycle, involving completion of DNA replication, cohesion between sister chromatids, resolution of any kind of DNA linkages between them and subsequent chromosome compaction. At the heart of the protein complexes controlling the progression of, and the interplay between these important events, appears the highly conserved Structural Maintenance of Chromosomes (SMC) family of proteins. Found in archaeal, prokaryote and eukaryote species, Smc proteins contribution to chromosome dynamics is present even before the evolutionary acquisition of histone-related structural regulation and thus, the elucidation of their precise network of activities and interaction is of much importance in order to fully understand chromosome metabolism.

Architecture of the Smc Proteins

Electron microscopy and sequence analysis have revealed that Smc proteins are large (approximately 110 to 170 kDa), and their primary structure is composed by five discrete domains: The amino- and carboxy-terminal globular domains containing

the Walker A (GxxGxGKS/T) and Walker B or DA-Box (ΦΦΦΦD, where Φ is any hydrophobic residue) nucleotide-binding motifs respectively, while the central domain is composed of a flexible "hinge" sequence flanked by two long coiled-coil motifs arranged in an anti-parallel fashion generating a two-armed, symmetrical structure (Melby et al., 1998; Hirano et al., 2001) (Figure 1-1, A). Crystallographic studies indicate that, due to this tridimensional conformation, both Walker A and Walker B motifs make close contact and generate a catalytic domain for ATP binding and hydrolysis, which is similar to that present in other ATP-binding cassette (ABC) ATPases, such as RAD50 (Melby et al., 1998). This catalytic domain is required for the ability of Smc proteins to bind DNA specifically through their carboxy-terminal domain, as indicated by the observations of Smc1 and Smc2 protein activity in *Saccharomyces cerevisiae* (Akhmedov et al., 1998).

Archeal and bacterial organisms possess a single Smc protein conforming a homodimer (Hirano and Hirano, 1998; Melby et al., 1998). However, eukaryotes are characterized by the expression of at least six different Smc proteins, each one interacting with an Smc partner in a heterodimeric conformation: Smc1-Smc3, Smc2-Smc4 and Smc5-Smc6. The two Smc proteins associate in an antiparallel alignment through their respective hinge domains, generating a big V-shaped heterodimer with each coiled-coil arm of ~50 nm long. (Fig. 1-1, B) (Melby et al., 1998; Anderson et al., 2002). Engagement and disengagement of head domains by ATP binding and hydrolysis respectively, as well as the opening and closing of the hinge

interphase, modulate the dynamic interaction of the Smc proteins with DNA (Hirano et al., 2001; Hirano and Hirano, 2004).

Interestingly, different studies have revealed that intermolecular association between individual Smc heterodimers occur and that this dynamic interaction can be both, ATP-dependent or independent (Hirano et al., 2001; Stray and Lindsley, 2003; Hirano and Hirano, 2004). Smc heterodimers, in association with particular sets of non-Smc subunits, composed the fundamental group of Smc holocomplexes characterized by their critical contribution to the regulation of chromosome structure and function.

The Smc1/3 Cohesin Complex

Composition of the cohesin complex

Genetic screen of mutants in *Saccharomyces cereviseae* with precocious sister chromatid separation lead to the identification of the Smc1-Smc3 heterodimer and at least two non-Smc subunits (Scc1/Mcd1/RAD21 and Scc3/SA/STAG) as members of the cohesin complex (Michaelis et al., 1997), and subsequent biochemical and electron microscopy analysis indicated the structural and functional conservation of the complex among different species including humans (Figure 1-2, A) (Losada et al., 1998; Toth et al., 1999; Losada et al., 2000; Sumara et al., 2000). Scc1 is member of the kleisin

family of proteins and binds to the head domains of Smc3 and Smc1 by its N terminus and C terminus respectively, thus serving as a "bridge" between the two Smc proteins and conferring the characteristic ring-like shape of the complex, which has been confirmed by electron microscopy and crystallography observations of purified cohesin complexes in vertebrates (Haering et al., 2002; Anderson et al., 2002; Haering et al., 2004). The HEAT-repeat domain protein Scc3 associates with Scc1, with two variants found in distinct cohesin complexes in somatic cells of higher eukaryotes (SA1/STAG1 and SA2/STAG2) which are differentially required for telomere and centromere cohesion, respectively (Carramolino et al., 1997; Losada et al., 2000; Canudas and Smith, 2009). Meiosis-specific subunits of cohesin are also present in different organisms, as in the case of the Smc1α in mammals, REC8, the paralog of Scc1/RAD21 subunit in yeast and mammals (Eijpe et al., 2003; Molnar et al., 1995; Watanabe and Nurse, 1999; Gutierrez-Caballero et al., 2011; Ishiguro et al., 2011; Lee and Hirano, 2011; Pezzi et al., 2000; Prieto et al., 2001), or the four Scc1 homologs in *C. elegans* with a differential regulation during development (Pasierbek et al., 2001).

Sister Chromatid Cohesion

The ATPase activity within the head domains of Smc1 and Smc3 regulates the loading of cohesin onto chromatin (Arumugam et al., 2003), but the timing of this chromatin association differs between species. While the complex associates with DNA at the end of G1 phase in yeast, vertebrate cells starts cohesin binding to DNA during

telophase once the nuclear envelope is reformed. (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Darwiche et al., 1999; Sumara et al., 2000; Gerlich et al., 2006). Studies in different organisms have revealed that the loading of cohesin onto DNA is dependent on the activity of the protein complex formed by Scc2 and Scc4, (Furuya et al., 1998; Ciosk et al., 2000; Gillespie and Hirano, 2004; Rollins et al., 2004; Takahashi et al., 2004; Bernard et al., 2006; Seitan et al., 2006; Watrin et al., 2006), and further analysis in yeast indicated that this DNA association requires the binding and hydrolysis of ATP by Smc1 and Smc3 (Arumugam et al., 2003; Weitzer et al., 2003), as well as a transient dissociation of the hinge domain between the two Smc proteins in order to allow passage of the DNA into cohesin's ring (Gruber et al., 2006).

As predicted by this ring entrapment model, site-specific cross-linking of the Smc1-Smc3 hinge, Smc1 head–Scc1-carboxy terminal and Smc3 head–Scc1-amino terminal interfaces traps circular sister minichromosome DNAs inside a covalently circularized cohesin ring (Haering et al., 2008). It remains to be clarified how Scc2-Scc4 regulates cohesin loading onto DNA at the mechanistic level, although it has been proposed that Scc2-Scc4 could stimulate the ATPase activity of Smc1 and Smc3 (Arumugam et al., 2003; Nasmyth and Haering, 2005a). Chromatin immunoprecipitation assays have revealed that in yeast, cohesin associates to AT-rich sequences located at intergenic regions as well as around the centromeric region, a binding profile that differ from that observed in Scc2-Scc4. (Gillespie and Hirano,

2004; Takahashi et al., 2004). This difference indicates that cohesin may be relocated to intergenic regions after Scc2-Scc4 mediates its loading onto chromosomes, consistent with a downstream movement along RNA polymerase II-transcribed genes (Lengronne et al., 2004; Schmidt et al., 2009). However, this is unlikely to be the case in higher eukaryotes, as ChIP-chip analysis in *Drosophila* indicated that Nipped-B (homolog of Scc2 in yeast) and cohesin share the same binding sites, which mainly correspond to transcribed regions and overlap with RNA polymerase II (Misulovin et al., 2008). Interestingly, Nipped-B was initially identified in *Drosophila* as a structural mediator of enhancer-promoter interactions (Rollins et al., 1999) and in the same line of evidence, mutations in cohesin subunits affect gene expression and appear linked to developmental defects in different species including humans (Donze et al., 1999; Benard et al., 2004; Krantz et al., 2004; Vega et al., 2005; Horsfield et al., 2007; Zhang et al., 2007; Pauli et al., 2008). In fact, recent analyses of the genome-wide distribution of cohesin by ChIP-sequencing in mammalian cells have shown that cohesin binding sites functionally overlap with a large proportion of the DNA binding sites of the enhancer-blocking insulator CTCF (Parelho et al., 2008; Rubio et al., 2008; Stedman et al., 2008; Wendt et al., 2008), thus revealing a non-canonical function of cohesin in the regulation of gene expression that occurs from G1 phase, a role that may be intimately linked to the evolutionary-acquired loading of the complex onto chromatin early during telophase.

DNA replication and sister chromatid cohesion establishment

Topological entrapment of DNA by cohesin is not sufficient to establish sister chromatid cohesion. A subsequent process is required for the stabilization of the complex during S-phase, as judged by the experiments in yeast demonstrating that cohesin dissociates from chromosomes if the Smc2-Smc4 complex is inactivated in G1 phase, but not after DNA replication, and furthermore, no significant exchange of the functional pool of cohesin occurs once DNA synthesis has concluded (Gerlich et al., 2006b; Haering et al., 2004; Bernard et al., 2008). It has been shown that the acetyltransferase Ctf7/Eco1, which interacts genetically with the sliding clamp PCNA (Pol30) and the replication factor C (RFC) complex (CTF18/DCC1/CTF8), is required for the establishment but not for the maintenance of cohesion in yeast as cells progress trough S-phase (Figure 1-2, B) (Skibbens et al., 1999; Toth et al., 1999; Bernard et al., 2008). Recent analysis indicates that Eco1 acetylates the cohesin subunit Smc3 as the replication fork passes cohesin binding sites (Ben-Shahar et al., 2008; Ünal et al., 2008; Zhang et al., 2008).

Non-acetylatable and acetylation-mimicking mutations of two conserved Smc3 acetylation sites further confirmed the critical role of this modification in the establishment of competent cohesion between sister chromatids. However, an additional factor called Wapl participates in the regulation of cohesin interaction with chromatin. Wapl is a conserved protein from yeast to humans, which is required for promoting cohesin dissociation from chromosomes; Wapl deletion in budding yeast make Eco1

dispensable for sister chromatid cohesion, suggesting that acetylation of Smc3 counteracts Wapl activity, among other possible roles in the establishment of cohesion. Point mutations in Smc3, as well as in Scc3 and Pds5 (a Wapl-interacting protein also required for efficient sister chromatid cohesion) have also shown to promote the establishment of cohesion in the absence of Eco1 (Gandhi et al., 2006; Kueng et al., 2006; Rowland et al., 2009; Sutani et al., 2009), probably by affecting the interaction surface required for the destabilization activity of Wapl on cohesin, although other functions of Smc3 acetylation in the establishment of cohesion cannot be discarded (Ben-Shahar et al., 2008; Rowland et al., 2009).

In vertebrate cells, an additional regulatory protein called Sororin has been recently found to regulate cohesin-DNA interactions. This protein, which was identified as a substrate of APC/C^{Cdh1}, a form of the APC/C that is active during mitotic exit and G1 phase, is dispensable for the association of cohesin with chromatin but it is critical for stable binding of cohesin complexes to DNA during G2 phase (Schmitz et al., 2007). DNA replication and cohesin acetylation promote binding of Sororin to cohesin, and Sororin in turn displaces Wapl from Pds5, thus inhibiting the ability of Wapl to dissociate cohesin from DNA. Correspondingly, in the absence of Wapl Sororin becomes dispensable for cohesion in *Xenopus* and Human cells (Schmitz et al., 2007; Nishiyama et al., 2010).

Cleavage of cohesin and sister chromatid segregation

Contrasting the situation in yeast where the bulk of cohesin is bound to chromosomes until metaphase, cohesion between sister chromatids in vertebrates is lost in two steps. First, during the so-called "prophase pathway", the bulk of cohesin associated to chromosome arms is removed during prophase and prometaphase, which is dependent on the hyperphosphorylation of Scc3-SA subunits, a modification that has been found to depend on the catalytic activity of the mitotic kinases Aurora B and Plk1 (Figure 1-2, C) (Sumara et al., 2002; Hauf et al., 2005; Losada et al., 2002). In a subsequent step, chromatids are fully separated at anaphase when separase is activated and cleaves the Scc1 that was kept at centromeres (Uhlmann et al., 1999, 2000). When the threonine residues in Scc3/SA2 -which become phosphorylated during the prophase pathway- are mutated to alanine, the dissociation of cohesin from chromosome arms is affected and a significant proportion of cohesin remain chromatin-bound, although separation of sister chromatids during anaphase is not affected, indicating that the additional cohesin associated to metaphase chromosomes is still efficiently removed by separase upon activation of the APC/C (Hauf et al., 2005).

Cohesin dissociation in prophase is also dramatically reduced after Wapl depletion in mammalian cells (Gandhi et al., 2006; Kueng et al., 2006) indicating that not only Scc3/SA2 phosphorylation but also Wapl activity, critically contribute to the prophase pathway of cohesin dissociation.

After prophase, the persistence of centromeric cohesion is a required condition for proper sister kinetochore biorientation on the mitotic spindle and subsequent pulling of the sister chromatids to the opposite poles (Goldstein, 1980; Kerrebrock et al., 1995; Kitajima et al., 2004; Rabitsch et al., 2004). The Shugoshin (Sgo1) protein has been recently found to recruit the protein phosphatase 2A (PP2A) complex to the centromeres, which in turn maintains unphosphorylated Rec8 at this region and make it refractory to cleavage by separase (Figure 1-2, C). Consistently, shugoshin and PP2A are required for maintaining cohesion in early mitosis in human cells and in meiosis I in yeast without activating the APC/C and separase (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006; Brar et al., 2006).

Therefore, shugoshin as well as additional protein regulators like the histone H3 threonine-3 kinase Haspin or prohibitin 2 (PHB2) are considered to protect cohesin from the prophase pathway, maintaining centromeric cohesion until separase activation in metaphase (Dai et al., 2006; Takata et al., 2007). The accumulated data indicate that the prophase pathway is, in any case, not essential for anaphase because depletion of Wapl (which compromises cohesin removal during prophase), only generates a delay in sister chromatid separation but eventually cohesin disappear from chromosome once they start to separate (Kueng et al., 2006), leading to the hypothesis that separase can cleave the extra amount of cohesin onto chromosomes and thus compensate any defect in the prophase pathway.

Once all chromosomes are bioriented on the mitotic or meiotic spindle, the spindle checkpoint is inactivated and the APC/C becomes active. This leads to ubiquitylation and subsequent degradation of numerous APC/C substrates, including securin and cyclin B, the separase inhibitor and the activator of Cdk1 respectively. During interphase and early mitosis separase is inhibited by its association with securin, which blocks the access of substrates to the active site of separase. (Hornig et al., 2002; Waizenegger et al., 2002). In vertebrate cells, separase is also inhibited by Cdk1-mediated phosphorylation and by physical binding to cyclin B; APC/C activation then promotes separase activity by inducing ubiquitylation and degradation of securin and cyclin B (Stemmann et al., 2001; Gorr et al., 2005; Huang et al., 2005; Huang et al., 2008), though proteolytic autocleavage of separase represent another level of regulation which is still poorly understood (Waizenegger et al., 2000).

Active separase cleaves Scc1 at two sites, leading to the dissociation of the cohesin ring from chromosomes and subsequent separation of sister chromatids (Uhlmann et al., 1999, 2000). In budding yeast, phosphorylation of Scc1 by Cdc5 (ortolog of Plk1) enhances the cleavage activity (Alexandru et al., 2001), and although only the 10% of cohesin (which represent cohesin remaining at the centromeres) is cleaved by separase in mammalian cells, this reaction is essential for sister chromatid separation as noncleavable mutants of Scc1 and deletion of separase genes induce

important defects in chromosome segregation (Waizenegger et al., 2000; Gerlich et al., 2006; Hauf et al., 2001; Kumada et al., 2006; Wirth et al., 2006). Remarkably, studies in different organisms showed that Rec8 is also cleaved by separase, an essential reaction for chromosome segregation in meiosis (Buonomo et al., 2000; Siomos et al., 2001; Kitajima et al., 2003b; Terret et al., 2003; Kudo et al., 2006).

After chromosome segregation, Scc1 is re-accumulated at the end of G1 phase in the next cell cycle when APC/C is inactivated and securin inhibits separase (Michaelis et al., 1997). During anaphase in yeast, Hos1 deacetylase reverse Smc3 acetylation after Scc1 cleavage. Hos1 inactivation leads to Smc3 proteins that remain acetylated after mitosis, which cannot be used for subsequent cohesion establishment in S phase, indicating that this process depends on de novo acetylation of Smc3 during DNA replication (Beckouët et al., 2010).

Recent analyses have determine that HDAC8 is the vertebrate Smc3 deacetylase, as loose of HDAC8 activity results in increased amounts of Smc3 with retained acetylation, a situation that decrease occupancy of cohesin localization sites as well as altered transcription seen in CdLs cell lines were HDAC8 gene is mutated (Deardorff et al., 2012).

Smc2/4 at the core of Condensin Complexes

Composition of the condensin complex

Condensin was first identified in *Xenopus laevis* as a five-subunit complex containing Smc2/CAP-E and Smc4/CAP-C heterodimer and three non-Smc subunits: CAP-D2, CAP-G, and CAP-H (Figure 1-3, A) (Hirano et al., 1997). Subsequent studies also characterized the complex in different organisms including humans (Sutani et al., 1999; Freeman et al., 2000; Schmiesing et al., 2000; Kimura et al., 2001) and in yeast, all the five subunits are essential for cell viability. CAP-D2 and CAP-G, share HEAT (Huntingtin, elongation factor 3, the A subunit of protein phosphatase 2A, TOR lipid kinase) repeats.

These repeats are present in a number of chromosomal proteins and are thought to generate a particular interphase for protein-protein recognition (Neuwald and Hirano, 2000; Kobe and Kajava, 2000). CAP-H, belongs to the kleisin family of proteins connecting the head domains of Smc2 and Smc4. *In vitro* studies using *Xenopus* egg extracts showed that the non-Smc subunits of condensin regulate the ATPase activity of the Smc2/4 heterodimer (Kimura and Hirano, 2000), but it remains to be determined how mechanistically these proteins regulate condensin binding and activity onto chromosomes.

Initial analysis determined that condensin associates with chromatin during mitosis and regulates chromosome condensation and segregation in yeast (Bhalla et al., 2002; Freeman et al., 2000; Lavoie et al., 2002; Lavoie et al., 2000; Ouspenski et al., 2000; Strunnikov et al., 1995; Saka et al., 1994; Sutani et al., 1999) Congruently, functional inactivation of condensin subunits in *Caenorhabditis*, *Drosophila* and *Xenopus* egg cell-free extracts indicated that condensin complex is required for the lateral compaction of mitotic chromosomes (Hagstrom et al., 2002; Bhat et al., 1996; Steffensen et al., 2001; Wignall et al., 2003; Hirano and Mitchison, 1994; Hirano et al. 1997).

The notion of condensin existing as a unique complex changed when a database search using the human CAP-D2 (hCAP-D2) sequence indicated that another distantly related protein (hCAP-D3) which also contain a HEAT repeat motif at its C-terminal region, was found to immunoprecipitate with the core Smc2/4 heterodimer in HeLa cells, together with two additional proteins called CAP-H2 –which is the kleisin subunit- and CAP-G2 which also contains HEAT repeats (Ono et al., 2003; Schleiffer et al., 2003).

This newly discovered complex was called condensin II (Ono et al., 2003; Yeong et al., 2003) and has been found in different species along with the original condensin complex (which became condensin I) (Figure 1-3, A). The ratio of the two complexes varies between the organisms, leading to the hypothesis that condensin II

contributes to additional levels of chromosome organization (Hirano, 2005). Interestingly, organisms undergoing a less dramatic process of mitotic chromosome condensation compared to metazoans do not posses condensin II as in the case of *S. pombe*, *S. cerevisiae*, *Aspergillus nidulans*, and *Neurospora crassa,* supporting the idea of a required contribution of condensin II to an specialized high-order chromosome organization.

Condensin II was first characterized in *C. elegans* and subsequent analysis determined that condensin I was also present in this organism. (Csankovszki et al., 2009). Additionally, *C. elegans* contains the dosage compensation complex (DCC), a specialized condensin complex composed by the Smc2 homologue (MIX-1), an Smc4 variant (DPY-27), and two non-Smc variants (DPY-26 and DPY-28), which are found only in nematodes (Chuang et al., 1994; Hagstrom et al., 2002) having a key role in X-linked dosage compensation (Ercan and Lieb, 2009). In prokaryotes, a condensin-like complex containing an Smc homodimer and two non-Smc subunits is also present, being involved in genomic organization and segregation (Graumann and Knust, 2009).

Condensin I and II regulation during the cell cycle

In vertebrates, condensin I is mainly maintained at the cytoplasm during interphase. The relocalization to the chromosomes occurs at the end of prophase after *n*uclear *e*nvelope *b*reak*d*own (NEBD), and is release again to the cytoplasm at late

telophase (Hirota et al., 2004; Ono et al., 2004; Gerlich et al., 2006). In contrast, condensin II appears nuclear during interphase is just until prophase when it seems to accumulate on chromatin until the end of telophase (Figure 1-3, B) (Hirota et al., 2004; Ono et al., 2004; Gerlich et al., 2006). Immunofluorescence analysis using specific antibodies against condensin I- and II revealed that both complexes are present at centromeres and axially along chromosome arms. Moreover, condensin I and II have distinct alternating localization patterns in chromosome axis, with some particular regions of overlapping (Ono et al., 2003). Subsequent analysis reported additional differences at the centromere with condensin I-enriched regions having a thinner and more internal appearance compared to the stronger centromere signals of condensin II (Ono et al., 2004).

Moreover, fluorescence recovery after photobleaching (FRAP) experiments, as well as quantitative time-lapse imaging showed that GFP-tagged condensin II stably binds to chromosomes throughout mitosis, while condensin I interacted dynamically with chromatin after completion of prophase compaction, reaching steady-state levels on chromosomes before congression. In the same study, condensin I-depleted cells showed chromosomes with normal compaction but mechanically labile, and failed to hold up spindle forces during alignment, while normal levels of condensin II were not required for chromosome stability.

The precise mechanism of condensin binding to chromosomes is still unclear. Not direct evidence about preferred sequences or epigenetic marks has been proved, though genome-wide mapping of condensin binding sites using ChIP-chip in budding and fission yeast revealed that condensin binding is largely unaltered throughout the cell cycle with a transient enrichment at the centromeres during mitosis. The analysis also indicated that the complex accumulates at sites of replication fork stalling after treatment with the replication inhibitor hydroxyurea (Wang et al., 2005; D'Ambrosio et al., 2008). Interestingly, condensin colocalize with the cohesin loader Scc2/4, and although no physical interactions between the complexes could be demonstrated, condensin and Scc2/4 binding was observed at all tRNA genes. In *S. cerevisiae*, condensin is also enriched at the rDNA locus during mitosis as well as in interphase, suggesting an specialized role in organizing this particular repetitive region, which seems to be also the case at the nucleolus in human cells (Lavoie et al., 2004; Freeman et al., 2000; Cabello et al., 2001).

Apart from this binding profile, several regulatory proteins have been involved in the binding of condensin to chromosomes. In human cells for instance, the A Kinase/anchoring protein (AKAP95) targets condensin I to chromosomes after NEBD (Eide et al., 2002). Additionally, depletion of condensin II subunits has shown to reduced significantly the premature chromosome condensation in early G1 phase and the delayed post-mitotic decondensation characteristic of the autosomal recessive primary microcephaly (MCPH1) patient cells carrying mutations in the gene MCPH1

encoding microcephalin. In contrast, depletions of condensin I do not reverse this cellular phenotype, thus providing another line of evidence that condensin I and II are regulated by distinct pathways (Trimborn et al., 2006). More recently, the *Drosophila* homologue of tumor suppressor gene pRb (RBF1) was found to bind dCAP-D3, an association that is required for efficient binding of dCAP-D3 to chromatin. This interaction was also observed in human cell lines, and interestingly, mutant alleles of dCap-D3 suppress cellular and developmental phenotypes induced by the overexpression of RBF1, suggesting a possible link between pRB/RBF1 and chromatin condensation, by which the functional inactivation of RB family members in human tumor cells may contribute to genome instability (Longworth et al., 2008).

Another level of regulation is mediated by the phosphorylation of several condensin components. In human cells, Smc4, CAP-D2, CAP-H and CAP-G possess a variety of phosphorylation sites in both interphase and mitosis. (Takemoto et al., 2004, 2006). Condensin I is phosphorylated by CK2 in interphase, but additional kinases including Cdc2/CDK1 are required for its phosphorylation during mitosis (Takemoto et al., 2006). Particularly, Cdk1-mediated phosphorylation of condensin II appears to be required for proper Polo kinase Plk1 to localize to chromosome axes through binding to CAP-D3, which in turns hyperphosphorylates the condensin II complex. This mitotic phosphorylation pattern is required for timely chromosome condensation during prophase, and appears to enhance DNA supercoiling activity, whereas CK2 phosphorylation suppresses supercoiling activity. Moreover, Aurora B-dependent

phosphorylation of condensin has been also found to be required for localization of the complex to chromatin in *Drosophila* (Giet and Glover, 2001), *C. elegans* (Hagstrom et al., 2002) and humans (Lipp et al., 2007).

Role of condensin complex in mitotic chromosome assembly

In vitro studies initially indicated that condensin critically contribute to chromosome condensation; however, subsequent analysis in different species has revealed that the most striking condensation defect in condensin mutants is related with the timing of prophase compaction rather than a direct effect in the structural pattern of chromosome assembly. Chicken DT40 cells positive for phosphorylated Histone H3S10 in G2/M showed no apparent chromosome condensation in the absence of Smc2 (Hudson et al., 2003). Similar results were also observed in *C. elegans* (Kaitna et al., 2002). However, the chromosomes in these cells underwent a nearly normal (though highly accelerated) condensation process in the 5 minutes following NEBD. In human cells the timing defect in chromosome condensation differs between condensin I and condensin II.

While condensin I-depleted cells do not showed any significant difference in prophase condensation, they take twice the time to induce chromosome compaction from prometaphase to anaphase following NEBD. Depletion of condensin II on the contrary, cause a marked reduction in the level of prophase condensation, but instead,

cells seems to progress from prophase to anaphase in a similar time frame of wild-type cells (Ono et al., 2004; Hirota et al., 2004).

Interestingly, cells depleted of condensin do not suffer a significant disruption in chromosome compaction, as the typical X-shaped molecule is generated under these conditions. Instead, condensin seems to be required for the robust chromosome architecture that provides sufficient mechanical strength to withstand the forces of the mitotic spindle. When condensin-depleted cells are exposed to hypotonic buffer lacking divalent cations, this situation results in a clear disruption in chromosome morphology, with a wispy and fuzzy appearance and chromosome arms relatively wider than wild type, though chromosome shape *per se* is maintained (Hudson et al., 2003; Ono et al., 2003; Vagnarelli et al., 2006).

In a more detailed analysis, the two condensin complexes have shown to play distinct roles in mitotic chromosome structure. Depletion of condensin I is responsible for the formation of swollen chromosomes, which are wider laterally and shorter axially, whereas condensin II generates more thinner and axially longer chromosomes, with a twisted and bent appearance, implying that condensin II but not condensin I is able to support mitotic chromosome rigidity, probably by establishing an initial chromosome axis around which condensin I can then arrange loops of chromatin (Ono et al., 2003; Green et al., 2012).

How exactly condensin regulates chromosome architecture is not clear yet, but it is important to note that loss of condensin induces the mislocalization of fundamental chromatin components including topoisomerase II, INCENP and KIF4A chromokinesin without affecting their protein levels (Hudson et al., 2003, 2008). In the case of topoisomerase II and INCENP, both proteins fail to partition into the chromosome scaffold fraction, which appears to be largely missing in the absence of condensin. The staining of the abundant scaffold protein KIF4A appeared diffuse and its axial concentration was lost after Smc2 depletion; but not when each condensin complex was depleted independently. In this case, chromosomes lacking condensin I had a more diffuse, wider scaffold staining of KIF4A, whereas chromosomes lacking condensin II displayed a tight axial staining that was also bent and twisted in line with the chromosome morphology (Hudson et al., 2008). These results indicated that a major function of condensin is to promote the correct association of nonhistone proteins with mitotic chromosomes, which is an essential condition for establishment of a robust chromosome structure.

Apart from its requirement in axial chromosome compaction, condensin have been also involved in the process of sister chromatid resolution in different organisms. Chromatid resolution is proposed to be achieved by cooperation between topoisomerase II and condensins (Coelho et al., 2003; Steffensen et al., 2001). In fact, the most prominent phenotype observed in the absence of condensin function is a clear defect in

chromosome segregation with characteristic anaphase bridges that impede the completion of cytokinesis (Hirano et al., 1986; Ono et al., 2004; Gerlich et al., 2006). This defect has been associated to a failure in resolving entangled DNA and/or protein-mediated DNA linkages leading to anaphase chromatin bridging (Hartl et al., 2008). In this context, condensin overwinding would be an important requirement for intertwine resolution by topoisomerase II and, together with the inhibition of transcription, it would contribute to chromosome condensation (Baxter et al., 2011).

A detailed examination of metaphase chromosomes lacking condensin in vertebrates also indicates that even though the structure of the kinetochores appears normal, sister kinetochore movements are uncoordinated due to loss of centromeric stiffness, which in turn leads to an increase in Mad2(+) kinetochores near the metaphase plate and a delayed anaphase onset. (Oliveira et al., 2005; Gerlich et al., 2006; Ribeiro et al., 2008). Additionally, it was recently demonstrated that during early anaphase in yeast, recoiling of a stretched chromosome region requires condensin and generates forces to remove residual cohesion. Under these conditions, residual sister chromatid cohesion during early anaphase is eliminated by the recoiling process facilitated by condensin, and this event is required to complete sister chromatid separation (Renshaw, 2010).

Mechanistic description of condensin activity

As in the case of cohesin, the ATPase catalytic activity of the two Smc subunits is essential for condensin function (Stray and Lindsley, 2003; Hudson et al., 2008). In vitro analyses using *Xenopus* egg and *C. elegans* extracts, condensin has shown to introduce positive supercoils into DNA in the presence of topoisomerase II, a process that requires ATP hydrolysis and cell cycle-specific phosphorylation of condensin (Kimura et al., 1997; Kimura et al., 1999; Hagstrom et al., 2002), and studies using electron spectroscopic imaging (ESI) of a single DNA molecule showed that condensin I can bind DNA in the absence of ATP, but it compacts the DNA only in an ATP-hydrolysis dependent manner (Bazett-Jones et al., 2002; Strick et al., 2004).

However, the holocomplex of condensin and not the SMC heterodimer alone exhibit DNA-stimulated ATPase activity (Kimura and Hirano, 1997; Kimura and Hirano, 2000; Sutani and Yanagida, 1997), suggesting the important contribution of the non-Smc subunits to condensin functions. *In vivo*, systematic mutagenesis of all known Smc2 ATPase domains was carried out in Smc2 conditional knockout cells indicated that ATP-binding, but not hydrolysis was required for the stable association of condensin with mitotic chromosomes (Hudson et al., 2008).

Recent studies have determined that condensin is able to encircle dsDNA in yeast, forming topological links in a way similar to the proposed model for cohesin-DNA interactions, rising the possibility that condensin could promote

chromatin folding and compaction by embracing and thus linking two distant chromatin segments (Cuylen et al., 2011), although a chiral looping model involving more than two segments is also viable (Stray and Lindsley, 2003; Stray et al., 2005; Hudson et al., 2008).

A previous model predicting that condensins could oligomerize or form a higher-order assembly to fold a chromatin fiber during the process of chromosome condensation (Swedlow and Hirano, 2003; Hirano, 2006; Graumann and Knust, 2009) was recently reintroduced into a novel general model of chromatin organization that has been proposed after several studies confirming that human mitotic HeLa chromosomes are mainly composed of irregularly folded nucleosome fibers rather than 30-nm chromatin fibers as it was long assumed (Nishino et al., 2012). In this updated model, condensin forming the chromosome axis holds the nucleosome fibers globally around the chromosomal center.

Self-assembled axial structures will be formed if condensins bind to specific sites on the genome and form loops that are able to interact with one another. Alternatively, as the loops would repel one another due to entropic effects, this may lead to the formation of regular loops that could be sufficient to generate a rod shape (Kleckner et al., 2004). The model also proposes that chromatin loops are collapsed and

irregularly folded to make particular domains. Nonetheless, future studies are required to elucidate the exact mechanism by which the ATP-binding and hydrolysis cycle of the Smc2/4 heterodimer might be coupled with condensin–DNA interactions as well as with condensin–condensin interactions.

The Unnamed Smc5/6 Complex

Phylogenetic analyses indicated that apart from the Smc1/3 and Smc2/4 heterodimers, eukaryotic cells possess two additional members of the Smc family, Smc5 and Smc6, that appeared at the core of a protein complex containing additional subunits recently characterized (Cobbe and Heck, 2000; Fousteri and Lehmann, 2000; Taylor et al., 2001). Smc6 was identified in early studies as the product of the Rad18 gene in screens for radiation-sensitive mutants of fission yeast (Lehmann et al., 1995) and subsequently, mutations in a number of the subunits of the Smc5/6 complex have confirmed the importance of these proteins in the DNA damage response (Fujioka et al., 2002; Harvey et al., 2004; Lehmann et al., 1995, McDonald et al., 2003; Pebernard et al., 2004).

Interestingly, unlike othe*r Rad g*ene products, Rad18/Smc6 as well as Spr18/Smc5 are essential for mitotic growth in *S. pombe* (Fousteri and Lehmann, 2000), implying that Smc5/6 complex participates in different levels of chromatin regulation beyond the DNA repair context (Bahler et al., 1998; Barker et al., 2002; Boddy et al., 2001). Apart

from the analyses in yeast, recent studies have lead to the description of Smc5, Smc6 and the other components of the complex in number of other eukaryotes, including *Saccharomyces cerevisiae*, *Xenopus laevis*, *Arabidopsis thaliana* and human (Boddy et al., 2000; Boddy et al., 2003; Carr et al., 2003; Chen et al., 2004).

Architecture of the protein complex

The Smc5/6 heterodimer associates with at least six non-Smc elements designated Nse1–Nse6 (Carr et al., 1993; Doe et al., 2002; Doe et al., 2000; Du et al., 2003; Eng et al., 1994; Freemont, 2000) (Figure 1-4). A very special feature of Smc5/6 is the ubiquitin and SUMO ligase activities carried by the Nse1 and Nse2 subunits respectively. Primary sequence analysis of Nse1 indicated the presence of a RING finger domain, common to ubiquitin E3 ligases, and in the case of Nse2 a Miz1/Siz1/PIAS/ARIP3 domain found in SUMO E3 ligases was also determined (Doe et al., 2002). In the case of Nse1, the RING-like motif is distinct from all previously described RING variants, and forms an evolutionary subclass found in Nse1 homologues across species.

A series of Nse1 mutations demonstrated that the NH-RING is not essential for vegetative growth, but constitutes a protein–protein interaction motif required for stable formation of the Nse1-Nse3-Nse4 heterotrimer, an structure required for the

recruitment/accumulation of the Smc5/6 complex at sites of DNA damage (Pebernard et al., 2008; Tapia-Alveal and O'Connell, 2011). It was also demonstrated that human and fission yeast Nse1 expressed in and purified from bacteria did not support ubiquitin E3 ligase activity *in vitro*. However, when the Nse3 subunit was added to Nse1 in the presence of an E2 ubiquitin-conjugating enzyme, a robust in vitro ubiquitin ligase activity was seen (Hazbun et al., 2003).

Nse3 is a melanoma-associated antigen gene (MAGE)-like protein and is also known as MAGE-G1 (Higgins et al., 1976). MAGE proteins are specifically expressed in certain cancers and are involved in the regulation of gene transcription and apoptosis (Hope et al., 2005). Moreover, biochemical and cellular assays demonstrated that MAGE proteins in general enhance the ubiquitin ligase activity of RING domain proteins, and congruently, it was recently demonstrated that MAGE-G1 promotes the activity of Nse1 in the presence of the MMS2 E2 ubiquitin-conjugating enzyme in vitro (Doyle et al., 2010).

