THE ROLE OF ESC2 AND DNA IN SMC5/6-MEDIATED SGS1-TOP3-RMI1 SUMOYLATION

by

Ashley S. Mutchler, B.S., M.S.

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Committee Members:

Xiaoyu Xue, Chair

Xiaolan Zhao

Tania Betancourt

Karen Lewis

Kevin Lewis

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ABSTRACT

Deoxyribonucleic acid (DNA) is arguably one of the most important biological materials. DNA contains the genetic material required to make up an organism and can be referred to as the body's instruction manual. The replication of DNA is essential for the growth and reproduction of cells and requires precise copying of the genome, which is challenged by many obstacles or damages such as DNA double-strand breaks (DSBs). There are several mechanisms to deal with DSBs and replication blockages, including homologous recombination (HR). HR leads to the formation of DNA intermediates such as Holliday junctions (HJ), which are processed by one of two mechanisms: resolution or dissolution. In double Holliday junction dissolution, Sgs1-Top3-Rmi1 (STR) complex in yeast creates non-crossover products, and this process is regulated by Mms21-Smc5/6 mediated sumoylation of STR in the S and G2 phases. While STR is known to work in conjunction with Smc5/6 and SUMO, the exact details and mechanisms of how Mms21-Smc5/6 mediated STR sumoylation is regulated remain vastly unknown. This dissertation seeks to further investigate the mechanism in which genome stability factor Esc2 acts to remove recombinational intermediates through regulating the Mms21-Smc5/6 mediated STR sumoylation. Here, we found that Esc2 specifically influences the Mms21 (SUMO E3) substrates associated with HJs or replication fork structures. Importantly, our results also showed that Esc2 enhances the sumoylation of STR complex both *in vitro* and *in vivo*, which is mediated by its SLD2 domain that interacts with Ubc9, and that this

interaction is essential in the dissolution of HJ and genome stability. Therefore, we suggest that Esc2 is a SUMO E2 cofactor that facilitates the sumoylation of Mms21 substrate STR in HJ dissolution. In addition, we found that the mid-region (MR) of Esc2 with HJ and replication fork binding activity also contributes to Sgs1 sumoylation, but does so through a DNA-independent manner. We also studied how STR sumoylation is regulated by DNA intermediates. We found that Sgs1 binding to DNA promotes its sumoylation, and Sgs1 sumoylation is enhanced when Esc2 is present. Ultimately, our data showed that Esc2 and DNA stimulated the Smc5/6-mediated sumoylation of the STR complex, which promoted the dissolution of DNA intermediates to maintain genome stability. Our results set a foundation for future studies on other potential binding partners of Ubc9 to better understand the efficiency of sumoylation, and also if sumoylation of Sgs1 affects its other roles in HR, such as end resection or D-loop assembly, to deepen the understanding of how sumoylation regulation of STR affects genome maintenance.

I. INTRODUCTION

DNA replication and DNA double strand break repair

Deoxyribonucleic acid (DNA) is arguably one of the most important biological materials. DNA contains the genetic material required to make up an organism and can be referred to as the body's instruction manual. The replication of DNA is essential for the growth and reproduction of cells and requires precise copying of the genome, which is challenged by many obstacles or damages such as DNA double-strand breaks (DSBs). A DSB occurs when both strands of the DNA are severed and is considered one of the most cytotoxic lesions (1). The inability to identify and control these lesions can lead to more problems like replication fork stalling, genome instability, and even chromosomal rearrangements leading to cancer and other diseases (2-4).

There are several mechanisms to deal with DSBs and replication blockages, including homologous recombination (HR) and nonhomologous end-joining (NHEJ). Briefly, NHEJ repairs DSBs in DNA without the need for a homologous template by rejoining the ends of broken DNA. This research focuses primarily on homology directed repair, specifically HR.

In HR, the Mre11-Rad50-Xrs2 (MRX) complex binds to the DNA on either side of the break, specifically to the 5'-end. The MRX complex is an integral part of controlling end resection as it recruits the Sae2 endonuclease protein and works together to trim back the 5'-ends to create 3'-overhangs of single-stranded DNA (ssDNA). End resection is continued by the Sgs1-Top3-Rmi1 (STR) complex and the Dna2 and Exo1 nucleases (5). The Sgs1 helicase unwinds the dsDNA, while Dna2 and Exo1 nucleases

cut the ssDNA produced by Sgs1. The STR complex also plays a role later on in dissolving DNA intermediates formed by HR (6,7).

These proteins also work closely with replication protein A (RPA), which coat 3'-ssDNA to protect them from nucleases and to prevent them from coiling back. Rad51, with the help of many other mediator proteins, then replaces the RPA and polymerizes on the 3'-ssDNA to form a presynaptic filament. This filament then begins to search for DNA sequences that are homologous to the 3'ssDNA. Once found, the filament invades the homologous DNA sequence in a process known as strand invasion. This process creates a displacement loop (D-loop) between the invading strand and the homologous chromosome. After D-loop formation, the HR pathway can be divided into either synthetic dependent strand annealing (SDSA) or double strand break repair (DSBR) pathways. In DSBR, a DNA polymerase is recruited and begins to extend the ends of the 3'-end involved in strand invasion. After second end capture, a double Holliday junction (dHJ) DNA intermediate is formed (6,7).

These dHJs are processed by one of two mechanisms: resolution or dissolution. The former is regulated by phosphorylation and requires the Mus81-Mms4 endonuclease complex, while the latter is regulated by protein sumoylation and requires the Sgs1-Top3-Rmi1 (STR) complex. In double Holliday junction dissolution, STR complex creates non-crossover products and is regulated by Mms21-Smc5/6 mediated sumoylation in the S and G2 phases (8,9).

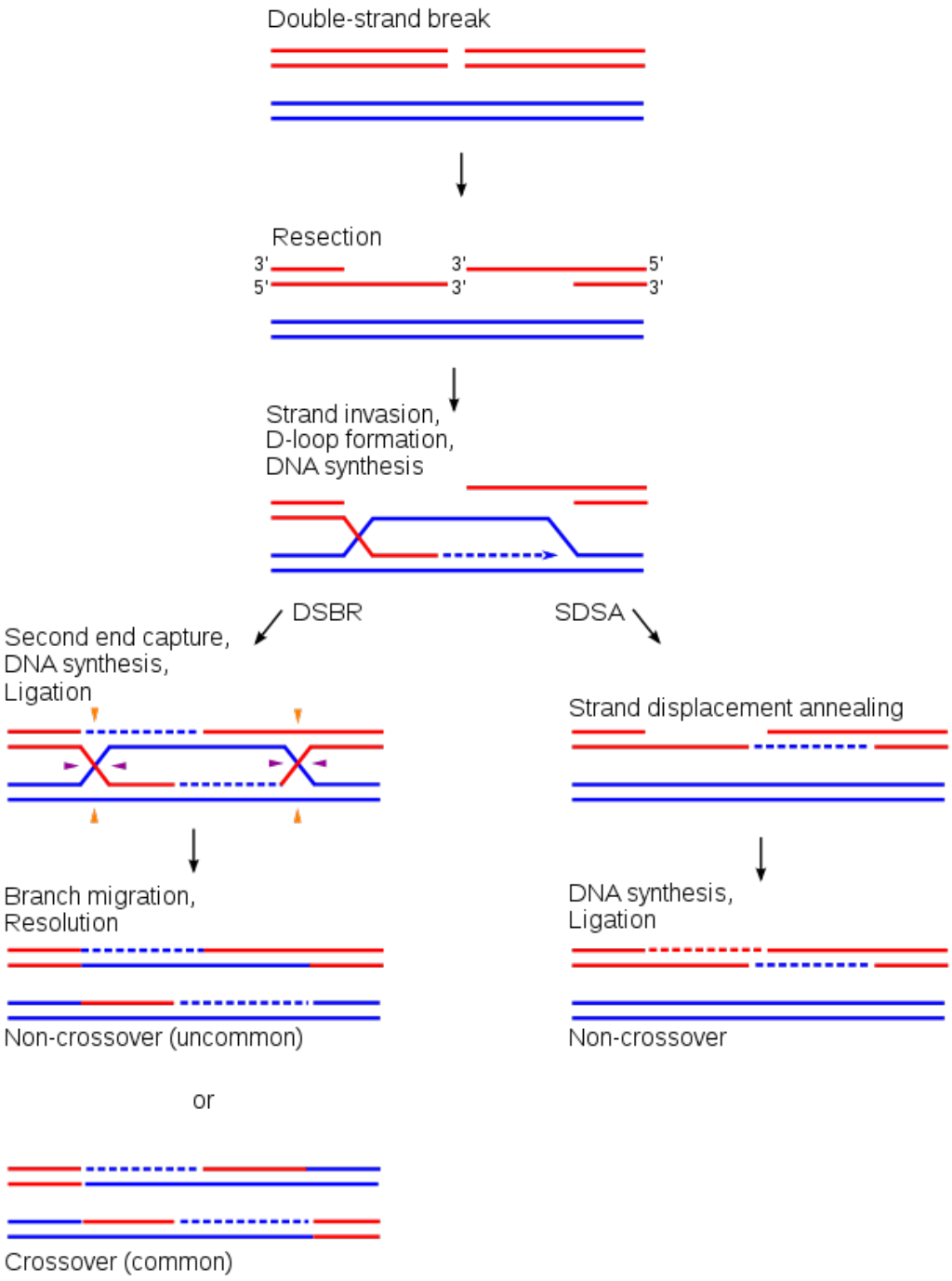

Figure 1: Homologous recombinational repair of DNA double stranded breaks (7).

There are other proteins involved in DNA damage repair. Proliferating cell nuclear antigen (PCNA) is one of these proteins, although its mechanism is not fully understood. PCNA is a ring-shaped complex that binds to DNA and enhances DNA synthesis by recruiting certain proteins such as DNA helicase Srs2 to the replication fork (10). The function of these proteins, and many other proteins involved in DNA damage repair, is also regulated by sumoylation, similar to the regulation of STR function in removing recombinational intermediates (11).

Protein sumoylation

Protein sumoylation is a post-translational modification which regulates numerous cellular processes, including DNA damage repair (12). It covalently modifies proteins with a small ubiquitin-like modifier (SUMO) or with a SUMO chain with the help of SUMO enzymes. Similar to ubiquitination (13), sumoylation requires the sequential action of the trio of the SUMO E1 activating enzyme, the SUMO E2 conjugating enzyme, and the SUMO E3 ligase. In budding yeast, these enzymes have been identified as the Aos1-Uba2 SUMO E1 heterodimer, the Ubc9 SUMO E2 enzyme, and three SUMO E3 enzymes (Siz1, Siz2, and Mms21) and participate in sumoylation for hundreds of substrates (14). Compared to ubiquitin enzymes, it is unclear how so few SUMO enzymes can modify such a large number of substrates.

The sumoylation process begins with the SUMO E1 activating enzyme undergoing an ATP-dependent activation of the SUMO C terminus (Figure 2). The C-terminal carboxyl group of SUMO attacks ATP, generating a SUMO C-terminal

adenylate. Next, cysteine's thiol group in the E1 attacks the SUMO adenylate, forming a thiolester bond. Next, the activated SUMO is then transferred to the SUMO E2 conjugating enzyme via its active cysteine site forming another thiolester bond. Finally, SUMO is then transferred to the substrate with the assistance of the SUMO E2 enzyme and one of the three SUMO E3 enzymes. The SUMO conjugated E2 serves as a donor when SUMO is transferred to a lysine residue on the substrate promoted by the SUMO E3s. Sumoylation is a reversible process. Enzymes in the Ulp family have been reported to cleave at the C terminus of SUMO (13).

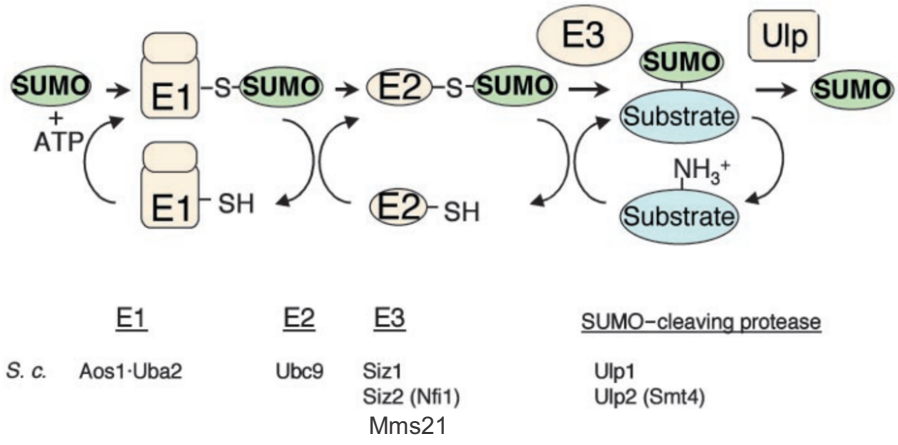

Figure 2: The SUMO conjugation pathway. Proteins are sumoylated by the sequential action of SUMO E1, E2, and E3 (13).

While many details of the sumoylation pathway are known, there are still many fundamental questions that remain unanswered, especially in the realm of DNA damage repair and how Smc5/6-mediated sumoylation of the STR complex facilitates in removing recombinational intermediates.

The Smc5/6 complex functions in DNA damage repair and protein sumoylation

One of the SUMO E3 ligase, Mms21, belongs to the Smc5/6 complex, which contains 8 subunits that work together in DNA repair (Figure 3). In budding yeast, the 8 subunits are Smc5, Smc6, Nse1, Nse2 (also referred to as Mms21 SUMO E3 ligase), Nse3, Nse4, Nse5, and Nse6. The pair of structural maintenance complexes (SMC) ATPases (Smc5/6) can fold back onto itself using a hinge domain to bring the N- and C-terminus together to form an ATPase that facilitates in DNA binding activity (15).

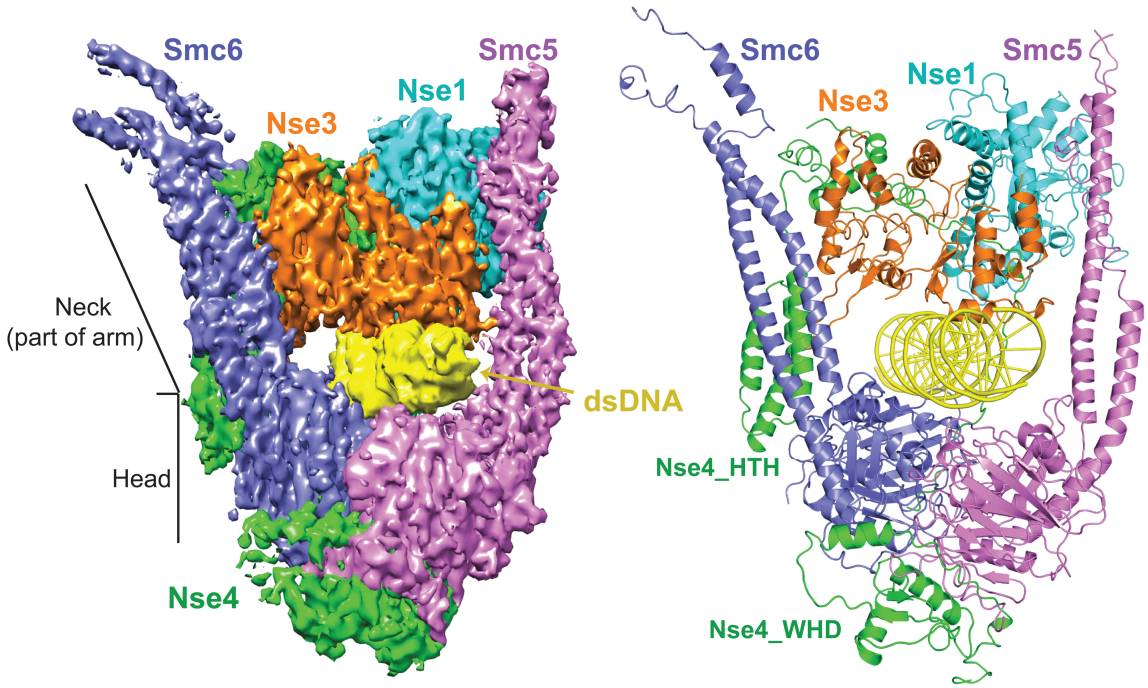

Figure 3: Cryo-EM structure of DNA-bound Smc5/6 complex. This figure shows the complex in an electron density representation (left) and in a ribbon representation (right), with components labeled and color-coded (16). Note that Nse2, which binds to the coiled-coil domain of Smc5, and Nse5-6, is not shown in the Smc5/6 complex structure.

The Smc5/6 complex was originally discovered in fission yeast (*S. pombe*), upon the realization that the Rad18 protein has structural similarities to SMC family proteins and forms a heterodimer with another SMC-like protein Spr18 (15,17,18). Smc5/6 was found to have 6 additional subunits, non-SMC elements (Nse), much later (19).

The Smc5/6 components have the ability to form several subcomplexes that may induce a conformational change of the complex or mediate distinct functions or interactions. One subcomplex is between Nse1, Nse3, and Nse4. Mutations in the Nse1-Nse3-Nse4 complex have been known to cause DNA damage sensitivity (20). Another subcomplex is formed between Nse5 and Nse6, although these subunits are not very conserved between species. In fission yeast, Nse5 and Nse6 are not even essential for viability (21). Additionally, Nse2 forms a subcomplex with Smc5, and its role in sumoylation will be examined in this research. Although these several subcomplexes are known to form the Smc5/6 complex, whether it interacts with its sumoylation substrates and how it regulates sumoylation are not known (8,15,22,23).

The Smc5/6 complex is known to have a variety of functions. Mutant strains of these *S. cerevisiae* genes were shown to have increased sensitivity to DNA-damaging agents, such as ultraviolet (UV) radiation, infrared radiation (IR), hydroxyurea (HU), and MMS (24,25). Additionally, Nse2 (Mms21 SUMO E3 ligase) mutants also have a hypersensitivity to MMS (26). For this reason, the Smc5/6 complex was presumed to have a role in DNA damage repair.

Since Smc5/6 is recruited to DSBs during the G2 and M phases, the Smc5/6 complex may facilitate sister chromatid cohesion during HR. HR requires homologous sequences to repair DSBs, and the preferred template is an intact sister chromatid, which

are present during the G2 and M phases, but not the G1 phase. Additionally, cells depleted of the Smc5/6 complex showed a decrease in HR, but not in NHEJ which does not require sister chromatids. The Smc1/3 cohesin is also recruited to DSBs and plays a role in holding together sister chromatids. When cells are depleted of both Smc5/6 and Smc1/3, they are susceptible to DNA damaging agents and have a decreased function for DNA damage repair, suggesting that they both play a role in HR (27,28).

Smc5/6 also functions in preserving stalled replication forks (Figure 4). Replication forks stalled at the S phase checkpoint are typically stabilized by the Mad3-Cds1 complex. When Cds1 is absent, fork collapsing occurs, which quickly enlists recombination proteins. In Smc5/6 mutant cells, recombination proteins are still able to be recruited to the collapsed forks, which suggests Smc/6 has a different function in this pathway. Smc5/6 was suggested to play a role in a late stage of HR (Figure 4) to avoid the accumulation of toxic recombination intermediates and to help restart replication by keeping the stalled fork in a recombination preferred conformation that promotes Rad52 association (25).

Additionally, Smc5/6 also restrains Mph1 function during replication fork repair. Mph1 is a helicase in budding yeast and is a homolog of the Fanconi anemia protein FANCM. It functions in an error-free bypass system in certain conditions of replicative stress. Cells that further deplete Mph1 in mutant Smc5/6 background show lower levels of growth defects and the accumulations of DNA intermediates when exposed to DNA damaging agents (29,30). In contrast, when Mph1 is overexpressed, these defects are increased. This suggests that Smc5/6 potentially plays a role in suppressing toxic Mph1 activity (29,30).

While Smc5/6 plays a role in DNA repair, it also facilitates chromosome stability and dynamics in cells that are undamaged. Chromatin immunoprecipitation (ChIP) analysis was used to study the localization of Smc5/6 during a normal cell cycle. It was found that Smc5/6 binds to ribosomal DNA (rDNA), among other things, and actually plays a role in maintaining rDNA integrity (31). rDNA is characterized by highly repetitive sequences and unidirectional replication. Smc5/6 mutants display rDNA nondisjunction phenotypes that arise from incomplete replication before segregation. Smc5/6 is also known to help repair DSBs in rDNA repeats, which occurs outside the nucleolus where recombinational proteins, such as Rad52, are recruited to the rDNA. In Smc5/6 mutants, high rates of extrachromosomal rDNA and recombinational proteins are found inside the nucleolus (32-34).

