SUMOylation at the centromere: The role of SUMOylation of DNA topoisoranase IIα C-terminal domain in the regulation of mitotic kinases in cell cycle progression.

By

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ABSTRACT

In many model systems, SUMOylation is required for proper mitosis; in particular, chromosome segregation during anaphase. It was previously shown that interruption of SUMOylation through the addition of the dominant negative E2 SUMO conjugating enzyme Ube9 in mitosis causes abnormal chromosome segregation in *Xenopus laevis* egg extract (XEE) cell-free assays, and DNA topoisomerase IIα (TOP2A) was identified as a substrate for SUMOylation at the mitotic centromeres. TOP2A is SUMOylated at K660 and multiple sites in the C-terminal domain (CTD). We sought to understand the role of TOP2A SUMOylation at the mitotic centromeres by identifying specific binding proteins for SUMOylated TOP2A CTD. Through affinity isolation, we have identified Haspin, a histone H3 threonine 3 (H3T3) kinase, as a SUMOylated TOP2A CTD binding protein. Haspin is important for phosphorylating H3T3 at the centromeres during M phase, which is essential for the recruitment of chromosomal passenger complex (CPC) to the centromere of chromatin for the proper progression of mitosis. However, the mechanism of Haspin localization on the centromere to target centromeric H3T3 was not clearly understood. We determined that Haspin is enriched at the centromeres of sister chromatids with TOP2A in a SUMO-dependent manner in mitotic XEE as interruption of SUMOylation caused a reduction in Haspin’s centromeric localization as well as in centromeric H3T3 phosphorylation (H3T3p). Mutations in TOP2A CTD SUMOylation sites or Haspin’s SUMO-interacting motif (SIM) reduced the binding interaction between TOP2A and Haspin on chromosomes. Haspin and SUMOylated TOP2A CTD interaction is also specific to mitotic XEE. A characteristic of Haspin during mitosis is that Haspin is hyperphosphorylated. We have discovered that T206 phosphorylation regulates TOP2A and Haspin binding interaction as a
T206A mutant had a reduction in binding, and the T206A SIM mutant caused a greater binding reduction. This dissertation shows a dual post-translational modification regulation of TOP2A-Haspin interaction that regulates Aurora B at the centromere. Having multiple means in regulating Haspin localization and activity allows for the cell to ensure proper timing in CPC localization for the progression of mitosis. Furthermore, TOP2 inhibitors caused an increase in the SUMOylation of TOP2A as well as a upregulation in the SUMO-binding proteins and Aurora B. Determining the role of TOP2A SUMOylation will help in understanding the mechanism for proper chromosome segregation during mitosis.
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I dedicate my dissertation at the University of Kansas to my graduate studies mentor, Dr. Yoshiaki Azuma. Dr. Azuma allowed me to join the lab in April of 2011 to continue on the DNA topoisomerase IIα project that was developed by a previous graduate student. Dr. Azuma pushed me into moving my research forward independently and provided intellectual discussion for my growth. With his teaching, I believe I have grown to think and act as a scientist.

Over the years of working in Dr. Azuma's lab, I have thought about my potential future of continuing my path in research on chromosome dynamics in Japan rather than in the United States. During my third year in the Azuma lab in 2014, Dr. Azuma and I attended the international SUMO conference that was held in Shanghai, China in May. To express my desire to potentially work in Japan, I initiated a conversation with a Japanese professor who was attending the conference. Dr. Azuma was an acquaintance of the Japanese professor, and the two professors recommended and started to plan my visit with a few professors in Japan as I was going to visit Japan on the way back from the conference. Visiting a few professors concreted my desire to work in Japan, and the few professors recommended me to attend the annual Japanese chromosome conference, which was to be held in December that year. After discussing with Dr. Azuma, he allowed me to attend the conference. However, not only did Dr. Azuma allow me to take time off to attend the conference, but he also notified a few professors in Japan that I was planning to attend. During the trip, I was able to present my research at the conference, discuss with other scientists that attended, notify many that I was interested in doing research in Japan, attend several labs at Japanese universities, and present in seminars. All in all, within my graduate student life at the University of Kansas, I attended a Japanese national conference,
visited nine labs in five universities and two national research institutes, and presented three seminars in Japan. For my future, I look forward to continuing on the path of science in Japan.

I would like to thank Dr. Mizuki Azuma who has supported my work and watched over my progress as a scientist from the beginning of my graduate student life in Dr. Yoshiaki Azuma's lab. She has always provided critical insight on my research as well as help me continue to enjoy my research. I would like to thank my committee members who has helped and guided me. They have always provided advice on issues that I needed to be careful on as I continue my path in research.

With that, I hope the work that I have done in the Azuma lab will help Dr. Azuma advance the lab's research.
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CHAPTER 1
INTRODUCTION TO SUMO AND DNA TOPOISOMERASES

The cell utilizes many different post-translational modifications in order to regulate its cellular processes in cell division and for survivability. These modifications range from phosphorylation to methylation to acetylation to ubiquitination to glycosylation and to many more. The studies of ubiquitination have led to many interesting discoveries in the protein degradation pathway as well as cellular activity (1-6). Interestingly, the discovery of ubiquitin-like protein modifications opened to many different fields of research (7). The discovery two decades ago of an ubiquitin-like protein, SUMO, or small ubiquitin-related modifier, has led to a surge of reports on the protein and its enzymes involved in the reversible modification of target proteins. In this dissertation, the post-translational modification that is primarily examined is the SUMO modification, or SUMOylation, and its role during cell division.

The discovery of SUMO

The SUMO gene was first identified through genetic screens suppressing the centromeric protein Mif2 (8). SUMO was then characterized as a binding protein to RAD51 and RAD52 (9). However, the SUMO proteins were found as a covalent binding protein of Ran GTPase-activating protein RanGAP1 in nuclear import studies in mammalian cells (10, 11). The SUMOylation of RanGAP1 allows for its interaction with RanBP2 of the nuclear pore complex on the cytoplasmic side (12).
Figure 1.1. Structures of ubiquitin and SUMO-1.

Ribbon structures of ubiquitin (blue) and human SUMO-1 (yellow) with an overlay of the two structures to show the similarities between the two proteins. Figure adapted from Dohmen, 2004 (13).

The name for the SUMO protein is derived from its characterization similar to ubiquitin. In fact, although the sequence identity between ubiquitin and SUMO1 is only around 18%, the structure of human SUMO1 obtained through NMR was determined to be similar to the crystal structure of ubiquitin (Fig. 1.1) (14, 15). Both proteins contain characteristics of a ββαββαβ fold with a di-glycine C-terminal tail. The N-terminal domain is unique to the SUMO structure,
which has implications for the binding of additional SUMO proteins to form a SUMO chain (16, 17).

SUMO is found in all eukaryotes. *Saccharomyces cerevisiae, S. pombe, C. elegans,* and *D. melanogaster* only contain one SUMO isoform (13, 18). Humans and other vertebrates, on the other hand, contain multiple isoforms. SUMO1, SUMO2, and SUMO3 have been seen to be ubiquitously expressed (19, 20). Interestingly, SUMO2 and SUMO3 have greater than 95% sequence identity while SUMO1 has less than 50% identity to the other two SUMO isoforms. Due to the almost identical sequence identity, SUMO2 and SUMO3 are considered indistinguishable and are often referred to as SUMO2/3. SUMO4 has also been discovered to be expressed primarily in the kidney, lymph node, and spleen but not much is yet known of its modification (20). While the SUMO modification is essential for cell viability in organisms including *S. cerevisiae, C. elegans,* and mice, disrupting the *S. pombe* SUMO (termed pmt3p) gene still leads to viable cells (21-24).

**SUMO E1 activating and E2 conjugating enzymes**

Like the ubiquitination pathway, the SUMOylation pathway is very much the same and contains E1, E2, and E3 enzymes. The heterodimer of Aos1 and Uba2 (SAE1 and SAE2 in budding yeast) was discovered to be the E1 SUMO-activating enzyme complex (22, 25-28). The E1 heterodimer interacts with SUMO by binding non-covalently to the SUMO, and through the use of ATP, forms a thioester bond between Uba2’s cysteine and SUMO (29). Interestingly, while E1 enzyme complex is essential for SUMO modification, in *S. pombe* that do not depend on SUMO for cell viability, deletion of the Aos1 orthologue Rad31 showed some SUMO conjugation occurring within the cells (22, 30, 31). This suggests that Uba2 which contains the
active site that binds to SUMO may have low SUMO-activating enzyme activity. Additionally, the level of Aos1 has been found to fluctuate during the cell cycle in humans while Uba2 levels stayed unchanged (26). These results suggest that the main regulatory mechanism of the E1-activating enzyme depend on the Aos1 subunit.

Similar to the E1 SUMO-activating enzyme complex, there has only been one E2 SUMO-conjugating enzyme discovered. Activated SUMO is transferred from the E1 enzyme to the E2 enzyme known as Ubc9 by forming thioester intermediates (32-34). Ubc9 is conserved between mammals and budding yeast, and replacement of the budding yeast Ubc9 with the mammalian version shows similar SUMO-conjugating activity (35). Interestingly, binding studies between Ubc9 and SUMO shows that Ubc9 does not discriminate between the different SUMO isoforms (36). The E2 enzyme can also target a subset of SUMO substrates by binding at their SUMOylation consensus sequence ψKxE, where ψ is a hydrophobic residue and x is any amino acid. Ubc9 can then conjugate SUMO to certain substrates without the involvement of the E3 ligase as seen with the Ubc9-mediated SUMOylation of RanGAP (37).

**SUMO E3 ligases**

In most cases, an E3 ligase is necessary to direct the SUMO protein and the E2 enzyme to the substrate for modification. E3 ligases interact with the Ubc9-SUMO complex and the substrate to direct the SUMO modification on the lysine of the substrate that is usually within the SUMOylation consensus site ψKxE. The first E3 ligases that were discovered were in *S. cerevisiae* known as Siz1 and Siz2 (38, 39). Siz1 mediates septin and PCNA SUMOylation and Siz2 overexpression could enhance the SUMOylation of Pds5 (38, 40-42). Homologous to the Siz proteins were protein inhibitor of activated STAT, or PIAS proteins. Both Siz proteins and
PIAS proteins contained an ubiquitin ligase RING-like domain, which was named Siz/PIAS RING, or SP-RING (38, 43, 44). Four genes encode five PIAS family proteins in mammals: PIAS1, PIASxα, PIASxβ, PIAS3, and PIASy/PIAS4 (45-51). Another SP-RING domain containing SUMO ligase is Mms21/Nse2 which is part of the Smc5/Smc6 complex (52-54). These SP-RING enzymes are thought to function depending on localization, cell cycle, and substrate specificity.

Three other types of proteins with E3 ligase activity exist. One type is the nucleoporin RanBP2/Nup358 (55). RanBP2 localizes on the cytoplasmic side of the nuclear pore complex and SUMOylates RanGAP. Its role from previous studies is to interact with SUMOylated RanGAP at the kinetochore for the attachment of microtubules (56-58). The third type of E3 ligase is the polycomb group protein PC2. Polycomb group proteins form large complexes that can mediate the silencing of transcription by modifying histones. PC2 could recruit another protein to large complexes to stimulate its SUMOylation (59-61). A fourth type of E3 ligase is the transcriptional co-repressor KAP1 that mediates SUMOylation through its zinc binding PHD finger (62).

![Figure 1.2. Mechanism of SUMOylation.](image)
SUMO binds to the cysteine of Aos1/Uba2 (E1) complex, which is then transferred to Ubc9 (E2). Ubc9 can SUMO-modify specific substrates such as RanGAP1 by itself, or with the help of the E3 ligase, it can SUMOylate other substrates. Figure adapted from Melchior et al., 2003 (63).

The process of SUMOylation

Conjugation of SUMO onto proteins primarily involves the activity of the E1 SUMO-activating enzyme, E2 SUMO-conjugating enzyme, and E3 SUMO ligase (Fig. 1.2) (63). The exception is that the E2 enzyme Ubc9 can also SUMO-modify certain proteins without the help of an E3 ligase (37). Using the mature form of SUMO, the E1 is activated by ATP to form the SUMO adenylate to allow for a thioester bond between SUMO and the cysteine of Uba2 (29). The E1-SUMO intermediate binds with Ubc9 to transfer the SUMO protein to the cysteine in the Ubc9 active site through a thioester bond (12, 32, 33, 64). E3 ligase can bind to its substrate and Ubc9 directly while binding to SUMO non-covalently. Binding to the substrate, Ubc9, and SUMO allows the E3 ligase to function as a platform to position the Ubc9-SUMO complex and the substrate for a favorable transfer of SUMO. The main domain that characterizes most E3 ligases is the SP-RING domain that forms a zinc finger (43). Through the E3 ligase, SUMO is transferred to the substrate often at a SUMOylation consensus site of ψKxE, where ψ is any large hydrophobic amino acid and x is any amino acid, which was originally found through the mapping of SUMOylated substrates RanGAP1, PML, p53, and others (65-70). SUMO conjugates onto the lysine of the substrate within the canonical site to finish the SUMOylation process. It is also possible for SUMO enzymes to conjugate SUMO isoforms to non-canonical SUMOylation sites, which is seen on the SUMOylation of *Xenopus laevis* DNA topoisomerase IIα at lysine 660 of the sequence RKEW (71).
**SUMO and SUMO-interacting motifs**

Not only does SUMO participate in covalent binding onto proteins, it can also interact with proteins through non-covalent interactions. A motif called the SUMO-interacting motif, or SIM, has been discovered to interact with SUMO through hydrophobic interactions (46, 72-74). This sequence mainly contains a short sequence of large hydrophobic residues that are flanked by acidic residues or serines. It is thought that the negative charges surrounding the hydrophobic core residues through acidic residues or phosphorylated serines could help stabilize the non-covalent interaction. NMR and X-ray studies show that SIMs form β-strands that bind to SUMO parallel to the α-helix and β-strand (72, 73, 75, 76). However, it was found that the interaction between the SUMO protein and the SIM sequence is relatively weak and requires high micromolar ranges of the proteins due to the short interaction surface (73, 76).

KAP1 is a co-repressor in gene silencing that has been reported to be SUMOylated (62). KAP1 SUMOylation has been shown to bind to both histone methyltransferase SETDB1 and CHD3 of the NuRD complex through their SIMs. Through the SUMO-SIM interaction, SUMOylated KAP1 could regulate SETDB1 histone methyltransferase activity for gene regulation. Another protein involved in transcriptional regulation is the transcriptional repressor Daxx. While Daxx is known to be SUMOylated, it also contains SIMs. Mutations in a SIM cause defective gene silencing activity (77). Interestingly, Daxx C-terminal SIM is also necessary for the UV irradiation-mediated apoptosis (78). Ubiquitin ligase RNF4 in mammals has also been found to recognize SUMOylated proteins. Its SUMO-SIM interaction helps in targeting proteins for degradation, making RNF4 part of the group of SUMO-targeted ubiquitin ligase, or STUbl.
Further studies in the role of SUMOylation will identify many other SUMO-binding proteins through SIMs.

Figure 1.3. Functions of SUMO.

A chart representing the possible functions of SUMO modification. SUMOylation can allow for specific localization of proteins, for protein interactions and protein complex formation, for enzyme regulation, and for targeting SUMO-conjugated proteins for degradation by the proteasome or the SUMO isoform covalently bonded to the lysine can prevent the ubiquitination of that lysine to prevent its targeting to the proteasome.

Figure adapted from Cubenas-Potts and Matunis, 2013 (82).

**SUMO-modified targets and the molecular consequences**

SUMOylation of proteins is similar to other post-translational modifications in that SUMO can regulate one or multiple characteristics or functions of its modified protein. Among
them are: localization, protein interaction, enzymatic activity, and protein degradation (Fig. 1.3) (82).

A recent report examined the role of the ubiquitin E3 ligase TRAIP/RNF206 (83). TRAIP has diverse functions by acting in cell cycle progression, DNA damage response, and DNA damage repair. However, how it localized to the nucleus remained unclear. Interestingly, TRAIP was found to be SUMOylated at five SUMO acceptor sites. Mutations in the SUMO acceptor sites to prevent its SUMOylation caused the cell to not retain TRAIP in the nucleus, suggesting that SUMOylation is necessary to keep TRAIP in the nucleus. This demonstrates that SUMO modification can regulate a protein’s localization.

SUMOylation of substrates also allows SUMO to function as a mediator for protein interactions and complexes. As explained in the section above, SUMOylation can cause novel protein interactions by allowing other proteins with SIMs to bind onto the surface of SUMO, as seen with the SUMO-targeted ubiquitin ligase RNF4 and SUMOylated KAP1 with SETDB1 and CHD3 (62, 79, 80). Another known SUMO-binding interaction is through PML bodies. PML bodies are SUMOylated and act as scaffolds to allow associated proteins to bind (84, 85).

While several SUMOylation targets are known to affect their regulation of transcription, such as Daxx and KAP1, SUMO can also modify proteins with enzymatic activities. AMP-activated protein kinase (AMPK) complex has a kinase activity in regulating cellular energy. The subunit AMPKβ2 is SUMOylated which enhances the trimeric AMPK complex kinase activity (86). Another enzyme SUMOylated is the human pancreatic glucokinase, which when SUMOylated, the glucokinase can increase its activity (87). However, SUMOylation does not only upregulate enzymatic activity, but can also play a role in the inhibition of catalytic activity. The E1 subunit Uba2/SAE2 contains the domain with the active site for the SUMO-activating
enzyme. However, its cysteine is surrounded by lysines that can be SUMOylated. While SUMOylation at the domain does not affect SUMO adenylation or the thioester bond between the E1 and SUMO, it can greatly inhibit its transfer of SUMO to the E2 conjugating enzyme (88).

The last known function of SUMO is its ability to promote or prevent protein degradation. As mentioned in the previous section, RNF4 is part of the family of SUMO-targeted ubiquitin ligase containing a SIM sequence (79-81). While ubiquitination is the key modification for the degradation of proteins, proteins that are targeted for degradation may first be SUMOylated. SUMOylation of those proteins can allow STUbI enzymes to bind in order to ubiquitinate the targeted proteins to be degraded by the proteasome. Additionally, since both SUMO isoforms and ubiquitin can covalently bind lysines, SUMOylation can prevent the target protein from becoming ubiquitinated at the same lysine as seen with PCNA at lysine 164 (41).

**SUMO proteases**

While SUMOylation is important for cell viability, removal of the SUMO modification is equally important. Among the first SUMO proteases identified were the ubiquitin-like proteases Ulp1 and Ulp2/Smt4. Ulp1 was identified as a cysteine protease in the processing of the C-terminal tail of SUMO to reveal its di-glycine end (89). A null ulp1 mutant in *S. cerevisiae* with mature SUMO peptides showed poor growth (89). Ulp2-mediated deconjugation of SUMO is also important for cells. A previous study examining the roles of mutant ulp2 and uba2 showed that a balance between SUMO conjugation and deconjugation is important for the proper function of SUMO (90, 91). A crystal structure of the catalytic domain of Ulp1 shows that it contains Cys-His-Asp residues in the active site similar to other cysteine proteases (31, 89). Ulp1
also contains a hydrophobic tunnel close to the active site that may recognize the SUMO di-glycine motif.

Figure 1.4. Comparison of SUMO proteases.

A schematic representation of the SUMO proteases in yeast (Ulp1/2) and mammals (SENP5). The catalytic domain (CD) is conserved in all SUMO proteases. Figure adapted from Kim and Baek, 2009 (92).

Mammals contain multiple SUMO proteases that share the C-terminal catalytic domain of Ulp1 (Fig. 1.4) (92, 93). These SUMO-specific proteases (SENP5) are characterized differently by their N-terminal domains which may allow for distinct functions, such as localization and target specificity. SENP1, SENP6, and SENP7 are found primarily in the nucleus (94-98), while SENP2 localizes in the nucleoplasmic side of the nuclear pore complex (99). SENP3 and SENP5 are reported to be found in the nucleolus (100, 101). Interestingly,
SENP6 and SENP7 catalytic domains are split into two domains each with an inserted sequence (102, 103). Biochemical analysis of the catalytic domains showed that they could process poly-SUMO2/3 chains, suggesting that the split catalytic domain functions in recognizing and processing SUMO chains (104).

**SUMOylation during mitosis.**

The process of SUMO modification during mitosis has essential roles in cell viability. Defects in the SUMOylation pathway such as the inhibition of SUMOylation or the mutations/deletion of SUMO processing enzymes has led to chromosome segregation defects (105-108). Azuma and colleagues were one of the groups to show, through the use of *Xenopus* egg extract (XEE), that mitotic chromosomes were defective in separating properly when SUMOylation was inhibited (Fig. 1.5) (105). In *S. cerevisiae*, SUMO modification deficiency led to short spindles and cohered sister chromatids during separation (22, 89, 91, 109, 110). Cells with mutations in the SUMO protease Ulp2 partially recovered from the deficiency (90, 91). However, knockdown of Ulp2 caused hypersensitivity to microtubule depolymerizing drugs and chromosome segregation impairment, suggesting that not only is SUMOylation necessary for proper mitosis, but deSUMOylation is also essential. The identification of the SUMO protein (Smt3 in budding yeast) and the SUMO protease Ulp2 (Smt4 in budding yeast), was through its connection to the centromeric protein Mif2, suggesting that SUMOylation has a role at the centromere and kinetochore regions (8).
Figure 1.5. Inhibition of SUMOylation during mitosis causes chromosome segregation defects in XEE.

A chromosome segregation assay using CSF mitotic Xenopus egg extract (XEE) without (Control) or with dominant negative E2 SUMO-conjugating enzyme Ubc9 (dn Ubc9) to inhibit SUMOylation. Tubulin is labeled in red while DNA is labeled in green. After 30 mins of sperm nuclei addition, chromosomes are aligned at the metaphase plate. Onset of anaphase soon after shows chromosomes segregating at 40 min and 45 mins in the control sample. However, addition of dn Ubc9 to inhibit global SUMOylation in the XEE causes chromosome segregation defects during anaphase (40 min and 45 min). Figure adapted from Azuma et al., 2003 (105).

Several proteins SUMOylated during mitosis have been characterized. The SUMOylation of RanGAP with RanBP2 helps at the kinetochore to attach microtubules (56, 58). Chromosome dynamic proteins cohesin and condensin are also seen to be SUMOylated (111). Pds5, while maintaining the cohesion of the sister chromatids, is SUMOylated to allow for its protein
degradation to dissolve the cohesion (42). Also among the mitotic proteins that have been found to be SUMOylated is the DNA topoisomerase type II (TOP2), and its known roles when SUMOylated will be explained later in this chapter as well as in later chapters (112).