Nse2 was originally described as *MMS21*, from a screen in *S. cerevisiae* for mutants sensitive to MMS and was later shown to be part of the Smc5/6 complex (Zhao and Blobel, 2005). Sequence analysis indicated that Nse2 belongs to the Siz/PIAS (SP) family of E3 SUMO ligases, due to the presence at its C-terminus of the characteristic catalytic SP-RING domain (Zhao and Blobel, 2005; Andrews et al., 2005; Potts and Yu, 2005). Despite the fact that Nse2 do not contain the SAR, Acinus and PIAS (SAP)

motif required for DNA binding present in all SP family ligases, the protein is able to bind chromatin through its interaction with the Smc5/6 complex (Jackson et al., 2001; Lindroos et al., 2006).

Nse2/MMS21 is able to SUMOylates itself in yeast and humans, as well as the budding yeast Ku70 and Smc5 (Zhao and Blobel 2005; Andrews et al., 2005; Potts and Yu, 2005), fission yeast Nse3, Nse4 and Smc6 (Pebernard et al., 2008; Andrews et al., 2005) and the human Rap1, SA2, Scc1, Smc6, Tin2, TRAX, Trf1 and Trf2 proteins (Potts et al., 2005; Potts et al., 2006; Potts and Yu, 2007). Experiments in yeast and human cells have demonstrated that Nse2 is indeed an E3 SUMO ligase that is required for efficient DNA repair (Zhao and Blobel 2005; Andrews et al., 2005; Potts and Yu, 2005).

Nse2, as in the case of the other members of the complex, is required for cell viability in yeast, but inactivating mutations of the Nse2 SP-RING as well as siRNA depletion of Nse2 in human cells, result in hypersensitivity towards different kind of DNA damage including hydroxyurea (HU), ionizing radiation (IR), methyl methanesulfonate (MMS) and ultraviolet (UV) light (McDonald et al., 2003; Pebernard et al., 2004; Rai et al., 2011).

The kleisin element Nse4 (initially described as Rad62) bridges the globular domains of Smc5 and Smc6 and also forms a subcomplex with Nse1 and Nse3 (Sergeant et al., 2005; Palecek et al., 2006). Two human homologs of Nse4 exists in human cells (hNSE4a and hNSE4b) and both Nse4 paralogs can form part of the Smc5/6 complex but only hNSE4a is expressed in somatic cells while hNSE4b is exclusively expressed in testis. Nse5 and Nse6 have been found to be present in the Smc5/6 complex only in yeast (Pebernard et al., 2006; Zhao and Blobel, 2005; Hazbun et al., 2003). While no conserved domains are found in the primary sequence of Nse5, Nse6 contains ARM/HEAT repeats also present in the cohesin loader Scc2 (Pebernard et al., 2006; Palecek et al., 2006), suggesting that, at least in yeasts, Nse6 might regulate Smc5/6 complex interactions with DNA.

All members of the Smc5/6 complex are required for viability in *S. Cerevisiae* (Zhao and Blobel, 2005), and all but Nse5 and Nse6 are essential in *Schizosaccharomyces pombe* (Pebernard et al., 2006). However, Smc5-deficient chicken DT40 cells are viable (Stephan et al., 2011), and siRNA-dependent depletion of Smc5/6 complex components has shown only moderate cell cycle delay without extensive cell death in human tissue culture cells (Potts and Yu, 2005; Behlke-Steinert et al., 2009; Taylor et al., 2008; Potts and Yu, 2006).

Assembly of the Smc5/6 complex

Several models based on biochemical and yeast-two-hybrid analyses have been proposed to define the interaction between the different subunits of the Smc5/6 complex. These studies revealed the formation of three subcomplexes in yeast: Smc5, Smc6 and Nse2 form one subcomplex, with Nse2 bound to the coiled-coil region of Smc5; Nse1, Nse3 and Nse4 form a second complex that interacts with the heads of Smc5 and Smc6 in fission yeast and only with the head of Smc5 in budding yeast, and a third subcomplex composed by Nse5 and Nse6 also binding to both Smc5 and Smc6 head domains in fission yeast, but associates to the Smc5/6 hinge domain in budding yeast (Pebernard et al., 2006; Sergeant et al., 2005; Palecek et al., 2006; Hudson et al., 2011).

In human cell lines, gel filtration analysis has suggested that all the components of the Smc5/6 complex are located exclusively in the complex except in a proportion of hNse1 and MAGEG1 that eluted with a much lower molecular weight, suggesting that these two proteins could form a separated heterodimer, although recent studies have also indicated that human Smc5 co-elutes from column fractions that contain Mms21 but lack Smc6 during mitosis (Behlke-Steinert et al., 2009). The fact that the subcomplex containing Nse1–Nse3 is associated with the ATPase head domain, and the SUMO ligase associates with the Smc5 arm prompts up the question of which could be the functional impact of these different molecular locations. The kleisin

interaction with the ubiquitin ligase could indicate that the ubiquitin ligase is controlled by the assembly of the full complex, while the SUMO ligase may also act independently of an Smc5/6 ring, supporting the notion of there being discrete Smc5/6 complex activities associated with the ubiquitin ligase or SUMO ligase functions.

Localization pattern of the Smc5/6 complex on the chromatin

The chromosomal localization of the Smc5/6 complex determined by ChIP-chip in budding yeast (Lindroos et al., 2006) indicates that during G1 phase, the complex is almost absent from the chromosomes except from a particular enrichment at telomeric sequences (and possibly at other heterochromatin domains not included in the sequence analysis), while cells arrested in S phase with HU showed the complex associated with regions around autonomously replicating sequences (ARS), indicating that Smc5/6 chromosome binding occurs just after DNA replication occurs, or that the complex is recruited to stalled replication forks that accumulate in the presence of HU.

Smc5/6 binding profile in G2/M indicates that the complex localizes to intergenic regions and is specially accumulated at centromeres, resembling the chromosomal localization of Cohesin (Glynn et al., 2004; Lengronne et al., 2004). Further analysis indicated that Smc5/6 binding to chromatin depends on the Scc2/4 loading complex as in the case of cohesin, although more recent studies by ChIP-Seq indicated that cohesin itself is responsible for the stable binding of Smc5/6.

Moreover, Smc5/6 accumulates at regions surrounding DNA double-strand breaks (DSBs) in G2/M in an Mre11-dependent manner, as well as at collapsed replication forks during S phase, when the complex is required for timely chromatid separation in the centromeric regions and prevents the accumulation of branched chromosome structures. In addition, a detailed analysis of Smc6 peaks by ChIP-chip and lately by ChIP-Seq showed that the frequency of arm interaction sites increases in response to chromosome lengthening, chromosome circularization, or inactivation of topoisomerase II, all having the potential to increase the number of sister chromatid intertwinings (Lindroos et al., 2006; Kegel et al., 2011)

In fission yeast, fluorescence microscopy and ChIP-chip indicated that Smc5/6 do not preferentially enriched at intergenic regions as in the case of budding yeast, but instead it binds along the entire length of the chromosomes, and while budding yeast smc5/6 showed maximal occupancy at centromeres in G2/M-phase of the cell cycle, in fission yeast the maximal occupancy occurred in S-phase during replication with a transient enrichment at the heterochromatic outer repeats of centromeres in an H3-K9 methylation-dependent manner.

A possible explanation for this discrepancy is the absence of centromeric heterochromatin in budding yeast compared to the case of fission yeast (Torres-Rosell et al., 2005). Methyl methanesulfonate (MMS) treatment in fission yeast induces the

accumulation of Smc5/6 at subtelomeres, in an Nse2 SUMO ligase-dependent, but H3-K9 methylation-independent manner, and it was proposed that Smc5/6 loads at all genomic tDNAs, a phenomenon that requires intact consensus TFIIIC-binding sites in the tDNAs (Pebernard et al., 2008). This localization is similar to that of the Smc2/4 condensin complex, which is localized to tDNAs in budding and fission yeast (D'Ambrosio et al., 2008).

Analysis in *Xenopus laevis* egg extracts has indicated that Smc5/6 was loads onto chromatin during DNA replication in a manner dependent on the initiation of DNA synthesis, and it dissociated from chromatin during mitosis. Induction of DSBs following replication did not significantly affect the amount of chromatin-associated Smc6, suggesting that the Smc5/6 complex is regulated during the cell cycle, presumably in anticipation of DNA damage that may arise during replication (Tsuyama et al., 2006).

Smc5/6 complex in the DNA damage repair response.

Mutations in genes encoding components of the Smc5/6 complex result in severe mitotic defects as well as in defective repair of DNA damage induced by IR, MMS and UV in different organisms including yeast, chicken, *Arabidopsis* and human cells (Lehmann et al., 1995; Taylor et al., 2001; McDonald et al., 2003; Pebernard et al., 2004; Potts et al., 2005; Taylor et al., 2008; Stephan et al., 2011; Potts and Yu 2006;

Watanabe et al., 2009). Epistasis analyses of the Smc5/6 complex have indicated that it participates at different stages of the Homologous Recombinational Repair pathway (HRR) of the DSB repair response (Figure 1-5). In fission yeast for instance, *smc6-X* and *rhp51*^{Rad51} double mutants do not present an enhanced sensitive to IR than *rhp51*^{Rad51} single mutants (Lehmann et al., 1995), consistent with the studies made in budding yeast, and the similar sensitivity to IR between Rad54(-/-) Smc5(-) double knockout and Rad54(-/-) observed in chicken cells (Stephan et al., 2011). Moreover, Smc5/6 is recruited to nuclease-induced DSBs in yeast and humans, an event that is MRN-dependent in budding yeast and secures efficient sister chromatid recombination at the break Lindroos et al., 2006; Potts and Yu 2006; De Piccoli et al., 2006). RNAi-mediated knockdown of Smc5 and Nse2 in human cells decreases sister chromatid HR, without affecting nonhomologous end-joining (NHEJ) or intra-chromatid, homologue, or extrachromosomal HR (Potts and Yu, 2006). In *Arabidopsis*, a mutant of *mim*, an Smc6 homolog present hypersensitivity to a variety of DNA-damaging agents and is defective in intrachromosomal homologous recombination in somatic cells (Mengiste et al., 1999).

Gross chromosomal rearrangements in the absence of functional Smc5/6 have been proposed to be the consequence of misregulated homologous recombination (De Piccoli et al., 2006). An example of this situation is the elevated loss of telomere marker and the chromosome instability observed in the *mms21Δsl* deletion mutant in budding yeast (Rai et al., 2011), indicating that the SUMO ligase activity, as well as the integrity

of the complex critically contribute to its repairing functions (Rai et al., 2011). Congruently, SUMOylation of multiple telomere-binding proteins, including TRF1 and TRF2 is necessary for the recruitment of telomeres to PML bodies where the alternative lengthening of telomeres (ALT) occurs, a mechanism that secures the maintenance of telomere length in a variety of human cancer cell lines (Potts and Yu, 2007). This evidence suggests that a role of Nse2 in the maintenance of telomere length is evolutionarily conserved (Zhao and Blobel, 2005; Chavez et al., 2010). It is interesting to note that mutations in Nse2 exclusively inactivating the SP-RING in budding and fission yeast (nse2-SA, mms21-11, mms21-CH, mms21Δsl, mms21-H202A, mms21-C221A) disrupt the HR repair process, but do not seem to affect cell viability in the absence of DNA damage, implying the specific requirement of the SUMO ligase activity of Nse1 for the repair functions of the Smc5/6 complex (McDonald et al., 2003; Pebernard et al., 2004; Zhao and Blobel, 2005; Andrews et al., 2005; Potts and Yu, 2005; Rai et al., 2011).

However, full Nse2 protein is necessary for normal cell cycle progression in *Arabidopsis*, where Nse2 mutation results in abnormal root development (Huang et al., 2009) thus, there seems to be significant differences in the requirement for Nse2 and the Smc5/6 complex in cell proliferation among the species.

In vitro analysis of the budding yeast has recently revealed that Smc5 protein preferentially binds to single-stranded, rather than double-stranded DNA, suggesting

that Smc5/6 may contribute in a different manner to DNA repair compared to the other SMC complexes (Roy et al., 2011), or that Smc5 itself may behave differently when bound to its Smc6 partner and Nse subunits in vivo.

What is then, the contribution of the Smc5/6 complex to the HR process? It has been observed that in the absence of Smc5/6, Rad51 foci formation still occurs at sites of DNA damage, but instead, cells accumulate X-shaped DNA molecules, suggesting that Smc5/6 do not affect the initial steps of HR but it is required for the efficient resolution of HR intermediates (Stephan et al., 2011, De Piccoli et al., 2006; Ampatzidou et al., 2006; Choi et al., 2010; Bermudez-Lopez et al., 2010) (Figure 1-5). Interestingly, the accumulation of chromosome linkages in Smc5/6 mutants lead to lethal mitosis, due to failure in chromosome separation (Ampatzidou et al., 2006; Choi et al., 2010; Bermudez-Lopez et al., 2010; Irmisch et al., 2009; Chen et al., 2009).

The fact that these mutants can entry mitosis even after DNA damage indicates that there is a marked defect in checkpoint responses in the absence of Smc5/6 function (Verkade et al., 1999). However, the expression of wild-type Smc6 in an *smc6-9* background, even as late as in metaphase, restores the ability of this mutant to resolve branched DNA molecules and prevents abnormal mitosis (Bermudez-Lopez et al., 2010), indicating a very high robustness and efficiency in the resolution step catalyzed by the Smc5/6 complex.

As mentioned before, Smc5/6 mutants are also sensitive to UV light (Lehmann et al., 1995; Pebernard et al., 2006). Epistasis analysis in *S. pombe* has indicated that a nucleotide excision repair (NER)-independent mechanism involving Smc5/6 activity and the HR process is required for efficient excision of UV-induced photoproducts (Lehmann et al., 1995). Rhp51^{Rad51} *nse6Δ* or Rhp51^{Rad51}*nse2-1* double mutants result in a similar sensitivity to UV light to that observed in *rhp51Δ* single mutants, whereas single mutants of Nse6 and Nse2 result in an increased sensitivity to UV compared to *rhp51Δ*mutant (Pebernard et al., 2006), indicating that the activities of Rhp51^{Rad51} in response to UV (and HU) are lethal when the functionality of Smc5/6 complex is compromised.

Roles of Smc5/6 in Replication Fork progression

Additional evidence supporting the idea that the activity of the Smc5/6 complex at stalled forks may involve the processing of recombinational structures comes from epistasis analysis in yeast. The sensitivity to DNA damage observed in Smc5/6 mutants in budding and fission yeast can be rescued after deletion of the MphI helicase (Chavez et al., 2010; Choi et al., 2010; Chen et al., 2009), which is a member of the FANCM family of helicases that work on different DNA structures such as stalled replication forks (for reversal activity), holiday junctions (branch migration activity) and D-loops, an activity required for efficient activation of the tumor

suppressor Fanconi Anemia (FA) pathway. In fact, direct interaction between the budding yeast MphI homolog and Smc5 has been observed *in vivo*, although this interaction is not required for efficient DNA repair (Chavez et al., 2010).

These results indicate that in the absence of Smc5/6, The FA pathway generates toxic DNA repair intermediates, but how mechanistically Smc5/6 regulates MphI activity is unknown (Chen et al., 1009). Another interesting observation is that the hypersensitivity to HU and the subsequent accumulation of X-shaped DNA molecules in fission yeast *smc6-74* mutants can be rescued by overexpression of the BRCT domain-containing Brc1 protein (Ampatzidou et al., 2006), which is required for efficient DNA repair during S phase (Verkade et al., 1999). It has been proposed that in the absence of functional Smc5/6 complex, accumulated HR intermediates can be repaired through alternative Brc1-dependent mechanisms (Ampatzidou et al., 2006), and actually, Brc1 appears essential for the viability of fission yeast *smc6* mutants (Verkade et al., 1999). Brc1 suppression of *smc6-74* lethality requires the Slx1/4 and Mus81/Eme1 nucleases that are able to cleave different types of DNA structures, such as stalled replication forks, 5′ and 3′ flaps, nicked holiday junctions and D-loops (Sheddy et al., 2005).

All these data suggest that Smc5/6 is necessary for the establishment and maintenance of specific DNA repair intermediates during DNA replication that can be

processed by alternative repair pathways, which become essential for cell survival when Smc5/6 function is compromised (Figure 1-6).

The fact that Smc5/6 is enriched at repetitive DNA loci, such as rDNA arrays, centromeres and telomeres (Stephan et al., 2011; Ampatzidou et al., 2006; Pebernard et al., 2008) and that the X-shaped DNA structures observed at the rDNA locus can be rescued by the deletion of *Rad52* in *smc6-9* and *smc5-6* mutants (Torres-Rosell et al., 2005) indicate that its activity is required at sites where spontaneous replication fork collapse may occur, or where DNA intermediate structures are likely to arise during the process of collapsed forks repair and re-start. Analyses of DNA damage repair process in yeast have recently indicated that DSBs within the rDNA loci induced by I-Sce-I or IR are excluded from the nucleolus in wild-type cells to promote their efficient repair, a delocalization process that do not occur in *smc6-9* and *nse5-1* cells (Torres-Rosell et al., 2007), although it is still to be determined how Smc5/6 controls DSB relocalization and efficient repair.

Smc5/6 regulation of higher-order chromosome structure

The fact that the Smc5/6 complex is required for the recruitment of telomeres to PML bodies, as well as the relocation of DSBs induced at rDNA loci to outside the nucleoli (Potts and Yu, 2007; Torres-Rosell et al., 2007) indicates a general activity of the Smc5/6 complex in the localization of damaged DNA probably by regulating

higher-order chromosomes structures –as in the case of the other Smc complexes- to allow DNA repair and recombination. Another implication of the Smc5/6 in general chromosome structure appears related to the sister chromatid cohesion.

In human cells, depletion of Nse2 or Smc5 induced a marked loss of sister chromatid cohesion during prometaphase (Behlke-Steinert et al., 2009), a situation also observed in Smc5-deficient chicken DT40 cells (Stephan et al., 2011) although a more modest decrease in sister chromatid proximity occurs in these cells. As indicated before, Smc5/6 complex is required for the relocalization of cohesin to the sites of DSB repair in HeLa cells (Potts and Yu, 2006) (Figure 1-6), and although this requirement was not observed in chicken cells, nether fission yeast or budding yeast (Strom et al., 2007; Outwin et al., 2009), the genome-wide cohesion induced by DSBs requires functional Smc5/6 in the particular case of budding yeast (Strom et al., 2007), and moreover, it has been recently shown that Nse2 actually SUMOylates the Scc1 subunit of cohesin in response to DSBs.

Strikingly, Smc6-74 mutants in *S. pombe* undergo lethal mitoses after HU-induced DNA damage due to a failure in the removal of chromosome-associated cohesin (Outwin et al., 2009). Cohesin is also abnormally retained in *smc6-74* and topoisomerase II double mutants, a situation that has been proposed to reflect an altered mitotic chromosome structure, rather than a DNA repair defect (Outwin et al., 2009;

Tapia-Alveal et al., 2011), although lethal, persistent sister chromatid association is mediated by DNA linkages, rather than cohesion in budding yeast (Bermudez-Lopez et al., 2010).

Moreover, a new concept indicating that Smc5/6 complex may be assisting replication fork rotation at a chromosome-level to unwind the supercoiling ahead of the replication fork (Figure 1-7) (Kegel et al., 2011) comes from the observation that *S. cerevisiae smc6* and *mms21* mutants have defects in the complete replication of longer chromosomes, due to the presence of interfering DNA linkages between the sister chromatids that prevent the release of superhelical tension during replication (Kegel et al., 2011). These alterations in chromosome topology could be considered as a system to control cohesin association to chromatin, as well as replication-associated recombination processes.

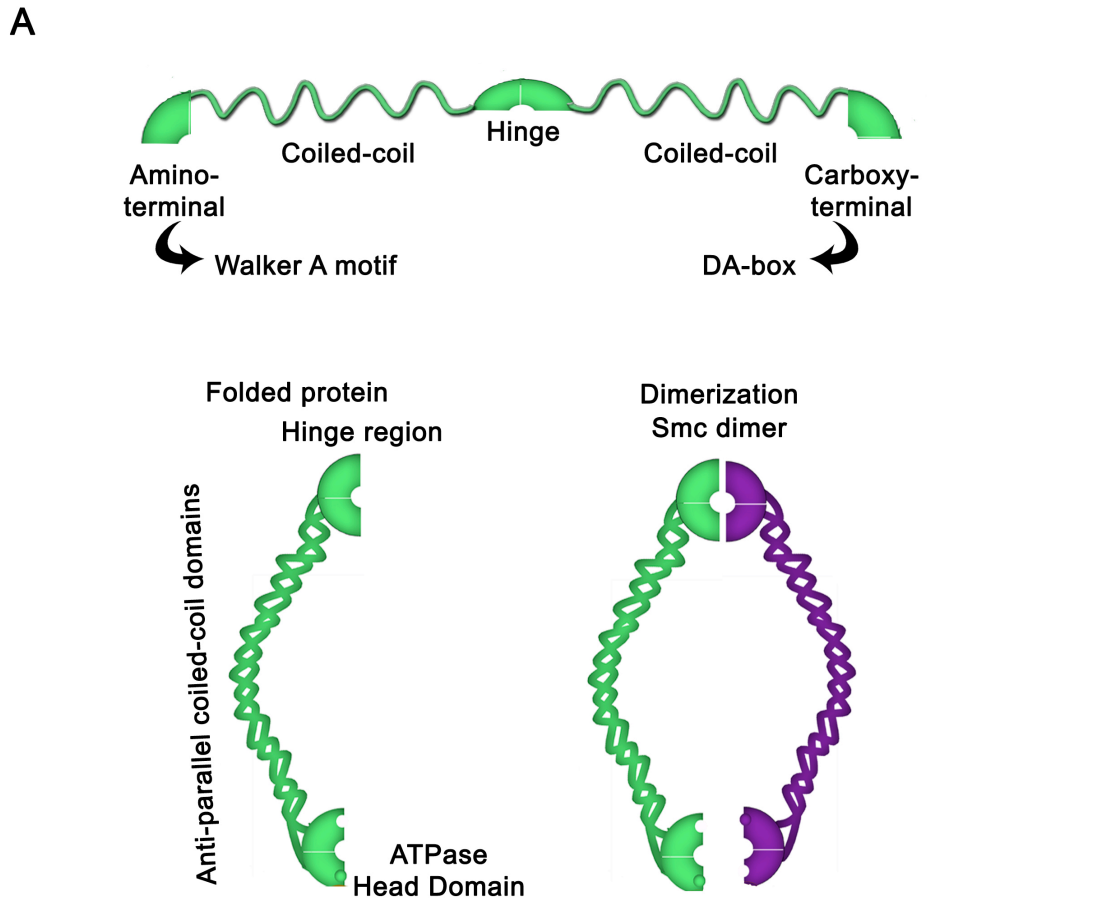
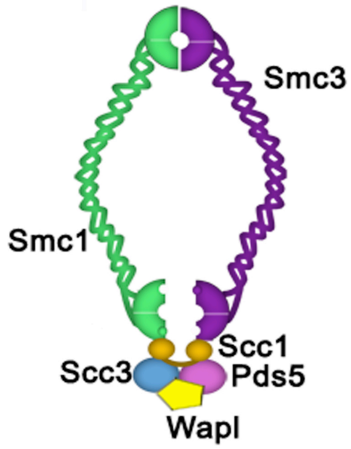
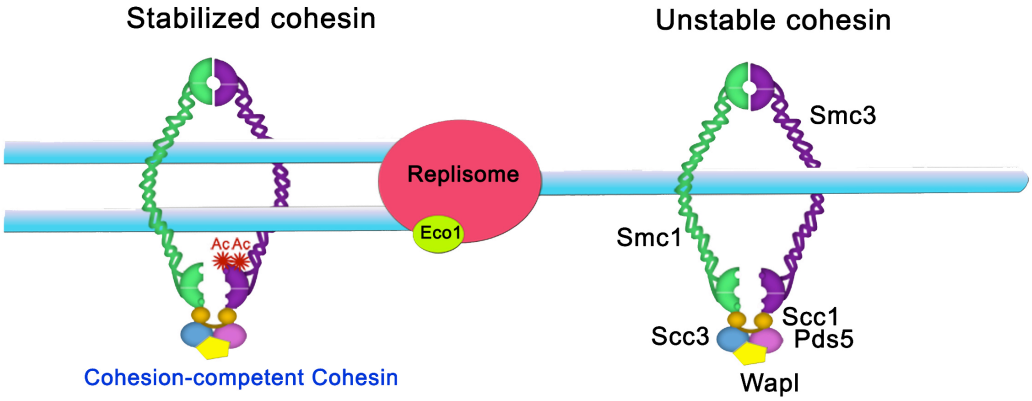

A
Hinge
Coiled-coil
Coiled-coil
Amino-
terminal
Carboxy-
terminal
Walker A motif
DA-box
Folded protein
Hinge region
Dimerization
Smc dimer
Anti-parallel coiled-coil domains
ATPase
Head Domain
B
Cohesin
Condensin
SMC5/6 Complex
Smc3
Smc1
Smc4
Smc2
Smc6
Smc5

Figure 1-1. Smc Protein Structure. A. Smc proteins contain N-terminal and C-terminal domains that fold back onto each other generating an ATPase "head" domain, connected to a central "hinge" domain via a long coiled-coil region. Dimerization of Smc proteins is mediated by the hinge domain, which also binds DNA. B. Eukaryotes possesses at least six Smc family members that form three heterodimers, which further associate with a particular set of non-Smc subunits: Smc1 and Smc3 constitutes the core of the cohesin complexes; Smc2 and Smc4 function as central components of the condensin complexes, and the third complex is composed by the Smc5 and Smc6 heterodimer along with six non-Smc elements (Nse).

A


Smc3
Smc1
Scc1
Scc3
Pds5
Wapl

B


Stabilized cohesin
Unstable cohesin
Replisome
Eco1
Ac Ac
Cohesion-competent Cohesin
Smc3
Smc1
Scc1
Scc3
Pds5
Wapl

C

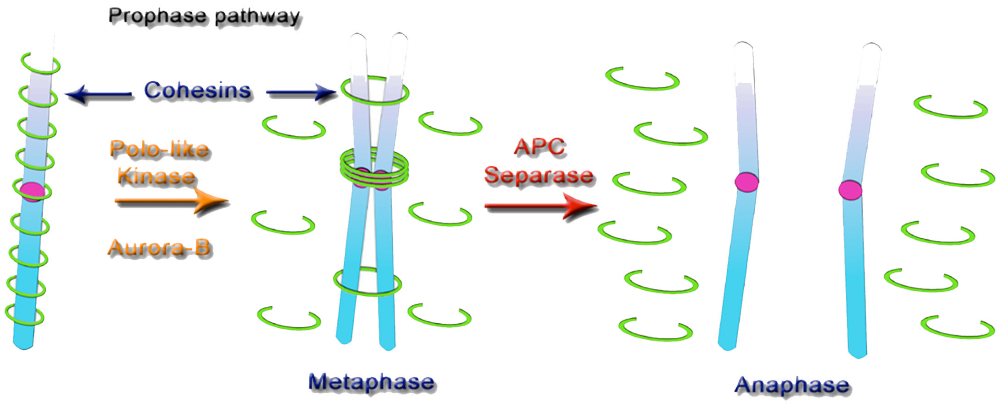

Prophase pathway
Cohesins
Polo-like Kinase
Aurora-B
APC
Separase
Metaphase
Anaphase

Figure 1-2. The cohesin complex. A. Cohesin consists of four essential subunits: Smc1 and Smc3, the kleisin RAD21/Scc1 and STAG/Scc3. The Smc1/3 heterodimer along with RAD21 form the ring-like structure. STAG protein interacts with RAD21 to complete the cohesin complex, and the cohesin regulators PDS5 and WAPL form a protein complex, which is associated to the cohesin complex by STAG interaction. B. Establishment of sister chromatid cohesion during DNA synthesis. The activity of Wapl to destabilize cohesin is counteracted by Eco1 (ESCO1 and ESCO2 in mammals). One possible model would be that the activity of Eco1 associates with the replication fork and thus Eco1 can acetylate the cohesin subunit Smc3 as the replisome pass through the cohesin ring. This acetylation serves as a "lock" function that confers stable association of cohesin to sister chromatids, thereby establishes sister chromatid cohesion. C. In vertebrates, the bulk of cohesin dissociates from chromosomes during prophase in a Polo-like kinase-dependent manner. A small amount of cohesin remains associated with metaphase chromosomes, predominantly at centromeres. At the metaphase-to-anaphase transition, the remaining fraction of chromatin-associated cohesin is cleaved by separase, which is removes cohesin completely from chromosomes. APC/C: anaphase-promoting complex.

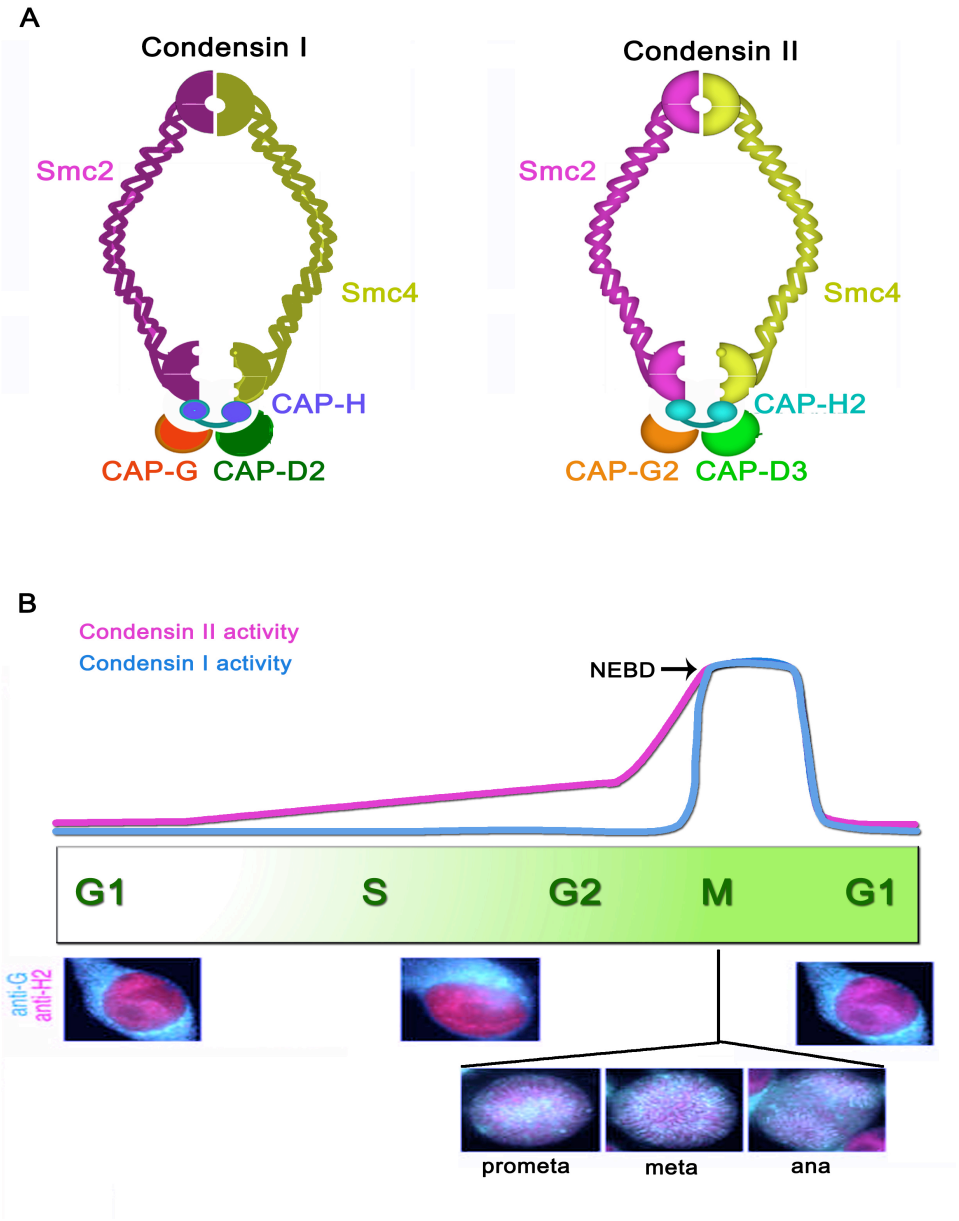


Figure 1-3. The condensin complex. A. Two paralogous condensin complexes — condensin I and condensin II — have been identified in many metazoans. The Smc2/4 heterodimer is present in both complexes but differ in the sets of non-SMC subunits. B. While condensin II is nuclear throughout interphase and mitotic prophase and is required for chromosome condensation in early prophase, condensin I localize to the cytoplasm in interphase and it associates with chromosomes only at the onset of prometaphase, when it is required for prometaphase and metaphase progression.

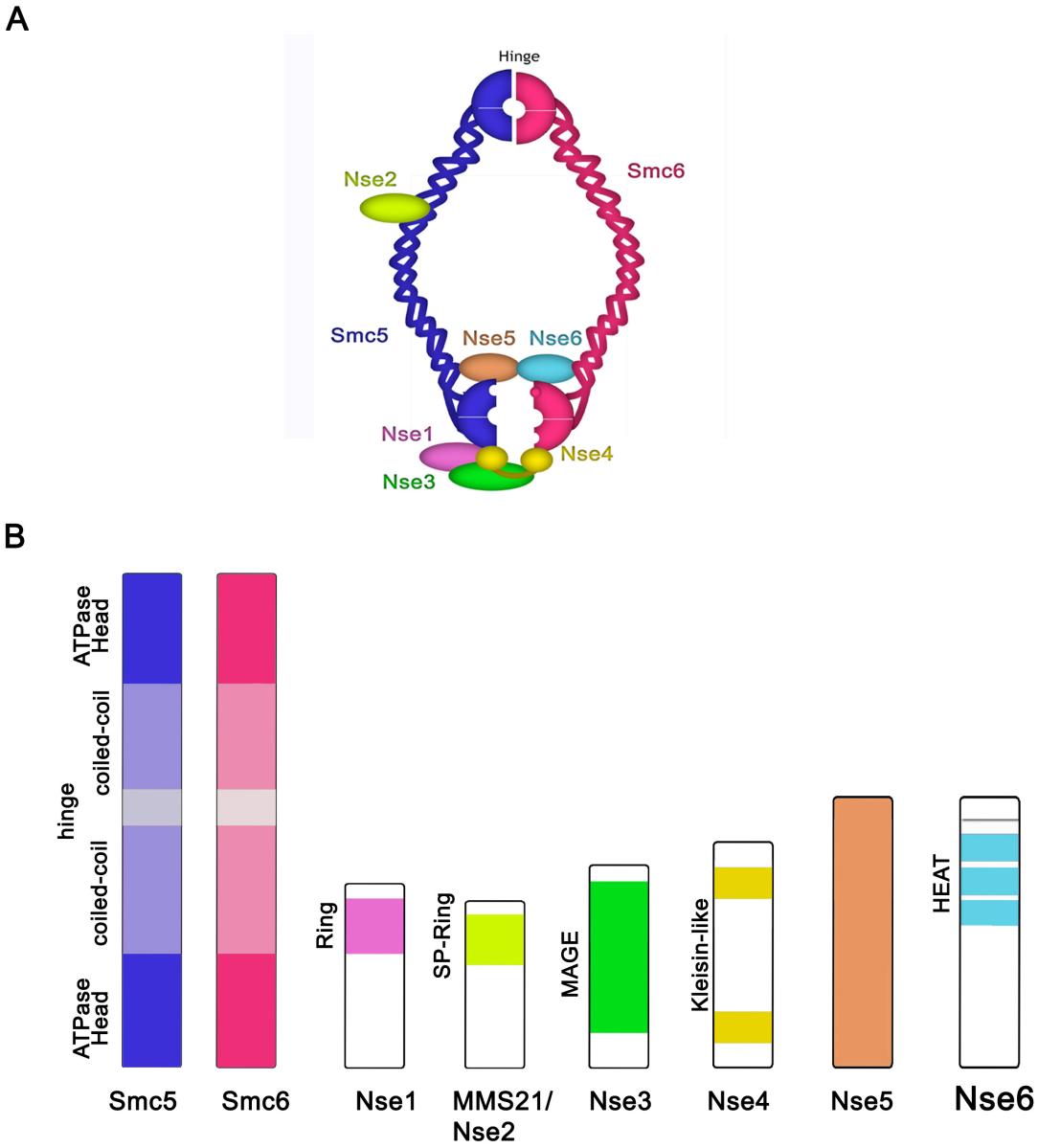


Figure 1-4. The Smc5/6 complex. A. The Smc5/6 complex contains eight subunits: structural maintenance of chromosomes (Smc)-5 and Smc6 plus six non-Smc element (Nse) subunits (Nse1–6). B. Structure of Smc5-6 subunits: The N- and C-terminal globular domains of each Smc subunit self-associate to generate an ATPase domain. Nse1 possesses a RING-finger ubiquitin E3 ligase. Nse2 contains 1 SP-RING-type zinc finger typical of the E3 small ubiquitin-related modifier (SUMO) ligases. Nse3 contains a MAGE (type II melanoma antigen) domain and Nse4 resembles a kleisin and may bridge the heads of Smc5 and Smc6. Nse5 and Nse6 (not present in vertebrate cells) also associate with the head domains, potentially forming a second bridge.

