COHESIN, THE SMC5/6 COMPLEX AND SUMO IN DNA REPAIR

APPROVED BY SUPERVISORY COMMITTEE

Hongtao Yu, Ph.D. (Mentor)

Yi Liu, Ph.D. (Chair)

Melanie Cobb, Ph.D.

David Chen, Ph.D.

DEDICATION

I would like to dedicate my thesis to my parents, Hong Zhang and Jun Wu, and my husband, Xiaosong Pan. My parents have always been there to support and love me whenever I was in difficulty. They showed me how to love by giving their unlimited love to me. They taught me how to be a nice person: humility, modesty, respectfulness, and generosity were what I learnt from my everyday life with them. They set the best example of family values, which will benefit my whole life. I sincerely appreciate everything they have done. My husband has been with me and encouraged me to pursue my dream through the last seven years. My life would never be so enjoyable without him. I thank him for all the support and love.

I would also like to acknowledge several people at UT Southwestern that have been instrumental in my graduate study. First and foremost, I would like to thank my mentor Dr. Hongtao Yu, for his guidance and support. His enthusiasm in science and unlimited energy deeply motivate me. He was always available and patient when I talked with him about my experimental progress. He cares about students, and gives a lot of advice. Second, I would like to thank Dr. Ryan Potts. He was like the second mentor to me, and provided me with much technical support and intellectual advice. At last, I would like to thank every former and present lab members for their kindness and help.

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by

NAN WU

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2012

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NAN WU, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2012

HONGTAO YU, Ph.D.

DNA double-strand breaks (DSBs) fuel cancer-driving chromosome translocations. Two related structural maintenance of chromosomes (Smc) complexes, cohesin and Smc5/6, promote DSB repair through sister-chromatid homologous recombination (SCR). Our results show that the Smc5/6 subunit Mms21 sumoylates multiple lysines of the cohesin subunit Scc1. Mms21 promotes cohesin-dependent SUMO accumulation at laser-induced DNA damage sites in S/G2 human cells. Cells expressing the non-sumoylatable Scc1 mutant (15KR) maintain sister-chromatid cohesion during mitosis, but are defective in SCR and sensitive to ionizing radiation (IR). Scc1 15KR is recruited to DNA damage sites. Depletion of Wapl, a negative cohesin regulator, rescues SCR defects of Mms21-deficient or Scc1 15KR-expressing cells. Expression of

the acetylation-mimicking Smc3 mutant does not bypass the requirement for Mms21 in SCR. We propose that Scc1 sumoylation by Mms21 promotes SCR by antagonizing Wapl at a step after cohesin loading at DSBs and in a way not solely dependent on Smc3 acetylation. Our results establish a new posttranslational regulatory mechanism of cohesin during DNA repair, and reveal both conserved principles and organism-specific features in cohesin regulation during sister-chromatid recombination.

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PRIOR PUBLICATIONS

Wu, N., Kong, X., Ji, Z., Zeng, W., Potts, P., Yokomori, K., Yu, H. (2012). Scc1 sumoylation by Mms21 promotes sister chromatid recombination through counteracting Wapl. Genes Dev *26*, 1473-1485.

Wu, N., and Yu, H. (2012). The Smc complexes in DNA damage response. Cell Biosci *2*, 5.

Ke, Y., Huh, J.W., Warrington, R., Li, B., Wu, N., Leng, M., Zhang, J., Ball, H.L., and Yu, H. (2011). PICH and BLM limit histone association with anaphase centromeric DNA threads and promote their resolution. EMBO J *30*, 3309-3321.

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LIST OF ABBREVIATIONS

ATM: ataxia telangiectasia mutated

ATR: ataxia telangiectasia related

A-NHEJ: alternative NHEJ

BER: base excision repair

BrdU: 5-bromo-2-deoxyuridine

ChIP: chromatin immunoprecipitation

ChIP-seq: ChIP followed by deep sequencing

DAPI: 4'-6-diamidino-2-phenylindole

DI: damage-induced

DNA-PK: DNA-dependent protein kinase

DNA-PK_{cs}: DNA-dependent protein kinase catalytic subunit

dox: doxycycline

DSB: double-strand break

DSGR: recombination-dependent daughter-strand gap repair

GFP: green fluorescent protein

HEAT: Huntington, elongation A subunit, TOR

HR: homologous recombination

HU: hydroxyurea

IF: immunofluorescence

IR: ionizing radiation

IVT: in vitro transcribed and translated

kb: kilobase

LOH : loss of heterozygosity

MAGE: melanoma-associated antigen gene

MMC: mitomycin C

MMS: methyl methanesulfonate

MRN: Mre11/Rad50/Nbs1

NHEJ: non-homologous end joining

NER: nucleotide excision repair

ns: nanosecond

NSE: non-SMC element

PCR: polymerase chain reaction

PFA: paraformaldehyde

PIAS: protein inhibitor of STAT

PIKK: phosphatidylinositol 3-kinase-like kinase

QPCR: quantitative PCR

rDNA: ribosomal DNA

RING: really interesting new gene

RNAi: RNA interference

ROS: reactive oxygen species

SCE: sister chromatid exchange

SCR: sister-chromatid homologous recombination

SENP: sentrin-specific protease

siRNA: small interfering RNA

Smc: structural maintenance of chromosomes

SSB: single-strand break

ssDNA: single-strand DNA

SUMO: small ubiquitin-related modifier

TLS: translesion synthesis

UV: ultraviolet

4-OHT: 4-hydroxytamoxifen

CHAPTER I: INTRODUCTION

DNA Repair

The genomic DNA within a cell experiences many types of damages daily. It's estimated that approximate 10,000 to 100,000 DNA lesions occur in a cell per day. These damages can result from exogenous factors, such as ultraviolet (UV) radiation, ionizing radiation (IR), and chemical carcinogens, or from endogenous factors, such as stalled replication forks due to replication stress, reactive oxygen species (ROS) generated by respiration, and spontaneous hydrolysis of nucleotide residues (Figure 1-1) (Giglia-Mari et al., 2011). Failure to properly repair DNA damages can result in cell death or genomic instability which may eventually lead to cancer.

DNA damage response

In response to DNA damage, cells elicit elaborate DNA damage responses (Figure1-1). For example, DNA damage checkpoints arrest cell cycle progression at various points, thus allowing more time for cells to execute DNA repair. These include the G1/S checkpoint, the intra-S checkpoint, and the G2/M checkpoint (Zhou and Elledge, 2000). Checkpoint-dependent cell cycle arrest is mediated by members of the phosphatidylinositol 3-kinase-like kinase (PIKK) family. In humans, three PIKK family kinases, ataxia telangiectasia mutated (ATM), ataxia

telangiectasia related (ATR) and DNA-dependent protein kinase (DNA-PK) are activated by DNA damage, and their activation induces the activation of many cell-cycle regulators, including p53, Chk1, and Chk2 (Abraham, 2004).

Cells use different repair mechanisms to deal with different kinds of DNA lesions, including base excision repair (BER), nucleotide excision repair (NER) and DNA double-strand break (DSB) repair (Figure 1-1) (Giglia-Mari et al., 2011). A persistently stalled replication fork can eventually form a DSB, which is highly toxic to genome stability. Therefore, two DNA damage tolerance mechanisms have evolved: translesion synthesis (TLS) and recombination-dependent daughter-strand gap repair (DSGR) (Li et al., 2002; Scully et al., 2000). If the damage is too severe, cells will trigger senescence or apoptosis.

DNA double-strand break repair

DNA DSB is the most deleterious form of DNA damages and can cause chromosome translocations, a major class of cancer-driving mutations. There are two major DSB repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Sonoda et al., 2006). Because HR requires a sister chromatid as template, this kind of repair pathway is preferred in S/G2 after DNA replication. In contrast, NHEJ can occur in any cell cycle.

The initial steps in DSB sensing

DNA damage repair proteins are divided into several groups, according to their roles in DNA damage response: sensors, transducers, mediators, and effectors (Figure 1-2) (Coster and Goldberg, 2010). The Mre11/Rad50/Nbs1 (MRN) complex serves as the DSB sensor in the case of DSB repair. Mre11 and Rad50 are highly conserved from eubacteria, to archaea, to eukaryotes, while Nbs1 is a eukaryote-specific protein (Williams et al., 2007). Electron microscopy and crystallography studies show that the MRN complex exist as an $M_2R_2N_2$ assembly (Hopfner et al., 2002; Hopfner et al., 2001; Hopfner et al., 2000; Stracker and Petrini, 2011; Williams et al., 2007). Rad50 uses its zinc hook and coiled coil domains to bridge DSBs and facilitate DNA end resection by Mre11 (Williams et al., 2007). Nbs1 has an ATM kinase interaction domain, which transmits the DNA damage signal to the DSB repair transducer ATM (Falck et al., 2005). ATM then phosphorylates adjacent histone H2AX, building a domain with the DNA damage specific marker γH2AX (Rogakou et al., 1999). Mediators facilitate transducer to amplify the damage signal and transmit it further to effectors, which contains many DNA repair proteins, checkpoint kinases, and proteins involved in apoptosis. MDC1, as a mediator, binds to γH2AX and phosphor-ATM, which allows a local enrichment of ATM and phosphorylation of more H2AX. This positive feedback enables the spreadings of the damage signal to megabases flanking a DSB (Coster and Goldberg, 2010).

Non-homologous end joining

The NHEJ process includes three steps: end capturing, end processing, and ligation (Weterings and Chen, 2008). The Ku70/80 heterodimer is in responsible for DSB end capturing. The interaction of Ku70/80 with DNA brings the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) to the DSB (Walker et al., 2001). Once the two DSB ends are captured by two Ku70/80 and two DNA-PK_{cs}, the DNA ends are ready to be processed (Weterings and Chen, 2008). The association of DNA-PK_{cs} with Ku and DNA is sufficient to activate the kinase activity of DNA-PK_{cs}. To date, the exact role and *in-vivo* target of DNA-PK_{cs} in NHEJ are not clear. The most studied substrate of this kinase is itself. Mutating the autophosphorylation sites of DNA-PK_{cs} results in defects in DSB repair and sensitivity to IR, indicating the functional importance of DNA-PK_{cs} autophosphorylation (Chan et al., 2002; Cui et al., 2005; Ding et al., 2003; Soubeyrand et al., 2003). It has also been shown that DNA-PK_{cs} autophosphorylation can occur in trans, suggesting that autophosphorylation occurs only when the two DSB ends are tethered by DNA-PK_{cs} (Meek et al., 2007). It is possible that the unphosphorylated DNA-PK_{cs} protects DSB ends from premature degradation or ligation. Once both DSB ends are coupled with DNA-PK_{cs}, the non-compatible DNA ends are processed. There are two NHEJ pathways: classical-NHEJ and alternative NHEJ (A-NHEJ) (Figure 1-2). In the classical pathway, the Artemis nuclease or polymerases are recruited to the DNA-

PK_{cs} coupled DSB ends to process them into ligatable termini that are repaired by the Ligase IV/XRCC4 complex. Alternatively, in the case of DSB ends with short complementary overhangs, Ligase IV/XRCC4 and XLF/Cernunos regulate the re-joining of one single-strand DNA end to the other DNA end, followed by polymerase filling (Weterings and Chen, 2008). Deletion or changes of a few nucleotides at the DSBs occur frequently during end processing. Therefore, the NHEJ pathway is an error-prone DSB repair pathway.

Homologous recombination

HR is a key DSB repair pathway and requires an undamaged DNA template: sister or homologous chromosomes. Because HR between homologous chromosomes can result in loss of heterozygosity (LOH), DSB repair with HR between sister chromatids (SCR) is preferable during the mitotic cell cycle.

The HR mechanism in DSB repair could be divided into three major steps: end resection, strand invasion, and double Holliday junction resolution (Figure 1-3) (Wyman and Kanaar, 2006). MRN and CtIP carries out the initial 5'-3' end resection process. Both Rad50 and Nbs1 enhance the endonuclease activity of Mre11 in vitro, and tethering the endonuclease CtIP to MRN restricts end resection to the proximity of the DSB (Paull and Gellert, 1998, 1999; Sartori et al., 2007; Trujillo et al., 1998). The extensive resection is finished by Exo1 or STR-Dna2. The reason for additional resection is to form long single-strand DNA

(ssDNA) which is required for checkpoint activation and repair fidelity by preventing HR between repeats (Symington and Gautier, 2011). The ssDNA 3' overhangs are quickly bound and stabilized by RPA. For the next step in HR, RPA coated on ssDNA is replaced with the recombinase Rad51 (Symington and Gautier, 2011). BRCA2 and Rad52 are required for Rad51 loading (Symington and Gautier, 2011; Wyman and Kanaar, 2006). Then the Rad51-ssDNA filament promotes strand invasion into the intact sister chromatid, generating a D-loop (Sugawara et al., 1995). Intuitively, the strand invasion step needs cohesin to bring two sister chromatids into proximity. In humans, the cohesin complex has been shown to be recruited to DSBs by immunofluorescence (IF) and chromatin immunoprecipitation (ChIP) (Kim et al., 2002a; Potts et al., 2006). The D-loop intermediate serves as a template for DNA synthesis by polymerase to repair the missing nucleotides. After DNA synthesis, strand ligation results in a double Holliday junction, the resolution of which depends on junction resolvases or the combination of a helicase and a topoisomerase. Resolution by junction resolvases results in crossover and non-crossover recombinants, while resolution by helicase and topoisomerase gives rise to non-crossover recombinants (Wyman and Kanaar, 2006). In bacteria, this step is accomplished by RuvABC complex or the RecG protein. In eukaryotes, the double Holliday junction is resolved by the BLM/TopoIII complex or cleaved by the endonucleases Gen1, Mus81/Eme1, ERCC1 or Slx1/Slx4 (Schwartz and Heyer, 2011).

The SMC Complexes in DNA Damage Response

The highly conserved structural maintenance of chromosomes (Smc) proteins regulate chromosome architecture and organization from bacteria to human. Most prokaryotes have a single Smc protein which forms a homodimer, while there are at least six Smc family members, Smc1-6, that form three heterodimers in eukaryotic organisms (Figure 1-4) (Losada and Hirano, 2005). Smc1 and Smc3 form the core of the cohesin complex which maintains sister-chromatid cohesion during mitosis to ensure accurate chromosome segregation (Nasmyth, 2002). Smc2 and Smc4 constitute the condensin complexes that promote chromosome condensation (Hirano, 2005). Smc5 and Smc6 form a complex that plays critical roles in DNA repair (Lehmann, 2005; Potts, 2009).

The Smc proteins contain about 1,000 amino acids and share similar domain structures. The ATPase domain of each Smc protein is separated into N- and C-terminal halves by a long linker. The two nucleotide-binding Walker A and Walker B motifs reside in the two different ATPase halves. The Smc linker folds into an intramolecular antiparallel coiled coil and allows the N-terminal ATPase half of an Smc protein to fold back to its C-terminal ATPase half and create a single globular ATPase head (Figure 1-4). The hinge domain at one end of the coiled coil mediates the heterodimerization of eukaryotic Smc protein (Hirano, 2006; Losada and Hirano, 2005; Nasmyth and Haering, 2005). The two ATPase

heads at the other end of the coiled coil can transiently interact with each other to bind and hydrolyze ATP. As revealed by electron microscopy, the Smc heterodimers can adopt different conformations, including V-shaped dimer and ring-like structures, possibly depending on the nucleotide-binding states of their ATPase heads (Anderson et al., 2002; Melby et al., 1998). Each Smc heterodimer associates with non-Smc subunits to form functional Smc complexes.

The genomic DNA with a cell experiences many types of damage daily. These damages can result from exogenous factors, such as ultraviolet (UV) radiation, ionizing radiation (IR), and chemical carcinogens, or from endogenous factors, such as stalled replication forks due to replication stress. In response to DNA damage, cells elicit elaborate DNA damage responses. For example, DNA damage checkpoints arrest cell cycle progression at various points, thus affording more time for cells to execute DNA repair. Failure to properly repair DNA damage can result in cell death or genomic instability which may eventually lead to cancer (Sancar et al., 2004).

The role of the Smc5/6 complex in DNA repair has long been appreciated. Emerging evidence in recent years has established that, in addition to their fundamental roles in chromosome segregation and organization, cohesin and condensin are also required for DNA damage checkpoints and DNA repair.

The Smc1/3 cohesin complex

The cohesin complex is composed of four evolutionarily conserved subunits, Smc1, Smc3, and two non-Smc proteins named Scc1 and Scc3 (Table 1-1) (Losada and Hirano, 2005; Onn et al., 2008). Vertebrate cells contain two Scc3 proteins, called SA1 and SA2. The N- and C-terminal regions of Scc1 link the head domains of Smc3 and Smc1, respectively, forming a tripartite ring. Scc3 is predicted to be a HEAT repeat-containing protein and interacts with Scc1 to further strengthen the ring structure of cohesin. Cohesin has been proposed to topologically embrace DNA and chromatids inside its ring. Other cohesin-binding proteins include Wapl, Pds5, and Sororin (in metazoans) that associate with cohesin in a sub-stoichiometric manner. Their interactions with cohesin are regulated during the cell cycle. Wapl negatively regulates cohesin association with chromatin while Sororin stabilizes cohesin on chromatin. Pds5 appears to have dual functions in cohesin regulation.

Cohesin and sister-chromatid cohesion

The major function of cohesin, as its name indicates, is to regulate sister-chromatid cohesion. Cohesin is loaded by the cohesin loader Scc2/4 complex in telophase and G1 prior to DNA replication (Figure 1-5). The loaded cohesin then becomes cohesive during DNA replication and has been proposed to topologically embrace both sister chromatids inside its ring to establish sister-chromatid cohesion. The mechanism by which cohesin is converted to the cohesive state

during DNA replication is not completely understood, but it requires the acetylation of Smc3 by the Eco1 family of acetyltransferases (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009; Unal et al., 2008; Zhang et al., 2008). In vertebrates, Smc3 acetylation enables the binding of Sororin to Pds5, which counteracts Wapl's ability to remove cohesin from chromatin (Nishiyama et al., 2010; Rankin et al., 2005; Schmitz et al., 2007). Because Sororin homologs have not been found in yeast, how Smc3 acetylation makes cohesin refractory to Wapl in yeast remains to be determined.

Timely dissolution of sister-chromatid cohesion is required for proper chromosome segregation in mitosis. In yeast, cohesin is cleaved by the protease separase at the metaphase–anaphase transition to trigger sister-chromatid separation. In humans, most cohesin on chromatid arms is removed by Wapl in prophase, and this process is facilitated by Plk1-dependent phosphorylation of SA2 (Figure1-5) (Gandhi et al., 2006; Hauf et al., 2005; Kueng et al., 2006). Only a small amount of cohesin remains associated with the centromeres and is protected from Wapl and Plk1 by the shugoshin–PP2A complex (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006). This centromeric pool of cohesin is cleaved by separase at metaphase to allow sister-chromatid separation (Figure 1-5) (Uhlmann et al., 2000).

Cohesin and DNA repair

In addition to its function in sister-chromatid cohesion, cohesin plays critical roles in DNA damage response (Figure 1-6). In fact, cohesin's role in DNA repair was discovered before the discovery of its function in sister-chromatid cohesion. The cohesin subunit Scc1 was first identified as Rad21, whose mutation rendered *S. pombe* cells hypersensitive to UV or IR (Birkenbihl and Subramani, 1992; Phipps et al., 1985). Later studies further confirmed a role of cohesin in DNA repair in several organisms, including *S. cerevisiae*, chicken, and humans (Atienza et al., 2005; Bauerschmidt et al., 2010; Schar et al., 2004; Sjogren and Nasmyth, 2001; Sonoda et al., 2001). These studies further pinpointed a specific function of cohesin in DNA double-strand break (DSB) repair through homologous recombination (HR).

In *S. cerevisiae*, cohesin and its positive regulators, including Scc2, Pds5, and Eco1, are all required for DSB repair during G2 (Sjogren and Nasmyth, 2001). Thus, it is not the cohesin complex per se but rather damage-induced functional sister-chromatid cohesion that is required for DNA repair. Moreover, in addition to observing the expected premature sister-chromatid separation phenotype in a conditional Scc1-deficient chicken DT40 cell line, Sonoda *et al.* also observed a marked defect in DNA repair in these cells (Sonoda et al., 2001). The Scc1-deficient cell line exhibited increased chromosome aberrations in S/G2 and a reduced frequency of sister-chromatid exchange (SCE). Finally, human cells with cohesin subunits depleted by RNA interference (RNAi) also exhibited

DNA repair and SCE defects (Atienza et al., 2005; Bauerschmidt et al., 2010; Potts et al., 2006). Taken together, these data establish a crucial role for cohesin in DSB repair through sister-chromatid HR during S/G2 phases of the cell cycle.

What is the function of cohesin in HR? HR-mediated DSB repair requires an undamaged DNA template. During the mitotic cell cycle, sister chromatids are the preferred template for HR, as HR between homologues may lead to the loss of heterozygosity. It is generally believed that sister-chromatid cohesion at or near a DSB helps to keep the DSB and the undamaged sister chromatid at close proximity, thereby promoting strand invasion and sister-chromatid HR. Although this notion makes intuitive sense, it remains to be formally tested experimentally. Consistent with a specific requirement for cohesin in sister-chromatid HR, cohesin is not required for certain forms of HR in *S. cerevisiae*, including intrachromosomal gene conversion (Unal et al., 2004). Cohesin is also not required for processes that occur in all forms HR, such as end resection and the formation of single-strand DNA at DSBs. Furthermore, cohesin suppresses DNA damage-induced recombination between homologous chromosomes in yeast (Covo et al., 2010). Finally, cohesin coordinates DSB repair pathway choice between HR and non-homologous end joining (NHEJ) through interaction with the Rad52 pathway (Schar et al., 2004). These results are consistent with a specific role of cohesin in sister-chromatid HR. It is possible that the elevation of NHEJ and HR between homologues seen in cohesin-deficient cells is simply due

to repair pathway competition. Alternatively, cohesin may actively suppress NHEJ and HR between homologues through unknown mechanisms.

Recruitment of cohesin to DSBs

A direct role of cohesin in DNA repair is further supported by its recruitment to DSBs, which was shown by both immunofluorescence (IF) and chromatin immunoprecipitation (ChIP). First, the Yokomori group detected the recruitment of cohesin to laser-induced DNA damage sites in human cell (Kim et al., 2002a). A laser microbeam was used to create DNA damage at discrete sites in the cell, and the recruitment of DNA repair proteins to these sites was monitored by IF. Shortly after radiation, cohesin and the MRN component Mre11 were detected at these sites. The recruitment of cohesin to these DNA damage foci was limited to S/G2 phases of the cell cycle, and dependent on Mre11 and Rad50. Furthermore, Rad50 was co-immunoprecipitated with cohesin subunits from human cells in S/G2, supporting a model in which MRN recruits cohesin to DSBs.

The recruitment of cohesin to DSBs is further supported by ChIP studies in yeast and human cells (Potts et al., 2006; Strom et al., 2004; Unal et al., 2004). In these cases, specific restriction endonucleases were used to cut defined sites in the genome to generate DSBs. In the study by the Koshland group (Unal et al., 2004), cohesin enrichment around a DSB was detected with ChIP in G2/M phases, but

not in G1 phase, of the cell cycle in *S. cerevisiae*, and required Scc2/4. In addition, proteins with well-known functions in HR, such as Mec1, Tel1, Rad53, Mre11, and γH2A, are all required for the establishment of the DSB-specific cohesin domain. In an accompanying study, the Sjögren group reported that cohesin was recruited to 50-kb genomic regions flanking a DSB induced during G2/M in yeast (Strom et al., 2004). They also showed that Scc2/4 was necessary for cohesin accumulation and DNA repair. Curious of the molecular function of cohesin recruitment to DSBs, the authors designed an elegant experiment to test whether functional cohesion was generated after induction of DSB. Indeed, they showed that cohesin recruited to the DSB was able to establish sister-chromatid cohesion in G2 phase after DNA replication. Finally, using ChIP, our lab showed that cohesin was also recruited to an I-SceI-induced DSB in human cells (Potts et al., 2006). Thus, cohesin recruitment to DSBs is a conserved process of the DNA damage response in organisms from yeast to man.

DNA damage-induced sister-chromatid cohesion

Both the Sjögren and Koshland groups found in yeast that DNA damage induced sister-chromatid cohesion not only at the DSB site but also throughout the genome after genome duplication in G2 (Strom et al., 2007; Unal et al., 2007). This damage-induced (DI) cohesion was controlled by the DNA damage response factors (Mec1, Tel1, Mre11, and γH2A) and cohesin regulators (Scc2, Eco1, and

Smc6), but was independent of DNA replication. Overexpression of Eco1, but not an Eco1 mutant lacking its acetyltransferase activity, bypassed the requirement for DSBs in DI-cohesion generation in G2/M (Unal et al., 2007). These results suggested that the activity of Eco1 was limiting in undamaged G2/M cells, and DNA damage response reactivated its activity.

How does DNA damage response augment Eco1's activity after DNA replication? What is the upstream signal? Does the signal regulate Eco1 or its substrate? What is the Eco1 substrate? An excellent study by the Koshland group began to answer these questions (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). They showed that phosphorylation of the cohesin subunit Scc1 (also known as Mcd1) by Chk1 at S83 was critical for DI cohesion. Substitution of S83 to alanine (S83A) inhibited DI cohesion, while substitution of S83 to aspartic acid (S83D) to mimic phosphorylation generated cohesion during G2/M, even in absence of a DSB or Chk1. Functional Eco1 was, however, still required for cohesion in S83D cells. Moreover, genetic evidence suggested that K84 and K210 of Scc1 were the Eco1-dependent acetylation sites in response to the DSB. Mutation of these two lysines to glutamine to mimic acetylation bypassed the requirement for the DSB or the acetyltransferase activity of Eco1 in DI cohesion. Furthermore, Smc3 acetylation by Eco1 was uniquely required for S-phase cohesion, but not for DI cohesion. Both Smc3 acetylation and Scc1 acetylation appeared to counteract the function of Wapl to establish cohesion. Collectively,

their results support a model in which phosphorylation of Scc1 at S83 by Chk1 makes Scc1 a better substrate for Eco1, which then acetylates K84 and K210 of Scc1 to establish DI-cohesion (Figure 1-6). Eco1 thus has distinct substrates in S-phase cohesion and DI cohesion. Future biochemical experiments are needed to confirm the phosphorylation and acetylation of Scc1 in response to the DSB.

So far, direct evidence for DI cohesion is only available in budding yeast. Several lines of indirect evidence suggest that DI cohesion might be a conserved mechanism in higher organisms. First, the critical cohesion-establishment factor, Sororin, is required for efficient DSB repair during G2 in HeLa cells (Schmitz et al., 2007). Sororin RNAi cells showed a marked increase of DNA breaks, compared to control cells. In addition, decreased inter-sister-chromatid distances were observed after DSB induction in chicken DT40 cells, consistent with the establishment of DI cohesion (Dodson and Morrison, 2009). Moreover, X rays enhanced sister-chromatid alignment in plants (Watanabe et al., 2009). Finally, ChIP followed by deep sequencing (ChIP-seq) revealed that IR triggered an Esco1-dependent increase of Smc3 acetylation and a genome-wide reinforcement of cohesin binding at pre-existing sites in human cells (Kim et al., 2010).

Future experiments are obviously needed to firmly establish DI cohesion as a conserved mechanism during DNA damage response in all eukaryotes. Moreover, even if DI cohesion exists in higher eukaryotes, the posttranslational modifications that regulate DI cohesion might be different from that of yeast. For

example, DNA damage-induced Scc1 acetylation could not be detected in human cells (Kim et al., 2010). The sites of Scc1 acetylation are also not conserved in human Scc1.

Cohesin and DNA damage checkpoint activation

In addition to its direct role in HR repair, cohesin is involved in DNA damage checkpoint activation (Figure 1-6). Several studies established the role of cohesin in intra-S-phase checkpoint activation in human cells (Garg et al., 2004; Kim et al., 2002b; Kitagawa et al., 2004; Yazdi et al., 2002). In response to DNA damage [e.g. IR, UV, and hydroxyurea (HU)], ATM or ATR phosphorylated two residues, S957 and S966, of Smc1, and phosphorylation of these two sites was required for S-phase checkpoint activation. In addition to Smc1, Smc3 was also phosphorylated by ATM at S1083 in response to IR, and S1083 phosphorylation was similarly required for the intra-S phase checkpoint (Luo et al., 2008). The Smc1/3 functions in the intra-S checkpoint are apparently mediated by the intact cohesin, not through a separate Smc1/3-containing recombination complex (Watrin and Peters, 2009).