**DNA topoisomerase family**

Topoisomerases are essential enzymes found in all organisms for the processing of the topology of nuclear DNA. These enzymes regulate the double stranded helical DNA by winding the double helices for either the creation or removal of DNA tangles (113-116). Two different mammalian classes of topoisomerase have been found – type I and type II – and within each types are different isoforms of the enzyme. Interestingly, both topoisomerase types I and II are able to be modified by SUMO proteins (112, 117).

Type I topoisomerases (TOP1) act as monomeric enzymes by creating single-stranded breaks in the DNA (115, 118, 119). Through these breaks, TOP1 can control the rotation of the helix at and around the breaks it creates. Because of this reaction, TOP1 can only wind or relax supercoiled DNA and cannot resolve DNA entanglement. On the other hand, type II topoisomerases (TOP2) function as dimers in resolving the topology of dsDNA through the hydrolysis of ATP to introduce temporary double-stranded DNA (dsDNA) cleavages in its DNA strand passage activity ([Fig. 1.6](#)) (113, 120-123). To do so, it binds to the dsDNA using active tyrosines for covalent bonding with the DNA phosphate backbone to form phosphotyrosyl bonds (116, 118, 121, 122). After capturing and cleaving the dsDNA, it passes an additional dsDNA through the cleaved opening of the first dsDNA and through the C-gate. Once the second dsDNA passes through, the cleaved dsDNA is ligated together by reversing the cleavage reaction and TOP2 dissociates from the re-ligated DNA. Because TOP2s can function with different
topologies of DNA, TOP2s can contribute to supercoiling as well as create or resolve tangled DNA.

Figure 1.6. Mechanism of DNA topoisomerase II activity.

A cartoon representation of the DNA topoisomerase II (TOP2) activity. TOP2 acts as a homodimer and opens the N-gate to allow in the first dsDNA strand (G segment, blue), which binds to the active site. It binds another strand (T segment, red) for transfer and creates a closed N-gate intermediate. Hydrolysis of ATP will allow TOP2 to create a dsDNA break on the G segment and pass the T segment through to the C-gate. The C-gate opens to release the T segment, and TOP2 ligates the dsDNA break together, allowing TOP2 to start the mechanism over again. Figure adapted from Nitiss, 2009 (123).
**DNA topoisomerase II isoforms**

Many processes within the eukaryotic cell cycle require the function of TOP2 isoforms to change the topology of the DNA. These processes include DNA replication, transcription, chromosome condensation and decondensation, and chromosome segregation (114, 115, 124). While yeast and Drosophila only contain one form of TOP2, many other eukaryotes including humans contain α and β isoforms (TOP2A and TOP2B, respectively) (125). Sequence comparison between the two isoforms reveals around 70% identity with the majority of the differences found in the C-terminal region of the enzymes (115, 126-130). While the isoforms are very similar, they each have different cellular functions with TOP2A being the major essential isoform for survival.

Although both isoforms have equivalent topoisomerase activity, TOP2B cannot compensate for the loss of TOP2A, suggesting non-redundant functions in the cell (125, 131-133). TOP2A functions during DNA replication to remove the tension in the DNA caused by the replication fork, and TOP2A is essential for proper chromosomal segregation during mitosis (114, 123, 124, 131, 134-136). While the reason for the importance of TOP2A during mitosis is not completely clear, TOP2A may condense and decondense chromosomes as well as help separate the sister chromatids. On the other hand, TOP2B is suggested to be the isoform involved in transcription (132, 137). However, the physiological role for TOP2B has not been determined rigorously.

While TOP2A activity is important in the cell, it is essential to have a fine balance between the two enzymatic functions – DNA cleavage and DNA ligation – to ensure that DNA damage does not occur (Fig. 1.7) (113, 120, 138, 139). If the cleavage activity is too low, the cell can undergo quiescence and cause mitotic failure. However, if the cleavage activity is too high,
TOP2A will introduce dsDNA breaks into the chromosomes, which can trigger apoptosis, or programmed cell death. On a rare occasion, the DNA fragments created by dsDNA breaks could fuse to form translocations, which could later induce cancer (140, 141). Therefore, TOP2A activity needs to be regulated and this regulation occurs through expression levels, post-translational modifications, and interacting proteins.

Figure 1.7. The balance of TOP2 DNA cleavage activity.

TOP2 DNA cleavage activity is followed by the ligation of the cleaved DNA in order to ensure its enzymatic activity does not cause DNA damage. Inhibitors of TOP2 can cause a decrease in the cleavage complex formed by TOP2 which could result in quiescence and cell death. TOP2 inhibitors and poisons can also cause an increased cleavage activity compared to ligation activity, causing DNA damage, apoptosis or translocations causing leukemia. Figure adapted from Deweese and Osheroff, 2009 (138).
The roles of DNA topoisomerase II inhibitors

While the balance between TOP2 DNA cleavage activity and ligation activity needs to be tightly regulated to ensure normal cell survival and proliferation, scientists have taken advantage of manipulating this balance through the discovery of chemical drugs that inhibit the TOP2 ligation activity but not the DNA cleavage activity. Etoposide, also known as VP-16 and derived from *Podophyllum peltatum*, was one of the first compounds for clinical use that targeted TOP2 (142, 143). Cells undergo apoptosis when they sense DNA damage that is deemed unreparable. Etoposide takes advantage of this mechanism by inhibiting TOP2 ligation activity after cleaving the DNA, leaving a large number of dsDNA breaks throughout the genome (144-146). Inhibitors of TOP2 ligation activity such as etoposide also referred to as TOP2 poisons, have been some of the most common drugs used clinically in cancer treatment targeting leukemia, lung cancer, breast cancer, lymphomas, and sarcomas by causing the cancerous cells to accumulate DNA damage to induce apoptosis in those cancerous cells (142, 143, 147). Therefore, TOP2 has been a popular target for the development of various chemical derivatives for scientific and clinical purposes.

The discovery of TOP2 poisons such as etoposide has helped in the battle to treat cancer cells. However, while these drugs are often used against cancer, they have also been seen to cause specific types of leukemia. Around 2% of patients treated with etoposide have developed acute myeloid leukemia or acute promyelocytic leukemia in the five years following treatment (113, 120, 140, 141). It is suggested that the dsDNA damage produced by the inhibitor-treated TOP2 allows for chromosomal translocation of the MLL gene (mixed lineage leukemia) on chromosomal region 11q23 or the PML gene (promyelocytic leukemia) on chromosome 15 with
the RARA gene (retinoic acid receptor α) on chromosome 17 (140, 141, 148, 149). Infants with leukemia that have never been clinically treated with TOP2 poisons also have been seen to have a translocation of the MLL gene, suggesting that the maternal consumption of foods with natural TOP2 poisons is associated with these cancers.

In addition to TOP2 poisons that inhibit TOP2 DNA ligation activity, other drugs target other regions and activities of TOP2. The bisdioxopiperazine compound merbarone is a different chemical compound compared to etoposide in that merbarone targets the ATPase activity that is necessary to initiate TOP2 activity, and thus, prevents both the DNA cleavage and ligation activity altogether (150). The dexrazoxane compound ICRF-187 and bisdioxopiperazine compound ICRF-193 trap the topoisomerase bound to the DNA during the ligation of the dsDNA cleavage (151-155). Interestingly, while each of these inhibitors target different steps in TOP2 activity, they seem to interact within similar regions of the catalytic core (150).

**DNA topoisomerase II and SUMOylation**

In the year 2000, both TOP2 isoforms were discovered to be SUMOylated (112). Interestingly, TOP2 inhibitors have been reported to cause an upregulation in the SUMOylation of both isoforms of TOP2 (112, 156, 157). Previous reports have shown that SUMOylation is essential for chromosomal segregation during mitosis, and among the proteins at the centromere that are SUMOylated during mitosis is TOP2A (8, 105, 106, 108, 109, 136, 158-160). SUMOylation of TOP2A has been shown to be essential for the resolution of chromosomes (107, 136, 158, 159). The SUMOylation of *Xenopus laevis* TOP2A at lysine 660 can inhibit its decatenation activity, and SUMOylation of the C-terminal domain allows for novel interaction with DNA damage checkpoint protein Claspin (71, 161). SUMOylation at K660 during early
mitosis is suggested to greatly inhibit TOP2A activity from untangling the centromeric tangled DNA to ensure that the sister chromatids are cohered together before the onset of anaphase. The SUMO-targeted ubiquitin ligase RNF4 also triggered SUMOylated proteins when it is exposed to etoposide (162). However, why the TOP2 inhibitors cause the upregulation in TOP2 SUMOylation remains unclear.

**DNA topoisomerase II and mitotic checkpoints**

TOP2 has been determined to be directly involved in defining checkpoints in the cell cycle. The decatenation checkpoint at the G2/M transition allows for the entry into mitosis to be delayed until the chromosomes within the cell have been disentangled (163). The NoCut checkpoint during late mitosis delays the abscission of the forming daughter cells until the chromosome bridges that could form when separating the sister chromatids are resolved (164). However, there is evidence that TOP2 functions in a mitotic checkpoint that can arrest the cells during metaphase, which is thought to be the spindle assembly checkpoint (SAC). Addition of TOP2 inhibitors such as etoposide and ICRF-193 to HeLa cells have been reported to cause a DNA damage response and the activation of the SAC for metaphase arrest (165-167). The TOP2 inhibitor ICRF-187 or TOP2A depletion in *Drosophila* S2 cells caused a reduction of the Aurora B kinase, a key regulator of the SAC, on mitotic chromosomes, suggesting that TOP2A may be involved in the recruitment of Aurora B to the chromosomes (168).

The potential mechanism of how TOP2A may cause the activation of SAC in the presence of TOP2 inhibitors may be determined through the TOP2A decatenation activity. Since TOP2 inhibitors prevent TOP2A from completing the decatenation of the DNA, stalling the decatenation activity may initiate the signal to promote metaphase arrest. While this may occur,
several studies have shown that type II topoisomerasers C-terminal domain acts as a mitotic checkpoint signaling mechanism. In budding yeast, a slow ATP hydrolysis TOP2 mutant showed mitotic checkpoint activation and cell cycle delay (169). However, the removal of the C-terminal domain of the top2 mutant prevented the delay from occurring. Additionally, a SUMOylation-resistant top2 mutant that mutates the C-terminal SUMOylation sites caused pre-anaphase spindle extensions and kinetochore separations in budding yeast (136). This suggests that the C-terminal domain of TOP2 is important for mitotic checkpoint activation and that the SUMOylation of the C-terminal domain may play a role in checkpoint signaling. However, how TOP2 C-terminal domain functions as a checkpoint signal for the mitotic checkpoint remained unclear.

Several previous studies on mitotic SUMO led to the conclusion that TOP2A may play a major role in the chromosome segregation process (71, 107, 136, 158, 159). However, how SUMOylated TOP2A functions biochemically or mechanistically during mitosis to promote proper chromosome segregation remained unclear. This dissertation will discuss a novel interaction between SUMOylated TOP2A and an Aurora B regulator protein Haspin identified through the Xenopus egg extract model. Aurora B is a necessary enzyme promoting proper chromosome segregation, and our results suggest that SUMOylated TOP2A plays a role in a pathway for the recruitment of Aurora B during early mitosis (170). The discovery of this novel function of SUMOylated TOP2A uncovers the underlying connection between TOP2A and Aurora B in regards to proper chromosome segregation.

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CHAPTER 2

TOP2A C-TERMINAL DOMAIN SUMOYLATION REGULATES PROTEIN INTERACTION OF SUMO-BINDING PROTEINS

INTRODUCTION

A role of SUMO modification during mitosis was first established in yeast where it was shown that Ubc9 was required for M-phase cyclin degradation, and thus, for metaphase-to-anaphase progression (1). Budding yeast SUMO, also known as SMT3, was found to be important for chromosome segregation (later confirmed in XEE assays), and centromeric SUMOylation was one of the key processes involved in proper mitotic progression (2-8). These results show that SUMOylation plays a major role during mitosis. While SUMOylated proteins have been identified, we do not yet understand why the SUMOylation of those proteins is important during mitosis.

One method that can be used to advance our knowledge of the molecular mechanism of SUMOylation during mitosis is through the use of XEE assays. XEE is a cell-free ex vivo model system that can be manipulated to study the cell cycle (9). Xenopus laevis eggs are harvested, lysed, and the soluble contents are collected to create the extract. Since Xenopus eggs are unfertilized and arrested during metaphase, the soluble contents contain all of the factors (cytostatic factors) necessary for mitosis and cell progression. After the role of SUMO in budding yeast (Smt3) was reported, Azuma et al. used XEE to show that mitotic SUMOylation is important for chromosome segregation (2, 10-15). Among the substrates that are SUMOylated during mitosis is DNA topoisomerase IIα (TOP2A), as reported by both Bachant et al. in budding yeast and Azuma et al. in XEE (2, 3). SUMOylation of TOP2A has been found to be
necessary for centromere resolution in HeLa cells and for chromosome segregation and genome stability in budding yeast (5, 16). While Takahashi et al. used budding yeast to identify the SUMOylated sites in the TOP2 C-terminal domain (CTD), there are technical difficulties, such as cell viability, in studying the biochemical function of TOP2 SUMOylation using budding yeast and even cell cultures due to the essential function of TOP2 in DNA replication (17). However, those technicalities can be overcome with the use of the cell-free ex vivo XEE system.

Figure 2.1. SUMOylation of TOP2A at CTD sites does not greatly inhibit TOP2A decatenation activity. (Top panel) A schematic representation of Xenopus laevis TOP2A with the different domains. The ATPase domain is necessary for DNA cleavage and ligation. The transducer domain is necessary for clamping DNA. The TOPRIM domain is a domain conserved in primases. The domain A contains the WHD repeat and the tower motif for DNA binding. Reported SUMOylated lysines sites are at K660 at the catalytic core and three sites (K1235, K1276, and K1298) in the C-terminal domain (18, 19). (Bottom panel) Graph of the TOP2A
decatenation activity using wildtype (WT), K660R mutant, 3KR mutant (K1235R, K1276R, and K1298R), and 4KR (all four lysine sites). The graph shows how much catenated kDNA remained by comparing SUMOylated versions of TOP2A activity to the non-SUMOylated TOP2A activity to determine the fold of inhibition due to SUMOylation. Graph was adapted from Ryu et al. 2015 (19).

While eukaryotic TOP2 isoforms can be modified with both SUMO1 and SUMO2/3, TOP2A has been reported to be modified primarily by SUMO2/3 during mitosis in Xenopus laevis (2, 20, 21). In Xenopus laevis, the SUMOylation sites of TOP2A have been found to be located at K660 in the catalytic core and at K1235, K1267, and K1298 in the CTD (Fig. 2.1) (18, 19). SUMOylation of K660 was found to inhibit TOP2A activity. However, recently, we have reported that the SUMOylation of the Xenopus CTD of TOP2A does not regulate its decatenation activity, suggesting that the SUMOylation of the CTD has a different role than regulating TOP2A enzymatic activity (Fig. 2.1) (19). One role of CTD SUMOylation has been resolved within the same report by using XEE to test to see if the CTD SUMOylation provided protein interactions with TOP2A assessed with a pull-down assay (Fig 2.2). We have identified DNA damage checkpoint protein Claspin as a TOP2A SUMO-binding protein through pull-down assay and mass spectrometry (19). Additionally, we have found many other proteins binding specifically to SUMOylated CTD instead of the non-SUMOylated CTD. However, many of those proteins have not been identified or confirmed to be SUMO-binding proteins. Therefore, to determine the identities of the other TOP2A SUMO-binding proteins, we isolated pull-down samples of SUMOylated TOP2A CTD in XEE for additional mass spectrometry analysis and confirmed their binding through immunoblotting. Our findings support the notion that SUMOylation of TOP2A CTD regulates its interaction with SUMO-binding proteins.
Figure 2.2. SUMOylated TOP2A XEE pull-down assay.

A schematic representation of the XEE pull-down assay using TOP2A CTD SUMOylation in vitro assay to prepare SUMOylated CTD (CTD-SUMO). CTD and CTD-SUMO are bound onto agarose beads and incubated in XEE to bind proteins. Incubated beads are isolated, washed, and treated with SENP2 catalytic domain to remove SUMO modification on the CTD which will remove SUMO-binding proteins from the beads. Binding proteins in the solution are then eluted to separate the proteins from the beads with the CTD and can be analyzed.

RESULTS

SUMOylation of Xenopus laevis TOP2A CTD regulates protein-protein interactions in vitro

While TOP2A CTD SUMOylation was reported to not regulate TOP2 enzymatic activity, it has been shown to be able to allow protein-protein interaction with the DNA damage checkpoint protein Claspin (19). Additionally, the SUMOylation of TOP2 has also been reported to be
important for its interaction with Polo-like kinase interacting checkpoint helicase PICH (22). Therefore, SUMOylated TOP2A could regulate binding of other proteins. For the comprehensive identification of SUMOylated TOP2A CTD-binding proteins, recombinant *Xenopus laevis* TOP2A CTD modified with SUMO2 (CTD-SUMO) by *in vitro* SUMOylation was prepared as previously reported, and was used to pull down proteins from XEE (19). Proteins pulled down with the beads were eluted with urea after being digested by the SUMO protease SENP2 catalytic domain (Fig. 2.3). SENP2 cleaves the conjugated SUMO2 protein from the modified TOP2A CTD, which allows for the pulled down proteins to dissociate from the TOP2A CTD-bound beads as well as eliminated the high molecular weight contaminants of SUMOylated CTD in the samples. Proteins were eluted with urea from the TOP2A CTD-bound agarose beads after digestion, and urea-eluted proteins were precipitated with trichloroacetic acid. Samples from each step were run on a SDS-PAGE gel to separate the proteins by size to analyze by silver staining to observe the differences between the proteins eluted from TOP2A CTD and from CTD-SUMO. Lanes 1 and 2 show the SUMOylation status of TOP2A CTD that were used to pull down proteins from XEE. Lanes 3 and 4 are samples that were eluted from the beads with urea while lanes 5 and 6 are the proteins precipitated after urea elution with trichloroacetic acid (TCA). Lanes 7 and 8 are the proteins that were not eluted from the beads. There were a few proteins that were eluted preferentially from the CTD pull-down sample, as seen in lane 3. However, when examining lane 4, many protein bands of different molecular weights were seen eluted from CTD-SUMO. This is also the case when the urea elution samples were precipitated with TCA and then analyzed. This suggests that TOP2A CTD can mediate protein interaction by creating novel binding sites when SUMOylated. Interestingly, many of the protein bands found in the CTD-SUMO urea elution sample that were not found in the CTD sample have a molecular
weight of 100 kDa or higher. It is also interesting to note that there is a distinct protein band (marked with a red asterisk) found specifically in the non-SUMOylated CTD samples, which suggests that SUMOylation of the CTD may also prevent the interaction of at least one protein with TOP2A.

**Figure 2.3. SUMOylation of TOP2A CTD regulates protein interaction in XEE.**

Silver stain of the pulled-down proteins using TOP2A CTD. S-tagged non-SUMOylated (CTD) and CTD SUMOylated (CTD-SUMO) *in vitro* were bound to S-agarose beads and incubated with CSF XEE for pull-
down assay. After incubation with the SENP2 catalytic domain (SENP2 CD), proteins were eluted with urea (Urea elution) and precipitated with trichloroacetic acid (TCA precip.). Lanes 1 and 2 represent 5% of the S-tagged CTD and CTD-SUMO bound onto S-agarose beads as bait. Proteins in each fraction were visualized with silver stain. After elution samples were the proteins remaining on S-agarose beads. TCA precip. fractions were subjected for protein identification by LC-MS/MS. The red asterisk (*) shows a protein band in the non-SUMOylated CTD sample that does not appear in the CTD-SUMO sample.

Mass spectrometry determines identities of proteins binding specifically to SUMOylated TOP2A CTD

To identify the proteins that were pulled down from XEE, the TCA precipitated proteins from the urea-eluted protein samples from both non-SUMOylated TOP2A CTD (CTD) and TOP2A CTD SUMOylated with SUMO2 (CTD-SUMO) were subjected for a mudpit LC-MS/MS analysis. Proteins identified by LC-MS/MS from CTD and CTD-SUMO were compared between the two samples to remove common proteins that were identified. The LC-MS/MS analysis identified multiple peptides of 61 proteins that were pulled down with SUMOylated TOP2A CTD but not with the non-SUMOylated CTD (Table 1). Proteins identified include known SUMO-binding proteins such as histone methyltransferase SETDB1 and SUMO targeted ubiquitin ligase RNF4 (23, 24). Also among the identified candidate proteins that were pulled down with CTD-SUMO was mitosis-specific histone kinase Haspin and chromatin remodeling factor ISWI. Interestingly, Claspin and PICH, which were previously identified as a TOP2A CTD SUMO-binding protein in XEE pull-down assays, were not any of the proteins identified through the mudpit LC-MS/MS analysis, which could be due to the small sample size (19).
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Table 2.1. SUMOylated TOP2A CTD-specific binding proteins identified through LC-MS/MS analysis.

Urea eluted and trichloroacetic acid precipitated pull-down samples using non-SUMOylated TOP2A CTD and SUMOylated TOP2A CTD were analyzed by LC-MS/MS analysis. Identified proteins from both samples were compared, and the proteins with multiple peptides found and that were specifically pulled down with SUMOylated TOP2A CTD were listed. The first column states the reference identification number, the second column states the protein description, and the third column shows the number of peptides for the protein found using LC-MS/MS analysis.

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**Identified proteins bind to SUMOylated TOP2A in vitro**

To determine whether the proteins identified by LC-MS/MS analysis of the pull-down samples of SUMOylated TOP2A CTD in XEE were correctly identified as TOP2A SUMO-
binding proteins, pull-down samples from CTD and CTD-SUMO were subject to immunoblotting using antibodies targeting specific proteins (**Fig. 2.4**). Among the proteins listed from LC-MS/MS analysis that were tested are SETDB1, ISWI, and Haspin. SETDB1 is a histone methyltransferase of H3K9 and is associated with heterochromatin. Here, we find that SETDB1 can bind to TOP2A CTD. However, when the CTD is SUMOylated, SETDB1 is pulled down from the XEE more abundantly. SETDB1 has been reported to be a SUMO-binding protein of KAP1, and this result supports SETDB1 as a promiscuous SUMO-binding protein (25). ISWI, or imitation SWI, is a chromatin remodeling factor, and similar to SETDB1, is pulled down in more abundance with CTD-SUMO than the non-SUMOylated CTD.

![Diagram showing protein binding](image)

**Figure 2.4.** Proteins bind more abundantly or specifically to SUMOylated TOP2A CTD.
The SENP2-digested pull-down samples from XEE using recombinant non-SUMOylated TOP2A CTD (CTD) and SUMOylated CTD (CTD-SUMO) were analyzed by immunoblotting for SETDB1, ISWI, Haspin, and Claspin. CSF lane represents 0.75% of the volume of XEE used for each pull-down sample.