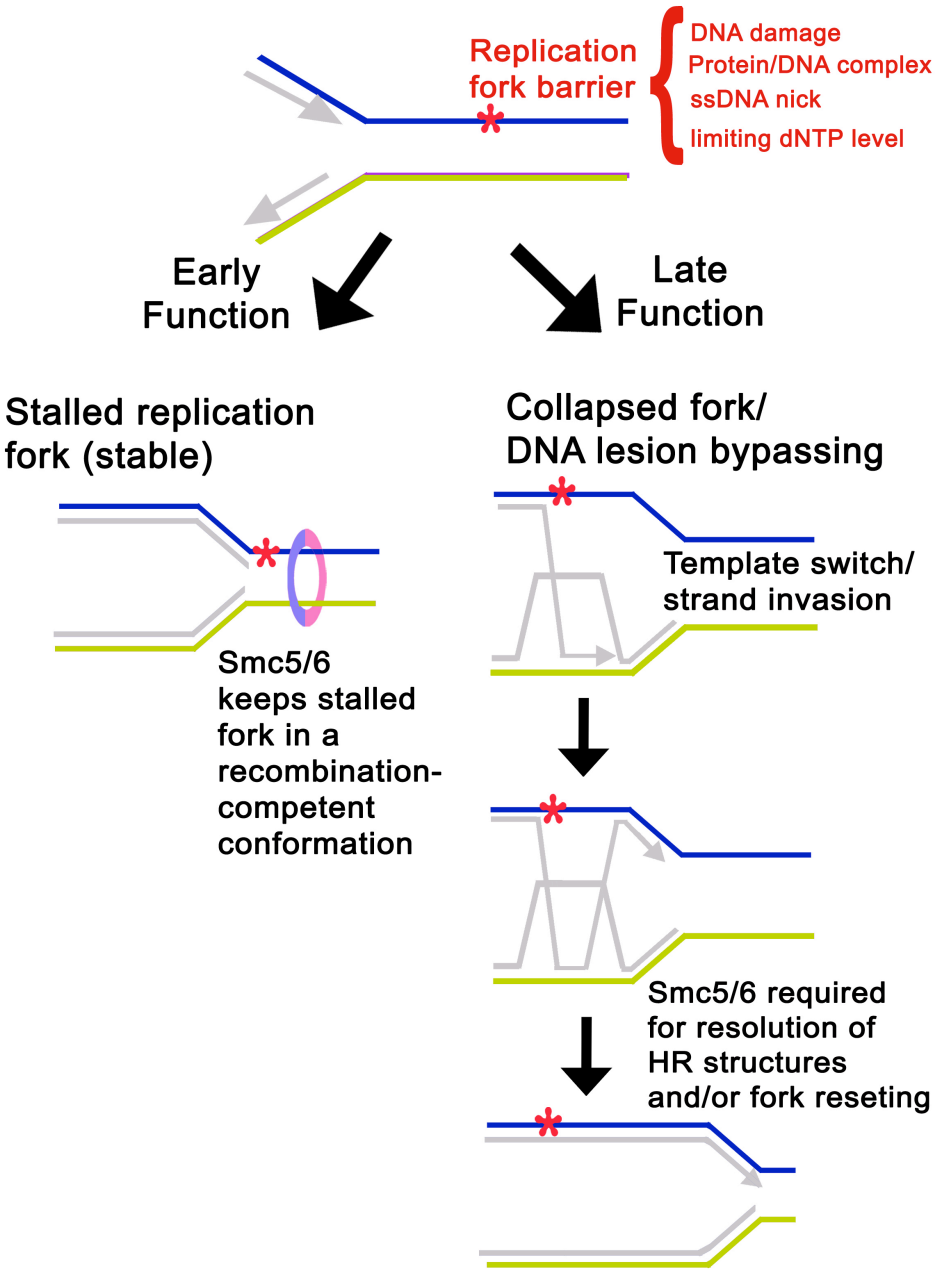


Figure 1-5. Dual function of the Smc5/6 complex in response to replication stress. Stabilized stalled replication forks are kept in a recombination-competent conformation by the Smc5/6 complex. This facilitates Rad52 association and replication restart (early function, left). Smc5/6 seems to be also required at a later stage for HR repair and/or restoration of collapsed replication forks and for bypassing DNA lesions (late function).

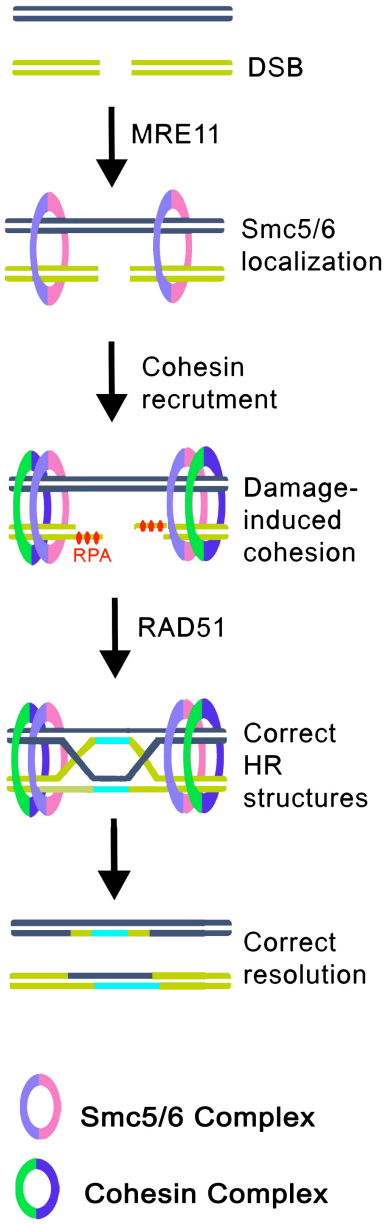


Figure 1-6. The Smc5/6 complex localizes to sites of DNA damage and promotes the recruitment of cohesin to the sites of double strand breaks (DSBs). This Smc5/6-mediated recruitment of cohesin to the DSB is crucial for holding the sister chromatids in close proximity to permit RAD51-dependent strand invasion and exchange using the sister chromatid as the repair template.

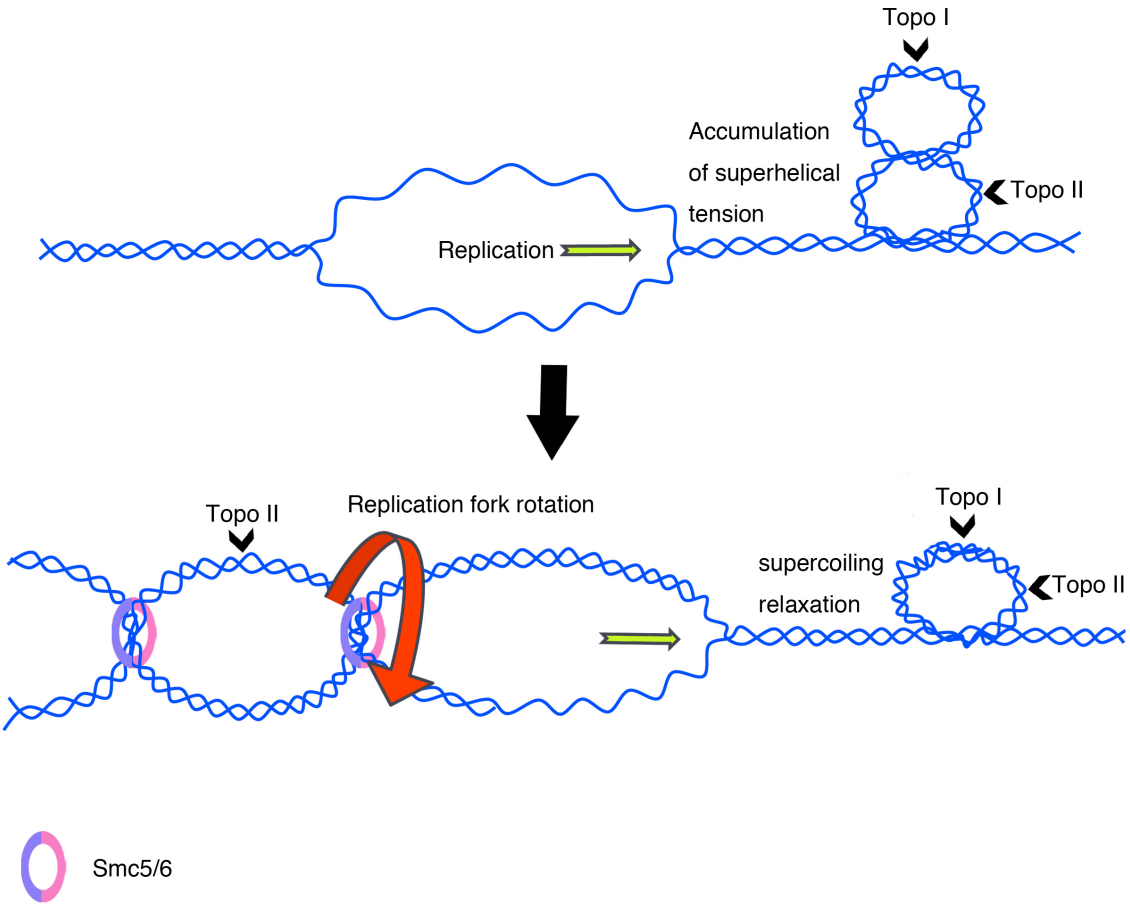


Figure 1-7. The movement of the fork generates positive supercoiling ahead of the replisome, where the topoisomerases (Top1 and Top2) resolve the DNA topological tension. A proposed strategy to avoid accumulation of supercoiled DNA ahead of the replication is that advancing fork could rotate with the turn of the helix. Fork rotation would then lead to pre-catenane formation behind the replication fork, which are detected and bound by Smc5/6 and in a subsequent step, Top2 can resolve them before mitosis.

CHAPTER II: SMC5/6 COMPLEX DYNAMICS DURING THE CELL CYCLE

INTRODUCTION

Accurate DNA replication and repair as well as faithful chromosome segregation are critical for safeguarding genome stability. Essential to these processes are the highly conserved structural maintenance of chromosomes (Smc) complexes, which regulate important aspects of chromosome architecture and organization during cell division. Smc complexes are composed of particular SMC protein heterodimers and a variety of non-SMC subunits. Smc1-Smc3 Cohesin is required for proper sister chromatid pairing until anaphase onset when bipolar attachment to the mitotic spindle is achieved, and is also involved in the DNA damage repair by homologous recombination during G2 phase (Michaelis et al., 1997; Sjogren and Nasmyth, 2001).

Smc2-Smc4 Condensin is essential for mitotic chromosome condensation, and appear as two distinct protein complexes, condensin I and condensin II in higher eukaryotes (Hirano et al., 1997; Ono et al., 2003). The third complex containing the Smc5-Smc6 heterodimer has been implicated in the DNA-damage response, as genetic analysis predominantly in fission and budding yeasts indicated that components of the Smc5/6 complex function in the homologous recombination repair pathway (McDonald et al., 2003; Harvey et al., 2004; Pebernard et al., 2004) and also participates in the

maintenance of DNA damage checkpoint signals (Verkade et al., 1999; Harvey et al., 2004).

Chromatin association pattern of Smc complexes is very dynamic and reveals the multiple roles of each complex in chromosome metabolism at the different stages of the cell cycle. In vertebrates, cohesin is loaded onto chromosomes during telophase and G1 following reformation of the nuclear envelope, and this binding is mediated by the Scc2 and Scc4 proteins (also called NippedBL/Mau2) (Losada et al., 1998; Darwiche et al., 1999; Sumara et al., 2000; Gerlich et al., 2006). During S phase, cohesion establishment occurs between replicated chromatids, and as cells progress from S phase to G2 phase, cohesin then slides away from the Scc2/4 complex to accumulate between genes that are transcribed in converging directions. Cohesin is maintained on chromosomes during G2 and then it is removed in mitosis by the prophase pathway and the cleavage of Scc1 by separase, a process required to allow sister chromatids separation in anaphase. Condensin I and condensin II complexes, which are present in mammalian cells show a different intracellular distribution and also a different timing in their association to chromosomes in mitosis. While condensin II is nuclear throughout interphase and mitotic prophase thus participating in an early stage of chromosome condensation within the prophase nucleus, condensin I is cytoplasmic in interphase, and its association with chromosomes occurs only after the nuclear envelope breaks down in prometaphase. After nuclear envelope breakdown (NEBD), condensins I and II cooperatively support mitotic chromosome assembly and proper sister chromatids

resolution before metaphase in order to ensure faithful segregation in anaphase.

Much less is known, however, about the behavior of the Smc5/6 complex during the cell cycle. An initial immunofluorescence analysis indicated that Smc6 protein was enriched at repetitive sequences like telomeres and ribosomal DNA in budding yeast (Torres-Rosell et al., 2005). Subsequent analyses of the chromosomal association pattern by ChIP-chip and ChIP-Seq (Lindroos et al., 2006) have revealed that the bulk of Smc5/6 is absent from G1 chromosomes except for the telomeric regions where the protein showed a modest enrichment. In cells arrested in S phase by hydroxyurea treatment (HU), the complex appeared enriched around early replicating origins, indicating that the complex is deposited on chromosomes as soon as replication occurs, in a manner that was suggest to be dependent on Scc2/4 as in the case of cohesin. Smc5/6 is most abundant on chromosomes in G2/M, showing an enrichment at the centromeric regions, and Smc6 peaks were mainly detected in intergenic regions, resembling the chromosomal localization of Cohesin (Glynn et al., 2004; Lengronne et al., 2004). Interestingly, the frequency of arm interaction sites increased as the length of the chromosomes increased, which has the potential to increase the number of sister chromatid intertwinings (SCIs). Thus, the length of chromosomes influences the need of superhelical tension release in budding yeast, and Smc5/6 was proposed to be required to reduce the accumulation of positive supercoils ahead of the replication machinery by assisting fork rotation through sequestration of sister chromatid intertwinings. Once

chromosome separation has occurred, Sm5/6 chromatin association goes back to G1-levels in telophase (Kegel et al., 2011).

ChIP-chip analysis in fission yeast indicated that Smc5/6 is transiently enriched at the heterochromatic outer repeats of centromeres in an H3-K9 methylation-dependent manner following hydroxyurea treatment, and during an unperturbed S phase, while methyl methanesulfonate (MMS) treatment induced the accumulation of Smc5/6 at subtelomeres, in an Nse2 SUMO ligase-dependent, but H3-K9 methylation-independent manner. Moreover, it was shown that Smc5/6 loads at all genomic tDNAs, and that the consensus TFIIIC-binding sites in the tDNAs is required for Smc5/6 binding (Pebernard, et al., 2008).

In human cells, early analysis on Smc5/6 localization showed that human Smc5 and Smc6 are nuclear proteins partially associating with DNA (Lehmann et al., 2001). In ALT cancer cell lines (which use telomere homologous recombination (HR) to elongate telomeres) Smc5/6 complex localizes to the pole bodies and is required for targeting telomeres to APBs. Telomere-binding proteins TRF1 and TRF2 are SUMOylated by Nse2/MMS21, a process that is required for APB formation. Smc5/6 depletion causes inhibition of the homologous recombination pathway, generating telomere shortening and senescence in ALT cells (Potts and Yu, 2007).

Here we present a detailed examination of the intracellular localization of human Smc5

and Smc6 during the cell cycle. Time-course analysis by chromatin fractionation, immunofluorescence, and live-cell imaging in RPE-1 (Human retinal pigment epithelial cell line) and HeLa cell lines indicated that these proteins associate with chromatin during interphase and are then significantly removed from the nucleus once chromosome condensation took place, revealing a similar pattern to that observed previously for the mammalian cohesin complex.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody was raised against the peptide sequence MATPSKKTSTPSPQPSKRALPRDPSSEVPC of Smc5 (Monoclonal Antibody Research Institute, Sapporo, Japan) and then purified using a protein G affinity column (MabTrap Kit; Amersham Biosciences, Piscataway, NJ). Other antibodies used were as follows: SMC6L1 (M01, Clone 2E6; Abnova, Taiwan), SMC5 (18038, Abcam, Cambridge, UK), Cyclin B (610219; BD Pharmingen, Lexington, KY), GFP (290, Abcam, Cambridge, UK), DNA topoisomerase IIα (74715; Abcam, Cambridge, UK), α-tubulin (B512; Sigma, St. Louis, MO), and phospho-Histone H3 (S10) (9701; Cell Signaling Technology Inc., USA).

Cell synchronization, inhibitor treatment, and cell lines

HeLa and RPE-1 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 environment. HeLa cells were synchronized by double thymidine arrest (24 h in the presence of 2 mM thymidine, 8 h release, 12 h in the presence of 2 mM thymidine) and were collected every 3 h after the second release for cell cycle progression analysis.

For G2/M-phase arrest, asynchronous RPE-1 cells were treated with 0,9 mM RO-3306 (Enzo Life Sciences) for 24 h. For enrichment of cells in prometaphase, 50 ng/ml nocodazole was added 7 h after release from the second thymidine block for 1 or 2 h; RPE-1 cells were treated with 15 mM STLC (TCI, Tokyo, Japan) for 2 h after release from RO-3306 arrest or 31 h after release from serum starvation for 2 or 3 h, and then recovered by mitotic shake off. The HeLa cell line stably expressing EGFP-tagged Smc5 protein was generated by fusing EGFP in frame with the C terminus of Smc5 in a bacterial artificial chromosome vector. The plasmid DNA was purified and transfected into HeLa cells using the FuGENE 6 reagent (Promega, Madison, WI). Stably expressing cell clones were selected in a complete medium containing G418, and expression of the tagged transgene was verified by fluorescence microscopy and immunoblotting.

Generation of Kyoto HeLa cells expressing EGFP-Smc5

LB medium supplemented with chloramphenicol (15 μg/ml) was used to inoculate with *E. coli* culture. After culturing for additional 2 h at 37°C with shaking at 1000 rpm, cells are centrifuge for 30 sec at 11,000 rpm in a cooled microfuge centrifuge and the pellet was resuspended with chilled ddH2O, mixed with 0.75ug of Red/ET Recombination protein expression plasmid pRedET and electroporated at 1350 V, 10 F, 600 Ohms. After resuspension into LB media, the sample was incubated at 30°C for 70 min, shaking at 1000 rpm. Cells were then plated on LB agar plates containing Tetracycline

(3 μg/ml) plus Chloramphenicol (15 μg/ml) for the BAC, and incubated at 30°C for at least 15 h. the linear DNA fragment containing the EGFP-neo cassette was introduced into cells containing the Red/ET Recombination protein expression plasmid by electroporation and recombination. The cultures were then streaked onto LB agar plates containing Chloramphenicol (15 μg/ml), Kanamycin (15 μg/ml) and Tetracycline (3μg/ml). Transformed colonies were used to replicate and purify the DNA sequence EGFP-Smc5 BAC transgene, which was used for transfection into HeLa Kyoto cells, and positively expressing EGFP-Smc5 colonies were confirmed by EGFP fluorescence microscopy and western blotting.

Chromatin fractionation

To isolate chromatin, cells were resuspended (4×10^7 cells/ml) in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 0.5 mg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride). Triton X-100 (0.1%) was added, and the cells were incubated for 5 min on ice. Nuclei were pelleted by low-speed centrifugation (5 min, 1590 G, 4°C). The supernatant containing the cytoplasmic fraction was further clarified by high-speed centrifugation (15 min, 3977 G, 4°C) to remove cell debris and insoluble aggregates. Nuclei were washed once and then resuspended in buffer A containing Triton X-100 (0.1%) and analyzed by immunoblotting.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with ice-chilled 100% methanol for 20 min at −20°C or with 4% paraformaldehyde in 0.137 M sodium phosphate buffer (pH 7.4). Fixed cells/chromosomes were permeabilized with 0.5% Triton X-100 in PBS and incubated with 3% bovine serum albumin in PBS for at least 1 h. Cells were incubated with the primary antibodies overnight at room temperature followed by incubation with secondary antibodies for 35 min together with 0.1 ìg/ml 4,6-diamidino-2-phenylindole (DAPI). The secondary antibodies used in this study were Alexa Fluor 488 and 568 goat anti–rabbit IgG, Alexa Fluor 488 and 568 goat anti-mouse, and Alexa Fluor 568 goat anti–human IgG (Molecular Probes, Carlsbad, CA). Cells were washed twice and mounted in Prolong Gold anti-fade mounting reagent (Invitrogen). Images were acquired on a Zeiss Imager Z.1 microscope equipped with epifluorescence and a Cool Snap HQ CCD camera (Photometrics Ltd, Tucson, AZ). 3D projections of the Immunofluorescence images were carried out using the 3D Volume viewer tool in ImageJ software (Wayne Rasband, NIH).

Live-cell imaging analysis

Cells were placed in CO_2-independent medium without phenol red (Gibco-BRL, Grand Island, NY) on chambered coverslips (Lab-TekTM, Nunc, Rochester, NY), and the chamber lids were sealed with silicone grease. Images were captured every 3 min, with

100-ms exposure times, through a 100×/1.40 NA Plan Apochromat oil objective lens mounted on an inverted microscope (IX-71; Olympus, Tokyo, Japan) equipped with a Cool Snap HQ CCD camera. A series of projected images of three *z*-sections with 5.0-mm intervals were analyzed. For data analysis, obtained images were processed using ImageJ software (Wayne Rasband, NIH).

Immunoprecipitation

Asynchronized HeLa cells expressing EGFP-tagged Smc5 were lysed in immunoprecipitation buffer (20mM Tris-HCl at pH 7.5, 150 mM NaCl, 20 mM β-glycerophosphate, 5 mM $MgCl_2$, 0.1% NP-40, protease inhibitors [Complete Mini EDTA-free, Roche], 1 mM DTT), supplemented with 100 nM okadaic acid, 2 mM Na_3VO_4, 10 mM NaF, and 0.25 U/L benzonase nuclease (Novagen), for 20 min on ice. Cell extracts, after removing the insoluble fraction by centrifugation at 15,000 rpm for 30 min at 4°C, were used for immunoprecipitation. 10 μL of protein A (Bio-Rad) beads coupled to antibodies were incubated with cell extracts for about 2 h at 4°C, then washed three times with immunoprecipitation buffer and three times with 0.05% TBS-Tween20.

Chromatin IP

Cells were cross-linked with 1% formaldehyde for 10 min, quenched with 125 mM glycine, and prepared for ChIP as previously described (Wendt et al., 2008). ChIP was

performed as previously described using SMC6L1 (M01, Cl7one 2E6; Abnova, Taiwan) (Wendt et al., 2008). In brief, cross-linked cell lysates solubilized by sonication were incubated with protein A Dynabeads (Dynal), cross-linked with the antibodies for 14 h at 4 °C. After this, beads were washed several times and eluted with elution buffer (50 mM Tris, 10 mM EDTA and 1% SDS) for 20 min at 65 °C. The eluates were incubated at 65 °C overnight to reverse crosslinks and then treated with RNaseA and then with proteinase K. The samples were further purified by phenol–chloroform extraction and an extra purification step using a PCR purification kit (Qiagen).

RESULTS

Cell cycle localization pattern of human Smc5 and Smc6 by Immunofluorescence and Chromatin Fractionation

To examine the behavior of the Smc5/6 complex during the cell cycle, we initially analyzed the immunostaining pattern of Smc5 and Smc6 in fixed asynchronous RPE-1 cells (Figure 2-1). Both proteins appeared mainly associated with the nucleus during interphase. Smc5/6 nuclear staining included the nucleoli compartment in a subset of cells and in addition, one of the commercial monoclonal antibody against Smc6 was also able to detect a foci pattern in some interphase nuclei, corresponding to cells transiting G1 phase based on their negative nuclear staining of topo IIα which is a marker of nuclei containing replicated chromatin (Figure 2-2, B).

However, in cells transiting mitosis, the Smc5 and Smc6 signals vastly dissociated from chromatin until late telophase/early G1 phase, when their nuclear staining was visible again. When pre-extraction with PBS-Triton X-100 was carried out in immunofluorescence analysis, the nuclear association of Smc5 during interphase and its dissociation during mitosis was clearly observed (Figure 2-2, A). This cell cycle behavior of Smc5/6 mainly coincides with that of the Smc3's cohesin complex, while it differs from that of the Smc2's condensin complexes (Figure 2-3, A).

In addition, RPE-1 cells arrested at G2 phase by treatment with an ATP-competitive inhibitor of Cdk1, RO-3306, were subjected to chromatin fractionation and western blot. This assay confirmed that Smc5 and Smc6 associated with chromatin during G2 phase with two independent antibodies for each protein (Figure 2-4, A). However, when the cells were allowed to proceed to mitosis and were then arrested at mitosis by further incubation in medium containing STLC, a potent inhibitor of Eg5 that causes monopolar spindles (DeBonis et al., 2004), the majority of both Smc5 and Smc6 proteins moved to the cytoplasmic fraction.

Intriguingly, in both HeLa and RPE-1 western blots, one of the commercial polyclonal antibody against Smc5 (18038, Abcam) was able to detect an slight band-shift in the gel mobility of the protein, and in the case of Smc6, one of the commercial monoclonal antibody (M01, Cl7one 2E6; Abnova) showed reduced band intensity in western blotting in total lysates, as well as in the chromatin-bound and cytoplasmic fractions in mitotic extracts.

When cells arrested in mitosis by nocodazole treatment were subjected to western blot in a time course analysis after release from mitotic arrest, the commercial antibody was able to detect the Smc6 protein again, indicating that the reduced band intensity is specific to the mitotic phase (Figure 2-4, B). We considered that the inability

of this antibody to detect Smc6 could be due to a possible mitotic degradation of the protein, or conversely, due to a mitotic-specific modification such as phosphorylation by mitotic kinases that could affect the antibody-epitope interaction. However, the reduction in band intensity of Smc6 observed with this commercial antibody was not rescued by treating cells with Plk1 or Aurora B inhibitors, neither by treatment with the MG132 inhibitor which blocks proteasome-mediated protein degradation (Figure 2-4, C and D).

Based on these results, and the fact that the reduction of band intensity is only observed with one specific antibody (Figure 2-4, A) it is conceivable that the reduction in band intensity observed with this antibody could represent another type of modification during mitosis (acetylation, SUMOylation, etc.) that may affect the epitope recognition by the antibody during this stage.

Importantly, the Scc2/4 complex (Nipbl/Mau-2 in humans), which is the loading factor of cohesin, has been also proposed to be the loading factor of Smc5/6 complex in budding yeast (Lindroos et al., 2006). However, depletion of Nipbl by siRNA treatment, did not induce any change in the chromatin association pattern of Smc5/6 in HeLa cells (Figure 2-3, C), indicating that the binding of Smc5/6 to chromosomes may be differentially regulated in human cells.

Live-cell Imaging Analysis of HeLa cells stably expressing EGFP-Smc5 protein.

In order to monitor the cell cycle behavior of Smc5/6 in more detail, we generated HeLa cells stably expressing EGFP-tagged Smc5 protein (Figure 2-5, A). Human BAC Smc5 transgene was modified by homologous recombination-mediated introduction of the EGFP sequence into the C-term of the gene. After confirmation by DNA sequencing, BAC DNA fragments were purified from *E. coli* colonies and transfected into Kyoto HeLa cells. Subsequently, cells stably expressing EGFP-tagged Smc5 protein were identified by EGFP fluorescence microscopy and western blotting, and analyzed by live-cell imaging (Figure 2-6, A). The expression of the EGFP-Smc5 protein was comparable to the endogenous protein, and its physiological activity was confirmed by immunoprecipitation, which revealed its interaction with the endogenous Smc6 (Figure 2-5, B and C).

EGFP-Smc5 showed constant nuclear association during interphase, dispersion through the cell immediately after nuclear envelope breakdown, and re-enrichment in the nucleus at the beginning of G1 phase. Chromatin fractionation of EGFP-Smc5 HeLa cells at different time points after release from double thymidine block, also indicated a reduction in the nuclear association of Smc5/6 in cells transiting mitosis as in the case of endogenous Smc6 (Figure 2-6, B). When cells were analyzed by immunofluorescence using a GFP antibody, we were able to detect a very slight

signal on mitotic chromosomes, which may correspond to the minor accumulation of Smc5/6 detected by the different antibodies used to detect Smc5/6 in the chromatin fraction by western blotting (Figure 2-7).

Together, these results suggest that the function of the Smc5/6 complex in chromatin dynamics may be primarily required during interphase, in a pattern similar to that observed for cohesin complex in human cells.

DISCUSION

We have established that, in human cells, Smc5/6 associates with chromatin during interphase but it is then removed extensively from the nucleus in mitosis. Our immunostaining analysis of endogenous Smc5 and Smc6 proteins, as well as the live cell imaging using EGFP-tagged Smc5 indicated that the dissociation of a vast proportion of the complex from the chromatin is rapid and occurs soon after the nuclear envelope breakdown (NEBD) occurs. EGFP-Smc5 was then recovered within the nuclei of daughter cells at telophase/early G1 phase, similar to what is observed for the human cohesin complex.

Smc5 and Smc6 Immunofluorescence indicated that both proteins accumulate at the nucleolus in many interphase cells, and the association of Smc6 to the ribosomal DNA was further confirmed by Smc6 ChIP-qPCR. These results are consistent to previous studies indicating that Smc5/6 chromatin binding is enriched at the rDNA sequences in yeast (Stephan et al., 2011; Ampatzidou et al., 2006; Pebernard et al., 2008). Moreover, the immunostaining of Smc6 using the Abnova's Smc6 monoclonal antibody revealed that some cells also presented a foci staining pattern, which may correspond to the G1 phase based on the negative staining of these nuclei by topoisomerase IIα, which associates to replicated chromatin. It has been reported that an antibody that recognizes a phosphorylated form of Smc6 was also able to detect a

foci-like pattern of the protein in immunofluorescence analysis, and that these foci colocalize with those observed for the SC35 splicing factor (Taylor et al., 2001). It will be important in the future to clarify the nature of this Smc6 foci formation during interphase, and whether they actually represent the sites were the pre-mRNA splicing occurs.

The cell cycle behavior of EGFP-Smc5 was also observed in a time-course chromatin fractionation, and confirmed in RPE-1 cells enriched at G2 phase and mitosis subjected to chromatin fraction. In both cases, Smc5 and Smc6 proteins were displaced from the nuclear fraction, in mitotically enriched samples,. Interestingly, only the commercial polyclonal antibody against Smc5 (Abcam) was able to detect a slight bandshift of the protein in mitotically enriched samples, indicating a mitosis-specific modification of the protein, although further analysis will be required to define the nature and the mediators of this modification.

Although Smc6 became hardly detectable in mitotic-enriched sample in immunoblotting by using the Abnova's monoclonal antibody, the second commercial available monoclonal antibody (Santa Cruz) did not detect such a reduction in the protein levels. Moreover, it is unlikely that Smc6 alone become unstable during mitosis, because the stability of Smc5 and Smc6 was found to be interdependent and a proteasome inhibitor MG132 did not restore the band intensity. A possible explanation for the effect observed using this particular antibody would be that a mitosis-specific modification of

Smc6 hinders the antibody access.

Although our immunofluorescence analysis was unable to detect any signal of Smc5 or Smc6 proteins within condensed chromosomes, it should be noted that low levels of the both proteins are still detectable by western blot in the nuclear fraction of mitotic samples, and the live-cell imaging of EGFP-Smc5 showed a dispersion of the protein throughout the cell, without a conspicuous exclusion from the nucleus. Thus, we can not rule out the possibility that this minor proportion of Smc5/6 accounts for a particular nuclear function of the complex in mitosis which, nevertheless, do not represent the major role in chromatin dynamics predicted to take place during interphase.

We have shown that the behavior of Smc5/6 during the cell cycle in human cells differs from that observed in other species such as budding and fission yeast or in *Xenopus* (Lindroos et al., 2006; Tsuyama et al., 2006). The fact that the complex associates with chromatin already in early G1 phase implies that the Smc5/6 complex is differentially regulated in human cells and has probably acquired additional functions before DNA replication. Interestingly, the localization pattern of Smc5/6 resembles that of cohesin complex and thus, in light of cohesin's function before S phase (Wendt et al., 2008), Smc5/6 might be also involved in the regulation of chromatin structure required to control gene expression. To find out the prospective function of Smc5/6 in G1 phase, and how might cohesin and Smc5/6 coordinate to shape chromatin await further investigations.

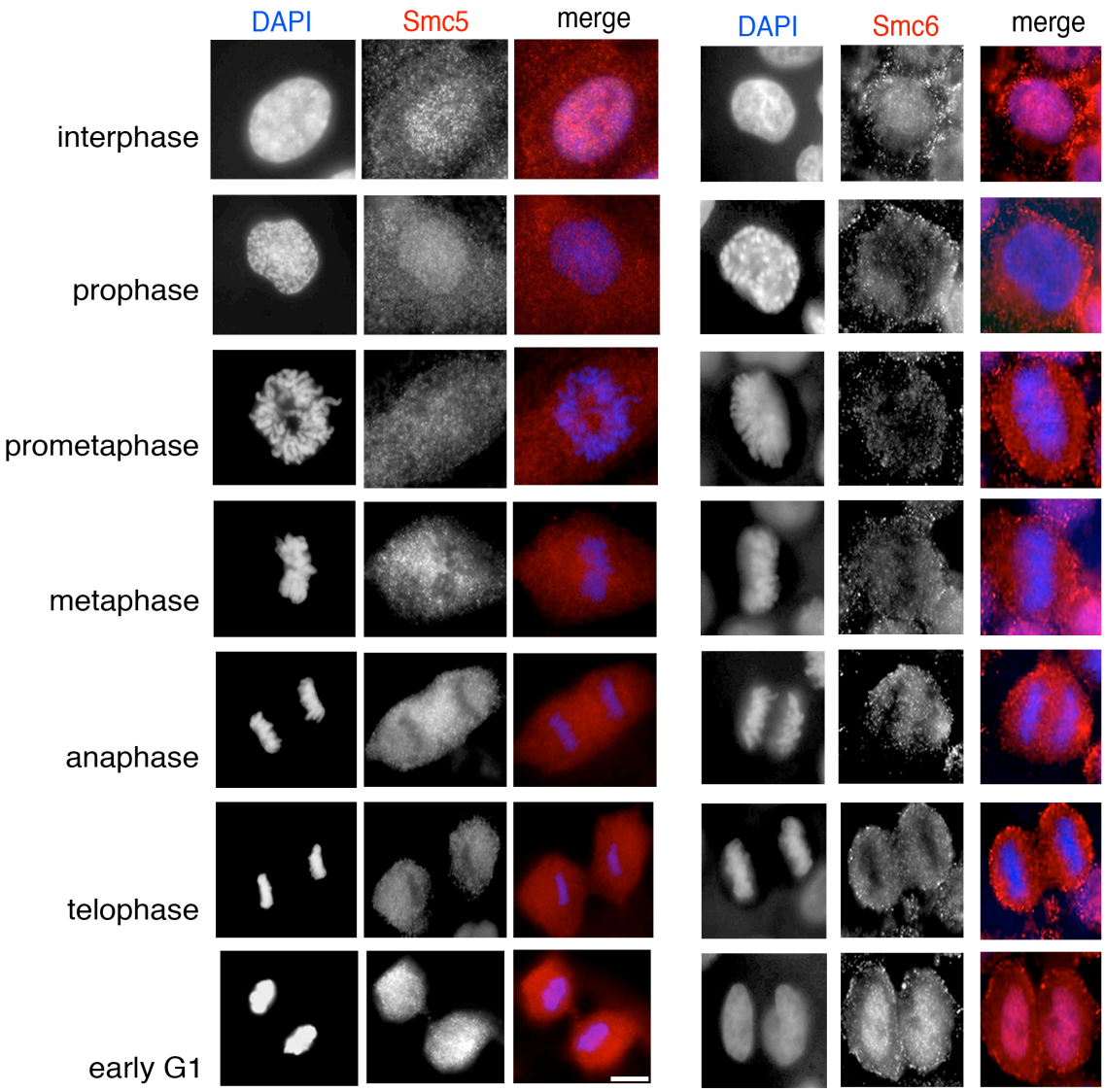


Figure 2-1. Chromatin association of Smc5 and Smc6 during the cell cycle. RPE-1 cells were fixed with 4% paraformaldehyde and stained with Smc5 or smc6 antibody (red). DNA was stained with DAPI (blue). Chromatin staining of Smc5 and Smc6 is absent from prophase, but it is recovered in the nuclei of daughter cells at late telophase/G1. Bar = 10 μm.

