Mitotic SUMOylation: Unraveling the role of DNA Topoisomerase IIα SUMOylation and PIASy SUMO E3 ligase in mitosis

By

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ABSTRACT

A post-translational modification with SUMO (SUMOylation) can regulate various cellular events such as DNA replication, repair, transcription and cell cycle regulation. Many studies have indicated that SUMOylation is crucial for proper cell cycle progression. With three important enzymes, E1 activating enzyme, E2 conjugating enzyme, and E3 ligase, SUMOylation is mechanistically very similar to ubiquitination. Though, SUMOylation can affect a substrates' cellular localization, enzymatic activity, or can mediate protein-protein interaction. Using *Xenopus* egg extracts (XEEs) we have shown that disruption of mitotic SUMOylation causes chromosome segregation defects. Our group has identified DNA topoisomerase IIα (Topo IIα) as one of the important mitotic proteins for SUMOylation. SUMOylated Topo IIα C-terminus (CTD) interacts with Haspin kinase and recruits chromosome passenger complex (CPC) to the mitotic centromeres. In yeast and mammalian cells, the catalytic disruption of Topo IIα is reported to induce a delay in mitosis. However, the molecular mechanism for this mitotic delay is not well understood. In this dissertation, I have provided a molecular insight for this mitotic delay. An additional study had provided important evidence that blockage of Topo IIα enzymatic activity results in the hyper SUMOylation of Topo IIα. We have determined that Topo IIα CTD SUMOylation behaves like a signal transducer to induce a mitotic delay when Topo IIα is catalytically disrupted. Mutation in CTD SUMOylation sites has abrogated the mitotic delay. Further, we have shown that disruption of Topo II strand passage reaction (SPR) results in increased Topo IIα SUMOylation and Aurora B mobilization on chromosome arms. This is a conserved mechanism in XEEs and mammalian cells. Aurora B is a catalytic component of CPC and its precise centromeric recruitment is essential for

timely metaphase to anaphase transition. Aurora B mislocalization on chromosomes utilizes the Haspin-H3T3P pathway and is a key factor for the mitotic delay. Further, to understand the role of SUMOylation more clearly in human cells we have targeted one of the important SUMOylation enzymes PIASy SUMO E3 ligase. Earlier in XEEs, we found that PIASy is an essentially important E3 ligase for mitotic SUMOylation. Next, we sought to examine if PIASy has a conserved role in human cells. To address this question, we have established Tet-ON inducible ectopic expression of PIASy and SUMO interacting motif (SIM) mutants in human cells. Our results suggest that PIASy is an important E3 ligase that mediates mitotic SUMOylation in human cells. Altogether, this dissertation research expands our understanding of the significance of SUMOylation during mitosis.

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Finally, I dedicate this dissertation to my parents and husband for their immense love, support, and belief. Without that I would not have been able to chase my dreams.

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ABBREVIATIONS

SUMO - Small ubiquitin like modifier

XEEs - *Xenopus* egg extracts

SIM - Sumo interacting motif

SENP - SENP proteases

Topo IIα - Topoisomerase IIα

PARP1- Poly (ADP-ribose) polymerase 1

PICH - Polo-like kinase 1-interacting checkpoint helicase

SPR - Strand passage reaction

CTD - C-terminus domain

CPC - Chromosome passenger complex

CENP-A - Centromeric protein A

HCG - Human chorionic gonadotropin

CSF - Cytostatic factor

Py-S2 WT - PIASy SENP2 catalytic domain wild type

Py-S2 Mut - PIASy SENP2 catalytic domain mutant

H3T3P – Histone H3 threonine 3 phosphorylation

CHAPTER -1 Introduction

Post-translational modification is the conjugation of a chemical group or small molecule to a specific sequence of a target protein. Post-translational modification generates diversity among proteins following their translation. The post-translational modification of a protein can regulate its structure, function, and localization in different ways. There are many well-studied post-translational modifications such as phosphorylation, methylation, acetylation, or ubiquitination that are important for cellular activity. A unique protein modification involves the covalent attachment of a ubiquitin-like protein SUMO (**S**mall **U**biquitin-like **Mo**difier, **Fig 1.2**) is referred to as SUMOylation. SUMOylation regulates several biological processes and is a comparatively less explored post-translational modification. This dissertation primarily focuses on SUMOylation and its novel role during mitosis. Chapter 1 is largely focused on the introduction to SUMOylation, DNA Topoisomerase IIα (Topo IIα) SUMO modification, and methods to study SUMOylation used in this dissertation.