Immunoblotting analysis of the pull-down samples confirmed that Haspin bound specifically with the SUMOylated form of TOP2A CTD (Fig. 2.4). Haspin phosphorylates histone H3 at threonine 3 at the centromere during early mitosis to allow for the recruitment of Aurora B and the chromosomal passenger complex to the centromere (26, 27). Haspin was previously reported to interact with Pds5, and the deletion of Pds5 could cause a reduction in H3T3 phosphorylation and Aurora B localized at the centromeres (28, 29). Therefore, Pds5 interaction with Haspin is suggested to be important for Haspin activity at the centromere. However, neither Xenopus Pds5a nor Pds5b were pulled down with either CTD or CTD-SUMO, suggesting that Pds5 is not involved in the protein interaction between SUMOylated TOP2A CTD and Haspin (Fig. 2.5). While not among the list from the LC-MS/MS analysis, Claspin also binds specifically to CTD-SUMO, which confirms the TOP2A-Claspin protein interaction reported in the previous study (19).
Recombinant S-tagged TOP2A CTD proteins were subject to an in vitro SUMOylation assay with (+ATP) or without ATP (-ATP) to produce non-SUMOylated CTD (CTD) and SUMOylated CTD (CTD-SUMO) with SUMO2, and were bound onto S-agarose beads. Both CTD and CTD-SUMO bound onto S-agarose beads were incubated with CSF XEE for the pull-down assay, and the samples were analyzed by immunoblotting for Pds5a and Pds5b. SENP2-digested S-tagged CTD was used as the loading control for the bait used in the pull-down assay. CSF lane represents 0.75% of the volume of XEE used for each pull-down sample.

**Human TOP2A CTD SUMOylation allows for protein interaction**

We have determined that the SUMOylation of *Xenopus laevis* TOP2A CTD provides novel protein-protein interactions with SUMO-binding proteins. To see if this interaction is also found with *Homo sapiens* TOP2A CTD (Hs TOP2A CTD), we first looked to see if it was
possible to SUMOylate Hs TOP2A CTD in a similar fashion. We used recombinant Hs TOP2A CTD (1195-1535 a.a.) in an in vitro SUMOylation assay (Fig. 2.6A). Interestingly, Hs CTD had similar molecular weight shifts compared to the SUMOylated Xenopus CTD (Xl), suggesting that the human CTD can be SUMOylated in a similar fashion.

To see if the SUMOylated human CTD could interact with the SUMO-binding proteins similar to the Xl CTD, a pull-down assay was formed using non-SUMOylated Hs CTD and SUMOylated CTD in XEE, and the samples were later analyzed for several of the known Xenopus TOP2A SUMO-binding proteins: Claspin, Haspin, and PICH (Fig. 2.6B). SUMO-binding proteins of the Xenopus TOP2A CTD were found to be pulled down specifically when the Hs CTD was SUMOylated beforehand. The cross-species protein interaction suggests that the SUMOylation of the human TOP2A CTD may function similar to the Xenopus TOP2A CTD in allowing protein interactions.

To identify the SUMOylation sites in the human CTD, the Xenopus laevis SUMO acceptor lysine sites in CTD were used to map the sites (19). We discovered three lysines that could potentially be SUMOylation sites in the human CTD at K1240, K1267, and K1286 that were similar to the SUMOylation sites in the TOP2A CTD for Xenopus laevis (19). To see whether these three lysines were able to be SUMOylated, we used recombinant fragments of the wildtype Hs TOP2A CTD or a mutant fragment containing lysine-to-arginine mutations at the three sites for an in vitro SUMOylation assay (Fig. 2.6C). While the wildtype CTD was able to be SUMOylated over time, CTD 3KR mutant was not SUMOylated at a similar extent, suggesting that the lysines that were mutated could be SUMOylated.
Figure 2.6. Human TOP2A CTD can be SUMOylated and bind to SUMO-binding proteins in XEE.
A) *Xenopus laevis* (Xl) TOP2A CTD (1222-1579 a.a.) and *Homo sapiens* (Hs) TOP2A CTD (1195-1535 a.a.) were subject for an *in vitro* SUMOylation assay over time and analyzed by immunoblotting. B) Human TOP2A CTD (CTD) and SUMOylated CTD (CTD-SUMO) were incubated in XEE for a pull-down assay, and samples were isolated for immunoblotting. C) Human wildtype (WT) TOP2A CTD or 3KR mutant (containing K1240R, K1267R, and K1286R) were subject for an *in vitro* SUMOylation assay over time and analyzed by immunoblotting.

**DISCUSSION**

TOP2A has been reported to be one of the major substrates for SUMOylation during mitosis in XEE (2, 30). Previous studies of TOP2A have shown that SUMOylated TOP2A plays an important role at the centromere in resolving sister chromatids (3, 5, 31). SUMOylation of K660 can negatively regulate TOP2A decatenation activity through *in vitro* assay (18). In budding yeast, three SUMOylation sites have been mapped to the C-terminal region and mutations in those sites at the CTD causes chromosome separation abnormalities as well as genome instability (16, 31). However, we have recently shown that SUMOylation of TOP2A CTD does not affect its enzymatic activity unlike K660, but allows for Claspin to interact with it during mitosis (19). In this chapter, we have shown that there are other proteins that are TOP2A-binding proteins in a SUMO-dependent manner. This finding suggests that TOP2A SUMOylation may regulate other protein interactions during mitosis, including a mitosis-specific enzyme Haspin. Since SUMOylation of TOP2A is mitosis-specific and occurs primarily at the centromeres, the interaction between TOP2A and Haspin may suggest that SUMOylation of TOP2A can regulate mitosis progression through Haspin due to the importance of Haspin activity in the recruitment of the chromosomal passenger complex for essential Aurora B activity at the centromere for
mitotic progression (26, 27, 32). However, the interaction seen between TOP2A and the SUMO-binding proteins are through in vitro assays, and therefore, further experiments are necessary to determine whether these interactions occur on the chromosomes during mitosis.

While a previous study showed Claspin as a TOP2A CTD SUMO-binding protein through mass spectrometry analysis and western blot of XEE pull-down assay, Claspin was not identified in the list from the mudpit LC-MS/MS analysis using the same pull-down assay, potentially due to a small sample size (19). PICH was also not listed as a TOP2A CTD SUMO-binding protein in the mudpit LC-MS/MS analysis (22). This suggests that the list of proteins identified by LC-MS/MS analysis is not all of the proteins that could bind to CTD-SUMO. Additional experiments of the pull-down and mass spectrometry may find novel SUMO-binding proteins and is necessary to establish a complete database of binding proteins. Identifying more proteins may help in the comprehensive understanding of the function of SUMOylation TOP2A CTD during mitosis.

**MATERIALS AND METHODS**

*DNA constructs, site-directed mutagenesis, recombinant protein expression, and antibodies.*

For recombinant *Xenopus laevis* and human TOP2A CTD, *X. laevis* TOP2A CTD DNA sequence (1,222-1,579 a.a.) and human DNA (1195-1535 a.a.) were subcloned into pET30a (EMD Millipore) while SENP2 catalytic domain (CD, 363-589 a.a.) and PIASy cDNAs were subcloned into pET28a vectors (EMD Millipore) with an N-terminal His-tag. For E1 complex (Aos1/Uba2 heterodimer), Uba2 and Aos1 cDNAs were subcloned into pRSF Duet vector (EMD Millipore) and expressed together in *E.coli*. Both wild type and dominant-negative form of Ubc9
(dnUbc9-C93S/L97S) were subcloned into pT7-7 vectors (from M. Dasso of NIH (33)) and SUMO2-GG was subcloned into pGEX4T-1 (GE Healthcare) with an N-terminal GST tag. All proteins were expressed in BL21 (DE3) or Rosetta 2 (DE3) bacteria either at 15°C in the 2xYT media containing 5% glycerol and 2.5% ethanol (for TOP2A CTD, SENP2 catalytic domain, PIASy and E1 complex) or 30°C in 2xYT media (for Ubc9 and SUMO2-GG). Proteins with His-6 tag (TOP2A CTD, PIASy, SENP2 CD) were extracted by lysing cells in the buffer (500 mM NaCl, 1 mM MgCl₂, 25 mM Hepes [pH7.8], 5% glycerol, 1 mM PMSF, and 0.5% Triton X-100) with 0.1mg/mL lysozyme (Sigma-Aldrich). His-6 tagged proteins were purified using Cobalt affinity beads (Talon Beads from Clontech) from soluble fractions after centrifugation at 25,000xg for 40 minutes. Proteins were eluted with imidazole and imidazole-eluted fractions were further separated by ion-exchange columns. For E1 complex purification, cells were lysed as above except with 150 mM NaCl concentration. The E1 complex containing imidazole elutions were loaded onto a SUMO1 affinity column (GST-SUMO1-GG conjugated to NHS-sepharose from GE Healthcare) in the presence of ATP, and bound E1 complex was eluted by DTT-elution buffer (100 mM NaCl, 1 mM MgCl₂, 30 mM Tris [pH 8.8], 5% glycerol, and 10 mM DTT). E1 complex containing DTT-eluted fractions were further purified by anion exchange column. GST-SUMO2-GG was extracted from *E.coli* cells by lysis method mentioned above and captured on Glutathione-sepharose beads (GE Healthcare). Bound beads were incubated with thrombin to cleave the GST tag to elute untagged SUMO2-GG. Eluted SUMO2-GG was further purified by anion exchange column followed by sephacryl S-100 gel filtration (GE Healthcare). Ubc9 proteins were extracted by sonication in 50 mM NaCl lysis buffer. Soluble fraction after centrifugation was loaded onto the anion exchange column. Collected Ubc9 proteins were separated by cation exchange column followed by sephacryl S-100 gel
filtration. All proteins were concentrated with buffer exchanged to 100 mM NaCl, 1 mM MgCl₂, 20 mM HEPES [pH 7.8], 5% glycerol, and 0.5 mM TCEP by the centrifugal concentrator (Amicon Ultra, ThermoFisher). The protein concentrations were measured using the Bradford method (Bio-Rad), with BSA as the standard. Purified proteins were snap frozen with liquid nitrogen and stored at -80°C. Mutations in the human CTD for K1240R, K1267R, and K1286R were generated by site-directed mutagenesis using a QuikChange II XL kit (Agilent) according to the manufacturer's protocol and verified by DNA sequencing.

Antibodies used for the study are as follows. For immunoblotting, rabbit anti-SUMO2/3 polyclonal antibody (1:1000) and rabbit polyclonal antibody against the TOP2A C-terminus region (1,358-1,579 a.a.) (1:1000) were prepared as previously described (2, 18, 34). Rabbit anti-ISWI polyclonal antibody and rabbit anti-SETDB1 polyclonal antibodies were prepared with the *Xenopus laevis* ISWI 1-223 a.a. and SETDB1 1-124 a.a. peptides. Rabbit anti-Haspin polyclonal antibody for *Xenopus laevis* was gifted by Dr. H. Funabiki (27). Rabbit polyclonal antibodies of anti-Pds5a and anti-Pds5b for *Xenopus laevis* were gifted by Dr. T. Hirano at RIKEN (35). Commercial antibody used was a S-protein-HRP (EMD Millipore; 1:2000) antibody.

*XEE, immunodepletion/add-back assay, in vitro SUMOylation, and pull-down assays.*

Low-speed extracts of *X. laevis* eggs arrested in metaphase with cytostatic factor (CSF XEE) and de-membraned sperm nuclei were prepared following standard protocols (9, 36).

*In vitro* SUMOylation reactions were incubated at 25°C for 2 hr. unless otherwise indicated. SUMO2 was used in the reactions. The reactions contained 15 nM E1, 6 µM SUMO2-GG, 500 nM S-tagged TOP2A CTD, 2.5 mM ATP, 30 nM Ubc9, and 15 nM PIASy. Reaction buffers were composed of 20 mM Hepes, pH 7.8, 100 mM NaCl, 5 mM MgCl₂, 0.05% Tween 20,
5% glycerol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 1 mM DTT. The reactions were stopped with half volumes of 3X SDS-PAGE sample buffer, and the samples were resolved on 8–16% Tris-HCl gradient gels by SDS-PAGE and then analyzed by Western blotting with HRP-conjugated S-tag (EMD Millipore). For pull-down assay, SUMOylation reaction was done with 40 nM E1, 24 µM SUMO2-GG, 4 µM S-tagged TOP2A CTD, 2.5 mM ATP, 80 nM Ubc9, and 40 nM PIASy for 2 hr. before binding onto S-agarose beads (EMD Millipore) overnight in 4°C for use in pull-down assays. Non-SUMOylated CTD was prepared by incubating with everything above but without ATP.

The XEE pull-down assays were performed as described previously with 10 mM iodoacetamide addition in buffers to prevent deSUMOylation activity in the XEE (34). XEEs were diluted two times the volume with PD-buffer (20 mM sodium phosphate pH7.8, 18 mM β-glycerol phosphate [pH 7.5], 5 mM MgCl₂, 50 mM NaCl, 5% glycerol, 1 mM DTT, and 10 mM iodoacetamide), and diluted XEEs were centrifuged at 25,000g for 45 min at 4°C. Equal volume of the PD-buffer supplemented with 0.2% Tween 20 and 0.2% Triton X-100 was added to the supernatants and incubated with S-tagged TOP2A CTD-bound or SUMOylated TOP2A CTD-bound S-agarose beads for 1 hours at room temperature. After washing with PD-buffer, the beads were incubated in the dilution buffer (20 mM sodium phosphate [pH 7.8], 18 mM β-glycerol phosphate [pH 7.5], 5 mM MgCl₂, 50 mM NaCl, and 5% glycerol) containing 35 µg/mL SENP2-CD for 45 minutes at room temperature to cleave conjugated SUMO2 from TOP2A CTD and to dissociate pulled down proteins from the beads. SDS-PAGE samples were prepared by adding a half volume of 3X SDS-PAGE sample buffer to beads suspension. All samples were separated on 8-16% Tris-glycine gels (ThermoFisher) by SDS-PAGE and analyzed with silver staining or immunoblotting. For the preparation of samples for LC/MS-MS analysis, pull-down samples and
the soluble fractions were isolated using spin columns, washed with urea and precipitated with trichloroacetic acid. Samples were subjected to LC/MS-MS analysis for protein identification (performed by Dr. S.P. Gygi at Harvard Medical School in Boston, MA).

REFERENCES


CHAPTER 3
SUMOYLATION OF DNA TOPOISOMERASE IIα REGULATES HISTONE H3 KINASE HASPIN AND H3 PHOSPHORYLATION IN MITOSIS

INTRODUCTION

During mitosis, the major checkpoint that can cause an arrest at metaphase to stop or delay the progression of mitosis to separate the sister chromatids is known as the spindle assembly checkpoint (SAC) (1-4). If the microtubules are not properly attached or under improper tension at the kinetochores, cell division could result in improper chromosome segregation and genomic instability that could lead to aneuploidy or multi-nucleated daughter cells. In order to prevent these abnormalities from occurring, cells can sense the absence of correct kinetochore-microtubule attachment and activate the SAC in order to delay the metaphase-to-anaphase progression to allow the problem to be fixed. Therefore, the assembly of the centromeric proteins and kinetochore are necessary during early mitosis in order to ensure proper microtubule attachment and proper tension of the kinetochore-microtubules in order to progress from metaphase to anaphase and complete mitosis.

Many proteins necessary for chromosome segregation localize at the centromeres (5). The centromere is the last region in which the sister chromatids are connected. Many proteins specifically localize to the centromeres during mitosis, such as the histone H3 variant CENP-A as well as the constitutive centromere-associated network (CCAN) proteins which are other CENP proteins that interact at the inner kinetochore (Fig. 3.1) (6). Attached to CCAN is the kinetochore protein complexes known as the KMN network (derived from Knl1 complex, Mis12 complex, and Ndc80 complex) (7-9). These kinetochore protein complexes that form at the outer kinetochore work together to attach microtubules that extend out of the spindle poles during
early mitosis. If the microtubules do not properly attach to the KMN complex, then the spindle assembly checkpoint activates in order to delay mitotic progression to allow time for proper attachment.

Figure 3.1. Representation of the centromere and kinetochore structure.

A simplified schematic representation of the organization at the centromere and kinetochore (KT) involving microtubules (MT), CENP-A nucleosomes (CA), H3 nucleosomes (H3), KMN complex (Knl1 complex, Mis12 complex, and Ndc80 complex), and the constitutive centromere-associated network (CCAN). Figure was adapted from Pesenti et al. 2016 (6).
Among the proteins that can sense and activate the spindle checkpoint are the mitotic-specific kinases and their regulators. These kinases play a role in specific pathways to ensure chromosomes segregate properly to prevent aneuploidy. Among the mitotic kinases, Aurora B plays a central role in the activation of the spindle checkpoint in response to improper kinetochore-microtubule attachment and tension during early mitosis (7, 10-14). Aurora B kinase functions in a complex called the chromosomal passenger complex (CPC). Other members of the CPC are: INCENP, Borealin, and Survivin. Each CPC member assists in Aurora B kinase activity or the localization of Aurora B at the centromeres during early mitosis (15-17). Several mechanisms for CPC recruitment at mitotic centromeres exist in eukaryotes involving either heterochromatin protein HP1 or two histone modifications (18). The two mechanisms that rely on histone modifications involve mitosis-specific phosphorylation of histone tails. Bub1-dependent phosphorylation of H2A threonine 120 (serine 121 in fission yeast) allows for its interaction with Shugoshin proteins, which can then interact and recruit the CPC by binding with Borealin (19, 20). The other mechanism is the recruitment of CPC to the centromere through the activity of histone H3 kinase Haspin, which phosphorylates histone H3 at threonine 3 (H3T3) for its direct interaction with the BIR domain of Survivin (21, 22). Haspin inhibitors cause a reduction of Aurora B activity, chromosome alignment defects, and a compromised spindle checkpoint, which suggests the importance of Haspin in the recruitment of Aurora B kinase (23). A previous study suggests that the cohesin-associated factor Pds5 may help target Haspin to chromosomes in fission yeast (20). However, the mechanism of how Haspin localizes onto the chromosomes to target centromeric histone H3 in vertebrates remains unclear. Because CPC recruitment at mitotic centromeres utilizes multiple molecular mechanisms, it suggests that different signals can control specific pathways.
Budding yeast DNA topoisomerase II (TOP2), the type II topoisomerase that is similar to other eukaryotic topoisomerase isoform TOP2A, has a critical role during mitosis for resolving tangled genomic DNA by its strand-passaging enzymatic reaction (24). Inhibition of TOP2 enzymatic activity could activate cell cycle checkpoints, including the DNA damage checkpoint due to double-stranded breaks mediated by TOP2 (25). A proposed mechanism of TOP2-initiated G2 arrest is the binding of MDC1 to the phosphorylated TOP2 C-terminal domain (26). More recently, Furniss et al. showed that specific mutations of TOP2 that alter its strand-passaging reaction at specific steps could induce a Mad2-dependent delay in mitosis in budding yeast (27). Interestingly, this checkpoint activation requires the C-terminal domain (CTD) of TOP2, which suggests that TOP2 CTD has a critical role in controlling cell cycle progression, and the domain could serve as a signal transducer for cell cycle checkpoints. Notably, TOP2 has been reported to be involved in Aurora B activation, suggesting that TOP2 can control mitotic checkpoints via Aurora B (28).

While topoisomerase II isoforms could be modified with both SUMO1 and SUMO2/3, topoisomerase IIα (TOP2A) has been reported to be modified primarily by SUMO2/3 during mitosis in Xenopus laevis (29-31). Recently, we have uncovered that SUMOylation of TOP2A CTD facilitates novel interaction with DNA damage checkpoint adaptor protein Claspin in Xenopus egg extracts (32). Claspin binds to Chk1, a kinase known to activate Aurora B by phosphorylating serine 311 in human cells (33, 34). Therefore, SUMOylated TOP2A could be involved in Aurora B activation by Chk1 recruitment via Claspin. In addition to the potential Aurora B regulation by Claspin, we have identified Haspin as a binding protein of SUMOylated CTD by comprehensive LC-MS/MS analysis. In the present chapter, I will demonstrate that both Haspin and phosphorylated H3T3 (H3T3p) were less abundant on mitotic chromosomes when
TOP2A SUMOylation was prevented (35). Robust binding of Haspin to SUMOylated TOP2A required Haspin's SUMO-interacting motifs (SIMs) and the phosphorylation of Haspin, and mutations in both T206 and SIMs caused Haspin to not properly localize at mitotic centromeres. Altogether, our results show that SUMOylated TOP2A regulates the targeting of active Haspin to mitotic centromeres for the phosphorylation of H3T3. We propose that this novel mechanism of Haspin recruitment mediated by SUMOylated TOP2A CTD may be another molecular mechanism that regulates the progression of mitosis by regulating Aurora B at the mitotic centromeres.

RESULTS

*Haspin localizes at mitotic centromeres for histone H3 threonine 3 phosphorylation in a SUMOylation-dependent manner*

SUMOylation has previously been reported to be essential for proper chromosome segregation during mitosis (30, 36-38). Inhibition of mitotic SUMOylation resulted in defective centromeric localization of Claspin (32). Because TOP2A SUMOylation occurs primarily at the centromere during mitosis (39-41) and can regulate the centromeric localization of Claspin as previously reported, we hypothesized that the localization of Haspin is dependent on the SUMOylation occurring on the mitotic chromosomes. To address this, we first examined whether mitotic SUMOylation in XEE can regulate the binding of Haspin on the chromosomes. We inhibited mitotic SUMOylation specifically through the addition of dominant negative mutant E2 enzyme Ubc9 (dnUbc9) in XEE after the completion of DNA replication and before the onset of mitotic induction (Fig. 3.2A). When SUMO modification was present on the mitotic chromosomes, Haspin bound onto mitotic chromosomes prominently (Fig. 3.2B). However,
inhibiting SUMOylation with the addition of dnUbc9 reduced the levels of Haspin bound on the mitotic chromosomes by 50% (Fig. 3.2C). Also, H3T3p was reduced by 22% on the SUMOylation-inhibited mitotic chromosomes. This suggests that the reduction in Haspin on the chromosomes without SUMOylation occurring may reduce activity of Haspin on the chromosomes. Immunofluorescence staining of H3T3p on the mitotic chromosomes showed that inhibiting SUMOylation reduced its centromeric signal by 31% (Fig. 3.2D and 3.2E). To determine whether the localization of Haspin on the mitotic chromosomes is affected by the inhibition of SUMOylation, we expressed Haspin-GFP in XEE with the addition of Haspin-GFP mRNA. The co-localization of Haspin-GFP with centromeric SUMO2/3 and CENP-A (Fig. 3.3A) indicated that Haspin localizes at the centromere to phosphorylate H3T3 as suggested by previous studies (42, 43). However, the inhibition of SUMOylation caused a reduction of Haspin-GFP at mitotic centromeres, with signal intensity at 21% compared to when SUMOylation was present (Fig. 3.3B). From these results, we conclude that mitotic SUMOylation contributes to the centromeric Haspin localization as well as the phosphorylation of centromeric H3T3. Interestingly, while exogenous Haspin-GFP expression also showed the localization of Haspin on the chromosomal arm regions, the inhibition of SUMOylation reduced those signals as well.
Figure 3.2. SUMOylation on mitotic chromosomes regulates Haspin binding and H3T3 phosphorylation.