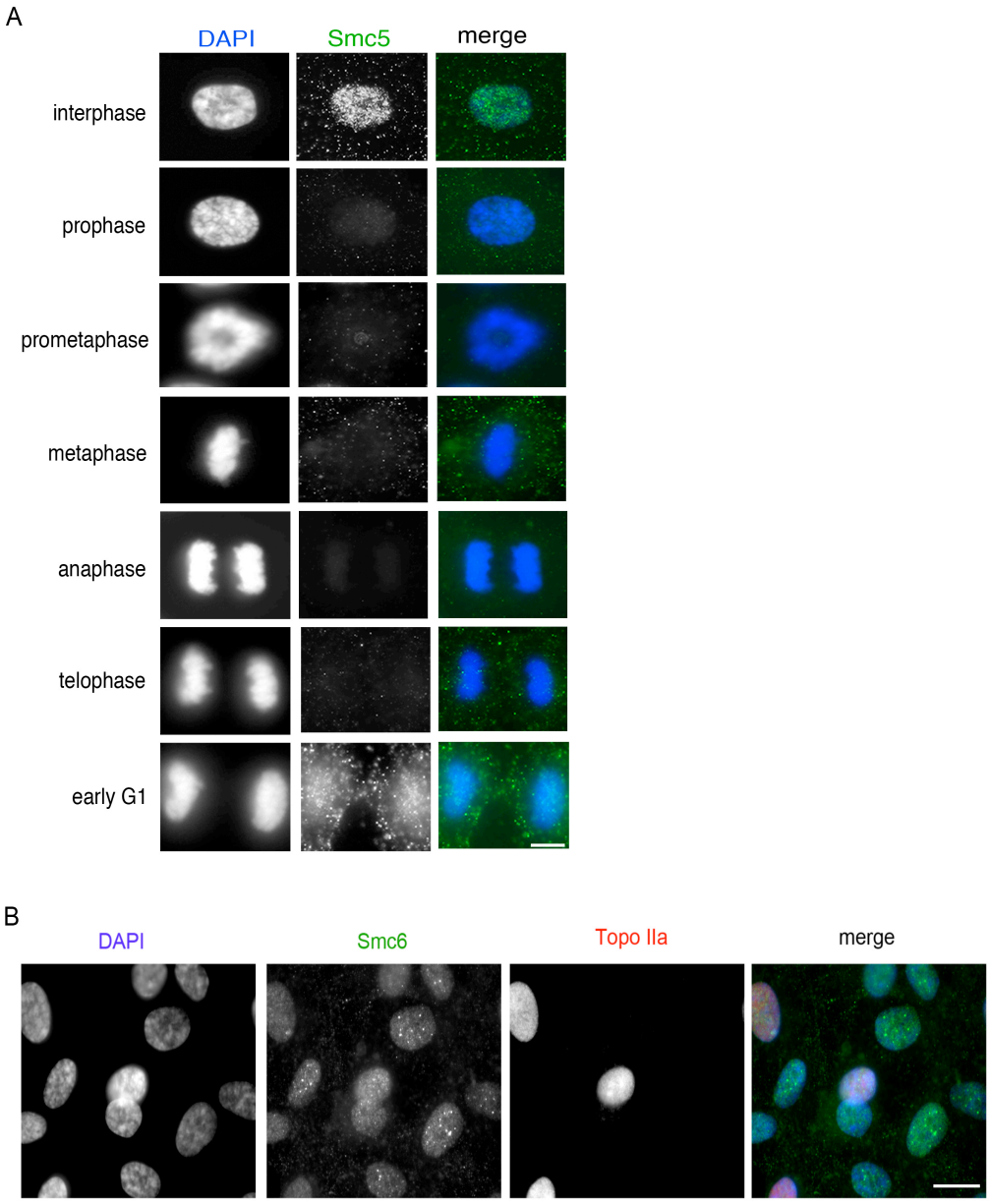


Figure 2-2. A. RPE-1 cells were subjected to pre-extraction treatment with PBS-Triton X100 (0,2%) and then fixed with 4% paraformaldehyde and stained with Smc5 antibody (green) and DNA was stained with DAPI (blue). These conditions allowed a clear observation of the dissociation of the protein from chromatin during mitosis. Bar = 10 μm. B. Immunostaining of Smc6 in asynchronized cell culture using the monoclonal commercial antibody (Abnova, Green) reveals a foci pattern in cells transiting G1 phase, based on the negative staining of topo IIα (red). DNA was stained with DAPI (blue). Bar = 20 μm.

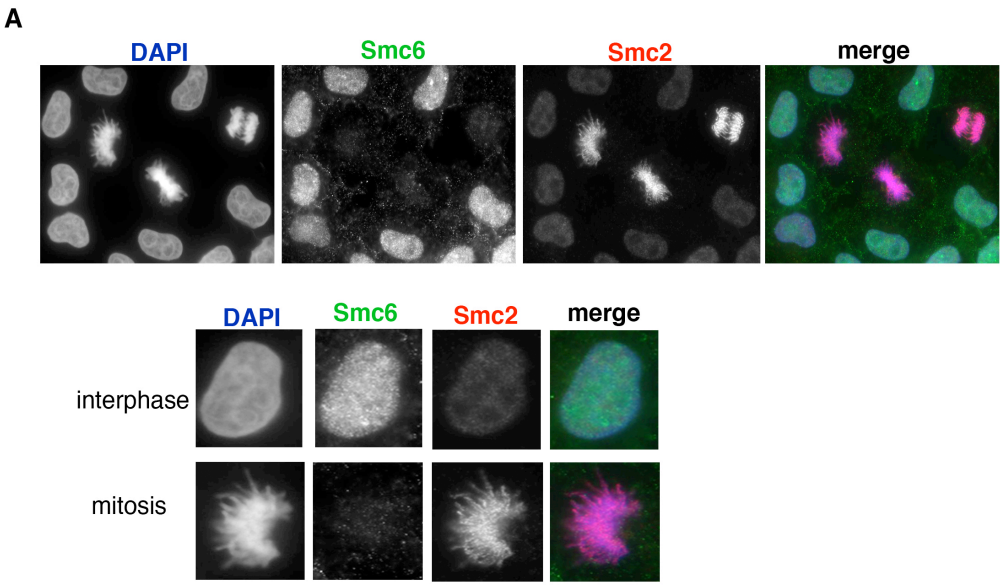

A
DAPI
Smc6
Smc2
merge
DAPI
Smc6
Smc2
merge
interphase
mitosis
B
DAPI
Smc6
Smc3
merge
DAPI
Smc6
Smc3
merge
interphase
mitosis
C
TOTAL
CYT
NUC
NIPBL siRNA
+
-
+
-
+
-
NPBL
Smc6
Tubulin

Figure 2-3. Comparative chromatin association of Smc complexes. A. Asynchronized HeLa cells were subjected to immunofluorescence for condensin's Smc2 immunofluorescence (red) and Smc6 (green). B. Same conditions of A but Smc6 protein localization is compared to that of cohesin's Smc3 (red). DNA was stained with DAPI (blue). C. Chromatin fractionation assay in HeLa cells depleted of the cohesin loading factor NPBL. Note that Smc6 nuclear localization is not affected by the absence of NPBL indicating that this protein do not participate in the loading of the Smc5/6 complex as proposed in budding yeast.

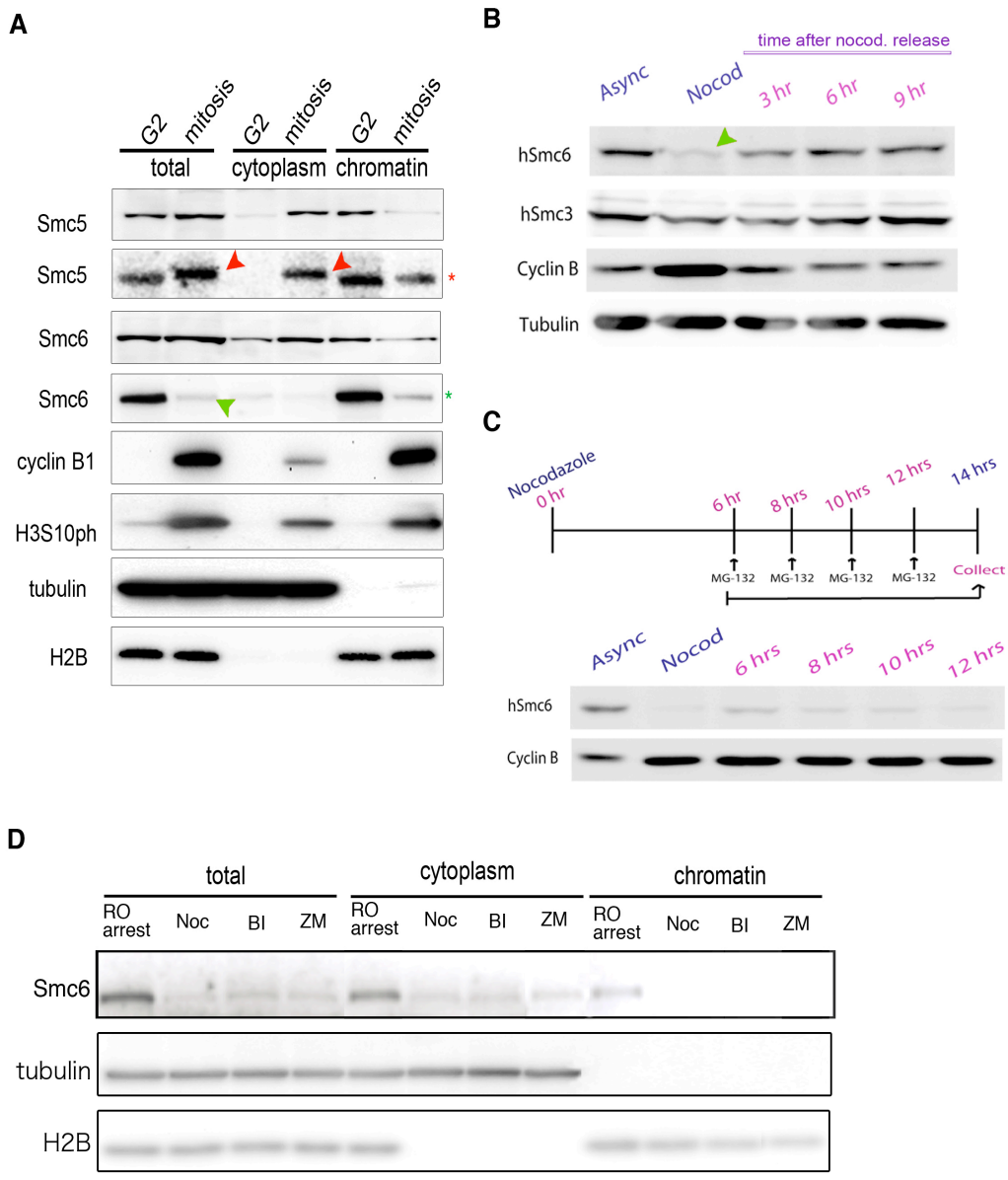
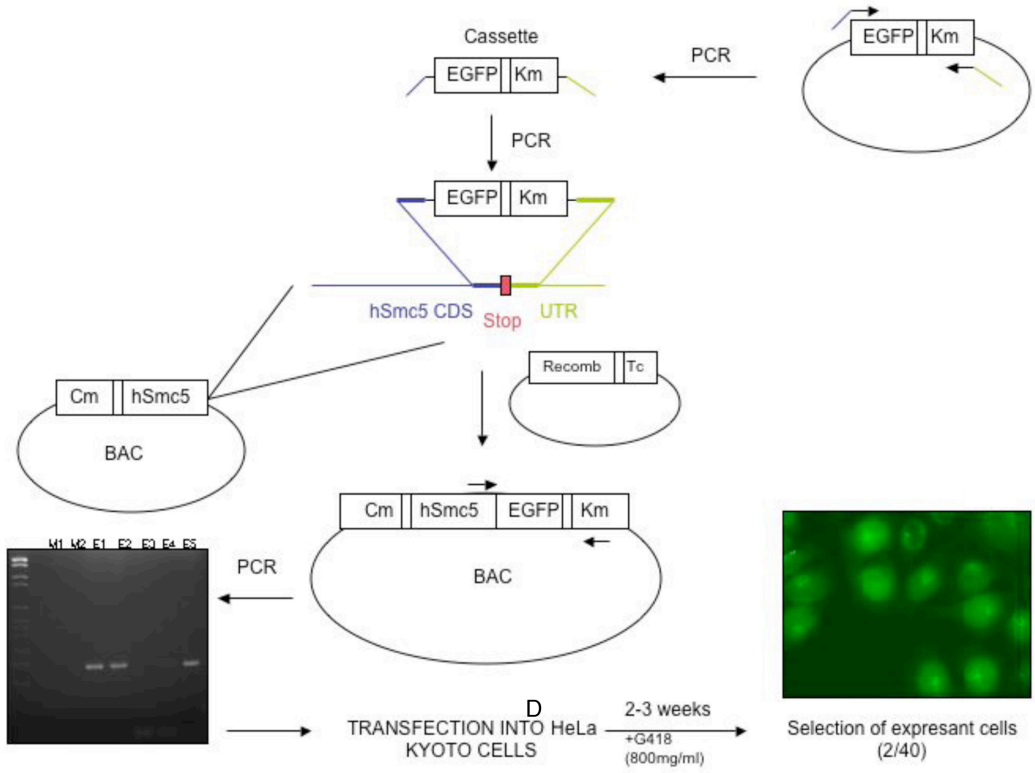


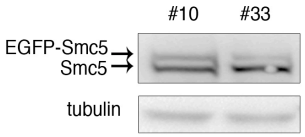
Figure 2-4. Analysis of Smc5 and Smc6 proteins by immunoblotting. A. RPE-1 cells arrested at G2 by a 24-h treatment with RO-3306 and a culture that was enriched for cells in mitosis by *S*-trityl-L-cysteine (STLC) treatment for 2 h after release from G2 arrest were subjected to chromatin fractionation and immunoblotting. Expression of cyclin B1 and phosphorylation of H3S10 were used as markers for mitosis. Red and green asterisks indicate the detection pattern using Abcam antibody against Smc5 and Abnova antibody against Smc6 respectively. Arrowheads point the mitotic band-shift in Smc5 and the decrease in band intensity in Smc6 detected with the above antibodies.

B. Recovery of Smc6 band intensity detected by Abnova Smc6 antibody after release from mitotic arrest by nocodazole treatment in HeLa cells. C. HeLa cells arrested in mitosis by nocodazole treatment where then treated with MG-132 in order to stop proteasome-dependent protein degradation. D. Chromatin fractionation assay in HeLa cells arrested at G2 by 4 hours treatment with RO-3306 6 hours after release from double thymidine block. The band intensity of Smc6 was compared to that observed in nocodazole-arrested cells, and nocodazole-arrested cells in the presence of BI 811283 or ZM 447439, Aurora B and Plk1 inhibitor, respectively.

A


Cassette
EGFP
Km
PCR
PCR
EGFP
Km
hSmc5 CDS
Stop
UTR
Recomb
Tc
Cm
hSmc5
BAC
Cm
hSmc5
EGFP
Km
BAC
PCR
D
TRANSFECTION INTO HeLa
KYOTO CELLS
2-3 weeks
+G418
(800mg/ml)
Selection of expresant cells
(2/40)

B


#10
#33
EGFP-Smc5
Smc5
tubulin

C

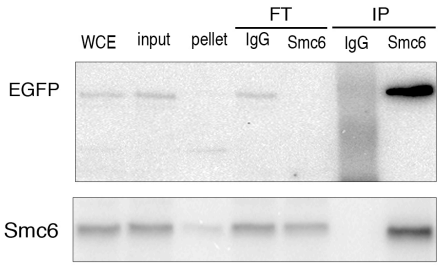
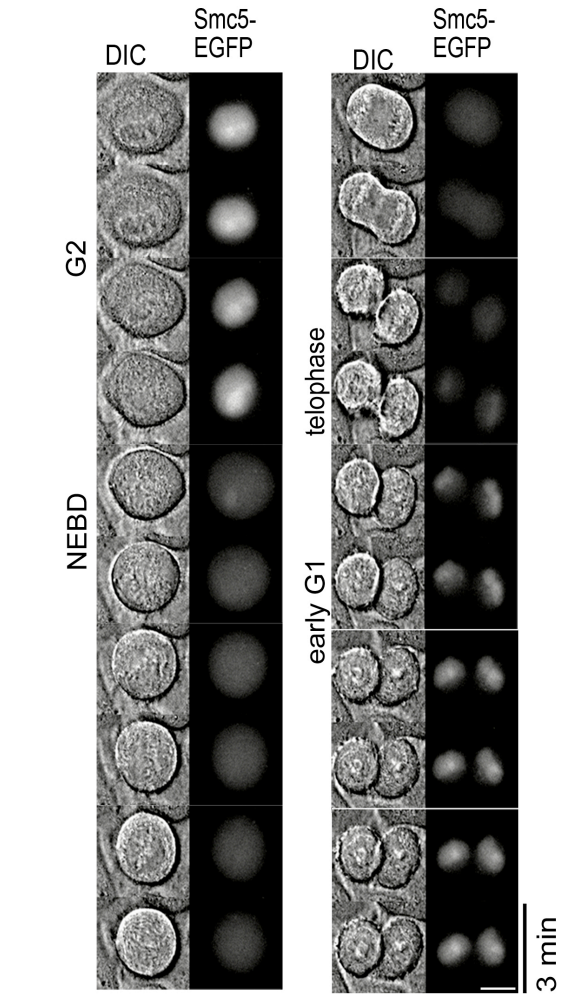

FT
IP
WCE
input
pellet
IgG
Smc6
IgG
Smc6
EGFP
Smc6

Figure 2-5. HeLa cells expressing EGFP-Smc5. A. Schematic representation of the BAC transgene system used to generate HeLa cell lines stably expressing EGFP-tagged version of Smc5 protein. B. Protein levels of endogenous Smc5 and EGFP-Smc5 detected by Smc5 antibody in two different HeLa cell lines that stably express EGFP-Smc5. Arrows indicate the band corresponding to the specified protein. Tubulin is used as a grading control. C. Immunoprecipitation of EGFP-Smc5 with endogenous Smc6 antibodies. Smc6 protein was immunoprecipitated from a whole cell extract prepared from asynchronized HeLa cells expressing EGFP-tagged Smc5 using monoclonal antibody (Abnova), or nonimmune IgG as a control, and resulting precipitates (IP) were analyzed by immunoblotting with the antibodies indicated. Input and pellet indicate the soluble and pellet fraction of the cell extract after high-speed centrifugation. FT indicates the soluble fractions (unbound) after incubation with the beads-antibody preparation.

A


DIC
Smc5-EGFP
DIC
Smc5-EGFP
G2
NEBD
telophase
early G1
3 min

B

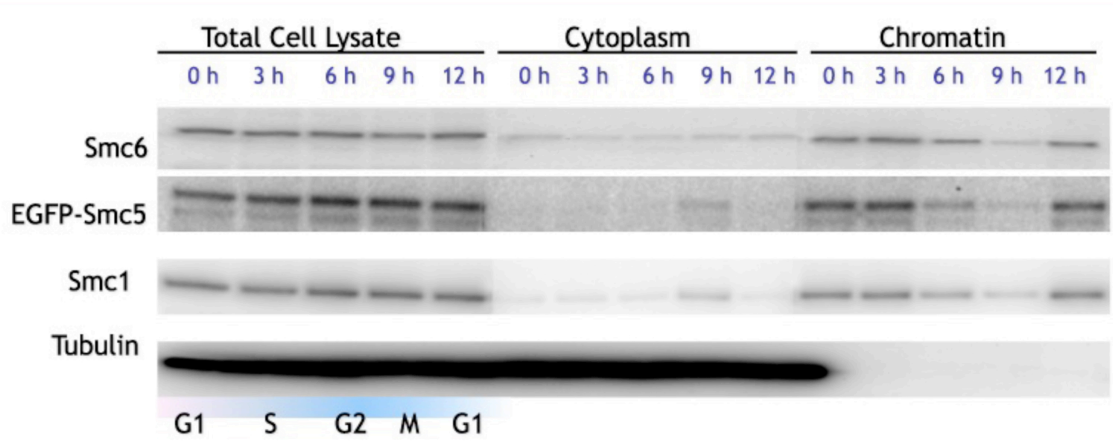

Total Cell Lysate
Cytoplasm
Chromatin
0 h 3 h 6 h 9 h 12 h 0 h 3 h 6 h 9 h 12 h 0 h 3 h 6 h 9 h 12 h
Smc6
EGFP-Smc5
Smc1
Tubulin
G1 S G2 M G1

Figure 2-6. HeLa cells expressing EGFP-Smc5. A. Cell cycle behavior of Smc5 analyzed by live-cell imaging. Time-lapse images of HeLa cells stably expressing EGFP-Smc5 were acquired at 3-min intervals. Fluorescence and differential interference contrast (DIC) images are shown for each time point. NEBD indicates nuclear envelope breakdown. Bar = 10 μm. B. EGFP-Smc5 cells were synchronized by double thymidine block and released intro fresh media. Samples were collected at different time points and subjected to chromatin fractionation and immunoblotting.

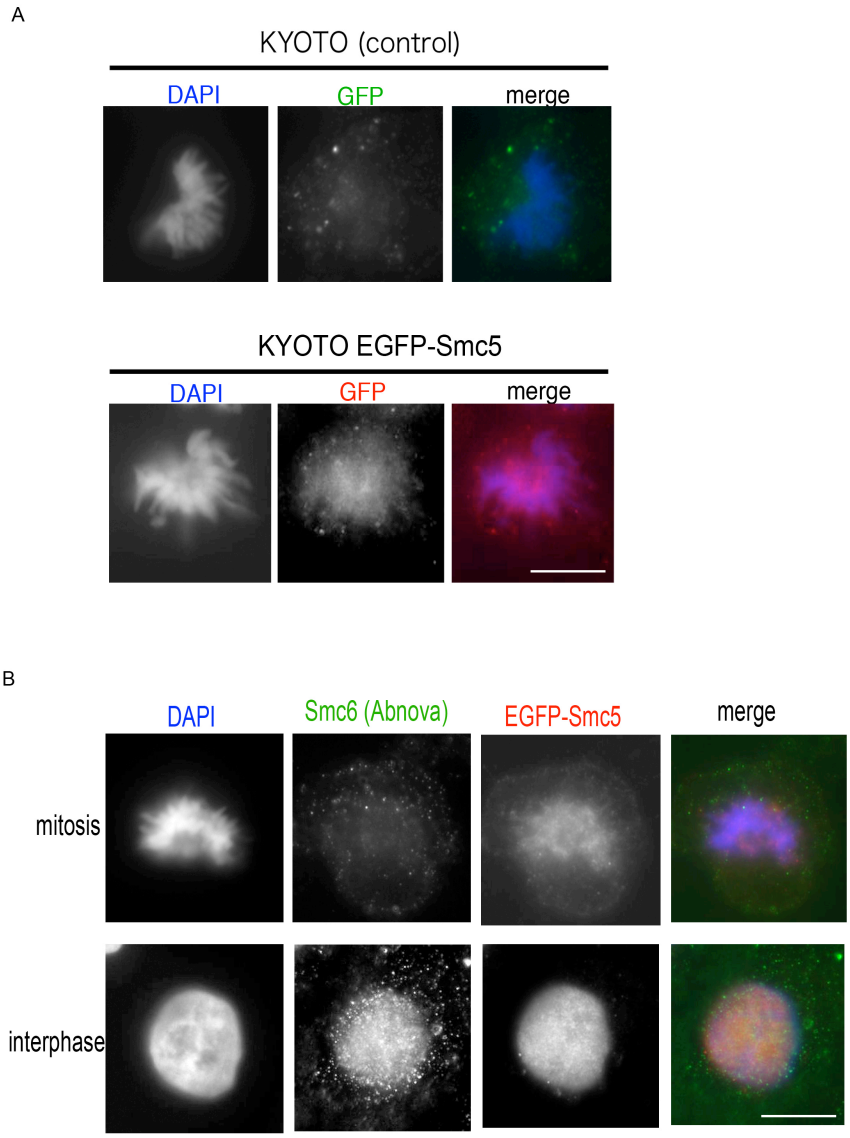


Figure 2-7. Cell cycle behavior of Smc5 analyzed by live-cell imaging. A. Time lapse images of HeLa cells stably expressing EGFP-Smc5 were acquired at 3-min intervals. Fluorescence and differential interference contrast (DIC) images are shown for each time point. NEBD indicates nuclear envelope breakdown. Bar = 10 μm. B. Chromatin fractionation assay in a time course analysis after release from double thymidine block.

CHAPTER III: SMC5/6 IS REQUIRED FOR NORMAL PROGRESSION OF DNA REPLICATION

INTRODUCTION

The faithful transmission of genetic material during successive cell divisions requires the accurate replication and package of parental DNA into chromatin. Failures during the semi-conservative replication of chromosomes in S-phase lead to mutation, epigenetic changes, and other chromosomal aberrations that ultimately generate genome instability. In eukaryotic cells, DNA replication forks are established at multiple origins on each chromosome, were the assembly of proteins called the replisome takes place and constitute a dynamic and precise machinery that enables efficient and complete synthesis of DNA. The replisome is composed by the replicative helicase, several DNA polymerases, a primase to initiate each new DNA fragment and other auxiliary factors. Replication forks progresses without difficulty until it encounters another fork coming in the opposite direction; at this point replisome is disassembled and replication termination occurs.

But the normal progression of replication forks can be hindered by a variety of obstacles that includes DNA lesions, insufficient nucleotides or particular regions in the genome, such as replication slow zones, that represent a challenge to replication fork movement and are associated with a high incidence of chromosomal rearrangements.

Natural replication-impeding sequences where certain proteins bind tightly to DNA as in the case of transcription machineries, pre-replicative complexes at origins of replication and telomeric protein complexes are also recognized as replication fork barriers (RFBs) and represent additional obstacles that can induce a much more slowly progression of the replicative helicase, a situation termed as replication fork stalling (Katou et al., 2003).

Cells have acquired several strategies to overcome the permanent challenge of replication stress and resume DNA synthesis, because persistent stalled forks are prone to collapse, a situation that severely compromises genome stability and cell viability. The DNA damage and replication checkpoints represent the mechanisms by which stalled replication forks are stabilized and restarted. In this context, checkpoint kinases response to altered structures of the fork upon replication fork stalling, such as large exposure of single-stranded DNA (Branzei and Foiani, 2005). Moreover, accumulating evidence indicates that homologous recombination (HR) is involved in bypass of some DNA damage during ongoing replication and plays critical roles in the rescue of stalled replication forks, which has been suggested to occur via a double-strand break (DSB) independent pathway through the mechanism of template switching (Lambert et al., 2010).

Members of the structural maintenance of chromosomes (SMC) family of proteins have been implicated in the regulation of homologous recombination. The Smc1/3 cohesin complex is required to maintain cohesion between sister chromatids during DNA replication, not only for ensuring their proper mitotic segregation but also to facilitate repair of DNA double strand breaks by HR. Most of the information regarding the function of the Smc5/6 complex has been obtained through the analysis of budding and fission yeast mutants. Smc6 protein (which together with Smc5 and the non-Smc elements subunits Nse1-6 shapes the Smc5/6 complex) was initially identified in a genetic screening of DNA repair mutations in Schizosaccharomyces pombe, and further analyses have revealed that components of the Smc5/6 complex function together with RAD51 in the repair of DSBs through HR (McDonald et al., 2003; Harvey et al., 2004; Pebernard et al., 2004; Mengiste et al., 1999).

In the same line of evidence, MMS21/Nse2 subunit, the SUMO ligase of the complex, have shown to SUMOylates Smc6 after DNA damage is induced, suggesting that the SUMOylation activity of MMS21/Nse2 is regulated by the cellular response to DNA damage and may contribute to the HR-related function of the Smc5/6 complex (Andrews et al., 2005).

In fission yeast, hypomorphic alleles of the Smc5/6 complex are not only hypersensitive to DNA-damaging agents, but also to replication arrest by inhibitors of

DNA synthesis, such as HU (De Piccoli et al., 2009), and importantly, inhibition of the homologous recombination pathway by deletion of RAD51 can partially rescue these defects (Branzei et al., 2006), suggesting that Smc5/6 may function downstream of RAD51, or alternatively, that the complex may be necessary for accurate RAD51-dependent strand invasion and exchange during HR-dependent rescue of replication fork stalling.

Smc5/6 mutants in yeast also exhibited an accumulation of chromosome linkages upon HU and MMS-induced replication arrest, leading to lethal mitosis due to failure in chromosome separation (Verkade et al., 1999; Harvey et al., 2004). This indicates not only a marked defect in checkpoint responses in the context of non-functional Smc5/6, but also a requirement of the complex in the resolution of DNA repair intermediates formed at stalled or collapsed replication forks. These DNA structures can be processed by alternative DNA repair pathways, which become essential for cell survival when Smc5/6 function is compromised (Choi et al., 2010; Chen et al., 2009).

Here, we analyzed the progression of DNA replication in human cells depleted of the Smc5/6 complex. We show that ablation of Smc5 and Smc6 markedly delays DNA synthesis and generates replication-related DNA damage response, which is alleviated by providing additional time to the cell in G2 phase to fully complete replication at particular genomic regions like centromeres and telomeres.

MATERIALS AND METHODS

Antibodies

A mouse monoclonal antibody was raised against the peptide sequence MATPSKKTSTPSPQPSKRALPRDPSSEVPC of Smc5 (Monoclonal Antibody Research Institute, Sapporo, Japan) and then purified using a protein G affinity column (MabTrap kit; Amersham Biosciences, Piscataway, NJ). Antibodies against other proteins were as follows: SMC6L1 (M01, Cl7one 2E6; Abnova, Taiwan), Smc6 (sc-365742, clone A-3; Santa Cruz Biotechnology, Santa Cruz, CA), SMC5 (18038, Abcam, Cambridge, UK), BLM (sc-7790, Santa Cruz Biotechnology Inc., Santa Cruz, CA), PICH (Clone 142-26-3, Millipore Corporation, Billerica, MA), and gamma-H2AX (A300-081A, Bethyl, Montgomery, TX).

Cell synchronization, inhibitor treatment, and cell lines

RPE-1 cells were cultured in Dulbecco's Modified Eagle's Medium (Wako, Tokyo, Japan) supplemented with 10% fetal calf serum (FBS, Equitech-Bio Inc., Tokyo, Japan), 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin ((Meiji Seika, Tokyo, Japan) at 37°C in a 5% CO_2 environment. RPE-1 cells were synchronized at G0/G1 by serum starvation for 96 h and then released into the cell cycle with fresh medium containing 10% fetal calf serum for further analysis. For G2/M-phase arrest, asynchronous RPE-1 cells were treated with 9.0 μM RO-3306

(Enzo Life Sciences, Plymouth, PA) for 12 h. For visualization of regions with newly synthesized DNA, cells were cultured in medium supplemented with 10 μM EdU kit (Invitrogen, Carlsbad, CA) for 0.5 h before fixation.

RNAi

siRNA sequences were as follows: Smc5 siRNA1, 5′-GGAACUUCAGCAGGGCUUUAAUAGUA-3′; Smc5 siRNA2, 5′-GGCAUUAUGUGAAGGCGAAAUAAUU-3′; Smc6 siRNA1, 5′-CAAAUUCUUCAUGAAAGCAACGCAA-3′; Smc6 siRNA2, 5′-GACCUAUCUUGAUCUGGAUAGUAAA-3′. Smc6 siRNA1 was used for ChIP-Seq analysis of topo IIa. Cells were transfected by incubating 50 nM duplex siRNA with Lipofectamine™ RNAiMAX (Invitrogen) in antibiotic-free growth medium. RNAi was performed concomitantly with the synchronization with thymidine or serum starvation, and 48 h of transfection with each siRNA was required for maximal knockdown of Smc5 and Smc6. For control transfections, the same annealing reaction was set up using without the presence of siRNA oligonucleotides.

PCC Assay

PCC was induced by adding Calyculin A (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) into culture media at a final concentration of 160 nM. After 1 h, cells were hypotonically swollen in a 40:60 mix (v/v) of PBS and distilled water for 5 min at room

temperature. Cells were fixed with freshly made Carnoy's solution (70% methanol, 30% acetic acid), dropped on glass slides, and dried. Slides were stained with 5% Giemsa stain, washed with water, air-dried, and mounted with Entellan embedding agent (Merck). For detection of incorporated EdU, the Click-iT EdU imaging kit (Invitrogen, Carlsbad, CA) was used. Briefly, the glass slides were incubated with a reaction solution containing fluorochrome-azide (Alexa Fluor 488) for 1 h at room temperature after which immunofluorescence staining was conducted.

Chromosome spreads

RPE-1 cells treated with STLC (15 mM) for 2–3 h were collected by mitotic shake-off and hypotonically swollen in a 40:60 mix (v/v) of PBS and distilled water for 5 min at room temperature. Cells were fixed with freshly made Carnoy's solution, dropped on glass slides, and dried. Slides were stained with 5% Giemsa stain, washed with water, air-dried, and mounted with Entellan embedding agent.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with ice-chilled 100% methanol for 20 min at −20°C or with 4% paraformaldehyde in 0.137 M sodium phosphate buffer (pH 7.4). Fixed cells/chromosomes were permeabilized with 0.5% Triton X-100 in PBS and

incubated with 3% bovine serum albumin in PBS for at least 1 h. Cells were incubated with the primary antibodies overnight at room temperature followed by incubation with secondary antibodies for 35 min together with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). The secondary antibodies used in this study were goat anti-rabbit IgG and goat anti-mouse IgG coupled to Alexa Fluor 488 or 568, and goat anti-human IgG coupled to Alexa Fluor 568 (Molecular Probes, Carlsbad, CA). Cells were washed twice and mounted in Prolong Gold anti-fade mounting reagent (Invitrogen). Images were acquired on a Zeiss Imager Z.1 microscope equipped with epifluorescence and a Cool Snap HQ CCD camera (Photometrics Ltd., Tucson, AZ). 3D projections of the immunofluorescence images were carried out using the 3D Volume viewer tool in ImageJ software (Wayne Rasband, NIH).