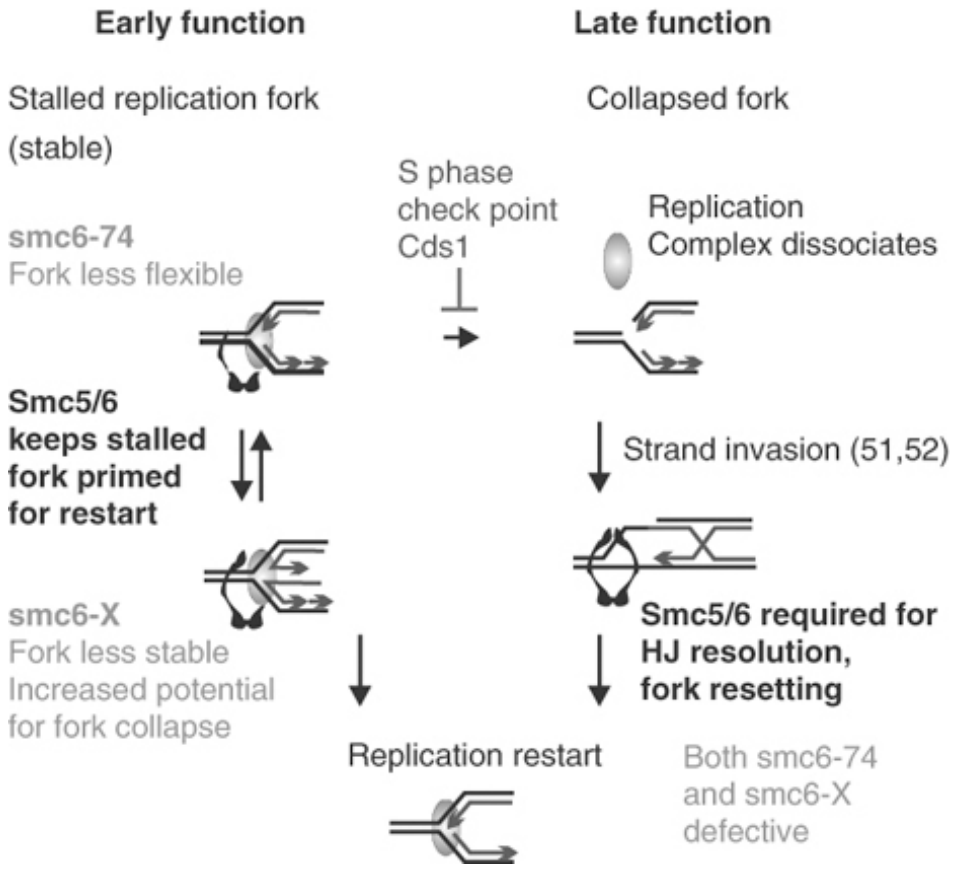

Figure 4: A schematic model for the early and late roles of Smc5/6 in stalled and collapsed forks. Cds1 stabilizes stalled replication forks and Smc5/6 keeps the fork in a conformation that prefers the restoration of replication. Smc5/6 acts during a later stage of recombination at collapsed forks (32).

As previously stated, Nse2 (Mms21) forms a subcomplex with Smc5, and its role in sumoylation will be examined in this research. Mms21 functions as SUMO E3 ligase in the sumoylation of DNA damage repair proteins. Although several subcomplexes are known to form from the Smc5/6 complex, whether it interacts with its sumoylation substrates and how it regulates sumoylation are not known (9,15,22,23).

Sgs1-Top3-Rmi1 complex functions in HR and its function relies on sumoylation modification

The STR complex is known to play many roles in various processes of DNA repair. It is made up of the Sgs1 helicase, Top3 topoisomerase, and Rmi1 cofactor. Sgs1 was originally identified as a suppressor of the slow-growth phenotype of Topoisomerase III (Top3) deletion (35). Sgs1 is a central piece of the HR process, as previously described. Briefly, it works together with Dna2 and Exo1 to promote end resection (36). Sgs1 also promotes active Rad51 homology search and strand invasion, which ultimately leads to D-loop formation (7). Most important to this research, Sgs1 helps to dissolve dHJ structures. Sgs1 helicase unwinds the DNA, while Top3 topoisomerase separates the strands creating non-crossover products. Rmi1 acts as a cofactor to Sgs1 and Top3 and helps to bind to HJ substrates (37).

Sgs1 is a member of the Rec Q family of 3'-5' DNA helicases, and is analogous to BLM in humans. Mutated BLM is associated with Bloom's syndrome, which is an autosomal recessive disorder that has a strong disposition to early onset cancer, as well as the development of multiple cancers (38). Cells lacking Sgs1 have shown to have higher levels of gross chromosomal rearrangements (GCRs) and are sensitive to DNA damaging agents such as methyl methanesulfonate (MMS) (39,40). Sgs1 works together with Top3, analogous to Topoisomerase-IIIα in humans. *S. cerevisiae* cells lacking Top3 have a severe growth defect and are also sensitive to MMS. Most Top3 defects can be suppressed by Sgs1 defects, supporting the model that Sgs1 activity creates toxic intermediates that are corrected by Top3 activity. Rmi1 acts as a cofactor to Sgs1 and Top3, and is analogous to Rmi1 and Rmi2 in humans. Cells lacking Rmi1 have been shown to accumulate DNA damage (41).

Although this research focuses on the sumoylation of the STR complex during dHJ dissolution, the STR complex also relies on its sumoylation during end resection in HR, specifically Sgs1. As previously stated, Sgs1 works with Dna2 and Exo1 endonucleases to facilitate HR end resection. In cells lacking Sgs1 and Exo1 (*exo1Δ sgs1*Δ), end resection is completely abolished. Finally, in cells where the Smc5/6 interaction with Sgs1 is impaired and where Sgs1 sumoylation is defective (*exo1Δ sgs1-SIM1-2*Δ, *exo1Δ sgs1-KR*), resection activity is decreased. This demonstrates that Sgs1 interaction with Smc5/6 and subsequently its sumoylation is necessary to carry out its role in end resection (42).

In regard to sumoylation of the STR complex during dHJ dissolution, it has been shown that when the STR complex is absent, there is an accumulation of DNA intermediates (43). While STR is known to work in conjunction with Smc5/6 and SUMO (Figure 5), the exact details and mechanisms of this interaction remain vastly unknown.

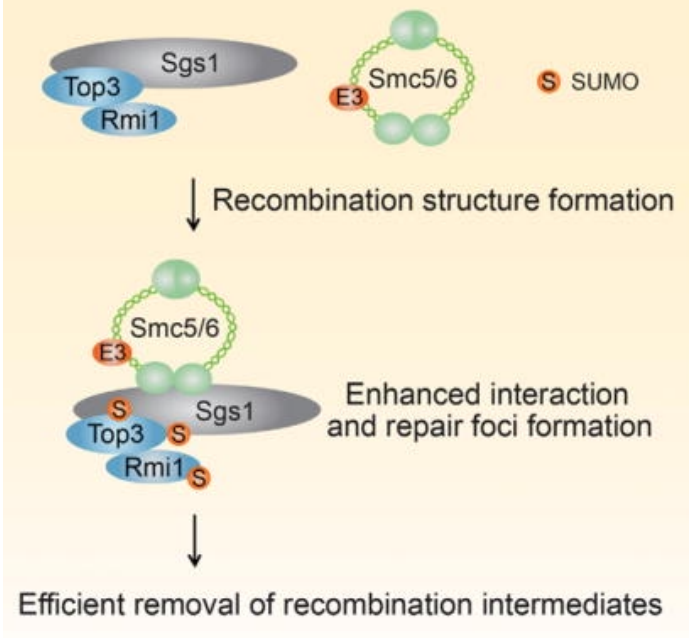

Figure 5: Schematic model of the role of the STR complex in DNA damage repair induced sumoylation. Smc5/6 complex interacts with and facilitates the sumoylation of the STR complex upon the formation of recombinational intermediates. The STR complex aids in the removal of these intermediates through its sumoylation, mediated by the Smc5/6 complex (9).

Esc2 genome stability factor

Budding yeast Esc2 is a scaffold protein functioning in DNA damage repair, but its exact role in sumoylation in unknown. The Esc2 ortholog in fission yeast, Rad60, has shown to be a regulator of SUMO E3, although it is more complex in budding yeast. Preliminary data has shown that mutants of Esc2 cause accumulation of recombinational intermediates such as HJs, proving that Esc2 supports DNA intermediate resolution

similar to Mms21 (44). When Esc2 is absent, cells are sensitive to DNA damaging agents such as MMS.

Interestingly, Esc2 contains two SUMO-like domains: SLD1 and SLD2 (Figure 6). In fission yeast, the SLD2 region is responsible for binding to the Ubc9 SUMO E2, which suggests a potential role for Esc2 in the sumoylation process (45). The budding yeast Esc2 also has a mid-region domain (MR) that is specific for DNA intermediates such as HJ and replication fork structures. *In vivo* data shows that Esc2 preferentially binds to HJ structures over ssDNA or dsDNA. By creating truncated Esc2 mutants, previous studies found that when aa 154-198 were missing, a 10-fold decrease of affinity towards DNA occurred, suggesting that the responsibility of DNA binding was found in this region (46).

The Mus81-Mms4 complex has been shown to work alongside the STR complex in resolving DNA intermediates. However, deletion of the Mus81-Mms4 complex does not attribute to a large amount of DNA intermediate accumulation, suggesting that its role may be limited (47,48). Previous studies show that Esc2 directly interacts with the Mus81-Mms4 complex and actually specifically enhances the activity of the complex in the resolution of DNA intermediates (49).

As previously stated, PCNA plays a pivotal role in DNA replication and damage repair, although its mechanism is not fully understood. PCNA is a ring-shaped complex that binds to DNA and enhances DNA synthesis by recruiting certain proteins such as DNA helicase Srs2 to the replication fork when sumoylated. This inhibits any unwanted recombination and promotes bypass synthesis. Esc2 can also interact with Srs2 through

the Srs2-SIM (sumo-interacting motif) domain, which allows it to interact with stalled replication forks and control the level of Srs2 (11).

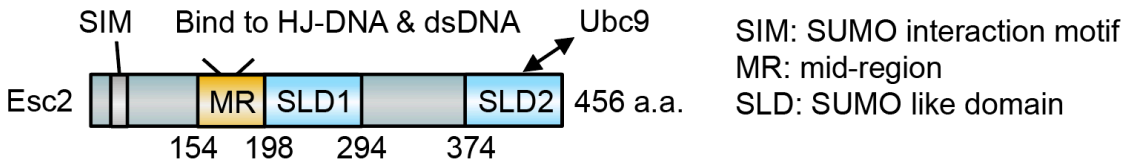

Figure 6: Schematic model of Esc2 protein domains SLD1, SLD2, and the MR domain. The SLD2 domain is responsible for binding to Ubc9 SUMO E2, while the MR is responsible for binding to DNA structures (50).

Summary

Protein sumoylation, as well as other post-translational modifications, are known to work in many DNA damage repair pathways. Most processes have been widely studied; however, knowledge of the role and mechanism of sumoylation of the STR complex in the dissolution of HJ mediated by the Smc5/6 complex still contains many gaps.

Our studies focus on genome maintenance proteins, such as genome stability factor Esc2 found in budding yeast and its genetic interaction with Smc5/6. While its involvement in sumoylation has been observed (51), its specific role and mechanism has yet to be established. Since depletion of Esc2 causes cells to be sensitive to DNA damaging agent MMS similar to that of Sgs1 depletion or Smc5/6 mutation, we

hypothesize that Esc2 will work together with STR and Smc5/6 in removing recombinational intermediates. This research seeks to further investigate the mechanism in which Esc2 acts to remove recombinational intermediates in conjunction with the Smc5/6 complex and the STR complex. This dissertation also examines the direct or indirect role of DNA intermediate structures, such as HJ or dsDNA, in STR sumoylation.

In this research, we investigate the role of Esc2 and its roles in DNA-damage induced sumoylation. We found that Esc2 specifically influences the Mms21 substrates associated with HJs or replication fork structures. Our results also show that Esc2 function in sumoylation is mediated by its SLD2 domain that interacts with Ubc9, and that this interaction is essential in the dissolution of HJ and genome stability. We suggest that Esc2 is a SUMO E2 cofactor that facilitates the sumoylation of Mms21 substrates in DNA intermediate dissolution. We also investigate how STR sumoylation is regulated and how Esc2 and DNA intermediates facilitate this process. We found that Sgs1 binding to DNA promotes its sumoylation, and is enhanced when Esc2 is present. Through generating Esc2 mutants that are defective in DNA binding, our results show that the MR of Esc2 does contribute to Sgs1 sumoylation, but does so through a DNA-independent manner, suggesting that it may play other roles in STR sumoylation. Ultimately, our data shows that Esc2, along with the STR complex and Smc5/6 complex, does promote the dissolution of DNA intermediates and helps to preserve genome integrity.

Materials science and protein sumoylation

Since Watson and Crick's revelation of the double helix structure of DNA in the 1950s, DNA has been at the center of biotechnological research, including molecular

biology and materials science. It is arguably one of the most important biological materials because it houses all of our genetic information and is one of the oldest naturally-occurring polymers. Its structural features can be utilized in many aspects of materials science. For example, DNA with a thiol modification can be used to bind to gold nanoparticles into aggregates that have useful optical and material properties that can be used for chemical sensors or spectroscopic enhancers. The addition of DNA allows for the aggregate to self-assemble and makes it possible to control, whereas synthesizing aggregates without DNA was very hard to control (52).

Precise copying of DNA is very important, otherwise it can lead to DNA damages. Exogenous factors such as ionizing radiation, ultraviolet radiation, carcinogens, and many endogenous factors can also lead to DNA damage in the polymer network (2, 53). Also, engineered nanomaterials that are included in commonly used items, such as cosmetics, deodorants, and even toothpastes, have been linked to DNA damage (54). The inability to identify and control these complications can lead to more problems at the cellular level like replication fork stalling, genome instability, and even chromosomal rearrangements leading to cancer and other diseases. There are many different DNA damage repair pathways, each requiring tight regulation to ensure timely activation and inhibition. One of these repair pathways involves post-translational modifications via sumoylation. While fundamental DNA damage repair pathways are widely studied, there are still many unanswered questions regarding the regulation of sumoylation.

In addition to DNA, the modification of proteins in these experiments places this research in the realm of materials science. The basis of materials science is to understand the relationships between the properties of a material and how they influence the

structure and performance of that material. According to the Texas State MSEC Student Handbook, the purpose of the characterization of a material is for "using, changing, or enhancing inherent properties to create or improve end products." Proteins are polypeptides composed of amino acids and play many roles in the function of biological systems. In this research, these proteins undergo a post-translational modification known as sumoylation. Sumoylation is the polymeric addition of SUMO molecules for the purpose of regulating and managing certain processes, specifically to this research, HR and the dissolution of DNA intermediates. In addition, polypeptide sequence of various proteins involved in DNA damage repair are mutated to study their effect on the sumoylation pathway. These mutations and their effect on the pathway will hopefully shed light on the function of some of these proteins that remain largely unknown. In doing so, DNA damage repair, and ultimately the replication and synthesis of DNA, might be more understood.

It is known that there are many post-translational modifications that occur in proteins to alter cellular functions including ubiquitination, glycosylation, phosphorylation, sumoylation, and others (55). This research to better understand the sumoylation process may by applicable across many different scientific fields, including the healthcare field.

Recently, studies have shown that expression of human SUMO E1, SUMO E2, and SUMO E3s is enhanced in certain cancers. For example, Ubc9 levels are increased in ovarian cancer, whereas PIAS3 (human SUMO E3) levels are increased in breast, prostate, and lung cancer (56). Additionally, sumoylation has been associated with

cardiac disease (57-59), neurodegenerative disease (60-63), and regulating immune responses (64-67).

In this research, poly-sumoylated proteins (material) were analyzed in their function to remove HJ intermediates in cells. In addition, different structured DNA, a kind of polymer structure, were analyzed for their ability to stimulate certain protein sumoylation both *in vitro* and in cells. In the future, it will be interesting to further analyze how protein sumoylation alters its function, and how DNA or other engineered materials will affect protein sumoylation in cells.

The versatility of sumoylation across many different fields and knowledge of the sumoylation process has the potential to facilitate advancement in these fields, with one of the most important being drug-target design. The sumoylation pathway and its enzymes have been studied as a potential target to clinical anti-cancer therapeutics (68). This study on the mechanism of sumoylation enzymes and how they interact with each other may also help with understanding the mechanisms of certain diseases, and have the potential to make a large impact in the world of material science.

II. MATERIALS AND METHODS

Yeast strains and plasmids

All yeast strains are derivatives of W1588-4C, a *RAD5* derivative of W303 (*MATa ade2-1 can1-100 ura3-1 his3-11,15 leu2-3,112 trp1-1 rad5-535*). Standard procedures were used for cell growth, media preparation, epitope tagging at endogenous loci, and spot assays. Yeast strains and plasmids used are listed in Tables 1 and 2, respectively.

Table 1: Yeast strains used in this study.

Name	**Genotype**	**Source**
X7559-8A	*SGS1-9myc::KAN 8His-SMT3::TRP1 ESC2-10FLAG::KAN*	Li et al. 2021
X7704-5A	*SGS1-9myc::KAN 8His-SMT3::TRP1 esc2Δ::KAN*	Li et al. 2021
X7555-5B	*TOP3-TAP::HIS3 8His-SMT3::TRP1 ESC2-10FLAG::KAN*	This study
X7705-1C	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X7556-13B	*RMI1-TAP::HIS3 8His-SMT3::TRP1 ESC2-10FLAG::KAN*	This study

X7706-11B	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X7816-2A	*POL2-3HA::KAN ESC2-10FLAG::KAN*	This study
X8357-3B	*POL2-3HA::KAN esc2Δ::KAN*	This study
X8374-1C	*ADH-3HA-MCM3::NAT 8His-SMT3::TRP1*	This study
X8374-4A	*ADH-3HA-MCM3::NAT 8His-SMT3::TRP1; esc2Δ::KAN*	This study
T439-2	*YKU70-TAF::KAN*	Lab collection
X7710-5A	*YKU70-TAF::KAN esc2Δ::KAN*	This study
X4641-2A	*SMC5-TAP::TRP1 8His-SMT3::TRP1*	This study
X7501-5C	*SMC5-TAP::TRP1 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X8341-3B	*SMC6-13Myc::HIS3 8His-SMT3::TRP1*	This study
X8341-4A	*SMC6-13Myc::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X7708-3A	*NSE4-13myc::HIS3 8His-SMT3::TRP1*	This study
X7708-3D	*NSE4-13myc::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study

X7709-2C	*SMC1-TAP::HIS3 8His-SMT3::TRP1*	This study
X7709-2A	*SMC1-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X7591-2A	*SMC3-TAP::HIS3 8His-SMT3::TRP1*	This study
X7591-8A	*SMC3-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X8430-3D	*SMC2-3HA::KAN 8His-SMT3::TRP1*	This study
X8430-1C	*SMC2-3HA::KAN 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X7592-1C	*SMC4-myc::HIS3 8His-SMT3::TRP1*	This study
X7592-8D	*SMC4-myc::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X5602-10C	*SGS1-3HA::LEU2 ESC2-13myc::KAN*	This study
X5602-7C	*SGS1-3HA::LEU2 ESC2-13myc::KAN SMC5-TAP::TRP1*	This study
X5766-8A	*SGS1-3HA::KAN*	This study
X5766-8B	*SGS1-3HA::KAN SMC5-TAP::TRP1*	This study
X5573-22A	*SGS1-3HA::LEU2 SMC5-TAP::TRP1 esc2Δ::KAN*	This study

X7561-1A	*SGS1-9myc::KAN 8His-SMT3::TRP1 esc2-SLD1m-10FLAG::KAN*	This study
X7562-5A	*SGS1-9myc::KAN 8His-SMT3::TRP1 esc2-SLD2m-10FLAG::KAN*	This study
X7551-4A	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2-SLD1m-10FLAG::KAN*	This study
X7553-2C	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2-SLD2m-10FLAG::KAN*	This study
X7557-3B	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2-SLD1m-10FLAG::KAN*	This study
X7558-5C	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2-SLD2m-10FLAG::KAN*	This study
X8356-14C	*POL2-3HA::KAN esc2-SLD1m-10FLAG::KAN*	This study
X7566-1D	*POL2-3HA::KAN esc2-SLD2m-10FLAG::KAN*	This study
X8439-1C	*ADH-3HA-MCM3-HA::NAT 8His-SMT3::TRP1 esc2- SLD1m::KAN*	This study

X8440-4A	*ADH-3HA-MCM3-HA::NAT 8His-SMT3::TRP1 esc2-SLD2m- 10FLAG::KAN*	This study
X8393-16C	*SGS1-9myc::KAN 8His-SMT3::TRP1 ESC2-SLD2Δ-SUMO- 10FLAG::KAN*	This study
X8433-2-13C	*SGS1-9myc::KAN 8His-SMT3::TRP1 ESC2-SLD2Δ-SUMO- D68R-10FLAG::KAN*	This study
X8409-13A	*TOP3-TAP::HIS3 8His-SMT3::TRP1 ESC2-SLD2Δ-SUMO- 10FLAG::KAN*	This study
X8435-7B	*TOP3-TAP::HIS3 8His-SMT3::TRP1 ESC2-SLD2Δ-SUMO- D68R-10FLAG::KAN*	This study
X8394-6A	*RMI1-TAP::HIS3 8His-SMT3::TRP1 ESC2-SLD2Δ-SUMO- 10FLAG::KAN*	This study
X8434-3A	*RMI1-TAP::HIS3 8His-SMT3::TRP1 ESC2-SLD2Δ-SUMO- D68R-10FLAG::KAN*	This study
X8021-2-2C	*mms4Δ::KAN*	This study
X8021-2-2D	*esc2-SLD2m-10FLAG::KAN*	This study
X8021-2-2B	*mms4Δ::KAN esc2-SLD2m-10FLAG::KAN*	This study