A) Schematic method for the preparation of mitotic replicated chromosomes from *Xenopus* egg extracts. B) Mitotic replicated chromosomes prepared as in Figure 1A with (Cont.) or without (+dnUbc9) mitotic SUMOylation. Isolated chromosomes were analyzed by immunoblotting with indicated antibodies. Histone H3 was used as a loading control for the mitotic replicated chromosomes. C) Quantification of Haspin and H3T3p levels on the mitotic replicated chromosomes, as seen in A, relative to levels of Cont. chromosomes from three independent experiments (n=3) with levels normalized by histone H3 levels. Error bar represents SD. Asterisk represents statistical difference by student t-test (* P < 0.05). D) Mitotic replicated chromosomes prepared from CSF XEEs with (Cont.) or without (+dnUbc9) mitotic SUMOylation were subjected to immunofluorescence staining with antibodies as indicated with Hoescht 33342. White bar indicates 10 µm. E) Quantification of H3T3p signal intensity at the mitotic centromeres, as seen in C, relative to signal intensities of Cont. centromeres from three independent experiments (n=3, 40 centromeres per n) with levels normalized by CENP-A. Error bar represents SD. Asterisk represents statistical difference by student t-test (*** P < 0.001).
Figure 3.3. SUMOylation regulates centromeric Haspin localization during mitosis.

A) Haspin-GFP mRNA was supplemented in XEEs for protein expression (upper panels), and mitotic replicated chromosomes prepared without or with dnUbc9 were subjected to immunofluorescence staining with indicated antibodies with Hoescht 33342. β-tubulin was used as a loading control for Haspin-GFP.
expression levels in XEE. White bar indicates 10 µm. B) Quantification of centromeric Haspin-GFP signal intensity, as seen in A, relative to signal intensities of +Haspin-GFP centromeres from three independent experiments (n=3, 50 centromeres per n) with levels normalized by CENP-A. Error bar represents SD. Asterisk represents statistical difference by student t-test (** P < 0.01).

SUMOylation contributes to the localization of Aurora B kinase on mitotic chromosomes

Haspin phosphorylates histone H3T3 to recruit Aurora B and the CPC to the mitotic centromeres (22, 43). Because SUMOylation on the mitotic chromosomes can regulate the both the localization of Haspin and its activity on H3T3 at the centromere, we looked to see whether Aurora B was also affected when SUMOylation was inhibited. Immunoblotting analysis of Aurora B on replicated mitotic chromosomes indicated that the inhibition of mitotic SUMOylation reduced both Aurora B and autophosphorylated Aurora B T248 (T232 in humans) levels on the mitotic chromosomes (Fig. 3.4A). Quantification of Aurora B levels revealed Aurora B levels were reduced by 20% while phosphorylated and activated Aurora B levels were reduced by 35% on the mitotic chromosomes with the addition of dnUbc9 (Fig. 3.4B). Furthermore, immunofluorescence staining of the mitotic chromosomes showed that the inhibition of SUMOylation reduced Aurora B localization at the centromeres (Fig. 3.4C). Consistent with the immunoblotting results, the immunofluorescence signal intensity of Aurora B at the centromere was significantly reduced with a reduction of 34% (Fig. 3.4D). These results suggest that mitotic SUMOylation could regulate centromeric Aurora B localization as well as the amount of activated Aurora B on the mitotic chromosomes.
**Figure 3.4. Inhibition of SUMOylation reduces Aurora B kinase on mitotic chromosomes.**

A) Mitotic replicated chromosomes isolated as in A) with (+dnUbc9) or without dnUbc9 (Cont.) were subject to immunoblotting. Histone H3 was used for the loading control for the mitotic chromosomes. B) Quantification of the Aurora B and Aurora B T248p levels on the mitotic chromosome, as seen in B, relative to levels of Cont. chromosomes from three independent experiments (n=3) with levels normalized by histone H3 levels. Error bars represent SD. Asterisk represents statistical difference by student t-test (* P < 0.05). C) Mitotic replicated chromosomes prepared as in A) with or without dnUbc9 (Cont.) were subjected to immunofluorescence staining with antibodies as indicated with Hoescht 33342. White bar indicates 10 µm. D) Quantification of the Aurora B signal intensity at mitotic centromeres, as seen in D, relative to signal intensities of Cont. centromeres from three independent experiments (n=3, 50 centromeres per n) with levels normalized by CENP-A signal. Error bars represent SD. Asterisk represents statistical difference by student t-test (* P < 0.05).

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**TOP2A C-terminal SUMOylation regulates Haspin binding and histone H3 threonine 3 phosphorylation on mitotic chromosomes**

Although inhibition of mitotic SUMOylation reduced the binding of Haspin and H3T3p levels on the chromosomes, dnUbc9 addition inhibits not only the SUMOylation of TOP2A in XEE but other proteins that are known to be SUMOylated at the mitotic centromeres as well (44, 45). To address whether SUMOylation of TOP2A CTD is responsible for the regulation of Haspin, we prepared mitotic chromosomes using recombinant TOP2A WT or 3KR (where all three known SUMO acceptor lysines on the CTD were mutated to arginine) (32) by removing endogenous TOP2A from the XEE through immunodepletion while adding back the recombinant TOP2A (Fig. 3.5A and 3.5B). Chromosomes were assembled in TOP2A-replaced CSF XEE, and Haspin and H3T3p levels were analyzed on the chromosomes by immunoblotting. When
endogenous TOP2A was replaced by recombinant TOP2A WT, endogenous Haspin and H3T3p on the mitotic chromosomes were reduced by 39% and 36%, respectively, in the presence of dnUbc9 (Fig. 3.5B and 3.5C). However, mitotic chromosomes from TOP2A 3KR-replaced XEE also showed reduction of both Haspin and H3T3p with levels reduced by 68% and 36%, respectively. Chromosomes with TOP2A 3KR with dnUbc9 present showed slightly further reduction with Haspin levels reduced by 76% and H3T3p levels reduced by 56%, but the difference was not statistically significant when compared to levels on the chromosomes without the dnUbc9 addition. These results suggest that SUMOylation of TOP2A CTD substantially contribute to binding of Haspin on mitotic chromosomes, and the binding of Haspin is critical for the prominent phosphorylation of H3T3. Interestingly, the TOP2A 3KR-replaced XEE with the addition of dnUbc9 revealed further reduction of both Haspin and H3T3p, which suggests that dnUbc9 addition may affect an additional recruitment mechanism of Haspin on the chromosomes other than through the SUMOylation of the C-terminal region of TOP2A.

A.
Figure 3.5. SUMOylation of TOP2A CTD regulates Haspin binding and H3T3 phosphorylation on mitotic chromosomes.

A) A schematic representation of the primary structure of *Xenopus laevis* TOP2A. Three lysines indicated in the C-terminal domain (CTD) were mutated to arginine for a TOP2A mutant that could not be SUMOylated in
the CTD (3KR). B) Endogenous TOP2A in CSF XEE was immunodepleted and replaced with either recombinant full-length T7-TOP2A wild type (WT) or 3KR (left panels). β-tubulin was used as a loading control of TOP2A levels in CSF XEE. Mitotic chromosomes assembled in TOP2A-replaced CSF XEEs were analyzed by immunoblotting with indicated antibodies (right panels). Histone H4 was used as a loading control for mitotic chromosomes. C) Quantification of Haspin and H3T3p levels on the mitotic chromosomes, as seen in B, relative to levels of TOP2A WT chromosomes from three independent experiments (\(n=3\)) with levels normalized by histone H4 levels. Error bar represents SD. Statistical differences by student t-test are represented with an asterisk (* \(P < 0.05\), ** \(P < 0.01\)).

**SUMOylated DNA Topoisomerase IIa interacts with Haspin through Haspin SIMs**

While we have identified that the SUMOylation of TOP2A CTD was important for the robust binding of Haspin on the mitotic chromosomes, we next looked to see what was important for Haspin to allow for the interaction to occur. Because a SUMO-interacting motif (SIM), a short sequence of large hydrophobic residues, can allow proteins to directly interact with SUMO on SUMOylated proteins (32, 46-48), we analyzed Haspin’s primary sequence using a SIM prediction program to determine whether Haspin possessed any SIMs (49). We identified two potential SIMs near the N-terminal region of Haspin at amino acids 343-346 (VICL) and 364-367 (VLCL) (**Fig. 3.6A**). To determine whether these sequences were important for Haspin's interaction with SUMOylated TOP2A, we created a double SIM mutant (2-SIM) Haspin construct in pTGFC70 with a GFP-tag and a 3' UTR of xKid (50). Using mRNA created from the construct, we expressed either the wild type (WT) Haspin-GFP or Haspin-GFP 2-SIM in XEE separately at similar levels, and the Haspin-GFP-expressed CSF XEE were subjected to pull-down assays with CTD-SUMO (**Fig. 3.6B**). Immunoblotting analysis showed that the
expressed Haspin 2-SIM bound 48% less to CTD-SUMO than Haspin WT (Fig. 3.6C). This indicates that the SIMs contribute to the robust binding of Haspin to SUMOylated TOP2A CTD. However, while mutations in the SIMs reduced the binding of Haspin to CTD-SUMO, it did not completely eliminate its binding capability, which suggests that another factor may be involved in the interaction.
Figure 3.6. Haspin binding to TOP2A CTD is dependent on SUMOylation and SIMs.

A) A schematic representation of the primary structure of *Xenopus laevis* Haspin. SIMs are located at a.a. 343-346 (VICL) and 364-367 (VLCL). Point mutations in each SIM are indicated in red for the disrupted SIM mutant protein (2-SIM). B) The mRNAs of GFP-tagged wild type (WT) or 2-SIM Haspin were supplemented in XEE to express Haspin-GFP, and Haspin-GFP-expressed CSF XEEs were subjected for the pull-down assay with S-tagged CTD SUMOylated (CTD-SUMO) through *in vitro* SUMOylation assay and bound onto S-agarose beads (middle panel). After SENP2-CD incubation, CTD-SUMO-bound Haspin-GFP was analyzed by immunoblotting (right panel). SENP2-digested S-tagged CTD was used as a loading control for the bait used in the pull-down assay. CSF XEE lanes represent 0.5% of the volume of the Haspin-GFP-expressed CSF XEE used for each pull-down sample. C) Quantification of the pulled down Haspin-GFP levels by CTD-SUMO, as seen in D, relative to Haspin-GFP WT levels from three independent experiments (*n*=3) with levels normalized by CTD levels. Error bar represents SD. Asterisk represents statistical difference by student t-test (*** P < 0.001).
Mitosis-specific phosphorylation of Haspin T206 regulates binding to SUMOylated TOP2A CTD

While mutating the two SIMs reduced Haspin 2-SIM levels bound onto SUMOylated TOP2A CTD through pull-down assays, it did not completely eliminate the interaction (Fig. 3.6B and 3.6C). This suggests that while the SUMOylation of TOP2A CTD is essential for the binding of Haspin, another factor contributes to the robust binding between SUMOylated TOP2A and Haspin. Interestingly, the molecular weight of Haspin-GFP is increased in the pull-down sample compared to the Haspin-GFP expressed in the XEE. The molecular weight shift suggests a post-translational modified form of Haspin bound onto the SUMOylated CTD. Haspin has been reported to be phosphorylated specifically during mitosis at multiple sites by kinases such as Cdk1 and Plk1 to activate Haspin (50-52). To determine whether the cell cycle-specific phosphorylation of Haspin contributes to the interaction of Haspin with SUMOylated TOP2A, we performed pull-down assays using either mitotic CSF XEE or interphase XEE expressing Haspin-GFP at similar levels. We utilized exogenous Haspin instead of endogenous Haspin in XEE due to difficulty in detecting endogenous Haspin in XEE and to eliminate the possibility of different Haspin expression levels between mitotic CSF XEE and interphase XEE. As a previous study reported (50), exogenous Haspin in mitotic CSF XEE showed a larger molecular weight than the Haspin in interphase XEE due to mitotic phosphorylation (Fig. 3.7A). Haspin-GFP was not detected in the pulled down fractions from non-SUMOylated TOP2A CTD in CSF or interphase XEE. However, when CTD-SUMO was used to pull down Haspin, the interphase form of Haspin-GFP was 73% less abundant compared to mitotic CSF Haspin-GFP (Fig. 3.7B). This result suggests that, since mitotic Haspin bound much more abundantly to SUMOylated CTD than the interphase form of Haspin, the cell cycle-specific phosphorylation of Haspin can
regulate its stable interaction with SUMOylated TOP2A. The initial phosphorylation for Haspin kinase activation is mediated by Cdk1 at threonine 206 in *Xenopus laevis* and threonine 128 in *Homo sapiens* (50, 52). T206 acts as a priming site that, when phosphorylated by Cdk1, promotes Plk1 binding for subsequent phosphorylation which leads to Haspin activation. Because the mitotic phosphorylation of Haspin may play a critical role in its interaction with SUMOylated TOP2A, we examined how a T206A mutation affected Haspin binding to CTD-SUMO (Fig. 3.7C). Haspin-GFP WT, T206A, 2-SIM, and a combined T206A/2-SIM mutant were expressed in XEEs separately at similar levels with Haspin-GFP mRNA addition (Fig. 3.7D). CTD-SUMO pulled down Haspin 2-SIM at 57% of WT, similar to what was observed in Figure 3.6C, while Haspin T206A was pulled down less at 15% of WT levels (Fig. 3.7E). The combined T206A/2-SIM mutant showed slightly lower levels pulled down at 9% of WT. These results suggest that phosphorylation of T206 greatly contributes to the stable interaction between Haspin and SUMOylated TOP2A, more so than the SIMs, in the *in vitro* pull-down assays.
Figure 3.7. Cell cycle-dependent Haspin T206 phosphorylation regulates SUMOylated TOP2A CTD-Haspin interaction.

A) Either CSF XEE or interphase XEE (Int.) expressing Haspin-GFP (left panels) were used in pull-down with S-tagged non-SUMOylated (CTD) and SUMOylated CTD (CTD-SUMO) bound to S-agarose beads (middle panel), and Haspin-GFP binding was analyzed by immunoblotting (right panels). β-tubulin was used as a loading control for Haspin-GFP levels in XEE (loading 0.5% of the volume of XEE used in each pull-down sample). SENP2-digested S-tagged CTD was used as the loading control for the bait used in the pull-down assay. B) Quantification of the pulled down Haspin-GFP levels with CTD and CTD-SUMO, as seen in A, relative to levels from the pull-down sample using CSF XEE with CTD-SUMO from three independent
experiments (n=3) with levels normalized by TOP2A CTD levels. Error bar represents SD. Asterisk represents statistical difference by student t-test (** P < 0.01). C) A schematic representation of Xenopus laevis Haspin mutants. Threonine 206 (T206) was mutated to alanine (T206A) to eliminate the mitotic phosphorylation site. T206A/2-SIM indicates the combined T206A and the double SIM mutations. D) Expressed wild type (WT), T206A, 2-SIM, and T206A/2-SIM Haspin-GFP in CSF XEE (upper panel) were used in pull-down assays with S-tagged CTD and CTD-SUMO bound onto S-agarose beads with Haspin-GFP binding analyzed by immunoblotting (lower panel). β-tubulin was used as a loading control for Haspin-GFP levels in XEE (loading 0.5% of the volume of XEE used in each pull-down sample) (upper panel). SENP2-digested S-tagged CTD was used as the loading control for the bait used in the pull-down assay (lower panel). E) Quantification of the pulled down Haspin-GFP levels with CTD and CTD-SUMO, as seen in D, relative to Haspin-GFP WT levels of CTD-SUMO from three independent experiments (n=3) with levels normalized by TOP2A CTD levels. Error bar represents SD. Statistical differences by student t-test are represented with asterisks (* P < 0.05, ** P < 0.01).

**Haspin T206 and SIMs regulate its centromeric localization on mitotic chromosomes**

Because both T206A and SIM mutations reduced the binding of Haspin to SUMOylated TOP2A CTD, we looked to determine whether these mutations also affected the centromeric localization of Haspin through immunofluorescence using Haspin-GFP WT, T206A, 2-SIM, and T206A/2-SIM expression with the addition of mRNA. To be sure that all four proteins are expressed at similar levels, different mRNA concentrations for each Haspin-GFP forms were added into XEEs and the chromosomes from XEEs with similar expression levels of Haspin-GFP (lanes 2, 5, 8, 11) were compared (Fig. 3.8A). Analysis of the centromeric Haspin-GFP signals showed a clear reduction in the centromeric Haspin localization with the mutant forms. Relative to WT Haspin-GFP levels, T206A mutant had Haspin-GFP levels at the centromeres
reduced to 44% while the 2-SIM mutant was reduced to 46% (Fig. 3.8B). Combining the mutations for the T206A/2-SIM mutant reduced the Haspin-GFP signal intensity further at the centromeres to 23%. This suggests that T206 phosphorylation and the SIMs contributes to the binding of Haspin to SUMOylated TOP2A CTD through an additive effect.

Altogether, our results suggest that the mitotic chromosomal binding of Haspin at the centromeres can be regulated by its interaction with TOP2A. This interaction occurs at the C- terminal region of TOP2A and is mediated by SUMOylation on TOP2A, Haspin SIMs, and the mitotic phosphorylation on Haspin.
Figure 3.8. Haspin SIMs and the phosphorylation of Haspin contribute to its localization at mitotic centromeres.

A) Mitotic replicated chromosomes prepared from Haspin-GFP-expressed XEEs with different mRNA concentrations of Haspin WT, T206A, 2-SIM, or T206A/2SIM mutant were subject to immunofluorescence staining. Immunofluorescence staining of chromosomes from XEE with similar levels of expressed Haspin-GFP is shown and compared (WT lane 2, T206A lane 5, 2-SIM lane 8, and T206A/2-SIM lane 11). β-tubulin was used as a loading control for Haspin-GFP levels in CSF XEE (upper panels). White bar indicates 10 μm.

B) Chromosomes from XEE with similar levels of expressed Haspin-GFP were quantified using the centromeric Haspin-GFP signal intensity, as seen in A, relative to signal intensities of Haspin-GFP WT from three independent experiments (n=3, 30 centromeres per n) with levels normalized by SUMO2/3. Error bar represents SD. Statistical differences by student t-test are represented with asterisks (* P < 0.05, ** P < 0.01).

DISCUSSION

Because Aurora B acts as a key mitotic regulator at the centromere during early mitosis, regulation of the recruitment of the CPC is essential for proper mitotic progression. Haspin has
been reported to contribute in the CPC recruitment (22, 43). The importance of Haspin can be seen through the use of Haspin inhibitors that caused a reduction of Aurora B activity and chromosome alignment defects and a compromised spindle checkpoint signaling activity (23).

However, the mechanism for the localization of Haspin remained unclear in vertebrates. Our results implicate a novel interaction between TOP2A and Haspin for the centromeric Haspin localization that is mediated by two different modifications: 1) the phosphorylation of Haspin required for kinase activation, and 2) the SUMOylation of TOP2A C-terminal region (Fig. 3.9). When both TOP2A and Haspin have been modified, active Haspin is recruited by SUMOylated TOP2A and binds to the vicinity of centromeric histone H3 to phosphorylate H3T3. H3T3p then allows for CPC to localize at the centromeres while Bub1-mediated phosphorylation of H2A T120 also contributes in the recruitment of the CPC via Shugoshin proteins (19, 53). However, mutating Haspin T206 is suggested to eliminate both Cdk1-dependent phosphorylation as well as Plk1-dependent phosphorylation (50). Therefore, it remains unknown as to whether the phosphorylation of T206 mediated by Cdk1 or the phosphorylation of the sites mediated by Plk1 on Haspin is important for its interaction with TOP2A. It also remains unclear how phosphorylated T206 and potentially other phosphorylated sites on Haspin contribute to the interaction with SUMOylated TOP2A structurally. Without SUMOylation, TOP2A and Haspin do not bind, which suggests that SUMOylation is essential for the two proteins to interact. However, even without the SIM sequences, Haspin, though not robustly, can bind to SUMOylated TOP2A. This could be because: 1) SUMOylation of TOP2A can cause structural change in the CTD which exposes a surface for phosphorylated Haspin to interact with; 2) phosphorylation of Haspin creates phospho-regulated SIMs (54) that interact directly with the SUMO protein more tightly; or 3) the phosphorylation-dependent Haspin conformational
changes suggested by Gheniou et al. allows it to bind to SUMOylated TOP2A (50). Future studies involving Haspin phosphorylation site mutants may provide insight on the specific site or sites that mediates this protein interaction.

Figure 3.9. Model for centromeric Haspin recruitment by DNA Topoisomerase IIα.
1) Centromeric DNA Topoisomerase IIα is SUMOylated (S) at the C-terminal domain by SUMO E3 ligase PIASy while Haspin is phosphorylated (P) by Cdk1 at T206 during the onset of mitosis. Plk1 binds to phosphorylated T206 to phosphorylate other sites on Haspin to create active Haspin kinase. 2) SUMOylated Topoisomerase IIα recruits active Haspin to the centromere to allow for the phosphorylation of histone H3 (purple) at threonine 3 (T3). Phosphorylated T3 recruits CPC members to the centromere through direct interaction with Survivin. H2A (dark purple) T120 phosphorylation mediated by Bub1 additionally contributes to the recruitment of CPC members to the centromere through the binding of Shugoshin 1/2 (Sgo1/2) that interacts with Borealin.

While our results show that the TOP2A CTD SUMOylation can regulate the binding of Haspin on the mitotic chromosomes by using TOP2A 3KR mutant, the addition of dnUbc9 to the XEE with TOP2A 3KR mutant showed a greater reduction in both Haspin and H3T3p levels on mitotic chromosomes than without the presence of dnUbc9 even though the difference was not statistically significant. This suggests that while TOP2A SUMOylation can regulate Haspin binding, other SUMOylated proteins on the mitotic chromosomes may also function to allow for the binding of Haspin, and thus, also affect H3T3p levels on the chromosomes. Additionally, Haspin binding on the mitotic chromosomes and the centromeric Haspin localization were not completely eliminated when SUMOylation was inhibited nor when both T206 and the SIMs were mutated together. This indicates that there could be other mechanisms for Haspin binding on mitotic chromosomes that are independent of SUMOylation, such as through the interaction with the cohesin cofactor Pds5 (20).