FACS analysis

Cells were harvested by trypsinization, fixed in 70% ethanol, stained with propidium iodide solution at a final concentration of 50 µg/ml, and subjected to fluorescence-activated cell sorting (FACS) analysis on FaCScalibur using Cell Quest (Becton Dickinson) software.

RESULTS

Depletion of Smc5/6 do not induce cell death

To gain insight into the function of the human Smc5/6 complex in regulating chromatin dynamics during the cell cycle, we aimed to deplete both Smc5 and Smc6 proteins by using short-interference RNA treatment with two independent oligonucleotides for Smc5 and two for Smc6. More than 80% of both proteins was depleted after 72 h of transfection, a pattern observed in three different cancer cell lines (HeLa, A529 and HCT116) as well as RPE-1 retinal pigment epithelial cell line (Figure 3-1). Depletion of Smc5 caused a decrease in the Smc6 protein levels and vice versa, consistent with the idea that these proteins are stable as a protein complex. Immunofluorescence microscopy also verified the reduction of Smc5 or Smc6 staining by treating cells with their specific siRNAs (Figure 3-2, A and B).

It was previously shown that Smc5/6-depleted yeast cells die during an aberrant mitosis, due to persistent linkages between sister chromatids as chromosome separation occurs in anaphase (Verkade et al., 1999; Harvey et al., 2004). We were not able to observe, however, such lethality after Smc5/6 depletion in human cell lines. Instead, Smc5/6-depleted cells exhibited a lower proliferation rate compared to control cells (Figure 3-2, C). This observation let us to speculate that a complete depletion of Smc5/6

in human cells could induce a cell cycle progression delay and/or a post-mitotic cell cycle arrest, rather than the mitosis-related cell death observed in yeast.

Prolonged S-phase delay in Smc5/6-depleted cells

We then aimed to investigate how cell cycle progression was affected by the absence of the Smc5/6 complex. HeLa cells were then depleted of Smc5/6 during double thymidine block and released into media containing 5-ethynyl-2'-deoxyuridine (EdU) to determine the efficiency of DNA synthesis. EdU incorporation assay showed that Smc5/6-depleted cells actually presented a slower progression of DNA replication compared to control cells (Figure 3-3, A).

We also strategically depleted Smc5 and Smc6 proteins in RPE-1 cells during a serum starvation-dependent cell cycle synchronization at G0/G1 (Figure 3-3, B), and carried out a detailed analysis of cell cycle progression as cells were re-stimulated in serum-containing media (Figure 3-4). Flow cytometry in synchronous cell populations were analyzed for their DNA content every 3 hours (Figure 3-4, B) after release from serum starvation. Cells depleted of Smc5 or Smc6 showed significant delay in the progression of DNA synthesis by 2.5-folds when compared to the controls.

To gain insight about the stage of DNA synthesis at which this delay is taking place, as well as to characterize the structural details of the deficient replication progression in the absence of Smc5/6, cells released from serum starvation were treated with Calyculin A, an inhibitor of type 1 and 2A protein phosphatases that induces premature chromosome condensation (PCC). Thus, we were able to differentiate cells from early to late S phase as well as G1 and G2 phase (Figure 3-4, A and C). In both control and Smc5/6-depleted cells, at 12 h after release from serum starvation, almost all cells exhibited the characteristic G1-like condensed chromosomes; at 26 h, the majority of control cells started to accumulate nuclei exhibiting DNA replication, particularly at late S phase, or contained fully replicated G2-like chromosomes; by 30 h, the late-S and G2-like nuclei represented the majority of the population.

In contrast, Smc6 siRNA-treated cells showed almost no accumulation of G2-like nuclei even at 26 h; instead, the nuclei at this time point exhibited a variety of DNA replication stages, from early to late S-phase, and had just started to generate G2-like chromosomes at 30 h. As late as 34 h after release from serum starvation, a significant proportion of Smc5/6-depleted cells were still undergoing DNA replication.

Importantly, when EdU pulse-labeling was carried out for 0.5 h prior to PCC induction in order to visualize regions of ongoing replication, G2 chromosome structures -which

showed the characteristic well-defined sister chromatids- in Smc5/6-depleted cells showed a high frequency of persistent ongoing replication, particularly at centromeres and telomeres, consistent with the late-replicating nature of these domains, suggesting that DNA replication was delayed at these sites in the absence of Smc5/6 (Figure 3-5, A and B).

Replication-dependent DNA damage in Smc5/6-depleted cells

In yeast, the Smc5/6 complex has been implicated in the stabilization of stalled replication forks and the restart of collapsed replication forks (Irmisch et al., 2009; Ampatzidou et al., 2006), raising the possibility that this complex may have a similar if not identical role in human cells. Thus, we first examined the extent of replication-related DNA damage in Smc5/6-depleted cells by looking at γH2AX and BLM foci, which are formed at stalled and/or collapsed replication fork sites (Li et al., 2004; Davalos and Campisi, 2003; Shimura et al., 2008) (Figure 3-6).

A time-course analysis during the S and G2 phases indicated no obvious enrichment of γH2AX and BLM foci in the control cells at any time point analyzed. However, Smc5/6-depleted cells showed a marked and progressive increase in foci formation. (Figure 3-6 A and B), indicating a significant proportion of stalled or collapsed replication forks in the absence of Smc5/6, comparable to the extend of the

γH2AX and BLM foci formed in the presence of low doses of Aphidicolin (a small-molecule inhibitor that directly blocks the activity of the replicative DNA polymerases) which leads to replication fork stalling.

Importantly, when Smc5/6-depleted cells were provided with a longer G2 phase by treating them with RO-3306 at 30 h after release from serum starvation for additional 12 h, most of the γH2AX/BLM foci disappeared (Figure 3-6, C). These data indicated that, in the absence of the Smc5/6 complex, DNA replication progresses at a much slower rate, with the concomitant generation of replication-related DNA damage, and that this perturbed replication fork progression can be overcome if more time is provided to cells to overcome DNA replication stress and fully complete DNA synthesis.

DISCUSION

The mitotic lethality of the Smc5/6 complex deficiency in yeast is consistent with its requirement for the completion of DNA synthesis, as well as for the maintenance of checkpoint signaling. Although our study indicated that depleting Smc5/6 did not represent lethality in human cells, they did exhibit increased levels of replication-related DNA damage, indicating that replication fork stalling and/or collapse occur frequently in Smc5/6-depleted cells, and that these conditions directly relate to the slower processing of DNA replication (Figures 3-4, 3-5 and 3-6).

Moreover, the fact that cell growing and mitotic index decreased after depletion of Smc5/6 strongly suggest that the majority of these cells underwent a post-mitotic arrest. Significantly, the frequency of the replication-related DNA damage was markedly reduced when cells were subjected to G2 arrest and thereby allowed extra time to complete DNA replication before mitosis (Figure 3-6, C).

In yeast, Smc5/6 is enriched at repetitive DNA loci, such as rDNA arrays, centromeres and telomeres (Stephan et al., 2011; Ampatzidou et al., 2006; Pebernard et al., 2008). Localization of the Smc5/6 at these loci may indicate that its activity is required at sites where spontaneous replication fork collapse may occur, or where

complex recombinational structures are likely to arise upon the initiation of repair of collapsed forks. Similarly, we found that Smc5- and Smc6-depleted human cells contained persisting active replication at centromeres and telomeres in G2-like PCC-induced nuclei, which could represent the source of possible intertwinings and linkages between sister chromatids in mitotic chromosomes.

A revealing observation was that cells depleted of Smc5/6, which were provided with extra time in G2 phase by RO-3306 treatment showed a marked reduction in γH2AX/BLM foci, indicating that the replication-related DNA damage arising in these cells can be overcome if they have sufficient time to repair and re-start replication fork movement and consequently for fully complete DNA replication.

Further analysis will be required to determine the precise localization and protein interaction pattern of the human Smc5/6-complex in the context of DNA replication stress, in order to elucidate the mechanistic contribution of the complex to the regulation of replication fork progression.

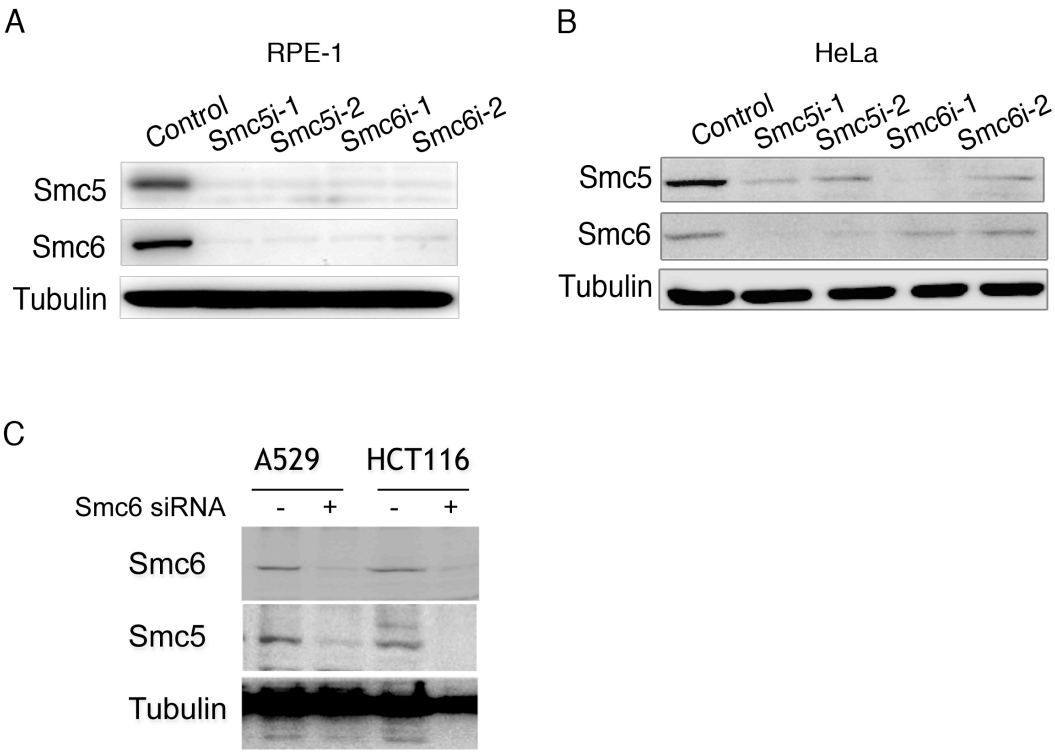
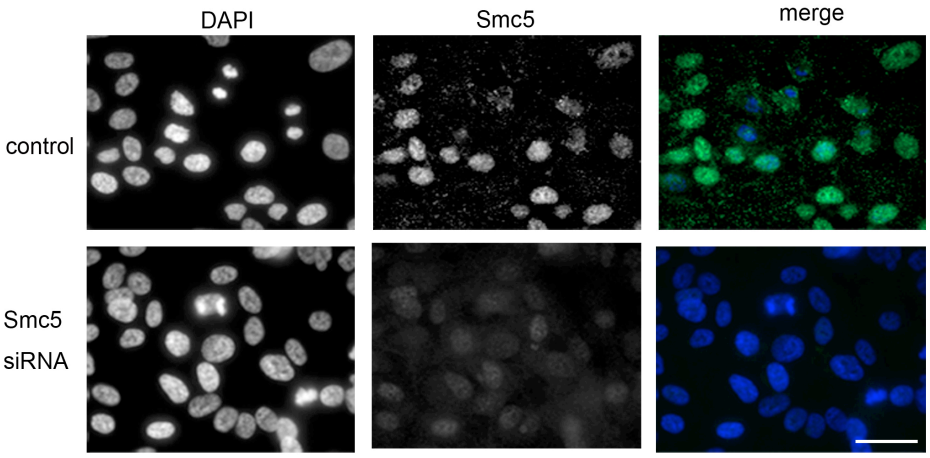
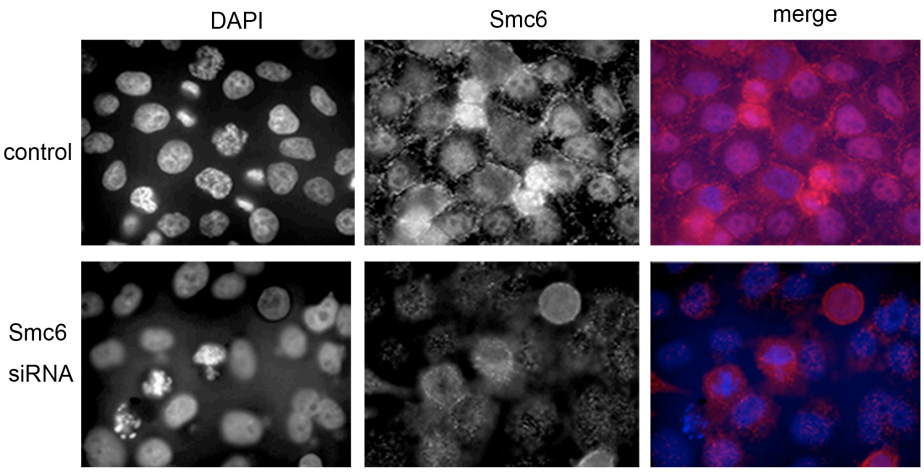


Figure 3-1. RNA interference (RNAi) mediated depletion of Smc5 and Smc6 in Human cells. A. Logarithmically proliferating RPE-1 cells were transfected with indicated siRNA, and whole-cell extracts were analyzed by immunoblotting 72 h after the transfection. Tubulin staining was shown as a loading control. Note that Smc6 become unstable in Smc5 depleted cells, and vice versa. B and C show same conditions as in (A) but for HeLa, A529 and HCT116 cell lines, respectively.

A


DAPI
Smc5
merge
control
Smc5
siRNA

B


DAPI
Smc6
merge
control
Smc6
siRNA

C

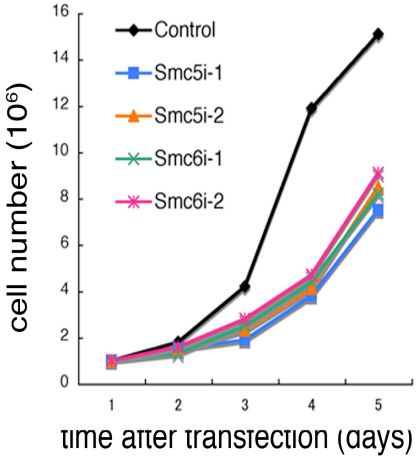
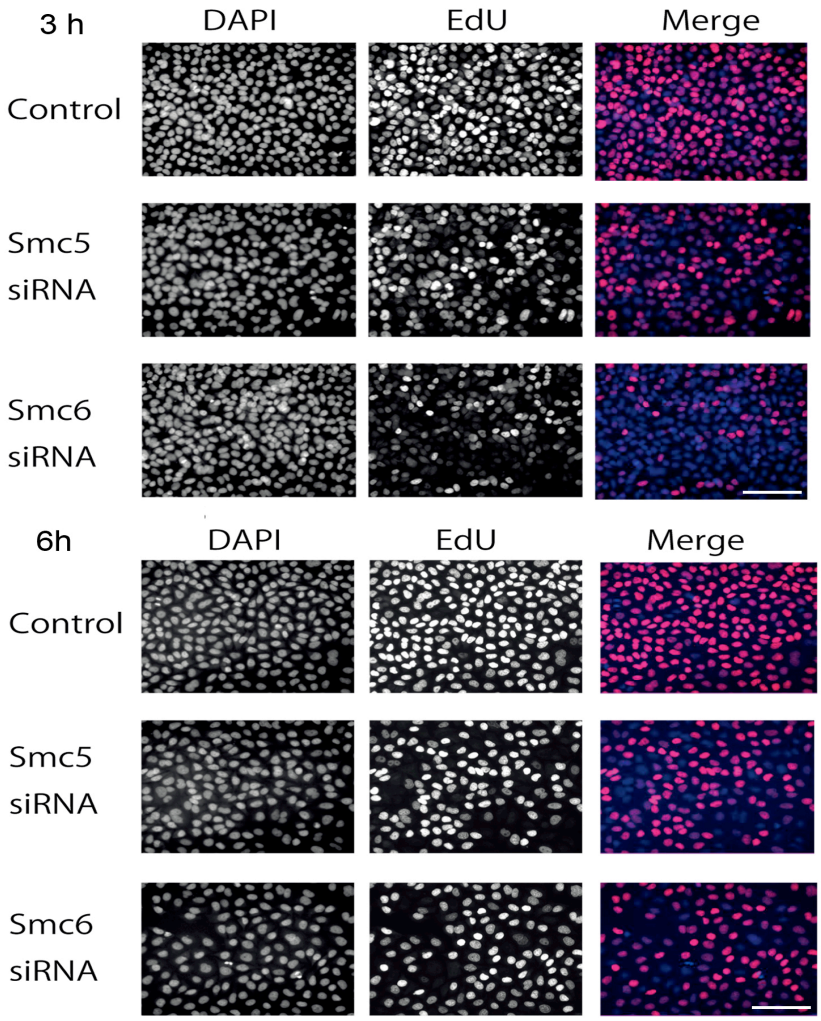

Control
Smc5i-1
Smc5i-2
Smc6i-1
Smc6i-2
cell number (10⁶)
time after transfection (days)
0
2
4
6
8
10
12
14
16
1
2
3
4
5

Figure 3-2. Immunofluorescence analysis in Smc5 or Smc6 depleted cells. A and B. After transfection with the indicated siRNA (72 h), HeLa cells were fixed and stained with an antibody against Smc5 (green, A), Smc6 (red, B) and DNA was counterstained with DAPI (blue). Bar = 20 μm. C. Cell number of the control and the Smc5- and Smc6-depleted cells was monitored for 5 days after siRNA treatment.

A


3 h
DAPI
EdU
Merge
Control
Smc5
siRNA
Smc6
siRNA
6h
DAPI
EdU
Merge
Control
Smc5
siRNA
Smc6
siRNA

B

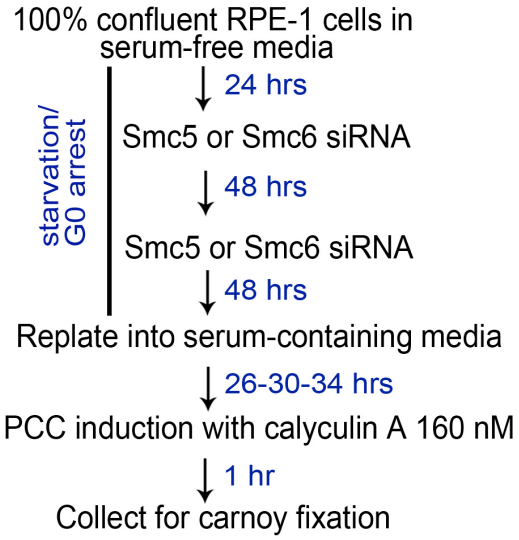
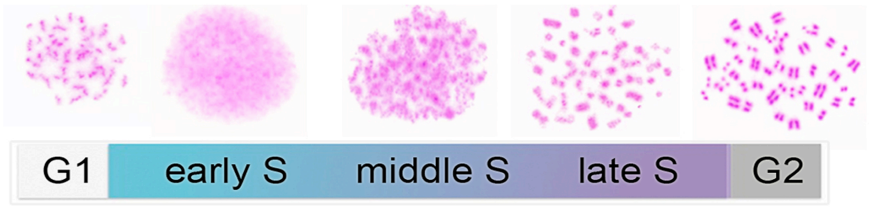
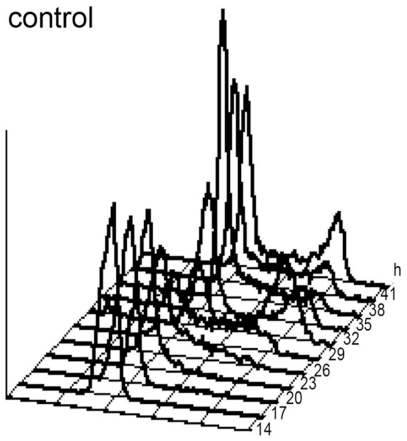

100% confluent RPE-1 cells in serum-free media
24 hrs
starvation/ G0 arrest
Smc5 or Smc6 siRNA
48 hrs
Smc5 or Smc6 siRNA
48 hrs
Replate into serum-containing media
26-30-34 hrs
PCC induction with calyculin A 160 nM
1 hr
Collect for carnoy fixation

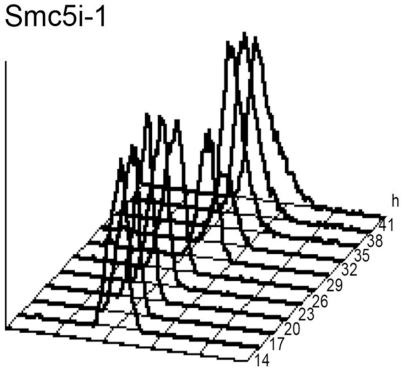
Figure 3-3. DNA synthesis is delayed in Smc5- and Smc6-depleted cells. A. HeLa cells transfected with siRNAs against Smc5 or Smc6 were synchronized by double thymidine block, then released and EdU pulse-labeled for 30 m after 3 h and 6 h. Representative immunofluorescence microscopy images of DNA (DAPI) and EdU (red) are shown. Bar = 100 μm. B. Experimental settings combining RNAi with cell cycle synchronization, followed by EdU pulse labeling and PCC analysis with the subsequent time-course analysis.

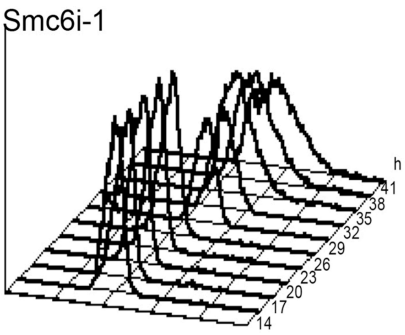
A


G1
early S
middle S
late S
G2

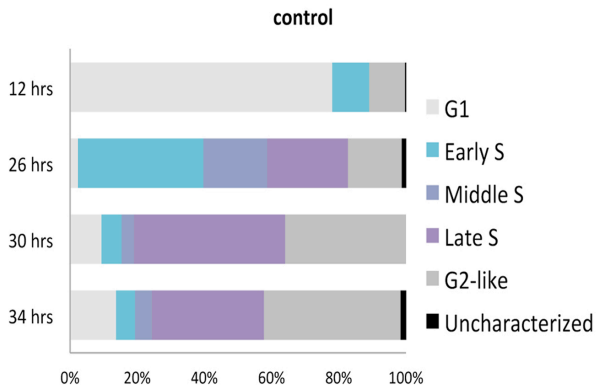
B

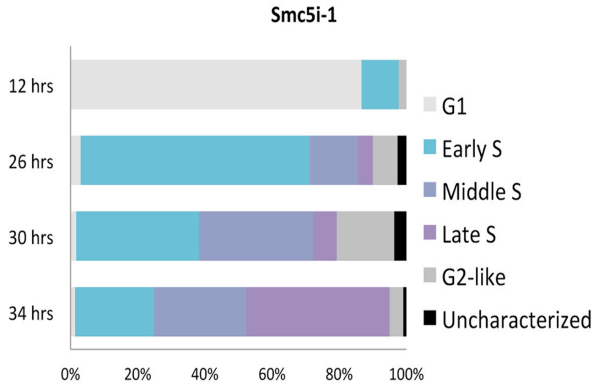

control
h
41
38
35
32
29
26
23
20
17
14


Smc5i-1
h
41
38
35
32
29
26
23
20
17
14


Smc6i-1
h
41
38
35
32
29
26
23
20
17
14

C


control
12 hrs
26 hrs
30 hrs
34 hrs
0%
20%
40%
60%
80%
100%
G1
Early S
Middle S
Late S
G2-like
Uncharacterized


Smc5i-1
12 hrs
26 hrs
30 hrs
34 hrs
0%
20%
40%
60%
80%
100%
G1
Early S
Middle S
Late S
G2-like
Uncharacterized

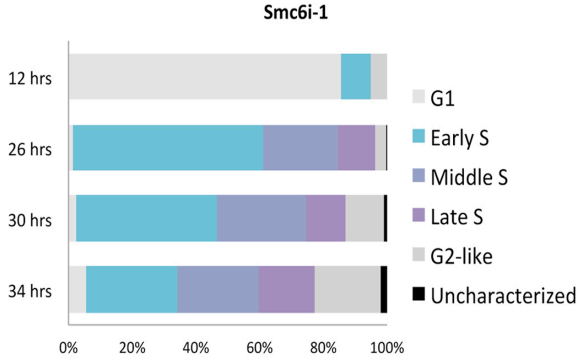
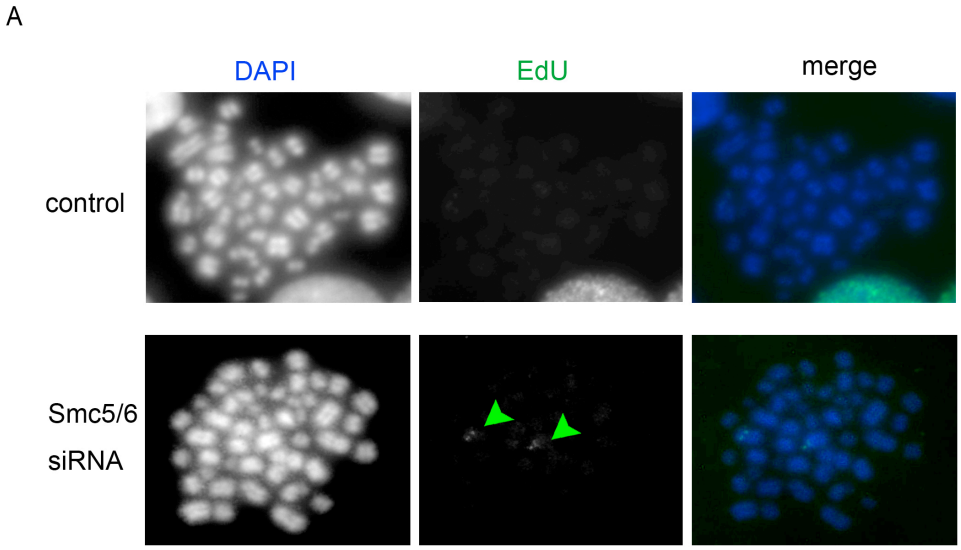
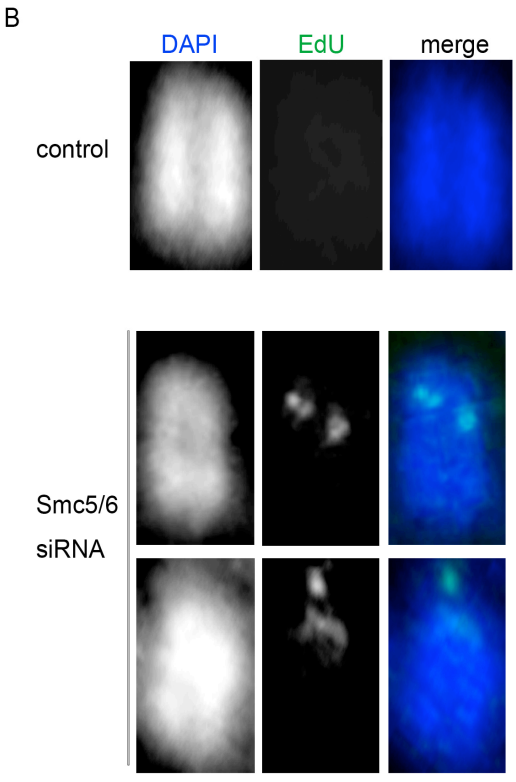

Smc6i-1
12 hrs
26 hrs
30 hrs
34 hrs
0%
20%
40%
60%
80%
100%
G1
Early S
Middle S
Late S
G2-like
Uncharacterized

Figure 3-4. Probing different stages of DNA replication using FACS and PCC assay. A. Representative images of PCC-induced nuclei transiting G1 phase, early, middle and late S phase, and G2 phase. B. RPE-1 cells transfected with an siRNA against Smc5 or Smc6 or a control mock were collected at indicated time points after release from serum starvation, and cell-cycle distribution was determined by flow cytometric analysis of DNA content. C. PCC-induced nuclei from RPE-1 cells transfected with an siRNA against Smc5 or Smc6 or a control mock were treated with hypotonic buffer, fixed, spread on slideglasses, and stained with Giemsa solution. Representative images for indicated cell cycle stages are shown. The band graphs represent the average proportion of cells at different interphase stages in control or siRNA-treated samples at the indicated time points after the release from serum starvation between three independent experiments.


A
DAPI
EdU
merge
control
Smc5/6
siRNA


B
DAPI
EdU
merge
control
Smc5/6
siRNA

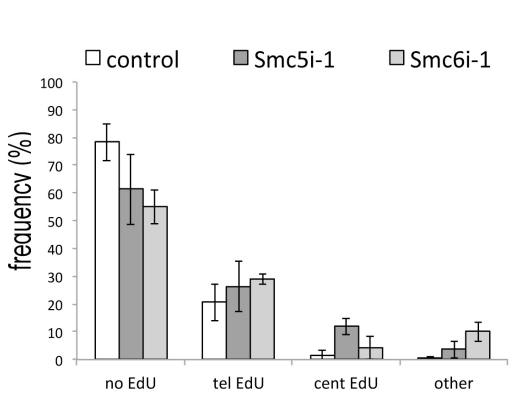

C
control
Smc5i-1
Smc6i-1
frequency (%)
100
90
80
70
60
50
40
30
20
10
0
no EdU
tel EdU
cent EdU
other

Figure 3-5. Slow progression of DNA replication in Smc5/6-depleted cells. A. RPE-1 cells transfected with an siRNA against Smc5 or Smc6 or a control mock were released from serum starvation, followed by EdU pulse-labeled for 30 min and PCC induction before carnoy fixation. G2-like chromosomes are stained with DAPI (blue) and regions of ongoing replication are detected by the Click it EdU detection system (green, Invitrogen, Carlsbad, CA). B. Frequently observed G2-like chromosomes in Smc6 siRNA-treated cells. Right panels show merged images. Bar = 1 μm. C. Frequency of PCC-induced nuclei at G2 showing persistent EdU staining. One hundred metaphases were analyzed for each sample in C and D, respectively. Bar graph shows mean ± SD from three independent experiments; P value <0.05;*P value 0.01 to 0.05; two-tailed student's t-test.

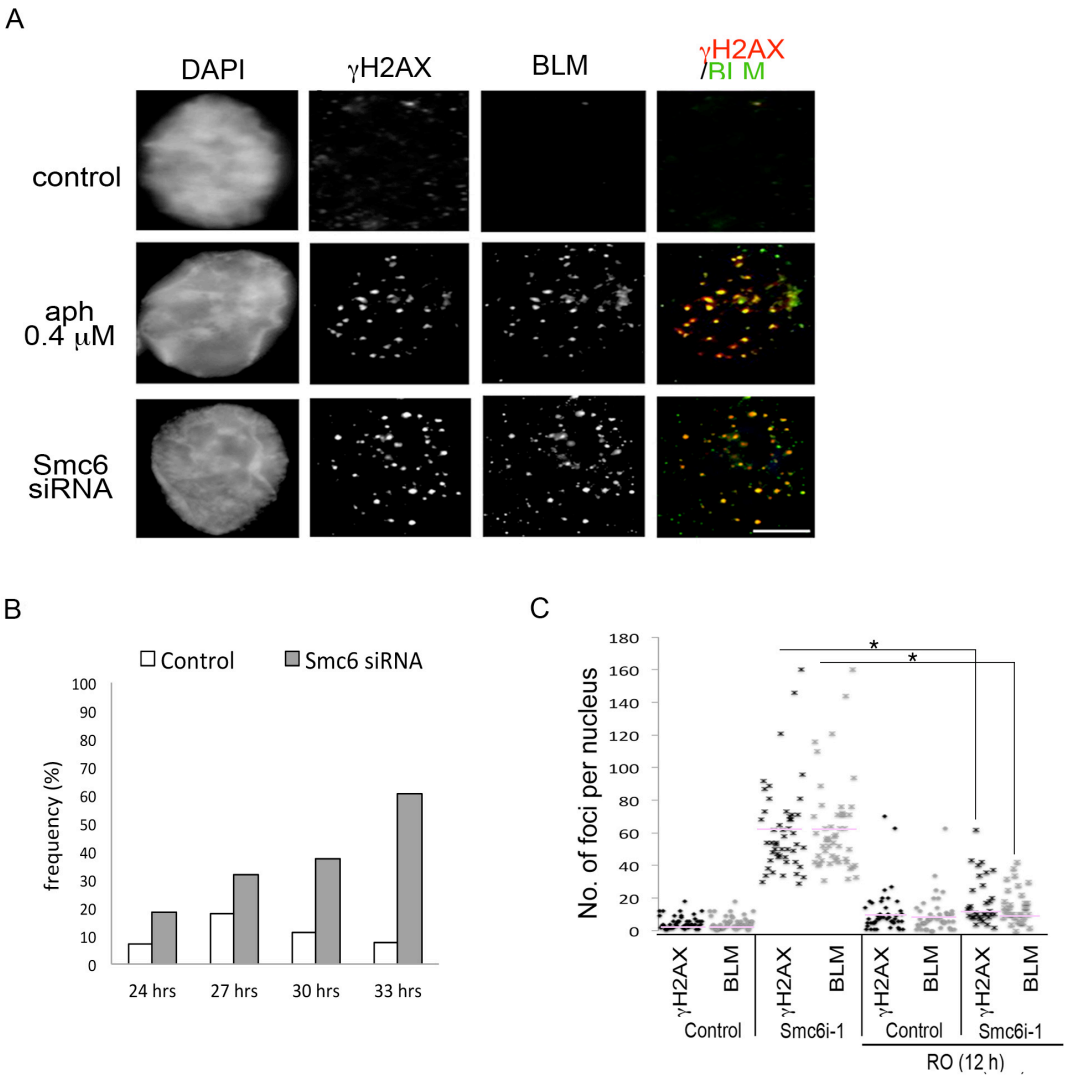


Figure 3-6. Replication-related DNA damage. RPE-1 cells transfected with an siRNA against Smc5 or Smc6 or a control mock were analyzed at the specified time points after release from serum starvation. A. Representative immunofluorescence of γH2AX (red) and BLM (green) foci formation in control cells (top panels) and in cells depleted of Smc6 (bottom panels). DAPI staining is shown in blue. A positive control for γH2AX and BLM staining is provided by low doze of aphidicolin treatment (aph 0.4 μM; middle panels). Bar = 10 μm. B. Frequency of γH2AX-positive cells at indicated time points after the release from serum starvation. C. Frequency of γH2AX-positive cells at 33 h-time point, with our without additional RO3306 treatment for 12 h and, which was further classified according to the number of foci per cell as indicated (n> 31 per condition). Pink bars denote means of foci number per cell.

CHAPTER IV: SMC5/6 FUNCTION IN HIGHER-ORDER CHROMOSOME STRUCTURE

INTRODUCTION

Dynamic structural changes within the chromatin are tightly regulated throughout the cell cycle in order to ensure a variety of genome functions such as DNA replication and repair, regulation of RNA transcription and chromosome segregation, all of which are crucial for cell viability. Although many questions remain unanswered about the mechanism by which the chromatin fiber is packaged during mitosis, increasing evidence suggests that mitotic chromosome assembly is a progressive and dynamic process involving the cooperative functions of key structural and regulatory chromatin components.

During interphase, chromosomes occupy distinct spaces within the nucleus where sister chromatid cohesion takes place to ensure that newly synthesized sister DNA sequences remain connected to each other. In subsequent steps, sister chromatids are assembled into juxtaposed cylindrical structures in a process called chromosome condensation, prior to their alignment on mitotic spindles. Both cohesion and condensation depend on the structural maintenance of chromosomes (Smc) protein complexes characterized by the core V-shaped heterodimer and the unique sets of associated proteins that define the structure of the three main eukaryotic complexes:

cohesin, condensin and Smc5/6.