How cohesin phosphorylation activates intra-S phase checkpoint to block DNA synthesis in response to DNA damage remains unclear at present and awaits the identification of the downstream effectors of Smc1/3 phosphorylation. These phosphorylation events may directly recruit proteins essential for checkpoint

activation to damage sites. Alternatively, phosphorylation of cohesin may affect its dynamic association with chromatin through regulating the ATPase activities of Smc1/3. This in turn may allow cohesin to act as barriers on chromatin and directly slow down DNA replication. Regardless of the mechanism, cohesin's role in S-phase checkpoint activation highlights a two-way crosstalk between cohesin and DNA replication.

Recently, cohesin has also been implicated in the G2/M DNA damage checkpoint in human cells (Watrin and Peters, 2009). Depletion of Scc1, but not Sororin, by RNAi caused defective 53BP1 recruitment to DNA damage foci and weaker Chk2 activation in G2. Because Sororin is required for functional cohesion, this result suggested that the G2/M checkpoint function of cohesin could be uncoupled from its function in sister-chromatid cohesion. Consistent with this notion, cohesin was also required for Chk2 activation in G1 prior to DNA replication (Figure 1-6).

Separase-mediated cohesin cleavage in DNA repair

An interesting study in *S. pombe* implicated a requirement for cohesin cleavage by separase in DNA repair (Nagao et al., 2004). It was shown that the separase inhibitor securin was essential for the proper repair of DNA damage induced by UV and IR. Expression of a non-cleavable Scc1 or inactivation of separase impaired DNA repair during G2, suggesting that the DNA repair

functions of securin and separase acted through the cleavage of cohesin. Whether cohesin cleavage by separase is required for DNA repair in other organisms remains to be determined. It is also unclear how separase becomes active in G2 and how its activity is presumably restricted to DNA damage sites.

The Smc2/4 condensin complexes

Condensin complexes are five-subunit complexes that regulate chromosome organization and condensation during mitosis and meiosis in eukaryotic cells. They are responsible for folding chromatin fibers into highly compact chromosomes to ensure their faithful segregation. In vertebrates, there are two types of condensin complexes: condensin I and condensin II (Table 1-1) (Hirano, 2006). Condensins I and II share two core subunits, Smc2/CAP-E and Smc4/CAP-C, but differ in the other three non-Smc subunits. Condensin I contains CAP-D2, CAP-H, and CAP-G while condensin II contains CAP-D3, CAP-H2, and CAP-G2 (Figure 1-4) (Ono et al., 2003). The two condensin complexes have apparently different roles in chromosome organization (Hirano, 2004). Depletion of condensin I produces a swollen chromosome shape while depletion of condensin II produces a curly shape. Depletion of both results in the formation of cloud-like chromosomes with a fuzzy appearance. In addition to condensin I and II, *C. elegans* has a specialized condensin-like Smc complex that regulates dosage compensation. This complex is composed of Smc2, an Smc4

variant called DPY-27, and three non-Smc proteins, DPY-26, DPY-28, and CAPG-1 (Csankovszki et al., 2009; Hirano, 2005).

Condensins in checkpoint activation and DNA repair

The first finding to reveal condensin's role in DNA repair came from a study in *S. pombe* (Aono et al., 2002). In this study, Aono *et al.* isolated a temperature-sensitive mutant of *cnd2*, a non-Smc subunit in the condensin complex (Aono et al., 2002). In addition to the expected mitotic chromosome condensation defects, this mutant exhibited elevated sensitivity to UV, HU, and methyl methanesulfonate (MMS), and a defect in Cds1 (the fission yeast ortholog of Chk2) activation. These results established a role of condensin in the replication checkpoint control and DNA repair. In a subsequent study, the same group identified a new condensin-binding protein called Cti1, using the hinge domain of Cut3 (Smc4) as the bait in a yeast two-hybrid screen (Chen et al., 2004). Overexpression of Cti1 suppressed the UV and HU sensitivity of the Cnd2 mutant, suggesting that Cti1 positively regulated the DNA repair function of condensin.

The two condensin complexes in humans are also involved in DNA repair. Condensin I has been shown to play a role in DNA single-strand break (SSB) repair by interacting with the PARP1–XRCC1 complex (Heale et al., 2006; Kong et al., 2011). Condensin I does not appear to play a significant role in DSB repair.

By contrast, a recent study showed that condensin II was involved in HR repair of DSBs to maintain genome integrity (Wood et al., 2008). Furthermore, depletion of condensin II only affected HR repair of IR-induced DSBs, but not the activation of the G2/M checkpoint.

Condensins and rDNA stability

Aside from its direct role in DNA repair to maintain genomic stability, condensin prevents unwanted intrachromosomal HR at the rDNA locus and controls rDNA stability in yeast (Tsang et al., 2007a; Tsang et al., 2007b; Tsang and Zheng, 2009). Condensin regulates rDNA condensation during interphase upon nutrient starvation. This sub-chromosomal DNA compaction likely inhibits intrachromosomal HR at this locus, reduces the production of extrachromosomal rDNA circles, and protects the integrity of the rDNA array. In the absence of condensin, Rad52 improperly localizes to the nucleolus. Deletion of Rad52 rescues the cell lethality under nutrient starvation caused by condensin inactivation. Thus, condensin-dependent nucleolus exclusion of Rad52 provides one mechanism for the regulation of rDNA stability by condensin.

rDNA stability is critical for normal nucleolar function and ribosome biogenesis. Dysregulation of ribosome biogenesis has been linked to cancer and other human diseases. In the future, it will be important to determine whether the rDNA protection function of condensin is conserved in higher organisms,

including humans. Along this vein, both *Xenopus* and human condensins have been shown to be associated with nucleolus during interphase (Cabello et al., 2001; Uzbekov et al., 2003).

The Smc5/6 complex

Unlike cohesin and condensins which have other major non-DNA repair functions in chromosome biology, the Smc5/6 complex is primarily required for DNA repair. The Smc5/6 complex is composed of Smc5, Smc6, and several non-SMC elements (Nse), including Nse1-6 (Table 1-1). Nse4 bridges the ATPase head domains of Smc5 and Smc6 (Figure 1-4), possibly in a manner similar to the role of Scc1 in linking Smc1 and Smc3. Nse1 interacts with Nse3, and both Nse1 and Nse3 bind to Nse4. Mms21/Nse2 does not bind to the ATPase head domains of Smc5/6, but interacts with the coiled-coil region of Smc5 (Duan et al., 2009; Potts, 2009). Intriguingly, two of the Nse proteins have enzymatic activities. Nse1 contains a RING domain commonly found in ubiquitin ligases and forms an active ubiquitin ligase with the MAGE (melanoma-associated antigen gene) protein Nse3 (Doyle et al., 2010). Mms21 contains an SP-RING domain and has small ubiquitin-like modifier (SUMO) ligase activity (Andrews et al., 2005; Potts and Yu, 2005; Zhao and Blobel, 2005). The SUMO ligase activity of Mms21 has been shown to be crucial for DNA damage repair and targets several substrates in different organisms from yeast to man.

The Smc5/6 complex and DSB repair by HR

The *Smc6* gene was initially identified by its ability to correct the radiation sensitivity of the *rad18-X* mutant isolated in a screen for radiation-sensitive mutations in the fission yeast *S. pombe* (Lehmann et al., 1995; Nasim and Smith, 1975; Phipps et al., 1985). Hypomorphic alleles of the Smc5/6 complex exhibit defects in the repair of DNA damage caused by a broad spectrum of agents, including IR, UV, MMS, mitomycin C and HU (Andrews et al., 2005; Lehmann, 2005; Pebernard et al., 2008; Pebernard et al., 2006; Santa Maria et al., 2007; Taylor et al., 2008). In *S. pombe*, Smc6 is required for DSB repair induced by IR and G2/M checkpoint activation (Verkade et al., 1999). Epistasis analysis further suggests a role of the Smc5/6 complex in HR, since *rad18, nse1, and nse2* are epistatic with *rhp51* (the fission yeast Rad51, a key HR protein) in response to IR (Lehmann et al., 1995; McDonald et al., 2003).

Consistent with these studies in the fission yeast, inactivation of the Smc5/6 complex in budding yeast, plants, chickens, and humans all results in sister-chromatid HR defects (De Piccoli et al., 2006; Mengiste et al., 1999; Potts et al., 2006; Stephan et al., 2011; Watanabe et al., 2009). In keeping with the role of the Smc5/6 complex in HR, it is recruited to HO-induced DSBs in budding yeast and I-SceI-induced DSBs in human cells, as revealed by ChIP experiments (De Piccoli et al., 2006; Lindroos et al., 2006; Potts et al., 2006). Moreover, the

Smc5/6 complex is only enriched at or around the HO-induced DSB in G2/M, but not in G1 when the sister chromatid is absent. Mre11 but not Mec1 and Rad53, are required for Smc6 recruitment to DSBs in yeast (Lindroos et al., 2006). Similar to cohesin depletion, depletion of the Smc5/6 complex in human cells reduced SCE. Co-depletion of both cohesin and the Smc5/6 did not further reduce SCE, suggesting that they acted in the same pathway to promote sister-chromatid HR (Figure 1-7) (Potts et al., 2006). Future studies are required to address the mechanisms by which the Smc5/6 complex promotes HR between sister chromatids.

The Smc5/6 complex and stalled replication forks

A second function of the Smc5/6 complex in DNA repair is the repair of collapsed replication forks. Smc6 localizes to collapsed replication forks in budding yeast (Lindroos et al., 2006). Inactivation of Smc5/6 caused accumulation of X-shaped HR intermediates that could be formed by the regression of stalled replication forks in rDNA. Furthermore, the SUMO ligase activity of Mms21 is required for preventing the accumulation of the X-shaped DNA molecules at damaged replication forks (Branzei et al., 2006), although the relevant substrate of Mms21 in this process is unknown. Overexpression of BRC1 (a BRCT domain protein required for DNA repair during S phase) or the bacterial resolvase RuvA rescued the replication-arresting defects of *nse5*, *nse6*, and *smc6*

mutants in *S. pombe* (Lee et al., 2007; Pebernard et al., 2006; Sheedy et al., 2005). The structure-specific endonucleases Slx1/4 and Mus81/Eme1 are required for the BRC1-mediated suppression of Smc5/6 mutant phenotypes. Moreover, inactivation of the Mph1 helicase suppressed the accumulation of aberrant recombination intermediates in *smc6* and *mms21*/*nse2* mutants in *S. cerevisiae* (Chen et al., 2009). Smc5/6 has also been shown to facilitate the resolution of sister-chromatid linkages during mitosis (Bermudez-Lopez et al., 2010; Chavez et al., 2010). Finally, the Smc5/6 complex is required for loading RPA and Rad52 onto stalled replication forks to maintain them in recombination-competent configurations (Irmisch et al., 2009). Collectively, these investigations indicate that the Smc5/6 complex promotes HR-dependent rescue of stalled replication forks by stabilizing them in recombination-component configurations and by facilitating the resolution or preventing the formation of certain recombination intermediates (Figure 1-7).

The Smc5/6 complex and rDNA integrity

Similar to condensin, the Smc5/6 complex is required for the maintenance of rDNA stability in budding yeast (Figure 1-7). The Smc5/6 complex accumulates at rDNA regions in budding yeast (Lindroos et al., 2006). It is enriched in the nucleolus (Torres-Rosell et al., 2005b). Inactivation of Smc5 or the Mms21 SUMO ligase activity results in fragmented and irregularly shaped

nucleoli (Torres-Rosell et al., 2005a; Zhao and Blobel, 2005), indicating a role of the Smc5/6 complex in maintaining rDNA integrity. The repair of DSBs in the rDNA locus occurs outside the nucleolus. To preserve the repetitive sequence of the rDNA array, DSB recognition, end resection, and RPA binding happen within the nucleolus. On the other hand, binding of the key downstream HR proteins Rad51 and Rad52 to these DSBs are excluded from the nucleolus. Cells harboring mutations in the Smc5/6 complex exhibit Rad52 foci at the DSBs in the nucleolus and elevated numbers of extrachromosomal rDNA circles (Torres-Rosell et al., 2007). These findings suggest that the Smc5/6 complex mediates Rad52 nucleolar exclusion, thereby suppressing the recombinational loss of rDNA repeats to ensure rDNA stability (Figure1-7).

Sumoylation in DNA Double-Strand Break Repair

Small ubiquitin-related modifier (SUMO) was identified as a reversible protein modifier in the 1990s. Sumoylation regulates substrate localization, activity, and protein-protein interaction. It is involved in many cellular processes, including gene transcription, DNA replication, and transport between the nucleus and cytoplasm. Recently, increasing evidence links sumoylation to DNA DSB repair.

The SUMO pathway

The SUMO proteins are conserved in all eukaryotes, from yeast to human. They share less than 20% sequence identity with ubiquitin, and there are at least four SUMO isoforms (SUMO1-4) in humans. SUMO2 and SUMO3 share 97% identity with each other and 50% identity with SUMO1 (Gareau and Lima, 2010; Geiss-Friedlander and Melchior, 2007). Because of the extremely high sequence similarity, SUMO2 and SUMO3 are often referred as SUMO2/3. SUMO1-3 are expressed in all tissues, while SUMO4 is only expressed in the kidney, lymph node and spleen (Guo et al., 2004). It is not clear so far if SUMO4 could be processed and conjugated to substrate. The mechanisms by which SUMO1 and SUMO2/3 are conjugated to substrates are the same, but the consequences of their conjugation are somewhat different. For example, they have distinct substrates, respond to different signals, and are removed by different isopeptidase enzymes. In addition, SUMO2/3 form SUMO chains on target proteins, whereas SUMO1 does not form chains efficiently. The embryonic-lethality of SUMO1 knockout mice also supports that SUMO1 and SUMO2/3 carry out non-redundant functions (Alkuraya et al., 2006).

SUMO conjugation and deconjugation

The sumoylation reaction is similar to the ubiquitination reaction in two ways. First, an isopeptide bond is formed between the C-terminal glycine residue of SUMO or ubiquitin and the ε-amino group of a lysine residue on the target

protein. Second, both reactions are catalyzed by enzyme cascades, although the actual enzymes are different for the two modifications.

The SUMO proteins are translated as immature forms and require processing before they can be conjugated to substrates (Figure 1-8). This processing is accomplished by SUMO-specific isopeptidases, known as sentrin-specific proteases (SENPs) in humans. SENPs remove several C-terminal residues of SUMO and expose the di-glycine motif. The mature SUMO proteins are then activated by a SUMO-specific E1 activating enzyme, which consists of the Aos1-Uba2 heterodimer, in an ATP-dependent manner (Figure 1-8). A thioester bond between the C-terminus of SUMO and the active-site cysteine of Uba2 is formed. Next, SUMO is transferred to the SUMO-specific E2 conjugating enzyme Ubc9, resulting in a thioester bond between the C-terminus of SUMO and the active-site cysteine of Ubc9. Ubc9 then transfers SUMO to the target protein, forming an isopeptide bond between the C-terminus of SUMO and a lysine residue on the target. This step usually occurs with the help of a SUMO E3 ligase, but in some cases, SUMO is conjugated to substrates by Ubc9 alone through recognition of a ψKXD/E (ψ is a large hydrophobic residue) consensus motif that contains a lysine acceptor residue. Sumoylation is a reversible post-translational modification. The SENP family of proteases can efficiently cleave the isopeptide bond between SUMO and its target. The released SUMO and target are ready to undergo another round of modification.

There are only a few SUMO E3 ligases identified, most of which are characterized by the presence of an SP-RING motif, similar to the RING domain in ubiquitin E3 ligases (Hochstrasser, 2001). A SUMO ligase can catalyze sumoylation in two ways: it can bring the Ubc9–SUMO thioester and substrate into a complex, or it can stimulate Ubc9 to discharge SUMO to substrate. The known SP-RING type SUMO ligases include Siz1, Siz2, and Mms21, and the protein inhibitor of STAT (PIAS) family proteins such as PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy (PIAS4). This type of SUMO E3 ligases is thought to interact with substrates and Ubc9-SUMO and bring them into close proximity. The E3-substrate interaction is particularly important for directing SUMO conjugation to lysine residues not located in a ψKXD/E consensus motif. A second type of SUMO E3 ligases is represented by RanBP2, which does not have an SP-RING domain (Kirsh et al., 2002). It brings the Ubc9-SUMO and substrate into an optimal position to catalyze the discharge of SUMO. A third type of SUMO ligases includes HDAC4 and the polycomb protein Pc2 (Geiss-Friedlander and Melchior, 2007). Their mechanisms of action are not yet clear.

Effects of sumoylation

The potential consequences of sumoylation are changes in substrate localization, activity, stability, and protein-protein interactions. Addition of a single SUMO or SUMO chains on the target protein could mask a previously

exposed binding site, provide a new binding surface, or cause a conformational change within the molecule (Figure 1-9). Cells utilize these versatile changes on the same protein to perform multiple functions in response to different environmental stress. For example, some proteins are only sumoylated under certain circumstances, such as 53BP1 sumoylation in response to DNA damage. Sumoylation is also limited by cellular localizations, which may be caused by the restricted localization of the E3 ligase. Other modifications may serve as a stimulus for sumoylation. For instance, phosphorylation of MEF2C stimulates its sumoylation. Meanwhile, modifications, such as acetylation or ubiquitination happening on the same lysine residue, could compete with protein sumoylation. Therefore, sumoylation is a highly regulated cellular process and tightly controlled by time and space.

Sumoylation in response to DNA damage

Sumoylation is involved in many aspects of DNA repair. For example, in base excision repair (BER), one of the DNA glycosylases, thymidine DNA glycosylase is sumoylated and the modification changes its cellular localization and affinity towards histone acetyltransferase CREB-binding protein (CBP) (Hardeland et al., 2002; Takahashi et al., 2005). In nucleotide excision repair (NER), the sumoylation of XPC promotes its stability and recycling from repair complexes (Wang et al., 2005). Another example is that the sumoylation of

XRCC4 in non-homologous end joining (NHEJ) is required for DSB repair, because mutation of the sumoylation site leads to radiation sensitivity (Yurchenko et al., 2006).

DNA DSB ends are recognized by the MRN complex, which recruits and activates ATM. ATM then phosphorylates histone H2AX, which attracts MDC1. The recruited MDC1 is phosphorylated by ATM and plays an essential role in spreading the damage signal. Among the many proteins recruited by MDC1, RNF8 and HERC2, two ubiquitin E3 ligases, and Ubc13, an ubiquitin E2-conjugating enzyme carry out the ubiquitination of H2A to form the K63-linked poly-ubiquitin chains (Figure 1-10). Ubiquitinated histones then recruit RNF168, another ubiquitin E3 ligase. RNF168 amplifies histone polyubiquitination around DSB to a level that is sufficient for the recruitment of 53BP1 and BRCA1 (Bekker-Jensen and Mailand, 2011; Zlatanou and Stewart, 2010). Two SUMO E3 ligases, PIAS1 and PIAS4, promote the sumoylation of BRCA1 and 53BP1, which is also required for their recruitment to DSBs (Galanty et al., 2009; Morris et al., 2009) (Figure 1-10). The PIAS1-dependent sumoylation of BRCA1 seems to promote the ubiquitin E3 ligase activity of the BRCA1-BARD1 heterodimer. Meanwhile, PIAS4-dependent sumoylation mediates RNF168 retention at the damaged chromatin by a yet unknown mechanism (Galanty et al., 2009; Morris et al., 2009).

RPA, an ssDNA binding protein, interacts with the 3' resected DNA overhangs. RPA consists of three subunits, RPA1 (RPA70), RPA2 (RPA32) and RPA3 (RPA14). RPA70 is sumoylated on its lysine residues K449 and K577, and the sumoylation is enhanced by replication-induced DSB. A non-sumoylatable RPA mutant shows a delay in Rad51 foci formation and defect in HR in vivo (Dou et al., 2010). Therefore, RPA70 sumoylation may serve as a mediator for Rad51 recruitment to the resected DSB ends (Figure1-10).

Both Rad51 and Rad52 have been shown to bind to SUMO and the SUMO E2 conjugating-enzyme Ubc9 (Dou et al., 2010; Kovalenko et al., 1996; Ouyang et al., 2009; Shen et al., 1996a; Shen et al., 1996b) (Figure 1-10). Consistently, inactivation of Ubc9 results in a dramatic inhibition of Rad51 foci formation at the damaged sites (Saitoh et al., 2002). Sumoylation of Rad52 is induced by methyl methanesulfonate (MMS) treatment in S phase and is critical for HR in yeast. Under conditions that generate DNA DSBs, the non-sumoylatable Rad52 mutant is quickly degraded. Thus, DNA damage-induced sumoylation of Rad52 mediates its stability and protects it from degradation (Sacher et al., 2006). Moreover, the sumoylated Rad52 is excluded from the nucleolus. As a consequence, the HR repair could only occur outside of the nucleolus (Torres-Rosell et al., 2007). This mechanism prevents unwanted recombination between repetitive sequences and protects rDNA stability.

Rad51 directs strand invasion and recombination. The newly synthesized intermediates form a double Holliday junction which needs to be resolved by the BLM/TopoIII complex or cleaved by the endonucleases Gen1, Mus81/Eme1, or Slx1/Slx4. In vitro, BLM has been shown to displace Rad51 from ssDNA and is capable to unwind the D-loop formed by Rad51 invasion (Bugreev et al., 2007). In contrast to its role in the late stage of HR, BLM also promotes the formation of Rad51 coupled ssDNA filament in the early step (Ouyang et al., 2008). Therefore, BLM has entirely opposite roles in the early and late steps of HR. The switch between the two opposing functions of BLM is controlled by sumoylation, which enhances the binding between Rad51 and BLM and switches BLM into a pro-recombination state to promote Rad51 function (Ouyang et al., 2009).

The Smc5/6 complex has multiple functions in DNA damage response (De Piccoli et al., 2009; Potts, 2009). It is recruited to DSBs and is critical for their repair through SCR in yeast and human (De Piccoli et al., 2006; Lindroos et al., 2006; Potts et al., 2006). The Smc5/6 complex component Mms21 is a SUMO E3 (Andrews et al., 2005; Potts and Yu, 2005; Zhao and Blobel, 2005). Although it is clear that the SUMO ligase activity of Mms21 is required for DNA repair, the critical targets of Mms21 in DSB repair have not been established. My research presented in this dissertation fills this important gap in our knowledge by identifying the cohesin subunit Scc1 as a critical Mms21 target in SCR.

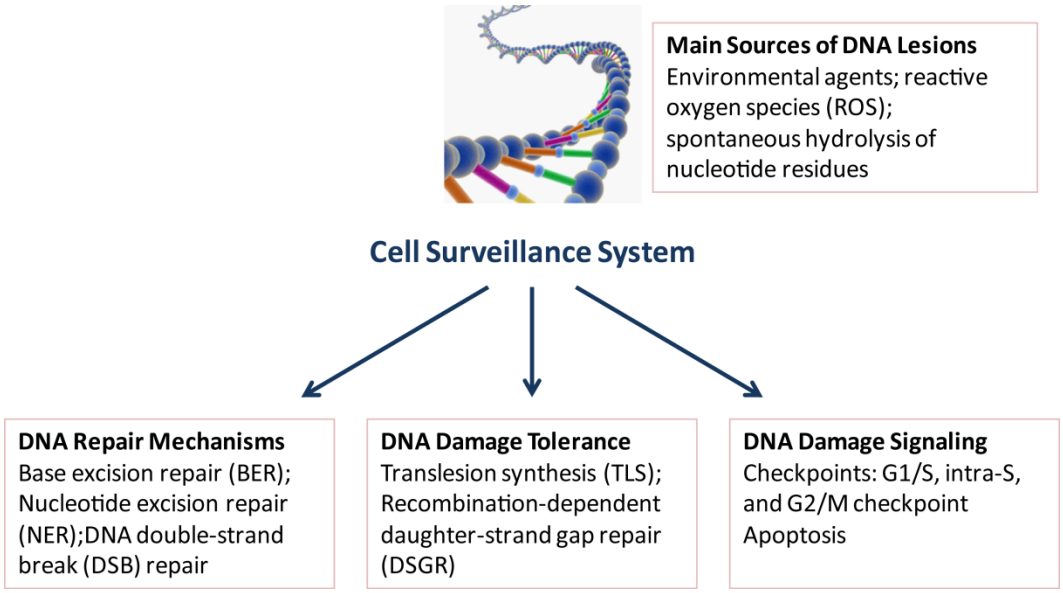

Figure 1-1. Human cells have a powerful DNA damage surveillance system
A human cell encounters more than 10,000 DNA lesions per day. These DNA damages mainly from three factors: environmental agents, reactive oxygen species (ROS), and spontaneous hydrolysis of nucleotide residues. In response to different kinds of DNA damages, cells elicit different repair pathways, such as base excision repair (BER), nucleotide excision repair (NER) and DNA double-strand break (DSB) repair. There are three DNA damage checkpoints according to different cell cycles: the G1/S checkpoint, the intra-S checkpoint and the G2/M checkpoint. In addition, cells evolve two DNA damage tolerance mechanisms: translesion synthesis (TLS) and recombination-dependent daughter-strand gap repair (DSGR). If the DNA damage is too severe, cells will trigger apoptosis.

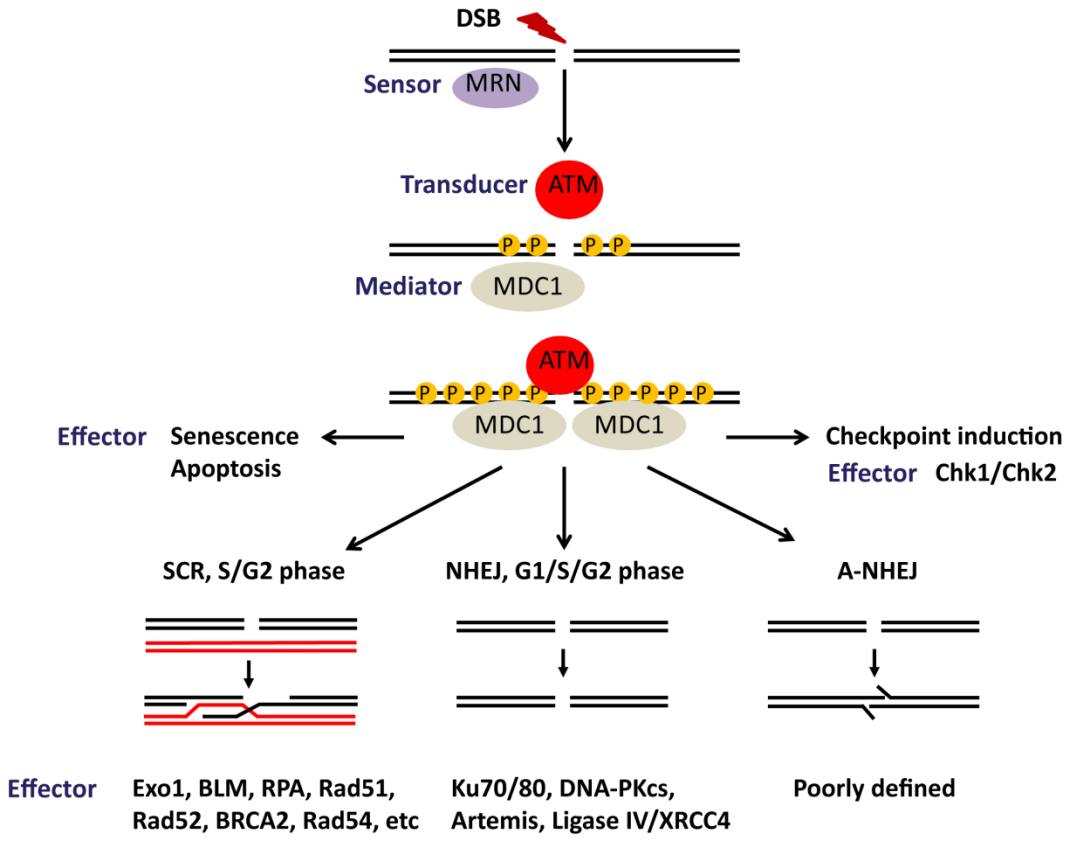

Figure 1-2. Non-homologous end joining (NHEJ) and homologous recombination (HR) are the two major pathways for DNA DSB repair
DNA damage repair proteins are divided into several groups, according to their roles in the DNA damage response: sensors, transducers, mediators and effectors. The Mre11/Rad50/Nbs1 (MRN) complex is the DSB sensor, which recruits the transducer ATM to phosphorylate H2AX to build a long γH2AX domain. MDC1 acts as the mediator and interacts with γH2AX and phosphor-ATM, which allows a local enrichment of ATM and phosphorylation of more H2AX. This positive feedback spreads the damage signal to megabases flanking a DSB. There are two major DSB repair pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). Because HR requires a sister chromatid as template, this kind of repair pathway is preferred in S/G2 after DNA replication. In contrast, NHEJ can occur in any cell cycle. Effectors contain many DNA repair proteins, checkpoint kinases, and proteins involved in apoptosis.