A recent analysis in budding yeast shows H3T3p-mediated Aurora B (Ipl1 in budding yeast) localization at the mitotic centromeres are conserved, and mislocalization of Aurora B is
observed in yeast with a truncated form of TOP2 lacking the CTD (55). Rescue of the H3T3p-dependent Aurora B localization in the truncated TOP2 mutant yeast by H3T3E substitution supports the conserved role of the CTD in the regulation of Aurora B localization in eukaryotes. Previous study has shown that TOP2A contributes in the regulation of Aurora B activity at the centromeres in somatic cells with TOP2 inhibitor treatment (28). TOP2 inhibitors have also been known to increase the SUMOylation of TOP2A in mitotic HeLa cells (29). Our results suggest that the SUMOylation-dependent regulation of Haspin may explain the molecular mechanism of the TOP2 inhibitor-dependent regulation of Aurora B. In addition, a recent study has indicated that specific mutations of TOP2 in budding yeast which obstructs the strand-passaging enzymatic reaction of TOP2 at different enzymatic steps can induce mitotic checkpoint activation (27). This mitotic checkpoint activation required the C-terminal region of TOP2, suggesting that TOP2 CTD can provide a signal to the mitotic checkpoint machinery. An intriguing future question is whether the SUMOylated TOP2A CTD and Haspin interaction is involved in the checkpoint activation caused by the strand-passaging reaction mutant TOP2.

MATERIALS AND METHODS

**DNA constructs, site-directed mutagenesis, recombinant protein expression, and antibodies.**

For recombinant full-length *Xenopus laevis* TOP2A proteins, cDNAs were subcloned into a pPic3.5 vector (ThermoFisher) that had a calmodulin-binding protein (CBP)-T7 tag sequence and were expressed in the GS115 strain of *Pichia pastoris* yeast. CBP-T7 tagged TOP2A proteins were extracted by grinding frozen yeast cells with dried ice followed by the addition of lysis buffer (150 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 30 mM Hepes [pH7.8]), and purified by
Calmodulin agarose (GE Healthcare) affinity chromatography and by anion exchange column (GE Healthcare) as previously described (41, 44). TOP2A CTD (1,222-1,579 a.a.) was subcloned into pET30a (EMD Millipore) while SENP2 catalytic domain (CD, 363-589 a.a.) and PIASy cDNAs were subcloned into pET28a vectors (EMD Millipore) with an N-terminal His-tag. For E1 complex (Aos1/Uba2 heterodimer), Uba2 and Aos1 cDNAs were subcloned into pRSF Duet vector (EMD Millipore) and expressed together in *E.coli*. Both wild type and dominant-negative form of Ubc9 (dnUbc9-C93S/L97S) were subcloned into pT7-7 vectors (from M. Dasso of NIH (56)) and SUMO2-GG was subcloned into pGEX4T-1 (GE Healthcare) with an N-terminal GST tag. All proteins were expressed in BL21 (DE3) or Rosetta 2 (DE3) bacteria either at 15°C in the 2xYT media containing 5% glycerol and 2.5% ethanol (for TOP2A CTD, SENP2 catalytic domain, PIASy and E1 complex) or 30°C in 2xYT media (for Ubc9 and SUMO2-GG). Proteins with His-6 tag (TOP2A CTD, PIASy, SENP2 CD) were extracted by lysing cells in the buffer (500 mM NaCl, 1 mM MgCl2, 25 mM Hepes [pH7.8], 5% glycerol, 1 mM PMSF, and 0.5% Triton X-100) with 0.1mg/mL lysozyme (Sigma-Aldrich). His-6 tagged proteins were purified using Cobalt affinity beads (Talon Beads from Clontech) from soluble fractions after centrifugation at 25,000xg for 40 minutes. Proteins were eluted with imidazole and imidazole-eluted fractions were further separated by ion-exchange columns. For E1 complex purification, cells were lysed as above except with 150 mM NaCl concentration. The E1 complex containing imidazole elutions were loaded onto a SUMO1 affinity column (GST-SUMO1-GG conjugated to NHS-sepharose from GE Healthcare) in the presence of ATP, and bound E1 complex was eluted by DTT-elution buffer (100 mM NaCl, 1 mM MgCl2, 30 mM Tris [pH 8.8], 5% glycerol, and 10 mM DTT). E1 complex containing DTT-eluted fractions were further purified by anion exchange column. GST-SUMO2-GG was extracted from *E.coli* cells by lysis method mentioned
above and captured on Glutathione-sepharose beads (GE Healthcare). Bound beads were incubated with thrombin to cleave the GST tag to elute untagged SUMO2-GG. Eluted SUMO2-GG was further purified by anion exchange column followed by sephacryl S-100 gel filtration (GE Healthcare). Ubc9 proteins were extracted by sonication in 50 mM NaCl lysis buffer. Soluble fraction after centrifugation was loaded onto the anion exchange column. Collected Ubc9 proteins were separated by cation exchange column followed by sephacryl S-100 gel filtration. All proteins were concentrated with buffer exchanged to 100 mM NaCl, 1 mM MgCl₂, 20 mM HEPES [pH 7.8], 5% glycerol, and 0.5 mM TCEP by the centrifugal concentrator (Amicon Ultra, ThermoFisher). The protein concentrations were measured using the Bradford method (Bio-Rad), with BSA as the standard. Purified proteins were snap frozen with liquid nitrogen and stored at -80°C.

*Xenopus laevis* Haspin cloned into a pTGFC70 plasmid with a C-terminal GFP tag and a 3’ UTR sequence of xKid was a gift from Dr. H. Funabiki at Rockefeller University. SIMs were predicted using the GPS-SUMO prediction program (49). Mutations in the SIMs at a.a. 343-346 (VICL to AICA) and a.a. 364-367 (VLCL to ALCA) and the T206A (threonine to alanine) mutation were generated by site-directed mutagenesis using a QuikChange II XL kit (Agilent) according to the manufacturer's protocol and verified by DNA sequencing. Wild type and mutant Haspin mRNA were obtained by *in vitro* transcription reaction with mMMESSAGE mMACHINE SP6 kit (ThermoFisher) from pTGFC70 plasmid as previous reports described (22, 50). The pTGFC70 plasmids of wild type and mutant Haspin-GFP were first linearized with NotI restriction enzyme digestion. Linearized pTGFC70 plasmids were incubated with the mMMESSAGE mMACHINE SP6 kit transcription mixture for 3 hours at 37°C to synthesize
mRNA. The transcribed mRNAs were precipitated in LiCl for recovery and dissolved in nuclease-free H$_2$O.

Antibodies used for the study are as follows. For immunoblotting, rabbit anti-SUMO2/3 polyclonal antibody (1:1000) and rabbit polyclonal antibody against the TOP2A C-terminus region (1,358-1,579 a.a.) (1:1000) were prepared as previously described (30, 40, 41). Anti-Aurora B kinase rabbit polyclonal antibody (1:1000) was prepared by full-length *Xenopus laevis* Aurora B as the antigen. Rabbit anti-Haspin polyclonal antibody for *Xenopus laevis* was gifted by Dr. H. Funabiki (22). Commercial antibodies used for immunoblotting analysis were mouse monoclonal anti-GFP (JL-8) (Clontech; 1:1000), rabbit monoclonal anti-Aurora B kinase T232 phosphorylation (T248 in *Xenopus laevis*) (Cell Signaling Technology; 1:1000), rabbit polyclonal anti-histone H3 (Cell Signaling Technology; 1:1000), rabbit polyclonal anti-histone H4 (Abcam; 1:1000), rabbit polyclonal anti-phosphorylated H3T3 (H3T3p) (Abcam; 1:1000), S-protein-HRP (EMD Millipore; 1:2000), and mouse monoclonal anti-β-tubulin (Sigma-Aldrich; 1:1000). For immunofluorescence staining, anti-SUMO2/3 guinea pig polyclonal antibody (1:500) and chicken polyclonal anti-*Xenopus laevis* CENP-A (1:500) were prepared as previously described (30, 40, 41, 44) and anti-Aurora B rabbit polyclonal antibody (1:500) was used. Commercial antibodies used for immunofluorescence analysis were mouse anti-Topoisomerase II monoclonal antibody (MBLI, 1:1000), and rabbit polyclonal anti-histone H3T3p (Abcam; 1:25000). Primary antibodies were visualized by fluorescently labeled secondary antibodies (ThermoFisher): goat anti-mouse IgG Alexa 488 (1/500), goat anti-rabbit IgG Alexa 568 (1/500), goat anti-guinea pig IgG Alexa 678 (1/500), and goat anti-Chicken IgG Alexa 488 (1/500).
XEE, immunodepletion/add-back assay, in vitro SUMOylation, and pull-down assays.

Low-speed extracts of X. laevis eggs arrested in metaphase with cytostatic factor (CSF XEE) and de-membraned sperm nuclei were prepared following standard protocols (57, 58). Immunodepletions of endogenous TOP2A were performed with protein A-conjugated magnetic beads (ThermoFisher) (59). Equal volume of rabbit anti-TOP2A antibody (1 mg/mL) and Protein A Dynabeads suspension were incubated to capture the antibodies on Dynabeads. Anti-TOP2A-captured beads were blocked with 5% BSA containing CSF-XB (100 mM KCl, 0.1 mM CaCl$_2$, 2 mM MgCl$_2$, 5 mM EGTA, 50 mM sucrose, and 10 mM HEPES [pH 7.8]). To reach greater than 99% depletion of TOP2A from XEE, we used anti-TOP2A captured Dynabeads from initial Dynabead suspension at 1.1 µL suspension-to-1 µL XEE ratio (ie. 440 µL suspension of anti-TOP2A Dynabeads were used for immunodepletion in 400 µL of XEE in Figure 3.5B). XEE/Dynabeads mixture was incubated for 15 minutes at room temperature followed by 15 minute incubation on ice. For add-back experiments, purified recombinant T7-TOP2A proteins were added to immunodepleted extracts to similar levels to the endogenous TOP2A, which was confirmed by immunoblotting. Chromosome isolations were performed as previously reported (60). For chromosome isolation, interphase extract was first obtained by releasing metaphase-arrested XEE with 0.6 mM CaCl$_2$. Demembranated sperm nuclei were incubated in interphase XEE at 6000 sperm nuclei/µL and equal volume of CSF XEE was added to induce the onset of mitosis. Inhibition of mitotic SUMOylation was made by the addition of a dominant-negative form of Ubc9 (dnUbc9) at a concentration of 150 ng/µL to the interphase XEE as well as the CSF XEE right before the two XEEs are combined for the induction of mitosis from interphase. After incubation, XEE was diluted three times its volume by 0.5x CSF-XB supplemented with 18 mM β-glycerophosphate and 0.25 % Triton-X100, 10 mg/mL of protease inhibitors (leupeptin,
pepstatin, and chymostatin from Calbiochem), and 0.2 μM okadaic acid (Calbiochem). Diluted XEEs were layered onto dilution buffer containing 35% glycerol and centrifuged at 10,000g for 5 minutes at 4°C. Precipitated chromosomes were boiled in SDS-PAGE sample buffer, and the extracted proteins were subjected to immunoblotting with antibodies. Immunoblotting signals were acquired with Image Station 4000R (Carestream), and the signal levels were quantified by Image J software. Relative levels were calculated by measuring the signal levels of each protein band, by normalizing values to the loading controls indicated in each figure, and by taking the mean and standard deviation of three independent experiments for each assay. Statistical significance of the difference was calculated by t-test of the means.

*In vitro* SUMOylation reaction was done by incubating 40 nM Aos1/Uba2, 80 nM Ubc9, 40 nM PIASy, 24 μM SUMO2-GG, 4 μM S-tagged TOP2A CTD, and 2.5 mM ATP for 2 hr. at 25°C before binding onto S-agarose beads (EMD Millipore) overnight in 4°C for use in pull-down assays. Non-SUMOylated CTD was prepared by incubating with the mixture above but without ATP.

The XEE pull-down assays were performed as described previously with 10 mM iodoacetamide addition in buffers to prevent deSUMOylation activity in the XEE (40). XEEs were diluted two times the volume with PD-buffer (20 mM sodium phosphate pH7.8, 18 mM β-glycerol phosphate [pH 7.5], 5 mM MgCl₂, 50 mM NaCl, 5% glycerol, 1 mM DTT and 10 mM iodoacetamide), and diluted XEEs were centrifuged at 25,000g for 45 min at 4°C. Equal volume of the PD-buffer supplemented with 0.2% Tween 20 and 0.2% Triton X-100 was added to the supernatants and incubated with S-tagged TOP2A CTD-bound or SUMOylated TOP2A CTD-bound S-agarose beads for 1 hours at room temperature. After washing with PD-buffer, the beads were incubated in the dilution buffer (20 mM sodium phosphate [pH 7.8], 18 mM β-glycerol.
phosphate [pH 7.5], 5 mM MgCl$_2$, 50 mM NaCl, and 5% glycerol) containing 35 µg/mL SENP2-CD for 45 minutes at room temperature to cleave conjugated SUMO2 from TOP2A CTD and to dissociate pulled down proteins from the beads. SDS-PAGE samples were prepared by adding a half volume of 3X SDS-PAGE sample buffer to beads suspension. All samples were separated on 8-16% Tris-glycine gels (ThermoFisher) by SDS-PAGE and analyzed with silver staining or immunoblotting. In the case of pull-down assays with Haspin-GFP-expressed XEE, interphase XEE with 10 ng/µL Haspin-GFP mRNA was incubated for 150 minutes at room temperature and then returned to mitotic phase by adding equal volume of CSF XEE, or kept in interphase XEE for the pull-down experiment in Figure 3.7A. Protein expression levels in XEEs after incubation with mRNA were checked through immunoblotting and adjusted accordingly with additional volumes of the original CSF XEE or interphase XEE to achieve similar protein expression concentrations before being used for pull-down assays. Immunoblotting signals were acquired by Image Station 4000R (Carestream), and signal levels were quantified by Image J software. Relative levels were calculated by measuring the signal levels of each protein band, by normalizing the values to the recombinant TOP2A CTD levels, and by taking the mean and standard deviation of three independent experiments for each assay. Statistical significance of the difference was calculated by t-test of the means.

**Immunofluorescence analysis of chromosomes.**

The mitotic chromosomes used for the immunofluorescence analysis were prepared as previously described (39). Replicated mitotic chromosomes were prepared by incubating demembranated sperm chromatin at 1000 sperm nuclei/µL in interphase XEE by adding 0.6 mM CaCl$_2$ followed with the induction of mitosis with the addition of equal volume of CSF XEE. To
inhibit SUMOylation, dnUbc9 was added at 150 ng/μL to both the interphase XEE and CSF XEE before they were combined to induce the onset of mitosis. XEE containing mitotic chromosomes was diluted by three times the volume with IF-dilution buffer (0.5x CSF-XB containing 18 mM β-glycerophosphate and 250 mM sucrose) and equal volume of fixation buffer (IF-dilution buffer with 4% p-formaldehyde) followed by an incubation for 10 minutes at room temperature. Fixed samples were layered on top of 8 mL of 40% glycerol cushion in glass tubes with cover slips. The chromosomes were spun down onto the coverslips by centrifuging at 6,000g for 20 minutes at room temperature. Chromosomes on the coverslips were post fixed with 1.6% p-formaldehyde in PBS for 5 minutes at room temperature. The specimens were blocked with PBS containing 5% BSA and 2.5% cold-fish gelatin and subjected to immunostaining with the antibodies. The localization of Haspin on mitotic chromosomes was observed by GFP signals from exogenously expressed Haspin-GFP prepared from mRNA addition to XEE (50). For Haspin-GFP expression from mRNA, Haspin-GFP mRNA was incubated in interphase XEE at room temperature for 60 minutes at a concentration of 20 ng/μL (Fig. 3A) or at multiples concentrations at 20 ng/μL, 40 ng/μL, and 60 ng/μL (Fig. 7A). Afterwards, demembraned sperm nuclei were added to allow for DNA replication. After the completion of DNA replication, equal volume of CSF XEE was added and incubated for 45 minutes for mitotic CSF XEE with Haspin-GFP. DNA was stained with Hoechst 33342 dye (EMD Millipore), and the samples were mounted using Vectashield H-1000 medium (Vector Laboratory). All images were acquired by using the Nikon Plan Apo 100x/1.4 oil objective lens on a Nikon TE2000-U microscope with a Retiga SRV CCD camera (QImaging) operated by Volocity imaging software (Improvision) at room temperature. Photoshop CS6 (Adobe) was used to process the obtained images from Volocity to show the signal intensities by adjusting overall intensity range levels equally within
independent experiments without any gamma adjustments as stated by JCB policy. Sizes were cropped and the resolution was adjusted to fit JCB policy. Quantification of fluorescent signals was through Image J and Adobe Photoshop CS6 software by measuring the signal intensity around CENP-A or SUMO2/3. Relative intensity of signals from indicated antibodies or GFP was normalized by CENP-A or SUMO2/3 signals. The mean of the signal intensities from multiple centromeres were calculated for each independent experiment, and the mean and standard deviation of three independent experiments were determined for each assay. Statistical significance of the difference was calculated by t-test of the means.

REFERENCES

CHAPTER 4
HYPER-SUMOYLATION OF TOP2A CTD BY TOP2 INHIBITOR CAUSES CLASPIN AND AURORA B UPREGULATION

INTRODUCTION

Type II DNA topoisomerases (TOP2) function as dimers in resolving the topology of dsDNA through the hydrolysis of ATP to introduce temporary dsDNA cleavages in its DNA strand passage activity (1, 2). Afterwards, the cleaved dsDNA is ligated together and TOP2 dissociates from its DNA. This DNA strand passage activity to resolve DNA topology is conserved in eukaryotes involved in DNA replication, transcription, chromosome condensation and decondensation, and segregation (3-5). Interestingly, many eukaryotes contain α and β isoforms (TOP2A and TOP2B, respectively) while yeast and Drosophila only contain one (6). However, only TOP2A seems to be absolutely essential in the resolution of the DNA after replication as well as during mitosis in the separation of sister chromatids (7, 8).

Since TOP2 enzymes are directly involved in changing the overall DNA structure necessary for cell cycle progression, TOP2 has been the target of chemotherapeutic drugs for inducing apoptosis in cells (9-13). TOP2 inhibitors cause the enzyme to stall at catalytic intermediates to prevent the whole cycle of activities in the resolution of DNA topology. Among the TOP2 inhibitors, etoposide (VP-16) is an anti-tumor agent that is used to prevent the religation of the cleaved DNA by interacting at the catalytic core, and therefore, causes cells to undergo apoptosis in response to the dsDNA breaks (14-17). ICRF-193 is a TOP2 inhibitor that, while allowing the religation of the cleaved DNA, causes TOP2 to remain in the closed conformation by binding to the catalytic core, and ICRF-193 can induce apoptosis in several cell types (18-22). Merbarone is
also suggested to interact with TOP2 domains similar to the cleavage-enhancing agents and block DNA cleavage activity (23).

Interestingly, TOP2 inhibitors have a connection to SUMO modifications as a previous study has reported that TOP2 inhibitors cause an upregulation in the SUMOylation of both isoforms of TOP2 (24-26). SUMOylation is a post-translational modification that occurs in many different cellular processes, involving the conjugation of the small ubiquitin-like modifier (SUMO) protein to its substrate (27-31). The process of SUMO modification occurs in a multistep process through the E1 activating enzyme Aos1/Uba2, the E2 conjugating enzyme Ubc9, and the E3 SUMO ligase, which covalently binds SUMO onto a lysine of its substrate. While E1 and E2 enzymes are unique, there are several known E3 SUMO ligases, such as the members of the protein inhibitor of activated STAT (PIAS) family, the nuclear pore protein RanBP2, the polycomb protein Pc2, and PHD finger-mediated KAP1 (32-35). Previous reports have shown that SUMOylation is essential for chromosomal segregation during mitosis, and among the proteins at the centromere that are SUMOylated during mitosis is TOP2A (36-42). SUMOylation of TOP2A has been shown to be essential for the resolution of chromosomes (37, 38, 40). Previous reports state that SUMOylation of *Xenopus laevis* TOP2A at lysine 660 can inhibit its decatenation activity, and SUMOylation of the C-terminal domain (CTD) allows for novel interaction with the DNA damage checkpoint protein Claspin (43, 44). Additionally, SUMOylation of the TOP2A CTD can regulate the histone H3 kinase Haspin that is involved in the recruitment of Aurora B kinase and other chromosomal passenger complex (CPC) to the centromere during early mitosis, suggesting that SUMOylation of TOP2A is important for proper mitotic progression (45-51). While there are reported connections between SUMOylation and certain proteins such as hypermethylated in cancer Hic1 protein and the SUMO-targeted
ubiquitin ligase RNF4 when cells are treated with etoposide, the direct effect due to the hyperSUMOylation of TOP2A caused by the different TOP2 inhibitors remains unknown (52, 53). Examining the downstream pathways of the SUMOylation of TOP2A will help in understanding the molecular effects and consequences of the use of TOP2 inhibitors.

Because SUMOylated TOP2A plays an important role on the chromosomes during early mitosis, we looked into determining how TOP2 inhibitors could cause the increase in the SUMOylation of TOP2A and the biological consequences in the upregulation of SUMOylation. Here, we show that the TOP2 inhibitors cause an upregulation in the SUMOylation of TOP2A specifically at the C-terminal domain SUMO-acceptor lysine sites. When examining TOP2A SUMO-binding proteins Claspin and Haspin, we found that while the increased levels of Claspin did not change in localization at the centromeres, Aurora B, which is recruited by Haspin, was increased not only at the centromeres but also in the chromosomal arm regions with ICRF-193 addition but not with etoposide or merbarone addition. This suggests that each TOP2A inhibitor can act differently on TOP2A and its downstream targets.