The cohesin complex form a ring-like structure with an internal diameter of about 40 nm (Gruber et al., 2003), which is able to tether two replicated sister chromatids from S-phase –when sister chromatid cohesion is established- until prophase and prometaphase, when the bulk of the complex is removed from chromosome arms in a process called the "prophase pathway". Persisting sister chromatid cohesion at centromeres is destroyed through the cleavage of cohesin's Scc1/Rad21 subunit by separase (Uhlmann et al., 1999, 2000), thus releasing cohesin from chromatin.

Condensin complex is recognized as the major molecular effector of the higher-order reorganization of chromosomal DNA from a relaxed stage in interphase to the characteristic compact configuration during mitosis, which enables its physical segregation in anaphase. Vertebrate cells possess two different condensin complexes, known as condensins I and II (Ono et al., 2003) that share the Smc2/CAP-E and Smc4/CAP-C heterodimer but differ in their associated non-SMC regulatory subunits. During interphase, condensin I is localized within the cytoplasm until prophase, and accesses the chromosomes after nuclear envelope breakdown (NEBD) in prometaphase. In contrast, condensin II is already nuclear during interphase and participates in early stages of chromosome condensation in prophase. Then, together with condensin I, condensin II helps shaping chromosomes, whose structure becomes clearly visible after prometaphase. Detailed analyses (Kireeva et al., 2008) have shown that hierarchical

folding of the chromatin in prophase represents the early stage of chromosome condensation, time at which Smc2/CAP-E protein appeared in the peripheral regions of chromosomes. Once NEBD occurs, Smc2/CAP-E gradually accumulates into the axial region of chromatids until metaphase, coincident with the recruitment of condensin I to chromosomes. Thus it was hypothesized that condensin II initiates chromosome condensation by hierarchical folding of the chromatin, while condensin I assist in the resolution and stability of shaped chromosomes by forming a core axial structure within the chromatids.

Another protein previously found as a component of this "axial scaffold" is DNA topoisomerase II (topo IIα), one of the most abundant non-histone protein components of mitotic chromosomes. Although it was initially thought that topo IIα was tightly associated with metaphase chromosomes, it was then demonstrated that topo IIα was actually easily extracted in physiological buffers from *in vitro* assembled chromosomes without any evident modification in chromosome structure (Hirano and Mitchison, 1993) indicating a very dynamic interaction of the protein with chromatin. Silver impregnation assay revealed that inhibition of topo IIα prevents the separation of sister axial structures observed in metaphase and anaphase from an initially single folded midline structure observed at earlier stages of mitosis (Gimenez-Abian et al., 1995). Immunolocalization of topo IIα also revealed chromatid cores analogous to those seeing with silver staining, suggesting a role of topo IIα in defining the chromosomal axial domain during transition through mitosis. Due to the fact that the formation of

topo IIα–containing axes and the chromatin binding property of topo IIα was shown to be dependent on the progression of DNA replication (Cuvier and Hirano, 2003), it has been proposed that topo IIα may be implicated in an early step of chromatin organization involving the formation of chromosome axis after DNA synthesis as part of a intricate molecular assembly already started in interphase that is then followed by the condensin-mediated compaction during mitosis.

Despite the advances in understanding cohesin and condensin dynamics in the context of chromosome architecture, little is known about the participation of the Smc5/6 complex in this structural network. Mutations in any of the components of these Smc complexes result in severe mitotic aberrations as well as defective DNA damage repair. Smc5/6 is recognized as an important component of the homologous recombinational repair pathway of DNA double-strand breaks (DSBs); it is required for the recruitment of telomeres to PML bodies, as well as the relocation of DSBs induced at rDNA loci to outside the nucleoli (Potts and Yu, 2007; Torres-Rosell et al., 2007), indicating a probable general activity of the complex in the regulation of higher-order chromosome structures required for the proper localization of damaged DNA and subsequent recombinational repair processes.

Additionally, the Smc5/6 complex has been also implicated in sister chromatid cohesion, as depletion of Smc5 or the Nse2 subunits induced a marked loss

of sister chromatid cohesion during prometaphase in HeLa cells (Behlke-Steinert et al., 2009). In striking contrast, *S. pombe* Smc6-74 mutants with HU-induced DNA damage undergo lethal mitoses due to a failure in removing chromosome-associated cohesin, although analyses in budding yeast indicated that the lethal, persistent sister chromatid association is mediated by DNA linkages, rather than cohesin (Bermudez-Lopez et al., 2010). Consistently, it was recently proposed that Smc5/6 assists chromosome-level rotation to unwind the supercoiling produced during DNA replication particularly in long chromosomes (Kegel et al., 2011). It is then conceivable that such alterations in chromosome topology may have an impact in controlling sister chromatid cohesion as well as different aspects of the large-scale chromatin re-organization required for mitotic chromosome assembly.

Here we show that in human cells, the Smc5/6 complex plays an important role in chromosome architecture and segregation. Depletion of Smc5 and Smc6 results in aberrant mitotic chromosomal structures characterized by a "curly" deformation of sister chromatids with an additional high incidence of DNA damage-related phenotypes in cancer HeLa cells, and immunofluorescence analysis indicated an impaired chromosomal axial localization of topo IIα and the condensin Smc2 under these conditions.

MATERIALS AND METHODS

Antibodies

Antibody to Smc2 was generously provided by Jan-Michael Peters (Research Institute of Molecular Pathology, Vienna, Austria). Antibodies against other proteins were as follows: topoisomerase IIα (74715; Abcam, Cambridge, UK), BLM (sc-7790, Santa Cruz Biotechnology Inc., Santa Cruz, CA), PICH (Clone 142-26-3, Millipore Corporation, Billerica, MA).

Cell synchronization, inhibitor treatment, and cell lines

HeLa and RPE-1 cells were cultured in Dulbecco's Modified Eagle's Medium (Wako, Tokyo, Japan) supplemented with 10% fetal calf serum (FBS, Equitech-Bio Inc., Tokyo, Japan), 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin ((Meiji Seika, Tokyo, Japan) at 37°C in a 5% CO_2 environment. RPE-1 cells were synchronized at G0/G1 by serum starvation for 96 h and then released into the cell cycle with fresh medium containing 10% fetal calf serum for further analysis. For enrichment of cells in prometaphase, 50 ng/ml nocodazole was added 7 h after release from the second thymidine block for 1 or 2 h; RPE-1 cells were treated with 15 mM *S*-trityl-L-cysteine (STLC; TCI, Tokyo, Japan) for 2 h after release from RO-3306 arrest or 31 h after release from serum starvation for 2 or 3 h, and then recovered by mitotic

shake-off. For chromosomal phenotype analysis with Giemsa stain (Merck, Darmstadt, Germany), HeLa cells were harvested 11 h after release from the second thymidine block.

RNAi

siRNA sequences were as follows: Smc5 siRNA1, 5′-GGAACUUCAGCAGGGCUUUAAUAGUA-3′; Smc5 siRNA2, 5′-GGCAUUAUGUGAAGGCGAAAUAAUU-3′; Smc6 siRNA1, 5′-CAAAUUCUUCAUGAAAGCAACGCAA-3′; Smc6 siRNA2, 5′-GACCUAUCUUGAUCUGGAUAGUAAA-3′. Smc6 siRNA1 was used for ChIP-Seq analysis of topo IIα. Cells were transfected by incubating 50 nM duplex siRNA with Lipofectamine™ RNAiMAX (Invitrogen) in antibiotic-free growth medium. RNAi was performed concomitantly with the synchronization with thymidine or serum starvation, and 48 h of transfection with each siRNA was required for maximal knockdown of Smc5 and Smc6. For control transfections, the same annealing reaction was set up using without the presence of siRNA oligonucleotides.

Chromosome spreads

RPE-1 cells treated with STLC (15 mM) for 2–3 h were collected by mitotic shake-off and hypotonically swollen in a 40:60 mix (v/v) of PBS and distilled water for 5 min at room temperature. Cells were fixed with freshly made Carnoy's solution, dropped on glass slides, and dried. Slides were stained with 5% Giemsa stain, washed with water, air-dried, and mounted with Entellan embedding agent.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with ice-chilled 100% methanol for 20 min at −20°C or with 4% paraformaldehyde in 0.137 M sodium phosphate buffer (pH 7.4). Fixed cells/chromosomes were permeabilized with 0.5% Triton X-100 in PBS and incubated with 3% bovine serum albumin in PBS for at least 1 h. Cells were incubated with the primary antibodies overnight at room temperature followed by incubation with secondary antibodies for 35 min together with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). The secondary antibodies used in this study were goat anti-rabbit IgG and goat anti-mouse IgG coupled to Alexa Fluor 488 or 568, and goat anti-human IgG coupled to Alexa Fluor 568 (Molecular Probes, Carlsbad, CA). Cells were washed twice and mounted in Prolong Gold anti-fade mounting reagent (Invitrogen). Images were acquired on a Zeiss Imager Z.1 microscope equipped with epifluorescence and a Cool Snap HQ CCD camera (Photometrics Ltd., Tucson, AZ). 3D projections of the

immunofluorescence images were carried out using the 3D Volume viewer tool in ImageJ software (Wayne Rasband, NIH).

PNA Probes

The peptide nucleic acid (PNA) probe for telomeric sequences is a ready-to-use probe included in the Telomere PNA FISH Kit/Cy3 (K5326 from DAKO, Glostrup, Denmark). The hybridization buffer contained 70% formamide.

Fluorescence *in Situ* Hybridization (FISH)

Chromosome preparations of HeLa were made using standard procedures. The hybridization was performed according to the manufacturer's instructions. In brief, this procedure includes the following steps: slides were washed twice with TBS buffer (Tris-buffered saline, pH 7.5), treated with proteinase K for 10 minutes at room temperature, fixed in ethanol and air-dried. PNA probes were denaturated together with the slide at 80°C for 3 minutes and hybridized for 30 to 60 minutes at room temperature. After extensive washing, first at 65°C and then at room temperature, the slides were mounted with antifade solution (Vector Laboratories, Burlingame, CA).

RESULTS

Abnormal chromosome morphology in Smc5- and Smc6-depleted RPE-1 cells

In order to determine the contribution of the Smc5/6 complex to human chromosome structure, RNA interference treatment was carried out using two independent oligonucleotides against Smc5 and two against Smc6 in RPE-1 cells synchronized at G0/G1 by serum starvation. Smc5/6-depleted cells were then serum re-stimulated, arrested subsequently in mitosis by *S*-trityl-L-cysteine (STLC) treatment and chromosome spreads were prepared and visualized by Giemsa staining (Figure 4-1).

In control cells, chromosomes had the characteristic rod-shaped structure and sister chromatids remained tightly connected at the centromeric region. In the Smc5- and Smc6-depleted cells, however, a high proportion of metaphase cells exhibited an abnormal chromosomal conformation characterized by a "curly" appearance. Asymmetrical distribution of condensed chromatin toward the end of the chromosomes was also observed in a sub-population of the siRNA-treated cells. In addition, ~25% of chromosomes exhibiting the curly phenotype also showed a cohesion defect in which sister chromatids appeared in a side-by-side arrangement (Figure 4-1). These

frequencies were reproduced using two additional oligonucleotides against Smc5 and Smc6 respectively (Figure 4-2).

Smc5/6 depletion induces chromosome mis-segregation

We then aimed to investigate the fate of chromosome segregation in the absence of Smc5/6. Immunofluorescence analysis revealed that Smc5- and Smc6-depleted cells showed a high frequency of lagging chromosomes and anaphase bridges, the majority of which were positive for Plk1-interacting checkpoint helicase (PICH) and Bloom syndrome helicase (BLM) immunostaining (Figure 4-3), indicating that they mainly represent centromeric unwound catenane structures (Baumann et al., 2007).

Moreover, immunostaining of a centromeric antigen by CREST antisera indicated that post-mitotic micronuclei were formed containing single or multiple chromosome fragments (Figure 4-3, B and C), supporting the hypothesis that chromosome loss/breakage occurred during cell division. Thus, the presence of Smc5 and Smc6 during the preceding interphase appeared to be a prerequisite for proper chromosome assembly and segregation in subsequent mitosis.

Defective axial localization of topo IIα and condensin in the absence of the Smc5/6 complex

We then aimed to determine whether the abnormal "curly" chromosome morphology observed in Smc5- and Smc6-depleted cells could be reflecting a deficiency in higher-order chromatin structure. The main constituents of the so-called dynamic "chromosome scaffold" correspond to the DNA topoisomerase IIα and the two condensin complexes present in humans (condensin I and condensin II), colocalizing at the axes of metaphase chromatids (Maeshima et al., 2003). Thus, we examined the binding pattern of topo IIα and Smc2 (a common component of both condensin complexes) in chromosome spreads of Smc5 and Smc6 siRNA–treated and control RPE-1 cells by immunofluorescence (Figure 4-4 and 4-5).

Staining in the control cells showed a normal beaded/coil-like pattern along the axis of each chromatid, with an intense signal of topo IIα concentrated at the centromeres, as previously observed (Maeshima et al., 2003). However, both Smc5- and Smc6-depleted cells showed an abnormal distribution of topo IIα along the chromosome axis, resembling the curly appearance of chromatids observed in Giemsa staining, with an additional striking enrichment toward the end of the chromosome arms - which was typically seen in chromosomes with hypocondensed centromeres - in an opposite fashion to the centromeric enrichment of topo IIα in control cells (Figure 4-4).

When we analyzed the chromosomal localization of Smc2, an irregular distribution of the protein along the chromatid lengths with less defined axial structure was observed in Smc5/6-depleted cells compared with control cells (Figure 4-5). A 3D projection reconstitution further confirmed the aberrant distribution of condensin in Smc5/6-depleted cells. In some cases, we observed that the telomeric staining of Smc2 appeared to be connected between the sister chromatids, giving the image of "closed" chromosomes, and further analysis by telomeric FISH confirmed the high incidence of linkages between sister telomeres in Smc5/6-depleted cells (Figure 4-6, B).

Enigmatically, some chromatids with the intense distal staining showed a double axis–like staining for Smc2, particularly in chromosomes with the hypocondensed-centromere phenotype. Telomeric FISH also showed that Smc5/6-depleted cells usually contained chromatids with two spots of telomeric signal, which probably correspond to the double axis-like staining of Smc2 (Figure 4-6, B).

A further structural analysis of the chromosomal axis by double staining of topo IIα and Smc2 revealed that the characteristic barber pole–like alternate distribution of these proteins (Maeshima et al., 2003) was frequently disrupted in Smc5- and Smc6-depleted cells (Figure 4-6, A). Together, these results strongly suggested that, as in the case for other Smc-containing complexes, the Smc5/6 complex is crucial for

mitotic chromosome assembly, and the cellular localization of the complex argues a specific role for this complex during interphase.

Abnormal chromosome morphology in Smc5- and Smc6-depleted Kyoto HeLa cells

When Smc5 and Smc6 were depleted in HeLa Kyoto cells after siRNA combined with double thymidine block, we were also able to observe the curly phenotype and the cohesion defect with telomeric linkages between sister chromatids detected in RPE-1 cells (Figure 4-7); however, in HeLa cells the curly abnormal structure was accompanied by a variety of additional chromosome rearrangements such as chromatid gaps, chromosome breaks (affecting only few or the whole set of chromosomes), complex exchange structures and telomeric fusions, all of them known to be product of unrepaired DNA damage arising during DNA replication.

Moreover, the phenotype characterized by centromeric hypocondensation which was found in RPE-1 cells was barely observed in HeLa cells depleted of Smc5/6, but instead, we detected an strictly high incidence of centromeric repulsion between sister chromatids (Figure 4-7, A and C). We then classified these observations into four groups, representing different ¨phenotypic stages" in which a more severe

replication-related DNA damage correlated with a more severe curly deformation, sister chromatid cohesion defect and centromeric repulsion (Figure 4-7).

Altogether, these abnormalities accounted for about 80% of the observed metaphases in Smc5/6-depleted cells. Importantly, immunofluorescence analysis confirmed that topo IIα axial staining also presented a "curly" pattern in these cell line, but additionally, it also appeared almost complete excluded from the centromeres (determined by CREST4 staining), a situation that strikingly contrasted to the centromeric enrichment of topo IIα observed in control cells (Figure 4-8).

These results indicate that in HeLa cell line, the absence of the Smc5/6 complex also affected chromosome structure and topo IIα axial localization as in the case of RPE-1 cells, but in this cancer cell line, an additional and severe disruption in centromeric chromatin assembly is generated, characterized by the repulsion between sister chromatids –revealing a premature loss of cohesion exclusive to these regions- and also by the nearly complete absence of topo IIα centromeric localization. Importantly, the higher incidence of chromosome aberrations in this cancer cell line indicates a more profound defect in the DNA damage response in the absence of Smc5/6 compared to RPE-1 cells.

DISCUSION

The Smc5/6 complex has been implicated in DNA repair, chromosome replication and chromosome segregation in different model species (De Piccoli et al., 2009) but so far, a possible role in regulating high level chromatin organization in normal cell cycle conditions had not been into consideration. Here, we have presented evidence indicating that the absence of the Smc5/6 complex in human cells generates an abnormal "curly" chromatid conformation, and an asymmetrical distribution of condensed chromatin toward the end of the chromosomes during mitosis.

This study suggests that the function of Smc5/6 is essential to form an intact axial structure of mitotic chromosomes in human cell lines. Topo IIα tended to accumulate at the distal arms and showing fewer or not centromeric staining in Smc5- and Smc6-depleted RPE-1 and HeLa cells, in opposition to the regular enrichment of the protein at this region in untreated cells (Figure 4-4). Both, the aberrant chromosome morphology as well as the abnormal distribution of topo IIα would be directly related to the high frequency of lagging chromosomes and anaphase bridges observed in Smc5/6-depleted cells, with the subsequent formation of post-mitotic micronuclei containing single or multiple chromosome fragments (Figure 4-3). Indeed, the majority of these anaphase bridges were PICH and BLM-positive (Figure 4-3, D-F), indicating

that they mainly represent centromeric unwound catenane structures, a hallmark of inefficient topo IIα decatenation activity at the centromeres (Baumann et al., 2007).

Moreover, our immunofluorescence results clearly show that not only topo IIα but also Smc2 –a component of human condensin complexes- showed aberrant chromosome staining in Smc5/6-depleted cells. Under these conditions, Smc2 protein presented a disorganized distribution along the axis of mitotic chromosomes, sometimes accompanied by a double axis–like pattern of Smc2 staining, which is a very intriguing observation (Figure 4-5). This condition reflects a fundamental defect in the global program of nucleosome fiber folding around the chromosome center, a process that is regulated by condensins and has recently been termed the "dynamic melted nucleosome" model of higher-order chromosome assembly (Nishino et al., 2012). Additional analyses are required to elucidate the mechanisms by which this abnormal conformation is generated in the context of Smc5 and Smc6 depletion.

The observation of an abnormal axial distribution of condensin in the context of Smc5/6 depletion supports the idea of a possible coordination between topo IIα and condensin chromosomal localization, or alternatively, it could indicate an additional interplay between condensin and the Smc5/6 complex in coordinating chromosome assembly. Indeed, it was recently found that condensin II promotes structural reorganization of duplicated chromosomes immediately after the replication (Ono et al., 2013), and thus, if a coordinated function of Smc5/6 and condensin do actually exist, it is plausible to conceive it as taking place during DNA replication. Further analyses are

required to elucidate the mechanisms by which the abnormal distribution of condensin is generated in the context of Smc5 and Smc6 depletion.

Importantly, although RPE-1 and HeLa cells shared the curly chromosomal phenotype as well as the curly topo IIα axial staining along chromosome arms in the absence of Smc5/6, they also presented several different chromosomal conformations. While RPE-1 showed the curly phenotype accompanied by centromeric hypocondensation, in the case of HeLa cells this uneven condensation along chromosomes was barely seeing in HeLa but conversely, they presented a high incidence of premature sister chromatid separation. Among these cohesion defects, we detected completely separated sister chromatids, but also an interesting conformation in which cohesion has been lost along the entire length of the sister chromatids except at the telomeric region where chromatids remained tightly linked (somehow resembling the "close chromosomes" observed with condensin immunostaining in RPE-1 cells).

An interesting observation was the frequent loss of centromeric cohesion observed in Sm5/6-depleted HeLa but not RPE-1 cells. The fact that topo IIα centromeric enrichment is absent in these chromosomes, and that in fact topo IIα binding is lost from regions positive for CREST signal indicate that this abnormal conformation is not merely a consequence of uncohesed sister centromeres but implies a

more complex disorder in chromatin assembly that may include condensation and decatenation defects.

In this respect, centromeric repulsion is a known hallmark of cells harboring mutations in the ESCO2 gene that result in the disruption of the acetyltransferase domain of the protein (Skibbens et al, 2005). This indicates that cohesin acethylation and subsequent cohesion establishment during S phase importantly contributes to centromeric structure and the determination of the primary constriction within mitotic chromosomes.

There is also increasing evidence that links disruption of the cohesin complex or the cohesion pathway to the activity of several proteins involved in the DNA damage repair. Mutations in Werner syndrome helicase (WRN) and BRCA1 protein result in precociously separated sister chromatids especially at heterochromatin regions, a remarkably similar phenotype to the one observed in Smc5/6-depleted HeLa cells in our study. These findings were interpreted to involve a “decatenation” checkpoint function (Franchitto et al., 2003), although it has been proposed that the deficient activity of these helicases directly contribute to the loss of sister chromatid cohesion as it has been observed in yeast (Skibbens 2005). Significantly, many of these proteins exhibit similar functions in stalled fork re-initiation and/or fork stabilization, indicating that replication-related DNA damage pathways may be critical components of cohesion

establishment (Xu et al., 2004; Bjergbaek et al., 2005) and thus, a direct link between replication-related DNA damage response and maintenance of centromeric structure and function may also exist.

Indeed, the Smc5/6 complex has been recently found to regulate replication-related recombinational events at centromeres in the absence of DNA damaging agents in yeast (Yong-Gonzalez et al., 2012) and in addition, the SUMO ligase activity of Nse2 appeared to promote the SUMOylation of two kinetochore proteins affecting their interaction with mitotic spindles. These results suggest that the Smc5–Smc6 complex regulates both recombination and kinetochore SUMOylation to facilitate chromosomal maintenance during growth.

Our studies have revealed the importance of the Smc5/6 complex in maintaining the integrity of centromeric chromatin assembly, although it is still to be determined whether Smc5/6 exerts the same recombination and SUMOylation-dependent regulatory functions at these chromosomal regions in human cells.

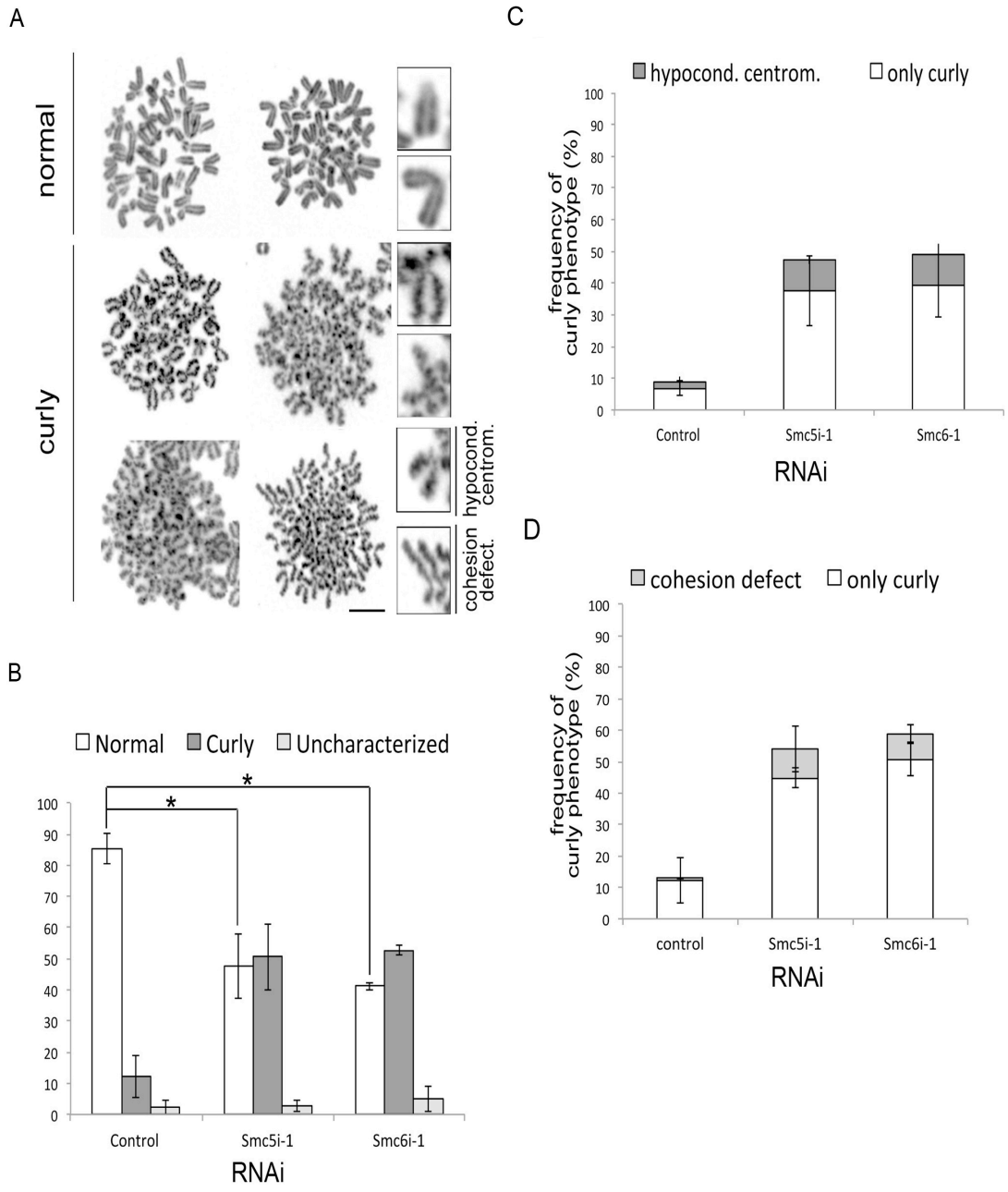


Figure 4-1. RNA interference (RNAi) mediated depletion of Smc5 and Smc6 in RPE-1 cells. A. RPE-1 cells transfected with siRNAs against Smc5 or Smc6 were synchronized by serum starvation for 144 h, allowed to recover in serum-containing medium, and then treated with STLC to induce arrest in mitosis. Cells were harvested, and Giemsa-stained chromosome spreads were prepared. Bar = 10 μm. B. Incidence of mitotic cells with curly chromosomes in cells transfected with indicated siRNAs. (C, D) Additional defects observed within the curly chromosomes in cells depleted of Smc5 or Smc6. Proportion of cells with hypercondensation of distal arms (C) and those with

cohesion defect (D) were shown in the histogram. For B – D, 300 cells per indicated condition were examined. Bar graph shows mean ± SD from three independent experiments; P value <0.05;*P value 0.01 to 0.05; **P value 0.001 to 0.01, two-tailed student's t-test.

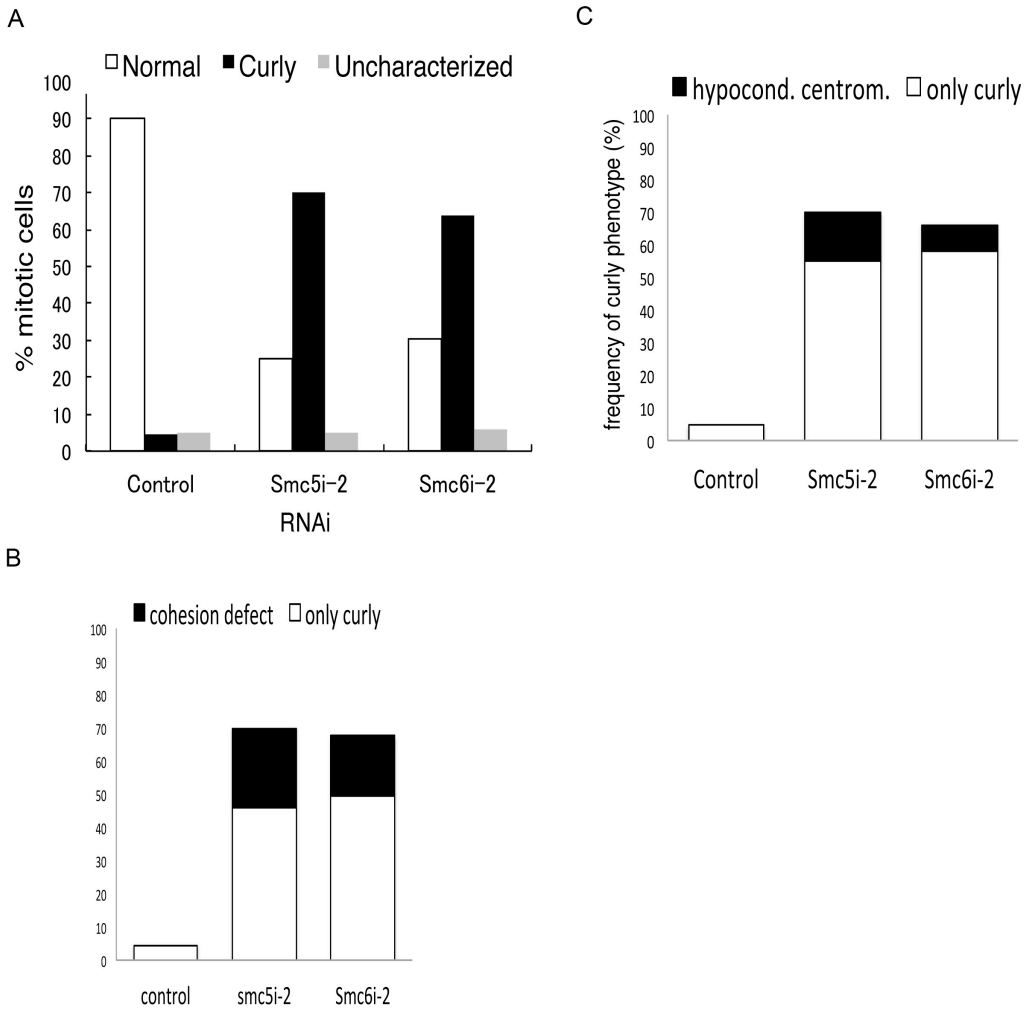


Figure 4-2. Abnormal chromosome morphology in Smc5/6-depleted cells. (A) RPE-1 cells transfected with another two independent siRNAs against Smc5 or Smc6 were synchronized by serum starvation for 144 h, allowed to recover in serum-containing medium, and then treated with STLC to induce arrest in mitosis. Cells were harvested, and Giemsa-stained chromosome spreads were prepared. The incidence of mitotic cells with abnormal chromosome structure is indicated. (B and C). Additional defects observed within the curly chromosomes in cells depleted of Smc5 or Smc6. Proportion of cells with hypercondensation of distal arms (B) and those with cohesion defect (C) were shown in the histogram.

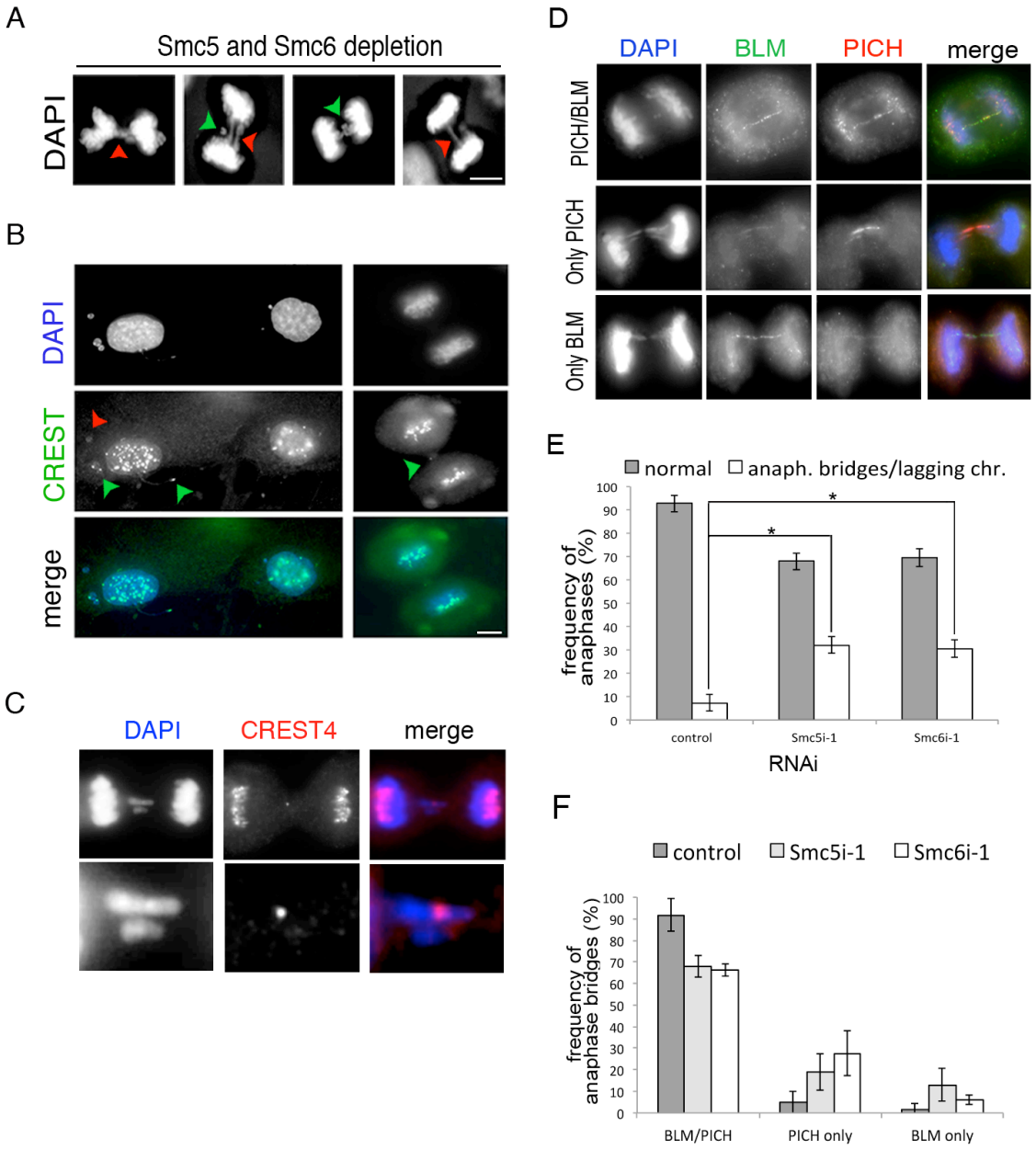


Figure 4-3. Depletion of Smc5 or Smc6 disrupts chromosome segregation. (A). Example images of the anaphase bridges (red arrowheads) and lagging chromosomes (green arrowheads) frequently seen in Smc5/6-depleted cells. Bar = 10 μm. B and C. Examples of the formation of micronuclei in Smc5- and Smc6-depleted cells. Green arrows indicate micronuclei containing the centromere marker CREST, and red arrows indicate micronuclei negative for the CREST signal. Bar = 10 μm. D. Representative images of PICH (red) and BLM (green) immunofluorescence in anaphase bridges. DNA

was counterstained with DAPI (blue). Bar = 10 μm. E. Frequency of anaphase bridges and lagging chromosomes within the anaphase chromosomes observed in the control and siRNA-treated cells. Three hundred anaphases were analyzed per condition. Bar graph shows mean ± SD from three independent experiments; P value <0.05; **P value 0.001 to 0.01, two-tailed student's t-test. F. Frequency of anaphase bridges positive for PICH and BLM, PICH only, or BLM only. One hundred anaphase bridges were analyzed for each sample.