X8009-9D	*slx4Δ::NAT*	This study
X8009-9B	*slx4Δ::NAT esc2-SLD2m-10FLAG::KAN*	This study
X7768-2C	*YEN1-TAP::HIS3 8His-SMT3::TRP1*	This study
X7768-14D	*YEN1-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	This study
X7549-10B	*SAW1-TAP::HIS3*	This study
X7549-3C	*SAW1-TAP::HIS3 esc2Δ::KAN*	This study
X7556-9D	*ESC2-10FLAG::KAN*	This study
X7816-2A	*ESC2-10FLAG::KAN POL2-3HA::KAN*	This study
X3598-15d	*SMC5-myc::HIS3 POL2-3HA::KAN*	Lab collection
X8275-1B	*SMC5-myc::HIS3 POL2-3HA::KAN esc2Δ::KAN*	This study
X5706-5-11D	*SMC5-myc::HIS3*	This study
X8424-2-13B	*ESC2-SLD2Δ-SUMO-10FLAG::KAN*	This study
X8424-2-13C	*mms4Δ::KAN ESC2-SLD2Δ-SUMO-10FLAG::KAN*	This study
X8470-13A	*ESC2-SLD2Δ-SUMO-D68R-10FLAG::KAN*	This study

X8470-13B	*mms4Δ::KAN ESC2-SLD2Δ-SUMO-D68R-10FLAG::KAN*	This study
X8471-14A	*slx4Δ::NAT ESC2-SLD2Δ-SUMO-10FLAG::KAN*	This study
X8472-14B	*slx4Δ::NAT ESC2-SLD2Δ-SUMO-D68R-10FLAG::KAN*	This study
X7782-2D	*SGS1-9myc::KAN 8His-SMT3::TRP1 esc2-Δ154-198aa-10FLAG::KAN*	This study
X8391-8C	*SGS1-9myc::KAN 8His-SMT3::TRP1 esc2-5E-10FLAG::KAN*	This study
X7555-5B	*TOP3-TAP::HIS3 8His-SMT3::TRP1 ESC2-10FLAG::KAN*	Li et al. 2021
X7705-1C	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	Li et al. 2021
X7783-2B	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2-Δ154-198aa-10FLAG::KAN*	This study
X8408-7B	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2-5E-10FLAG::KAN*	This study

X7556-13B	*RMI1-TAP::HIS3 8His-SMT3::TRP1 ESC2-10FLAG::KAN*	Li et al. 2021
X7706-11B	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2Δ::KAN*	Li et al. 2021
X7784-3D	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2-Δ154-198aa-10FLAG::KAN*	This study
X8392-1C	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2-5E-10FLAG::KAN*	This study
X7556-9D	*ESC2-10FLAG::KAN*	Li et al. 2021
X8020-2-11C	*esc2Δ::KAN*	This study
X8022-2-10A	*esc2-Δ154-198aa-10FLAG::KAN*	This study
X8423-5D	*esc2-5E-10FLAG::KAN*	This study
X8021-2-7D	*esc2-SLD2m-10FLAG::KAN*	This study
X8448-12D	*esc2-SLD2m-5E-10FLAG::KAN*	This study
X8423-4C	*mms4Δ::KAN*	This study
X8019-12A	*mms4Δ::KAN ESC2-10FLAG::KAN*	This study

X8020-9A	*mms4Δ::KAN esc2Δ::KAN*	This study
X8022-8B	*mms4Δ::KAN esc2-Δ154-198aa-10FLAG::KAN*	This study
X8423-4B	*mms4Δ::KAN esc2-5E-10FLAG::KAN*	This study
X8021-14A	*mms4Δ::KAN esc2-SLD2m-10FLAG::KAN*	This study
X8448-12C	*mms4Δ::KAN esc2-SLD2m-5E-10FLAG::KAN*	This study
T2199-8	*esc2-sim-10FLAG::KAN*	This study
X8806-19A	*mms4Δ::KAN esc2-sim-10FLAG::KAN*	This study
X8614-1A	*SGS1-9myc::KAN 8His-SMT3::TRP1 esc2-sim-10FLAG::KAN*	This study
X8603-6C	*TOP3-TAP::HIS3 8His-SMT3::TRP1 esc2-sim-10FLAG::KAN*	This study
X8602-1B	*RMI1-TAP::HIS3 8His-SMT3::TRP1 esc2-sim-10FLAG::KAN*	This study
X8890-16B	*mms4Δ::KAN esc2-5E-10FLAG::KAN rad51Δ::LEU2*	This study
X8888-3B	*mms4Δ::KAN esc2Δ::KAN rad51Δ::LEU2*	This study

Table 2: Plasmids used in this study.

Name	Vector information	Source
	pOAD	Lab collection
pXZ170	pOAD-Nse1	Lab collection
pXZ89	pOAD-Mms21	Lab collection
p2	pOAD-Nse3	Lab collection
pXZ212	pOAD-Nse4	Lab collection
pXZ188	pOAD-Nse5	Lab collection
pXZ166	pOAD-Nse6	Lab collection
pXZ189	pOAD-Smc5	Lab collection
pXZ171	pOAD-Smc6	Lab collection
pXZ217	pOAD-Sgs1	Lab collection
pXZ549	pOAD-Top3	Lab collection
pXZ558	pOAD-Rmi1	Lab collection

pXZ93	pOAD-Ubc9	Lab collection
pXZ220	pOAD-Smt3	Lab collection
	pOBD	Lab collection
pXZ434	pOBD-Esc2	Lab collection
pXZ890	pOBD-Esc2-SLD1m	This study
pXZ891	pOBD-Esc2-SLD2m	This study
	pGEX-6P-1-Esc2	Sebesta et al. 2017
	pGEX-6P-1-Esc2-SLD2m	This study
	pGEX-6P-1-Esc2-SLD2Δ-Su	This study
	pGEX-6P-1-Esc2-SLD2Δ-SuDR	This study
	pFastBac-HTB-Flag-Sgs1	Niu et al. 2010
pLK79	pET11c-V5-Top3	Niu et al. 2010
	pGEX-6P-Rmi1	Niu et al. 2010
pXZ114	pET15b-Mms21	Duan et al. 2009

pXZ115	pET28a-Smc5	Duan et al. 2009
p588	pET28a-4xSmt3	Gillies et al. 2016
	pESC-Trp-Myc-Smc5	Niu et al. 2010
	2μ-His_9-Strep-Tactin-Smc6	Niu et al. 2010
pXZ998	pRSFDuet-GST-Aos1	Zhao and Blobel 2005
pXZ999	pET22-Uba2	Zhao and Blobel 2005
p541	pET21a-Smt3	Lab collection
pXZ893	pET21a-Smt3-D68R	This study
G1827	pET-Ubc9	Lab collection
	pGEX-6p-1-Esc2-MRΔ	This study
	pGEX-6p-1-Esc2-5E	This study
G1371	pRSF-Nse5	This study
Y140	pGEX-6P-1-Nse6	This study

DNA substrates

The HJ and dsDNA substrates were made by annealing the 80-mer oligos listed in Table 3 as pictured in Figure 8. The annealed substrates were then gel purified and concentrated in TE buffer (10 mM Tris-Cl, 1 mM EDTA, ph 8.0). For DNA mobility shift assays, one of these 80-mer oligos was ^{32}P-labeled, and the substrates were made following the same procedure.

^{32}P labeling of DNA oligos

The 5'-radiolabeling of oligos was carried out as follows. Briefly, a total of 5 µg of H3 (Table 3), and 5 µL of radiolabeled γ-ATP (Perkin Elmer) were incubated with T4 polynucleotide kinase (40 units) in 1X PNK Buffer (70 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT) at 37°C for 1.5 h. The kinase catalyzes the transfer of the radiolabeled phosphate from ATP to the 5'-hydroxyl terminus of the oligo. Next, the temperature is increased to 65°C to deactivate the kinase. Labeled oligos are purified using a Micro Bio-Spin P6 column (Bio-Rad).

Table 3: Exact sequences of oligos used to generate HJ and dsDNA substrates.

Oligo sequences	
H3	5'-TTGATAAGAGGTCATTTGAATTCATGGCTTAGAGCTTAATTGCTGAATCTGGTGCT GGGATCCAACATGTTTTAAATATG-3'
H4	5'-CATATTTAAAACATGTTGGATCCCAGCACCAGATTCAGCAATTAAGCTCTAAGCCA TGAATTCAAATGACCTCTTATCAA-3'
H5	5'-CATATTTAAAACATGTTGGATCCCAGCACCAGATTCAGCATACGTTACCGATCGTA CGTTCGATGCTGGCTACTGCTAGC-3'
H7	5'-GCTAGCAGTAGCCAGCATCGAACGTACGATCGGTAACGTAGTCGATTATCGAGAT CAAGCTAGCATAGCCATAGCGCGAC-3'
H8	5'-GTCGCGCTATGGCTATGCTAGCTTGATCTCGATAATCGACATTAAGCTCTAAGCCA TGAATTCAAATGACCTCTTATCAA-3'

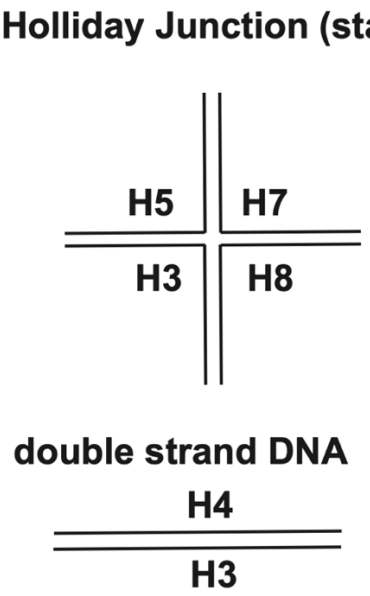

Figure 7: Schematic of HJ and dsDNA substrates made from oligos previously listed.

Protein expression and purification

Expression and purification of most recombinant proteins used in *in vitro* analysis were carried out following previously published protocols. These include Flag-Sgs1 (69), V5-Top3/GST-Rmi1 (70), Mms21/Smc5 complex (71), Myc-Smc5/His9-Strep-Tactin-Smc6 complex (72), Esc2 and its mutants (46), Smt3, Smt3-D68R, Ubc9, Aos1-Uba2 (19), and 4XSmt3 (73). STR complex was assembled *in vitro* using 1:1 mol ratio of purified Flag-Sgs1 and V5-Top3/GST-Rmi1 complex.

Briefly, A FLAG tag was inserted after the $(His)_6$ tag in the pFastBac-HTB vector (Invitrogen) and the Sgs1 coding sequence was subsequently cloned in frame 5' to these tag sequences. A bacmid was generated in the E. coli strain DH10Bac (Invitrogen) and used to transfect insect cells to obtain and amplify a baculovirus. Tagged Sgs1 expression was done in High Five (Invitrogen) insect cells. Sgs1 purification began by lysing cells in extraction buffer (K buffer containing 300 mM KCl, 1 4x-protease inhibitor tablet, 1 mM PMSF). Lysate was incubated with 2 mL of Anti-Flag M2 affinity gel (Sigma-Aldrich), washed with washing buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 500 mM KCl), and eluted with elution buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 500 mM KCl) containing 200 µg/mL 3x FLAG peptide (Apex Bio Technology). The eluate containing Sgs1 was incubated with 2 mL of Ni Sepharose 6 Fast Flow (GE Healthcare), washed with washing buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 500 mM KCl), an elution buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 500 mM KCl) containing 200 mM imidazole. Peak fractions were pooled and concentrated in an Ultracel-10K concentrator (Amicon) before storing at -80°C.

Many Esc2 variants are used in this research, and are summarized in Table 4.

Table 4: Summary of Esc2 variants and their mutations.

Esc2 variant	**Mutations**
Esc2-SLD1m	D268A, I287Y
Esc2-SLD2m	D447A, D449A
Esc2-SLD2Δ-Su	SLD2 domain replaced with SUMO moiety
Esc2-SLD2Δ-SuDR	SLD2 domain replaced with SUMO moiety containing D68R mutation
Esc2-MRΔ	MR truncated (154aa – 198aa)
Esc2-5E	K179E, K182E, K183E, K197E, R198E

Briefly, coding sequence of Esc2 and its mutants was synthesized and cloned into the pGEX-6p-1 vector. Esc2 proteins were expressed in Rossetta cells. Cells were grown at 37 °C to OD_{600} = 0.8, and protein expression was induced by the addition of 0.2 mM IPTG at 16 °C for 16 h. Esc2 purification began by lysing cells with extraction buffer. Lysate was incubated with Q Sepharose fast flow resin (GE Healthcare) and connected to an AKTA pure system for elution. The column was washed with washing buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT) containing 150 mM KCl and developed with a 25-mL gradient of 150–650 mM KCl. Pooled samples were incubated with Glutathione Sepharose 4B resin (GE Healthcare), washed with washing buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01%

Igepal, 1 mM DTT, 300 mM KCl), and eluted using elution buffer (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT, 300 mM KCl) containing 10 mM reduced glutathione. Finally, the pooled samples were separated into two halves. One half of the GST-Esc2 sample was loaded onto a Mono Q column (Mono Q 5/50 GL, Sigma-Aldrich) for elution. Peak fractions were pooled and concentrated in an Ultracel-10K concentrator before storing at -80°C. The other half of the GST-Esc2 sample was incubated with 100-200 µg PreScission protease (Xue lab stock) at 4 °C overnight, to cleave the GST off. Next, the sample was diluted with buffer K (20 mM KH_2PO_4, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT) and loaded onto a 1-ml Mono Q column (GE Healthcare) equilibrated in buffer K containing 100 mM KCl. Esc2 were eluted with a 20-ml gradient of 100–500 mM KCl in buffer K. Peak fractions were pooled and concentrated in an Ultracel-10K concentrator before storing at -80°C.

GST pull-down assay with Esc2, Ubc9, SUMO, and SUMO chain

The interaction between Esc2 and Ubc9 was done by performing a GST pull-down assay. 2.2 µM of GST-tagged Esc2 was incubated with 3.6 µM of Ubc9 in 30 µL of T buffer (25 mM Tris-Cl at pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.01% Igepal, 1 mM DTT) supplemented with 80 mM KCl for 30 minutes at 4°C. The protein mixture was incubated with 10 µL of Glutathione Sepharose 4B resin (GE Healthcare) for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 µL of T buffer with 80 mM KCl. Finally, the bound proteins were eluted with 20 µL of 2% SDS. Samples from the supernatant (S), wash (W), and eluate (E) were analyzed by SDS-PAGE.

To evaluate Esc2 interaction with SUMO or SUMO chain, the process is nearly identical. 2.2 μM of GST-tagged Esc2 was incubated with 5.7 μM of Smt3 or 4X-Smt3 in 30 μL of T buffer supplemented with 80 mM KCl for 30 minutes at 4°C. The protein mixture was incubated with 10 μL of Glutathione Sepharose 4B resin for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 μL of T buffer with 80 mM KCl. Finally, the bound proteins were eluted with 20 μL of 2% SDS. Samples from the supernatant (S), wash (W), and eluate (E) were analyzed by SDS-PAGE.

GST pull-down assay with Esc2, Esc2-SLD2m and Ubc9

The binding preference of SLD2 domain to Ubc9 were examined using a GST pull-down assay that utilized both wildtype-Esc2 and a version of Esc2 with a mutation in the SLD2 domain (Esc2-SLD2m) that would be defective for Ubc9 interaction. The Esc2-SLD2m mutant was designed by structural analysis of a SUMO-Ubc9 complex (74). 2.2 μM of GST-tagged Esc2 or GST-tagged Esc2-SLD2m was incubated with 3.6 μM of Ubc9 in 30 μL of T buffer supplemented with 80 mM KCl for 30 minutes at 4°C. The protein mixture was incubated with 10 μL of Glutathione Sepharose 4B resin for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 μL of T buffer with 80 mM KCl. Finally, the bound proteins were eluted with 20 μL of 2% SDS. Samples from the supernatant (S), wash (W), and eluate (E) were analyzed by SDS-PAGE.

Sumoylation assay with Smt3, Smt3-D68R, and Mms21/Smc5

An *in vitro* sumoylation assay was establishd to examine the effect of Mms21/Smc5 in STR sumoylation. 20 nM of the STR complex was incubated with 2.2

μM Smt3 (or Smt3-D68R), 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 40 nM Mms21/Smc5 (E3) in buffer R (Hepes-Na (pH 7.0), 5 mM $MgCl_2$, 72 mM KCl, and 0.1 mM DTT) for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 5 minutes, 25 minutes, and 60 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag (Sigma-Aldrich A8592) and anti-CBP antibodies (Santa Cruz Biotechnology SC33000).

Sumoylation assay with Esc2, Esc2-SLD2m, and Ubc9

Next, Esc2 and Esc2-SLD2m was used to stimulate *in vitro* sumoylation and to confirm the role of the SLD2 Ubc9 binding site. 20 nM of the STR complex was incubated with 2.2 μM Smt3 (or Smt3-D68R), 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), 40 nM Mms21/Smc5 (E3), with or without 300 mM Esc2 (or Esc2-SLD2m) in buffer R for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 1 minutes, 5 minutes, 25 minutes, and 60 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag and anti-V5 antibodies (Rockland 600-401-378).

GST pull-down assay with Esc2, Esc2-SLD2Δ-Su, Esc2-SLD2Δ-SuDR, and Ubc9

To test the binding affinity of Ubc9 with Esc2-SLD2Δ-Su and Esc2-SLD2Δ-SuDR, a GST-pull-down assay was performed. 2.2 μM of GST-tagged Esc2 (or Esc2-SLD2Δ-Su, Esc2-SLD2Δ-SuDR) was incubated with 3.6 μM of Ubc9 in 30 μL of T buffer supplemented with 80 mM KCl for 30 minutes at 4°C. The protein mixture was

incubated with 10 µL of Glutathione Sepharose 4B resin for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 µL of T buffer with 80 mM KCl. Finally, the bound proteins were eluted with 20 µL of 2% SDS. Samples from the supernatant (S), wash (W), and eluate (E) were analyzed by SDS-PAGE.

Sumoylation assay with Esc2, Esc2-SLD2Δ-Su, Esc2-SLD2Δ-SuDR

Finally, to evaluate the effect of Esc2-SLD2Δ-Su and Esc2-SLD2Δ-SuDR on STR sumoylation, a sumoylation assay was performed. 20 nM of the STR complex was incubated with 2.2 µM Smt3 (or Smt3-D68R), 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), 40 nM Mms21/Smc5 (E3), with or without 300 mM Esc2 (or Esc2-SLD2Δ-Su, Esc2-SLD2Δ-SuDR) in buffer R for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 10 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag and anti-CBP antibodies.

Gross chromosomal rearrangement assay

Cells were washed and serial dilutions were plated on synthetic complete (SC) medium and FC medium containing canavanine and 5-FOA. At least nine cultures were examined for each genotype. GCR rates were calculated as m/NT using the following formula: $m \times (1.24 + \ln[m]) - NFC = 0$, where m is mutational events, NFC is the number of colonies on FC plates, and NT is the number of colonies on SC plates. The upper and lower 95% confidence intervals (95% CI) were calculated as described (75). A two-tailed

Mann-Whitney test was performed as described previously (76) using GraphPad Prism version 7.

Sumoylation assays with HJ and Mms21/Smc5

To test if HJs could stimulate STR sumoylation in the presence or absence of HJ or SUMO E3, a sumoylation assay was performed. 20 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 40nM HJ, with or without 25 nM Mms21/Smc5 (E3) in buffer $R^{low\ salt}$ (45 mM Hepes-Na (pH 7.0), 5 mM $MgCl_2$, 65 mM KCl, and 0.1 mM DTT) for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 5 minutes, 25 minutes, and 60 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag and anti-V5 antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

To test the additive effects of HJ and SUMO E3, a sumoylation assay was performed. 20 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 40 nM HJ, with or without 25 nM Mms21/Smc5 (E3) in buffer $R^{low\ salt}$ for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag and anti-V5 antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

DNA mobility shift assay with STR complex, HJ, and dsDNA

To confirm interaction between STR complex and DNA (HJ or dsDNA), a DNA mobility shift assay was performed. 5-40 nM STR was incubated with 5 nM radiolabeled HJ or dsDNA at 30°C for 10 min in 10 µL buffer D (35 mM Tris-HCl (pH 7.5), 1 mM DTT, 100 µg/ml BSA, 5 mM $MgCl_2$, and 130. mM KCl). The reaction mixtures were mixed with DNA loading buffer, and the resulting mixtures were then resolved in 6.5% polyacrylamide gels in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Gels were dried onto Whatman DE81 paper (Whatman International Limited) and analyzed in a Typhoon 5 system (Cytiva). The gels were further quantified by ImageQuant (GE Healthcare).

Sumoylation assay with dsDNA and Mms21/Smc5

To test the additive effects of dsDNA and SUMO E3, a sumoylation assay was performed. 20 nM of the STR complex was incubated with 2.2 µM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 40 nM dsDNA, with or without 25 nM Mms21/Smc5 (E3) in buffer $R^{low\ salt}$ for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

Sumoylation assay with Esc2 and HJ

A sumoylation assay was performed to test the additive effects of Esc2 and HJ on Sgs1 sumoylation. To better quantify the influence of Esc2 on Sgs1 sumoylation, higher salt concentration was used to increase the stringency of the sumoylation reaction. 30 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 300 nM Esc2, with or without 40 nM HJ in buffer $R^{high\ salt}$ (45 mM Hepes-Na (pH 7.0), 5 mM $MgCl_2$, 80 mM KCl, and 0.1 mM DTT) for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

Sumoylation assay with Esc2 and dsDNA

A sumoylation assay was performed to test the additive effects of Esc2 and dsDNA on Sgs1 sumoylation. 30 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 300 nM Esc2, with or without 40 nM dsDNA in buffer $R^{high\ salt}$ for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

DNA mobility shift assays of Esc2, Esc2-5E, Esc2- MRΔ with HJ, and dsDNA

To test the preference of Esc2-WT to either HJ or dsDNA, a DNA mobility shift assay was performed. 5-80 nM Esc2-WT was incubated with 5 nM radiolabeled HJ or dsDNA at 30°C for 10 min in 10 μL buffer D (35 mM Tris-HCl (pH 7.5), 1 mM DTT, 100 μg/ml BSA, 5 mM $MgCl_2$, and 130. mM KCl). The reaction mixtures were mixed with DNA loading buffer, and the resulting mixtures were then resolved in 7% polyacrylamide gels in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Gels were dried onto Whatman DE81 paper (Whatman International Limited) and analyzed in a Typhoon 5 system (Cytiva). The gels were further quantified by ImageQuant (GE Healthcare).