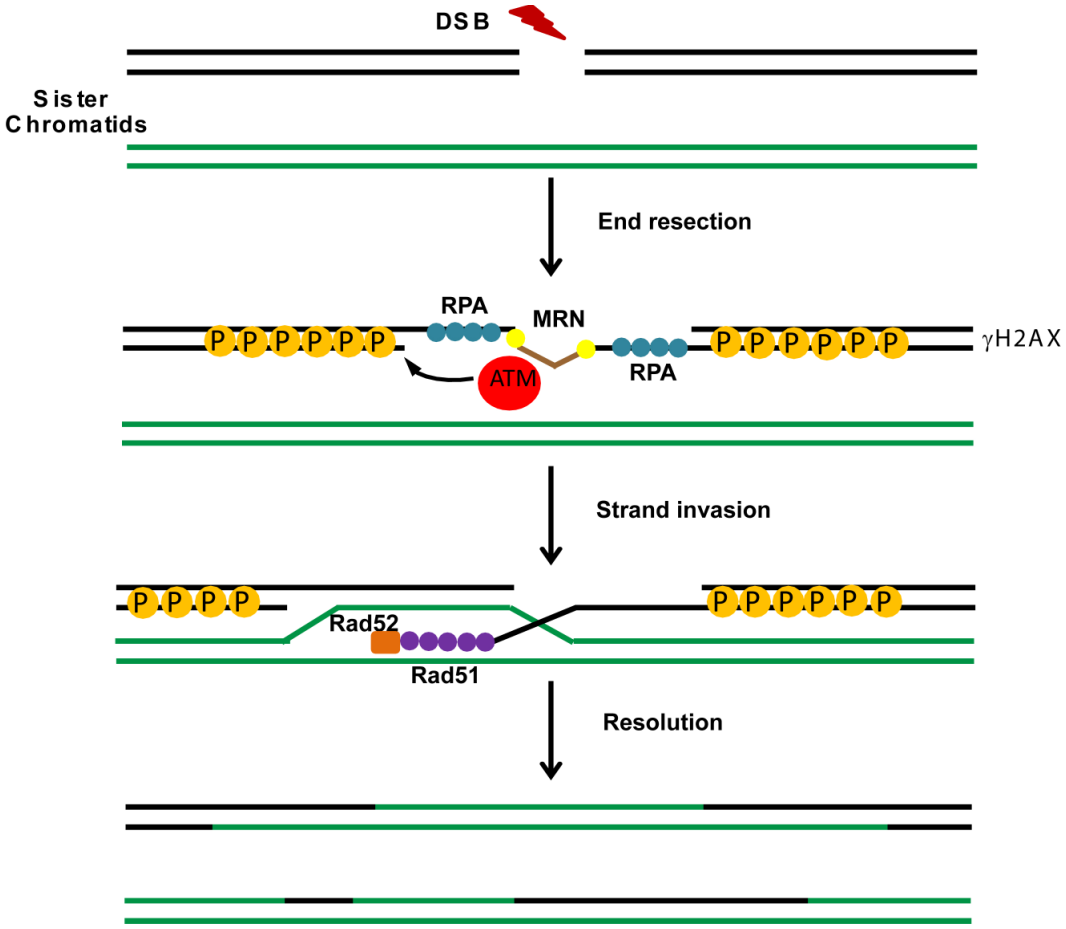

Figure 1-3. Homologous recombination (HR) mediated DNA DSB repair
HR is a key DSB repair pathway and requires an undamaged DNA template: sister or homologous chromosomes. Because HR between homologous chromosomes can result in loss of heterozygosity (LOH), DSB repair with HR between sister chromatids (SCR) is preferable. MRN complex binds to the DSB ends and recruits ATM, which mediating many downstream events, including phosphorylation of H2AX. The DSB ends are processed to form 3' single-strand DNA overhangs coated with RPA. After this step, RPA is replaced by Rad51 with the help of Rad52. The Rad51 coated single-strand filament promotes strand invasion into the intact sister chromatid. After DNA synthesis by polymerases, the strand ligation results in a double Holliday junction. Finally, the junction is resolved by junction resolvases or helicase and topoisomerase.

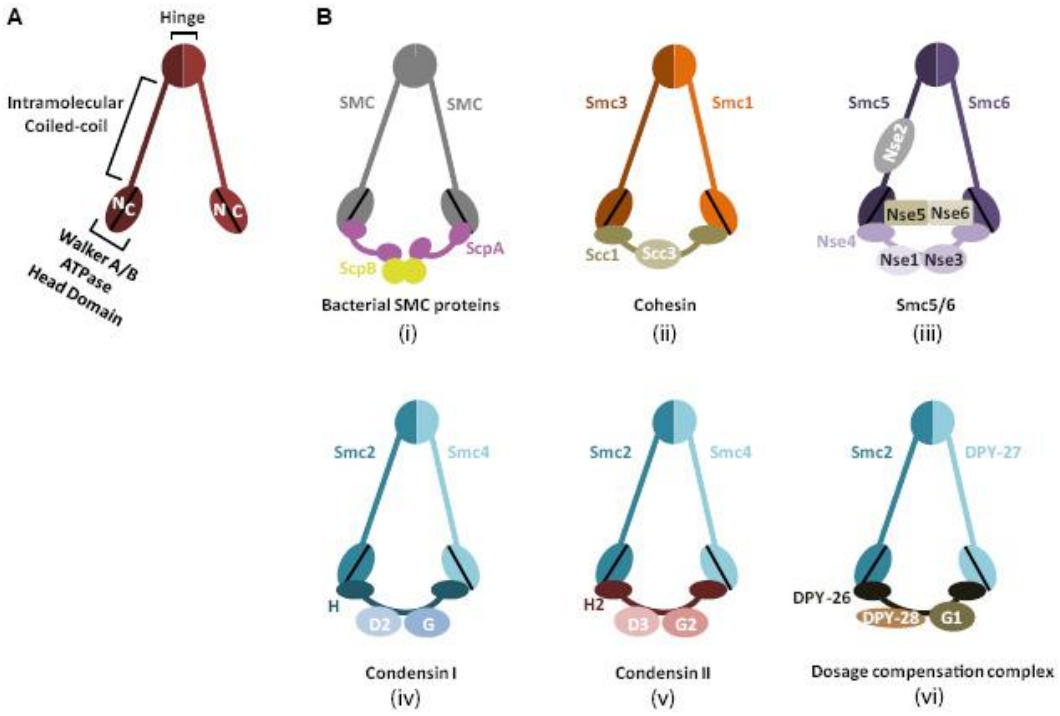

Figure 1-4. Architecture of the Smc complexes

(A) The core of each Smc complex is formed by two Smc proteins. Each Smc protein contains an ATPase head domain, a hinge domain, and an intramolecular antiparallel coiled coil that connects the two. The hinge domain mediates the dimerization of Smc proteins. (B) Various Smc complexes from bacteria and eukaryotes. Each Smc complex is composed of a specific Smc dimer and several non-Smc subunits. (i) The bacterial Smc complex from *Bacillus subtilis*. (ii) The Smc1/3 cohesin complex. (iii) The Smc5/6 complex. (iv) The condensin I complex. H, D2, and G stand for CAP-H, CAP-D2, and CAP-G, respectively. (v) The condensin II complex. H2, D3, and G2 stand for CAP-H2, CAP-D3, and CAP-G2, respectively. (vi) The condensin-like dosage compensation complex in *C. elegans*. DPY-27 is an Smc4 variant.

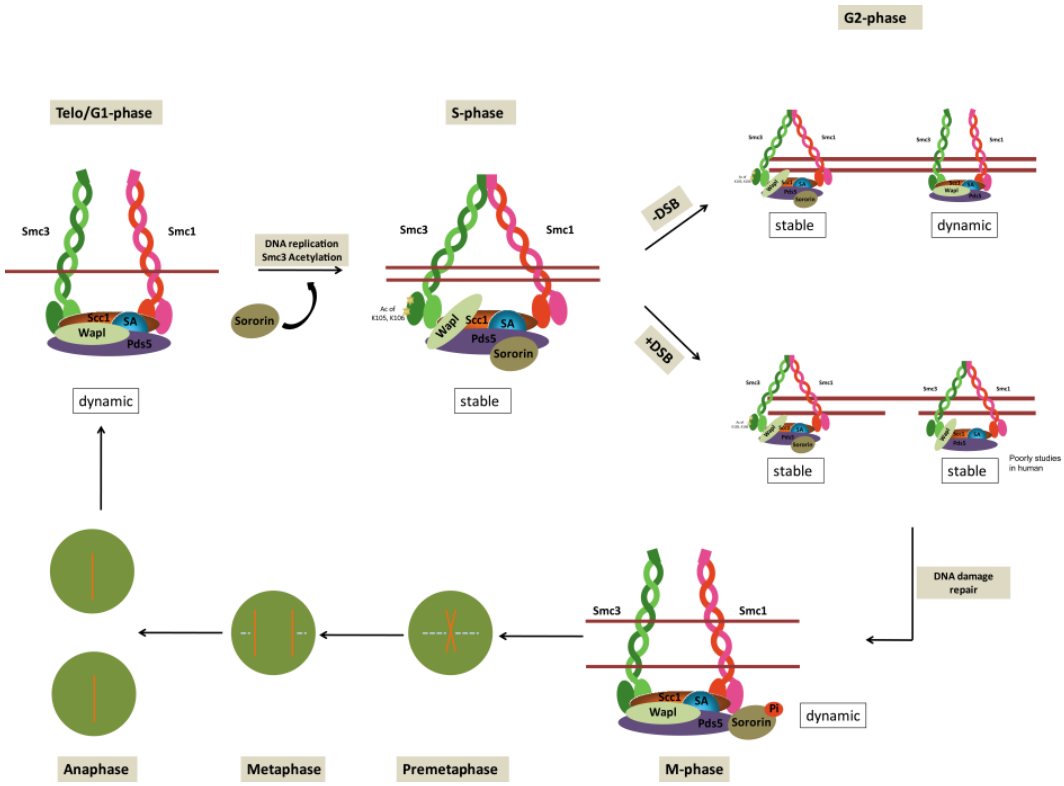

Figure 1-5. The cohesin cycle in a human cell

Cohesin is loaded by the Scc2/4 complex in telophase and G1 prior to DNA replication. The loaded cohesin then becomes cohesive during DNA replication and has been proposed to topologically embrace both sister chromatids inside its ring to establish sister-chromatid cohesion. The mechanism by which cohesin is converted to the cohesive state during DNA replication is not completely understood, but it requires the acetylation of Smc3 by Esco1/2. Smc3 acetylation enables the binding of Sororin to Pds5, which counteracts Wapl's ability to remove cohesin from chromatin. After DNA replication, the Esco1/2 activity becomes limited. Cohesin is still dynamically loaded onto chromatin; however, it does not become cohesive, and is quickly removed by Wapl. In budding yeast, it is reported that DNA DSBs trigger genome wide cohesion establishment. However, the mechanism in humans is not clear yet. After cells enter mitosis, most cohesin on chromatid arms is removed by Wapl in prophase, and this process is facilitated by Plk1-dependent phosphorylation of SA2. Only a small amount of cohesin remains associated with the centromeres and is protected from Wapl and Plk1 by the shugoshin–PP2A complex. This centromeric pool of cohesin is cleaved by separase at metaphase to allow sister-chromatid separation.

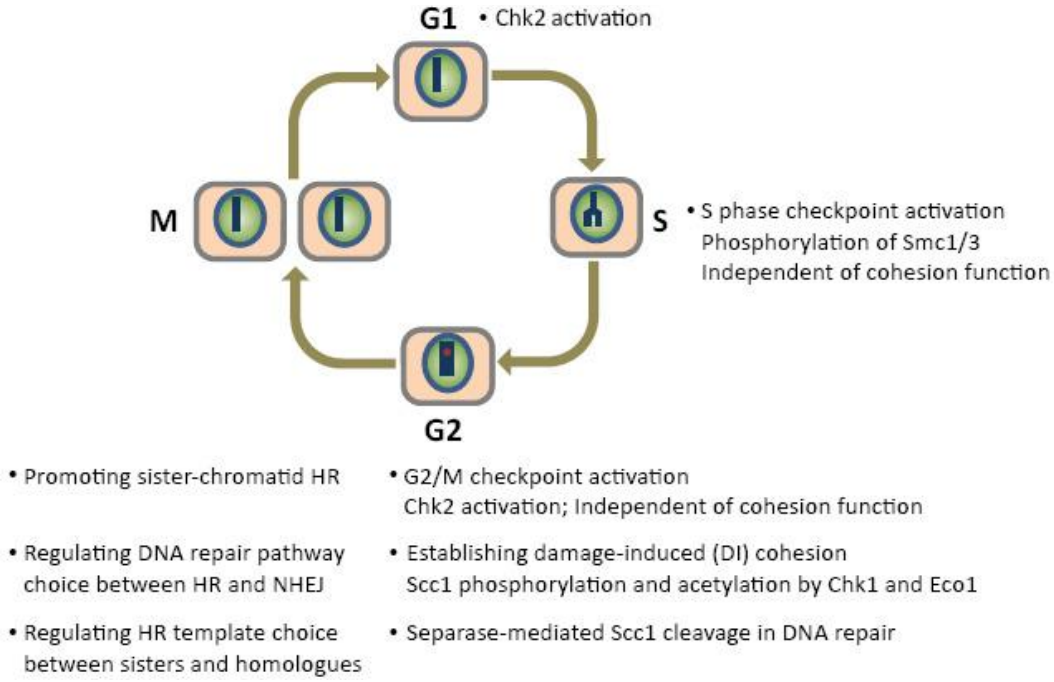

Figure 1-6. Functions of cohesin in DNA damage response during the cell cycle

The function of cohesin in promoting DSB repair through sister-chromatid homologous recombination (HR) has been established in multiple organisms ranging from yeast to man. Most of the other proposed functions are only demonstrated in specific organisms, and their evolutionary conservation needs to be further tested.

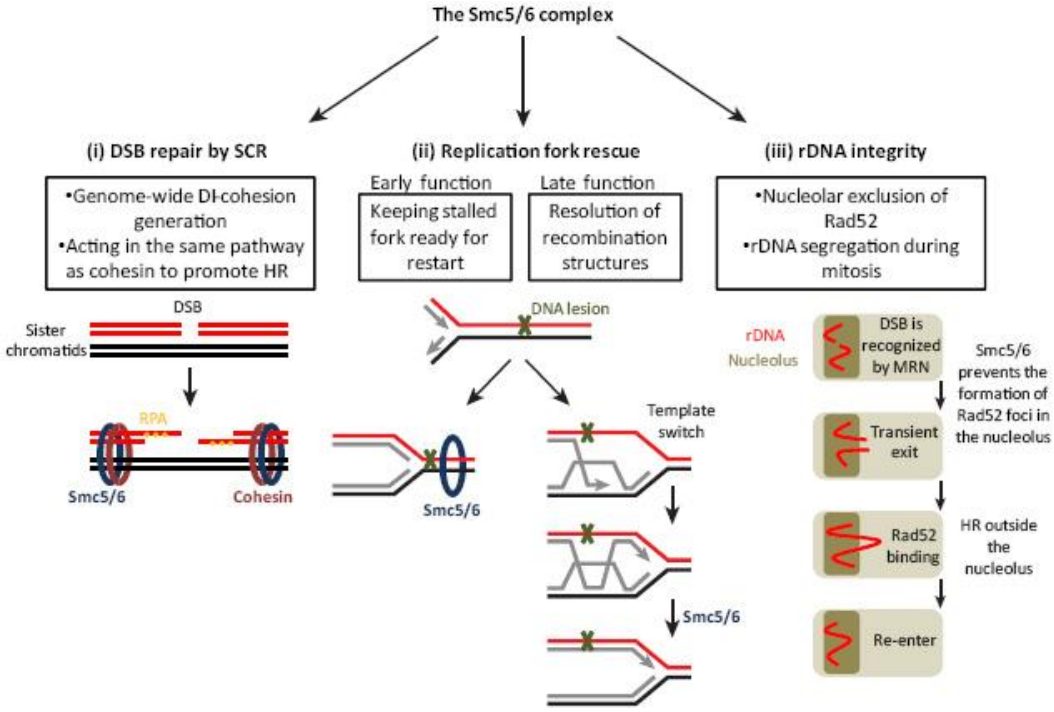

Figure 1-7. Functions of the Smc5/6 complex in DNA repair and rDNA maintenance

The Smc5/6 complex plays multiple roles in DNA repair. First, it is required for sister chromatid homologous recombination (SCR). It regulates genome-wide DSB induced cohesion generation in budding yeast. It is also required for SCR in humans. Genetic study suggests the Smc5/6 complex facilitate SCR in the same pathway with cohesin. Second, the Smc5/6 complex promotes HR-dependent rescue of stalled replication forks. It functions in two ways: stabilizing the stalled replication forks in recombination-component configurations and facilitating the resolution or preventing the formation of certain recombination intermediates. Lastly, the Smc5/6 complex is essential for rDNA stability in budding yeast.

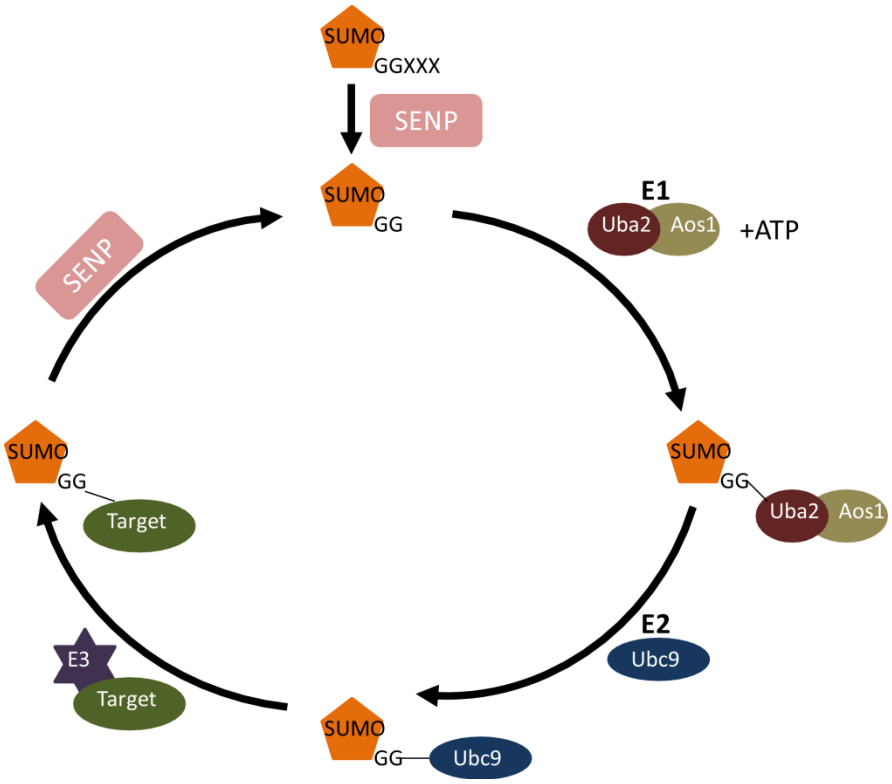

Figure 1-8. The sumoylation cycle
Before conjugation to a substrate, the C-terminal residues of a SUMO protein have to be cleaved by SENP to reveal the di-glycine motif. The mature SUMO is activated by SUMO-specific E1 activating enzyme heterodimer Aos1-Uba2 in an ATP-dependent manner. Next, SUMO is transferred to the SUMO-specific E2 conjugating enzyme Ubc9. At last, Ubc9 transfers SUMO to the target protein, forming an isopeptide bond between the C-terminal glycine residue of SUMO and a lysine residue on the target. This step usually occurs with the help of a SUMO E3 ligase, but in some cases, SUMO is conjugated to the substrate by E2 alone. Sumoylation is a reversible post-translational modification. SENP family of proteases can efficiently cleave the isopeptide bond between SUMO and its target.

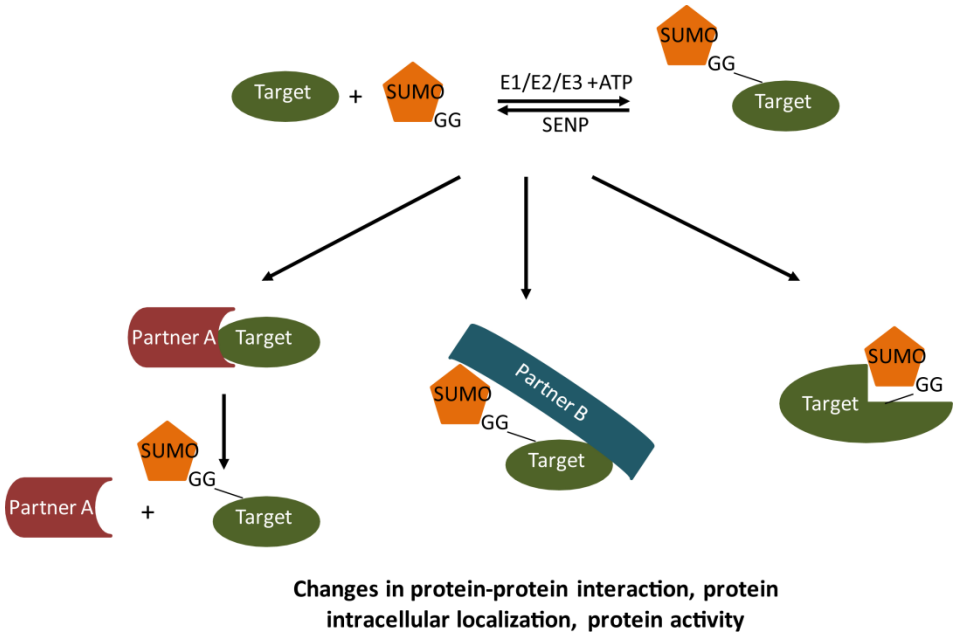

Figure 1-9. Molecular consequences of sumoylation
Sumoylation is a reversible post-translational protein modification. The sumoylation reaction is catalyzed by SUMO specific E1, E2, E3 enzymes and requires ATP. The de-sumoylation reaction is facilitated by SUMO isopeptidase SENP. Addition of a single SUMO or SUMO chains on the target protein potentially causes three types of changes: masking a previous binding site on the target protein, creating a new binding surface on the target protein, or generating a conformational change within the molecule.

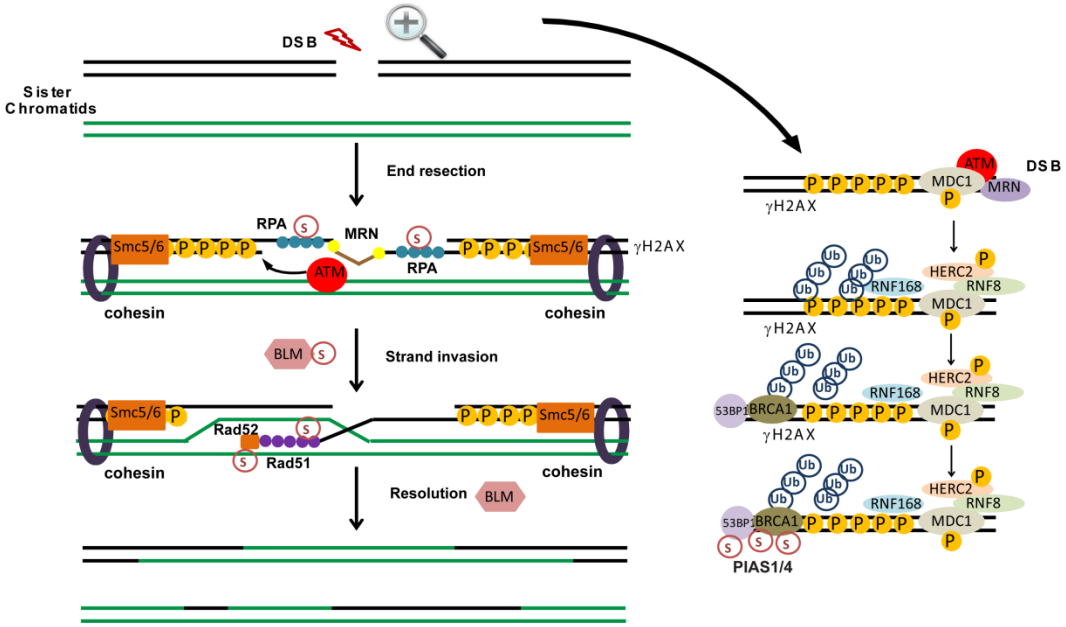

Figure 1-10. Sumoylation in DNA damage response
Many factors in the DNA DSB repair pathway are sumoylated. PIAS1-dependent sumoylation of BRCA1 promotes its recruitment and ubiquitin ligase activity. PIAS4-dependent sumoylation of 53BP1 promotes its recruitment and RNF168 retention on the damaged chromatin. RPA70 sumoylation is required for Rad51 recruitment. Rad51 and Rad52 sumoylation are essential for HR. Sumoylation of BLM in the early step of HR promotes Rad51 filament formation. The Smc5/6 complex is recruited and critical for HR. The Smc5/6 complex component Mms21 is a SUMO specific E3 ligase. Although it is clear that the SUMO ligase activity of Mms21 is required for DNA repair, the critical targets of Mms21 in DSB repair have not been established.

Table 1-1. Components of the SMC complexes and regulatory proteins in different organisms

	S.cereviae	*S.pombe*	*H.sapiens*
Cohesin	Smc1	Psm1	Smc1
	Smc3	Psm3	Smc3
	Mcd1/Scc1	Rad21	Scc1/Rad21
	IRR1/Scc3	Psc3	SA1/STAG1,SA2/STAG2
Cohesin regulatory factors	Scc2	Mis4	NIPBL
	Scc4	Ssl3	MAU2/Scc4
	Eco1/Ctf7	Eso1	Esco1/EFO1, Esco2/EFO2
	Pds5	Pds5	Pds5A, Pds5B
	Rad61/Wpl	Wpl1	Wapl
	-	-	Sororin
Condensin	Smc2 (I&II)	Cut14	CAP-E
	Smc4 (I&II)	Cut3	CAP-C
	Brn1	Cnd2	CAP-H (I)
	Ycs4	Cnd1	CAP-D2 (I)
	Ycs5	Cnd3	CAP-G (I)
	-	-	CAP-D3 (II)
	-	-	CAP-G2 (II)
	-	-	CAP-H2 (II)
Smc5/6	Smc5	Spr18/Smc5	Smc5
	Rhc18/Smc6	Rad18/Smc6	Smc6
	Nse1	Nse1	Nse1
	Mms21/Nse2	Nse2	Nse2
	YDR228W/Nse3	Nse3	Nse3
	Qri2/Nse4	Rad62/Nse4	Nse4
	YML023C/Nse5	Nse5	-
	Kre29/Nse6	Nse6	-

CHAPTER II: SCC1 SUMOYLATION BY MMS21 PROMOTES SISTER CHROMATID RECOMBINATION THROUGH COUTERACTING WAPL

Introduction

DNA double-strand break (DSB) is the most deleterious form of DNA damage and can cause chromosome translocations, a major class of cancer-driving mutations. Homologous recombination (HR) is a key DSB repair pathway and requires an undamaged DNA template: sister or homologous chromosomes. Because HR between homologous chromosomes can result in loss of heterozygosity (LOH), DSB repair with HR between sister chromatids (SCR) is preferable during the mitotic cell cycle. Two related structural maintenance of chromosomes (Smc) complexes, cohesin and the Smc5/6 complex, play critical roles in SCR-dependent repair of DSBs (De Piccoli et al., 2009; Potts, 2009; Watrin and Peters, 2006).