SUMOylation: The process of SUMO conjugation

SUMOylation is a conserved post-translational modification among eukaryotes and mechanistically very similar to ubiquitination. SUMOylation requires three enzymes: E1 SUMO-activating enzyme, E2 SUMO-conjugating enzyme, and E3 SUMO ligase [1]. The SUMO molecules are initially processed by SUMO proteases (Ulps/SENPs) to produce mature SUMO. A precursor SUMO is cleaved in its C-terminal region to generate a C-terminal di-glycine **(Fig 1.1)**. The first step of the SUMO conjugation is the activation of E1 enzyme by ATP which forms a SUMO-adenylate conjugate [2]. Next, SUMO is

transferred from the E1- SUMO complex to the E2 conjugating enzyme (Ubc9). At this step, the SUMO moiety forms a thioester bond with a cysteine (C93) present in the active site of E2 enzyme [3–6]. Finally, SUMO is conjugated to the substrate at the ε- amino group of lysine with the aid of E3 ligase. E3 ligase acts as an adapter protein in SUMO attachment to a target protein [7–10]. Previous studies have indicated that E3 ligase is important for substrate recognition in *vivo*. However a few substrates can be SUMOylated by E2 enzyme without E3 ligase [11].

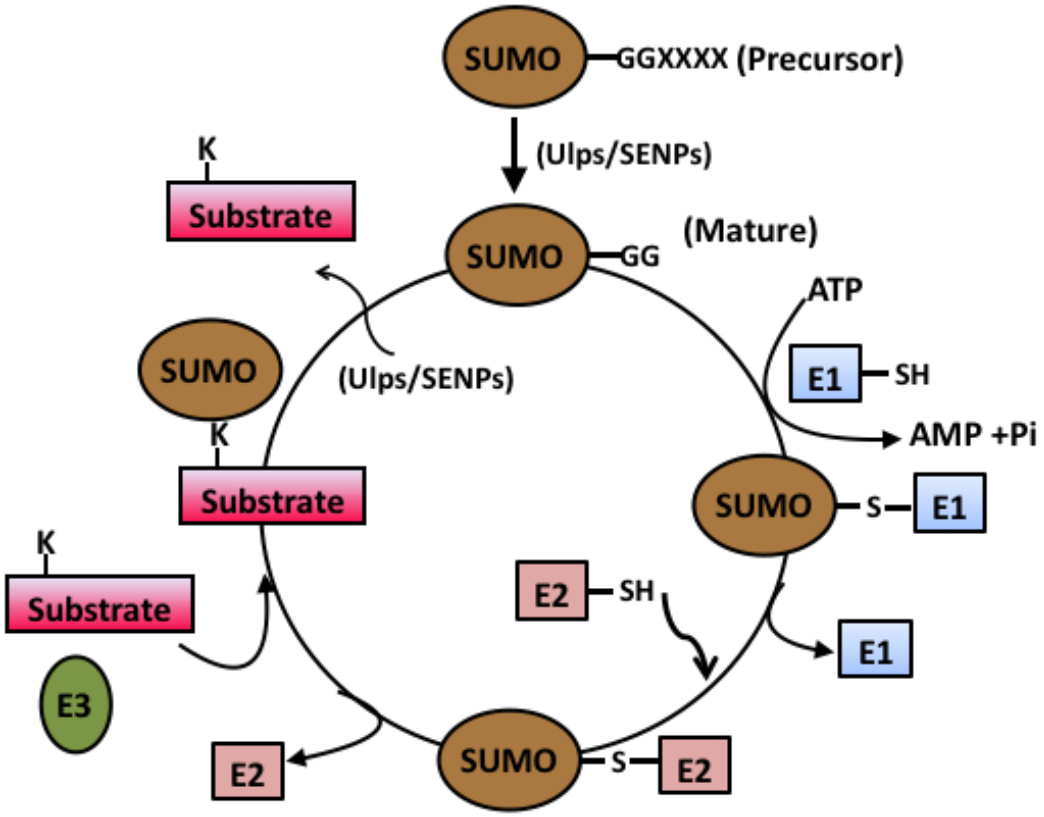

Figure 1.1 SUMOylation pathway. SUMOylation of substrates require three distinct classes of enzymes E1 activating enzyme, E2 conjugating enzyme, and E3 ligases. SUMOylation is a reversible post-translational modification. During SUMOylation, SUMO is conjugated to the substrate by forming an isopeptide bond between a ε- amino group of lysine and the carboxyl group of the C-terminus di-glycine of SUMO. For details, refer to text.