To test the preference of Esc2-5E to either HJ or dsDNA, a DNA mobility shift assay was performed. 5-80 nM Esc2-5E was incubated with 5 nM radiolabeled HJ or dsDNA at 30°C for 10 min in 10 μL buffer D (35 mM Tris-HCl (pH 7.5), 1 mM DTT, 100 μg/ml BSA, 5 mM $MgCl_2$, and 130. mM KCl). The reaction mixtures were mixed with DNA loading buffer, and the resulting mixtures were then resolved in 7% polyacrylamide gels in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Gels were dried onto Whatman DE81 paper (Whatman International Limited) and analyzed in a Typhoon 5 system (Cytiva). The gels were further quantified by ImageQuant (GE Healthcare).

To test the DNA binding ability of mutant Esc2-MRΔ, a DNA mobility shift assay was performed. 5-80 nM Esc2-MRΔ was incubated with 5 nM radiolabeled HJ or dsDNA at 30°C for 10 min in 10 μL buffer D (35 mM Tris-HCl (pH 7.5), 1 mM DTT, 100 μg/ml BSA, 5 mM $MgCl_2$, and 130. mM KCl). The reaction mixtures were mixed with DNA

loading buffer, and the resulting mixtures were then resolved in 7% polyacrylamide gels in TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Gels were dried onto Whatman DE81 paper (Whatman International Limited) and analyzed in a Typhoon 5 system (Cytiva). The gels were further quantified by ImageQuant (GE Healthcare).

Sumoylation assay with Esc2-5E and HJ

A sumoylation assay was done to test the effect of the Esc2-5E mutant on Sgs1 sumoylation in the presence of HJ. 30 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 300 nM Esc2-WT (or Esc2-5E), with or without 40 nM HJ in buffer $R^{high\ salt}$ for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

Sumoylation assay with Esc-5E and dsDNA

A sumoylation assay was done to test the effect of the Esc2-5E mutant on Sgs1 sumoylation in the presence of dsDNA. 30 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 300 nM Esc2-WT (or Esc2-5E), with or without 40 nM dsDNA in buffer $R^{high\ salt}$ for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15

minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

Sumoylation assays with Esc2, Esc2-5E, Esc2-SLD2m, and Esc2-MRΔ

Sumoylation assays were performed that compared the effect of Esc2, Esc2-5E, Esc2-SLD2m, and Esc2-MRΔ with no DNA present. 30 nM of the STR complex was incubated with 2.2 μM Smt3-D68R, 50 nM Aos1-Uba1 (E1), and 280 nM Ubc9 (E2), with or without 300 nM Esc2-WT (or Esc2-5E, Esc2-SLD2m, Esc2-MRΔ) in buffer $R^{high\ salt}$ for 10 minutes on ice. The sumoylation reaction took place at 30°C and was initiated upon the addition of 5 mM ATP. Samples were taken at 2 minutes, 5 minutes, and 15 minutes. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag antibodies. For quantification, this experiment was repeated, and sumoylated Sgs1 was quantified using ImageQuant (GE Healthcare).

GST pull-down assay with Esc2, Esc2-5E, Esc2-SLD2m, Esc2-MRΔ, and Ubc9

A pull-down assay was performed to see if the Esc2-MR domain mutants also abolished Esc2 interaction with Ubc9. 2.2μM of GST-tagged Esc2 (or Esc2-5E, Esc2-SLD2m, Esc2-MRΔ) was incubated with 3.6 μM of Ubc9 in 30 μL of T buffer supplemented with 80 mM KCl for 30 minutes at 4°C. The protein mixture was incubated with 10 μL of Glutathione Sepharose 4B resin (GE Healthcare) for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 μL of T buffer with 80 mM KCl.

Finally, the bound proteins were eluted with 20 µL of 2% SDS. Samples from the supernatant (S), wash (W), and eluate (E) were analyzed by SDS-PAGE.

Spot assay

Cells were grown in YPD until OD600 reached 0.2. Then cells underwent a 10-fold serial dilution in water. Next, diluted cells were incubated on YPD plates and YPD plus MMS plates and grown at 30°C for 2 days. Pictures were taken every day.

GST pull-down assay with Smc5/6 complex and STR complex

A pull-down assay was performed to test the interaction between the Smc5/6 complex and the STR complex. 0.3 µg of Sgs1 and 0.3 µg of the TR complex was incubated with 0.3 µg of the Smc5/6 complex in 30 µL of T buffer supplemented with 80 mM KCl for 30 minutes at 4°C. The protein mixture was incubated with 10 µL of Glutathione Sepharose 4B resin for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 µL of T buffer with 80 mM KCl. Finally, the bound proteins were eluted with 20 µL of 2% SDS. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-Flag, anti-CBP, and anti-GST antibodies.

GST pull-down assay with Mms21/Smc5 complex and STR complex

Another pull-down assay was performed to test a specific subcomplex of the Smc5/6 complex. 0.3 µg of Sgs1 and 0.3 µg of the TR complex were incubated with 0.3 µg of the Mms21/Smc5 subcomplex in 30 µL of T buffer supplemented with 80 mM KCl

for 30 minutes at 4°C. The protein mixture was incubated with 10 µL of Glutathione Sepharose 4B resin for 30 minutes at 4°C. The resin was washed for a total of 4 times with 200 µL of T buffer with 80 mM KCl. Finally, the bound proteins were eluted with 20 µL of 2% SDS. Samples were analyzed by SDS-PAGE and subsequently immunoblotted using anti-His and anti-GST antibodies.

Purification of Nse5/Nse6

To determine which component of the Smc5/6 complex is responsible for the interaction with STR, this requires the purification of each component of the Smc5/6 complex, including Mms21/Smc5, Smc5/6, Nse5-Nse6, and Nse1-Nse3-Nse4, to be used independently in future GST pull-down assays. Mms21/Smc5 was already purified using a published protocol (71), as was Smc5/6 (72).

Nse5/Nse6 was expressed and purified using the following protocol. The Nse5-WT with N-terminal His-SUMO-Ulp1 plasmid (G1371, pRSF-Nse5) and Nse6-full length with N-terminal GST Precission plasmid (Y140, pGEX-6P-1-Nse6) was co-transformed into BL21(DE3) *E. coli* strain. Next, the cells were heat-shocked in a 42°C water bath for 45 seconds and subsequently placed on ice for 2 minutes. After incubation, 0.2 mL of room temperature LB medium was added. The cells were shaken at 200 rpm for 45 minutes. Finally, cells were spread on LB plates with ampicillin and kanamycin added. Plates were incubated overnight at 37°C. The next day, colonies were inoculated in 120 mL of LB medium with ampicillin and kanamycin added. These cells were incubated overnight while shaking at 37°C. The following day, 20 mL of cells were added to 5-2 L flasks with 1 L of LB with ampicillin and kanamycin added. The flasks

were incubated at 37°C until the OD reached approximately 0.7. Then, 200 mM of IPTG was added to each flask to induce protein expression. The temperature of the incubator was lowered to 16°C and the cells were incubated overnight at 200 rpm. Finally, the media was centrifuged at 5000 rpm for 10 minutes at 4°C to pellet down the expressed protein. The pellet was stored at -80°C until the start of purification.

Upon the start of purification, the Nse5/6 pellet was incubated with a lysis buffer (T buffer containing 200 mM KCl, 1 4x-protease inhibitor tablet, 1 mM PMSF) at 4°C. The solution was then sonicated and subsequently ultracentrifuged at 40,000 rpm for 45 minutes at 4°C. The supernatant was incubated with 2 mL of Nickel resin (Ni Sepharose 6 Fast Flow, GE Healthcare) for 1 hour with rotation at 4°C. The column was washed twice with 50 mL of T buffer supplemented with 200 mM KCl and once with 50 mL of T buffer supplemented with 200 mM KCl and 30 mM imidazole. The complex was eluted using 12 mL of T buffer containing 200 mM KCl and 200 mM imidazole. The samples were pooled and incubated with 250 µg Ulp1 enzyme overnight with rotation at 4°C. Upon confirmation of Ulp1 cleavage, the sample was incubated with 2 mL of Glutathione Sepharose 4B resin at 4°C with rotation for 1 hour. The resin was washed three times with 50 mL of T buffer supplemented with 200 mM KCl. Nse5/6 was eluted with 10 mL of T buffer containing 200 mM KCl and 15 mM reduced glutathione. Samples were pooled, and half were incubated overnight with 150 µg of Precission Protease enzyme. For the half that were left uncleaved, the sample was run through a premade Superdex 200 gel filtration column (Sigma-Aldrich). The sample was concentrated down to 0.5 mL using an Ultracel-10K concentrator and loaded onto the column attached to the ATKA pure system. The complex was eluted using a preset software protocol and the sample

was concentrated down, aliquoted and stored at -80°C. The half of the sample that was incubated with Precission Protease was dialyzed to remove any unwanted molecules from the buffer. Then, the sample was incubated with 0.5 mL of Glutathione Sepharose 4B resin at 4°C for 1 hour with rotation. Then the column was washed one time with 3 mL of T buffer containing 200 mM KCl. The sample at this point was approximately 5 mL. That sample was concentrated down to 0.5 mL using an Ultracel-10K concentrator and loaded onto the Superdex 200 gel filtration column in T buffer supplemented with 200 mM KCl. The complex was pooled and concentrated down, aliquoted and stored at -80°C.

III. RESULTS AND DISCUSSION

Esc2 interacts with Ubc9, but not SUMO or SUMO chain in vitro

Preliminary data revealed key information in the role of Esc2 in sumoylation and its relationship with Mms21. It was found that in cells that were depleted of Esc2, the sumoylation of all three STR subunits was reduced (Figure 8). This provides a possible explanation for the role of Esc2 in reducing these levels of modified proteins.

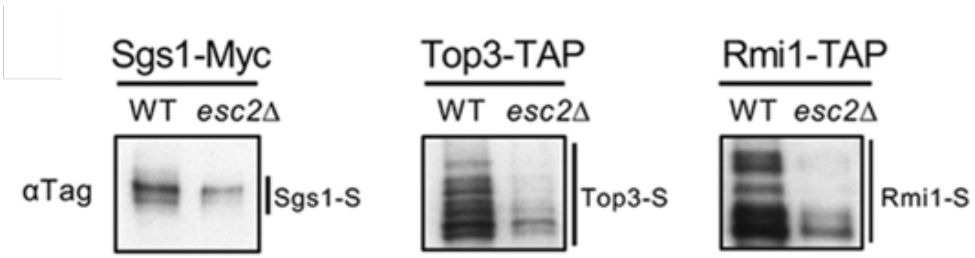

Figure 8: Esc2 stimulates STR sumoylation. Cells that are depleted of Esc2 show reduced levels of sumoylation in STR subunits.

Another interesting finding is that cells depleted of Esc2 did not reduce sumoylation levels of the Siz E3 substrates that bind to HJ, replication fork, or other DNA intermediates (Figure 9). Yen1 is a HJ resolution enzyme, PCNA is a replication fork associated protein, and Saw1 binds to DNA flap structures. This data suggests that Esc2 is not a general E3 regulator, but instead it specifically contributes to the sumoylation of Mms21 substrates that associate with HJ and replication fork structures.

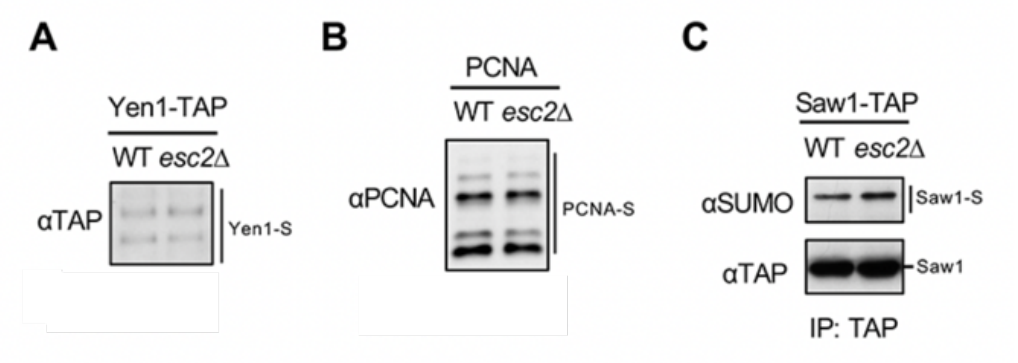

Figure 9: Esc2 is not a general E3 regulator. Cells depleted of Esc2 did not reduce sumoylation of Siz E3 substrates Yen1 (A), PCNA (B), or Saw1 (C).

We also found that there was no interaction between Esc2 and any of the subunits of the Smc5/6 complex or the STR complex via a yeast-two-hybrid assay (Figure 10). However, Esc2 did interact with Ubc9 and weakly with Smt3.

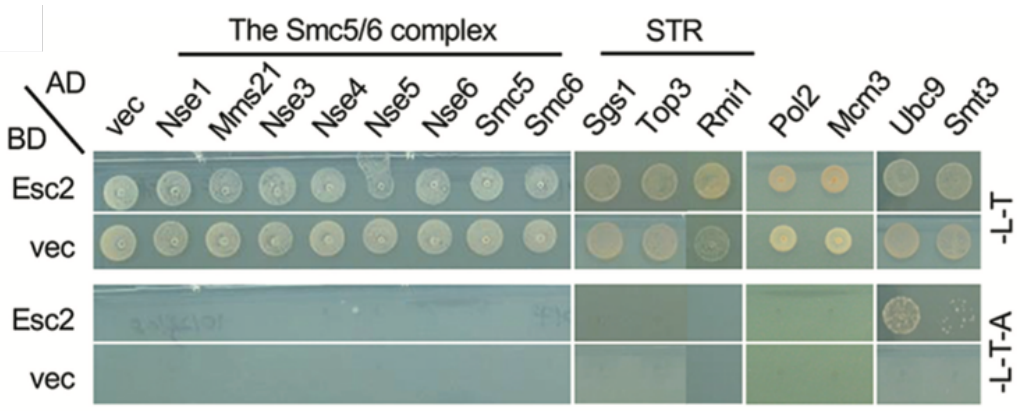

Figure 10: Yeast-two-hybrid data showing that Esc2 interacts with Ubc9 and Smt3, but not with the Smc5/6 complex or the STR complex. (AD) Gal4 activation domain, (BD) Gal4 DNA binding domain, (vec) vector. SC-Leu-Trp media (-L-T) select for BD and AD plasmids, while SC-Leu-Trp-Ade media (L-T-A) report for positive interactions.

Based on this data, pull-down assays using purified proteins were performed to further examine the interaction found in the yeast-two-hybrid assay. The interaction between Esc2 and Ubc9 was analyzed by a GST pull-down assay. GST-tagged Esc2 was incubated with Ubc9 and Glutathione Sepharose 4B resin. GST alone was used as a control to test if it could pull-down Ubc9. We found that GST-Esc2, but not GST alone, could bind to Ubc9 (Figure 11A).

To evaluate Esc2 interaction with SUMO, a separate GST-pull down assay was performed. GST-tagged Esc2 was incubated with Smt3 (yeast SUMO) or 4X-Smt3 and Glutathione Sepharose 4B resin. Again, GST alone was used as a control. A 4X-SUMO chain was used because weak binding to SUMO can often be enhanced by using a SUMO chain (77). However, no interaction between Esc2 and SUMO or the SUMO chain was

seen (Figure 11B). This shows that Esc2 binds directly to Ubc9. The SUMO interaction by Esc2 could not be detected by *in vitro* pull-down assay. These findings also raise the possibility that Esc2 controls sumoylation via this exclusive interaction and not with other substrates.

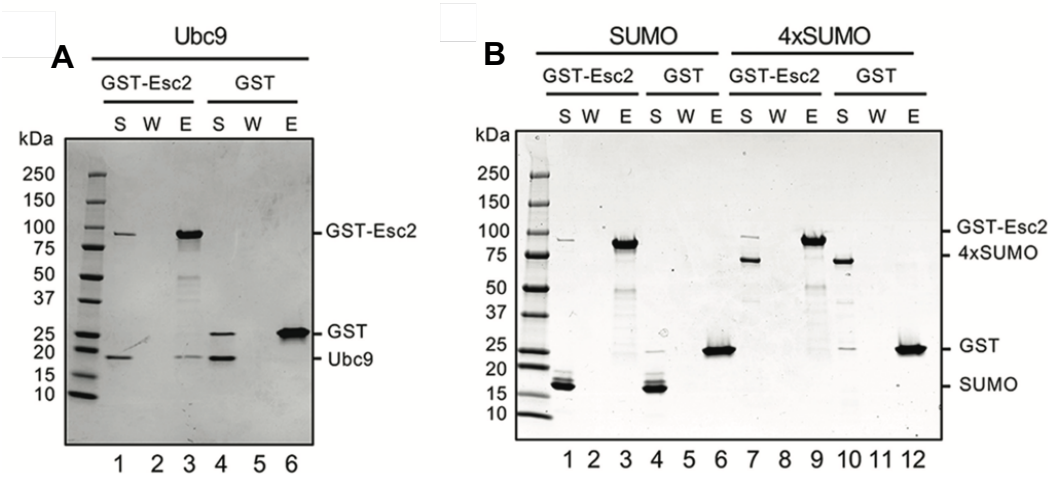

Figure 11: GST pull-down assay showing Esc2 interacts with Ubc9, but not with SUMO or 4X-SUMO *in vitro*. Esc2 binds to Ubc9 (A), but not SUMO or 4X-SUMO (B). Esc2 was bound to glutathione beads and tested to see their ability to pull-down Ubc9, SUMO, or 4X-SUMO. Proteins were examined by SDS-PAGE and subsequent Coomasie blue staining and de-staining. Supernatant (S), Wash (W), and Eluate (E).

Esc2-SLD2 domain and Ubc9 interaction is crucial for STR sumoylation

Esc2 contains two SUMO-like domains, SLD1 and SLD2. SLD2 has more sequence similarity to SUMO and also folds similarly to SUMO. In fission yeast, both SLD2 and SUMO bind to the Ubc9 backside, which is opposite from the Ubc9 active site

that forms a thiolester bond with SUMO (78,79). We wonder if SLD1 or SLD2 in budding yeast have the similar function to interact with Ubc9. Our collaborator generated an Esc2 variant, Esc2-SLD1m, by mutating the aspartic acid residue D268 to alanine and the isoleucine residue I287 to tyrosine. Esc2-SLD1m was compared to Esc2-SLD2m in a yeast-two-hybrid assay (Figure 12). Mutating the SLD1 domain did not diminish the interaction with Ubc9, while the mutation in the SLD2 domain did abolish the interaction between Esc2 and Ubc9.

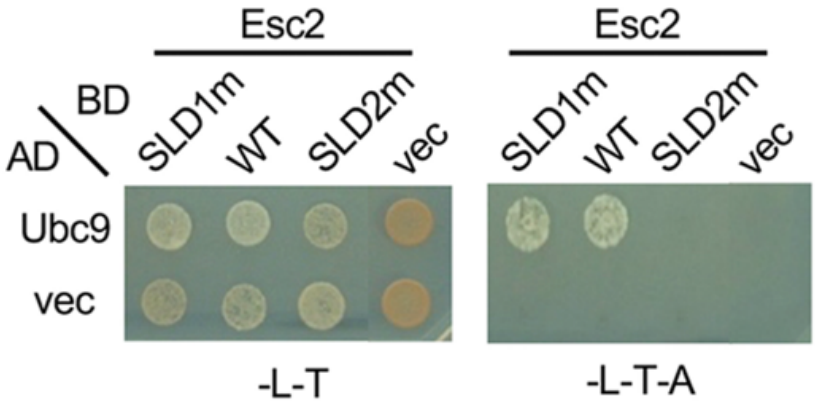

Figure 12: Yeast-two-hybrid data showing the SLD2 domain is responsible for Ubc9 interaction. Esc2-SLD1m did not abolish the interaction with Ubc9, while Esc2-SLD2m did abolish the interaction with Ubc9.

A pull-down assay was done to confirm this finding. The assay showed that wildtype Esc2 is able to pull-down Ubc9, whereas the mutated Esc2-SLD2m had no interaction with Ubc9 (Figure 13). This confirms that the SLD2 domain of Esc2 is

responsible for the Esc2-Ubc9 interaction and that the Esc2-SLD2m mutant effectively disrupts this interaction.

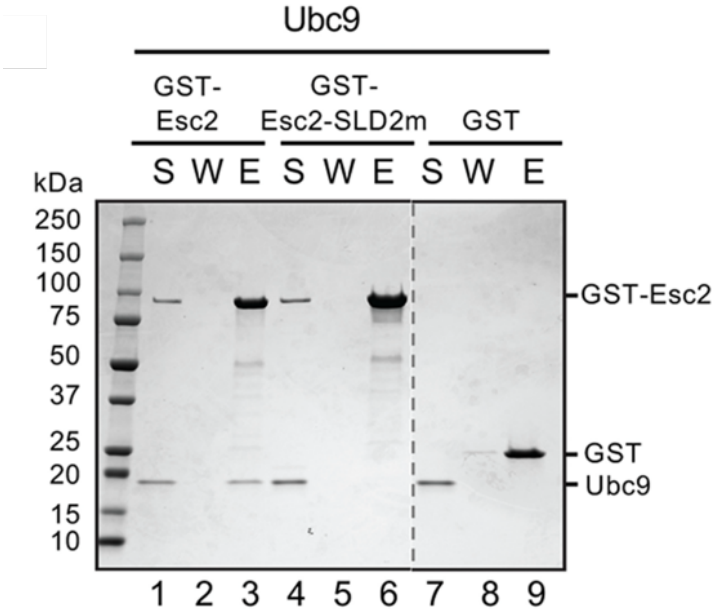

Figure 13: GST pull-down assay showing Esc2-SLD2m loses interaction with Ubc9. Esc2 was able to pull-down Ubc9, while Esc2-SLD2m was unable to interact with Ubc9.