RESULTS

TOP2 inhibitors increase SUMOylation of TOP2A on mitotic chromosomes

TOP2A is SUMOylated during early mitosis and TOP2A SUMOylation is suggested to be important for mitotic progression in multiple model systems (36-38, 40, 42-44). Agostino et al. reports that topoisomerase II inhibitors etoposide (VP-16), ICRF-193, and ICRF-187 can increase SUMOylation of TOP2A in HeLa cells (24). To see if this upregulation is conserved in other vertebrates, we looked to see the immediate effects of TOP2 inhibitors on the
SUMOylation on mitotic condensed chromosomes using the ex vivo cell free *Xenopus laevis* egg extract assay (Fig 4.1A). Several bands of SUMO2/3 are found on the mitotic chromosomes from the control DMSO-treated XEE at around 180 kDa and above (Fig. 4.1B). When 1 µM ICRF-193, 10 µM etoposide, or 10 µM merbarone was incubated with the mitotic chromosomes for 10 mins, SUMO2/3 bands were darker and had bands of higher molecular weight, which suggests an upregulation of SUMO2/3 modification on mitotic chromosomes. While the majority of TOP2A on mitotic chromosomes are seen to be unmodified by SUMO2/3, a few increased molecular weight TOP2A bands on the control DMSO-treated chromosomes are seen, which have previously been reported to be the SUMOylated form of TOP2A (36, 54). However, there were increases in the molecular weight bands of TOP2A on the chromosomes when TOP2 inhibitors were present for 10 mins. These increased molecular weight bands of TOP2A seem to be additional SUMOylated TOP2A as the addition of dominant negative E2 SUMO enzyme Ubc9 (dnUbc9) to inhibit SUMOylation on mitotic chromosomes also eliminated many of those bands.

While TOP2A localizes along the axis of the condensed chromosomes, SUMO2/3 modification is visible at the mitotic centromeres, suggesting that centromeric TOP2A is primarily SUMOylated during mitosis (43, 54). Since TOP2 inhibitors cause an upregulation of SUMO-modification on TOP2A, we looked to see if SUMO2/3 localization was affected (Fig 4.1C). While the control DMSO-treated chromosomes showed a focus of SUMO2/3 at the centromeres, the mitotic chromosomes treated with TOP2 inhibitors showed SUMO2/3 not only increased at the centromeres but also showed localization in the chromosomal arm regions. These results suggest that TOP2 inhibitors causes an upregulation of SUMOylated TOP2A on
the mitotic chromosomes and may even cause TOP2A at chromosomal arm regions which are normally not seen primarily SUMOylated to be modified with SUMO2/3.

Figure 4.1. TOP2 inhibitors upregulate TOP2A SUMOylation on mitotic chromosomes.
A) A schematic representation of the isolation of mitotic replicated chromosomes in the presence of a TOP2 inhibitor. Interphase XEE is induced with calcium solution and demembranated sperm nuclei is added to the XEE to allow for the replication of the chromosomes. After replication, CSF XEE is added to induce mitosis to condense the chromosomes. Condensed replicated chromosomes are treated with TOP2 inhibitor for 10 min. before isolation and analysis. Inhibition of SUMOylation occurs by adding dnUbc9 to interphase XEE and CSF XEE before the induction of mitosis. B) Mitotic replicated condensed chromosomes treated with or without dnUbc9 were isolated after treatment with TOP2 inhibitor (1 µM ICRF-193, 1 µM etoposide (Etop.), and 10 µM Merbarone (Merb.)) for 10 min. and analyzed by immunoblotting. Histone H4 is used as a loading control of the chromosomes. C) Mitotic replicated condensed chromosomes with or without dnUbc9 were isolated after 10 min. treatment of TOP2 inhibitor (1 µM ICRF-193, 1 µM etoposide (Etop.), and 10 µM Merbarone (Merb.)) for immunofluorescence staining. White bar indicates 10 µm.

**TOP2 inhibitors upregulate the SUMOylation of TOP2A at the C-terminal domain SUMO-acceptor sites**

TOP2 inhibitors can cause TOP2A to be stalled in certain intermediate conformations when TOP2A changes conformation during its enzymatic activity. Etoposide causes TOP2A to be structurally stalled with the cleaved dsDNA while ICRF-193 causes TOP2A to crosslink with the ligated DNA to keep TOP2A in the closed conformation (2, 18, 21). Since TOP2 inhibitors increased SUMOylation on the mitotic chromosomes, we looked to determine if the inhibitors that stall TOP2A in certain conformations could directly cause the upregulation in TOP2A SUMO2/3 modification. To do so, we first examined how TOP2 inhibitors affected the in vitro SUMOylation assay using E1 Aos1/Uba2, E2 Ubc9, E3 SUMO ligase PIASy, SUMO2, and either full-length wildtype TOP2A (WT) or 4KR mutant with four lysines (K660, K1235, K1276, and K1298) that we have previously reported to be SUMOylated on *Xenopus laevis* mitotic
chromosomes substituted with arginine so that they cannot be modified (Fig. 4.2A) (43, 44). In
the presence of TOP2 inhibitor ICRF-193 or etoposide, the highest TOP2A band (the third
SUMO-modified TOP2A) was increased compared to the control DMSO-treated sample after 30
min. as well as after 60 min. (the fourth SUMO-modified TOP2A) (Fig. 4.2B). This suggests that
both ICRF-193 and etoposide can directly increase the rate of SUMOylation of TOP2A.
Interestingly, the 4KR TOP2A mutant was not found to be modified after 30 mins, and while
SUMOylated 4KR bands were found in the presence of the TOP2 inhibitors after 60 mins, they
were comparable to the DMSO-treated control SUMOylation, suggesting that the 4KR mutations
can prevent the increased SUMOylation caused by the inhibitors.

Because TOP2 inhibitors cause TOP2A to be stalled in certain intermediate conformations,
the increased rate of SUMOylation may be due to the SUMOylation of new lysines that are
normally unexposed throughout most of TOP2A intermediate conformations during its
decatenation activity. Therefore, we looked to see if the upregulation of TOP2A SUMOylation
was due to new lysines being SUMOylated or whether SUMO2/3 were forming SUMO chains at
the previously reported SUMOylation sites. To do so, we replaced the endogenous TOP2A in
XEE through TOP2A immunodepletion and added back either recombinant TOP2A WT or 3KR
mutant that mutates just the three SUMOylation sites (K1235, K1267, and K1298) at the C-
terminal domain (CTD). Mitotic chromosomes in the presence of DMSO or TOP2 inhibitors
were analyzed. The mitotic chromosome with the addition of ICRF-193 or etoposide showed
more modified recombinant TOP2A WT compared to the DMSO-treated chromosomes (Fig.
4.2C). However, when endogenous TOP2A was replaced with the TOP2A 3KR mutant, the
mutant TOP2A did not show the high molecular weight shifted bands that was seen with the
TOP2A WT even when TOP2 inhibitors were present. This suggests that the TOP2 inhibitors are
causing an upregulation of SUMOylation of TOP2A at the known SUMO-acceptor lysine sites in the CTD in the form of SUMO chains rather than on additional lysines.
**Figure 4.2. Upregulation of TOP2A SUMOylation occurs at the C-terminal domain.**

A) A schematic representation of TOP2A. Four lysines have been mapped to be SUMOylated: one in the catalytic core and three sites in the C-terminal domain (CTD). B) Recombinant SUMOylation enzymes E1 (Aos1/Uba2), E2 (Ubc9), PIASy (E3), SUMO2-GG, and TOP2A (WT or 4KR) were mixed in the presence of a DNA plasmid with DMSO or a TOP2 inhibitor for an *in vitro* SUMOylation assay and analyzed at 30 and 60 min. C) Endogenous TOP2A was depleted from CSF XEE and replaced with recombinant TOP2A (WT or 3KR). Chromosomes incubated in TOP2A-replaced XEE that were treated with DMSO or TOP2 inhibitor (1 μM ICRF-193 and 1 μM etoposide (Etop.)) for 10 min. were isolated and analyzed by immunoblotting. Histone H4 serves as a loading control for the isolated chromosomes.

**TOP2 inhibitors do not directly affect the *in vitro* activity of SUMOylation enzymes.**

To see if the TOP2 inhibitors could directly affect the SUMO enzymes’ SUMOylation activity, we tested the effect of TOP2 inhibitors on *in vitro* SUMOylation of the C-terminal domain (CTD) of TOP2A. TOP2 inhibitors ICRF-193 and etoposide are only known to interact with TOP2A in the catalytic core or N-terminal gate and not the CTD (55). Using an *in vitro* SUMOylation FRET assay with CyPET-tagged SUMO2-GG or SUMO2-G and YPet-tagged TOP2A CTD, we found that the fluorescent signal increased over time when SUMO2-GG was used instead of SUMO2-G (Fig. 4.3A and 4.3B). This is due to the fact that two glycines at the tail of SUMO2 are needed to conjugate onto its target (56). Therefore, an energy transfer to fluoresce the YPET tag can only occur when the stimulated CyPET tagged SUMO2 is conjugated to the YPet-TOP2A CTD. We then tested the effects of the TOP2 inhibitors on the SUMOylation activity using the FRET assay. Results from the FRET assay showed that in 30 min. of SUMOylation activity, ICRF-193, etoposide, and merbarone did not cause a shift in the fluorescence signal even when the concentrations of the inhibitors were increased (Fig 4.3C).
This suggests that the upregulation in the SUMOylation of full-length TOP2A seen both *in vitro* and *ex vivo* are not due to the inhibitors directly affecting the SUMO enzymes themselves.

Figure 4.3. SUMOylation enzymes are not affected by TOP2 inhibitors *in vitro*. 
A) A schematic representation of the *in vitro* SUMOylation FRET assay involving YPet-TOP2A CTD and CyPet-SUMO2. CyPet tagged to SUMO2 is specifically excited at 414 nm. If the YPet tagged to the CTD is adjacent by conjugation, the CyPet tag can emit at 475 nm to excite the YPet tag to emit light at 530 nm. B) Fluorescence emission graph of the YPet tag on TOP2A CTD at 530 nm from an *in vitro* SUMOylation FRET assay over time using either CyPet-SUMO2-G or CyPet-SUMO2-GG. Fluorescent levels represented are the mean of three independent experiments. Error bar represents the standard deviation. C) Fluorescent emission graph of the YPet tag on TOP2A CTD at 530 nm from an *in vitro* SUMOylation FRET assay for 30 min. in the presence of a TOP2 inhibitor (ICRF-193, etoposide, or merbarone) at different concentrations. Fluorescent levels represented are the mean of three independent experiments which were normalized with the 0 uM concentration (DMSO). Error bar represents the standard deviation. Figures 4.3B and C are courtesy of Caitlin Hilliard.

**SUMO-dependent binding proteins and their downstream targets are increased on mitotic chromosomes**

We have previously reported two proteins that bound SUMOylated TOP2A during mitosis: DNA damage checkpoint protein Claspin and Histone H3 kinase Haspin (44, 50). Both Claspin and Haspin bind onto SUMOylated TOP2A at the centromeres which allows for Haspin to phosphorylate histone H3 to recruit the chromosomal passenger complex (CPC) involving Aurora B kinase to the centromeres while Claspin is thought to interact with Chk1 in the phosphorylation and the activation of Aurora B at the centromeres (57-61). Etoposide has been previously reported to cause known SUMO-binding protein and SUMO-targeted ubiquitin ligase RNF4 to be upregulated as a response (53). Since the SUMOylation of TOP2A was upregulated with the addition of TOP2 inhibitors, we looked to see if the SUMOylated TOP2A binding proteins were upregulated as well. We have reported that Claspin localizes at the centromeres
with SUMO2/3 and addition of TOP2 inhibitors caused the centromeric Claspin signal intensity to increase (Fig. 4.4A) (44). Interestingly, even though the SUMO2/3 signal is also seen in the chromosomal arm regions when the TOP2 inhibitors were present, the Claspin signal did not move into the arm regions. To see if the interaction between SUMOylated TOP2A and Haspin was also affected with the addition of the TOP2 inhibitors, we looked downstream of its pathway at Aurora B. Aurora B is recruited to the centromeres in a SUMO-dependent manner due to the Haspin-dependent phosphorylation on histone H3 (50). A previous study shows that TOP2 inhibitor ICRF-187 was able to cause Aurora B reduction in cells (62). However, the addition of ICRF-193 caused Aurora B to increase on mitotic centromeres and localized Aurora B on the chromosomal arm regions while neither etoposide nor merbarone seem to noticeably affect Aurora B level or its localization (Fig. 4.4B). To determine if the increase in Aurora B levels on the centromeres and chromosomal arm regions by ICRF-193 was due to the upregulation of SUMOylation, mitotic chromosomes initially treated with dnUbc9 were analyzed in the presence of ICRF-193. The addition of dnUbc9 prevented the robust increase in Aurora B levels on the chromosomal arm regions and the centromeres, suggesting that the increase in Aurora B on the mitotic chromosomes depends on the upregulation of SUMOylation on the chromosomes by the presence of ICRF-193 (Fig 4.5). These results suggest that SUMOylated TOP2A-binding proteins are also affected by TOP2 inhibitors possibly due to the increase in SUMOylated TOP2A. Also, specific TOP2 inhibitors may work differently in affecting SUMOylated TOP2A-binding proteins as Aurora B was only upregulated on the chromosomes in the presence of ICRF-193. Interestingly, while it was reported that Aurora B levels at the centromere are reduced with the addition of dnUbc9 to inhibit SUMOylation, Aurora B levels were not reduced on the
DMSO-treated chromosomes with dnUbc9, suggesting that DMSO may somehow affect Aurora B levels at the centromeres.

Figure 4.4. TOP2 inhibitors affect the downstream of SUMOylated TOP2A differently.
A) Mitotic replicated condensed chromosomes were isolated after 10 min. treatment of TOP2 inhibitor (1 µM ICRF-193, 1 µM etoposide (Etop.), and 10 µM Merbarone (Merb.)) for immunofluorescence staining with Claspin, CENP-A, and SUMO2/3. White bar indicates 10 µm. B) Mitotic replicated condensed chromosomes were isolated after 10 min. treatment of TOP2 inhibitor (1 µM ICRF-193, 1 µM etoposide (Etop.), and 10 µM Merbarone (Merb.)) for immunofluorescence staining with Aurora B, CENP-A, and SUMO2/3. White bar indicates 10 µm.

Figure 4.5. ICRF-193 mediated Aurora B upregulation depends on the upregulation of SUMOylation on the mitotic chromosomes.
Mitotic replicated condensed chromosomes were isolated from XEE in the presence or absence of dnUbc9 after 10 min. treatment of DMSO or ICRF-193 for immunofluorescence staining with Aurora B, CENP-A, and SUMO2/3. White bar indicates 10 µm.

DISCUSSION

A previous study has shown that TOP2 inhibitors cause an upregulation of SUMOylation of TOP2A in HeLa cells (24). However, how and to what extent TOP2 inhibitors affect the SUMOylation of TOP2A has remained unclear. In our study, we have shown that TOP2 inhibitors ICRF-193, etoposide, and merbarone cause an upregulation of TOP2A SUMOylation while not affecting the actual SUMOylation machinery. TOP2A SUMOylation on mitotic chromosomes can be inhibited when the C-terminal SUMO-acceptor lysine sites were mutated, suggesting that the upregulation in SUMOylation occurs at the C-terminal domain sites.

When examining the downstream proteins of the upregulated SUMOylated TOP2A, we interestingly found that while ICRF-193 and etoposide both affect TOP2A at the catalytic core or N-gate, they affect the downstream pathway of SUMOylated TOP2A differently. With the addition of TOP2 inhibitors, SUMO2/3 is increased not only at the centromeres, but also on the chromosomal arm regions, which suggests that the hyperSUMOylation caused by the TOP2 inhibitors also affects TOP2A on the chromosomal arm regions. However, while Claspin levels increased at the centromeres, TOP2 inhibitors did not cause a change in Claspin’s centromeric localization. Interestingly, ICRF-193 alone caused an upregulation of Aurora B, an indirect target of the TOP2A SUMO-binding protein Haspin on the chromosomes. It was thought that TOP2A inhibitors in the cell caused metaphase arrest due to DNA damage (63-65). The results from this study show that each TOP2 inhibitor can cause different effects on the cell besides
DNA damage. With Aurora B being upregulated on the chromosome in the presence of ICRF-193, the spindle assembly checkpoint that Aurora B regulates may also be affected by the TOP2 inhibitor, and therefore, affect cell cycle progression. A previous study reported effects of the different TOP2 inhibitors during mitosis. Skoufias et al. reported that high concentrations of etoposide cause chromosomal DNA damage, and resulted in metaphase arrest while ICRF-193 at certain concentrations can cause metaphase arrest without causing DNA damage (66). Therefore, it will be important to analyze whether DNA damage occurs within the 10 minute incubation of the TOP2 inhibitors at the used concentrations by examining the dsDNA break marker γ-H2A.X to determine if the Aurora B increase may result from DNA damage.

The difference in the localization between Aurora B and Claspin with the addition of TOP2 inhibitors is striking. The upregulation of Aurora B at the mitotic centromeres as well as the localization onto the chromosomal arm regions due to ICRF-193 addition is consistent with the hyperSUMOylation of TOP2A causing Haspin to be upregulated at the centromeres and the chromosomal arm regions for Aurora B recruitment. The addition of etoposide or merbarone did not cause the noticeable Aurora B upregulation that was seen with ICRF-193 addition. However, this could be due to the fact that the SUMOylation of TOP2A is upregulated more with the ICRF-193 addition compared to etoposide or merbarone addition. The addition of higher concentrations of etoposide or merbarone may increase the hyperSUMOylation of TOP2A to cause Aurora B upregulation on the mitotic chromosomes similar to levels seen with the addition of 1 µM ICRF-193. Claspin, on the other hand, increased at the centromeres with all three TOP2 inhibitors but did not localize into the chromosomal arm regions. This suggests that Claspin may need another factor besides the SUMOylated TOP2A to bind onto the chromosomes, and that factor may only be found at the mitotic centromeres. Additionally, since Claspin is a known
DNA damage checkpoint protein, Claspin may be specifically functioning at the centromeres in a SUMO-independent manner and as a DNA damage mediator protein due to the potential DNA breaks caused by TOP2A with the TOP2 inhibitors. Further studies are necessary to determine the factors in the different recruitment mechanisms of the SUMO-binding proteins. Additionally, other inhibitors that affect TOP2A may lead to different results, such as ICRF-187 which has been reported to negatively regulate Aurora B on the chromosomes (67). Further studies will show the consequences of these inhibitors on pathways that may be affected through the hyperSUMOylation of TOP2A.

MATERIALS AND METHODS

DNA constructs, site-directed mutagenesis, recombinant protein expression, and antibodies.

For recombinant full-length *Xenopus laevis* TOP2A proteins, cDNAs were subcloned into a pPIC3.5 vector (ThermoFisher) that had a calmodulin-binding protein (CBP)-T7 tag sequence and were expressed in the GS115 strain of *Pichia pastoris* yeast. CBP-T7 tagged TOP2A proteins were extracted by grinding frozen yeast cells with dried ice followed by the addition of lysis buffer (150 mM NaCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 30 mM Hepes [pH7.8]), and purified by Calmodulin agarose (GE Healthcare) affinity chromatography and by anion exchange column (GE Healthcare) as previously described (43, 68). TOP2A CTD (1,222-1,579 a.a.) and was subcloned into pET30a (EMD Millipore) and YPet-CTD and CyPet-tagged SUMO-G and SUMO2-GG were subcloned into pET28a vectors (EMD Millipore) while SENP2 catalytic domain (CD, 363-589 a.a.) and PIASy cDNAs were subcloned into pET28a vectors (EMD Millipore) with an N-terminal His-tag. For E1 complex (Aos1/Uba2 heterodimer), Uba2 and Aos1 cDNAs were subcloned into pRSF Duet vector (EMD Millipore) and expressed together in
E. coli. Both wild type and dominant-negative form of Ubc9 (dnUbc9-C93S/L97S) were subcloned into pT7-7 vectors (from M. Dasso of NIH (69)) and SUMO2-GG was subcloned into pGEX4T-1 (GE Healthcare) with an N-terminal GST tag. All proteins were expressed in BL21 (DE3) or Rosetta 2 (DE3) bacteria either at 15°C in the 2xYT media containing 5% glycerol and 2.5% ethanol (for TOP2A CTD, SENP2 catalytic domain, PIASy and E1 complex) or 30°C in 2xYT media (for Ubc9 and SUMO2-GG). Proteins with His-6 tag (TOP2A CTD, PIASy, SENP2 CD) were extracted by lysing cells in the buffer (500 mM NaCl, 1 mM MgCl2, 25 mM Heps [pH7.8], 5% glycerol, 1 mM PMSF, and 0.5% Triton X-100) with 0.1mg/mL lysozyme (Sigma-Aldrich). His-6 tagged proteins were purified using Cobalt affinity beads (Talon Beads from Clontech) from soluble fractions after centrifugation at 25,000xg for 40 minutes. Proteins were eluted with imidazole and imidazole-eluted fractions were further separated by ion-exchange columns. For E1 complex purification, cells were lysed as above except with 150 mM NaCl concentration. The E1 complex containing imidazole elutions were loaded onto a SUMO1 affinity column (GST-SUMO1-GG conjugated to NHS-sepharose from GE Healthcare) in the presence of ATP, and bound E1 complex was eluted by DTT-elution buffer (100 mM NaCl, 1 mM MgCl2, 30 mM Tris [pH 8.8], 5% glycerol, and 10 mM DTT). E1 complex containing DTT-eluted fractions were further purified by anion exchange column. GST-SUMO2-GG was extracted from E. coli cells by lysis method mentioned above and captured on Glutathione-sepharose beads (GE Healthcare). Bound beads were incubated with thrombin to cleave the GST tag to elute untagged SUMO2-GG. Eluted SUMO2-GG was further purified by anion exchange column followed by sephacryl S-100 gel filtration (GE Healthcare). Ubc9 proteins were extracted by sonication in 50 mM NaCl lysis buffer. Soluble fraction after centrifugation was loaded onto the anion exchange column. Collected Ubc9 proteins were separated by cation
exchange column followed by sephacryl S-100 gel filtration. All proteins were concentrated with buffer exchanged to 100 mM NaCl, 1 mM MgCl₂, 20 mM HEPES [pH 7.8], 5% glycerol, and 0.5 mM TCEP by the centrifugal concentrator (Amicon Ultra, ThermoFisher). The protein concentrations were measured using the Bradford method (Bio-Rad), with BSA as the standard. Purified proteins were snap frozen with liquid nitrogen and stored at -80°C.