The primary function of cohesin is to establish sister-chromatid cohesion in S phase and maintain this cohesion until the metaphase–anaphase transition (Nasmyth and Haering, 2009; Onn et al., 2008; Peters et al., 2008). Although cohesin is loaded onto chromosomes during telophase or G1, it only becomes cohesive during S phase. Cohesion establishment in S phase requires Smc3 acetylation by the acetyltransferase Eco1 at K112 and K113 in yeast (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009; Unal et al., 2008) or by the Eco1 homologs Esco1/2 at K105 and K106 in human cells (Hou and Zou, 2005; Zhang

et al., 2008). In human cells, Smc3 acetylation enables the chromatin binding of Sororin through Pds5, which protects cohesin from the cohesin inhibitor Wapl, thus establishing functional cohesion (Kueng et al., 2006; Nishiyama et al., 2010; Rankin et al., 2005).

The Smc5/6 complex has multiple functions in DNA damage response (De Piccoli et al., 2009; Potts, 2009). It is recruited to DSBs and is critical for their repair through SCR in yeast and humans (De Piccoli et al., 2006; Lindroos et al., 2006; Potts et al., 2006). The Smc5/6 complex component Mms21 is a ligase for small ubiquitin-like modifier (SUMO) (Potts and Yu, 2005; Zhao and Blobel, 2005). Although it is clear that the SUMO ligase activity of Mms21 is required for DNA repair (Potts and Yu, 2005; Zhao and Blobel, 2005), the critical targets of Mms21 in DSB repair have not been established.

In addition to its role in global sister-chromatid cohesion and chromosome segregation in mitosis, cohesin is loaded locally at DSBs to facilitate SCR in organisms from yeast to human. In yeast, cohesin is loaded de novo at DSBs in a process that requires the cohesin-loading complex Scc2/4 (Strom et al., 2004; Unal et al., 2004). DNA damage then induces ATR/Chk1-dependent phosphorylation of the cohesin subunit Scc1 at S83, which in turns enables its acetylation at K84 and K210 by Eco1 (Heidinger-Pauli et al., 2008; Heidinger-Pauli et al., 2009). Scc1 acetylation antagonizes Wapl to establish DNA damage-induced (DI) cohesion (Heidinger-Pauli et al., 2009; Strom et al., 2007; Unal et

al., 2007). Thus, Eco1 has distinct targets during replicative and DI cohesion establishment in yeast. The Smc5/6 complex is loaded at DSBs (De Piccoli et al., 2006; Lindroos et al., 2006). It is not required for the initial loading of cohesin at DSBs, but is required for DI cohesion establishment (Strom et al., 2007). The mechanism by which Smc5/6 contributes to DI cohesion establishment is unknown.

In human cells, both cohesin and the Smc5/6 complex are loaded at endonuclease induced DSBs and collaborate in the same pathway to promote SCR (Potts et al., 2006). In contrast to yeast, however, it is unclear whether DNA damage triggers local DI cohesion in human cells, and if so, how this cohesion is established. The Chk1 phosphorylation site (S83) and the Eco1 acetylation sites (K84 and K210) in yeast Scc1 are not conserved in human Scc1 (Figure 2-1), suggesting that if human cells establish DI cohesion, they might use a different mechanism to do so.

Results

Mms21 sumoylates Scc1 at multiple sites

I first verified that cohesin was required for SCR and DSB repair in human cells. For this purpose, I performed colony survival and sister-chromatid exchange (SCE) assays on HeLa Tet-On cells depleted of cohesin subunits by RNA interference (RNAi). Depletion of Smc1, Smc3, Scc1, or SA1/2 expectedly

rendered these cells sensitive to IR (Figure 2-2A and 2-2B). SCE is one form of SCR that involves the resolution of double Holliday junctions in a crossover-producing pathway (West, 2003). In the SCE assay, cells were incubated with the bulky nucleotide analog BrdU through two rounds of DNA replication and division. In the end, both DNA strands of one sister chromatid incorporated BrdU while only one strand of the other sister contained BrdU. The sister with two BrdU-containing strands was more weakly stained with acridine orange, a DNA-intercalating dye. SCE was induced by the topoisomerase I inhibitor camptothecin and then scored in metaphase spreads by counting the number of crossover events between sister chromatids. Both Scc1- and SA1/2-RNAi cells showed a decrease in SCE frequency (Figure 2-2C and 2-2D). Depletion of each cohesin subunit was confirmed by Western blots (Figure 2-2E). Depletion of a given cohesin subunit often reduced the protein levels of other subunits, suggesting that cohesin existed as a single functional entity. Therefore, cohesin is required for SCR and DSB repair in human cells. I note that cohesin depletion also caused sister chromatid separation in mitosis in a fraction of the metaphase spreads. I was only able to count SCE events in the cells that retained cohesion and presumably had incomplete cohesin knockdown. Thus, the decrease in SCE seen in cohesin RNAi cells was likely an underestimation.

Our lab had previously shown that the SUMO ligase activity of human Mms21 was required for DNA repair (Potts and Yu, 2005). Mms21 could

sumoylate Scc1 and SA2, two cohesin subunits (Potts et al., 2006). In yeast, Mms21 had been shown to sumoylate the cohesin subunits, Smc1 and Smc3 (Takahashi et al., 2008). To determine whether Mms21-dependent sumoylation of cohesin was functionally important, I decided to characterize the sumoylation of Scc1 by Mms21. I thus transfected HeLa cells with plasmids encoding Mms21 and His_6-GFP-SUMO1/2, lysed the cells with a protein-denaturing buffer, performed Ni^{2+}-beads pull-down from the lysates, and blotted the lysates and proteins bound to beads with anti-Scc1. As a positive control, I blotted the lysates and beads with anti-RanGAP1, a known SUMO conjugated protein (Matunis et al., 1998). RanGAP1 that was conjugated to His_6-GFP-SUMO1/2 was highly enriched by Ni^{2+}-beads pull-down, validating the assay (Figure 2-3). Several Scc1 bands were also enriched by Ni^{2+}-beads pull-down, indicating that they were Scc1–SUMO conjugates (Figure 2-3). Thus, the endogenous cohesin indeed underwent Mms21-dependent sumoylation. This sumoylation was not further enhanced by IR treatment (Figure 2-4). Despite repeated attempts, I could not detect sumoylation of endogenous cohesin by immunoblotting in G2 human cells with or without IR in the absence of Mms21 or SUMO overexpression (data not shown), presumably because only a small pool of cohesin was sumoylated.

I next performed sumoylation assays on ectopically expressed Scc1. I transfected HeLa cells with different combinations of plasmids encoding GFP-SUMO1, GFP-SUMO1 ΔGG (a SUMO mutant lacking the two C-terminal

glycines required for conjugation), GFP-Mms21, and Myc-Scc1, and blotted the cell lysates with anti-Myc (Figure 2-5A). Myc-Scc1 formed high molecular mass species, in the presence of GFP-SUMO1, but not GFP-SUMO1 ΔGG. Mms21 greatly increased the intensity of these Myc-Scc1 species. By contrast, the ligase-dead mutant of Mms21 (C215A) did not stimulate the production of the modified forms of Myc-Scc1 (Figure 2-5C). Moreover, when His_6-GFP-SUMO2 was co-transfected with Myc-Scc1 and Mms21 WT or C215A, these modified forms of Myc-Scc1 were absent in the Mms21 C215A sample and were highly enriched by Ni^{2+}-beads pull-down, indicating that these species were Scc1–SUMO conjugates (Figure 2-5D). Finally, another SUMO ligase PIASy failed to stimulate the sumoylation of Myc-Scc1 under the same conditions (Figure 2-5B). Thus, these results confirmed that Scc1 was sumoylated by Mms21 in human cells.

The Scc1–SUMO conjugates formed several discernible bands. To determine whether these conjugates contained SUMO chains or represented Scc1 sumoylation at multiple sites, I performed the sumoylation assay with GFP-SUMO1 K-less, a SUMO1 mutant with all lysines mutated to arginine (Gocke et al., 2005). A similar multi-band sumoylation pattern of Myc-Scc1 was observed with SUMO1 K-less (Figure 2-6). Because SUMO1 K-less could not form chains, this result indicated that Mms21 sumoylated multiple residues of Scc1. Likewise, both SUMO2 WT and K11R (a SUMO2 mutant that cannot efficiently form chains) produced Scc1 sumoylation patterns similar to SUMO1 (Figure 2-6),

indicating that Mms21 also catalyzed conjugation of SUMO2 to multiple sites in Scc1.

Construction of a non-sumoylatable Scc1 mutant

To elucidate the potential roles of Mms21-dependent sumoylation of Scc1 in DSB repair, I needed to create an Scc1 mutant that could no longer be sumoylated by Mms21. Sumoylation often occurs on lysines within ΨKXD/E (Ψ is a large hydrophobic residue and X is any residue) consensus motifs, which directly interact with Ubc9 (Gareau and Lima, 2010). Scc1 contained one such motif at K216 (Figure 2-12A). Scc1 K216R was, however, still efficiently sumoylated by Mms21 (Figure 2-12B), consistent with our finding that Mms21 sumoylated Scc1 at multiple sites.

To identify the Mms21 sumoylation sites of Scc1, I generated a series of truncation mutants of Scc1 (Figure 2-7A), and tested each fragment in the in vivo sumoylation assay (Figure 2-7B). Scc1I (residues 317-420) containing the SA-binding domain was the smallest fragment that retained Mms21-dependent sumoylation (Figure 2-7B). A SUMO ligase can catalyze sumoylation in two ways: it can bring the Ubc9–SUMO thioester and substrate into a complex, or it can stimulate Ubc9 to discharge SUMO to substrate (Gareau and Lima, 2010). When co-expressed in HeLa cells, Myc-Scc1 indeed bound to HA-Mms21 (Figure 2-8B). Scc1I was the smallest Scc1 fragment that retained both Mms21

binding and Mms21-dependent sumoylation (Figure 2-9). These results suggest that Mms21 (possibly as a part of the intact Smc5/6 complex) simultaneously binds to Scc1 and Ubc9–SUMO and brings them into close proximity, thereby facilitating Scc1 sumoylation.

I could not detect a physical interaction between the endogenous Scc1 and Mms21 in human cells with or without IR. I thus fractionated lysates of HeLa cells arrested in G2 and treated with or without IR on a gel filtration column (Figure 2-10). All cohesin subunits eluted in the same fractions. All tested Smc5/6 components also co-fractioned with one another. IR treatment did not significantly alter the fractionation profiles of cohesin, Smc5/6, and the cohesin regulators Pds5A/B and Wapl. Therefore, my data suggested that the bulk of Mms21 existed as a component of the Smc5/6 complex in human cells with or without DNA damage. It might sumoylate cohesin through a transient interaction between the two intact complexes. I cannot exclude the possibility that a small pool of Mms21 shuttles between Smc5/6 and cohesin complexes.

On the other hand, Mms21 stimulated the sumoylation of an Scc1 fragment (Scc1F) that could not bind to Smc1 or Smc3 (Figure 2-11A), suggesting that sumoylation of Scc1 by Mms21 did not require the intact cohesin. To test whether free Mms21 stimulated sumoylation of Scc1, I expressed and purified from bacteria the full-length human Mms21. Recombinant Mms21 stimulated the sumoylation of in vitro translated full-length Myc-Scc1 or Scc1F in the presence

of SUMO1, Aos1/Uba2 (E1), and Ubc9 (E2) (Figure 2-11B and 2-11C). Therefore, Mms21 is sufficient to sumoylate Scc1 in vitro, although cohesin sumoylation in vivo might be mediated through a transient interaction between the intact Smc5/6 and cohesin complexes.

Scc1I contained 14 lysines, some of which were conserved in metazoan (Figure 2-1). Mutation of all 14 lysines to arginines (14KR) in the context of the full-length Scc1 or deleting residues 317-420 greatly reduced Scc1 sumoylation (Figures 2-12B and 2-11D). Mutations of different subsets of these 14 lysines did not appreciably diminish Scc1 sumoylation (data not shown), suggesting that sumoylation of these lysines might be distributive, with many of them being potential SUMO acceptors. Finally, I created the Scc1 15KR mutant, which contained the K216R and 14KR mutations. Scc1 15KR was only weakly sumoylated in the presence of SUMO1 (Figure 2-12B). This weak sumoylation was not stimulated by Mms21. Thus, Scc1 15KR has lost its ability to be sumoylated by Mms21.

Scc1 15KR is functional in sister-chromatid cohesion during mitosis

To study the functions of Scc1 sumoylation, I generated stable HeLa Tet-On cell lines that expressed Myc-Scc1 WT or 15KR transgenes driven by a tetracycline-inducible promoter. Both Scc1 transgenes contained silent mutations to make them resistant to RNAi-mediated depletion. For subsequent experiments,

I chose two clones for each cell line: clones 9 and 31 for Myc Scc1 WT and clones 3 and 37 for Myc-Scc1 15KR. In the presence of doxycycline, these clones expressed Myc-Scc1 WT or 15KR at levels comparable to that of the endogenous Scc1 (Figure 2-13A). I first tested whether Scc1 15KR supported replicative cohesion establishment and proper chromosome segregation in mitosis. In the metaphase spread of a cell with normal cohesion, sister chromatids were paired at their centromeres and had an X shape while sister chromatids had lost their connection at centromeres and were not paired in cohesion-defective cells (Figure 2-13B). In the absence of doxycycline, depletion of the endogenous Scc1 caused premature sister-chromatid separation in more than 50% of mitotic cells in all four clones (Figure 2-13C). In the presence of doxycycline, expression of Myc-Scc1 WT or 15KR largely rescued the cohesion defect caused by Scc1 RNAi. Thus, Scc1 15KR supports proper sister-chromatid cohesion in mitosis, and by inference, is capable of establishing replicative cohesion in normal S phase. Consistently, Scc1 15KR could still bind to Smc1, Smc3, and SA2 (data not shown).

Scc1 15KR is defective in DSB repair and SCR

Next, I examined whether Scc1 15KR supported the DNA repair function of cohesin. In the absence of doxycycline, depletion of Scc1 by RNAi rendered cells expressing Myc-Scc1 WT or 15KR sensitive to IR (Figure 2-14).

Doxycycline-induced expression of Myc-Scc1 WT, but not 15KR, rescued the IR sensitivity caused by Scc1 depletion.

Depletion of Scc1 also reduced the frequency of SCE in these cells in the absence of doxycycline (Figure 2-15A and 2-15B). Induced expression of Myc-Scc1 WT restored the SCE frequency to that of the mock-depleted cells. By contrast, expression of Myc-Scc1 15KR did not rescue the SCE defect caused by Scc1 knockdown. These results indicate that Scc1 15KR is defective in DSB repair and SCR, and further suggest that Mms21-dependent sumoylation of Scc1 might be critical for DNA repair.

Scc1 sumoylation is not required for cohesin recruitment to DNA damage sites

Because the non-sumoylatable Scc1 15KR mutant was defective in DSB repair and SCR, I wished to examine whether Scc1 15KR was recruited to DNA damage sites. We collaborated with Dr. Kyoko Yokomori at University of California at Irvine. They helped us to examine the recruitment of Myc-Scc1 WT and 15KR to laser-induced DNA damage sites in fixed cells with immunofluorescence. HeLa Tet-On cells stably expressing Myc-Scc1 WT or 15KR were synchronized at S/G2 by a thymidine-arrest-release protocol. DSBs were generated along a straight line using nanosecond (ns) green laser microirradiation. This system was used previously to demonstrate S/G2-specific

and Mre11-Rad50-dependent cohesin recruitment to damage sites in human cells (Kim et al., 2002a). This Mre11-dependence in cohesin recruitment to DSBs was corroborated by chromatin immunoprecipitation (ChIP) analysis in yeast (Strom et al., 2004; Unal et al., 2004), supporting the physiological relevance of their laser system. Cells were fixed at 1 hr post damage, and stained with antibodies against Myc and the key HR protein, Rad51. Both Scc1 WT and 15KR were recruited to the laser-induced DNA damage foci, along with Rad51 (Figure 2-16). It is well established that cohesin loading to chromatin per se is insufficient to establish functional cohesion (Rolef Ben-Shahar et al., 2008; Rowland et al., 2009; Unal et al., 2008). Their result suggests that sumoylation of Scc1 does not affect cohesin recruitment to DSBs, but might affect its function at a later step during SCR.

Smc5/6 is dispensable for cohesin recruitment to DNA damage sites in human cells

Our lab had previously shown that the Smc5/6 complex was recruited to I-SceI endonuclease-induced DSBs by chromatin immunoprecipitation (ChIP) analysis and that depletion of Mms21 and Smc5 abolished cohesin recruitment to these sites in human cells (Potts et al., 2006), implicating Smc5/6 in cohesin recruitment. The fact that Scc1 15KR was still recruited to DNA damage sites was inconsistent with these previous results. Furthermore, in yeast, although the

Smc5/6 complex is required for DI cohesion establishment, it is not required for cohesin recruitment to DSBs (Strom et al., 2007). Finally, the Mms21 siRNA used in our previous work (siMms21-7) and certain Smc5 siRNAs (siSmc5-5) were recently shown by others to cause premature sister-chromatid separation (Behlke-Steinert et al., 2009), prompting the authors to suggest that Smc5 and Mms21 might have roles in replicative cohesion establishment or maintenance. We decided to re-investigate the role of Smc5/6 in cohesin recruitment to laser- and endonuclease-induced DNA damage sites in human cells.

First, I tested whether Smc5/6 was indeed required for proper chromosome segregation in human cells. I depleted Mms21 from HeLa cells using 7 siRNAs. Although 6 Mms21 siRNAs depleted Mms21 efficiently, only siMms21-3, siMms21-5, and siMms21-7 caused premature sister-chromatid separation (Figure 2-17D). Moreover, ectopic expression of Mms21 resistant to siMms21-7 failed to rescue the chromosome segregation defects in cells transfected with siMms21-7 (data not shown). Similarly, only 1 of the 6 Smc5 siRNAs and none of Smc6 siRNAs caused premature sister-chromatid separation (Figure 2-17E). Note that depletion of Smc5 also reduced the protein levels of Smc6, and vice versa. These results indicate that Smc5/6 and Mms21 are not required for replicative cohesion establishment and proper chromosome segregation during mitosis. siMms21-7 and siSmc5-5 may have depleted cohesin regulators through off-target effects.

Consistent with our previous ChIP analysis of endonuclease-induced DSB sites (Potts et al., 2006), Smc5 and Mms21 were recruited to laser-induced damage sites (Figure 2-18A). The localization of Mms21 to these sites was dependent on Smc5, suggesting that it was recruited as a part of the Smc5/6 complex. Scc1 depletion had no effect on Mms21 localization (Figure 2-18B), indicating that the Smc5/6 complex localized to damage sites independently of cohesin, consistent with the ChIP analysis (Potts et al., 2006). Transfection of siMms21-7 and siSmc5-7 diminished cohesin recruitment to laser-induced DNA damage sites (Figure 2-18D). By contrast, depletion of Smc5 and Mms21 with the new siRNAs (siSmc5-2 and siMms21-6) that did not cause premature sister-chromatid separation did not affect cohesin recruitment to DNA damage sites (Figure 2-18C and 2-18D). Depletion of Smc6 also did not prevent cohesin recruitment to damage sites (data not shown). Therefore, Smc5/6 and Mms21 are not required for the initial cohesin loading at laser-induced DNA damage sites.

I next examined cohesin recruitment to endonuclease-induced DSBs by ChIP in human cells (Berkovich et al., 2007). I transfected HeLa Tet-On cells with a plasmid encoding I-PpoI fused to estrogen receptor (ER-I-PpoI) and added 4-OHT to induce the nuclear translocation of ER-I-PpoI and DNA cleavage. Addition of 4-OHT indeed induced DNA damage in a time-dependent manner, as indicated by the increase in γH2AX and phospho-Chk1 signals which peaked at 12 hrs after 4-OHT addition (Figure 2-19A). I-PpoI has been shown to induce

DSBs in the native rDNA locus (Berkovich et al., 2007). I then performed ChIP experiments on cells treated with or without 12-hr 4-OHT treatment and measured the amounts of rDNA in the IPs using quantitative PCR (Figure 2-19B). There was basal level cohesin binding to the rDNA locus in the absence of DNA damage. I-PpoI-mediated DSB formation enhanced cohesin binding at this locus. Depletion of Smc5 and Mms21 with siSmc5-2 and siMms21-6 did not affect either the basal cohesin binding or the DSB-induced cohesin enrichment at the native rDNA locus. Thus, the Smc5/6 complex is dispensable for cohesin loading to endonuclease-induced DSBs in human cells. Taken together, our previous finding that depletion of Mms21 and Smc5 prevented cohesin loading at DSBs was due to off-target effects of the particular siRNAs used, and Mms21 and cohesin are recruited to DNA damage sites independently of each other.

Because cohesin was sumoylated by MMS21 in vitro and in human cells, we examined whether this occurred at damage sites. Since cohesin was recruited to damage sites only in S/G2 phase of the cell cycle (Kim et al. 2002), our collaborator helped us examine the SUMO signal at damage sites in S/G2 cells. A prominent SUMO2/3 signal was observed at damage sites, which was diminished by Smc5 and Mms21 depletion at this cell cycle stage (Figure 2-20A). Furthermore, cohesin depletion greatly diminished the SUMO2/3 signal at damage sites (Figure 2-20B). These results suggest that cohesin is the major target of Mms21-dependent sumoylation at damage sites. Taken together, our data

presented in the current study suggest that cohesin sumoylation is not required for the initial cohesin targeting to damage sites, but is important for a later function of cohesin in SCR.

Wapl depletion bypasses the requirement of Mms21 in SCR

Our data thus far support a role of Mms21-dependent sumoylation of Scc1 in promoting SCR, at a step after the initial cohesin recruitment. Because Wapl is a negative regulator of cohesin throughout the cell cycle (Figure2-21), I hypothesized that Mms21 promoted cohesin's function at DSBs through counteracting Wapl. I tested this hypothesis using three types of assays.

First, in the colony survival assay, Mms21 depletion rendered HeLa cells sensitive to IR while Wapl depletion had no effect (Figure 2-22A, 2-22B and 2-22C). Strikingly, co-depletion of Wapl along with Mms21 rescued the IR sensitivity of Mms21-RNAi cells. Second, in the SCE assay, co depletion of Mms21 and Wapl restored the SCE frequency to that of the mock-transfected cells (Figure 2-23A and 2-23B). Consistent with previous reports, sister chromatids were less resolved in cells depleted of Wapl alone and in cells depleted of both Wapl and Mms21 (Figure 2-23A).

Finally, a GFP-based gene targeting assay done by Dr. Patrick Ryan Potts further confirmed the antagonism between Mms21 and Wapl. In this assay, an artificial gene target, containing a mutated *GFP* gene with in-frame stop codons

and an I-SceI recognition site inserted, was stably integrated at a single genomic locus in 293 cells (Figure 2-24A) (Potts et al., 2006). These cells were then transfected with a repair plasmid that contained the *I-SceI* gene and a truncated *GFP* gene. Neither the mutated *GFP* gene integrated in the genome nor the truncated *GFP* gene on the repair plasmid could express functional GFP. Expression of I-SceI introduced a DSB within the integrated *GFP* locus. HR between the mutated *GFP* gene in the genome and the truncated *GFP* gene on the plasmid reconstituted a functional *GFP* gene. The percentage of GFP-positive cells as determined by flow cytometry then provided a quantitative measure of the frequency of gene targeting. Consistent with a previous study (Potts et al., 2006), depletion of Mms21 increased the percentage of GFP-positive cells in this assay, because inactivation of Mms21 decreased SCR, an HR pathway that was not expected to produce GFP-positive cells (Figure 2-24B). While Wapl depletion alone did not alter the gene targeting efficiency, co-depletion of Wapl nullified the increase in gene targeting rate caused by Mms21 depletion. Taken together, our results from all three assays are consistent with the notion that Mms21 promotes SCR by counteracting Wapl. Our results further implicate cohesin as a major downstream target of Mms21 in DSB repair in human cells.

If Scc1 is a key downstream target of Mms21 in this process, one would expect that depletion of Wapl should also rescue the SCR defect of cells expressing Scc1 15KR. Consistent with results in Figure2-15B, doxycycline-

induced expression of Myc-Scc1 15 KR did not rescue the SCE defect caused by Scc1 depletion (Figure 2-25A and 2-25B). Depletion of Wapl in these cells, however, restored SCE to normal levels. This result suggests that Scc1 sumoylation by Mms21 functionally opposes Wapl to promote SCR.

Expression of the Smc3 acetylation mimicking mutant fails to bypass the requirement for Mms21 in SCR

I next explored the mechanism by which Scc1 sumoylation counteracted Wapl. Mms21-dependent sumoylation occurs in the central region of Scc1. A similar region has been implicated in Wapl binding (Shintomi and Hirano, 2009). One possibility is that Scc1 sumoylation directly blocks Wapl binding to cohesin. On the other hand, recombinant purified N-terminal domain of Wapl could still bind to sumoylated Scc1 in vitro (Figure 2-26), disfavoring the possibility of direct inhibition.

In yeast, Eco1 acetylates Scc1 and is required for DNA damage-induced cohesion generation (Heidinger-Pauli et al., 2009). In human cells, replicative cohesion establishment during S phase requires Esco1/2-dependent acetylation of Smc3 at K105 and K106 (Zhang et al., 2008). Smc3 acetylation enables Sororin binding to Pds5A/B, which disrupts a binding interface between Wapl and Pds5A/B and inhibits Wapl's ability to release cohesin from chromatids (Nishiyama et al., 2010). Sororin has also been implicated in DNA repair

(Schmitz et al., 2007). Because our results described herein implicated Wapl as a downstream effector of Scc1sumoylation by Mms21 in SCR, I next sought to examine the potential interplays among Scc1 sumoylation, Smc3 acetylation, and Sororin in this process. Depletion of Esco1/2 or Sororin by RNAi caused IR sensitivity and SCE defect in HeLa cells, confirming their involvement in DNA repair (Figure 2-27 and 2-28).

A recent study showed that IR increased Smc3 acetylation at K105 and K106 in log phase human cells (Kim et al., 2010), suggesting that DNA damage caused an up-regulation of global sister-chromatid cohesio. I tested whether IR also increased global sister-chromatid cohesion post-replicatively in G2. If so, it would have provided a simple assay for me to examine the potential mutual regulation between Scc1 sumoylation and Smc3 acetylation following DNA damage. I arrested HeLa cells in G2 with the Cdk1 inhibitor RO3306, treated these cells with IR, harvested cells at different time points, and performed anti-Smc1 IP from cell lysates (Figure 2-29A). IR indeed created DNA damage, as indicated by increases in phospho-Chk1 and γH2AX levels. As expected, phosphorylation of Smc1 also increased. The level of Smc3 acetylation did not increase after IR, however (Figure 2-29A). There were also no discernible changes in the amounts of Wapl, Pds5A/B, and Sororin bound to cohesin. Similar results were also observed in the chromatin-bound pool of cohesin. Therefore, I

did not have evidence for a global increase in sister-chromatid cohesion following DNA damage in G2 human cells.

I next examined the relationship between Smc3 acetylation and Scc1 sumoylation. Consistent with a previous report (Zhang et al., 2008), I showed that doxycycline-induced ectopic expression of Smc3 QQ (an acetylation-mimicking Smc3 mutant with K105 and K106 mutated to glutamine), but not Smc3 WT, rescued the sister-chromatid cohesion defect of 293T cells depleted of Esco1/2 by RNAi (Figure 2-30A and 2-30B). This result confirmed that Smc3 QQ indeed functionally mimicked acetylation at these two sites and that Smc3 K105 and K106 are the major targets of Esco1/2 in replicative sister-chromatid cohesion. Expression of Smc3 QQ did not, however, rescue the SCE defect of Mms21-RNAi cells (Figure 2-30C). Thus, Smc3 acetylation might not be the sole critical downstream event of Mms21-dependent Scc1 sumoylation in SCR. Furthermore, Smc3 QQ expression did not rescue the SCE defect of Esco1/2 RNAi cells (Figure 2-30D), suggesting that Esco1/2 had target sites in addition to (or instead of) Smc3 K105 and K106 in DNA repair in human cells, similar to yeast Eco1.

Scc1 is the critical target of Eco1 in DNA repair (Heidinger-Pauli et al., 2009). Although the acetylation sites of yeast Scc1 are not conserved in human Scc1 and previous mass spectrometry analysis failed to uncover acetylation sites in human Scc1 (Kim et al., 2010), it remained formally possible that, in addition to abolishing Mms21-dependent sumoylation, Scc1 15KR also eliminated yet

unidentified acetylation sites on Scc1. I immunoprecipitated Myc-Scc1 WT and 15KR from lysates of HeLa Tet-On cells with or without IR treatment, and blotted the IPs with the antibody against several pan-specific acetyllysine antibodies. Two panacetyllysine antibodies detected bands that corresponded to the size of Myc-Scc1, suggesting that Scc1 was acetylated (Figure 2-31). Scc1 acetylation was, however, not stimulated by IR. Scc1 15KR was as efficiently acetylated as Scc1 WT. Thus, there was no evidence to indicate that the 15KR mutation adversely affected Scc1 acetylation. A pan-acetyllysine antibody also detected another cohesin-associated protein whose identity remained to be determined.