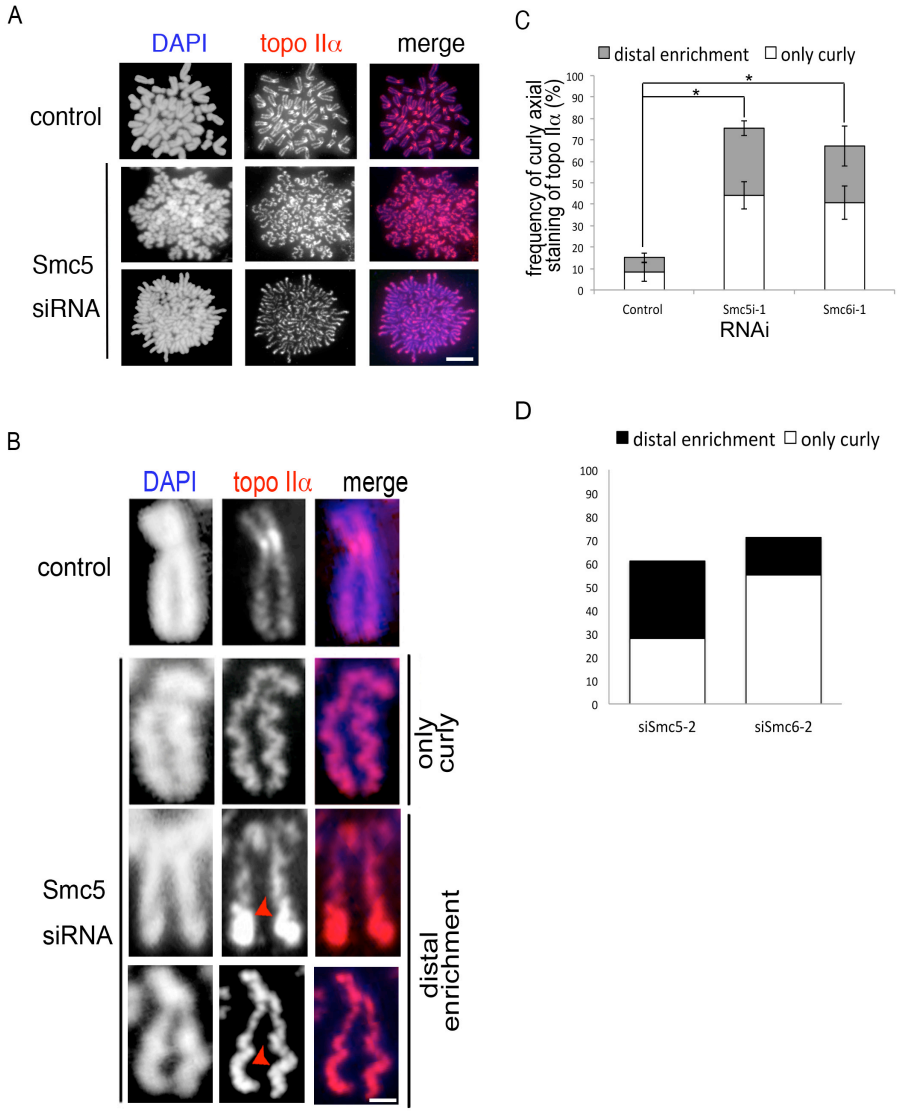


Figure 4-4. Chromosome axis deformation in Smc5/6-depleted cells. A. RPE-1 metaphase cells transfected with siRNAs against Smc5 or Smc6 or a control mock were subjected to hypotonic treatment, fixation, and staining for DNA (DAPI) and topo IIα. Right panels show the merged images of DAPI (blue) and topo IIα (red). Bar = 5 μm. B. Examples of individual chromosome morphologies observed in control and Smc5-depleted cells. In merged images in the right panels. DAPI staining is shown in blue and topo IIα in red. Red arrowheads point to the defects mentioned. Bar = 1 μm.

C. Frequency of curly axial staining of topo IIα with or without enrichment at distal chromosome regions. Three hundred metaphases were analyzed for each sample. Bar graph shows mean ± SD from three independent experiments; P value <0.05; **P value 0.001 to 0.01, two-tailed student's t-test. D. Frequency of curly axial staining of topo IIα with or without enrichment at distal chromosome regions.

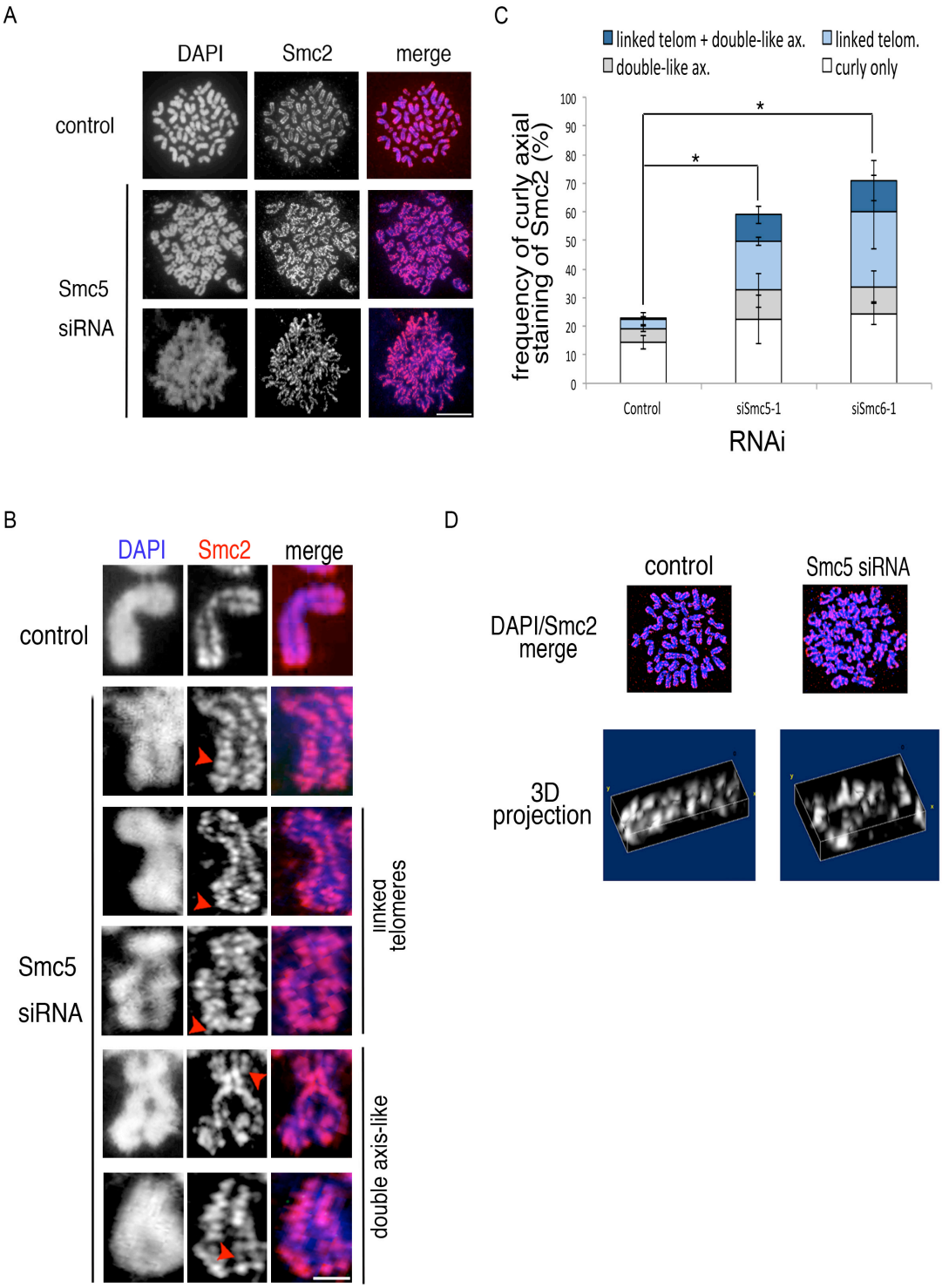

A
DAPI
Smc2
merge
control
Smc5
siRNA
C
linked telom + double-like ax.
linked telom.
double-like ax.
curly only
frequency of curly axial staining of Smc2 (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
Control
siSmc5-1
siSmc6-1
RNAi
B
DAPI
Smc2
merge
control
Smc5
siRNA
linked telomeres
double axis-like
D
control
Smc5 siRNA
DAPI/Smc2 merge
3D projection

Figure 4-5. A. Metaphase chromosome spreads were prepared from control and Smc5-depleted cells and stained with DAPI (blue) and anti-Smc2 (red). Bar = 5 μm. B. Images of individual chromosomes showing the linked telomeres and "double axis-like" staining observed in the Smc5/6-depleted cells. Smc2 staining is in red, and DAPI in blue. Bar = 1 μm. C. Frequency of disorganized axial Smc2 staining (white) or with at least one chromosome with additional "double axis-like" staining (grey), linked telomeres (light blue), or a combination of both linked telomeres and "double axis-like" staining (dark blue). Three hundred metaphases were analyzed for each sample. Bar graph shows mean ± SD from three independent experiments; P value <0.05;*P value 0.01 to 0.05; **P value 0.001 to 0.01, two-tailed student's t-test. D. 3D reconstruction of the Smc2 staining of metaphase chromosomes in the Smc5/6-depleted cells.

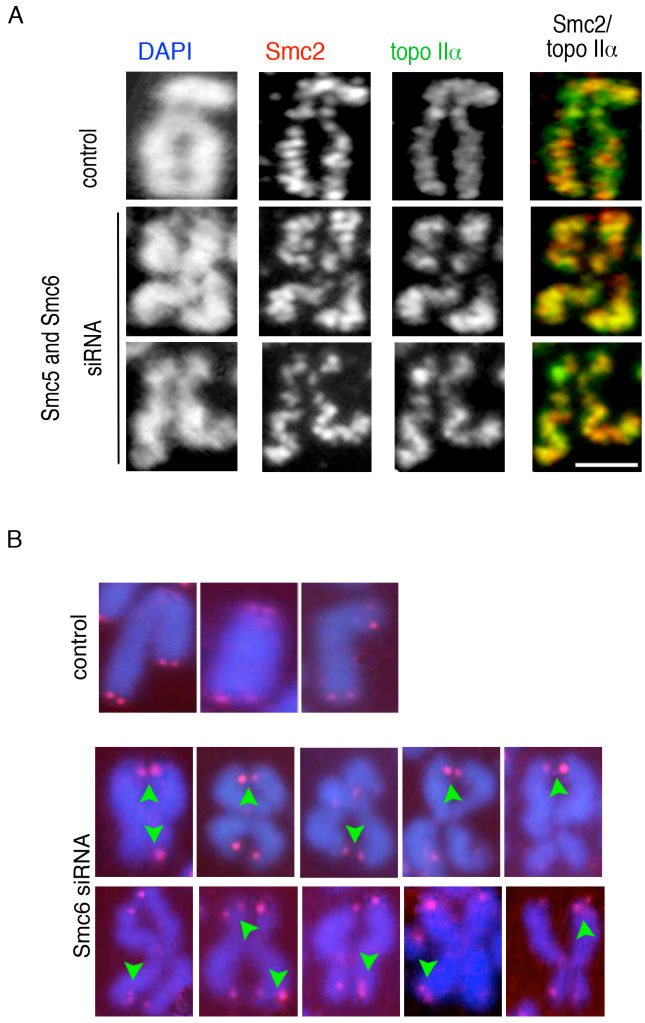
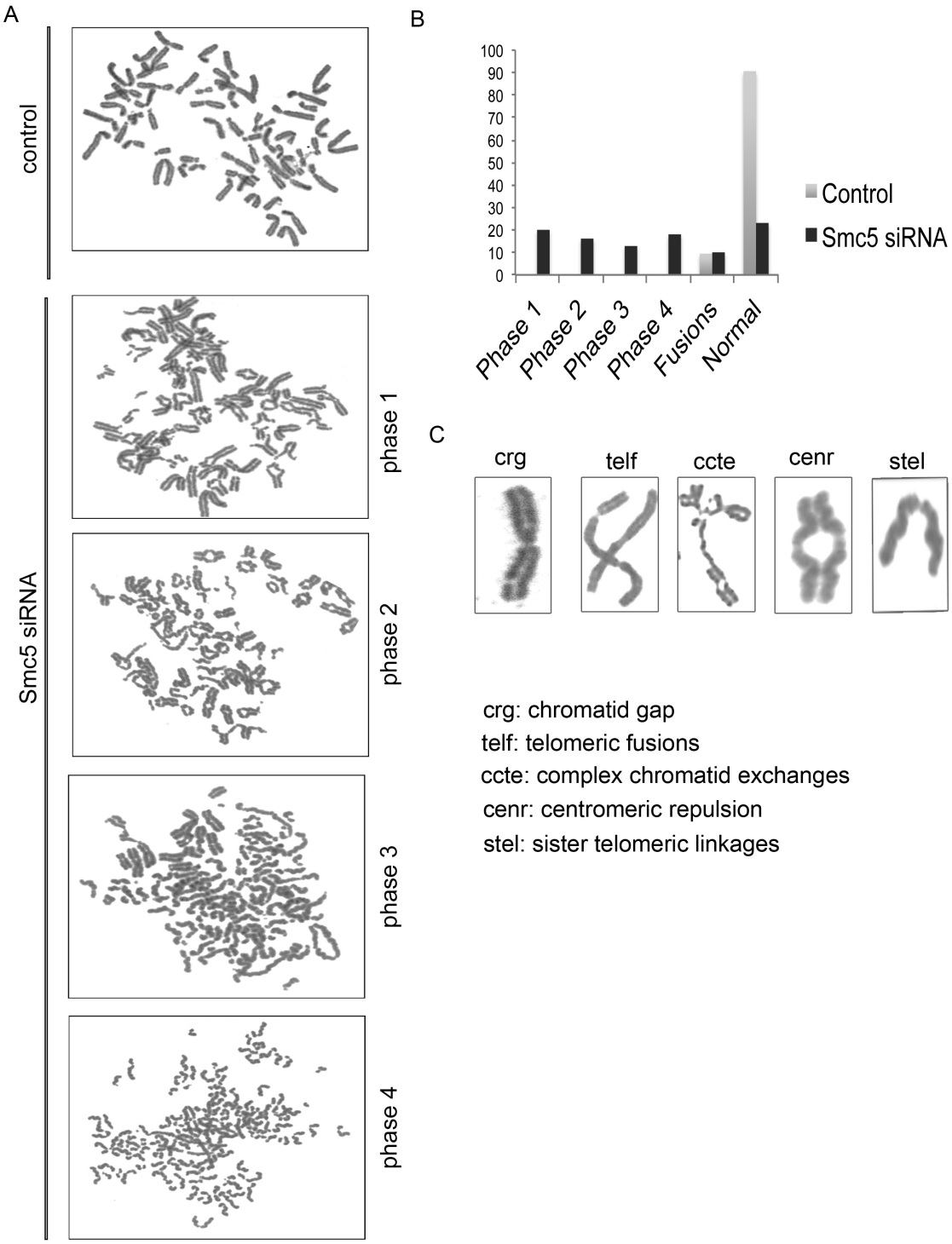


Figure 4-6. A. Representative examples of chromosomes observed in metaphase spreads from control and Smc5- or Smc6-depleted cells stained with DAPI, Smc2 and topo IIα, as indicated. In merged images, Smc2 is shown in red and topo IIα in green. Note that the characteristic barber pole–like alternate pattern of topo IIa and Smc2 distribution in control cells contrasts with the irregular distribution in Smc5/6-depleted cells. Bar = 1 μm. B. FISH images of RPE-1 cells using TelC-Cy3 (Telomere PNA FISH probe) (red) and DNA staining by DAPI (blue)/ Green arrowheads point linked sister telomeres and fragmented telomeric signals.



crg: chromatid gap
telf: telomeric fusions
ccte: complex chromatid exchanges
cenr: centromeric repulsion
stel: sister telomeric linkages

Figure 4-7. Chromosomal phenotype after depletion of Smc5 and Smc6 in HeLa cells. A. HeLa cells transfected with siRNAs against Smc5 were synchronized by double thymidine block, released and treated with nocodazole at 8 hour for another 4 hours to induce arrest in mitosis. Cells were harvested, and Giemsa-stained chromosome spreads were prepared. Phenotypes are classified into four phases based on the severity of the defects in chromosome structure. B. Incidence of mitotic cells with chromosome showing chromosome abnormalities as classified in (A) in cells transfected with indicated siRNAs. C. Individual chromosome phenotypes are further characterized as crg (chromatid gaps), telf. (telomeric fusion-like), ccte (complex chromatid exchanges), cenr (centromeric repulsion) or stel (sister telomeric linkages).

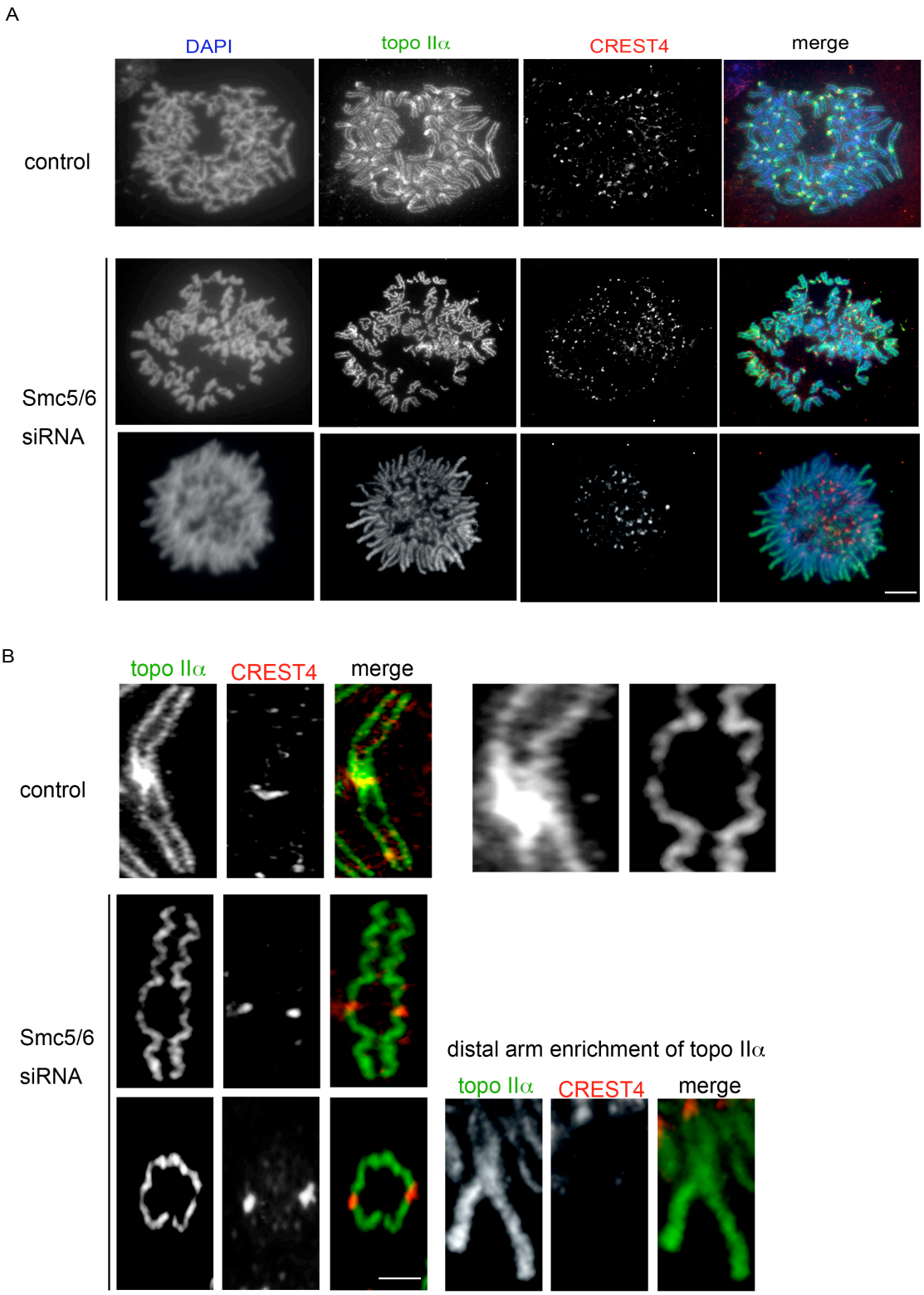

A
DAPI
topo IIα
CREST4
merge
control
Smc5/6
siRNA
B
topo IIα
CREST4
merge
control
Smc5/6
siRNA
distal arm enrichment of topo IIα
topo IIα
CREST4
merge

Figure 4-8. Chromosome axis and centromeric deformation in Smc5/6-depleted HeLa cells. A. HeLa metaphase cells transfected with siRNAs against Smc5 or a control mock were subjected to hypotonic treatment, fixation, and staining for DNA (DAPI, blue), CREST4 (red) and topo IIα (green). Bar = 5 μm. B. Examples of individual chromosome morphologies observed in control and Smc5-depleted cells. In merged images in the right panels. CREST4 staining is shown in red and topo IIα in green. Bar = 1 μm.

CHAPTER V: SMC5/6 COMPLEX LINKS REPLICATION PROGRESSION TO TOPO IIα CHROMATIN BINDING AND DISTRIBUTION

INTRODUCTION

Chromosome condensation is an important cellular mechanism by which chromatin fibers are disentangled and packaged into compact chromosome structures in mitosis. This process is critical for the subsequent chromosome congression and separation that occurs in metaphase and anaphase, respectively, which are essential in the maintenance of genome stability and cell viability. However, how precisely the pattern of higher-order chromatin organization is determined remain largely a mystery.

Early structural studies suggested that chromosome compaction is achieved after consecutive folding and coiling events, transforming the 10-nm "beads-on string" structure composed of nucleosome arrays, into a series of fibers approximately 30 nm, 200 nm, and 700 nm in diameter. (reviewed in Koshland and Strunnikov, 1996). However, recent studies have indicated that human chromosomes are mainly composed of irregularly folded nucleosome fibers rather than 30-nm chromatin fibers (Maeshima et al., 2010; Nishino et al., 2012). This new line of evidence suggests that the organization of mitotic chromosomes involves irregular arrangements of nucleosome fibers, and that a chromosomal axial structure corresponding to a dynamic protein

scaffold holds the nucleosome fibers globally around the center of the chromosome (Maeshima and Laemmli, 2003; Swedlow and Hirano, 2003; Ono et al., 2004; Hirota et al., 2004).

The discreet subset of proteins within the axial chromosome scaffold consists mainly of two major bands, termed Sc1and Sc2 (Lewis and Laemmli, 1982). Sc1 was later identified as topoisomerase II (Gasser et al., 1986), whose roles in chromosome condensation, and relaxation of negative supercoils and DNA catenations have been elucidated in subsequent studies (Uemura et al., 1987). Sc2 was identified as the Smc2 protein, member of the multi-subunit complex condensin (Maeshima et al., 2003), which has been shown to possess positive supercoiling activities *in vitro* and is thought to coil up chromosomes by introducing global positive writhe (Kimura et al., 1999). It is unlikely, however, that the complex is sufficient to compact chromosomes completely during mitosis, as genetic analyses in Drosophila have indicated that chromosome compaction can be achieved in the absence of condensin function (Bhat et al., 1996), thus raising the question about what additional processes may be required for the reorganization of chromatin in the transition from interphase to mitosis, and how these mechanisms are regulated in time and space.

In this context, the role of DNA replication in chromosome condensation has been largely overlooked. The relationship between DNA synthesis and higher-order chromatin organization was first proposed when several analyses revealed a correlation

between apparent chromosome fiber loop lengths and mean distances between replication origins (Marsden and Laemmli, 1979).

Supporting this notion, studies in *Drosophila* have indicated that mutations in components of the origin recognition complex (ORC), which serves as the platform upon which replication initiation complexes assemble, generated a mitotic arrest with aberrantly condensed chromosomes that failed to align at the metaphase plate and a poorly assembled mitotic spindle (Loupart et al., 2000; Pflumm and Botchan, 2001). In these mutants, the pattern of chromosomal replication was markedly altered, with some euchromatic regions replicating even later than heterochromatin (which is normally replicated after euchromatin).

Importantly, the abnormally late replicating regions of euchromatin exhibited the greatest problems in mitotic condensation. Similar results were obtained with mutant versions of MCM4, a subunit of the putative DNA replicative helicase, and PCNA, an essential cofactor for DNA synthesis, suggesting that DNA replication may be a requirement for proper chromosomal condensation.

Human cells possess two different condensin complexes, known as condensins I and II. While condensin is located in the cytoplasm from interphase to prophase, and binds to chromosomes after nuclear envelope breakdown (NEBD) in prometaphase, condensin II localizes to the nucleus during interphase, regulating early

stages of chromosome condensation in prophase, and also collaborating with condensin I to shape chromosomes after prometaphase. Interestingly, it was recently shown that condensin II changes its chromatin-binding property during S phase, and premature chromosome condensation (PCC) assays indicated that depletion of condensin II compromised PCC-driven sister chromatid resolution during S phase.

When mild replicative stress was induced, it partially impaired condensin II-dependent sister chromatid resolution, and further exacerbated phenotypes arising from condensin II depletion, such as the curly conformation of chromosome axes. A proposed explanation for these observations is that, as dormant origins are trigger in the context of reduced replication fork progression, the size of chromatin loops is also reduce (Courbet et al., 2008), which in turns restricts the distribution and/or action of condensin II during S phase, generating a distorted axes in metaphase chromosomes.

Finally, analyses on the chromatin dynamics of topoisomerase II in *Xenopus* egg extracts have indicated that topo II plays a crucial role in linking DNA replication to mitotic chromosome condensation (Cuvier & Hirano 2003). These study showed that the presence or absence of DNA replication affects the pattern of topo II-induced chromosome condensation during subsequent mitosis. Topo II accumulates on an axial structure when mitosis is induced only after DNA replication (G1 phase-induced cells failed to form topo II-containing axial structures), and this conformation can be visualized in the absence of condensin, which seems to be required in subsequent steps

of chromosome assembly. It was then speculated that the close association of topo II with the fork movement may prime the assembly of the subchromosomal axial structure during S-phase, which can only be visualized on mitotic entry, and that this replication-dependent reorganization of topo II distribution might also contribute to the removal of nonreplicative catenanes.

Here we present evidence indicating that the abnormal replication progression observed in Smc5/6-depleted cells interferes with the topo IIα re-localization onto chromosomes before entering mitosis. These defects may be directly related to the abnormal axial distribution of topo IIα and the mitotic chromosomal phenotype observed in Smc5/6 depletion and reveals an important aspect of the temporal harmonization of DNA replication and the determination of mitotic chromosome assembly.

MATERIALS AND METHODS

Antibodies

SMC6L1 (M01, Cl7one 2E6; Abnova, Taiwan), DNA topoisomerase IIα (D081-1, clone 8D2; MBL, Aichi, Japan), DNA topoisomerase IIα (74715; Abcam, Cambridge, UK), and anti-SMC1 antibody (21583; Abcam, Cambridge, UK).

Cell synchronization, inhibitor treatment, and cell lines

RPE-1 cells were cultured in Dulbecco's Modified Eagle's Medium (Wako, Tokyo, Japan) supplemented with 10% fetal calf serum (FBS, Equitech-Bio Inc., Tokyo, Japan), 0.2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin ((Meiji Seika, Tokyo, Japan) at 37°C in a 5% CO_2 environment.

RPE-1 cells were synchronized at G0/G1 by serum starvation for 96 h and then released into the cell cycle with fresh medium containing 10% fetal calf serum for further analysis. For visualization of regions with newly synthesized DNA, cells were cultured in medium supplemented with 10 μM EdU kit (Invitrogen, Carlsbad, CA) for 0.5 h before fixation.

PCC Assay

PCC was induced by adding Calyculin A (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) into culture media at a final concentration of 160 nM. After 1 h, cells were hypotonically swollen in a 40:60 mix (v/v) of PBS and distilled water for 5 min at room temperature. Cells were fixed with freshly made Carnoy's solution (70% methanol, 30% acetic acid), dropped on glass slides, and dried. Slides were stained with 5% Giemsa stain, washed with water, air-dried, and mounted with Entellan embedding agent (Merck). For detection of incorporated EdU, the Click-iT EdU imaging kit (Invitrogen, Carlsbad, CA) was used. Briefly, the glass slides were incubated with a reaction solution containing fluorochrome-azide (Alexa Fluor 488) for 1 h at room temperature after which immunofluorescence staining was conducted.

RNAi

siRNA sequences were as follows: Smc5 siRNA-1, 5′-GGAACUUCAGCAGGGCUUUAAUAGUA-3′; Smc6 siRNA-1, 5′-CAAAUUCUUCAUGAAAGCAACGCAA-3′;. Cells were transfected by incubating 50 nM duplex siRNA with Lipofectamine™ RNAiMAX (Invitrogen) in antibiotic-free growth medium. RNAi was performed concomitantly with the synchronization with thymidine or serum starvation, and 48 h of transfection with each siRNA was required

for maximal knockdown of Smc5 and Smc6. For control transfections, the same annealing reaction was set up using without the presence of siRNA oligonucleotides.

Immunofluorescence microscopy

Cells grown on coverslips were fixed with ice-chilled 100% methanol for 20 min at −20°C or with 4% paraformaldehyde in 0.137 M sodium phosphate buffer (pH 7.4). Fixed cells/chromosomes were permeabilized with 0.5% Triton X-100 in PBS and incubated with 3% bovine serum albumin in PBS for at least 1 h. Cells were incubated with the primary antibodies overnight at room temperature followed by incubation with secondary antibodies for 35 min together with 0.1 μg/ml 4,6-diamidino-2-phenylindole (DAPI). The secondary antibodies used in this study were goat anti-rabbit IgG and goat anti-mouse IgG coupled to Alexa Fluor 488 or 568, and goat anti-human IgG coupled to Alexa Fluor 568 (Molecular Probes, Carlsbad, CA). Cells were washed twice and mounted in Prolong Gold anti-fade mounting reagent (Invitrogen). Images were acquired on a Zeiss Imager Z.1 microscope equipped with epifluorescence and a Cool Snap HQ CCD camera (Photometrics Ltd., Tucson, AZ). 3D projections of the immunofluorescence images were carried out using the 3D Volume viewer tool in ImageJ software (Wayne Rasband, NIH).

ChIP-Seq Analyses

DNA from whole-cell extracts (WCE) and ChIP fractions was further sheared by sonication (Branson sonifier 250D), end-repaired, ligated to sequencing adapters and amplified according to the SOLiD Library Preparation kit Protocol (Applied Biosystems, Foster City, CA, USA). DNA purified from gel and amplified between 100 and 150 bp was sequenced on the Applied Biosystems SOLiD platforms (SOLiD 3 and 5500) in order to generate single-end 50-bp reads. Sequenced data of both ChIP fractions and WCE and aligned to the human genome (UCSC hg19) using Bowtie (Langmead et a., 2009), allowing three mismatches in the first 28 bases per read (-n3 option). All duplicate reads and those without unique alignment were removed from further analysis. ChIP-Seq and DNA sequencing data from this study have been deposited in the Sequence Read Archive database (http://www.ncbi.nlm.nih.gov/sra) under accession number SRS383137. We further analyzed only uniquely aligned reads. Each aligned read was extended to a predicted fragment length of 150 bp. Reads were summed in 100-kb windows along the chromosome, and the fold enrichment (ChIP/WCE) for each window was calculated.

RESULTS

Chromatin binding pattern of Smc6 and Topo IIα mislocalization upon Smc6 depletion during mitosis

In order to better understand the role of the Smc5/6 complex in chromatin organization as well as in the genome-wide regulation of topo IIα localization, we performed ChIP-Seq analysis of asynchronous HeLa cells and mitotically arrested cells transfected with control or Smc6 siRNA (Figure 5-1), and the chromatin immunoprecipitation was performed using commercial monoclonal antibodies against Smc6 (Abnova) and topo IIα (MBL). In the asynchronous sample, both Smc6 and topo IIα localized to binding sites along the entire length of the chromosomes with an apparent accumulation at regions of low gene density, in a similar profile HP1α chromatin binding obtained previously (Figure 5-1, D). Interestingly, within the 11633 Smc6 peaks detected, about 29% represented overlapped regions with cohesin's Rad21 binding sites obtained in previous ChIP-Seq analysis (Figure 5-1, C),

Moreover, the binding profile of topo IIα presented a dramatic reconfiguration once cells entered mitosis, with most of the protein concentrated at the centromeric and pericentromeric regions and a substantial decrease in the binding along the arms and telomeric regions, consistent with the topo IIα immunofluorescence microscopy.

Strikingly, in Smc6-depleted cells, topo IIα showed a markedly different mitotic chromatin-binding profile compared to that observed in control cells (Figure 5-2). Topo IIα enrichment at the centromeric/pericentromeric region was reduced in Smc6-depleted cells, and the general redistribution of topo IIα did not appear to take place, as the binding along the chromosome arms significantly resembled the pattern observed in asynchronous (interphase) cultured cells. Thus, these data indicated that the Smc5/6 complex is required for topo IIα chromosomal distribution during mitosis, which seems essential for proper axial conformation and general chromosome architecture.

Pre-mitotic topo IIα binding to chromatin is affected by the perturbed progression of replication in Smc5/6-depleted cells.

Our analysis indicated that mitotic chromosome structure was disrupted in the absence of Smc5/6. However, we also determined that the vast majority of the complex appeared to be excluded from mitotic chromosomes, and that the majority of the complex dissociates from chromatin as soon as nuclear envelope breakdown takes place. This conflictive situation led us to hypothesize that some aspect of Smc5/6 function before cells enter mitosis may alter chromosome organization later as they undergo condensation.

It has been proposed that a major determinant of the mitotic lethality of Smc5 and Smc6 mutants in fission yeast, apart from the permanent DNA linkages between sister chromatids, is a defective cohesin removal from chromosome arms (Outwin et al., 2009), which could reflect abnormal chromatin-binding dynamics of cohesin during interphase. However, we did not detect any persistent binding of cohesin on mitotic chromosomes after depletion of Smc5/6 in human cells (Figure 5-3), indicating that the abnormal mitotic phenotype in fission yeast seems mechanistically different from the chromosome structural defect we observed in human cells.

As it was shown before, monitoring of DNA replication by EdU incorporation, as well as by PCC and FACS analyses indicated that the progression of replication fork is slowed down in Smc5/6-depleted cells. Previous studies have revealed that preventing DNA replication perturbs the formation of topo IIα–containing axes and alters the chromatin-binding properties of topo IIa (Cuvier and Hirano, 2003). Given that Smc5/6 is required for the relocalization of topo IIα from interphase to mitotic binding sites (Figure 5-2), a plausible following question was to ask whether the role of Smc5/6 in regulating replication fork progression is functionally related with topo IIa chromatin binding during interphase and/or later in mitosis.

Then, we analyzed the pattern of topo IIα chromatin localization with respect to ongoing replication by immunostaining topo IIα in cells pulse-labeled with EdU for

0.5 h prior to PCC induction (Figure 5-4). Both control and Smc6-depleted cells showed a similar pattern of topo IIα and EdU staining in PCC-induced nuclei at early and middle S phase, with a characteristic progressive accumulation of topo IIα at particular chromatin domains that were, with rare exceptions, exclusive of the regions of EdU incorporation, representing nascent replicating chromatin regions.

In late S and G2 nuclei, however, several differences were observed between control and Smc5/6-depleted cells. First, in the absence of Smc5/6, there was an accumulation of late S phase nuclei presenting a distinctive pattern of EdU and topo IIα staining; although individual sister chromatids masses are formed, EdU-positive chromatin was found as defined linear staining in between these chromosome structures, which was barely seen in control cells (Figure 5-4 and Figure 5-5, B). This EdU-staining pattern was accompanied by a reduction in the distinct topo IIα chromatin-binding domains, which would be normally seen in late S phase. This dispersed pattern of topo IIa is typically observed in mid S phase.