The importance of the SLD2 domain was further examined using sumoylation assays in cells. It was found that Esc2-SLD2m acted like cells that were depleted of Esc2 in that they reduced STR sumoylation levels, while Esc2-SLD1m had no effect on STR sumoylation (Figure 14). This data suggests that the Esc2-Ubc9 interaction is key for the sumoylation pathway.

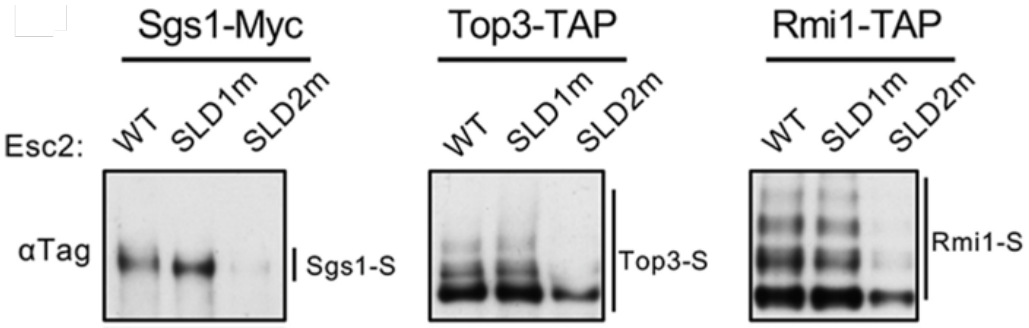

Figure 14: Esc2-SLD2m effectively disrupts STR sumoylation. The WT Esc2 and Esc2-SLD1m had no effect on sumoylation levels, while Esc2-SLD2m saw a reduction in STR sumoylation levels.

To test whether the SLD2 domain has a direct role in sumoylation, an *in vitro* sumoylation system for Sgs1 and Top3 was established. We have purified the SUMO, SUMO E1 (Aos1-Uba2), E2 (Ubc9), E3 (Mms21/Smc5), Sgs1 and Top3-Rmi1 complex and examined them for the *in vitro* sumoylation assay (Figure 15). Our sumoylation assay has shown that these purified proteins were active and good for the biochemical assays (Figure 16). The sumoylation assay also shows that the addition of SUMO E3 (Mms21/Smc5) greatly stimulated Sgs1 and Top3 sumoylation when compared to lanes where SUMO E3 is absent (Figure 16).

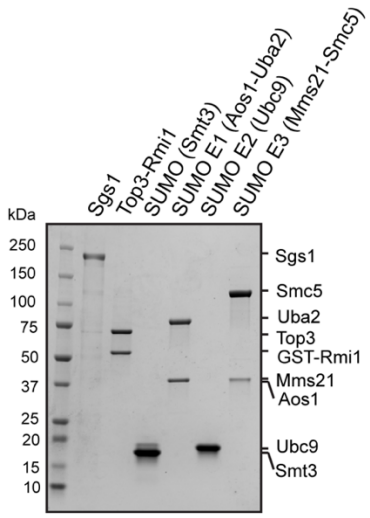

Figure 15: Purified sumoylation proteins were analyzed by SDS-PAGE. Purified SUMO, SUMO E1, E2, E3, Sgs1 and Top3-Rmi1 proteins were run on an SDS-PAGE gel and subsequently stained with Coomasie blue.

This data is consistent with *in vivo* findings and demonstrates that Mms21 can directly promote Sgs1 and Top3 sumoylation. Rmi1 was not sumoylated in this system, possibly because additional factors required for its sumoylation are not present in this assay. Sgs1 and Top3 were the focus for all further sumoylation assays.

Because SUMO and Esc2 compete for binding of Ubc9, a SUMO variant was created with a mutation at a key residue for binding to Ubc9 (D68R) to eliminate this interaction. The SUMO-DR mutant showed lower sumoylation levels than the wildtype SUMO. However, increased sumoylation levels can still be seen with the presence of SUMO E3 (Figure 16).

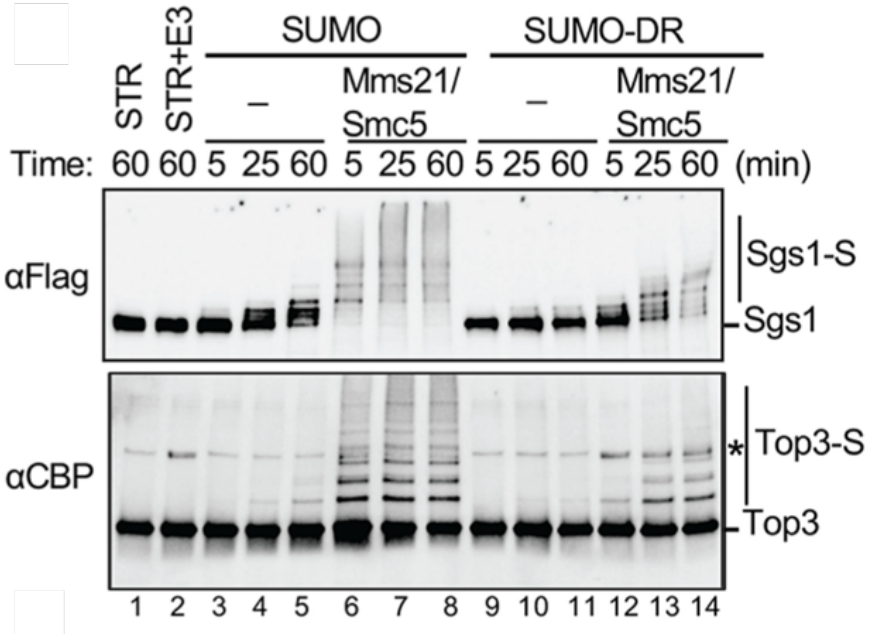

Figure 16: Mms21-Smc5 stimulates Sgs1 and Top3 sumoylation in the presence of SUMO or SUMO-D68R. *In vitro* sumoylation assays were performed by incubating purified STR complex with the SUMO E1, the SUMO E2, SUMO (or SUMO-DR), and ATP in the presence or absence of the Mms21-Smc5 SUMO E3 at 30°C for the indicated time. Sgs1 and Top3 sumoylation is still stimulated by Mms21-Smc5 with a reduced overall sumoyaltion in the presence of SUMO-DR. Asterisk represents a cross-reactivity band.

Next, Esc2 and Esc2-SLD2m were used to stimulate *in vitro* sumoylation of STR and to confirm the role of the SLD2-Ubc9 binding. This experiment showed that Esc2 alone did stimulate Sgs1 and Top3 sumoylation (Figure 17). For example, at the 5-minute time point, mono-sumoylation can be observed in the absence of Esc2. When Esc2 is

present, poly-sumoylation can be observed at the 5 minute time point. However, Esc2-SLD2m failed to stimulate significant sumoylation. This data indicates that Esc2 stimulates sumoylation and relies on SLD2 binding to Ubc9. It also suggests a direct role of Esc2 SLD2 binding in sumoylation.

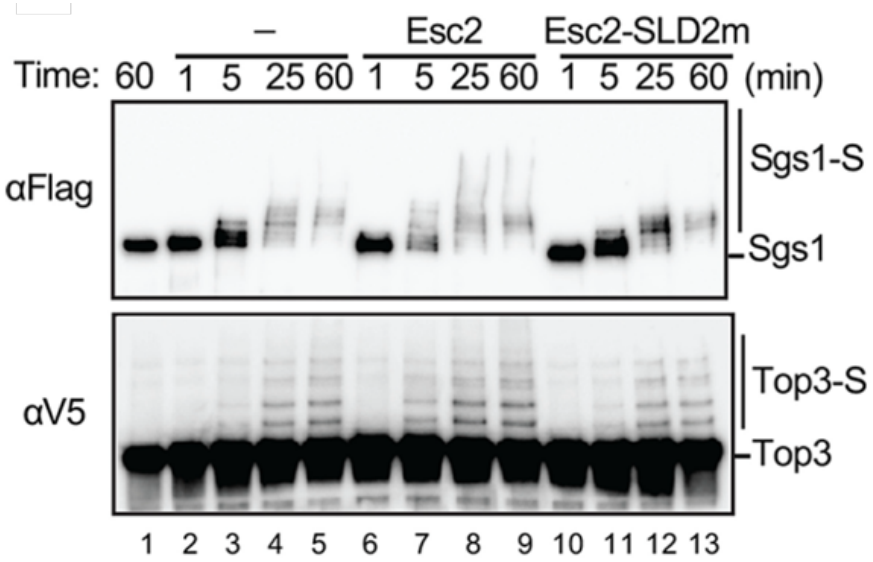

Figure 17: Sumoylation assay showing Esc2, but not Esc2-SLD2m, stimulates Sgs1 and Top3 sumoylation *in vitro*. Sumoylation assays were performed as in Figure 16 in the presence of Mms21-Smc5 and SUMO-DR.

Circular dichroism (CD) analysis was done to compare WT Esc2 and Esc2-SLD2m (data not shown) secondary structure. The profiles were similar, indicating that the D447A and D449A point mutations did not affect the overall protein folding. Biochemical assays were also done to confirm that Esc2-SLD2m maintained certain WT functions. A GST pull-down assay was done to confirm Esc2-SLD2m maintained its interaction with the Mus81/Mms4 complex (data not shown). Esc2 acts in parallel with

the Mus81/Mms4 complex, which acts in HJ resolution. This assay confirms that the mutations introduced did not alter the overall protein structure.

Previous studies suggest that SUMO binding to the Ubc9 backside can better orient the Ubc9 active site for efficient SUMO transfer. To test if SLD2 also does this, the binding affinity of Ubc9 with Esc2-SLD2Δ-Su and Esc2-SLD2Δ-SuDR were tested using a GST-pull down assay (Figure 18). Esc2-SLD2Δ-Su was able to pull down Ubc9, confirming interaction that is similar to wildtype Esc2. However, Esc2-SLD2Δ-SuDR lost this interaction. These results indicate that SLD2 uses the SUMO binding surface on the Ubc9 backside to enhance the SUMO E2 function.

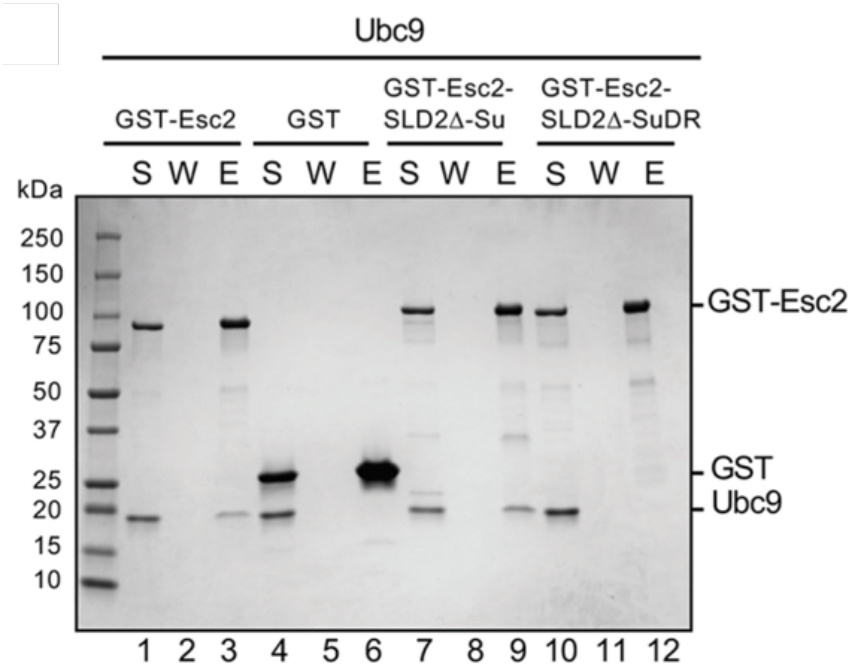

Figure 18: GST pull-down assay showing Esc2-SLD2Δ-Su maintains interaction with Ubc9, but Esc2-SLD2Δ-SuDR loses this interaction. Esc2-SLD2Δ-Su was able to interact with and pull-down Ubc9, while Esc2-SLD2Δ-Su abolished the interaction with Ubc9.

To evaluate the effect of Esc2-SLD2Δ-Su and Esc2-SLD2Δ-SuDR on STR sumoylation, a sumoylation assay was performed (Figure 19). Esc2-SLD2Δ-Su stimulated *in vitro* Sgs1 sumoylation similarly to Esc2. On the other hand, Esc2-SLD2Δ-SuDR showed very minimal stimulation of sumoylation of Sgs1, which is comparable to Esc2-SLD2m. This data suggests that Esc2 uses it SLD2 binding domain to promote STR sumoylation.

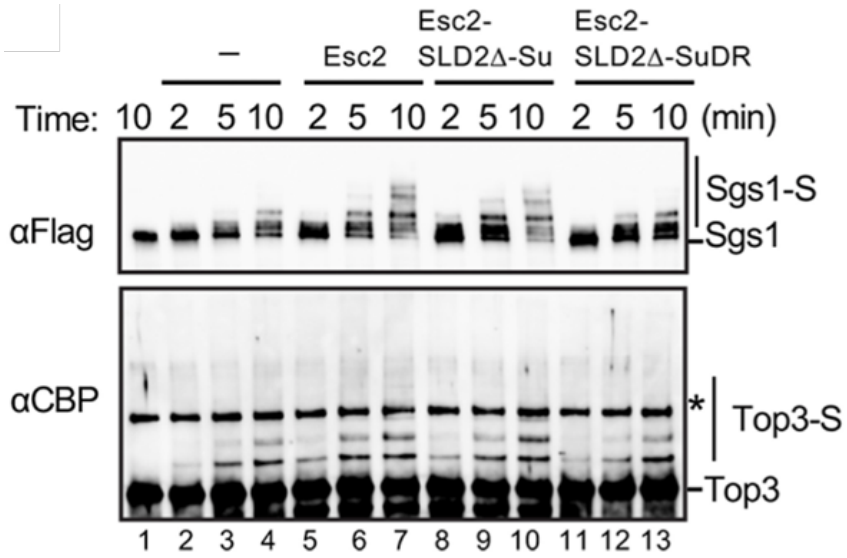

Figure 19: Sumoylation assay showing that Esc2-SLD2Δ-Su, but not Esc2-SLD2Δ-SuDR, stimulates Sgs1 sumoylation in STR complex. Esc2-SLD2Δ-Su acted like WT Esc2 in stimulating Sgs1 sumoylation. Esc2-SLD2Δ-SuDR did not stimulate Sgs1 and Top3 sumoylation. Asterisk represents a cross-reactivity band.

These *in vitro* findings were further examined by testing if Esc2, Esc2-SLD2m, Esc2-SLD2Δ-Su and Esc2-SLD2Δ-SuDR could maintain sumoylation levels in the Sgs1, Top3, and Rmi1 proteins in cells. Esc2 and Esc2-SLD2Δ-Su maintained sumoylation in

all STR units (Figure 20). However, Esc2-SLD2m and -SLD2Δ-SuDR showed decreased levels of sumoylation. This data shows that the SLD2 domain can be replaced by SUMO but not SUMO-DR, suggesting that SLD2 uses the SUMO binding surface of Ubc9 to enhance sumoylation.

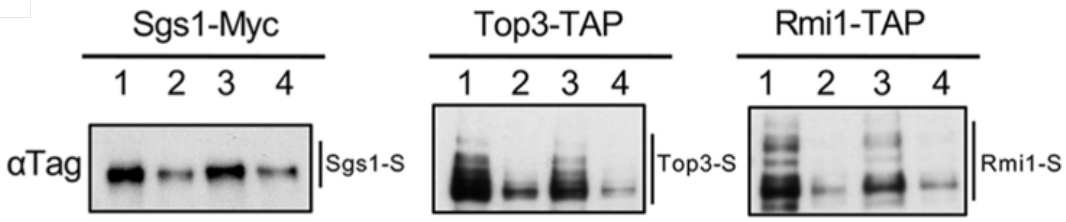

Figure 20: Sumoylation assay showing Esc2-SLD2Δ-SuDR reduced stimulation of sumoylation in cells. Lane designations (1) WT, (2) Esc2-SLD2m, (3) Esc2-SLD2Δ-Su, and (4) Esc2-SLD2Δ-SuDR. WT Esc2 and Esc2-SLD2Δ-Su showed similar STR sumoylation levels, while Esc2-SLD2m and Esc2-SLD2Δ-SuDR showed reduced levels.

Esc2 and Ubc9 interaction restricts accumulation of gross chromosomal rearrangements and recombinational intermediates

Moreover, we also examined the effect of the Esc2-Ubc9 interaction on genome maintenance. It is known that Esc2 promotes genome stability during normal growth and it limits HJs in the presence of methyl methanesulfonate (MMS), but it is unclear if the Esc2-Ubc9 interaction is required (44). Through gross chromosomal rearrangements (GCR) assays, our collaborator, Xiaolan Zhao's group, found that cells without Esc2 had a 23-fold increase in GCR rates when compared to cells with wildtype Esc2. The Esc2-SLD2m mutant cells had a three-fold higher GCR rate than cells with wildtype Esc2, which indicates that the Esc2-Ubc9 interaction does have some effect on genome

maintenance (Figure 21A). We also found that the Esc2-SLD2m mutant showed a two- to three-fold increase of X-shaped molecules (X-mols), which includes HJ structures, via DNA 2-dimensional (2D) agarose gel electrophoresis (Figure 21B). This data suggests that the Esc2-Ubc9 interaction may contribute to HJ clearance.

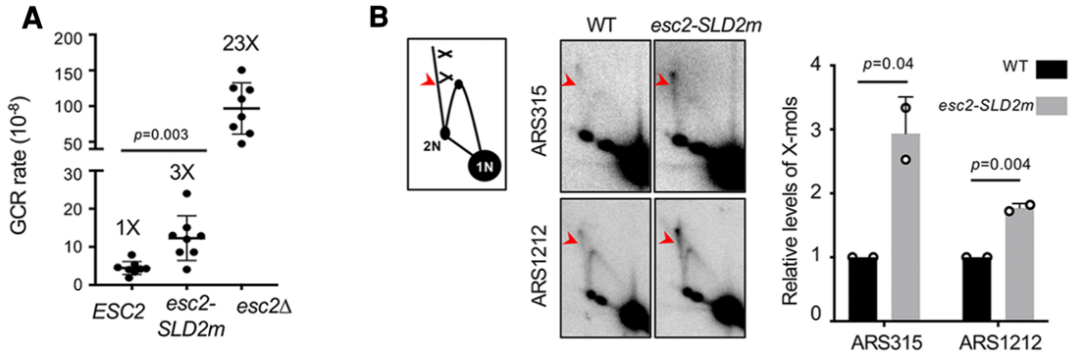

Figure 21: GCR rates and X-mols from 2-D agarose gel electrophoresis. (A) GCR assay data shows that the depletion of Esc2 leads to a 23-fold increase of GCR rates. Additionally, Esc2-SLD2m shows a three-fold increase in GCR rates when compared to wildtype Esc2. (B) Data from 2-D agarose gel electrophoresis. shows an increase in X-mols for Esc2-SLD2m. The graph on the right shows quantitative data for 2-D agarose gel electrophoresis.

Proposed model of Esc2 stimulation of substrate sumoylation

Based on the above data, a model for Esc2 stimulation of sumoylation can be proposed (Figure 22). The binding of Esc2 to the backside of Ubc9 through its SLD2 domain leads to a conformational change that aids in SUMO transfer and stimulates

sumoylation of certain Smc5-6-Mms21 E3 substrates. This occurs most likely at HJ and replication fork sites, ultimately contributing to HJ removal and genome stability.

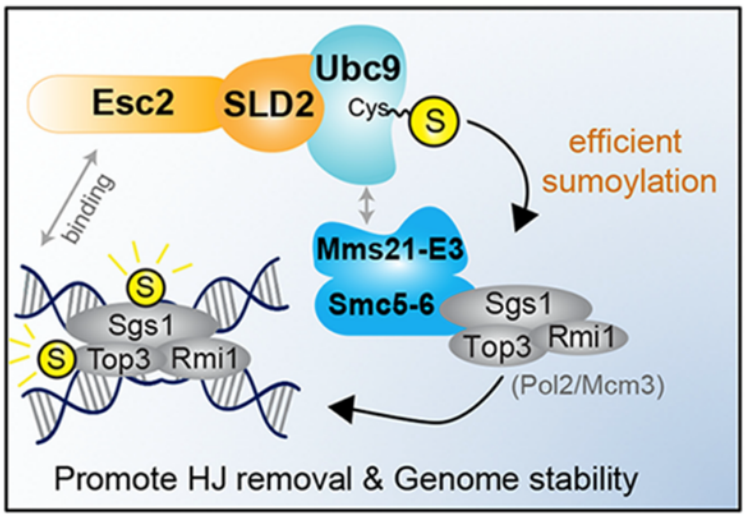

Figure 22: Working model of the stimulation of STR sumoylation by Esc2. Esc2 binding to the backside of Ubc9 through its SLD2 leads to the stimulation of sumoylation of a subset of Smc5-6-Mms21 E3 substrates, likely at HJ and replication fork sites, contributing to HJ dissolution and genome stability (51).