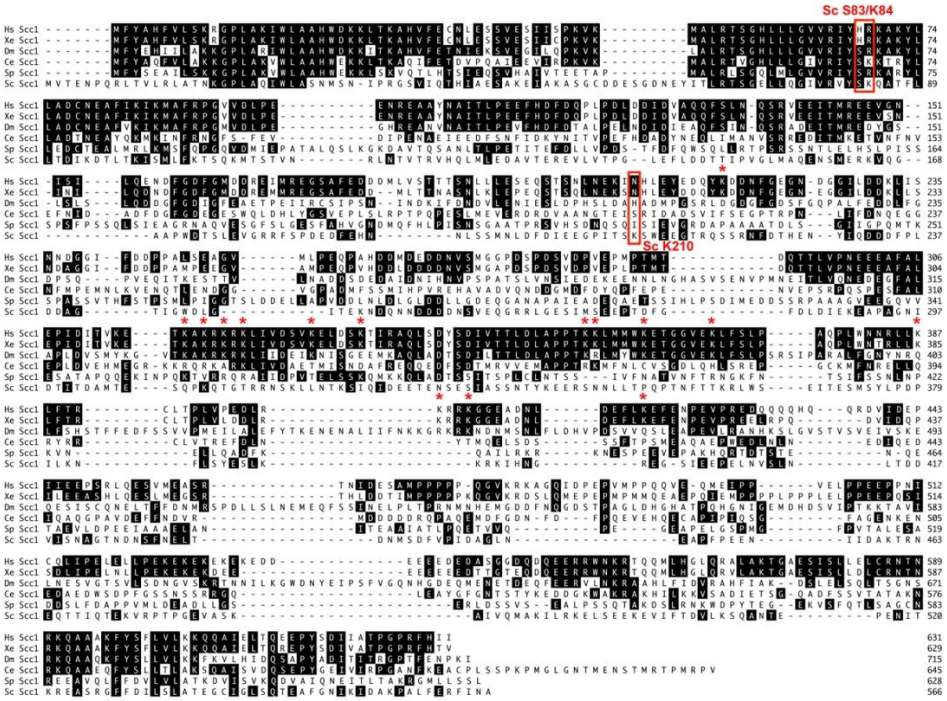

Figure 2-1. Sequence alignment of Scc1 protein from different species
The residues corresponding to S83, K84, and K210 in the budding yeast Scc1 which undergo DNA damage-induced posttranslational modifications are boxed. The 15 lysines mutated in human Scc1 in this study are indicated with asterisks.

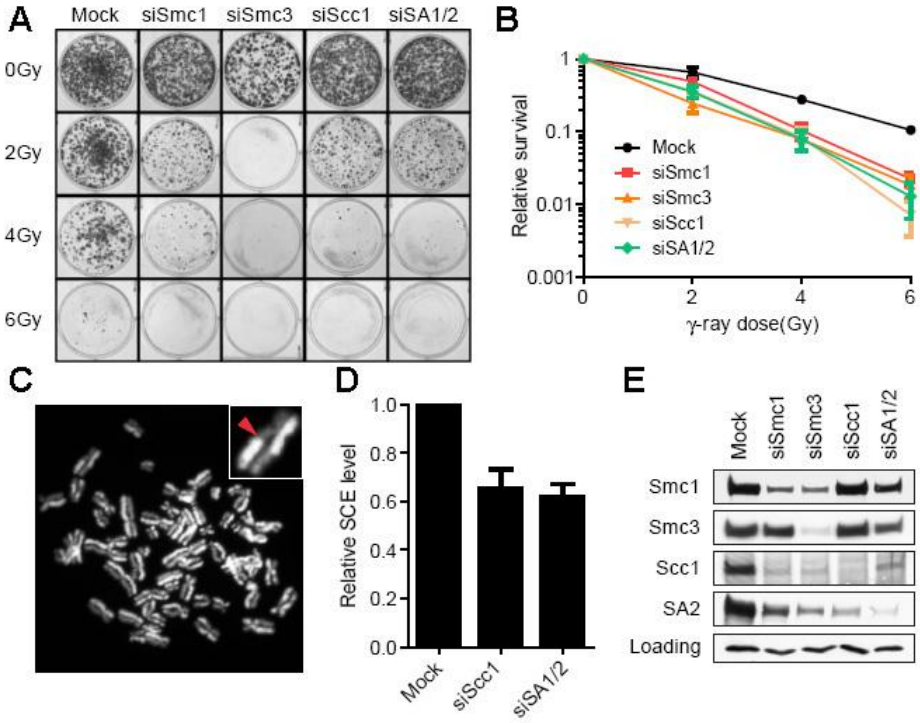

Figure 2-2. Cohesin is required for DSB repair and SCR
(A) Representative image of the IR colony survival assay. (B) IR colony survival assay of HeLa Tet-On cells transfected with the indicated siRNAs. Each data point represents the mean and standard deviation of values in two independent experiments, with duplicate samples in each experiment. (C) Representative image of the SCE assay. A pair of sister chromatids is magnified and shown in inset with the SCE event marked by arrowhead. (D) Quantification of the relative SCE levels of HeLa Tet-On cells transfected with the indicated siRNAs. The mean and standard deviation of data from three experiments are shown. About 30 cells were counted in each experiment. (E) Lysates of cells in (A) were blotted with the indicated antibodies. Note that depletion of a given cohesin subunit often reduced proteins levels of other subunits.

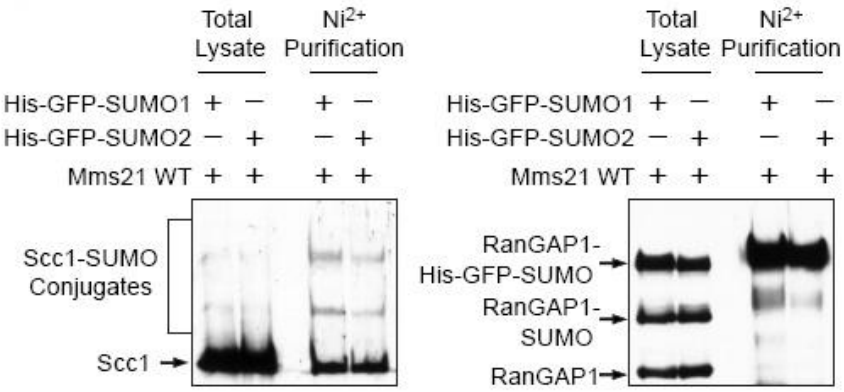

Figure 2-3. Endogenous Scc1 undergoes Mms21-dependent sumoylation
HeLa Tet-On cells transfected with Mms21 and His_6-GFP-SUMO1/2 plasmids were lysed with a protein denaturing buffer and subjected to Ni^{2+}-beads pull-down. The total lysates and pull-down were blotted with α-Scc1 (left panel) or α-RanGAP1 (right panel).

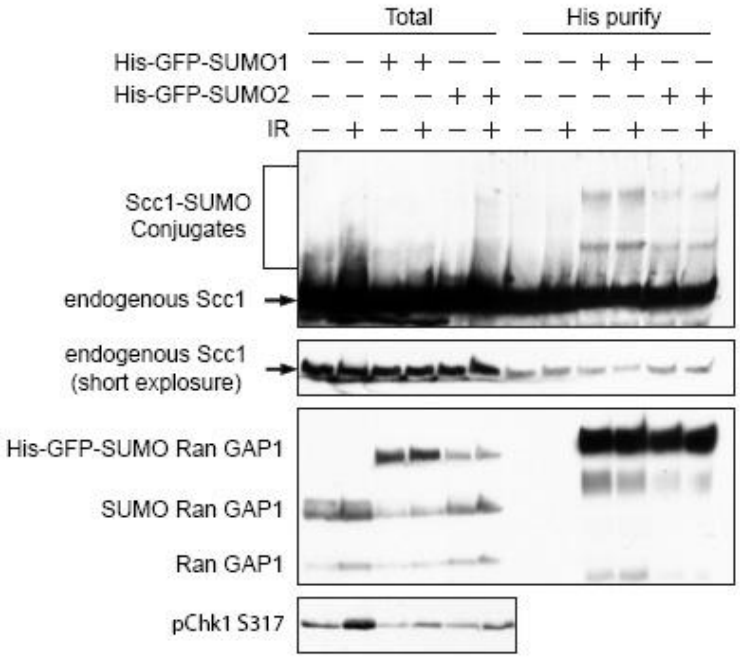

Figure 2-4. The endogenous Scc1 sumoylation is not enhanced by IR
HeLa Tet-On cells transfected with empty vector or His_6-GFP-SUMO1/2 plasmids were arrested in G2 by treating Cdk1 inhibitor RO3306 for 20 hrs, untreated or exposed with 8 Gy IR and recovered for 1 hr. The cells were then lysed with a protein denaturing buffer and subjected to Ni^{2+}-beads pull-down. The total lysates and pull-down were blotted with α-Scc1 (top panel) or α-RanGAP1 (bottom panel). Phosphor-Chk1 indicated IR indeed created DNA damage.

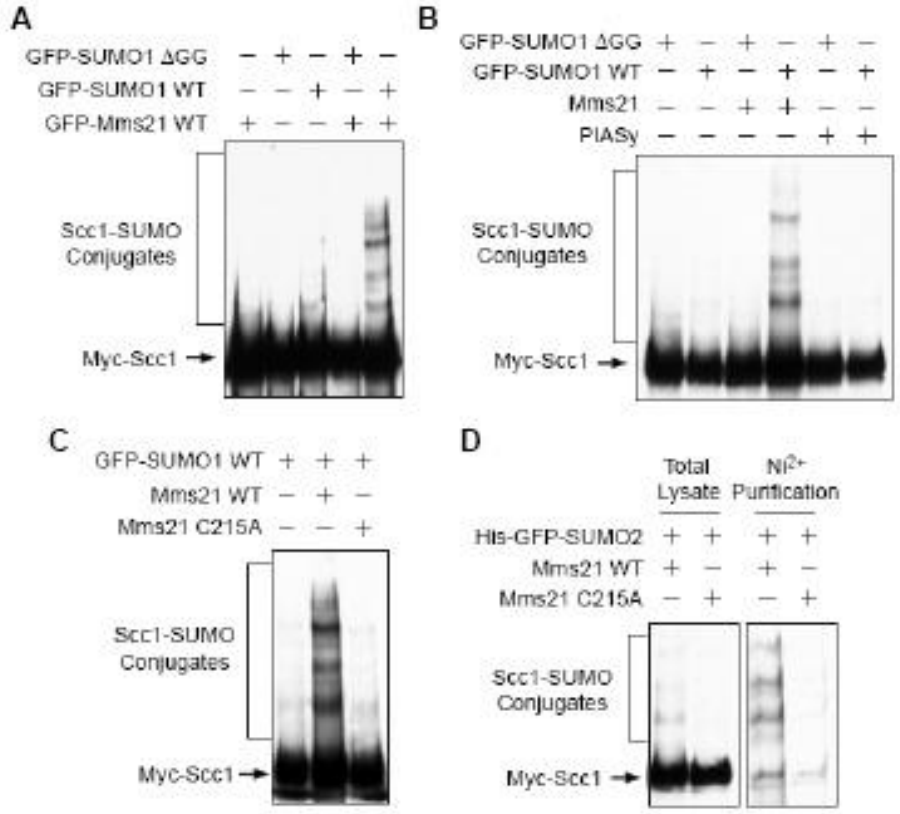

Figure 2-5. Mms21 stimulates cohesin sumoylation in human cells
(A) Mms21 enhances Scc1 sumoylation in human cells. Myc-Scc1 was co-expressed with GFP-SUMO1 WT or ΔGG in presence or absence of GFP-Mms21 in HeLa cells for 24 hrs. The cell lysates were blotted with α-Myc. The positions of unmodified and sumoylated Scc1 are labeled. (B) The Scc1 sumoylation is enhanced by Mms21 but not PIASy. Lysates of HeLa Tet-On cells transfected with the indicated plasmids were blotted with α-Myc. The positions of sumoylated Scc1 are labeled. (C) The ligase activity of Mms21 is required for Scc1 sumoylation. Lysates of HeLa cells transfected with the indicated plasmids were blotted with α-Myc. The C215A mutation abolished the ligase activity of Mms21.(D) HeLa Tet-On cells transfected with Myc-Scc1, Mms21 WT/C215A, and His_6-GFP-SUMO2 plasmids were lysed with a protein denaturing buffer and subjected to Ni^{2+}-beads pull-down. The total lysates (left panel) and pull-down (right panel) were blotted with α-Myc.

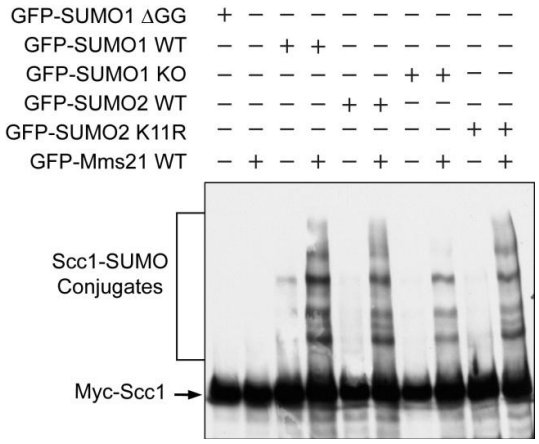

Figure 2-6. Mms21 sumoylates multiple residues of Scc1 in human cells
Myc-Scc1 was co-expressed with GFP-SUMO1 ΔGG, WT, KO or GFP-SUMO2 WT, K11R in presence or absence of GFP-Mms21 in HeLa Tet-On cells for 24 hrs. GFP-SUMO1 KO is a SUMO1 mutant with all lysines mutated to arginine. GFP-SUMO2 K11R is a SUMO2 mutant that cannot efficiently form chains. The cell lysates were blotted with α-Myc. The positions of unmodified and sumoylated Scc1 are labeled.

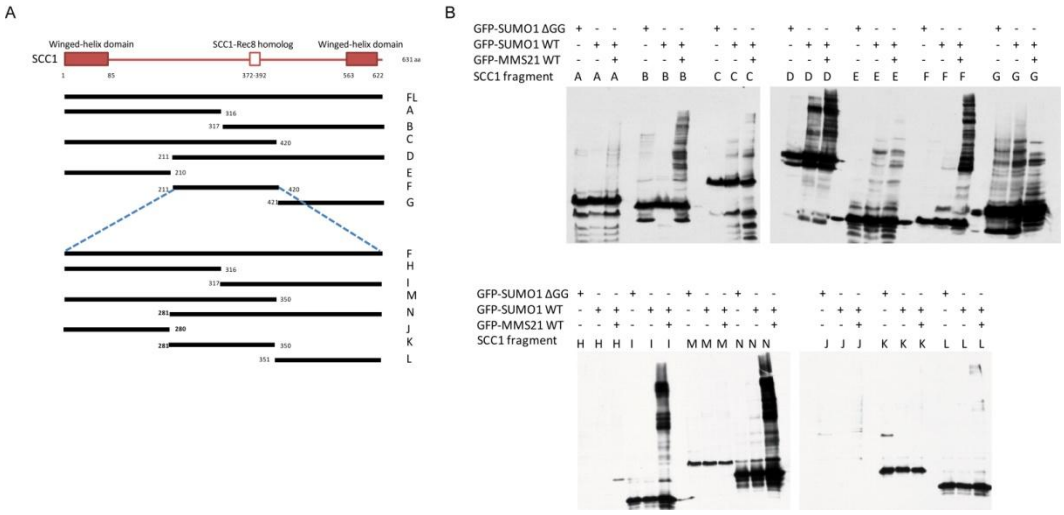

Figure 2-7. Mapping the Mms21-dependent sumoylation region of human Scc1

(A) Schematic drawing of Scc1 and its truncation mutants. The boundaries of each fragment are indicated. (B) Mms21 enhances several Scc1 truncation mutants sumoylation in human cells. Myc-Scc1 truncation fragments were co-expressed with GFP-SUMO1 WT or ΔGG in presence or absence of GFP-Mms21 in HeLa Tet-On cells for 24 hrs. The cell lysates were blotted with α-Myc. The positions of unmodified and sumoylated Scc1 are labeled.

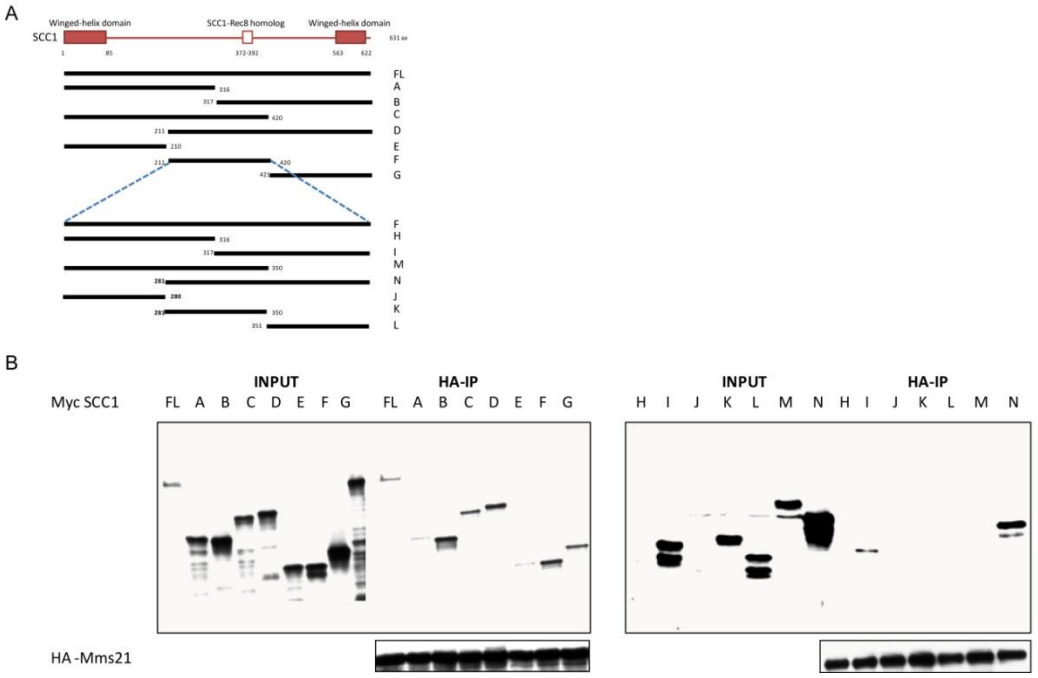

Figure 2-8. Mapping the Mms21-binding domains of human Scc1
(A) Schematic drawing of Scc1 and its truncation mutants. The boundaries of each fragment are indicated. (B) Myc-Scc1 truncation mutants and HA-Mms21 were co-expressed in HeLa Tet-On cells for 24 hrs. The cell lysates were subjected to α-HA IP.

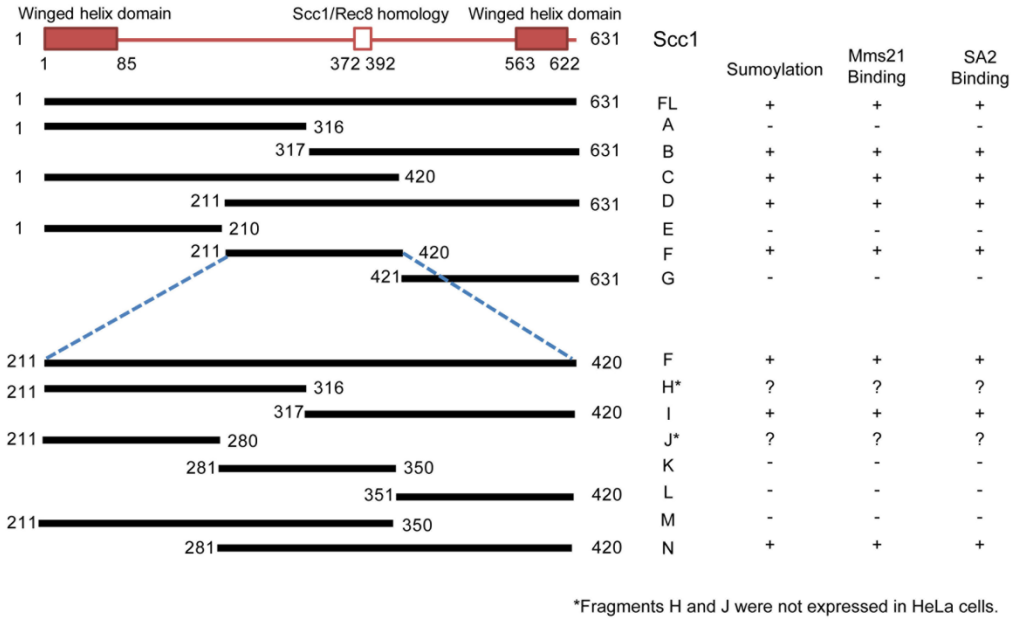

Figure 2-9. Mapping the Mms21-dependent sumoylation region and SA2/Mms21-binding domains of human Scc1

Schematic drawing of Scc1 and its truncation mutants. The boundaries of each fragment are indicated. Results of Mms21-dependent sumoylation, Mms21 binding, and SA2 binding of each fragment are tabulated on the right. Fragments H and J were not expressed in HeLa Tet-On cells and could not be tested.

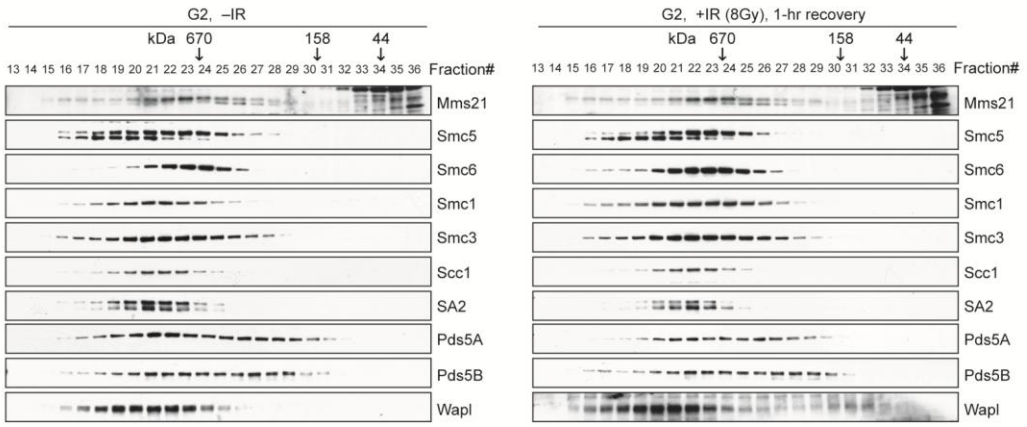

Figure 2-10. The fractionation profiles of the endogenous Smc5/6 and cohesin complexes are not altered by DNA damage

Lysates of HeLa Tet-On cells arrested at G2 either untreated or treated with IR were fractionated on a Superose 6 column. Fractions were blotted with the indicated antibodies. Fractionation positions of the native molecular mass markers are shown above.

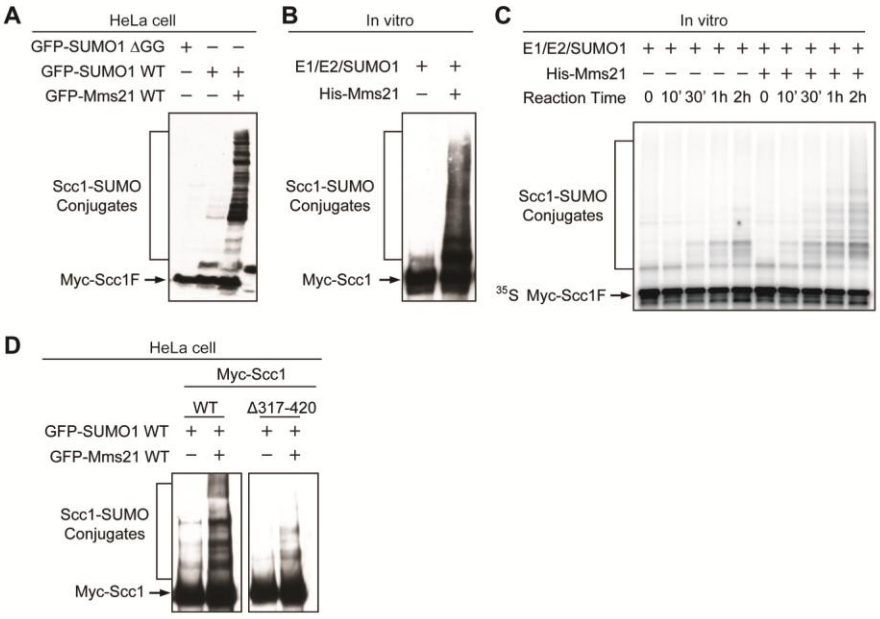

Figure 2-11. Recombinant Mms21 sumoylates Scc1 in vitro

(A) Myc-Scc1F (an Scc1 fragment containing residues 211-420) was co-expressed with GFP-SUMO1 WT or ΔGG in presence or absence of GFP-Mms21 in HeLa cells. The cell lysates were blotted with α-Myc. (B) Scc1 is sumoylated by Mms21 in vitro. In vitro translated Myc-Scc1 was incubated with SUMO1, Aos1–Uba2 (E1), Ubc9 (E2), and energy mix in the presence or absence of His_6-Mms21. The reaction mixtures were blotted with α-Myc. (C) Mms21 sumoylates Scc1F. Scc1F was in vitro translated in the presence of ^{35}S-methionine and incubated with SUMO1, Aos1–Uba2 (E1), Ubc9 (E2), and energy mix in the presence or absence of His_6-Mms21 for the indicated times. The reaction mixtures were resolved by SDS-PAGE followed by phosphor-imager analysis. (D) Lysates of HeLa cells transfected with the indicated plasmids were blotted with α-Myc. The positions of Myc-Scc1 and Scc1–SUMO conjugates are labeled.

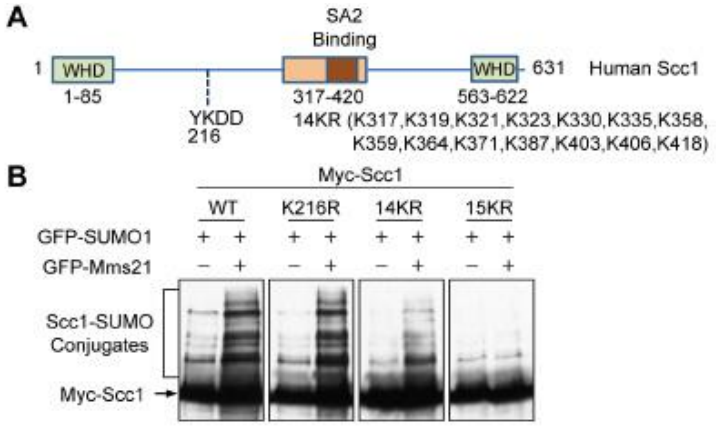

Figure 2-12. Making a non-sumoylatable Scc1 mutant

(A) Schematic drawing of the domains and motifs of human Scc1. The positions of two winged helix domains (WHD) required for Smc1/3 binding, the central conserved domain that binds to SA, and a consensus sumoylation motif at K216 are indicated. The Scc1 15KR mutations contain the K216R mutation and mutations of all 14 lysines in the central region (residues 317- 420) to arginines (14KR). (B) Myc-Scc1 WT and mutants were expressed together with GFP-SUMO1 in presence or absence of GFP-Mms21 in HeLa Tet-On cells. The cell lysates were blotted with α-Myc.