Additionally to our previous observation of a high incidence of persistent ongoing replication at centromeres and telomeres of PCC-induced G2 chromosome structures in Smc5/6-depleted cells, we also determined that, within the regions of active replication, topo IIα binding was clearly excluded, generating an irregular

staining pattern of the protein along G2 chromosomes, in strike contrast to control cells that showed the characteristic axial staining of topo IIα in well-defined sister chromatids with no evidence of ongoing replication by EdU staining (Figure 5-5, A).

Importantly, when Smc5/6-depleted cells were treated with RO-3306 at 30 h after release from serum starvation to arrest cells in G2 for additional 12 h, not only the γH2AX/BLM foci disappeared as mentioned before, but also the incidence of curly chromosome phenotype as well as the abnormal axial distribution of topo IIα was markedly reduced after release from RO-3306 treatment (Figure 5-5, C and D). Thus, it is reasonable to predict that the abnormal chromosome structure could be rescued by providing the cells more time to deal with the perturbed replication fork progression occurred during S phase.

Overall, these results indicated that depletion of the Smc5/6 complex markedly altered S phase progression, extending the time of DNA replication at particular regions. This delay critically affected the pattern of topo IIα accumulation on PCC chromosomes, and this abnormal binding of topo IIα during replication processes may be later reflected by the abnormal distribution of topo IIα in mitosis observed by immunostaining and by ChIP-Seq.

DISCUSION

Our study suggests that the function of Smc5/6 is essential to form an intact axial structure of mitotic chromosomes in human cell lines. ChIP-Seq analysis revealed that redistribution of topo IIα from interphase binding sites to the centromeric region in mitosis is blocked in Smc6-depleted cells, so it is conceivable that topo IIα tendency to accumulate at the distal arms in these cells –instead of the regular centromeric enrichment of the protein in untreated cells- may be causally related to the disturbed chromatin dynamics of the protein in the transition from interphase to mitosis, as well as with the abnormal "curly" appearance of sister chromatid and the characteristic unbalanced condensation between the chromosomal arms and centromeres in the absence of Smc5/6.

Our Smc6 ChIP-Seq analyses also revealed that the chromatin binding pattern of the protein during interphase coincides -although not totally- with the profile observed for topo IIα. This situation insinuates a functional relationship between Smc5/6 and topo IIα within particular chromatin domains before chromosome assembly takes place. Smc6 binding sites also partially overlap the cohesin binding sites, which suggest that the Smc5/6 complex may be also involved in some aspect of the cohesin pathway, which in turn would explain the incidence of premature loss of sister chromatid cohesion particularly at the centromeric region.

Nevertheless, a more detailed characterization of the DNA sequences found at the binding sites for both Smc6/5 and topo IIα is still needed, as so far our ChIP-Seq samples did not fulfill the conditions (optimal ChIP/WCE ratios) required to determine a sufficient amount of enrichment/peak calls for each dataset using bioinformatics tools.

How might Smc5/6 regulate topo IIα recruitment to chromosomes?

Previous study in *Xenopus* egg extracts by Cuvier and Hirano (2003) indicated that DNA replication allows topo IIα to be tightly associated with chromosomes such that it becomes detectable at the chromosomal axial structure. Our PCC assay is in agreement with these findings, as it showed that topo IIα association with chromatin increases as replication progresses and its accumulation at chromosome axes became unambiguously visible after replication and sister chromatid pairs are formed. Moreover, visualization of the nascent DNA by EdU pulse labeling revealed that regions of active replication always excluded topo IIα accumulation. These observations are consistent with the notion of a intimate relationship between a topo IIα-mediated subchromosomal axis construction and the process of DNA replication. This would explain why defective DNA replication in the absence of Smc5/6 disturbs proper chromosomal localization of topo IIα, which may consequently perturb its redistribution during mitotic chromosome assembly.

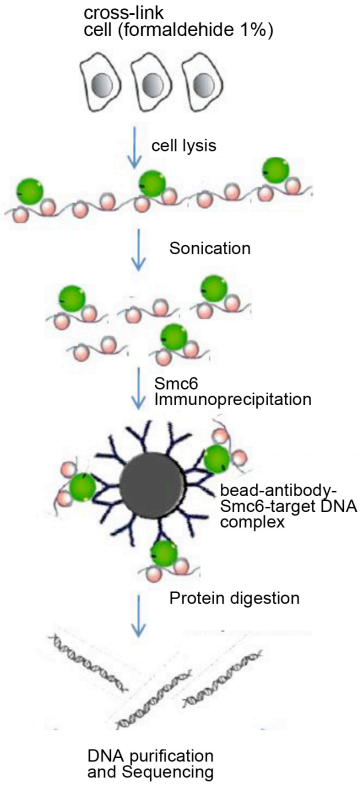
Smc5/6 links DNA replication to chromosome segregation

Our work suggests a model in which the Smc5/6 complex regulates replication fork progression and the replication-dependent reorganization of topo IIα distribution along chromosomes before cells enter mitosis. An important implication from these observations is that replication progression can directly affect chromosome assembly in mitosis. There is precedence to this hypothesis as mutations in components of the origin recognition complex, PCNA and MCM4, produce shorter and thicker chromosomes in *Drosophila* (Loupart, et al., 2000; Pflumm and Botchan, 2001). Significantly, the frequency of both, replication-related DNA damage and the curly chromosomal phenotype were markedly reduced when cells were subjected to G2 arrest and thereby allowed extra time to complete DNA replication before mitosis. It is therefore conceivable that the aberrant chromosome morphology in Smc5/6-depleted cells is directly related to a temporal disconnection between DNA replication and chromosome assembly.

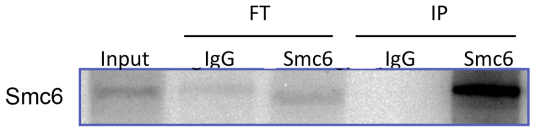
Delayed or incomplete replication within repetitive sequences such as the ribosomal DNA loci or telomeric regions is a common feature of Smc5 and Smc6 deficiency in other systems, which results in chromosome missegregation (Chavez et al., 2010). Similarly, we found that Smc5- and Smc6-depleted human cells contained persisting active replication at centromeres and telomeres, which results in intertwining and

linkages between sister chromatids in mitotic chromosomes. Both centromeres and telomeres are known as late-replicating regions in human cells and thus, the timing of DNA replication and the subsequent chromatin assembly at these domains may be challenged in the context of replication stress. Thus, it is tempting to speculate that the function of Smc5/6 becomes particularly crucial to maintain the stability of late-replicating regions in human genome.

A

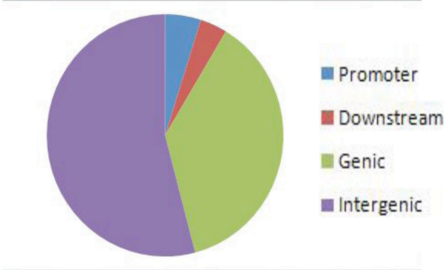


B



C

name	peak threshold	# peak	overlap with Rad21	%	±5kb	%
Smc6	5	11,633	1,358	11.67	3,344	28.75
	8	332	161	48.49	199	59.94
	10	66	46	69.70	50	75.76



D

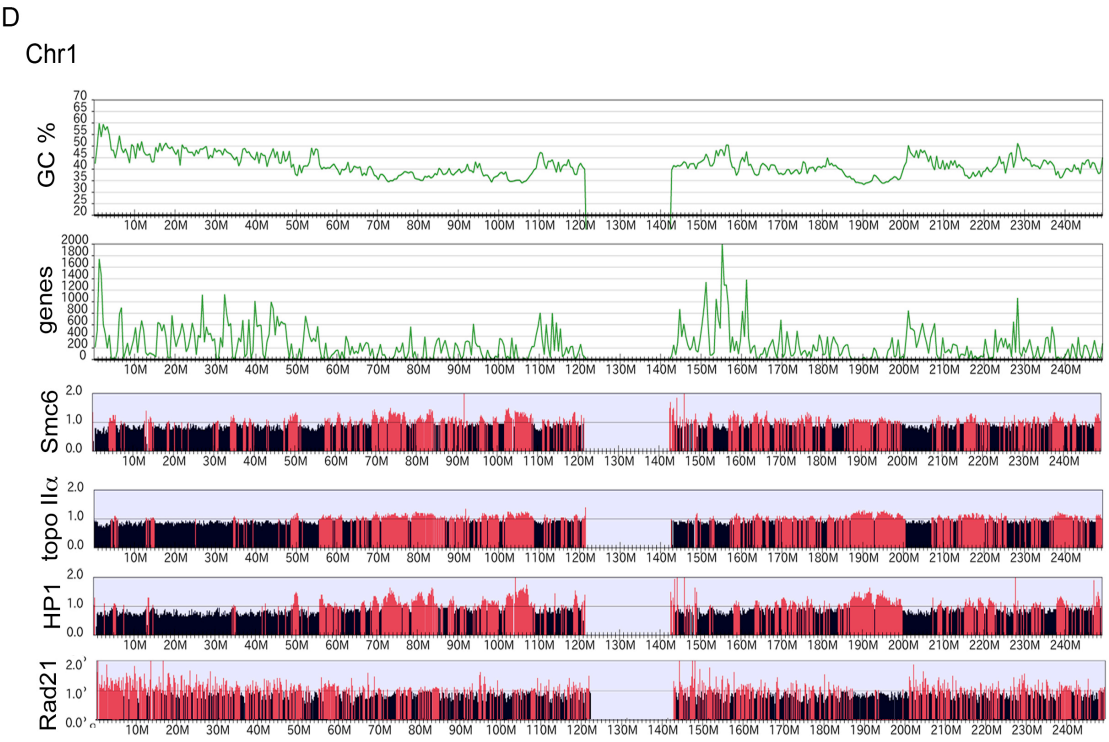
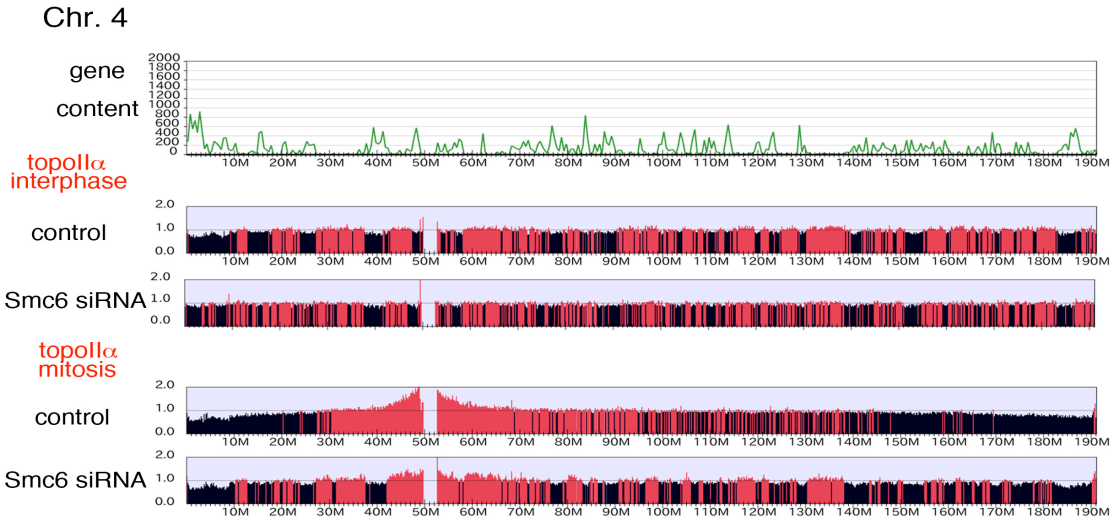
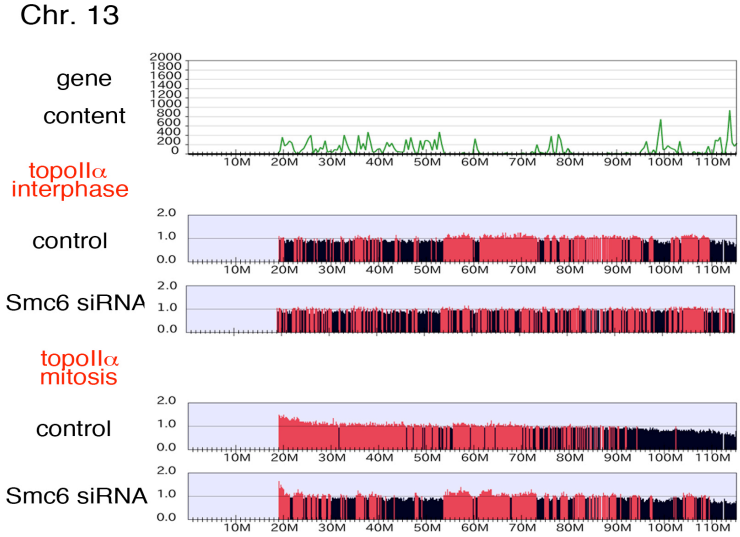


Figure 5-1. A. Schematic representation of Chromatin immunoprecipitation (ChIP) followed by high-throughput DNA sequencing (ChIP-Seq). B. Immunoblotting of asynchronized HeLa cells subjected to chromatin immunoprecipitation using Smc6 monoclonal antibody (Abnova) or IgG as control. C. Quantification of Smc6 binding peaks indicating the frequency of overlapping with cohesin's Rad21 binding sites and the nature of the binding regions classified as: Promoter, downstream, genic and intergenic regions. D. Uniquely aligned reads are summed in 100-kb windows along the chromosome for ChIP fractions and whole-cell extracts (WCEs) for Smc6, topo IIα, HP1α and Rad21. Windows in which the enrichment ratio of ChIP/WCE was higher than 1.0 are highlighted in red (see materials and methods). Gene content was calculated for every 500 kb. Representative results for the chromosome 1 are shown.


Chr. 4
gene content
topoIIα interphase
control
Smc6 siRNA
topoIIα mitosis
control
Smc6 siRNA


Chr. 13
gene content
topoIIα interphase
control
Smc6 siRNA
topoIIα mitosis
control
Smc6 siRNA

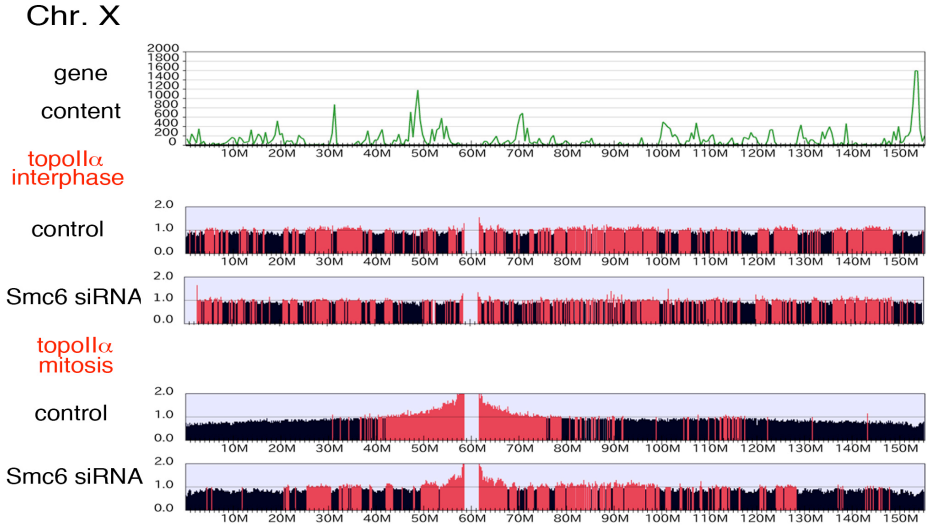

Chr. X
gene content
topoIIα interphase
control
Smc6 siRNA
topoIIα mitosis
control
Smc6 siRNA

Figure 5-2. Uniquely aligned reads defined as explained in figure 5-1 and representative results for chromosome 4, 13 and X are shown, exemplifying the requirement of Smc5/6 for the timed binding of topo IIα in interphase and the relocalization of topo IIα in mitosis.

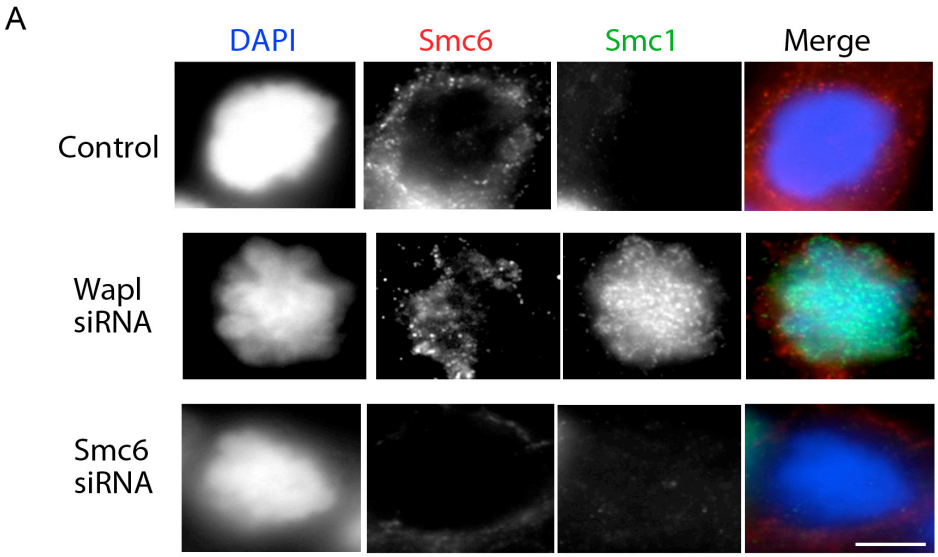


Figure 5-3. Immunofluorescence analysis of cohesin in mitotic chromosomes. RPE-1 metaphase cells transfected with an siRNA against Smc6 or the cohesin regulator WAPL were stained for DNA (DAPI; blue, left), Smc6 (red, center-left), and a cohesin subunit Smc1 (green, center-right). Right panels show merged images. Bar = 10 μm.

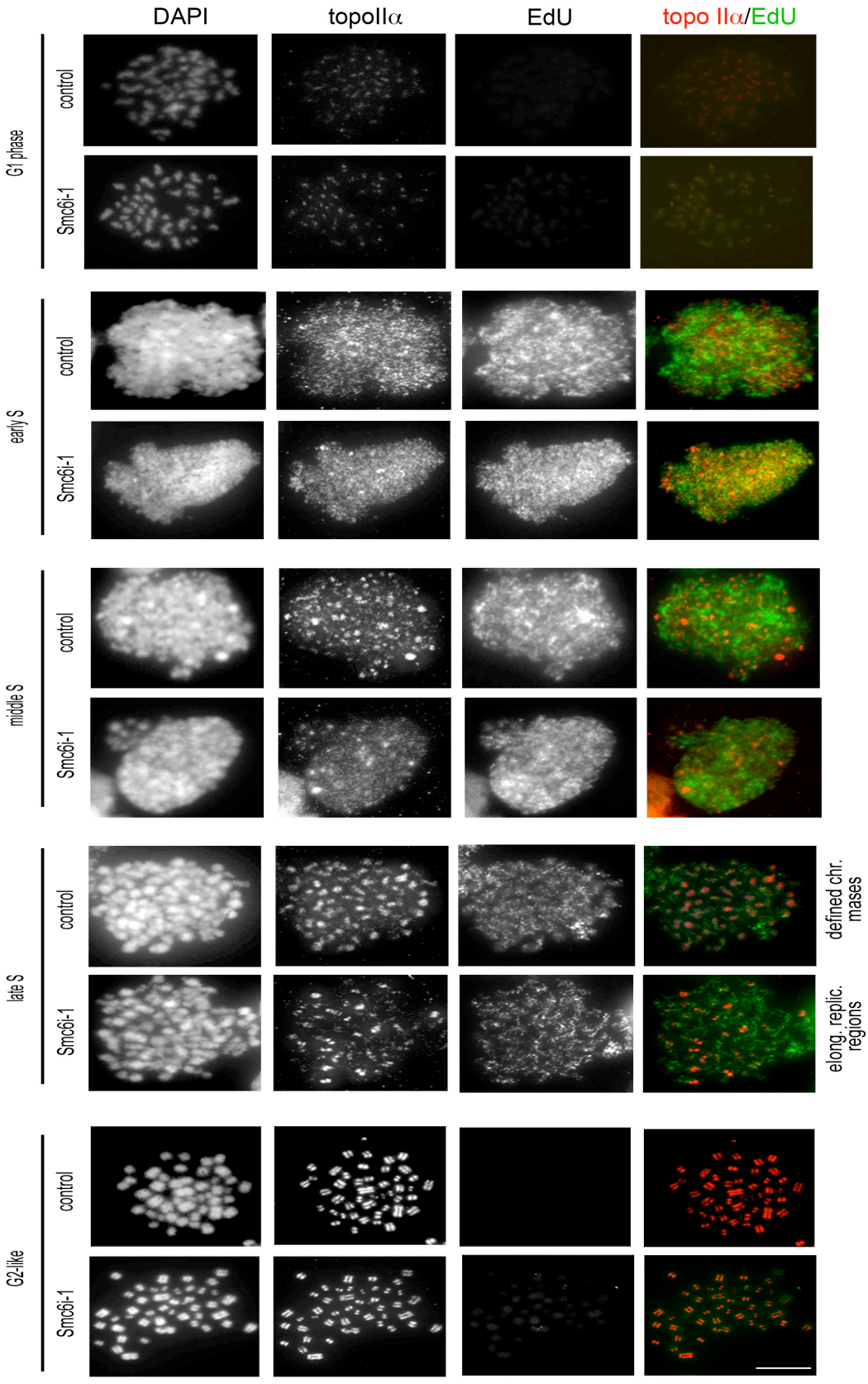

DAPI
topoIIα
EdU
topo IIα/EdU
G1 phase
early S
middle S
late S
G2-like
control
Smc6i-1
defined chr. mases
elong. replic. regions

Figure 5-4. Progression of DNA replication and loading of topo IIα onto sister chromatids in S phase. A. PCC analysis combining EdU pulse labeling and topo IIα immunolocalization. Immunofluorescence microscopy images of DNA (DAPI), topo IIα (red, in merged images) and EdU (green, in merged images) are shown. Note that late S phase can be characterized based on two properties, i.e., elongated replicating regions and well-defined chromatin masses. Right panels show merged EdU and topo IIα staining. Bar = 10 μm.

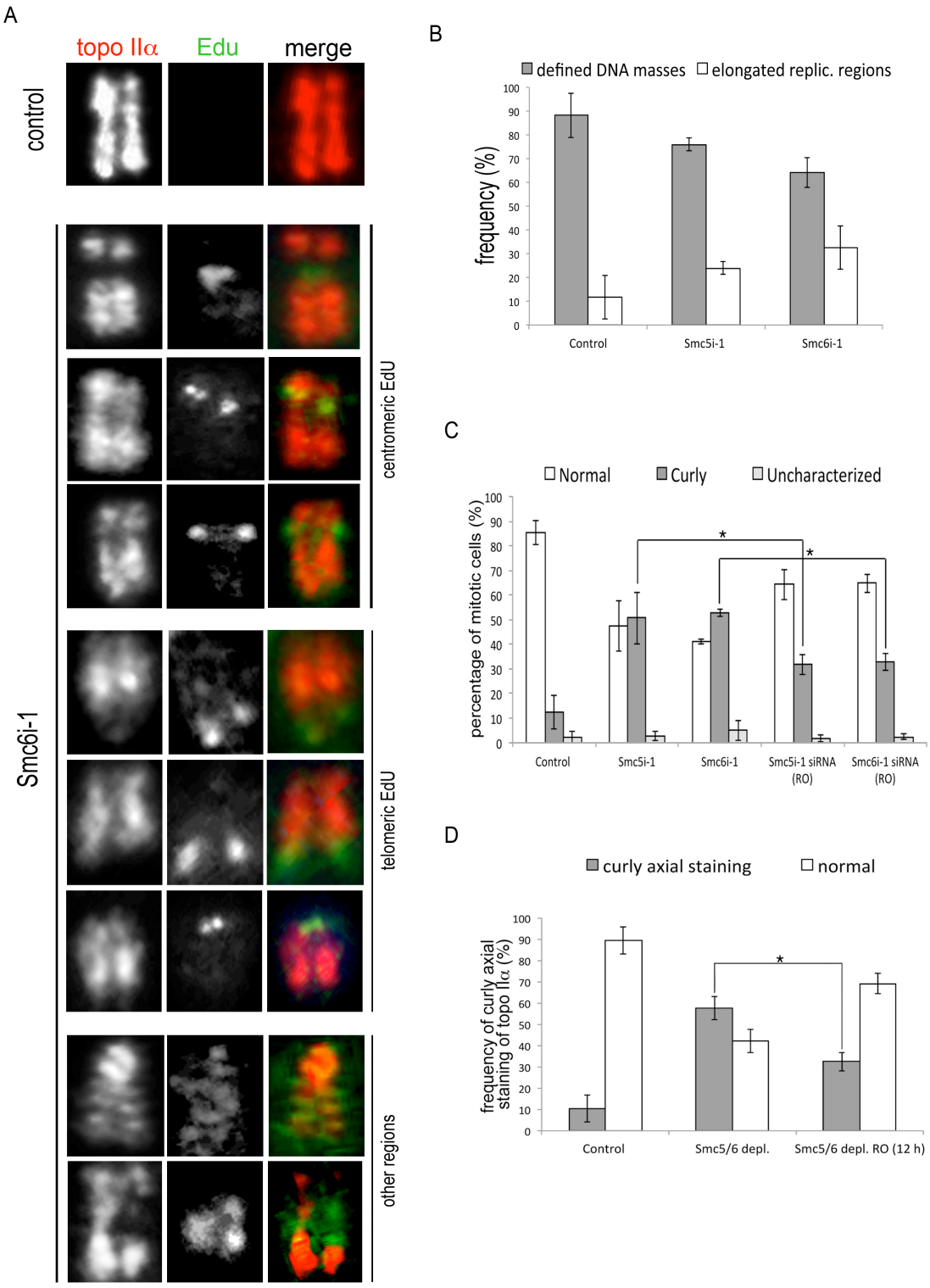

A
topo IIα
Edu
merge
control
Smc6i-1
centromeric EdU
telomeric EdU
other regions
B
defined DNA masses
elongated replic. regions
frequency (%)
Control
Smc5i-1
Smc6i-1
C
Normal
Curly
Uncharacterized
percentage of mitotic cells (%)
Control
Smc5i-1
Smc6i-1
Smc5i-1 siRNA (RO)
Smc6i-1 siRNA (RO)
D
curly axial staining
normal
frequency of curly axial staining of topo IIα (%)
Control
Smc5/6 depl.
Smc5/6 depl. RO (12 h)

Figure 5-5. A. Frequently observed G2-like chromosomes in Smc6 siRNA-treated cells. Note that EdU-positive extended regions of ongoing replication (green) excludes topo IIa axial localization (red). Right panels show merged images. Bar = 1 μm. B. The elongated replicating regions are more often seen in late S phase of Smc5/6 depleted cells. PCC-induced late S phase cells are assessed for the completion of DNA replication by counting the incidence of elongated replicating regions and defined chromatin masses. C. Frequency of curly axial staining of topo IIα in smc5 or Smc6-depleted cells in the presence or absence of RO for additional 12 h. Bar graph shows mean ± SD from three independent experiments; P value <0.05;*P value 0.01 to 0.05; **P value 0.001 to 0.01, two-tailed student's t-test.

CHAPTER VI: CONCLUSIONS AND PERSPECTIVES

THE SMC5/6 COMPLEX ASSOCIATES WITH CHROMATIN DURING INTERPHASE

We have established that, in human cells, the Smc5/6 complex associates with chromatin during interphase but it is then removed extensively from the nucleus in mitosis. Our live cell imaging analysis using EGFP-Smc5 combined with immunofluorescence analyses and chromatin fractionation assay of endogenous proteins, indicated that the dissociation of the complex from the chromatin is rapid and occurs soon after the nuclear envelope breakdown (NEBD) occurs. EGFP-Smc5 was then recovered within the nuclei of daughter cells at telophase/early G1 phase.

This behavior of Smc5/6 during the cell cycle in human cells differs from that observed in other species such as budding and fission yeast or in *Xenopus* (Lindroos et al., 2006; Tsuyama et al., 2006). The fact that the complex associates with chromatin already in early G1 phase implies that the Smc5/6 complex is differentially regulated in human cells and has probably acquired additional functions before DNA replication. In agreement with this, we were able to detect a foci-like distribution of Smc6 in G1 nuclei, which may correspond to sites where particular G1-specific functions of Smc5/6 take place. Interestingly, the cell cycle localization pattern of the Smc5/6 complex resembles that of cohesin complex and thus, in light of cohesin's function before S phase (Wendt

et al., 2008), Smc5/6 might be also involved in the regulation of chromatin structure required to control gene expression. Further analysis revealing the protein-protein interaction pattern of Smc5/6 in G1 phase will reveal the prospective function of the complex prior to DNA replication, as well as to clarify a possible interplay between cohesin and Smc5/6 to coordinately shape chromatin at this stage of the cell cycle.

To be noted, chromatin fractionation analysis of Smc5 indicated that a minor proportion of the protein can be still detected within the nuclear fraction during mitosis. Thus, it will be important to establish whether this minor protein population may have some particular role in mitotic chromosome architecture.

THE SMC5/6 COMPLEX PARTICIPATES IN CHROMOSOME ASSEMBLY

We have revealed that the depletion of Smc5 and Smc6 proteins do not only lead to a low proliferation rate within treated cells but also generates a clear abnormal structure of mitotic chromosomes, represented by a curly deformation of the sister chromatids and a hypercondensation of distal regions in chromosome arms. There was also a high incidence of cohesion defects characterized by a side-by-side placement of sister chromatids, or in the particular case of HeLa cells, by a premature separation of sister chromatids; both phenotypes are described in cells depleted of several cohesin components and cohesion's regulatory factors.

Based on the localization profile, these mitotic phenotypes presumably relate to the function of Smc5/6 during interphase, which makes an important contribution to higher-order chromatin organization upon entry into mitosis. Importantly, the absence of the Smc5/6 complex induces chromosomal segregation errors and often creates PICH- and BLM-positive anaphase bridges that indicate existence of unresolved, intertwining of sister DNAs particularly at centromeric regions. As these aberrant phenotypes have also been associated with the loss of topo IIα and condensin function (Spence et al., 2007; Gerlich et al., 2006), delocalization of topo IIα and condensin may be causally related to such segregation defects.

When Smc6 binding profile was assessed by ChIP-Seq, we were able to obtain a set of binding sites that overlapped to those previously determined for cohesin's Rad21. This finding indicates that the Smc5/6 complex may also contribute to the regulation of cohesin chromatin dynamics, which may causally explain the defects in sister chromatid cohesion observed in Smc5/6-depleted cells. Interestingly, the centromeric repulsion observed in HeLa cells, has been also attributed to defects in the decatenation checkpoint signaling in cells depleted for several recombination-related helicases such as BRCA1 or WRN.

However, recent interpretations have pointed out the possibility that these proteins may have also a direct contribution to the establishment and maintenance of sister chromatid cohesion during unperturbed cell cycle, as part of its role in the

regulation of DNA replication progression. Thus, it would be important to determine whether Smc5/6 complex deficiency affects sister chromatid cohesion by its contribution to the dissolution of DNA linkages between sister chromatids, by its regulatory role in cohesin binding to chromatin, or by a combination of these two conditions.

Importantly, HeLa cells also presented a more profound defect in DNA damage repair in the absence of Smc5/6 compared to RPE-1 cells, indicating a critical requirement of the complex for cell survival in conditions of replication stress which is typical of several cancer cell lines. Moreover, there also exist a proposed link between premature sister chromatid separation and aneuploidy, supported by the fact that sister chromatids which separate before their kinetochores are captured by microtubules, increase the probability of random chromatid segregation. Thus, our study suggests a possible contribution of the Smc5/6 complex to numerical chromosome instability, which is a hallmark of tumorigenesis.

RELOCALIZATION OF TOPO IIα IN MITOSIS DEPENDS ON SMC5/6

In this study we have shown that the Smc5/6 complex importantly contributes to the formation of an intact axial structure of mitotic chromosomes. After depletion of Smc5 and Smc6 proteins, we observed an accumulation of topo IIα at the distal regions of

chromosome arms, which contrast to the centromeric enrichment of the protein in control cells. Moreover, our ChIP-Seq analysis revealed that, Smc6 and topo IIα chromosomal binding shows a similar profile during interphase, with a particular accumulation at domains of low gene content. Moreover, we have also determined that in normal conditions, mitotic entrance induces a redistribution of topo IIα from interphase binding sites to the centromeric and pericentromeric region, and that depletion of Smc5/6 markedly blocks this relocalization process. We could reason that this defective relocalization of topo IIα caused characteristic unbalanced condensation between the chromosomal arms and centromeres in the absence of Smc5/6.

Our premature chromosome condensation assay (PCC) indicated that topo IIα association with chromatin increases as replication progresses and its accumulation at chromosome axes became visible after replication has been completed and sister chromatid pairs are formed. Moreover, the visualization of the nascent DNA by EdU pulse labeling revealed that regions of active replication always excluded topo IIα accumulation. These observations are consistent with the idea that the topo IIα-mediated subchromosomal axis construction is intimately coupled to the process of DNA replication. This would explain why defective DNA replication in the absence of Smc5/6 disturbs proper chromosomal localization of topo IIα, which may perturb its redistribution during mitotic chromosome assembly.

We were able to observe a variety of aberrant chromosome conformations including curly axes, fused telomeres and double axis, were induced by Smc5/6 depletion. These structures were found to be associated with abnormal distribution of condensin, which would reflect disorganized chromosomal axes caused by improper deposition of topo IIα. However, it was recently found that condensin II promotes structural reorganization of duplicated chromosomes immediately after the replication (Ono et al., 2013). Thus, a direct interplay between condensin and Smc5/6 during DNA replication to coordinate chromosome assembly may also exist. Additional analyses will be required to elucidate the mechanisms by which this abnormal conformation is generated in the context of Smc5 and Smc6 depletion.

SMC5/6 LINKS DNA REPLICATION TO CHROMOSOME SEGREGATION

In our analysis, Smc5/6-depleted cells exhibited increased levels of replication-related DNA damage, which indicates that replication fork stalling and/or collapse occur frequently in the absence of Smc5/6, and that these conditions directly relate to the slower processing of DNA replication. Significantly, the frequency of these damages as well as the curly chromosomal phenotype were markedly reduced when cells were subjected to G2 arrest and thereby allowed extra time to complete DNA replication before mitosis. It is therefore conceivable that the aberrant chromosome morphology in Smc5/6-depleted cells is directly related to a temporal disconnection between DNA replication and chromosome assembly.

Our work suggests a model in which the Smc5/6 complex regulates replication fork progression and the replication-dependent reorganization of topo IIα distribution along chromosomes before cells enter mitosis. An important implication from these observations is that replication progression can directly affect chromosome assembly in mitosis. There is precedence to this hypothesis as mutations in components of the origin recognition complex, PCNA and MCM4, produce shorter and thicker chromosomes in *Drosophila* (Loupart, et al., 2000; Pflumm and Botchan, 2001).

Delayed or incomplete replication within repetitive sequences such as the ribosomal DNA loci or telomeric regions is a common feature of Smc5 and Smc6 deficiency in other systems, which results in chromosome missegregation (Chavez et al., 2010). Similarly, we found that Smc5- and Smc6-depleted human cells contained persisting active replication at centromeres and telomeres, which results in intertwining and linkages between sister chromatids in mitotic chromosomes.

Both centromeres and telomeres are known as late-replicating regions in human cells and thus, the timing of DNA replication and the subsequent chromatin assembly at these domains may be challenged in the context of replication stress. Thus, it is tempting to speculate that the function of Smc5/6 becomes particularly crucial to maintain the stability of late-replicating regions in human genome.

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