HJ can stimulate sumoylation of Sgs1

To test if HJs could stimulate STR sumoylation in the presence or absence of HJ or SUMO E3, a sumoylation assay was performed. It was confirmed that Mms21/Smc5 did show moderate DNA-binding ability (Figure 23); however, other components of the assay, such as SUMO, SUMO E1, and SUMO E2, do not bind DNA. The sumoylation assay showed that in the absence of E3, HJ may stimulate mono-sumoylation of Sgs1 (Figure 24). When quantified, the assay showed that at 60 minutes, only about 20% of

Sgs1 was sumoylated when HJ was absent. When HJ was present, Sgs1 sumoylation increased to around 80%. This suggests that the interaction between Sgs1 and HJ does stimulate sumoylation. Furthermore, the presence of E3 (Mms21/Smc5) in the assay strongly enhanced the sumoylation of Sgs1, as expected. At only 5 minutes, already close to 40% of Sgs1 was sumoylated in the presence of E3 compared to 20% without E3.

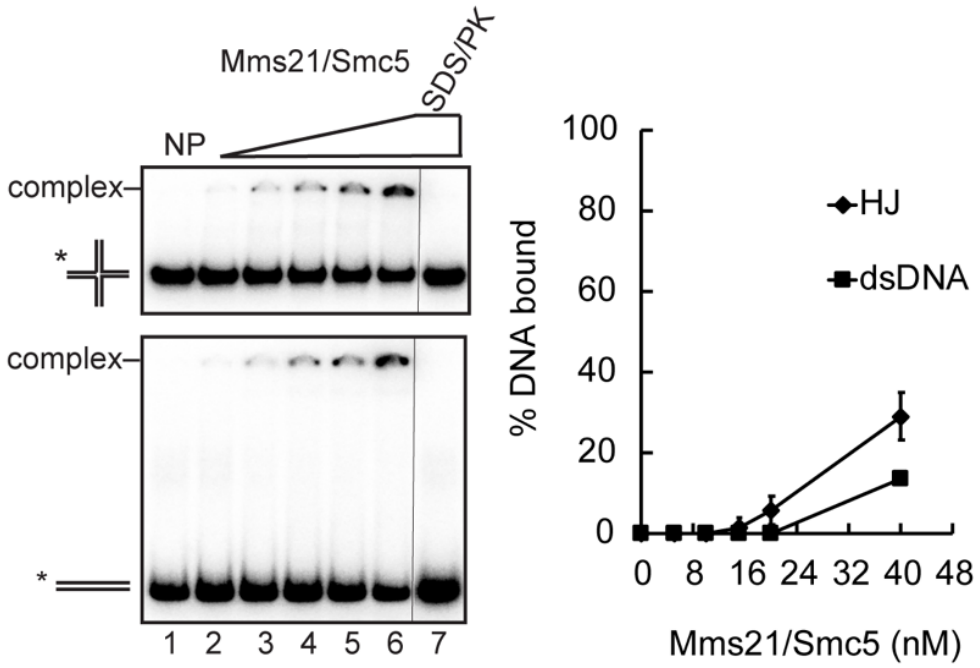

Figure 23: DNA mobility shift assay of Mms21/Smc5 shows interaction between SUMO E3 and both HJ and dsDNA. The results were quantified and plotted as mean ± SD (n=3 technical replicates). (NP) No protein, only DNA was present.

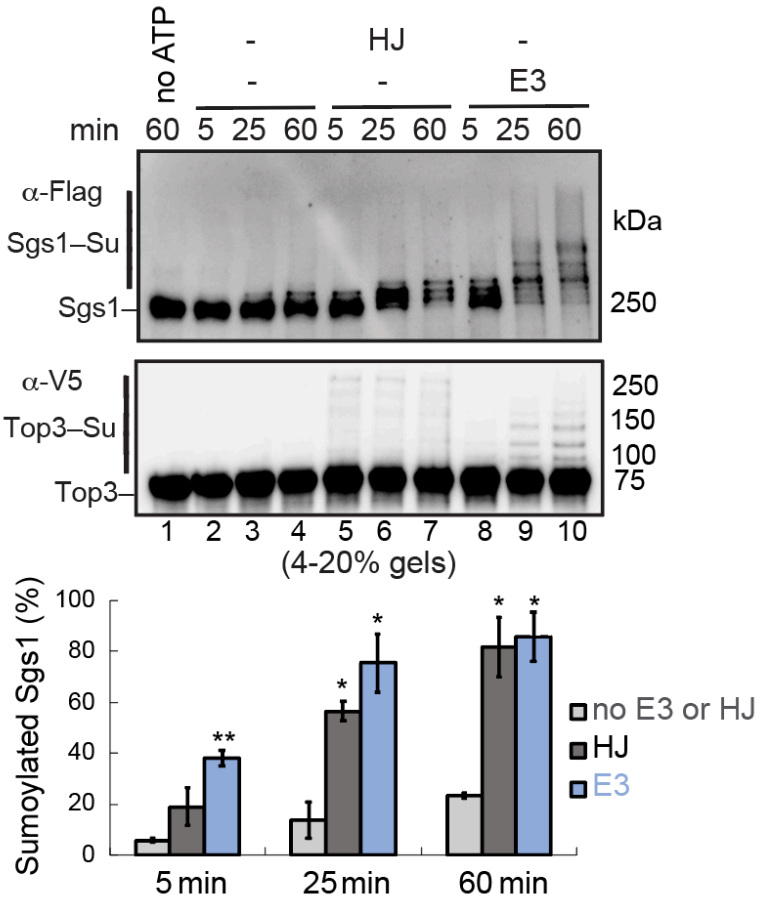

Figure 24: HJ alone is able to stimulate the sumoylation of STR. The presence of E3 alone was also enough to stimulate increased sumoylation of Sgs1. Sumoylation levels were quantified and organized in a bar graph. The addition of either HJ or E3 had increased sumoylated Sgs1 levels when compared to lanes with no E3 or HJ. The percentage of sumoylated Sgs1 was shown as mean ± SD (n = 2 technical replicates). *p < 0.05; **p < 0.01; ***p < 0.001.

DNA and SUMO E3 additively increase Sgs1 sumoylation

After examining the individual effects of HJ and SUMO E3 on Sgs1 sumoylation, an assay was done to examine their additive effects. Because enhanced sumoylation was

anticipated, the reaction time points were shortened to 2 minutes, 5 minutes, and 15 minutes. The additive effect of HJ and SUMO E3 was confirmed for Sgs1 sumoylation (Figure 25).

Upon quantification, it was found that after only 5 minutes of the reaction taking place, already almost 80% of Sgs1 had been sumoylated when both HJ and E3 were present. However, when only one component was present, the level of Sgs1 that was sumoylated was only around 30-50% sumoylated. In previous studies, it was found that Mms21/Smc5 can bind to dsDNA (80). Through a DNA mobility shift assay, it was shown that HJ can also bind to STR (Figure 26) and SUMO E3 (Figure 23). Therefore, it is possible that HJ binding to STR or E3 can contribute to Sgs1 sumoylation additively.

Top3 sumoylation was not robust, possibly because its sumoylation requires elements that are not included in this *in vitro* assay. For the same reason, Rmi1 was not sumoylated in this system, which has been previously discussed. Because of this, Sgs1 sumoylation was the main focus in subsequent testing.

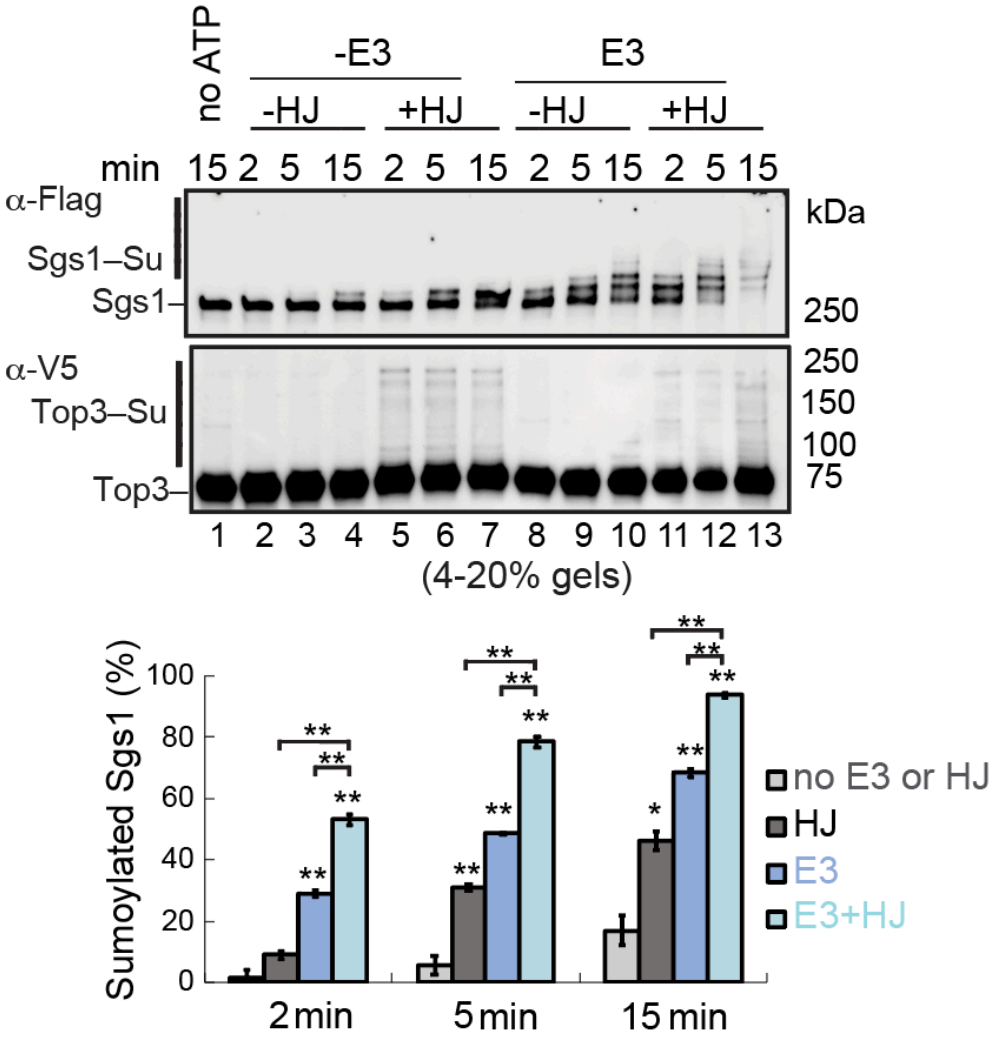

Figure 25: Sumoylation levels were increased with the addition of both HJ and E3, confirming their additive effect. Sumoylation levels were quantified and organized in a bar graph. The addition of both HJ and E3 had increased sumoylated Sgs1 levels when compared to lanes with just one component, or no component at all. The percentage of sumoylated Sgs1 was shown as mean ± SD (n = 2 technical replicates). *p < 0.05; **p < 0.01; ***p < 0.001.

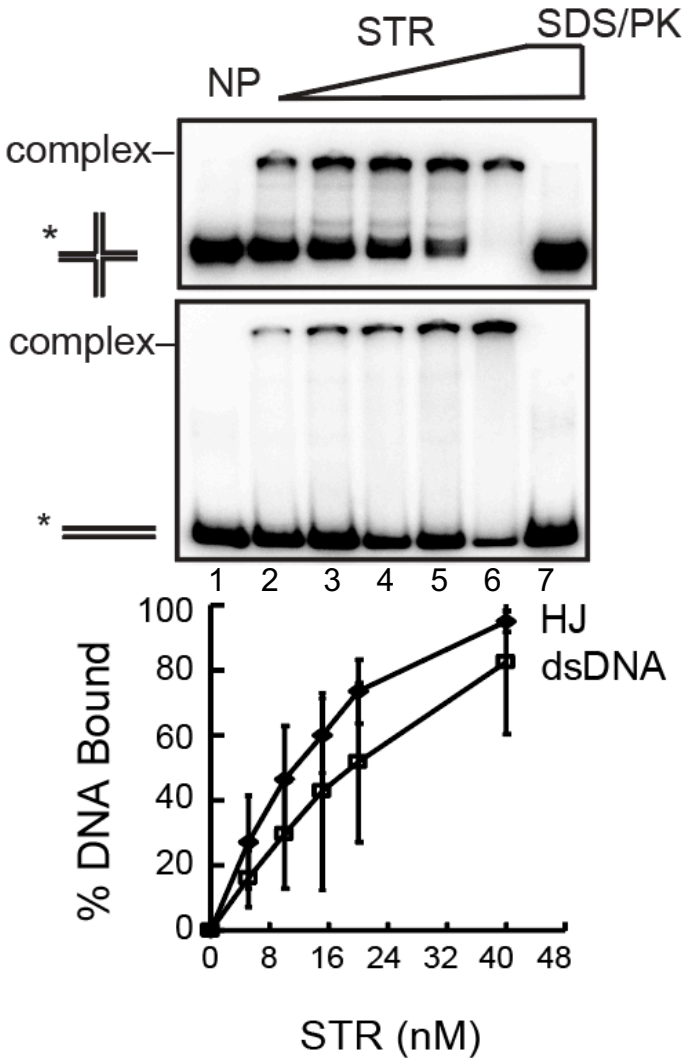

Figure 26: Purified STR complex was shown to bind to both HJ and dsDNA in this DNA mobility shift assay as indicated by the shifting bands. The results were quantified and plotted on a graph. The results were quantified and plotted as mean ± SD (n=3 technical replicates). (NP) No protein, only DNA.

Because HJ and dsDNA bind to STR similarly (Figure 26), dsDNA was also tested with or without E3 to compare effects on the sumoylation of Sgs1. It was found that dsDNA in the absence of E3 stimulated Sgs1 sumoylation (Figure 27) to comparable

levels with HJ in the absence of E3 (Figure 25). When quantified, dsDNA showed to sumoylate around 40% of Sgs1 within 15 minutes of the reaction taking place. dsDNA also showed additive effects when in the presence of E3. After quantification, it was shown that around 90% of Sgs1 was sumoylated when in the presence of both dsDNA and E3 after only 5 minutes, similar to HJ and E3. This data demonstrates that the dsDNA binding to STR or E3 can further stimulate Sgs1 sumoylation when in the presence of E3.

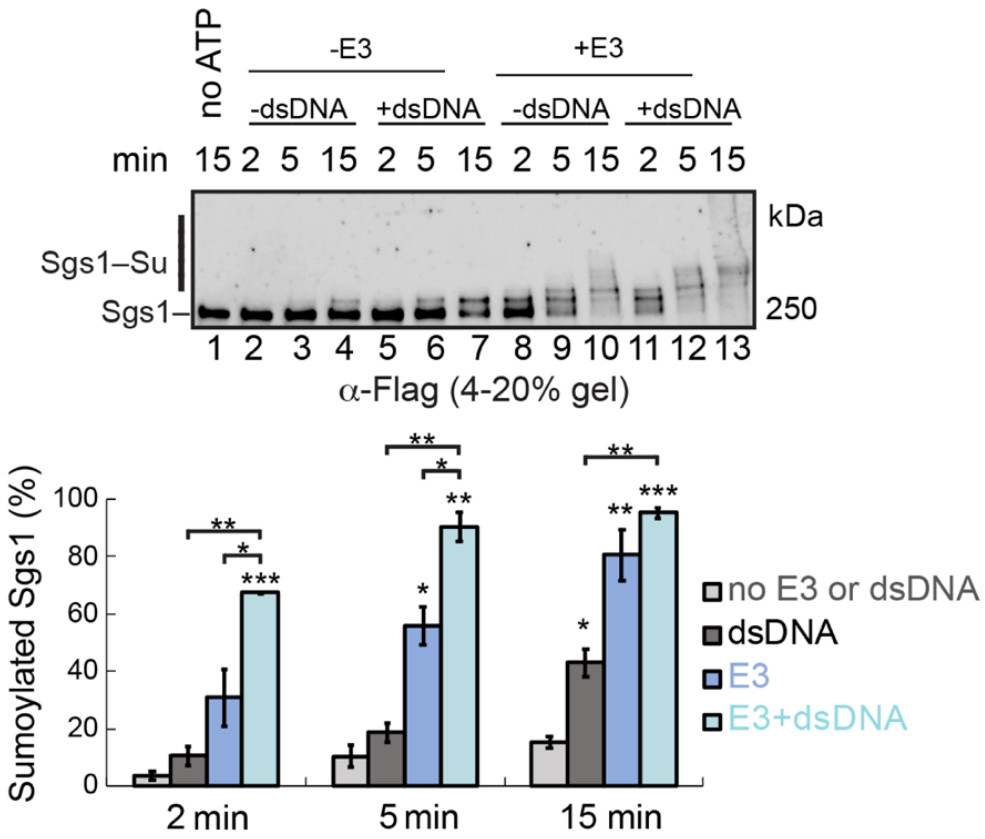

Figure 27: Sumoylation levels were increased with the addition of both dsDNA and E3, confirming their additive effect. Sumoylation levels were quantified and organized in a bar graph. The addition of both dsDNA and E3 had increased sumoylated Sgs1 levels when compared to lanes with just one component, or no component at all. The percentage of sumoylated Sgs1 was shown as mean ± SD (n=2 technical replicates). *p < 0.05; **p < 0.01; ***p < 0.001

DNA and Esc2 additively increase Sgs1 sumoylation

As previously stated Esc2 plays an important role in the regulation of STR sumoylation. It has two SUMO-like domains (SLD1 and SLD2), in which the role and importance have been discussed earlier in this dissertation. Esc2 also contains a mid-

region (Esc2-MR) that has been shown to have strong binding preferences to DNA. Specifically, Esc2-MR has shown to prefer HJ over dsDNA (11,46). It is known that both Esc2 and HJ stimulate Sgs1 sumoylation independently, and we wonder if HJ and Esc2 together could have an additive effect on Sgs1 sumoylation.

A sumoylation assay was performed to test the effects of Esc2 and HJ on Sgs1 sumoylation. It was confirmed that Esc2 alone can stimulate Sgs1 sumoylation. It was also confirmed that HJ alone can stimulate Sgs1 sumoylation. When quantified, it was found that around 45% of Sgs1 had been sumoylated in the presence of either Esc2 or HJ after 2 minutes of reaction (Figure 28).

This sumoylation assay also confirmed the additive effect of HJ and Esc2 on Sgs1 sumoylation levels. It was found that the addition of both HJ and Esc2 increase sumoylation levels. Upon quantification, more than 70% of Sgs1 was sumoylated after only 2 minutes in the presence of both HJ and Esc2.

This entire experiment was repeated, replacing HJ with dsDNA. Similar observations were observed, where after 2 minutes, around 45% of Sgs1 was sumoylated in the presence of either HJ or Esc2, and around 70% was sumoylated in the presence of both HJ and Esc2 (Figure 29). These results demonstrate that Esc2 along with either HJ or dsDNA can additively increase sumoylation of Sgs1.