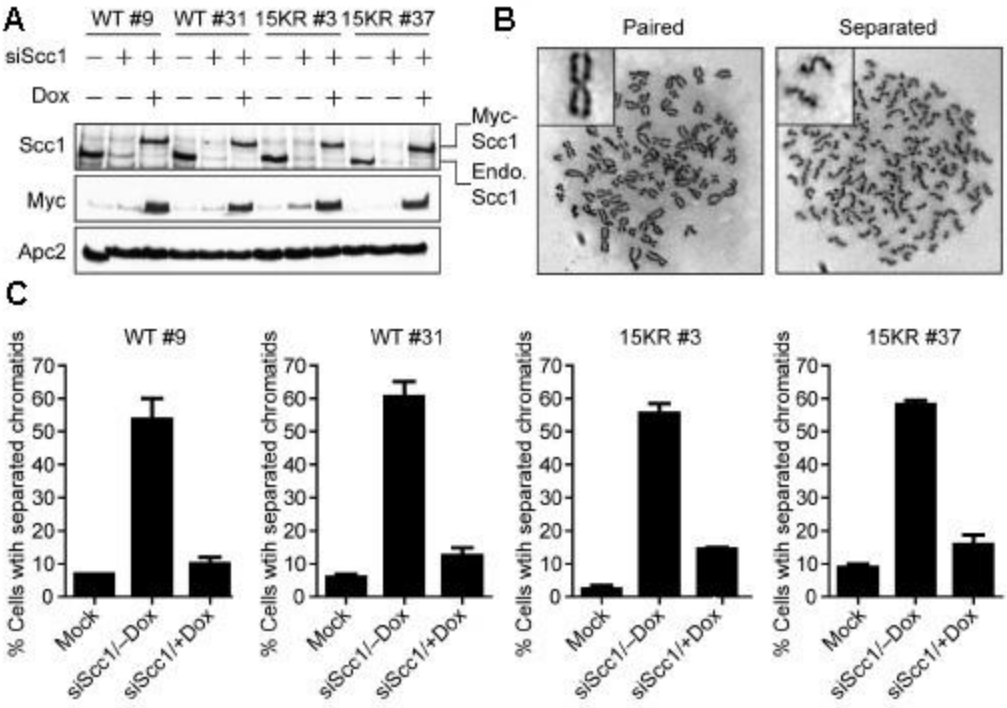

Figure 2-13. The non-sumoylatable Scc1 15KR mutant is functional in mitotic cohesion

(A) Construction of stable HeLa Tet-On cell lines that inducibly expressed Myc-Scc1 WT or 15KR. Two clones of each cell line cultured with or without doxycycline (Dox) were either mock transfected or transfected with siScc1. Cell lysates were blotted with the indicated antibodies. The positions of Myc-Scc1 or endogenous (Endo.) Scc1 are labeled. (B) Representative metaphase spreads of cells in (A) with paired (left panel) or separated (right panel) sister chromatids. (C) Quantification of the percentage of mitotic cells in (A) with separated sister chromatids. More than 200 cells in each sample were counted. The mean and standard deviation of data in two independent experiments are shown.

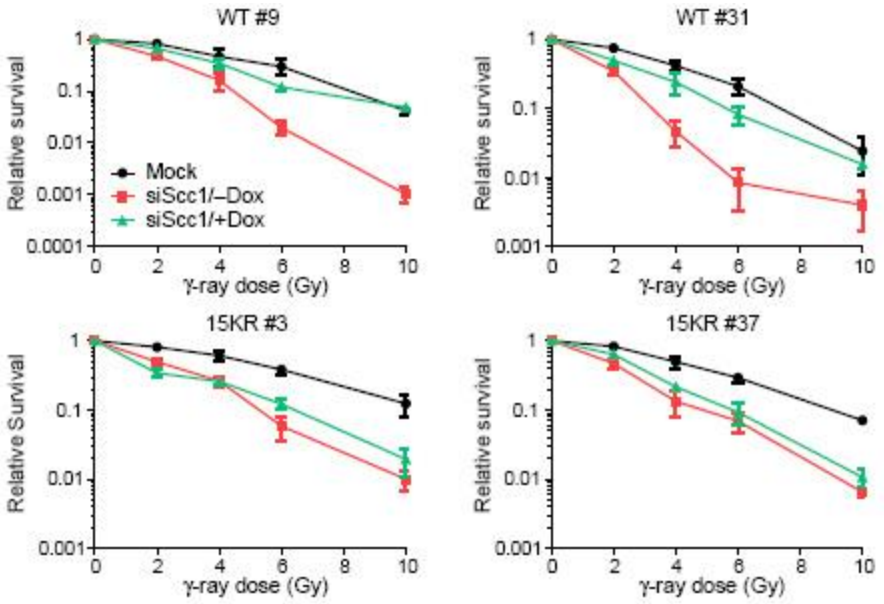

Figure 2-14. Cells expressing Scc1 15KR are sensitive to IR

Two clones each of HeLa Tet-On cell lines that inducibly expressed Myc-Scc1 WT or 15KR were cultured with or without doxycycline (Dox), mock transfected or transfected with siScc1, and irradiated with different doses of IR. The relative colony survival numbers are plotted against IR doses. Each data point represents the mean and standard deviation of values in two independent experiments, with duplicate samples in each experiment. The differences in the IR sensitivity seen in different clones of the Myc-Scc1-expressing lines after Scc1 RNAi are likely due to clonal variation.

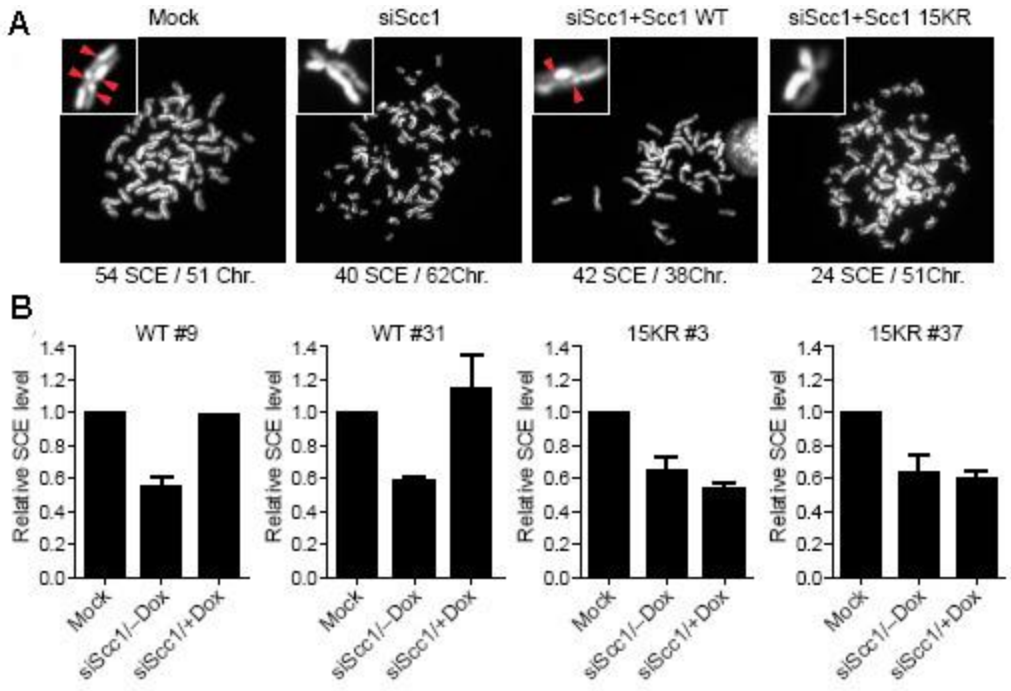

Figure 2-15. Cells expressing Scc1 15KR are defective in SCR
(A) Two clones each of HeLa Tet-On cell lines that inducibly expressed Myc-Scc1 WT or 15KR were cultured with or without Doxycycline (Dox), mock transfected or transfected with siScc1, and tested in the sister-chromatid exchange (SCE) assays. The numbers of SCE events and chromosomes in each image are shown below. A pair of sister chromatids for each image is magnified and shown in the inset with the SCE events marked by red arrowheads. (B) Quantification of the relative SCE levels of cells described in (A). The mean and standard deviation of data from two experiments are shown. About 30 cells were counted in each experiment

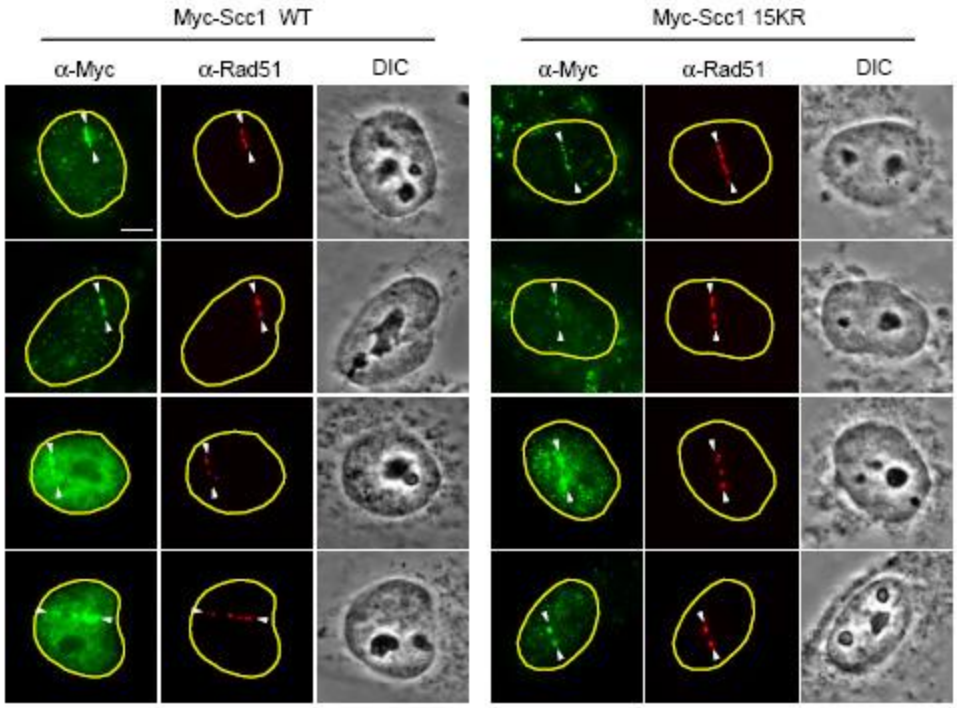

Figure 2-16. Scc1 15KR is recruited to laser-induced DNA damage sites
HeLa Tet-On cells expressing Myc-Scc1 WT or 15KR were cultured in the presence of doxycycline, synchronized at S/G2 by thymidine arrest and release, and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-Myc (green) and α-Rad51 (red). DIC, differential interference contrast. We irradiated 20 Myc-Scc1 WT-expressing and 20 Myc- Scc1 15KR-expressing cells. Among them, 16 Myc-Scc1 WT and 15 Myc-Scc1 15KR cells were Myc-positive. All Myc-positive cells in both groups exhibited enrichment of Myc signals at DNA damage sites, with 12 of 16 in the WT group and 11 of 15 in the 15KR group showing strong recruitment of Myc-Scc1 proteins to damage sites. Four representative cells of each cell line are shown. Scale bar, 10 μm.

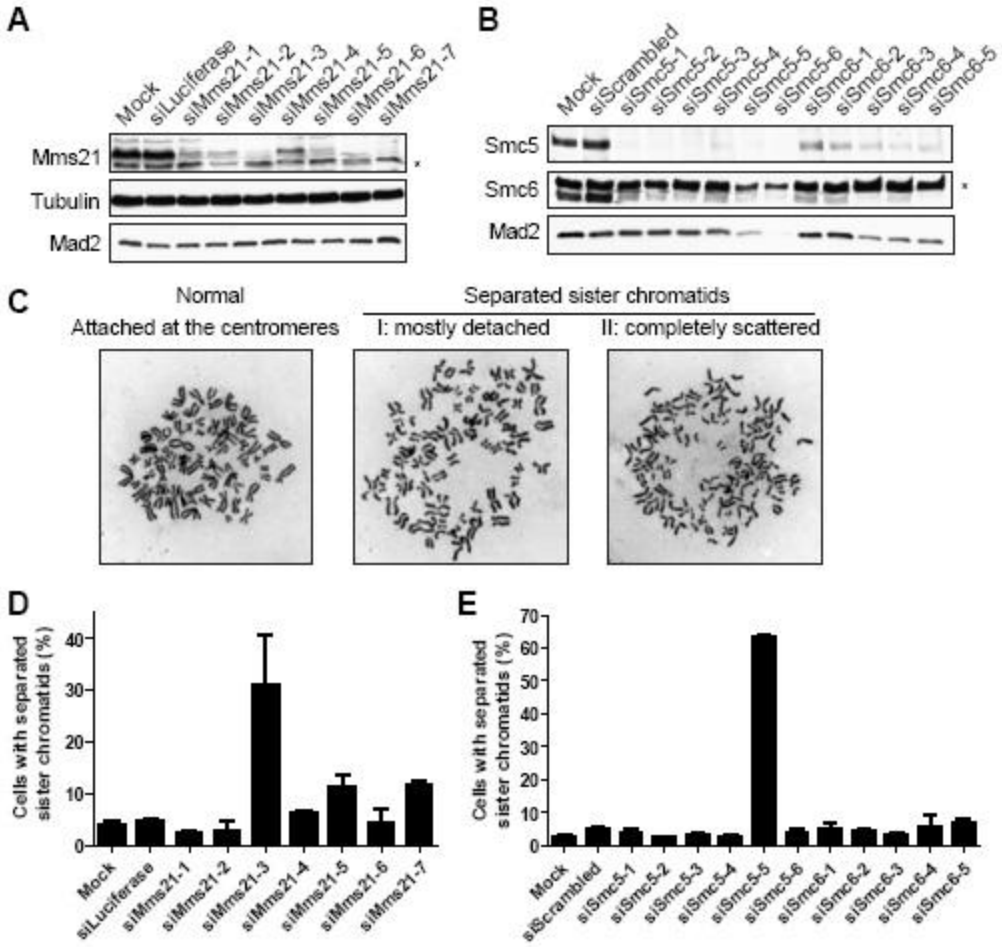

Figure 2-17. Certain Mms21 and Smc5 siRNAs cause premature sister-chromatid separation through off-target effects

(A) Lysates of HeLa cells transfected with the indicated siRNAs were blotted with α-Mms21, α-Tubulin and α-Mad2. A cross-reacting band in the α-Mms21 blot was denoted with an asterisk. (B) Lysates of HeLa cells transfected with the indicated siRNAs were blotted with α-Smc5, α-Smc6, and α-Mad2. A cross-reacting band in the α-Smc6 blot was denoted with an asterisk. (C) Representative metaphase spreads illustrating different types of chromosome morphology. In the metaphase spread of a cell with normal cohesion, sister chromatids were paired at their centromeres and had an X shape, while sister chromatids had lost their connection at centromeres and were not paired in cohesion-defective cells. (D) Quantification of the percentage of cells with separated sister chromatids. Cells were transfected with the indicated siRNAs and treated with nocodazole for 3 hrs to enrich mitotic cells. The average and standard deviations of two independent experiments are shown. (E) Quantification of the percentage of cells with

separated sister chromatids. Cells were transfected with the indicated siRNAs and treated with nocodazole for 3 hrs to enrich mitotic cells. The average and standard deviations of two independent experiments are shown.

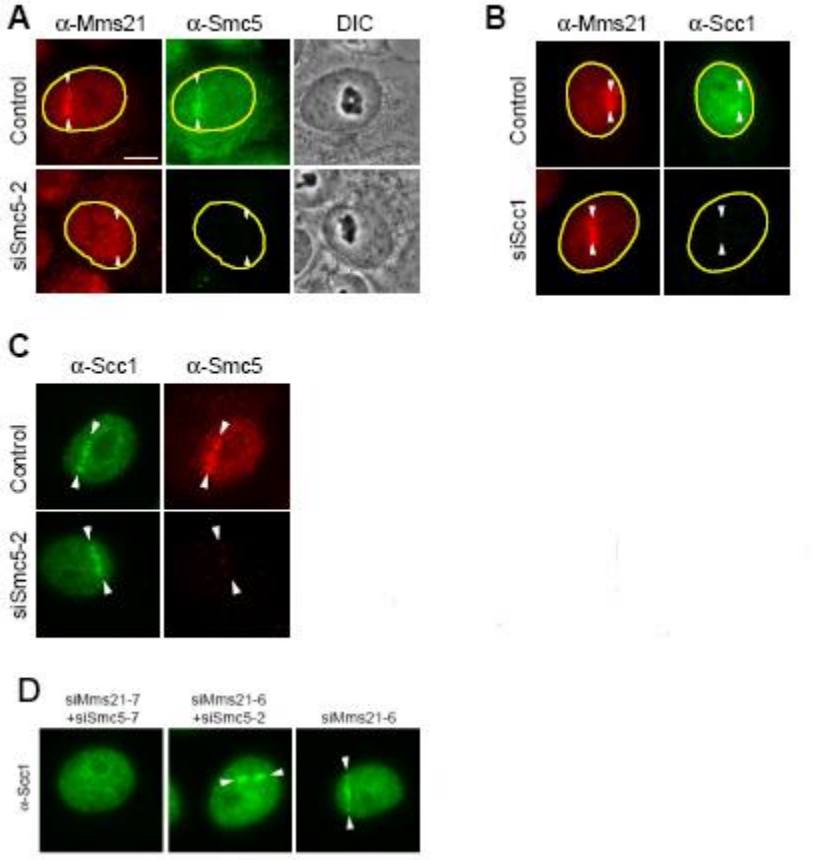

Figure 2-18. The Smc5/6 complex is dispensable for cohesin loading at laser-induced DNA damage sites

(A) HeLa Tet-On cells transfected with siControl or siSmc5-2 were synchronized at S/G2 by thymidine arrest and release, and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-Smc5 (green) and α-Mms21 (red). DIC, differential interference contrast. Scale bar, 10 μm. (B) HeLa Tet-On cells transfected with siControl or siScc1 were synchronized at S/G2 and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-Scc1 (green) and α-Mms21 (red). (C) HeLa Tet-On cells transfected with siControl or siSmc5-2 were synchronized at S/G2 by thymidine arrest and release, and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-Scc1 (green) and α-Smc5 (red). (D) HeLa Tet-On cells transfected with the indicated siRNAs were synchronized at S/G2 by thymidine arrest and release, and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-Scc1.

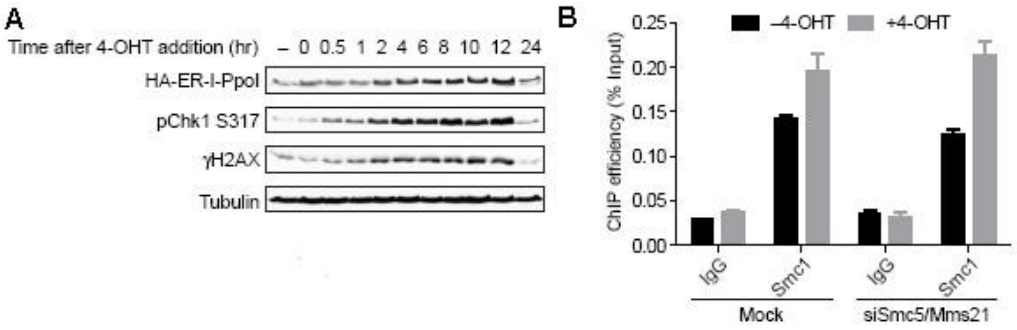

Figure 2-19. The Smc5/6 complex is dispensable for cohesin loading at DNA damage sites generated by an endonuclease I-PpoI

(A) ER-I-PpoI expression induces DNA damage in human cells. HeLa Tet-On cells were transfected with the ER-I-PpoI plasmid and then treated with or without 4-OHT for the indicated times. Total cell lysates were blotted with the indicated antibodies. (B) HeLa Tet-On cells were transfected with siControl or siSmc5-2/siMms21-6 and then transfected with the ER-I-PpoI plasmid. Cells were then treated with or without 4-OHT for 12 hrs and subjected to chromatin immunoprecipitation (ChIP) by IgG and α-Smc1 followed by Q-PCR analysis. The mean and standard deviation of two independent experiments are shown.

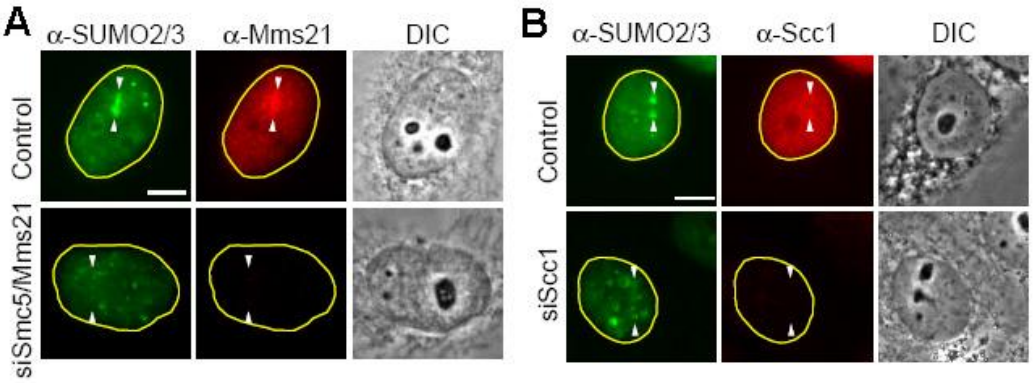

Figure 2-20. Smc5/6 and cohesin are required for SUMO2/3 accumulation at DNA damage sites in S/G2 human cells

(A) HeLa Tet-On cells transfected with siControl or siSmc5-2/siMms21-6 were synchronized at S/G2 and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-SUMO2/3 (green) and α-Mms21 (red). DIC, differential interference contrast. Scale bar, 10 μm. (B) HeLa Tet-On cells transfected with siControl or siScc1 were synchronized at S/G2 and subjected to laser irradiation along a straight line. At 1 hr after laser cutting, cells were fixed and stained with α-SUMO2/3 (green) and α-Scc1 (red). DIC, differential interference contrast. Scale bar, 10 μm.

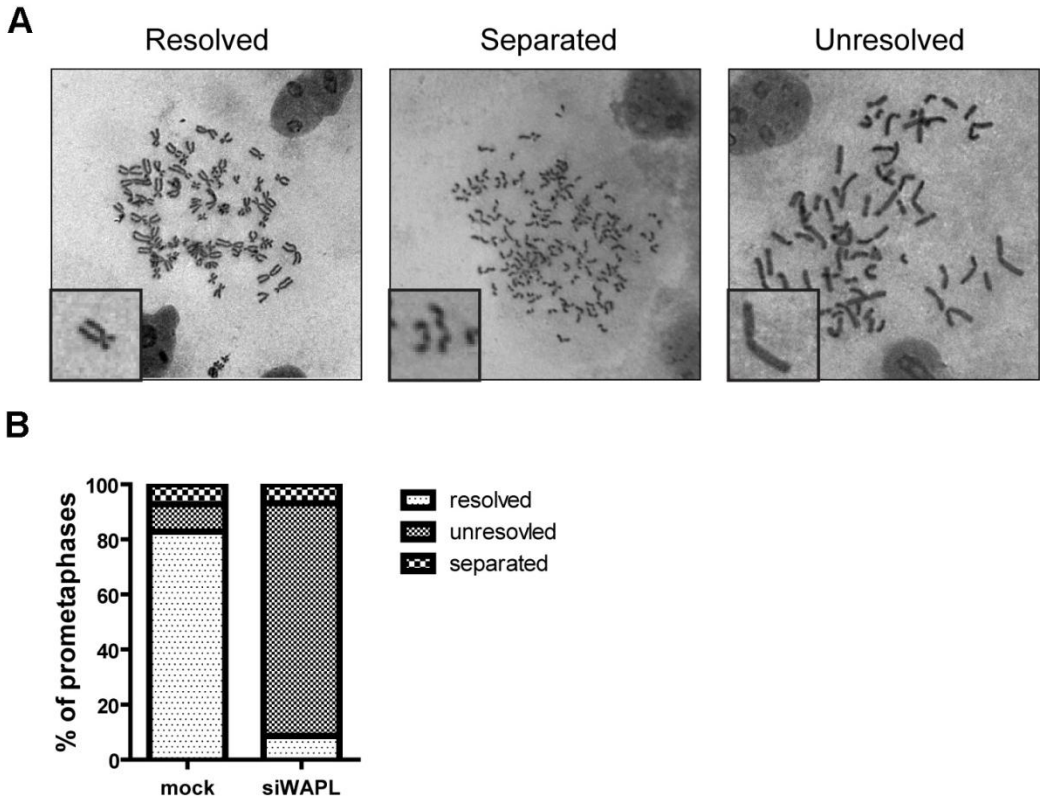

Figure 2-21. Wapl is required for sister chromatid resolution
(A) Representative metaphase spreads illustrating different types of chromosome morphology. (B) Quantification of the percentage of cells with different types of chromosome morphology. Cells were transfected with indicated siRNA for 48 hrs and then treated with nocodazole for 3 hrs to enrich mitotic cells. More than 200 cells in each sample were counted.

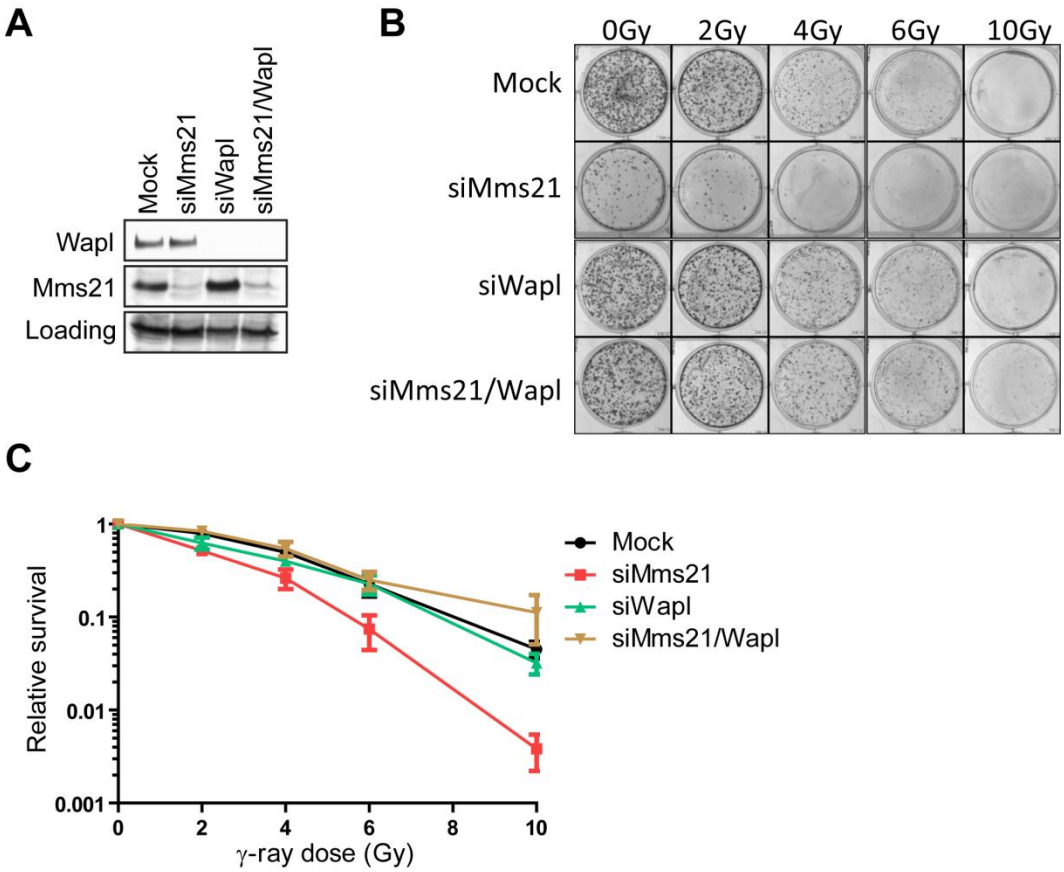

Figure 2-22. Wapl depletion rescues IR sensitivity of Mms21-RNAi cells
(A) Lysates of HeLa Tet-On cells transfected with the indicated siRNAs were blotted with α-Wapl and α-Mms21. A cross-reacting band in the α-Mms21 blot served as the loading control. (B) Representative image of the IR colony survival assay. (C) IR colony survival assay of cells described in (A). Each data point represents the mean and standard deviation of values in three independent experiments, with duplicate samples in each experiment.