Antibodies used for the study are as follows. For immunoblotting, rabbit anti-SUMO2/3 polyclonal antibody (1:1000) and rabbit polyclonal antibody against the TOP2A C-terminus region (1,358-1,579 a.a.) (1:1000) were prepared as previously described (36, 43, 70). Anti-Aurora B kinase rabbit polyclonal antibody (1:1000) was prepared by full-length Xenopus laevis Aurora B as the antigen. Rabbit anti-Haspin polyclonal antibody for Xenopus laevis was gifted by Dr. H. Funabiki (46). Commercial antibodies used for immunoblotting analysis were mouse monoclonal anti-GFP (JL-8) (Clontech; 1:1000), rabbit polyclonal anti-histone H4 (Abcam; 1:1000), S-protein-HRP (EMD Millipore; 1:2000), and mouse monoclonal anti-β-tubulin (Sigma-Aldrich; 1:1000). For immunofluorescence staining, anti-SUMO2/3 guinea pig polyclonal antibody (1:500) and chicken polyclonal anti-Xenopus laevis CENP-A (1:500) were prepared as previously described (36, 43, 68, 70) and anti-Aurora B rabbit polyclonal antibody (1:500) was used. Commercial antibodies used for immunofluorescence analysis were mouse anti-Topoisomerase II monoclonal antibody (MBLI, 1:1000). Primary antibodies were visualized by fluorescently labeled secondary antibodies (ThermoFisher): goat anti-mouse IgG Alexa 488 (1/500), goat anti-rabbit IgG Alexa 568 (1/500), goat anti-guinea pig IgG Alexa 678 (1/500), and goat anti-Chicken IgG Alexa 488 (1/500).

**XEE and immunodepletion/add-back assay.**
Low-speed extracts of *X. laevis* eggs arrested in metaphase with cytostatic factor (CSF XEE) and de-membraned sperm nuclei were prepared following standard protocols (71, 72). Immunodepletions of endogenous TOP2A were performed with protein A-conjugated magnetic beads (ThermoFisher) (73). Equal volume of rabbit anti-TOP2A antibody (1 mg/mL) and Protein A Dynabeads suspension were incubated to capture the antibodies on Dynabeads. Anti-TOP2A-captured beads were blocked with 5% BSA containing CSF-XB (100 mM KCl, 0.1 mM CaCl₂, 2 mM MgCl₂, 5 mM EGTA, 50 mM sucrose, and 10 mM HEPES [pH 7.8]). To reach greater than 99% depletion of TOP2A from XEE, we used anti-TOP2A captured Dynabeads from initial Dynabead suspension at 1.1 µL suspension-to-1 µL XEE ratio (ie. 440 µL suspension of anti-TOP2A Dynabeads were used for immunodepletion in 400 µL of XEE in Figure 5). XEE/Dynabeads mixture was incubated for 15 minutes at room temperature followed by 15 minute incubation on ice. For add-back experiments, purified recombinant T7-TOP2A proteins were added to immunodepleted extracts to similar levels to the endogenous TOP2A, which was confirmed by immunoblotting. Chromosome isolations were performed as previously reported (74). For chromosome isolation, interphase extract was first obtained by releasing metaphase-arrested XEE with 0.6 mM CaCl₂. Demembranated sperm nuclei were incubated in interphase XEE at 6000 sperm nuclei/µL and equal volume of CSF XEE was added to induce the onset of mitosis. Inhibition of mitotic SUMOylation was made by the addition of a dominant-negative form of Ubc9 (dnUbc9) at a concentration of 150 ng/µL to the interphase XEE as well as the CSF XEE right before the two XEEs are combined for the induction of mitosis from interphase. After incubation, XEE was diluted three times its volume by 0.5x CSF-XB supplemented with 18 mM β-glycerophosphate and 0.25 % Triton-X100, 10 mg/mL of protease inhibitors (leupeptin, pepstatin, and chymostatin from Calbiochem), and 0.2 μM okadaic acid (Calbiochem). Diluted
XEEs were layered onto dilution buffer containing 35% glycerol and centrifuged at 10,000g for 5 minutes at 4°C. Precipitated chromosomes were boiled in SDS-PAGE sample buffer, and the extracted proteins were subjected to immunoblotting with antibodies.

**Immunofluorescence analysis of chromosomes.**

The mitotic chromosomes used for the immunofluorescence analysis were prepared as previously described (54). Replicated mitotic chromosomes were prepared by incubating demembranated sperm chromatin at 1000 sperm nuclei/µL in interphase XEE by adding 0.6 mM CaCl$_2$ followed with the induction of mitosis with the addition of equal volume of CSF XEE. To inhibit SUMOylation, dnUbc9 was added at 150 ng/μL to both the interphase XEE and CSF XEE before they were combined to induce the onset of mitosis. XEE containing mitotic chromosomes was diluted by three times the volume with IF-dilution buffer (0.5x CSF-XB containing 18 mM β-glycerophosphate and 250 mM sucrose) and equal volume of fixation buffer (IF-dilution buffer with 4% p-formaldehyde) followed by an incubation for 10 minutes at room temperature. Fixed samples were layered on top of 8 mL of 40% glycerol cushion in glass tubes with cover slips. The chromosomes were spun down onto the coverslips by centrifuging at 6,000g for 20 minutes at room temperature. Chromosomes on the coverslips were post fixed with 1.6% p-formaldehyde in PBS for 5 minutes at room temperature. The specimens were blocked with PBS containing 5% BSA and 2.5% cold-fish gelatin and subjected to immunostaining with the antibodies. Afterwards, demembranated sperm nuclei were added to allow for DNA replication. After the completion of DNA replication, equal volume of CSF XEE was added and incubated for 45 minutes for mitotic CSF XEE with Haspin-GFP. DNA was stained with Hoechst 33342 dye (EMD Millipore), and the samples were mounted using Vectashield H-1000
medium (Vector Laboratory). All images were acquired by using the Nikon Plan Apo 100x/1.4 oil objective lens on a Nikon TE2000-U microscope with a Retiga SRV CCD camera (QImaging) operated by Volocity imaging software (Improvision) at room temperature. Photoshop CS6 (Adobe) was used to process the obtained images from Volocity to show the signal intensities by adjusting overall intensity range levels equally within independent experiments without any gamma adjustments. Sizes were cropped and the resolution was adjusted. Quantification of fluorescent signals was through Image J and Adobe Photoshop CS6 software by measuring the signal intensity around CENP-A or SUMO2/3.

**In vitro SUMOylation assay**

*In vitro* SUMOylation reactions were incubated at 25°C for the indicated time. The reactions contained 20 nM E1, 6 µM SUMO2-GG, 500 nM T7-TOP2A, 2.5 mM ATP, 40 nM Ubc9, and 20 nM PIASy. Reaction buffers were composed of 20 mM Hepes, pH 7.8, 100 mM NaCl, 5 mM MgCl$_2$, 0.05% Tween 20, 5% glycerol, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 1 mM DTT. DMSO or TOP2 inhibitors were mixed before incubation began. Reactions were stopped with half volume of 3x SDS sample buffer and analyzed on SDS-Page 4-12% Tris glycine gels and immunoblot. For FRET assay, CyPet-tagged SUMO2-G or SUMO2-GG was used in the reactions. The reactions contained 30 nM E1, 6 µM SUMO2-GG, 500 nM YPet-TOP2A CTD, 2.5 mM ATP, 30 nM Ubc9, and 60 nM PIASy. The reactions were stopped with 5 mM EDTA. The reactions are placed into well plates and excited at 414 mm and the emission at 530 mm is read 5 times and averaged with the Cary Eclipse Software. Addition of inhibitors at the indicated concentrations in the figures occurs before the start of the reaction and incubated for 30 mins.
REFERENCES


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CHAPTER 5
THE FUTURE OF TOP2A SUMOYLATION STUDIES

INTRODUCTION

SUMOylation of TOP2A has been previously reported to have a function at the centromere of the sister chromatids (1-3). SUMOylation on K660 was one of the first post-translational modifications reported to regulate TOP2A enzymatic activity (4). Disruption of the SUMOylation of lysine sites that have been found in the budding yeast TOP2 C-terminal domain (CTD) has also been linked to defects during mitosis (3, 5). However, none of these studies has explained the mechanistic function of SUMOylated TOP2A. Not until recently that the function of TOP2A SUMOylation during mitosis was explained biochemically.

The SUMOylation of TOP2A CTD has recently been shown to not alter TOP2A enzymatic activity (6). However, SUMOylated CTD can cause novel protein-protein interactions as shown in chapter 2. How SUMOylated TOP2A interacts with certain proteins has been explained by our research and in chapter 3 (6, 7). Among the TOP2A SUMO-binding proteins, Haspin shows a promising connection to the regulation of TOP2A SUMOylation for proper mitotic progression as Haspin is involved in the recruitment of Aurora B kinase to the centromere and kinetochore. Other proteins that interact with SUMOylated TOP2A may also be involved in this process. These proteins could be proteins that were identified with the recent mass spectrometry analysis explained in chapter 2. However, the recent mass spectrometry analysis of TOP2A SUMO-binding proteins did not identify a complete list of SUMO-binding proteins since Claspin, which was previously reported as a TOP2A CTD SUMO-binding protein,
was not found in the analysis. Another protein that was not identified in the recent mass spectrometry of TOP2A SUMO-binding proteins is Polo-like interacting checkpoint helicase, or PICH (8). Studies of PICH have shown that PICH cooperates with TOP2A in centromere disjunction during mitosis and can bind to TOP2A in a SUMO-dependent manner (9, 10). Further studies will determine whether the SUMOylation of TOP2A is important for its activity with PICH in regulating chromosome disjunction. If SUMOylation of TOP2A is necessary, it may explain the abnormal phenotypes that occur during chromosome segregation when SUMOylation is inhibited (5, 11-13). Therefore, further studies on not only the identification of TOP2A SUMO-binding proteins but on how SUMOylated TOP2A interacts and regulates these proteins are necessary to gain a better understanding of the role of SUMOylated TOP2A during mitosis. Several methods for the analysis of the biochemical function of SUMOylated TOP2A that will help our research will be explained in this chapter.

**SUMOylated TOP2A-Haspin protein interaction analysis**

The study presented in chapter 3 explains the importance of mitosis-specific phosphorylation on Haspin. Previous studies have shown that mutations at the initial phosphorylation site T206 in *Xenopus laevis* Haspin (T128 in humans) the phosphorylation of other sites mediated by Polo-like kinase 1, or Plk1, which prevented Haspin from becoming activated (14, 15). Interestingly, mutating threonine 206 to alanine greatly reduced Haspin's ability to bind to SUMOylated TOP2A *in vitro* and reduced Haspin localization at the mitotic centromeres. These results suggest that mitotic phosphorylation has a direct impact on the regulation of Haspin. However, mutating T206 not only prevents phosphorylation of that site but multiple other sites that require T206 phosphorylation as well. It remains unknown which
phosphorylation site is key for Haspin to bind SUMOylated TOP2A and localize on the mitotic centromeres. Further phosphorylation site mutation studies may reveal the key site.

One of the main XEE methods to study the roles of SUMO-binding proteins is to eliminate interactions with SUMO by adding dnUbc9 to XEE to inhibit SUMOylation. However, the addition of dnUbc9 inhibits global SUMOylation of proteins on the mitotic chromosomes which could affect other proteins that are regulated by SUMO modification, such as TOP2A at K660 (4). This suggests that a more targeted method is necessary for the study of the roles of SUMO-binding proteins instead of inhibiting all SUMOylation. One method is to utilize the known domains necessary for the SUMO-binding proteins to interact with SUMOylated substrates. The study in chapter 3 has shown that Haspin SIMs are important for Haspin binding and localization on the mitotic chromosomes. Mitosis-specific phosphorylation on Haspin has been mapped to the N-terminal region away from the kinase domain. Since the N-terminal domain (NTD) of Haspin contains the SIMs and the phosphorylation sites, these results suggest that the kinase domain located near the C-terminal end is not essential for Haspin to bind to and localize on the chromosomes (Fig. 5.1A). Expression of Haspin NTD (1-767 a.a.) mRNA in the XEE resulted in Haspin NTD localizing primarily at the mitotic centromere, suggesting that the NTD on Haspin is sufficient for the localization of Haspin (Fig. 5.1B). Overexpression of exogenous Haspin NTD may compete with the SUMO-binding proteins and prevent their binding onto SUMOylated proteins by binding onto the SUMOylated proteins itself. To test this, we expressed Haspin NTD in the XEE and examined whether the localization of Aurora B, which is normally recruited to the centromere in a SUMO-dependent manner by Haspin kinase activity, was affected. Interestingly, when Haspin NTD was expressed and localized at the mitotic centromeres, the level of Aurora B at the centromeres was reduced (Fig. 5.1C). This
suggests that Haspin NTD can outcompete the endogenous Haspin in binding onto SUMOylated proteins at the centromeres, and therefore, it can reduce endogenous Haspin kinase activity in the recruitment of Aurora B. Utilizing this method will allow for the study of SUMO-binding proteins without affecting the SUMOylation status of the proteins on the mitotic chromosomes.

Figure 5.1. Haspin NTD decreases the centromeric localization of Aurora B.
A) A schematic representation of Haspin and Haspin NTD. Haspin NTD is truncated at 737 a.a. to remove the kinase domain at the C-terminal end while still containing the phosphorylation site T206 and the SIMs. White bar indicates 10 µm. B) Haspin NTD-GFP mRNA was added in XEE, and mitotic replicated chromosomes were isolated from the Haspin NTD-GFP-expressed XEE for immunofluorescence staining for TOP2A and SUMO2/3. C) Haspin NTD-GFP mRNA was added in XEE, and mitotic chromosomes were isolated for immunofluorescence staining of Aurora B and SUMO2/3. White bar indicates 5 µm.

**TOP2A C-terminal domain phosphorylation and domain analysis**

Mutation of T206 into alanine is more than sufficient to reduce both Haspin binding on the SUMOylated TOP2A CTD and localization at the mitotic centromeres even when both the SUMO-modification and the SIMs are still intact. While the phosphorylation on Haspin may change the conformation of Haspin to a more suitable conformation to interact with SUMOylated TOP2A, it may also be that the phosphorylated sites interact specifically with a region of the CTD. A truncation analysis of the CTD may tell us which region of the CTD can interacts specifically with Haspin, and will, thus, be beneficial in understanding the different interaction mechanisms in which SUMO-binding proteins can bind to SUMOylated TOP2A.

In addition to the domains in the CTD that may be necessary for its interaction with SUMO-binding proteins, post-translational modification of the CTD may contribute to its ability to bind to those proteins. It has been previously reported that the TOP2A CTD is phosphorylated in the G2/M stage of the cell cycle and that the phosphorylation of T1524 in humans allows for DNA damage checkpoint protein MDC1 to bind to TOP2A in HeLa cells (16). Much as how phosphorylation on Haspin at T206 and other potential sites help Haspin binding to the SUMOylated TOP2A CTD, the phosphorylation of TOP2A in the CTD may additionally help
binding interactions. Interestingly, recombinant TOP2A CTD incubated in mitotic CSF XEE had an increase in its molecular weight when incubated at room temperature which was later determined to be caused by phosphorylation since incubation with Antarctic phosphatase caused it to shift back down (Fig. 5.2A and 5.2B). Domain analysis of TOP2A CTD shows that the upshifted band of TOP2A visibly disappears when the CTD is truncated by 52 amino acids at the C-terminal end, suggesting phosphorylation occurs at the C-terminal end of the TOP2A. Mass spectrometry analysis of the modified CTD reveals several potential sites of phosphorylation and further studies may reveal whether the phosphorylation of the CTD is essential for the binding of SUMO-binding proteins (Fig. 5.2C).
Figure 5.2. TOP2A CTD phosphorylation occurs within 1523-1579 amino acids.

A) A schematic representation of the truncated TOP2A CTD. Truncated CTD fragments were incubated in XEE and isolated to observe molecular weight shifts signifying modifications. The right column signifies which truncations were visibly shifted upwards in molecular weight when incubated in XEE compared to 4°C.

B) TOP2A full-length CTD, truncated CTD 1349-1579 amino acid, and truncated CTD 1240-1522 were incubated in XEE and isolated to see molecular weight shift. Isolated CTD were treated with Antarctic phosphatase (AP) for removal of phosphorylation.

C) Mass spectrometry analysis of TOP2A CTD. Green letters indicate no evidence for modifications, and the red highlights show amino acids within 1523-1579 that are necessary for visible molecular weight shift in XEE. Underlined amino acids are amino acids that could be phosphorylation.

TOP2 inhibitor analysis of SUMOylation on the chromosomes.
TOP2 inhibitors have previously been reported to cause an upregulation in the SUMOylation of TOP2A (17). However, the downstream effects of upregulated TOP2A SUMOylation remained unknown. In chapter 4, our study has shown that ICRF-193, etoposide, and merbarone can upregulate SUMOylation on mitotic chromosomes. While the focus of chapter 4 was to show the consequences of hyperSUMOylation of TOP2A, the increase in SUMOylated TOP2A may not account for all of the SUMO modification seen on the mitotic chromosomes. Surprisingly, our study revealed that the TOP2 inhibitors could also affect another known SUMOylated protein poly-(ADP ribose) polymerase 1 (PARP1), which is involved in DNA damage repair (18). While PARP1 is known to localize all over the chromosomes, a minority of PARP1 appears to be SUMOylated on the mitotic chromosomes (18). However, with the addition of the TOP2 inhibitors, we found that SUMOylated PARP1 increased on the mitotic chromosomes (Fig. 5.3A). These results suggest that the inhibitors could indirectly cause increases in the SUMOylation of other proteins on mitotic chromosomes, which may be revealed with further research.

Since TOP2A is not the only protein to be hyper-SUMOylated in the presence of TOP2 inhibitors, it may be possible that the inhibitors are affecting the binding of SUMOylation enzymes on the chromosomes. Therefore, we looked to see if the E3 SUMO ligase PIASy, which is known to be sole E3 enzyme responsible for SUMOylation on mitotic chromosomes in XEE (19, 20), was affected with the addition of the TOP2 inhibitors. When ICRF-193, etoposide, or merbarone were present with the mitotic chromosomes, PIASy binding on the chromosomes were increased in comparison with levels on the control DMSO-treated chromosomes (Fig. 5.3A). Interestingly, when dnUBc9 was added to inhibit SUMOylation, the amount of PIASy bound on the chromosomes was reduced and was comparable to the DMSO-treated
chromosomes. When determining whether the inhibitors affected PIASy localization, we found that, while PIASy normally localizes near mitotic centromeres as previously reported, the addition of TOP2 inhibitors caused PIASy to also localize in the chromosomal arm regions (Fig. 5.3B) (20). Interestingly, the inhibitors do not directly affect the SUMOylation activity as seen in chapter 4. While an increase of PIASy on the chromosomal arms can explain the upregulation of SUMO2/3 modification on the chromosomes in XEE, it remains a mystery how PIASy localized to the chromosomal arm regions to begin with to potentially SUMOylate proteins in the chromosomal arm regions when PIASy is normally seen localizing specifically at the mitotic centromeres. Therefore, further study is necessary to determine how TOP2 inhibitors are affecting the localization of PIASy.

A.
Figure 5.3. TOP2 inhibitors upregulate SUMOylation of PARP1 and PIASy binding on mitotic chromosomes.

A) Mitotic replicated chromosomes were treated with DMSO or TOP2 inhibitors (1 µM ICRF-193, 10 µM etoposide (Etop.), or 10 µM merbarone (Merb.)) for 10 mins before isolation from XEE and analysis by immunoblotting. Histone H3 was used as a loading control for the chromosomes. B) Mitotic replicated chromosomes treated with DMSO or TOP2 inhibitors (1 µM ICRF-193, 10 µM etoposide (Etop.), or 10 µM merbarone (Merb.)) for 10 mins were isolated from XEE and analyzed by immunofluorescence staining for TOP2A, SUMO2/3, and PIASy. White bar indicates 10 µm.
The study of TOP2 inhibitors reported in chapter 4 has shown that different TOP2 inhibitors can cause different downstream effects such as differential effects on Aurora B kinase localization and levels on the chromosomes due to the upregulation of SUMOylation. Additionally, the difference in the upregulation between Aurora B and Claspin on the chromosomes suggests that Haspin and Claspin act differently as TOP2A SUMO-binding proteins. This difference could be due to Claspin having an additional regulator for its chromosomal localization that happens to be at the centromeres while Haspin could bind to the hyperSUMOylated TOP2A on the chromosomal arm regions to recruit Aurora B to the arm regions. Another possibility is that, since Claspin is a DNA damage checkpoint protein, Claspin may function as a DNA damage response protein specifically at the centromeres independent of SUMOylation. There are also other known TOP2 inhibitors, such as ICRF-187, amsacrine, and doxorubicin. Studying how each of the TOP2 inhibitors affects SUMOylation of TOP2A and its downstream pathways may help our understanding of the role of SUMOylation, the consequences of TOP2 inhibitors, and the difference in the mechanism of recruitment between the SUMO-binding proteins.

*Moving TOP2A SUMOylation analysis to cell cultures.*

The SUMOylation of TOP2A has been shown to be required for proper centromeric resolution in HeLa cells and for chromosome segregation in budding yeast (1-3, 5). However, how SUMOylated TOP2A regulates those processes remains unclear. The experimental studies presented in the previous chapters involve the biochemical study of SUMOylated TOP2A, SUMO-binding proteins, and their downstream effects. Using what we have learned from these studies, we can work toward determining whether these biochemical functions of SUMOylated
TOP2A play a role in the processes of centromeric resolution and chromosome segregation as well as any other processes. In chapter 2, we have shown that the SUMOylation of *Xenopus laevis* TOP2A CTD provides novel protein-protein interactions with SUMO-binding proteins. Additionally, we have found sites in the human TOP2A CTD that can be mutated to affect its SUMOylation pattern. This suggests that the human TOP2A CTD may function in a similar fashion as the *Xenopus* TOP2A CTD. While the studies of SUMOylation sites and their effects have only been reported in yeast and in XEE, TOP2A mutant tetracycline-inducible cell culture using TetON cells can be established to observe the effects of mutating the human CTD SUMOylation sites (3-6, 21). This will allow for RNAi to deplete endogenous TOP2A while inducing exogenous RNAi-resistant TOP2A mutants as its replacement. By using inducible cell cultures as a tool, it may be possible to see the cellular consequences of preventing human TOP2A CTD SUMOylation. Examining how the mutations effect the SUMO-binding proteins identified from XEE assays and their downstream targets may also help us determine whether these downstream pathways are conserved between *Xenopus* and humans.

**PLACING THE PUZZLE PIECES TOGETHER IN TOP2A SUMOYLATION STUDIES**

The studies presented in the chapters of this dissertation demonstrate that SUMOylation of TOP2A CTD regulates protein interaction with SUMO-binding proteins. Among those binding proteins, Haspin is important for the recruitment of Aurora B and the chromosomal passenger complex (CPC) (22, 23). Haspin is known to phosphorylate centromeric histone H3 at threonine 3, and the phosphorylation at threonine 3 allows for Aurora B and the CPC to bind at the mitotic centromeres during early mitosis (24, 25). Then, Aurora B can regulate proteins
involved in microtubule attachment and the spindle assembly checkpoint to regulate the metaphase-to-anaphase transition (26). We have found that SUMOylated TOP2A plays a direct role in this pathway by acting as a scaffold at the mitotic centromeres to recruit Haspin to the proximity of centromeric histone H3 for its phosphorylation. However, not only have we found that mitotic SUMOylation is important for this protein interaction, we have also found that the phosphorylation of Haspin during early mitosis is also necessary for it to bind to the centromeres, and this provides a dual post-translational modification binding regulation mechanism.