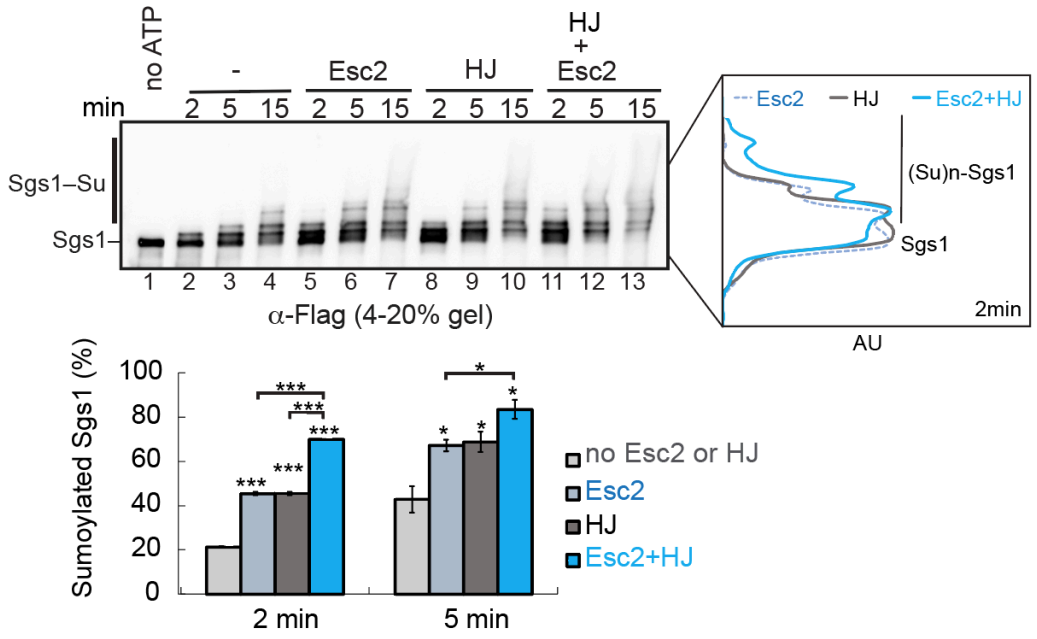

Figure 28: Esc2 and HJ can additively stimulate Sgs1 sumoylation. Results were quantified and organized in bar graph (below). Immunobloting information was also organized into a graph (right). The scans of lanes 5, 8, and 11 at 2 minutes show the appearance of poly-sumoylated Sgs1 forms in Esc2 and HJ presence. The percentage of sumoylated Sgs1 was shown as mean ± SD (n=2 technical replicates). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

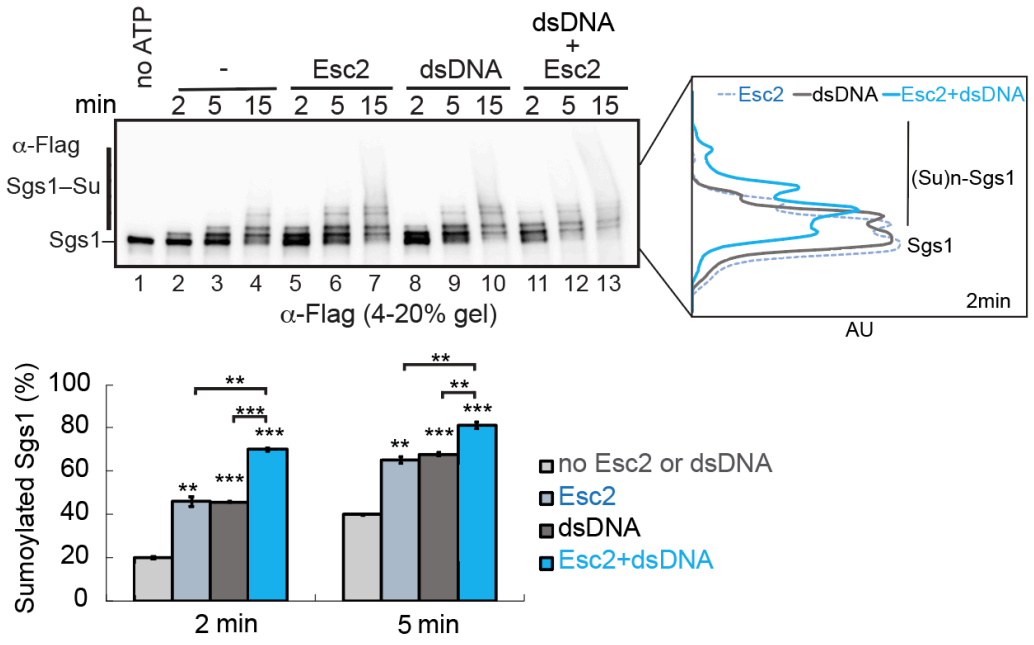

Figure 29: Esc2 and dsDNA can additively stimulate Sgs1 sumoylation. Results were quantified and organized in bar graph (below). Immunobloting information was also organized into a graph (right). The scans of lanes 5, 8, and 11 at 2 minutes show the appearance of poly-sumoylated Sgs1 forms in Esc2 and dsDNA presence. The percentage of sumoylated Sgs1 was shown as mean ± SD (n=2 technical replicates). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Esc2-MR promotes Sgs1 sumoylation independent of its DNA-binding ability

Although it was seen in previous *in vitro* experiments that HJ and dsDNA performed similarly in the sumoylation of Sgs1 (Figures 28, 29), a mobility shift assay was done to test Esc2 preference for HJ or dsDNA (Figure 30). It was found that Esc2 does prefer to bind HJ over dsDNA.

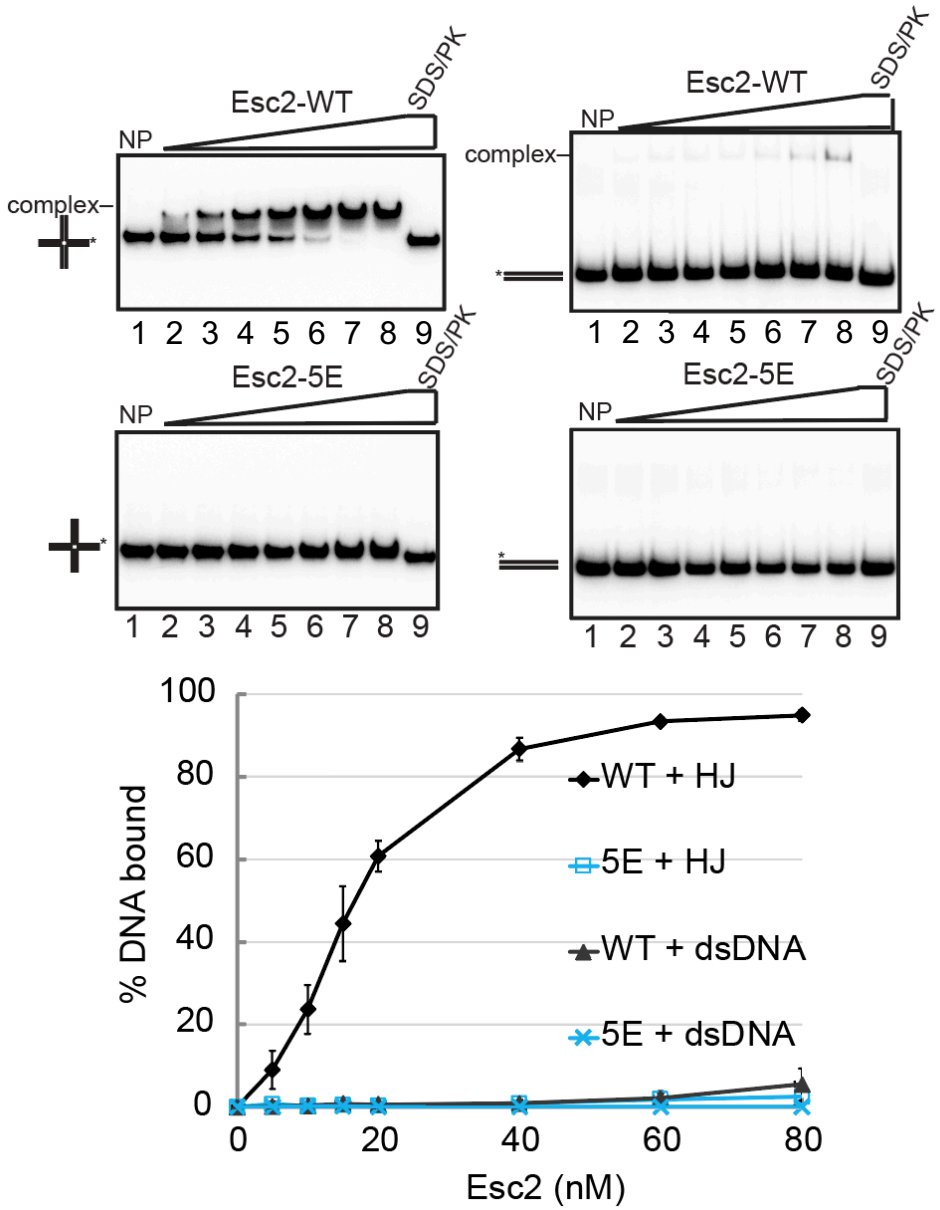

Figure 30: A DNA mobility shift assay was performed that showed Esc2-WT had a strong binding preference to HJ over dsDNA. Esc2-5E (mutant) was defective in binding both HJ and dsDNA. The mean ± SD from at least three independent experiments were plotted. (NP) No protein, only DNA.

This preference raised the possibility that Sgs1 sumoylation stimulation by Esc2 might be independent of its DNA binding abilities. To test this idea, an Esc2 mutant was made that deleted the entire mid-region (154aa – 198aa) and was expected to abolish Esc2 DNA binding ability (Esc2-MRΔ). This was confirmed through a DNA mobility shift assay (Figure 31).

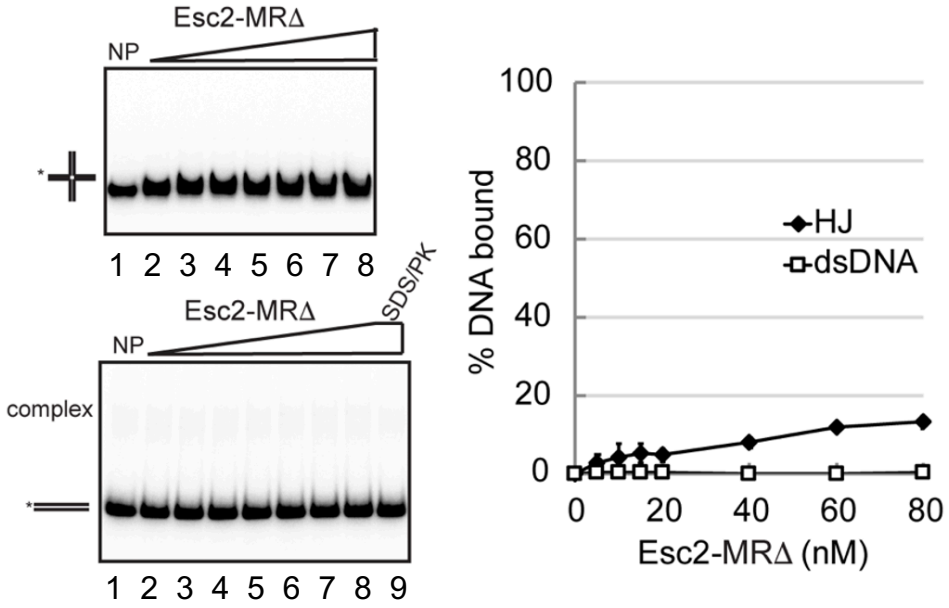

Figure 31: DNA mobility shift assay confirms the abolishment of Esc2 DNA binding activity in the Esc2-MRΔ mutant. Esc2-MRΔ does not bind to HJ or dsDNA. This data was quantified and organized into a graph. The mean ± SD from at least three independent experiments were plotted. (NP) No protein, only DNA.

However, deleting an entire domain could potentially affect protein folding, so another mutant was created with point mutations that were also expected to abolish Esc2 DNA-binding ability. Upon comparing sequence alignments among Esc2 orthologs, it was observed that there are several conserved lysine and arginine residues within the

Esc2-MR domain that could potentially be involved in DNA binding activity. The new mutant, Esc2-5E, has five point mutations (K179E, K182E, K183E, K197E, and R198E). A DNA mobility shift assay confirmed the abolishment of DNA binding activity with HJ and ds DNA (Figure 30).

To confirm the mutation does not change the overall structure of Esc2-5E, the size exclusion elution profiles of WT Esc2 and Esc2-5E were compared (data not shown). It was found that both proteins eluted at approximately the same volume, 12.11 mL for WT Esc2 and 12.02 mL for Esc2-5E. This indicates that both proteins have around the same apparent molecular weight in solution. Additionally, both peaks are tall and narrow, indicating mono dispersity. The small peak to the left represents larger contaminate proteins. It can be assumed that the protein is not aggregated because there is no peak at the void volume.

Additionally, a GST pull-down assay was done to confirm Esc2-5E and Esc2-MRΔ still maintained its interaction with Ubc9, which will be discussed later in this dissertation. Esc2 was found to be a cofactor to SUMO E2 Ubc9. This assay confirms that the mutations introduced did not alter the overall protein folding.

Next, a sumoylation assay was done to test the effect of the Esc2-5E mutant on Sgs1 sumoylation in the presence of DNA. If Esc2 DNA binding capabilities were essential for Sgs1 sumoylation, then it would be expected for sumoylation levels to be reduced in the presence of Esc2-5E and DNA, and remain the same in reactions without DNA. However, it was seen that Esc2-5E reduced levels of sumoylation in both the presence and absence of HJ (Figure 32) and dsDNA (Figure 33). Figure 34 visualizes how in the presence or absence of DNA, Esc2-5E maintains its reduced sumoylation

levels. This suggests that the Esc2-MR domain does contribute to STR sumoylation, but in a DNA-independent manner. This also raises the possibility that the Esc2-MR domain has some other unknown function.

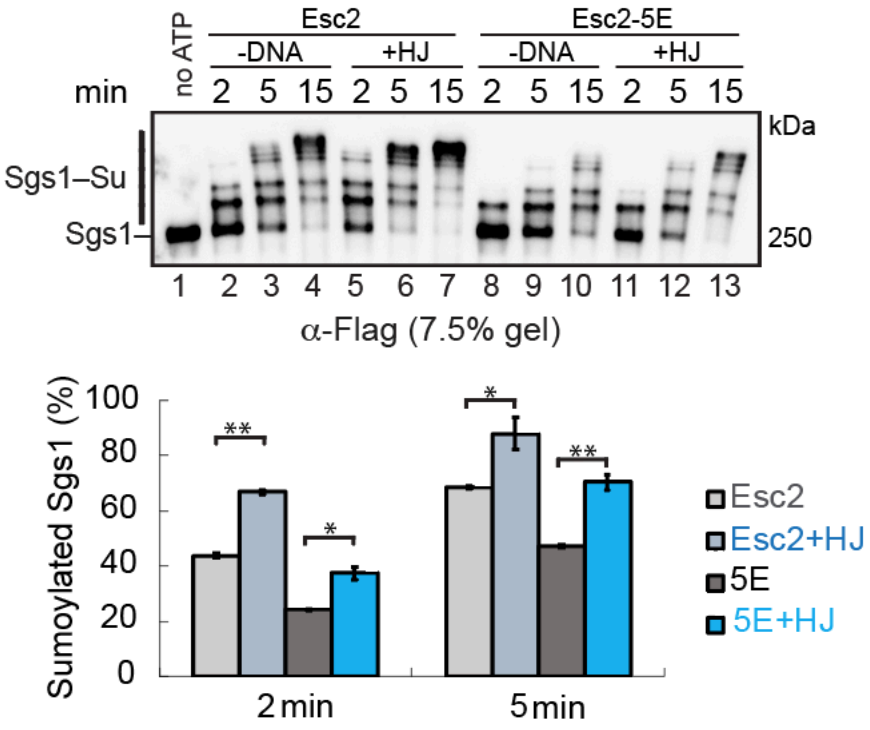

Figure 32: Sumoylation assay shows that Esc2-5E reduced sumoylation levels in both the presence and absence of HJ. This data was quantified and organized into a bar graph. Percentage of sumoylated Sgs1 showed mean ± SD (n=2 technical replicates). *p < 0.05; **p < 0.01; ***p < 0.001.

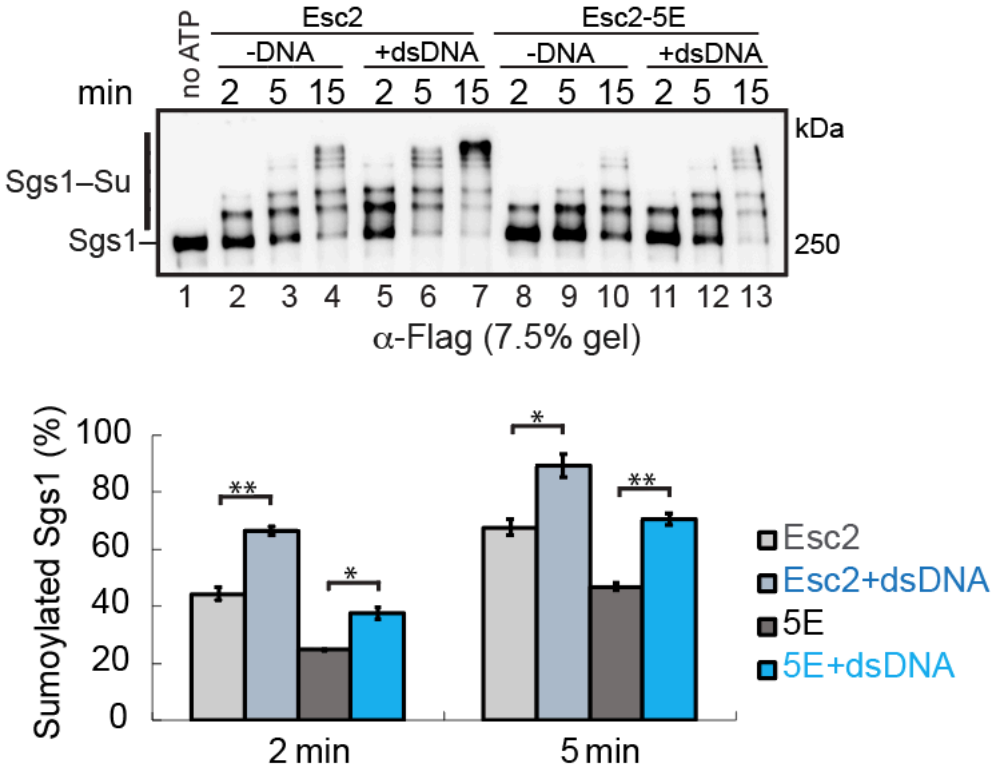

Figure 33: Sumoylation assay shows that Esc2-5E reduced sumoylation levels in both the presence and absence of dsDNA. This data was quantified and organized into a bar graph. Percentage of sumoylated Sgs1 showed mean ± SD (n=2 technical replicates). *p < 0.05; **p < 0.01; ***p < 0.001.

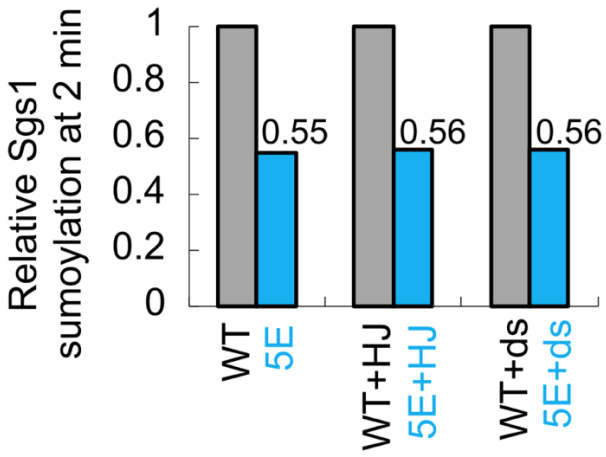

Figure 34: Quantified levels of Sgs1 sumoylation comparing WT Esc2 and Esc2-5E. Esc2-5E exhibited lower levels of sumoylation when compared to WT Esc2. Quantification was based on data shown in Figure 32 and Figure 33 at 2 min, setting the Sgs1 sumoylation level in reactions containing WT Esc2 as 1.00. 57 *p < 0.05; **p < 0.01; ***p < 0.001.

We wondered if the Esc2-MR domain is essential for regular Esc2 stimulation of Sgs1 sumoylation in the absence of DNA. Sumoylation assays were performed that compared the effect of Esc2, Esc2-5E, Esc2-SLD2m, and Esc2-MRΔ with no DNA present. It was shown that Esc2-5E and Esc2-MRΔ had no stimulation of Sgs1 sumoylation (Figure 35). When quantified, it was shown that in the presence of Esc2-WT, Sgs1 was sumoylated around 46% in 2 minutes. Whereas, in the presence of Esc2-5E, Esc2-MRΔ, and Esc2-SLD2m, Sgs1 was only sumoylated a little over 20% in 2 minutes comparable to that of no Esc2 condition. This data indicates that the Esc2-MR domain is essential for Esc2-based stimulation of Sgs1 sumoylation *in vitro*, and is not dependent on DNA for this stimulation.

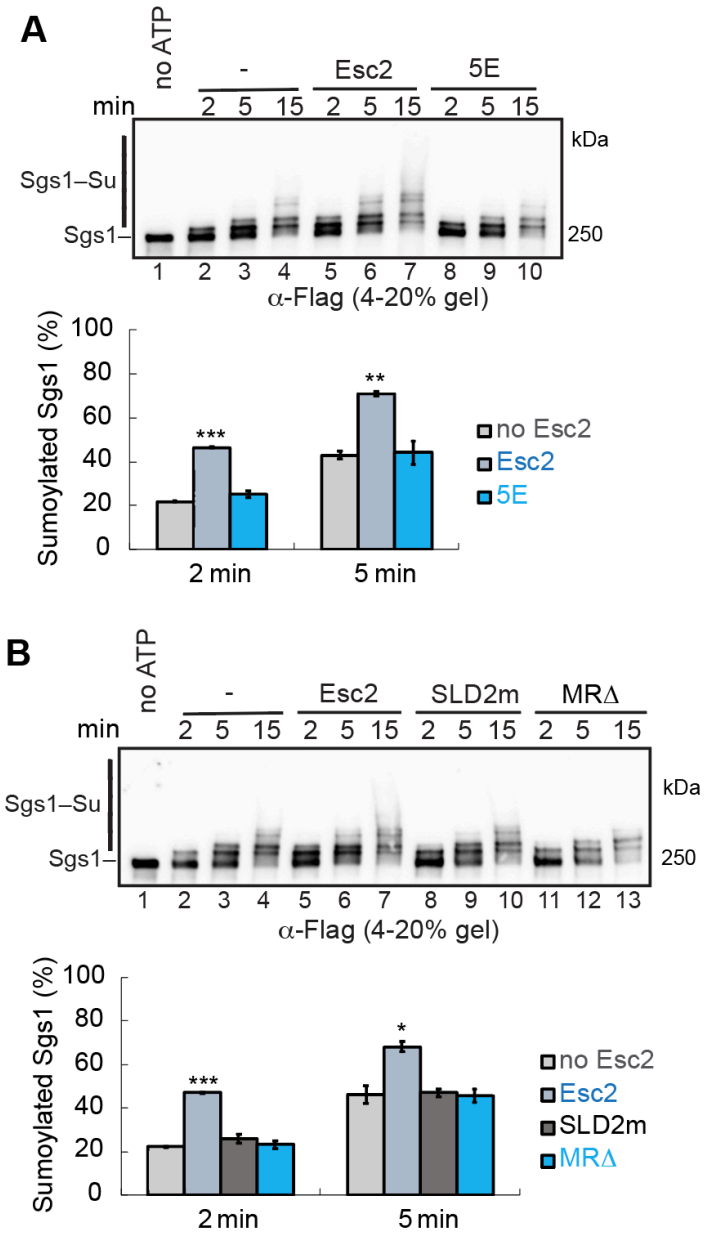

Figure 35: Sumoylation assay showing Esc2-5E, Esc2-SLD2m, and Esc2-MRΔ decreased sumoylation stimulation. Esc2-WT maintained normal levels of sumoylation, while (A) Esc2-5E, (B) Esc2-SLD2m, and Esc2-MRΔ showed decreased levels of Sgs1 sumoylation. Data was quantified and organized into a bar graph. The quantified percentage of sumoylated Sgs1 showed mean ± SD (n=2 technical replicates). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Esc2-MR domain mutants maintain Esc2 interaction with Ubc9

As previously stated, Esc2 uses its SLD2 domain to bind to the Ubc9 backside. Continuing from the previous finding that Esc2 SLD2m and Esc2-MRΔ both had similar decreased levels of Sgs1 sumoylation, a pull-down assay was performed to see if the Esc2-MR domain mutants also abolished Esc2 interaction with Ubc9. It was found that. Esc2-MRΔ and Esc2-5E still maintained interaction with Ubc9, while Esc2-SLD2m did not interact with Ubc9 (Figure 36). This provides evidence that the detected decrease in Sgs1 sumoylation levels with Esc2-MR mutants are not due to disrupting the Esc2-Ubc9 interaction.