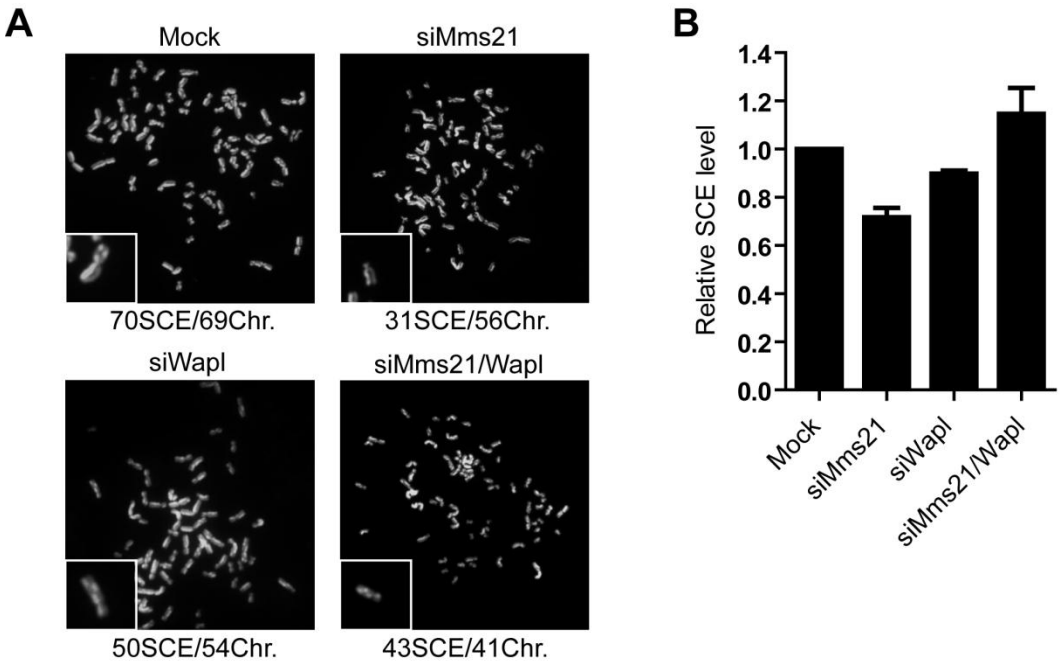

Figure 2-23. Wapl depletion rescues SCR defects of Mms21-RNAi in SCE assay

(A) Representative images from the SCE assay. HeLa Tet-On cells transfected with the indicated siRNA were performed SCE assay. The numbers of SCE events and chromosomes in each image are shown below. A pair of sister chromatids for each image is magnified and shown in the inset with the SCE events marked by red arrowheads. (B) Quantification of the relative SCE levels of cells in (A). The mean and standard deviation of data from three experiments are shown. More than 30 cells were counted in each experiment.

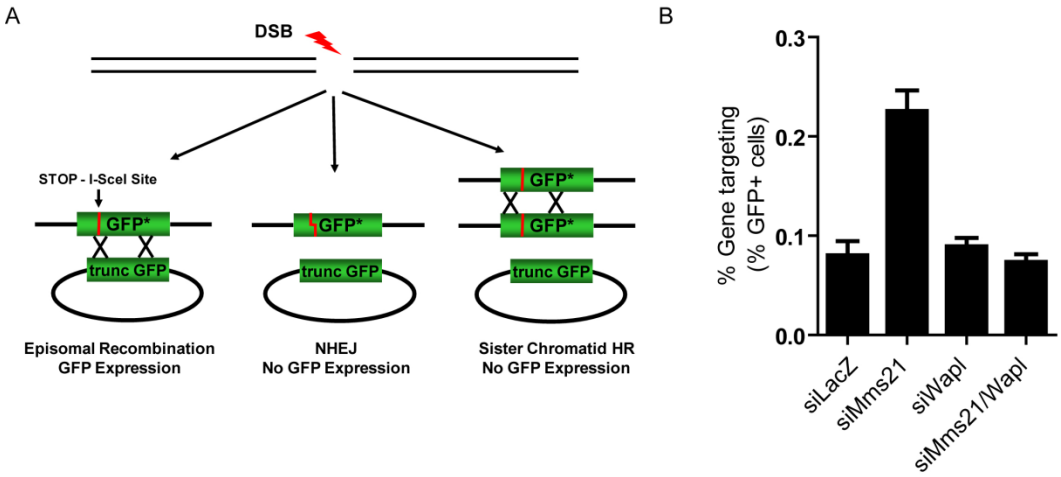

Figure 2-24. Wapl depletion rescues SCR defects of Mms21-RNAi cells in gene targeting assay

(A) Schematic drawing of the gene targeting assay. A GFP gene with an in-frame-stop codon and I-SceI endonuclease site was stably integrated into 293 cells to generate the 293/A658 cell line. Transfection of a plasmid expressing I-SceI and a truncated form of GFP induces a DSB at the I-SceI site. There are three repair pathways to repair the DSB: homologous recombination (HR) using the episomal plasmid as template, non-homologous end joining (NHEJ) and HR using sister chromatid as template. (B) Quantification of the normalized percentage of GFP-positive cells in the I-SceI based gene targeting assay of 293/A658 cells transfected with the indicated siRNAs. The cells were co-transfected with siRNAs and the repair plasmid. Three days later, cells were harvested and analyzed by FACS.

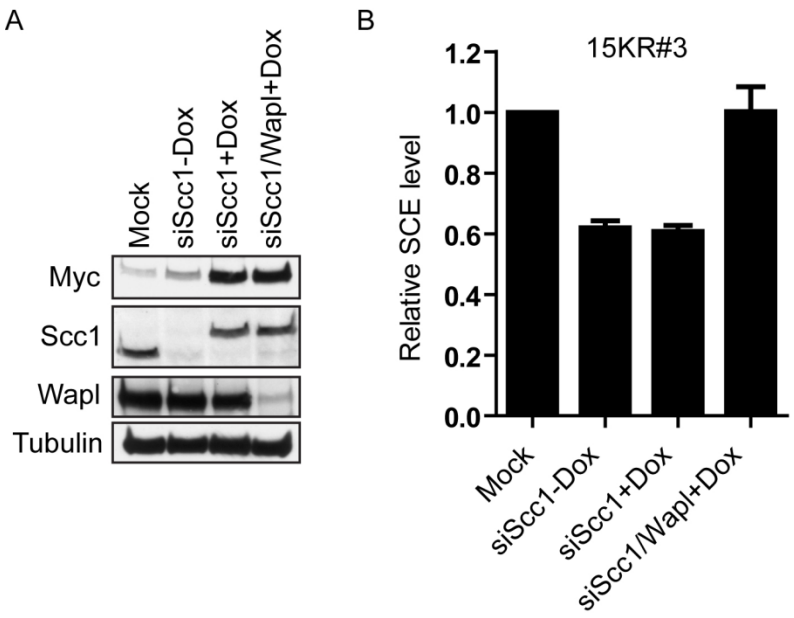

Figure 2-25. Wapl depletion rescues SCR defects of Scc1 15KR-expressing cells

(A) HeLa Tet-On cells expressing Myc-Scc1 15 KR (clone 3) cultured in the absence or presence of doxycycline (Dox) were transfected with the indicated siRNAs. Lysates were blotted with the indicated antibodies. (B) Quantification of the relative SCE levels of cells described in (A). The mean and standard deviation of data from two experiments are shown. About 30 cells were counted in each experiment.

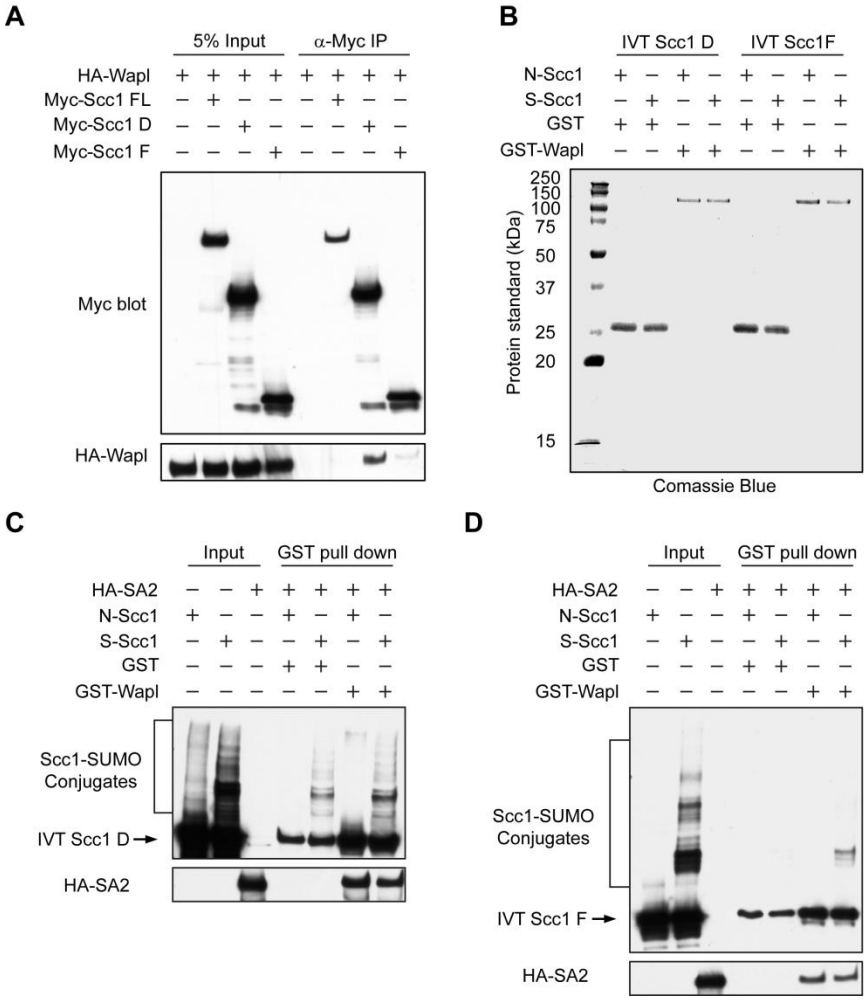

Figure 2-26. Recombinant purified N-terminal domain of Wapl binds to sumoylated Scc1 fragments in vitro

(A) Scc1 truncation fragment D and F interacts with full length (FL) Wapl in human cells. Myc-Scc1 FL, D or F fragments and HA-Wapl FL were co-expressed in HeLa Tet-On cells. The cell lysates were subjected to α-Myc IP. (B-D) In vitro binding assay of non-sumoylated/sumoylated Scc1 fragments and recombinant purified N-terminal domain of Wapl. Scc1 D and F fragment were in vitro translated in rabbit reticulocyte lysate, and then underwent Mms21 dependent sumoylation in vitro. Non-sumoylated Scc1 D and F fragments were used as control. N-Scc1, non-sumoylated Scc1; S-Scc1, sumoylated Scc1. GST protein or GST-N-terminal Wapl were used as bait protein to pull down non-sumoylated or sumoylated Scc1 fragments. In vitro translated HA-SA2 was added into the binding assay to enhance interaction between Scc1 and Wapl. A Comassie blue staining showed the amount of bait protein in each reaction (B).

The binding result of Scc1 D fragment and N-terminal Wapl was shown in (C). The binding result of Scc1 F fragment of N-terminal Wapl was shown in (D).

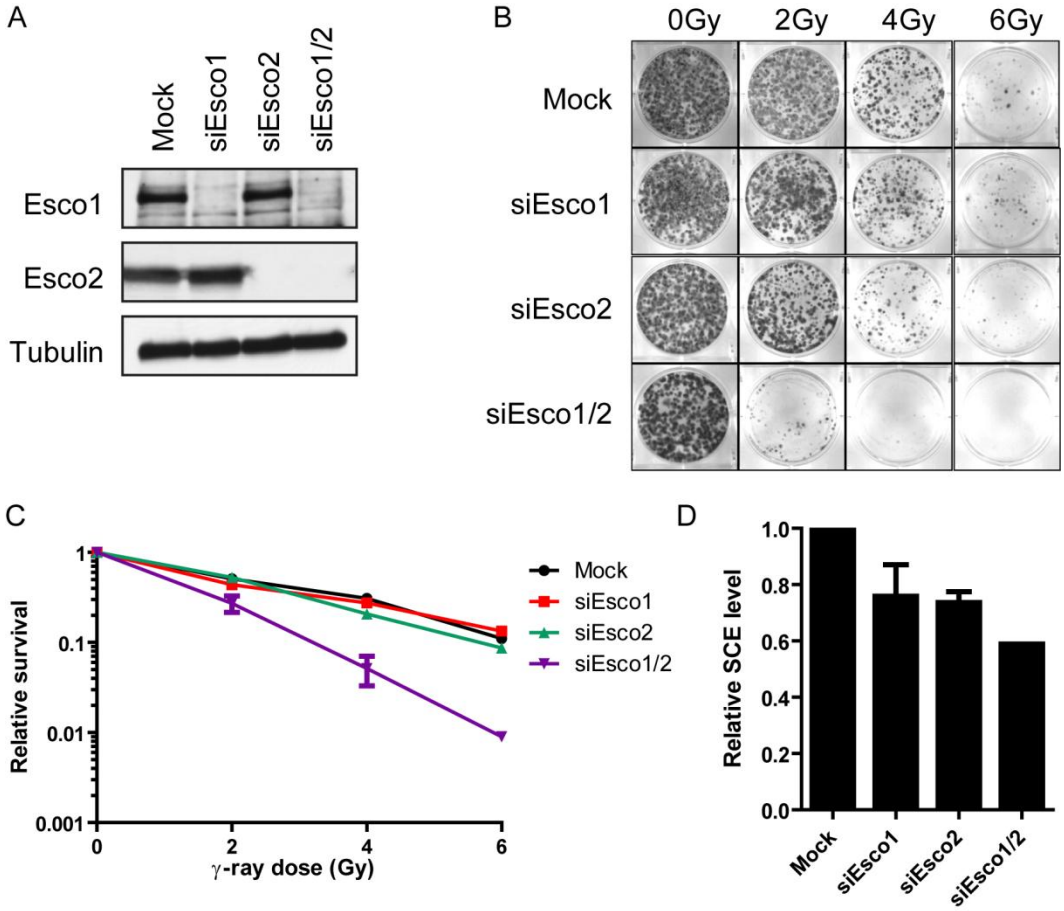

Figure 2-27. Esco1/2 are required for DSB repair and SCR
(A) Lysates of HeLa Tet-On cells transfected with the indicated siRNAs were blotted with the indicated antibodies. (B) Representative image of the IR colony survival assay. (C) IR colony survival assay of HeLa cells transfected with the indicated siRNAs. Each data point represents the mean and standard deviation of values in two independent experiments, with duplicate samples in each experiment. (D) Quantification of the relative SCE levels of HeLa Tet-On cells transfected with the indicated siRNAs. The mean and standard deviation of data from two experiments are shown. About 30 cells were counted in each experiment.

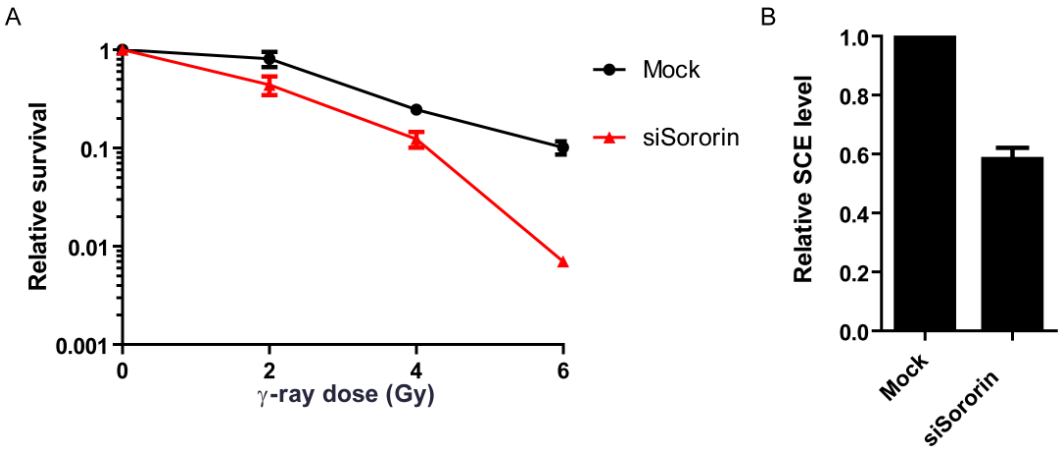

Figure 2-28. Sororin is required for DSB repair and SCR
(A) IR colony survival assay of HeLa Tet-On cells transfected with the indicated siRNAs. Each data point represents the mean and standard deviation of values in two independent experiments, with duplicate samples in each experiment. (B) Quantification of the relative SCE levels of HeLa Tet-On cells transfected with the indicated siRNAs. The mean and standard deviation of data from two experiments are shown. About 30 cells were counted in each experiment.

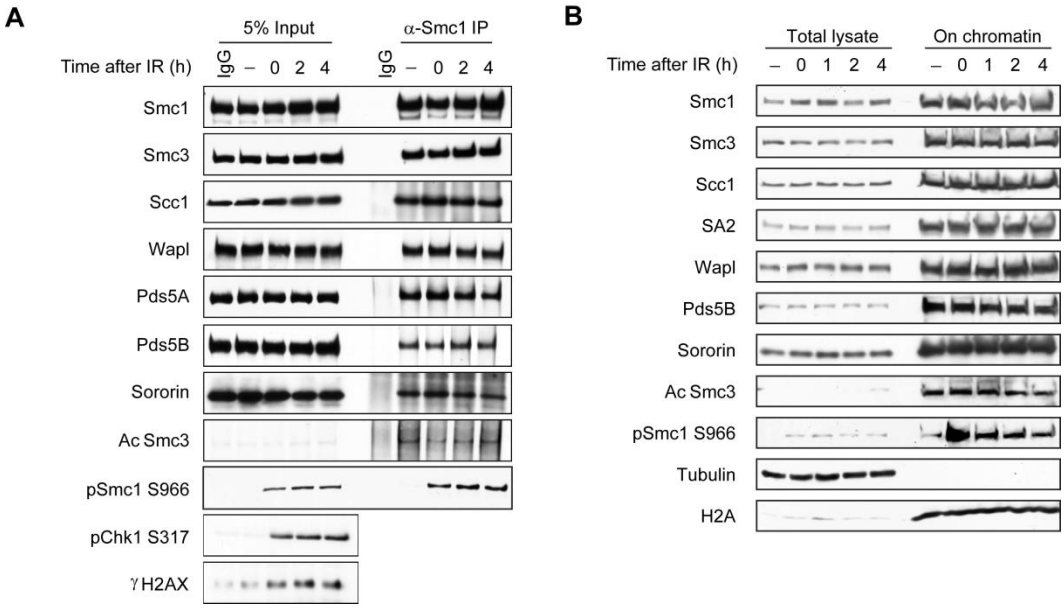

Figure 2-29. DNA damage does not alter global Smc3 acetylation or regulator binding to cohesin

(A) HeLa Tet-On cells were arrested in G2 by Cdk1 inhibitor RO3306 for 20 hrs, either untreated (–) or exposed to 8 Gy IR, and then harvested at different time points post IR. The cell lysates were subjected to IgG or α-Smc1 IP. Lysates and IP were blotted with the indicated antibodies. (B) DNA damage does not appreciably alter the chromatin-bound pool of cohesin and its regulators. HeLa Tet-On cells were arrested at G1/S by thymine, released into fresh medium for 3 hrs, treated with IR, and harvested at the indicated time points. Total lysates and the chromatin fractions of these cells were blotted with the indicated antibodies.

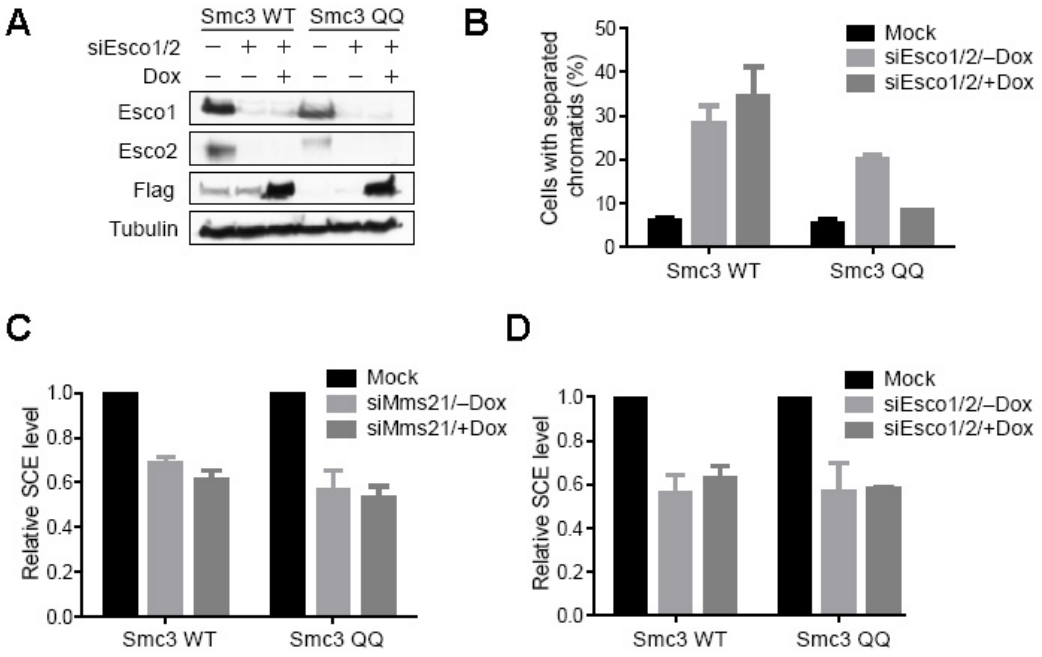

Figure 2-30. Expression of Smc3 acetylation-mimicking mutant does not bypass requirement for Mms21 in SCR

(A) 293T cell lines inducibly expressing Flag-Smc3 WT or K105Q/K106Q (QQ) were cultured in the absence or presence of doxycycline (Dox) and then either mock transfected or transfected with siEsco1/2. Lysates were blotted with the indicated antibodies. (B) Quantification of the percentage of mitotic cells in (A) with separated sister chromatids. The mean and standard deviation of three independent experiments are shown. (C) 293T cell lines inducibly expressing Flag-Smc3 WT or K105Q/K106Q (QQ) were cultured in the absence or presence of doxycycline (Dox) and then either mock transfected or transfected with siMms21. The relative SCE levels of these cells were quantified. The mean and standard deviation of data from two experiments are shown. About 30 cells were counted in each experiment. (D) Quantification of the relative SCE levels of cells in (A).The mean and standard deviation of data from two experiments are shown. About 30 cells were counted in each experiment.

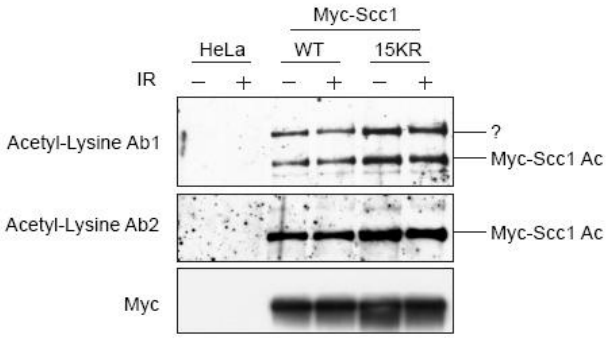

Figure 2-31. Scc1 15KR does not change Scc1 acetylation
HeLa Tet-On cells or cells expressing Myc-WT Scc1 and 15KR were arrested in G2 by RO3306 for 20 hrs and then untreated (-) or exposed to 8Gy IR. The cell lysates were subjected to α-Myc IP. The IP was blotted with the indicated antibodies. Acetyllysine Ab1 and Ab2 are from Immunechem (ICP0380) and Cell Signaling (9441), respectively.

Table 2-1. siRNAs used in this study

siMms21-1	5'-GCU GUU CAA UCU ACA AUA A-3'
siMms21-2	5'-GCA ACU AAA CCA UUA UGU A-3'
siMms21-3	5'-GCA CUU AGA AGG GCA AUU G-3'
siMms21-4	5'-CAA CUG GUU UCA UCU CCU U-3'
siMms21-5	5'-GGC AAA AGC GGA AGA AAA AdTdT-3'
siMms21-6	5'-GAC UGA AGU GAG UAG UGA AdTdT-3'
siMms21-7	5'-CUC UGG UAU GGA CAC AGC UdTdT-3' (Potts et al. 2006)
siSmc5-1	5'-CGA AAU AAU UGA UAA GCG A-3'
siSmc5-2	5'-GAA AGA AUU GAA CGG GUA A-3'
siSmc5-3	5'-GAA ACU UGU UAC CGA AUU A-3'
siSmc5-4	5'-GAA CAG GGA AGU CGA GCA U-3'
siSmc5-5	5'-AGA GAA AGU UGC AGC CUU AUU-3' (Behlke-Steinert et al. 2009)
siSmc5-6	5'-GUU UAA AGC UGA UGG AAC AdTdT-3'
siSmc5-7	5'-GAA GCA AGA UGU UAU AGA ATT-3' (Potts et al. 2006)
siSmc6-1	5'-AGA AAU AGA UAA UGC GGU U-3'
siSmc6-2	5'-GGA CAA AGA AAU UAA UCG A-3'
siSmc6-3	5'-CAG CAU AGA UGG AAG UCG A-3'
siSmc6-4	5'-CUU UAA AGC CAG UGU GUA U-3'
siSmc6-5	5'-CCA CAA GAA UGA AAC UCU AdTdT-3'
siScc1	5'-GGA AGA AGC AUU UGC AUU GUU-3'
siWapl	5'-CGG ACU ACC CUU AGC ACA AdTdT-3' (Kueng et al. 2006)
siLacZ	5'-GCG CCG AAA UCC CGA AUC UdTdT-3'
siLuciferase	5'-UCA UUC CGG AUA CUG CGA UUU-3'
siScrambled	5'-GGC AUC UUA GCC CGU ACU UUU-3'
siControl	AllStars negative control siRNA from Qiagen (#1027280)
siSmc1	Dharmacon ON-TARGETplus Set of 4
siSmc3	Dharmacon ON-TARGETplus Set of 4
siSA1	Dharmacon ON-TARGETplus Set of 4
siSA2	Dharmacon ON-TARGETplus Set of 4
siEsco1	Dharmacon ON-TARGETplus Set of 4
siEsco2	Dharmacon ON-TARGETplus Set of 4

CHAPTER III: METERIALS AND METHODS

Cell Culture, Transfection, and Synchronization

HeLa Tet-On, 293/A658, and 293T cells were grown in DMEM (Invitrogen) supplemented with 10% FBS, 100 μg/ml penicillin and streptomycin, and 2 mM L-Glutamine. Site-directed mutagenesis was performed using the QuikChange kit (Qiagen).

For plasmid transfections, cells were transfected at 50% confluency with the Effectene reagent (Qiagen) according to the manufacturer's protocols. For siRNA transfections, cells were transfected at 20% confluency with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocols. For plasmid and siRNA double transfections, cells were transfected at 80% confluency with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. To establish stable cell lines, HeLa Tet-On cells were transfected with pTRE2-Myc-based plasmids encoding siRNA-resistant Scc1 WT or 15KR with a C-terminal Myc_6 tag. Clones were selected in the presence of 200 μg/ml hygromycin B. Inducible expression was screened in the absence or presence of 1 μg/ml doxycycline (Invitrogen). 293T cell lines expressing Smc3 WT and Smc3 K105Q/K106Q (QQ) were kindly provided by Dr. Jun Qin (Baylor College of Medicine). For the functional rescue experiments, 1 μg/ml doxycycline was added into the medium to induce protein expression at 8 hrs before siRNA transfection.

To arrest cells in S/G2, cells were treated with 2 mM thymidine for 16 hrs and released into fresh medium for 5~6 hrs. To arrest cells in G2, cells were treated with the Cdk1 inhibitor RO3306 (EMD) at 10 μM for 20 hrs. The siRNAs used in this study were chemically synthesized by or purchased as pools from Dharmacon.