Many previous studies have shown that inhibition of mitotic SUMOylation causes chromosome segregation defects (5, 11-13). This suggests that mitotic SUMOylation is necessary for proper chromosome segregation. The pathway discovered involving SUMOylation of TOP2A CTD regulating Haspin at the centromeres may explain the abnormal phenotype many have reported when SUMOylation is misregulated since it would affect the centromeric localization of Aurora B which is important for proper chromosome segregation. However, Haspin may not be alone as a TOP2A SUMO-binding protein that regulates Aurora B kinase. The mitotic function of Claspin still remains unclear. However, during the DNA damage checkpoint, Claspin binds and interacts with checkpoint kinase Chk1 (27-31). A previous study has reported Chk1 to phosphorylate Aurora B at S331 during mitosis and this phosphorylation is necessary to activate Aurora B kinase (32). Since Chk1 is capable of binding to Claspin, it is possible that SUMOylated TOP2A CTD recruits Chk1 to the centromere through Claspin in order to allow Chk1 to phosphorylate Aurora B for Aurora B activation. In fact, Chk1 localization at the centromeres of Xenopus mitotic chromosomes is greatly reduced when SUMOylation is inhibited, suggesting that the localization of Chk1 is dependent on SUMO (Fig. 5.4A and 5.4B). Therefore, SUMOylation of TOP2A CTD may play a dual role in regulating
Aurora B both spatially in its localization through the Haspin-H3T3p recruitment pathway, and temporally in its timely activation through the Claspin-Chk1 signaling pathway. Further studies may determine the interaction between Claspin and Chk1 during mitosis.

Figure 5.4. Chk1 localizes at mitotic centromeres in a SUMO-dependent manner.

**A)** Recombinant T7-tagged Chk1 was added to XEE, and mitotic replicated chromosomes were isolated from XEE and analyzed by immunofluorescence staining for TOP2A, SUMO2/3, and T7-tag. Dominant negative Ubc9 (dnUbc9) was added to inhibit SUMOylation. White bar indicates 5 µm. **B)** Recombinant T7-tagged Chk1 was added to XEE, and mitotic replicate chromosomes treated with or without dnUbc9 were isolated for immunofluorescence staining of Claspin, T7, and SUMO2/3. White bar indicates 5 µm.
It is interesting to think about how SUMO modification can regulate two different functions of an enzyme depending on where the modification occurs on the protein. TOP2A K660 SUMOylation inhibits the decatenation activity necessary to resolve the cohesion of the sister chromatids to allow them to separate (4). On the other hand, TOP2A CTD SUMOylation allows Aurora B to regulate proteins at the centromere via the Haspin-H3T3p pathway for the spindle assembly checkpoint. While these two functions of SUMOylation appear to act opposite of each other at first glance, both functions work together for the same overall purpose of maintaining early mitosis. While the CTD SUMOylation allows for the regulation of Aurora B at the centromere, K660 SUMOylation helps maintain the cohesion of the tangled centromeric DNA until TOP2A is signaled to resolve the cohesion of the sister chromatids to allow their separation. That signal occurs at the onset of anaphase when deSUMOylation occurs by SUMO proteases (33). Additionally, the deSUMOylation will allow for Aurora B to be released from the centromeres to move to the midzone (34).

Since Aurora B functions as a key regulator at the centromeres and kinetochores to signal for spindle checkpoint activation to delay mitosis, proper regulation is necessary to make sure Aurora B is properly regulating the sister chromatid separation. One method to regulate Aurora B kinase activity is to control its localization at the centromere. The dual post-translational modification-mediated protein interaction between SUMOylated TOP2A and Haspin helps regulate Haspin activity, and more importantly, helps regulate Aurora B localization. In addition to the Haspin-H3 pathway, other interactions help localize Aurora B and the CPC to the centromere during mitosis. Phosphorylation of histone H2A at T120 mediated by Bub1 allows for Shugoshin proteins to bind and recruit the CPC at the centromeres through the Shugoshin-
Borealin interaction (35, 36). Alongside the two histone modifications is the interaction between the heterochromatin protein HP1 with INCENP to localize Aurora B at the centromeres (37). With several different pathways involved in localizing Aurora B and the CPC to the mitotic centromere, the question arises why the cell developed multiple layers of regulation for Aurora B localization.

One possibility is to make sure that Aurora B is acting at the centromeres at proper levels. Aurora B phosphorylates its substrates involved in centromeric and kinetochore functions by correcting microtubule attachment errors (38-42). Inhibition of Aurora B causes microtubule depolymerization, perturbed chromosome alignment, bypass of the spindle assembly checkpoint, and chromosome segregation defects (43-50). On the other hand, overexpression of Aurora B activity causes a constitutively active spindle checkpoint and continuous disruption of the attachments of microtubules to the kinetochore, which leads to improper chromosome segregation (51-55). In fact, overexpression of Aurora B is seen in different cancer cells and has become a target for the development of anti-cancer therapeutics (56). Therefore, there seems to be an upper threshold and a lower threshold for the level of Aurora B activity at the centromeres that is essential for proper mitotic progression. In order for the cell to achieve the proper level of activity within the threshold, it is possible that the cells utilize multiple pathways in the recruitment of Aurora B, or to minimize the detrimental effects when one of those recruitment pathways is defective.

Another possibility for having multiple regulation layers on the activity of Aurora B may relate to the proteins' roles outside of Aurora B recruitment. Bub1 is a kinase that is responsible for the localization of crucial spindle assembly checkpoint components, the Mad proteins (57, 58). The condensation of the chromosomes during early mitosis leads to heterochromatin
formation and gene silencing, which HP1 maintains by interacting with methylated histone H3 at K9 (59, 60). SUMOylation plays an important role for many of the proteins during mitosis. The SUMOylation of TOP2A, specifically at K660, inhibits TOP2A enzymatic activity to ensure that the centromeric DNA is kept entangled to maintain the cohesion of the sister chromatids before the onset of anaphase (4). Since each of the proteins have essential roles during mitosis, their normal performance during early mitosis may signal the recruitment of Aurora B to mitotic centromeres. Phosphorylation of histone H2A at T120 indicates an active Bub1 that is ready if the spindle checkpoint needs to be activated (35, 61). HP1 binding to INCENP in the CPC indicates that heterochromatin has formed from the condensed chromatin to silence gene activity for mitotic progression (37). SUMOylation of TOP2A CTD may signify that K660 is SUMOylated and that the cohesion of the sister chromatids at the centromeres is maintained before the sister chromatid need to be separated. These proteins, while playing a role in other mechanisms, may act as signaling proteins during mitosis to activate the separate pathways in recruiting Aurora B to the centromeres. Each pathway may partially accumulate centromeric Aurora B, and when all pathways are activated, the level of Aurora B at the centromere becomes the necessary amount for Aurora B activity to correct and maintain kinetochore-microtubule attachment to progress into anaphase properly.

The phenotypes caused by TOP2 inhibitors are often tied to DNA damage. In mitosis, TOP2 inhibitor-mediated DNA damage causes metaphase arrest, suggesting the activation of the spindle assembly checkpoint and DNA damage response (DDR) (62-64). However, ICRF-193 concentrations that do not cause DNA damage have also been reported to cause metaphase arrest (65). Mad2 seems to play a role in this type of metaphase arrest while Bub1 does not, suggesting activation of a checkpoint other than the spindle assembly checkpoint (62). This suggests that,
since TOP2 inhibitors causes hyperSUMOylation of TOP2A, the hyperSUMOylation may act as a signal for the activation of a delay through a mitotic checkpoint. HyperSUMOylation of TOP2A could cause the Haspin-H3T3p pathway to abundantly recruit Aurora B to the chromosomes to upregulate Mad2. However, it is still unclear whether this is the case. Interestingly, DNA damage checkpoint protein Claspin only increased at the centromeres and not on the chromosomal arm regions by TOP2 inhibitor addition during mitosis, suggesting that Claspin may function specifically to control the centromeric accumulation of Aurora B.

The results from the experimental studies presented in these chapters will help pave way into further studies that we hope will provide the knowledge necessary to fully understand the functions of SUMOylated TOP2A. However, even though the studies presented in these chapters have advanced our knowledge of SUMOylated TOP2A, our understanding of TOP2A and SUMOylation in connection to cell division can continue to expand. Many questions remain unanswered with new questions arising after each outcome. While the effects of TOP2A and SUMOylation in cell division and cancer cells are still unclear, further studies on the biochemical and phenotypical analysis of SUMOylated TOP2A will provide knowledge necessary to understand its roles during cell division.

MATERIALS AND METHODS

DNA constructs, site-directed mutagenesis, recombinant protein expression, and antibodies.

For recombinant full-length Xenopus laevis Chk1 proteins, cDNAs were subcloned into a pPIC3.5 vector (ThermoFisher) that had a calmodulin-binding protein (CBP)-T7 tag sequence and were expressed in the GS115 strain of Pichia pastoris yeast. CBP-T7 tagged Chk1 proteins
were extracted by grinding frozen yeast cells with dried ice followed by the addition of lysis buffer (150 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 30 mM Hepes [pH7.8]), and purified by Calmodulin agarose (GE Healthcare) affinity chromatography and by anion exchange column (GE Healthcare) as previously described (4, 18). Recombinant Xenopus laevis TOP2A CTD (1,222-1,579 a.a.) was subcloned into pET30a (EMD Millipore) while SENP2 catalytic domain (CD, 363-589 a.a.) and PIASy cDNAs were subcloned into pET28a vectors (EMD Millipore) with an N-terminal His-tag. For E1 complex (Aos1/Uba2 heterodimer), Uba2 and Aos1 cDNAs were subcloned into pRSF Duet vector (EMD Millipore) and expressed together in E.coli. Both wild type and dominant-negative form of Ubc9 (dnUbc9-C93S/L97S) were subcloned into pT7-7 vectors (from M. Dasso of NIH (66)) and SUMO2-GG was subcloned into pGEX4T-1 (GE Healthcare) with an N-terminal GST tag. All proteins were expressed in BL21 (DE3) or Rosetta 2 (DE3) bacteria either at 15°C in the 2xYT media containing 5% glycerol and 2.5% ethanol (for TOP2A CTD, SENP2 catalytic domain, PIASy and E1 complex) or 30°C in 2xYT media (for Ubc9 and SUMO2-GG). Proteins with His-6 tag (TOP2A CTD, PIASy, SENP2 CD) were extracted by lysing cells in the buffer (500 mM NaCl, 1 mM MgCl₂, 25 mM Hepes [pH7.8], 5% glycerol, 1 mM PMSF, and 0.5% Triton X-100) with 0.1mg/mL lysozyme (Sigma-Aldrich). His-6 tagged proteins were purified using Cobalt affinity beads (Talon Beads from Clontech) from soluble fractions after centrifugation at 25,000xg for 40 minutes. Proteins were eluted with imidazole and imidazole-eluted fractions were further separated by ion-exchange columns. For E1 complex purification, cells were lysed as above except with 150 mM NaCl concentration. The E1 complex containing imidazole elutions were loaded onto a SUMO1 affinity column (GST-SUMO1-GG conjugated to NHS-sepharose from GE Healthcare) in the presence of ATP, and bound E1 complex was eluted by DTT-elution buffer (100 mM NaCl, 1 mM MgCl₂, 30 mM Tris.
[pH 8.8], 5% glycerol, and 10 mM DTT). E1 complex containing DTT-eluted fractions were further purified by anion exchange column. GST-SUMO2-GG was extracted from E. coli cells by lysis method mentioned above and captured on Glutathione-sepharose beads (GE Healthcare). Bound beads were incubated with thrombin to cleave the GST tag to elute untagged SUMO2-GG. Eluted SUMO2-GG was further purified by anion exchange column followed by sephacryl S-100 gel filtration (GE Healthcare). Ubc9 proteins were extracted by sonication in 50 mM NaCl lysis buffer. Soluble fraction after centrifugation was loaded onto the anion exchange column. Collected Ubc9 proteins were separated by cation exchange column followed by sephacryl S-100 gel filtration. All proteins were concentrated with buffer exchanged to 100 mM NaCl, 1 mM MgCl₂, 20 mM HEPES [pH 7.8], 5% glycerol, and 0.5 mM TCEP by the centrifugal concentrator (Amicon Ultra, ThermoFisher). The protein concentrations were measured using the Bradford method (Bio-Rad), with BSA as the standard. Purified proteins were snap frozen with liquid nitrogen and stored at -80°C.

*Xenopus laevis* Haspin NTD cloned into a pTGFC70 plasmid with a C-terminal GFP tag and a 3’ UTR sequence of xKid. Haspin NTD mRNA was obtained by *in vitro* transcription reaction with mMMESSAGE mMACHINE SP6 kit (ThermoFisher) from pTGFC70 plasmid as previous reports described (14, 25). The pTGFC70 plasmids of Haspin NTD-GFP were first linearized with NotI restriction enzyme digestion. Linearized pTGFC70 plasmids were incubated with the mMMESSAGE mMACHINE SP6 kit transcription mixture for 3 hours at 37°C to synthesize mRNA. The transcribed mRNAs were precipitated in LiCl for recovery and dissolved in nuclease-free H₂O.

Antibodies used for the study are as follows. For immunoblotting, rabbit anti-SUMO2/3 polyclonal antibody (1:1000) and rabbit polyclonal antibody against the PIASy N-terminus
region (68-138 a.a.) (1:1000) were prepared as previously described. Rabbit anti-Haspin polyclonal antibody for \textit{Xenopus laevis} was gifted by Dr. H. Funabiki (25). Commercial antibodies used for immunoblotting analysis were mouse monoclonal anti-GFP (JL-8) (Clontech; 1:1000), rabbit monoclonal anti-Aurora B kinase T232 phosphorylation (T248 in \textit{Xenopus laevis}) (Cell Signaling Technology; 1:1000), rabbit polyclonal anti-histone H3 (Cell Signaling Technology; 1:1000), S-protein-HRP (EMD Millipore; 1:2000), and mouse monoclonal anti-β-tubulin (Sigma-Aldrich; 1:1000). For immunofluorescence staining, anti-SUMO2/3 guinea pig polyclonal antibody (1:500) and chicken polyclonal anti-\textit{Xenopus laevis} CENP-A (1:500) were prepared as previously described (4, 11, 18, 20), and anti-PIASy rabbit polyclonal antibody (1:500) was used (4, 11, 20). Commercial antibodies used for immunofluorescence analysis were mouse anti-Topoisomerase II monoclonal antibody (MBLI, 1:1000) and mouse monoclonal T7-tag antibody (ThermoFisher). Primary antibodies were visualized by fluorescently labeled secondary antibodies (ThermoFisher): goat anti-mouse IgG Alexa 488 (1/500), goat anti-rabbit IgG Alexa 568 (1/500), goat anti-guinea pig IgG Alexa 678 (1/500), and goat anti-Chicken IgG Alexa 488 (1/500).

**XEE, immunodepletion/add-back assay, and pull-down assays.**

Low-speed extracts of \textit{X. laevis} eggs arrested in metaphase with cytostatic factor (CSF XEE) and de-membraned sperm nuclei were prepared following standard protocols (67, 68). Immunodepletions of endogenous TOP2A were performed with protein A-conjugated magnetic beads (ThermoFisher) (69). Equal volume of rabbit anti-TOP2A antibody (1 mg/mL) and Protein A Dynabeads suspension were incubated to capture the antibodies on Dynabeads. Anti-TOP2A-captured beads were blocked with 5% BSA containing CSF-XB (100 mM KCl, 0.1 mM CaCl$_2$, 2
mM MgCl₂, 5 mM EGTA, 50 mM sucrose, and 10 mM HEPES [pH 7.8]). To reach greater than 99% depletion of TOP2A from XEE, we used anti-TOP2A captured Dynabeads from initial Dynabead suspension at 1.1 µL suspension-to-1 µL XEE ratio (ie. 440 µL suspension of anti-TOP2A Dynabeads were used for immunodepletion in 400 µL of XEE in Figure 5). XEE/Dynabeads mixture was incubated for 15 minutes at room temperature followed by 15 minute incubation on ice. For add-back experiments, purified recombinant T7-TOP2A proteins were added to immunodepleted extracts to similar levels to the endogenous TOP2A, which was confirmed by immunoblotting. Chromosome isolations were performed as previously reported (70). For chromosome isolation, interphase extract was first obtained by releasing metaphase-arrested XEE with 0.6 mM CaCl₂. Demembranated sperm nuclei were incubated in interphase XEE at 6000 sperm nuclei/µL and equal volume of CSF XEE was added to induce the onset of mitosis. Inhibition of mitotic SUMOylation was made by the addition of a dominant-negative form of Ubc9 (dnUbc9) at a concentration of 150 ng/µL to the interphase XEE as well as the CSF XEE right before the two XEEs are combined for the induction of mitosis from interphase. After incubation, XEE was diluted three times its volume by 0.5x CSF-XB supplemented with 18 mM β-glycerophosphate and 0.25 % Triton-X100, 10 mg/mL of protease inhibitors (leupeptin, pepstatin, and chymostatin from Calbiochem), and 0.2 µM okadaic acid (Calbiochem). In the case to test TOP2 inhibitor treatment (ICRF-193, etoposide, merbarone), the inhibitors or DMSO were added to the XEE for 10 minutes before the XEEs were diluted. Diluted XEEs were layered onto dilution buffer containing 35% glycerol and centrifuged at 10,000g for 5 minutes at 4°C. Precipitated chromosomes were boiled in SDS-PAGE sample buffer, and the extracted proteins were subjected to immunoblotting with antibodies. Immunoblotting signals were acquired with Image Station 4000R (Carestream).
In vitro SUMOylation assay was performed with 15 nM E1, 6 μM SUMO-2, 2.5 mM ATP, and 500 nM TOP2A CTD in reaction buffer (20 mM Tris (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 0.05% Tween 20, and 0.5 mM dithiothreitol) together with 30 nM Ubc9 (E2) and 30 nM PIASy (E3). Reactions were incubated at 25 °C for the indicated periods.

The XEE pull-down assays were performed as described previously with 10 mM iodoacetamide addition in buffers to prevent deSUMOylation activity in the XEE (20). XEEs were diluted two times the volume with PD-buffer (20 mM sodium phosphate pH7.8, 18 mM β-glycerol phosphate [pH 7.5], 5 mM MgCl₂, 50 mM NaCl, 5% glycerol, 1 mM DTT and 10 mM iodoacetamide), and diluted XEEs were centrifuged at 25,000g for 45 min at 4°C. Equal volume of the PD-buffer supplemented with 0.2% Tween 20 and 0.2% Triton X-100 was added to the supernatants and incubated with S-tagged TOP2A CTD-bound or SUMOylated TOP2A CTD-bound S-agarose beads for 1 hours at room temperature. After washing with PD-buffer, the beads were incubated in the dilution buffer (20 mM sodium phosphate [pH 7.8], 18 mM β-glycerol phosphate [pH 7.5], 5 mM MgCl₂, 50 mM NaCl, and 5% glycerol) containing 35 μg/mL SENP2-CD for 45 minutes at room temperature to cleave conjugated SUMO2 from TOP2A CTD and to dissociate pulled down proteins from the beads. SDS-PAGE samples were prepared by adding a half volume of 3X SDS-PAGE sample buffer to beads suspension. In the case of looking for the molecular weight shift of TOP2A CTD, non-SUMOylated forms were incubated in XEE at room temperature of 4°C and isolated. Antarctic phosphatase was incubated with the isolated TOP2A CTD. All samples were separated on 8-16% Tris-glycine gels (ThermoFisher) by SDS-PAGE and analyzed with silver staining or immunoblotting. Immunoblotting signals were acquired by Image Station 4000R (Carestream).
**Immunofluorescence analysis of chromosomes.**

The mitotic chromosomes used for the immunofluorescence analysis were prepared as previously described (19). Replicated mitotic chromosomes were prepared by incubating demembranated sperm chromatin at 1000 sperm nuclei/µL in interphase XEE by adding 0.6 mM CaCl₂ followed with the induction of mitosis with the addition of equal volume of CSF XEE. To inhibit SUMOylation, dnUbc9 was added at 150 ng/µL to both the interphase XEE and CSF XEE before they were combined to induce the onset of mitosis. XEE containing mitotic chromosomes was diluted by three times the volume with IF-dilution buffer (0.5x CSF-XB containing 18 mM β-glycerophosphate and 250 mM sucrose) and equal volume of fixation buffer (IF-dilution buffer with 4% p-formaldehyde) followed by an incubation for 10 minutes at room temperature. In the case of testing TOP2 inhibitor treatment (ICRF-193, etoposide, merbarone), the inhibitors or DMSO were added to the XEE for 10 minutes before the XEEs were diluted. Fixed samples were layered on top of 8 mL of 40% glycerol cushion in glass tubes with cover slips. The chromosomes were spun down onto the coverslips by centrifuging at 6,000g for 20 minutes at room temperature. Chromosomes on the coverslips were post fixed with 1.6% p-formaldehyde in PBS for 5 minutes at room temperature. The specimens were blocked with PBS containing 5% BSA and 2.5% cold-fish gelatin and subjected to immunostaining with the antibodies. The localization of Haspin NTD on mitotic chromosomes was observed by GFP signals from exogenously expressed Haspin NTD-GFP prepared from mRNA addition to XEE (14). For Haspin NTD-GFP expression from mRNA, Haspin NTD-GFP mRNA was incubated in interphase XEE at room temperature for 60 minutes at a concentration of 20 ng/µL. Afterwards, demembranated sperm nuclei were added to allow for DNA replication. After the completion of DNA replication, equal volume of CSF XEE was added and incubated for 45 minutes for mitotic
CSF XEE with Haspin NTD-GFP. DNA was stained with Hoechst 33342 dye (EMD Millipore), and the samples were mounted using Vectashield H-1000 medium (Vector Laboratory). All images were acquired by using the Nikon Plan Apo 100x/1.4 oil objective lens on a Nikon TE2000-U microscope with a Retiga SRV CCD camera (QImaging) operated by Volocity imaging software (Improvision) at room temperature. Photoshop CS6 (Adobe) was used to process the obtained images from Volocity to show the signal intensities by adjusting overall intensity range levels equally within independent experiments without any gamma adjustments.

REFERENCES