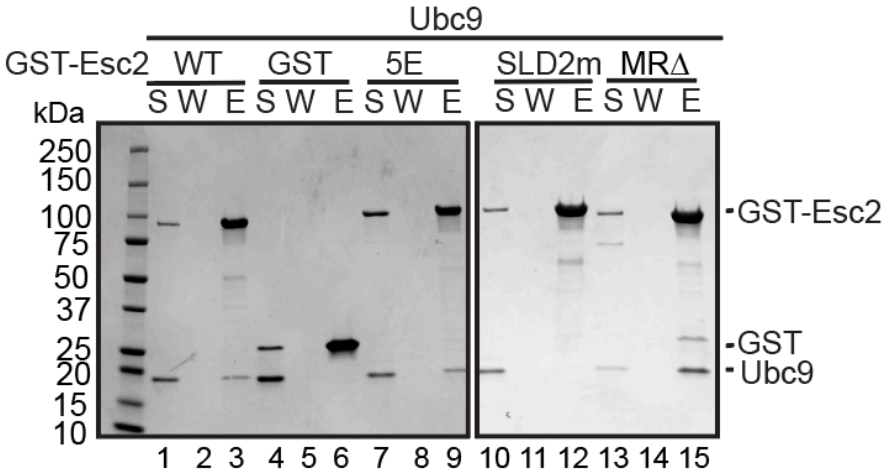

Figure 36: Pull-down assay showing that Esc2-5E and Esc2-MRΔ did not lose interaction with Ubc9. Esc2-SLD2m is consistent in its abolishment of Ubc9 interaction, while Esc2-5E and Esc2-MRΔ maintain this interaction with Ubc9. Purified GST, GST-Esc2 or its variant proteins bound to glutathione beads were examined for their abilities to pull down Ubc9. The assay was examined by SDS-PAGE, and pictures

of representative gels after Coomassie blue stain are shown. (S) Supernatant, (W) wash, (E) eluate.

Esc2-MR is crucial for STR sumoylation in vivo

Next, our collaborator utilized cell-based assays to examine the importance of the MR domain in Sgs1 sumoylation. It was found that cells with Esc2-5E and Esc2-MRΔ had reduced sumoylation levels when compared to WT cells. Cells depleted of Esc2 also had similar findings (Figure 37). This is in-line with *in vitro* findings that Esc2 and its MR domain are important for STR sumoylation.

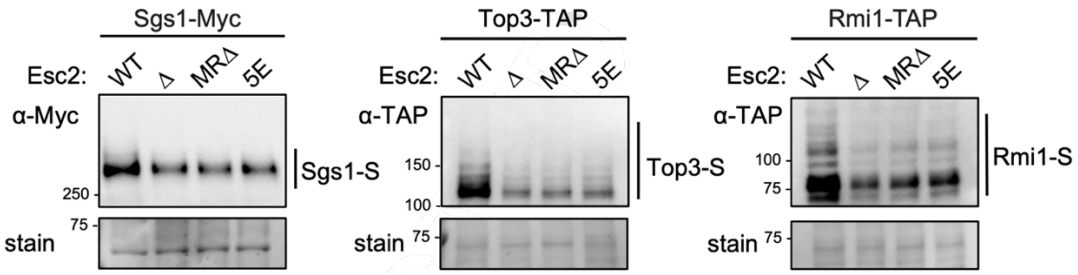

Figure 37: Sumoylation levels of STR subunits were reduced in cells with Esc2-MR mutants (Esc2-MRΔ and Esc2-5E) when compared to WT cells. Cells depleted of Esc2 (Δ) also showed reduced sumoylation levels.

Esc2 mutants worsen genotoxic sensitivity of cells

Our collaborator also examined the genotoxic sensitivity of various *esc2-MR* mutants (Figure 38). The HJ removal pathway mediated by the STR complex acts in parallel with the Mus81-Mms4-mediated HJ removal pathway, so it would be expected that *esc2-MR* mutants would have negative genetic interactions with *mms4* mutants. Data

showed that *esc2*Δ *mms4*Δ double mutants showed stronger sensitivity to MMS, and also slower growth than other mutants (Figure 38). When *esc2-5E* or *esc2-MR*Δ mutants were combined with *mms4*Δ mutants, MMS sensitive was also increased, but sensitivity was suppressed when introduced to a *rad51*Δ mutant, which suggests that the Esc2-MR is involved in STR-based HJ removal. *Esc2-5E* had a stronger phenotype than *esc2-sld2m*, possibly because it disrupted DNA binding as well as other unknown functions. Additionally, the *esc2-5E-SLD2m* combined mutation showed a worse phenotype than the single mutations. It is possible that the MR and SLD2 have separate roles in cells.

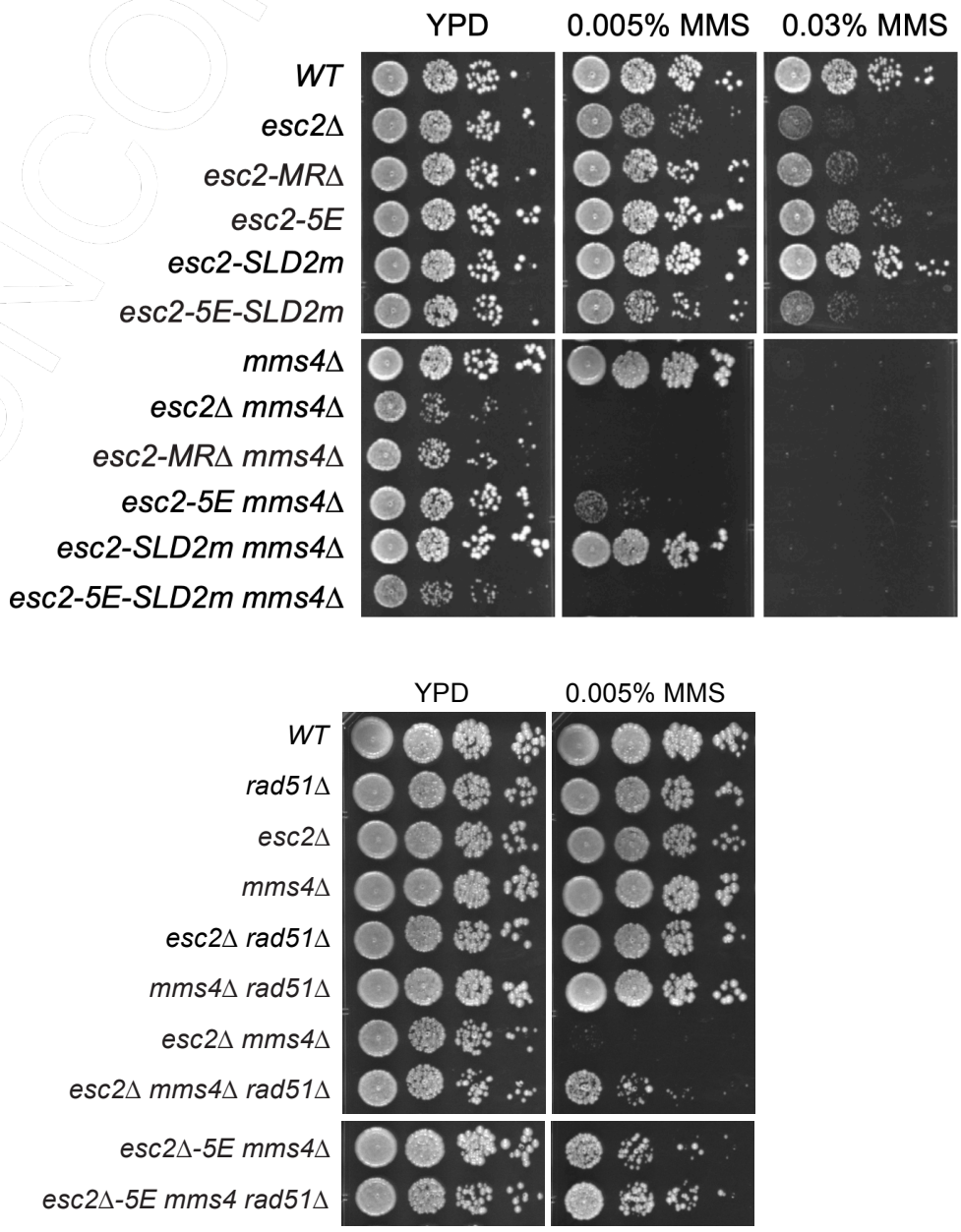

Figure 38: Genetic sensitivity of various *esc2* mutants. *Esc2* mutants combined with *mms4*Δ mutants showed to be more susceptible to MMS. Sensitivity was suppressed when introduced to a *rad51*Δ mutant, which suggests that the Esc2-MR is involved in STR-based HJ removal. Cells were spotted in 10-fold serial dilutions and grown for 2 days at 30°C.

Future directions

STR does interact with Smc5/6 in vitro

GST-pull downs were performed to detect interaction between the Smc5/6 complex and the STR complex (Figure 39A). This assay shows that STR alone can pull-down and interact with the Smc5/6 complex. This confirms that the Smc5/6 complex plays a direct role in STR sumoylation.

Another GST pull-down was performed to examine a subcomplex of Smc5/6 complex, the Mms21/Smc5 complex. This assay shows that STR can pull-down Smc5 and Mms21 (Figure 39B) . However, the signal for Smc5 seems stronger than the signal for Mms21. This data may suggest that the interaction occurs directly with Smc5.

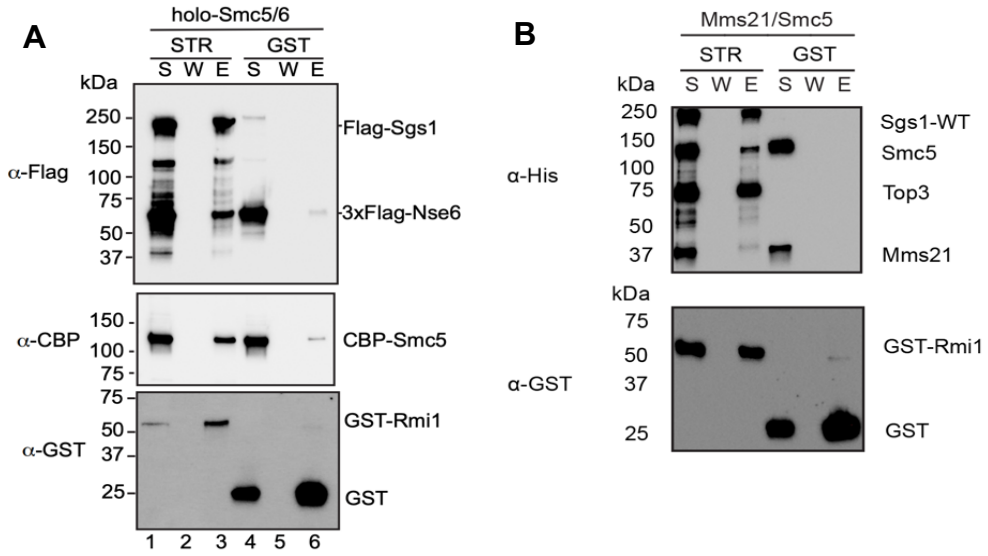

Figure 39: GST pull-down assay showing interaction between the STR complex and the Smc5/6 complex (A) and its subcomplex Mms21/Smc5 (B). STR was able to pull-down Nse6 and Smc5, subunits of the Smc5/6 complex. STR was also able to pull down Smc5 and, to a lesser extent, Mms21.

Purification of various components of the Smc5/6 complex

To determine which of the 8 components of the Smc5/6 complex contributes to the interaction with STR, each of the components or their subcomplexes has to be purified.

The purification of Nse5-Nse6 is a multiple step process. Nse5-Nse6 was co-expressed in BL21(DE3) cells and purified for future use (Figure 40). These proteins will be used in future GST-pull down and sumoylation assays to elucidate which component of the Smc5/6 complex is responsible for stimulating STR sumoylation.

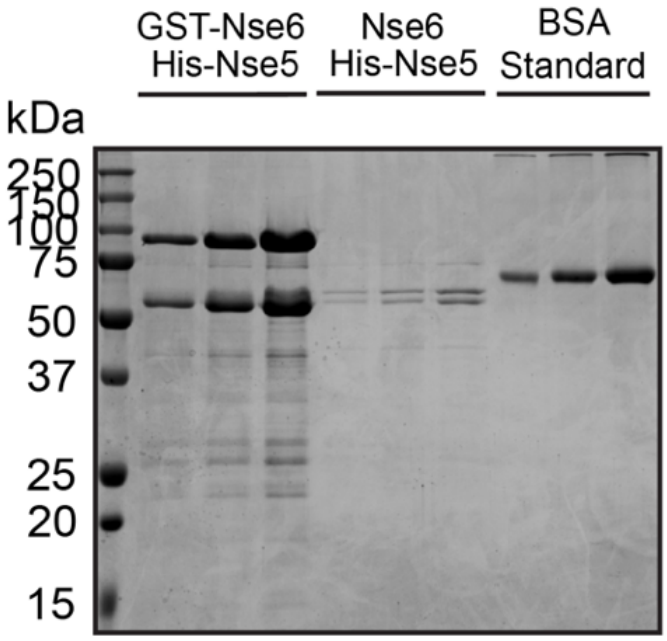

Figure 40: Final concentration of Nse5-Nse6 proteins. The concentration of GST-Nse5/6 was determined to be 0.25 µg/µL in T200. The concentration of Nse5/6 was determined to be 0.03 µg/µL in T200.

IV. CONCLUSION AND FUTURE DIRECTION

Protein sumoylation plays an important role in regulating many cellular processes, and it still remains unclear how the limited number of sumoylation proteins can efficiently modify a large number of substrates in the pathway. While previous studies have suggested that Esc2 may be a general SUMO E3 regulator, our data actually suggests that Esc2 can act as a SUMO E2 cofactor to enable specific substrate sumoylation. Our *in vivo* and *in vitro* data shows that Esc2 binds Ubc9 using its SLD2 domain in sumoylation stimulation. We also found that Esc2 regulates the sumoylation of a specific set of Mms21 substrates that associate with HJ and replication fork structures. This suggests that Esc2 regulation may be DNA structure-specific.

Our data also demonstrates that Esc2 interaction with Ubc9 is through its SLD2 domain, and not its SLD1 domain. An Esc2 variant with a mutation in the SLD1 domain did not abolish the interaction with Ubc9, while a mutation in the SLD2 domain did abolish the interaction. Furthermore, the Esc2-SLD2m mutant displayed a similar decrease in substrate sumoylation to that of cells deleted of Esc2. We also established an *in vitro* sumoylation system to further study the sumoylation of the STR complex, specifically Sgs1 and Top3, where we showed that Esc2 directly stimulates sumoylation dependent on its interaction with Ubc9. When the Esc2-SLD2 domain was replaced with a SUMO motif, it acted as wild type Esc2, further verifying the dependency of Esc2 on its Ubc9 interaction for sumoylation.

We also did studies on how the Esc2-Ubc9 interaction affects genome maintenance. As previously mentioned, cells with Esc2-SLD2 mutations have impaired sumoylation. Esc2-SLD2 mutations were further found to have an increased level of X-

shaped molecules found via DNA 2-dimensional (2D) agarose gel electrophoresis. Although Esc2 also interacts with the Mus81-Mms4 complex, these results are likely an impairment in STR-mediated HJ clearance, and not through the Mus81-Mms4 complex as Esc2-SLD2 mutant cells were still able to bind to the Mus81-Mms4 complex (data not shown). In addition, Esc2-SLD mutant cells that also lacked Mms4 were more sensitive to MMS than single mutants. To continue our study on genome maintenance, we found that Esc2-SLD2 mutant cells had elevated GCR rates. While HJ accumulation might not be accounted for in a GCR assay, the smaller increase in GCR rates of Esc2-SLD2 mutant cells when compared to cells without Esc2 is consistent with the milder phenotype of Esc2-SLD2m seen in other assays. This data indicates that Esc2 does have some effect on genome maintenance.

This research also looked at the STR complex and its role in dissolving HJ. Our data first showed that Sgs1 binding to DNA stimulates its sumoylation. Secondly, we found that HJ-based stimulation of Sgs1 sumoylation is further enhanced by Mms21/Smc5, a subcomplex formed from the larger Smc5/6 complex that includes the SUMO E3 ligase Mms21. It is also enhanced by the addition of Esc2, which shares the similar DNA-binding function with Mms21. The DNA binding site of Mms21 is unknown, but the DNA binding site of Esc2 is within its MR.

To test the role of the MR domain of Esc2 in Sgs1 sumoylation, we used two Esc2 mutants, Esc2-5E and Esc2-MRΔ, that are defective in the DNA-binding region in an *in vitro* sumoylation system. If Esc2 DNA binding capabilities were essential for Sgs1 sumoylation, then it would be expected for sumoylation levels to be reduced in the presence of Esc2-5E and DNA, and remain the same in reactions without DNA.

However, it was seen that Esc2-5E reduced levels of sumoylation in both the presence and absence of HJ and dsDNA. This suggests that the Esc2-MR domain does contribute to STR sumoylation, but in a DNA-independent manner. This also raises the possibility that the Esc2-MR domain has some other unknown function.

Our data also shows that Esc2-MR and Esc2-5E still maintained interaction with SUMO E2 Ubc9. This provides evidence that the detected decrease in Sgs1 sumoylation levels with Esc2-MR mutants is not due to disrupting the Esc2-Ubc9 interaction.

In vivo data correlated with our previous findings of the role of Esc2-MR in Sgs1 sumoylation. When using *mms4* synthetic interactions as a genetic readout, *esc2-MR*Δ had a stronger phenotype than *esc2-5E*. This might be explained by a stronger defect of deleting the whole domain, rather than point mutations. *esc2-5E* had a stronger phenotype than *esc2-sld2m*, possibly because it disrupted DNA binding as well as other unknown functions. Additionally, the *esc2-5E-SLD2m* combined mutation showed a worse phenotype than the single mutations. It is possible that the MR and SLD2 have separate roles in cells. As previously discussed, the MR is involved with DNA binding, as well as interacting with Srs2 and the Mus81-Mms4 complex, while the SLD2 is involved with SUMO E2 binding and sumoylation. However, it is unknown if the two domains collaborate or act independently.

We also performed a few assays to test the possibility of interaction between the STR complex and the Smc5/6 complex. GST-pull down assays confirmed the interaction between the complexes, although it is unclear which part of each complex is required for the interaction. The signal for Smc5 was moderately stronger than the signal for Mms21.

This data may suggest that the interaction occurs directly with Smc5, although further testing would have to be done to confirm this.

Work was done to begin to purify various subcomplexes of the Smc5/6 complex in order to further elucidate the role of the complex in sumoylation. Nse5/Nse6 was successfully purified, although the GST-cleaved sample was not produced in high yields.

In summary, we have shed light on how the SUMO E2 Ubc9 protein works in sumoylation and how it aids in genome maintenance. We have also provided evidence for the direct role of DNA in STR sumoylation and how the Esc2-MR may have other functions in sumoylation outside of its DNA-binding abilities. These findings deepen our understanding of the mechanism underlying the sumoylation regulation of the STR complex.

The versatility of sumoylation across many different fields and knowledge of the sumoylation process has the potential to facilitate advancement in many fields, including the healthcare field. Sumoylation has been linked to cancers, neurodegenerative diseases, and regulation of the immune system. The sumoylation pathway and its enzymes have also been studied as a potential target to clinical anti-cancer therapeutics (65). This study on the mechanism of sumoylation enzymes and how they interact with each other may also help with understanding the mechanisms of certain diseases, and have the potential to make a large impact in the world of material science.

In the future, research will focus on Mms21/Smc5 and its role as subunit of the larger Smc5/6 complex. Mms21/Smc5 was used in many of the *in vitro* experiments as SUMO E3. However, *in vivo*, the whole Smc5/6 complex that contains other subunits with their own functions is present along with Mms21/Smc5. These include the Nse5/6

complex, and the Nse1/3/4 complex Future experiments will focus on if these other components contribute to or affect the functions of Mms21/Smc5. More specifically, the Nse1/3/4 complex has shown to facilitate DNA binding to the Smc5/6 complex (16). We want to see if the DNA binding capabilities of other Smc5/6 components affect STR sumoylation.

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