SUMO molecules are often attached on the **ε-** amino group of the lysine residue in the consensus sequence ΨKxD/E. In this sequence Ψ represents a hydrophobic residue, K is a lysine residue, x is any amino acid followed by either aspartate or glutamate residue (D/E) [12,13]. However, a few substrates are SUMOylated at a lysine residue which is not part of this consensus sequence. Examples of non-canonical SUMOylation sites include PCNA K164 and Topo IIα K660 [14,15].

Discovery of SUMO (Small Ubiquitin-like Modifier)

SUMO molecules belong to the small ubiquitin-like protein family and are approximately 100 amino acids in length. The SUMO gene was first discovered more than two decades ago in *Saccharomyces cerevisiae* and named SMT3 (suppressor of Mif 2 protein 3). Mif2 is a homolog of human centromeric protein CENP-C [16,17]. Later, different groups studied SUMO and characterized it as a novel UBL1 (Ubiquitin-like protein 1) due to it being a distant homolog of ubiquitin protein. UBL1 was first reported to interact with RAD51 and RAD52 proteins (double-strand break repair proteins) in mammalian cells. UBL1 also shares significant sequence similarity with yeast SMT3 (yeast SUMO homologue) protein [18]. Further, UBL1 was identified to modify RanGAP1 (Ran GTPase-activating protein) and allow RanGAP1 to interact with the nuclear pore complex protein RanBP2 (Ran-GTP-binding protein 2) [19,20]. In 1997, Mahajan et al. renamed UBL1 SUMO-1 (Small Ubiquitin-like Modifier 1) and demonstrated that SUMO modification could be involved in various cellular processes including protein targeting. [20].

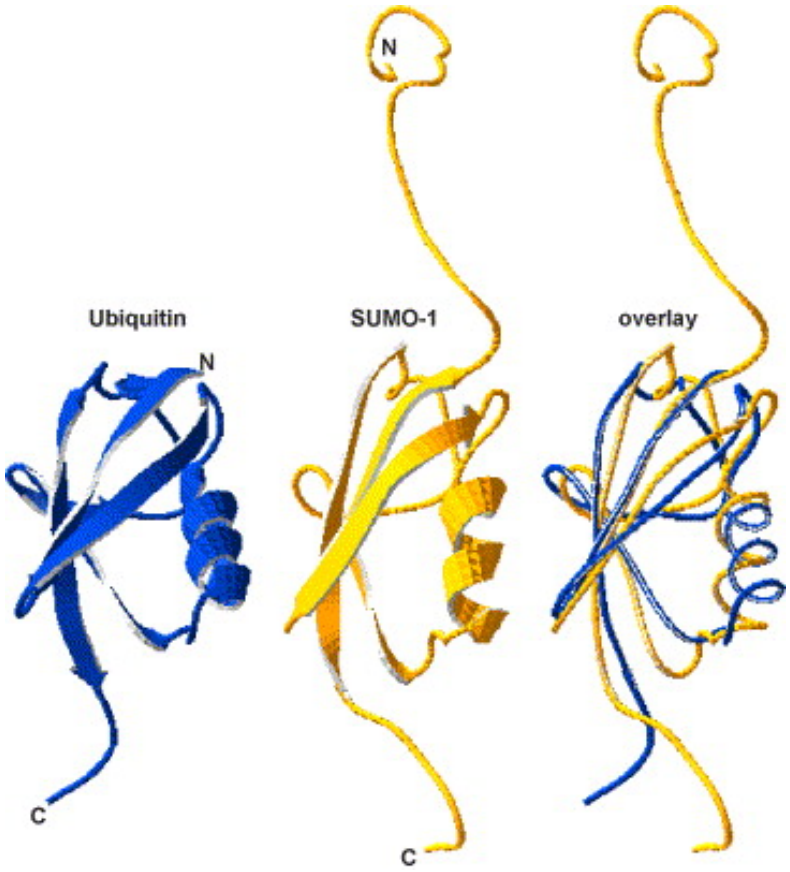

Figure 1.2 Structure of SUMO-1 and ubiquitin: Ribbon representation of SUMO and ubiquitin structure shows structure similarity between these two proteins. Figure adapted from Dohmen, 2004* [21].