Antibodies, Immunoblotting, and Immunoprecipitation

To generate antibodies against Scc1 and Wapl, fragments of Scc1 (residues 211-316) and Wapl (residues 601-1190) were produced in bacteria as His6-tagged fusion proteins and purified. The proteins were used to immunize rabbits at Yenzym Antibodies (South San Francisco, CA). Production of the α-Mms21 antibody was described previously (Potts and Yu, 2005). Antibodies against sororin, Esco1/2, and Smc3 K105Ac were gifts from Susannah Rankin (Oklahoma Medical Research Foundation), Hui Zou (UT Southwestern), and Jun Qin (Baylor College of Medicine), respectively. The commercial antibodies used in this study were: α-Myc (Roche, 11667203001), α-HA (Roche, 11666606001), α-Scc1 (Bethyl, A300-080A), α-FLAG (Stratagene, 200472), α-Smc1 (Bethyl, A300-055A), α-phospho-Smc1 S966 (Bethyl, A300-050A), α-Smc3 (Bethyl, A300-060A), α-SA2 (Santa Cruz Biotechnology, sc-81852), α-Smc6 (Bethyl, A300-237A), α-Smc6 (Santa Cruz Biotechnology, sc-365742), α-Smc5 (Bethyl, A300-236A), α-Pds5A (Bethyl, A300-089A), α-Pds5B (Bethyl, A300-537A), α-

Pds5B (Bethyl, A300-538A), α-Chk1 phospho-S317 (Cell Signaling, #2344), γH2AX (Millipore, 05-636), α-SUMO3 (ABGENT, AM1201a), α-RanGAP1 (Santa Cruz Biotechnology, sc-25630), α-acetylated-lysine Ab1 (Immunechem, ICP0380), and α-acetylated-lysine Ab2 (Cell Signaling, #9441).

For immunoblotting, cells were lysed in SDS sample buffer, sonicated, boiled, separated by SDS-PAGE, and blotted with the desired antibodies. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgGs (Amersham Biosciences) were used as secondary antibodies. Immunoblots were developed using the ECL reagent (Amersham Biosciences) according to the manufacturer's protocols. For immunoprecipitation, lysate was prepared in the lysis buffer containing 25 mM Tris-HCl pH 7.7, 50 mM NaCl, 0.1% (v/v) NP-40, 2 mM $MgCl_2$, 10% (v/v) glycerol, 5 mM NaF, 0.3 mM $NaVO_4$, 10 mM β-glycerophosphate, 1 mM DTT, and 1X protease inhibitor cocktail (Roche). Turbo Nuclease was also added. Cells were broken by passing through a small gauge needle 10 times. After incubating for 1 hr on ice and 10 min at room temperature (RT), all samples were centrifuged at 14,000 rpm for 20 min at 4℃. Protein A beads (Bio-Rad) and the desired antibody or antibody coupled beads were incubated with the supernatant for 3 hrs at 4℃ (final concentration of antibody at a concentration of 1 μg/ml). The beads were then washed four times with the lysis buffer. The proteins bound to the beads were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with the appropriate antibodies.

Covalent Coupling of Antibodies to Protein A Beads

Desired amount of Bio-Rad Affi-prep Protein A support was washed with PBS for three times. 1 mg antibody per 1 mL solid beads was added into washed beads, and gently rotated at RT for 1 hr. After incubation, beads were washed twice with 0.2 M sodium borate pH 9.0. 20 mM dimethyl pimelimidate in sodium borate buffer was incubated with beads for 30 min at RT to crosslink antibody with beads. The reaction was stopped by washing beads once in 0.2 M ethanolamine pH 8.0. Beads were then incubated with 0.2 M ethanolamine pH 8.0 for 2 hrs at RT, washed once with 0.1 M Glycine pH 2.5, and washed three times with PBS buffer. Finally, the beads were resuspended in PBS containing 0.01% sodium azide and stored at 4 ℃.

In Vitro Translation

Reaction mixture was incubated for 90 min at 30 ℃, and then snapped freeze and stored in -80 ℃. Each reaction included 100 μg plasmid, 5 uL SP6-TNT rabbit reticulocyte lysate (Promega) and 1.0 μL methionine.

In Vitro Sumoylation Assay

Scc1 substrates were in vitro transcribed and translated (IVT) from pCS2-Myc plasmids in reticulocyte lysate (Promega) in the presence of cold or ^{35}S

methionine. 1 µl of IVT protein was then mixed with 1 µg Aos1–Uba2, 0.1 µg Ubc9, 10 µg SUMO1, 1 µl energy mix (150 mM phosphocreatine, 20 mM ATP, 2 mM EGTA, 20 mM $MgCl_2$, pH 7.7) in the absence or presence of 0.5 µg His-Mms21 at 30℃ for the indicated time. Reaction mixtures were adjusted to 10 µl with XB buffer (10 mM HEPES, pH 7.7, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 100 mM KCl, and 50 mM sucrose). SDS sample buffer was added to stop the reactions. The samples were boiled and subjected to SDS-PAGE followed by immunoblotting or autoradiography.

Denature Purification

For the denaturing Ni^{2+}-beads pull-down from HeLa cell lysates to enrich SUMO conjugates, cells were incubated with the lysis buffer containing 6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 8.0, 5 mM imidazole, and 10 mM β-ME, sonicated, and mixed with Ni^{2+}-NTA beads for 2 hrs at RT. The beads were then washed with the following buffers: buffer I (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 8.0 and 10 mM β-ME), buffer II (8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 8.0 and 10 mM β-ME), buffer III (8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 6.3, 10 mM β-ME and 0.2 % (v/v) Triton X-100), buffer IV (8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 6.3, and 10 mM β-ME), and buffer V (8 M Urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-HCl pH 6.3, 10

mM β-ME and 0.1 % (v/v) Triton X-100). The proteins bound to beads were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with the appropriate antibodies.

Flow Cytometry

For cell cycle analysis, cells were harvested by trypsinization, washed with PBS, and fixed in 70% ethanol at -20℃ for up to 24 hrs. The fixed cells were rinsed with PBS once, resuspended in PBS/0.25% (v/v) Triton X-100, and left on ice for 5 min. After centrifugation, PBS/1% BSA containing α-phospho-Ser/Thr-Pro, MPM-2 antibody was added and incubated for 3hrs at RT. Cells were rinsed with PBS/1%BSA, centrifuged, and incubated with secondary antibody for 30 min at RT. During this step, cells needed to be protected from light. After incubation, cells were washed and stained with PI buffer (PBS containing 0.1% Triton X-100, 25 μg/mL propidium iodide, and 0.1 mg/mL RNase) for 30 min at RT. Cells were analyzed with a flow cytometer. Data were processed with FlowJo.

For gene targeting assay, 293/A658 cells were harvested by trypsinization, washed with PBS, resuspended in FACS buffer (1×PBS, 2% FBS, 1mM EDTA, 0.1% sodium azide) and then analyzed with a flow cytometer.

Laser Microirradiation and Fluorescent Image Analysis

Laser damage induction and image analysis were performed essentially as described (Kim et al., 2002a; Kong et al., 2009). Briefly, 532 nm of the second harmonic of a pulsed Nd:YAG laser beam (~2–3 μJ/pulse energy after objective; ~4–6 ns pulse duration; 7.5 Hz, Quantronix- Continuum Lasers, La Mesa, CA) was focused through a 100X oil objective (NA 1.3; Olympus) on a microscope (Model IX81; Olympus). The sample stage was repeatedly scanned for 2 min at a scanning rate of ~10 μm/s to create a line pattern of microirradiation inside the nucleus. The cells were incubated for 1 hr at 37℃, and then fixed in 4% formaldehyde (10 min at RT). Cells were permeabilized with 0.5% Triton X-100 for 5 min, and then immunostained with the appropriate antibodies. Five to seven cells were damaged in one plate, and each experiment was repeated at least three times.

Immunofluorescence

Fresh 1% paraformaldehyde (PFA) in PBS was made and used in each experiment. PFA was dissolved by stirring on hotplate inside beaker of water at heat setting 4-5 for 15-30 min. After dissolving, 1% PFA was cooled down to RT and then filtered through 0.45 μm filter. Cells were washed once with PBS, fixed in 1% PFA for 15 min, and then washed twice with PBS at RT. Then, cells were permeabilized with buffer I (0.2% Triton X-100 and 3% BSA in PBS) for 20 min at 4 °C, incubated with primary antibodies (2 μg/mL) in buffer I for 1 hr at RT,

and washed three times with buffer II (0.2% Triton X-100 in PBS). Secondary antibodies diluted in buffer I were incubated with cells for 30 min at RT. Cells were then washed with buffer II for three times, stained with DAPI (1 μg/mL) in PBS for 2 min, washed once again in PBS, and then mounted and covered by a coverslip. After 15-30 min air dry, the slides were sealed and stored at 4 ℃ in the dark.

Metaphase Spread

Cells were collected by trypsinization and pelleted. 1 mL of medium was left in the tube. 2 mL of tap water was then added to resuspend the cells. After a 5-min incubation, the fixation solution (1:3 v/v glacial acetic acid:methanol) was added. After centrifugation, cells were resuspended in the fixation solution and incubated for 10 min at RT and washed twice with the fixation solution. Cells were resuspended and stored at -20 ℃. To prepare the slides for Giemsa staining, 3 drops of fixed cells were dropped onto each slide (pre-treated with methanol) and air dried for 5 min. 5% Giemsa (EMD) in the Giemsa staining buffer (0.01 M PBS, pH 6.8) was used to stain the slides. The slides were washed twice with running water, dried at RT for 20 min, mounted in Entellan mounting media (Merck), and analyzed by microscopy.

Colony Survival Assay

HeLa Tet-On cells were transfected with the indicated siRNAs for 24 hrs, and then replated into 6-well plates with 500, 2,000, 10,000 and 40,000 cells per well. After one day, cells were exposed to a Cs-137 sealed irradiator to receive different doses of γ-ray (0, 2, 4, 6, 10 Gy), and then put back into culture for about 10 days to form colonies. At the day of staining, medium was removed, and crystal violet colony staining solution (0.5 g crystal violet, 27 mL 37% formaldehyde, 100 mL 10×PBS, 10 mL methanol in 1 L) was added to fix and stain colonies for 20 min at RT. After staining, dishes were washed under running water and air dried for 2 days. Colonies with more than 50 cells were manually counted. The numbers of colonies were normalized to the unirradiated sample.

Sister Chromatid Exchange (SCE) Assay

HeLa Tet-On cells were transfected with the indicated siRNAs for 24 hrs, and then replated and incubated in the presence of 100 μM BrdU and 2.5 nM camptothecin for 42 hrs (about two cell divisions). Colcemid (150 ng/ml) was added during the final 2 hrs to enrich for mitotic cells. Cell were collected by trypsinization and washed with PBS. Cells were swelled in 75 mM KCl for 12 min at 37℃, followed by centrifugation. Cell pellets were resuspended in the fixation solution for 20 min at 4℃. Cells were washed twice with the fixation solution, resuspended, and dropped onto cold slides. After 2-3 days, slides were stained with 0.05 mg/ml acridine orange (Molecular Probes) for 5 min, washed

under running water for 4 min, and mounted in the Sorenson buffer (0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.8) for 1 min. Slides were immediately viewed under the microscope. For each experiment, the numbers of crossover events and chromosomes were counted in about 30 mitotic cells. The numbers of SCE per 100 chromosomes were calculated. All data were normalized to the value of the mock-transfected sample.

Chromatin immunoprecipitation (ChIP) assay

HeLa Tet-On cells were transfected with ER-I-*Ppo*I expression plasmid for 24 hrs. In experiments involving siRNA, cells were transfected with siRNA for 24hrs before transfection with the ER-I-*Ppo*I expression plasmid. 1 μM 4-hydroxytamoxifen (4-OHT) was added into medium for 12 hrs to induce the enzyme after the 24hr-plasmid-transfection.

CHIP experiment was performed as described previously (Berkovich et al., 2008). Cells were crosslinked in 1 % formaldehyde for 10 min at RT with continued agitation, quenched with 0.125 M glycine for 5 min at RT, washed twice with cold PBS, and then scraped in cold PBS. The cell pellet was washed once with cold PBS containing 1 mM PMSF, resuspended in cell lysis buffer I (10 mM HEPES pH 6.5, 10 mM EDTA, 0.5 mM EGTA, 0.25 % Triton X-100, 1X protease inhibitor, 1X phosphatase inhibitor and 1 mM PMSF) and incubated on ice for 10 min, washed once with cell lysis buffered II (10 mM HEPES pH 6.5, 1

mM EDTA , 0.5 mM EGTA, 200 mM NaCl, 1X protease inhibitor, 1X phosphatase inhibitor and 1 mM PMSF) and then resuspended in nuclei lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 0.5 % SDS, 1X protease inhibitor, 1X phosphatase inhibitor and 1 mM PMSF) and incubated for 10 min on ice. Cells were sonicated at 50% duty cycle, seven times for 10 sec continuously in an ice water bath with 1 min breaks between sonications. The fragmented chromatin was then cleared by centrifugation and diluted 1:5 with the IP dilution buffer (1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl, 1X protease inhibitor, 1X phosphatase inhibitor and 1 mM PMSF). The diluted chromatin was precleared with blocked protein A sepharose beads (GE healthcare) for 4 hrs at 4℃. To prepare blocked protein A beads, incubate protein A beads in IP dilution buffer with 100 µg/ml tRNA, 1 mg/ml BSA at 4℃ for overnight (ON). 1% of the fragmented chromatin was used as the input sample. To the remaining precleared chromatin, 4 µg of the indicated antibodies and 25 µl blocked protein A beads were added and incubated for ON. Another 25 µl blocked protein A beads were added and rotated at 4℃ for 3 hrs on the second day. The chromatin bound beads were washed seven times with IP wash buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 0.7% deoxycholic acid, 0.5 M LiCl, 1% NP-40, 1X protease inhibitor, 1X phosphatase inhibitor and 1 mM PMSF) and once with TE. Chromatin was then eluted from the beads by incubating in elution buffer (0.1 M $NaHCO_3$ and 1 % SDS) at 65℃ for 20 min. Repeat and combine

both elutions in the same tube. The chromatin was then decrosslinked and proteins were degraded. DNA was purified through a QIAquick PCR purification kit (QIAGEN). Quantitative real-time PCR (QPCR) was perfomed using validated primers toward rDNA multiple I-*Ppo*I sites (5'-TGGAGCAGAAGGGCAAAAGC-3' and 5'-TAGGAAGAGCCGACATCGAAGG-3')

Fractionation

HeLa Tet-On cell lysate was prepared in the lysis buffer containing 5 μg/ml cytochalasin B, 25 mM Tris-HCl pH 7.7, 100 mM NaCl, 0.1% (v/v) NP-40, 2 mM $MgCl_2$, 10 % (v/v) glycerol, 5 mM NaF, 0.3 mM $NaVO_4$, 10 mM β-glycerophosphate, 1 mM DTT, and 1X protease inhibitor cocktail (Roche). Turbo nuclease was added as well. Cells were broken by passing through a small gauge needle for 20 times. After incubating on ice for 1 hr and 10 min at 37℃, all samples were centrifuged at 14,000 rpm for 1 hr at 4℃. The supernatant was filtered and fractionated on a Superose 6 column. The fractions were analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. The elution positions of the molecular protein standard are indicated.

In Vitro Binding Assay with GST-tagged Proteins as Baits

5 µL glutathione-agarose beads were used for each condition. The beads were first equilibrated with buffer QA (20 mM Tris-HCl pH 7.7, 100 mM KCl, 1mM $MgCl_2$, and 1 mM DTT) and then incubated with 3 µg of the GST or GST-tagged protein in a total volume of 50 µL at RT for 35 min with vigorous shaking. For the purpose of blocking, 460 µL of blocking solution (TBS+0.05% Tween 20+ 5% dry milk) was added to each reaction and incubated at RT for 40 min with shaking. The beads were then washed twice with blocking solution. 4 µL of in-vitro-translated target proteins was diluted with 45 µL of blocking solution for each reaction, added into the beads and incubated at RT for 1 hr with shaking. After binding, the beads were washed four times with TBST (TBS+0.05% Tween 20), quenched with SDS sample buffer, boiled and analyzed on SDS-PAGE gels. For ^{35}S-labeled target protein, the protein gel was dried and exposed to phosphoimager cassettes. For cold-methionine translated target protein, the protein gel underwent western blot.

CHAPTER IV: DISCUSSIONS AND FUTURE DIRECTIONS

In this study, we provide evidence to suggest that Mms21-dependent Scc1 sumoylation is necessary for SCR in human cells. It functionally opposes the negative cohesin regulator, Wapl, at a step after cohesin recruitment to DSBs. We further show that Smc3 acetylation is not the sole critical molecular event downstream of Scc1 sumoylation in DNA repair. Our study thus establishes a general framework for the function and regulation of cohesin in SCR in human cells.

Role of Scc1 Sumoylation by Mms21 in SCR

We have convincingly shown that Mms21 is sufficient to promote Scc1 sumoylation in vitro and in human cells. We also provide strong evidence to suggest that a small pool of endogenous cohesin is sumoylated at laser-induced DNA damage sites in an Smc5/6-dependent manner. On the other hand, we could not biochemically detect the sumoylation of endogenous cohesin without overexpression of Mms21 or SUMO under normal or DNA-damage conditions. The difficulty of detecting sumoylation of endogenous proteins is well documented. Only a handful of proteins are sumoylated at appreciable steady-state levels in human cells. The underlying reasons for the low steady-state levels of sumoylation are not understood, but have been attributed in part to the highly dynamic nature of this modification. Furthermore, our fractionation (Figure 2-10)

experiments show that DNA damage does not induce global changes in the composition of cohesin and its molecular interactions with known regulators. Therefore, our results suggest that only a small pool of cohesin at DSBs is sumoylated, providing a possible explanation for our failure to detect sumoylation of endogenous cohesin in cells with native levels of Mms21 and SUMO.

Scc1 sumoylation by over-expressed Mms21 and SUMO is not further stimulated by DNA damage (Figure 2-4). This result suggested that the SUMO ligase activity of the bulk Mms21 was not directly regulated by DNA damage. Cohesin sumoylation might be triggered by a transient interaction between cohesin and the Smc5/6 complex when both are independently recruited to DNA damage sites. On the other hand, we cannot exclude trivial explanations for the apparent lack of regulation of Mms21-dependent Scc1 sumoylation by DNA damage. For example, over-expressed Mms21 might not behave the same as the endogenous Mms21 in this regard. A definitive answer to this question awaits the development of a method that can selectively isolates DSB-bound cohesin from human cells in the future.

Although our results show that Scc1 15KR is defective in DSB repair and SCR, we cannot completely rule out the possibility that, in addition to abolishing Mms21-dependent sumoylation, the 15KR mutation affects other functions of Scc1. Scc1 15KR is still functional in maintaining mitotic sister-chromatid cohesion, indicating that it does not have gross structural defects. On the other

hand, yeast strains expressing Scc1 at 30% of wild-type levels are deficient in DNA repair (Heidinger-Pauli et al., 2010). By contrast, sister-chromatid cohesion and chromosome segregation remain normal even when Scc1 is reduced to 13% of wild-type levels in yeast cells. Similarly, certain siRNAs against Scc1 that depleted cohesin poorly caused IR sensitivity, but produced little defects in sister-chromatid cohesion in human cells (data not shown). Thus, partial inactivation of cohesin reveals its role in DNA repair while a more complete inactivation of cohesin is needed to reveal its function in sister-chromatid cohesion. A simple quantitative difference between the activities of Scc1 WT and 15KR could explain why Scc1 15KR is capable of supporting sister-chromatid cohesion, but fails to support proper DNA repair. Finally, because human Esco1/2 acetylate unknown substrates in DNA repair and because Scc1 acetylation is critical for DI cohesion in yeast, the 15KR mutation could conceivably eliminate a yet unidentified acetylation site. We do not have evidence for such a scenario, because Scc1 15KR does not alter Scc1 acetylation as detected by pan-acetyllysine antibodies.

How does Scc1 sumoylation by Mms21 contribute to SCR? We show that the Smc5/6 complex and Scc1 sumoylation are dispensable for cohesin recruitment to DSBs. Depletion of Wapl, a negative regulator of cohesin, rescues the IR sensitivity and SCE defect of Mms21-deficient or Scc1 15KR-expressing cells. Our results support a two-step model for cohesin regulation in SCR (Figure

4-1). In the first step, both cohesin and the Smc5/6 complex are independently recruited to DNA damage sites. The loaded cohesin is not yet cohesive and may be removed by Wapl. In the second step, a transient interaction between the loaded Smc5/6 and cohesin complexes enables Mms21-dependent sumoylation of Scc1 locally at DSBs. This local cohesin sumoylation counteracts Wapl, stabilizing cohesin around DSB and facilitating SCR.

Antagonism between Mms21 and Wapl

We do not have evidence that Scc1 sumoylation directly prevents Wapl binding to cohesin in vitro and in cells. Furthermore, Wapl does not completely dissociate from functional cohesin that has Smc3 acetylation and is bound to Pds5 and Sororin. How Wapl promotes cohesin release from chromatin and how Sororin antagonizes Wapl remain poorly understood. Without a mechanistic understanding of how Wapl works, biochemical studies on how Scc1 sumoylation affects Wapl function are premature. Our data thus only establish Wapl as a downstream effector of the Mms21 pathway, but do not prove a causative mechanistic link between the two. Future studies will be necessary to determine how Wapl promotes cohesin removal from chromatin. Crystallography studies on Wapl, Wapl-Pds5A/B-Scc1-SA2 complex, Wapl-Pds5A/B-Sororin-Scc1-SA2 complex will be appreciated and provide a lot of insights.

During the unperturbed S phase, Esco1/2 acetylates Smc3 at K105 and K106 in a process that is coupled to DNA replication (Zhang et al., 2008). Expression of the Smc3 acetylation-mimicking mutant rescues the sister-chromatid cohesion defect of Esco1/2-RNAi cells, but fails to bypass the requirement for Mms21 in SCR. Therefore, in the context of SCR, Smc3 acetylation is unlikely to be the sole downstream event that is regulated by Scc1 sumoylation. As discussed above, we cannot rule out the possibility that Smc3 QQ is not a perfect mimic of acetylation. It supports sister-chromatid cohesion, but does not support DNA repair, because the latter process requires larger amount of functional cohesin. On the other hand, because Sororin and Esco1/2 are required for DBS repair and SCR (Schmitz et al., 2007), it is conceivable that Scc1 sumoylation acts indirectly through Esco1/2 and Sororin to oppose Wapl function. Identification of other DNA-damage induced modifications on cohesin and/or its regulatory subunits will be essential for illustrating the DNA damage cohesin pathway.

Similarities and Differences of Cohesin Regulation in DNA Repair in Yeast and Humans

In yeast, it has been conclusively shown that DNA damage induces functional cohesion locally at DSBs and globally throughout the genome (Strom et al., 2007; Unal et al., 2007). Because we do not have a strategy to inactivate the

existing functional cohesin specifically in G2 human cells, we cannot definitively test whether DNA damage actually establishes functional cohesion at damage sites in human cells. Our results do, however, reveal common themes shared by yeast and human cells in terms of cohesin regulation in DNA repair. First, the involvement of cohesin in DNA repair is a multi-step process in both organisms. The mere loading of cohesin to damage sites is insufficient. Posttranslational modifications (Scc1 acetylation in yeast and sumoylation in humans) are necessary to make the loaded cohesin functional. Second, in both organisms, Wapl is the critical downstream effector. Posttranslational modifications of cohesin antagonize Wapl to promote SCR. Third, replicative cohesion establishment and the DNA repair function of cohesin require different modifications of cohesin and its regulators. In yeast, Eco1 targets Smc3 in replicative cohesion establishment, but targets Scc1 to establish DI cohesion. Likewise, Esco1/2 in humans also have distinct targets in replicative cohesion establishment and in DNA repair, although the target of Esco1/2 in the latter process remains to be identified.

There are also important differences in the mechanisms by which cohesin promotes DNA repair in yeast and human cells. The acetylation and phosphorylation sites in yeast Scc1 those are critical for DNA repair and DI cohesion are not conserved in human Scc1. Furthermore, Sororin is required for

DSB repair and SCR in human cells. Functional Sororin homologs have not been identified in yeast.

Conclusion and Perspective

In conclusion, our results establish a new posttranslational regulatory mechanism of cohesin during DNA repair, and reveal both conserved principles and organism-specific features in cohesin regulation during sister-chromatid recombination.

The Smc family of proteins has critical roles in the DNA damage response of organisms from yeast to man. The Smc1/3 cohesin complex promotes DNA double-strand break (DSB) repair through homologous recombination (HR) between sister chromatids, presumably by holding sister chromatids in proximity to help strand invasion. Cohesin is also required for DNA damage checkpoint activation. The condensin complexes are required for DNA damage checkpoint activation, DNA repair, and rDNA stability. The Smc5/6 complex facilitates DSB repair through HR between sister chromatids and does so in the same pathway as cohesin. The Smc5/6 complex has additional roles in DNA repair, including resolution of collapsed replication forks and rDNA maintenance.

Many outstanding questions still remain in this area. First, the detailed molecular mechanisms by which the Smc proteins mediate DNA repair are not understood. More needs to be learned about how the DNA repair functions of the

Smc complexes are regulated during the cell cycle. The coordination and crosstalk among the three Smc complexes in the DNA damage response need to be further examined. For example, both condensin and the Smc5/6 complex are required for rDNA stability in yeast. Is this function of condensin and Smc5/6 conserved in higher eukaryotes? Do these two complexes function in the same or different pathways? Future studies aimed at addressing these questions will greatly advance our understanding of the molecular mechanisms underlying chromosome maintenance and genome stability.

Mutations of the Smc complexes and their regulators have been linked to human diseases, including cancer. A better understanding of how these complexes protect genomic stability will help us understand the molecular basis of disease phenotypes and may ultimately lead to strategies that exploit the dysregulation of the Smc proteins to treat these human diseases.

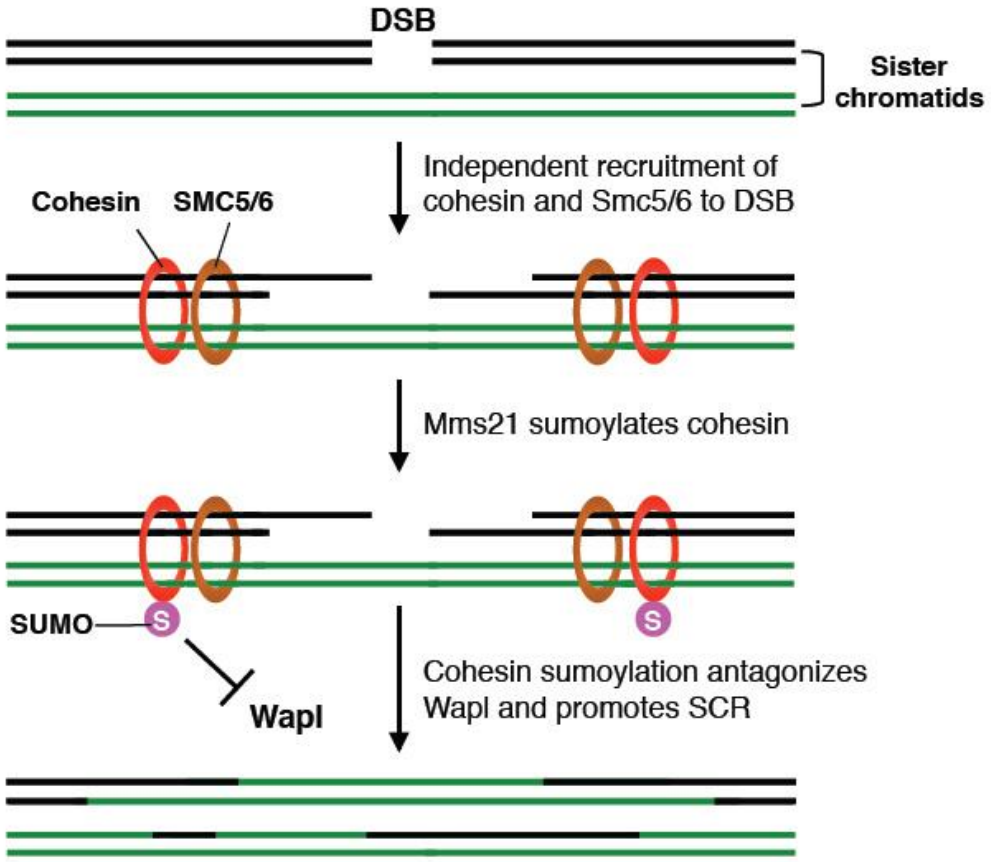

Figure 4-1. A two-step model for cohesin regulation in SCR
In the first step, both cohesin and the Smc5/6 complex are independently recruited to DNA damage sites. The loaded cohesin is not yet cohesive and may be removed by Wapl. In the second step, a transient interaction between the loaded Smc5/6 and cohesin complexes enables Mms21-dependent sumoylation of Scc1 locally at DSBs. This local cohesin sumoylation counteracts Wapl, stabilizing cohesin around DSB and facilitating SCR.

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