Despite having only 18 % sequence identity with ubiquitin, the three-dimensional structure of SUMO-1 is very similar to ubiquitin **(Fig 1.2)** [22]. The C-terminal glycine residues are conserved in SUMO and essential to form an isopeptide bond during SUMOylation. A notable difference between SUMO-1 and ubiquitin structure is the extended and flexible N-terminus that is absent in ubiquitin. The extended N-terminus is important for binding of additional SUMO molecules to form a SUMO chain [23,24].

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* "Reprinted from Biochim Biophys Acta (BBA) - Molecular Cell Research, 1695, Dohmen RJ, SUMO protein modification,113–131, (2004), with permission from Elsevier."

SUMO isoforms

In invertebrates, there is only one SUMO whereas vertebrates have four isoforms; SUMO-1, SUMO-2, SUMO-3, and SUMO-4. SUMO-1, -2, and -3 share around 50 % sequence identity with yeast SUMO paralogue Smt3. SUMO-1 represents approximately 46 % sequence identity with SUMO-2 and -3. SUMO-2 and SUMO-3 are 96 % identical to each other and almost indistinguishable, therefore together referred to as SUMO2/3. SUMO-1, -2, and -3 are the most explored isoforms and ubiquitously expressed. However, there is not much known about SUMO-4 and its expression is limited to tissues such as kidney, lymph node and spleen [25–27]. SUMO-1 and SUMO2/3 are attached to different substrates and SUMO 2/3 modified proteins play a role in cellular responses to environmental stress. One study showed that SUMO2/3 - modified proteins are found in a greater abundance than SUMO-1 - modified proteins in mammalian cellular extract [28]. Additionally, SUMO-1 and SUMO2/3 are localized differently during the cell cycle. SUMO-1 is primarily located to the nucleolus and nuclear envelope because RanGAP1, a primary SUMO-1 substrate, is abundantly present there [29]. On the other hand, SUMO2/3 are mostly distributed to nucleoplasm where it modifies various nuclear proteins such as Topo IIα [29].

SUMO E1 activating enzyme

The SUMO E1 activating enzyme is a heterodimer of Aos1 (activation of smt3p) and Uba2 (ubiquitin activating enzyme). Initially, Aos1 and Uba2 were discovered in budding yeast as SAE2 and SAE1 respectively [30]. Most organisms have only one SUMO E1 activating enzyme to modify all the substrates. The interaction between E1 heterodimer and SUMO is non-covalent, utilizes ATP, and forms a thioester bond between cysteine present in the

active site of Uba2 and SUMO. While in most organisms E1 heterodimer is essential for the SUMO modification, in *Saccharomyces pombe* deletion of Rad31 (an orthologue of Aos1) still showed SUMOylation within the cells [31,32]. This study had indicated that despite an active site present in the Uba2, it might have lower SUMO activating capacity and works more efficiently in conjugation with the Aos1 subunit. Although SUMO E1 activating enzyme function as a heterodimer, the expression level for Uba2 and Aos1 is different. Uba2 level is almost consistent throughout the cell cycle whereas Aos1 expression level is found to change substantially [33].

SUMO E2 conjugating enzyme

Similar to the E1 activating enzyme, yeast and vertebrates have only one SUMO E2 conjugating enzyme (Ubc9). The Ubc9 gene was initially discovered in *Saccharomyces cerevisiae* and originally thought to be one of the ubiquitin E2 enzymes due to 35 % sequence identity with them. The overall charge distribution of Ubc9 is distinct from ubiquitin E2 enzymes allowing specific interaction of Ubc9 with SUMO, not with ubiquitin [34]. Ubc9, with a more positively charged surface allows binding with negatively charged SUMO-1. So far, only one SUMO conjugating enzyme has been reported, unlike the ubiquitin pathway which has various E2 enzymes. In humans, a single Ubc9 is mapped to chromosome 16p13.3 and shares approximately 65% sequence identity with yeast Ubc9p. The primary sequence for Ubc9 protein is highly conserved across species.

Many studies in yeast have shown that Ubc9p (yeast Ubc9 homologue) is essential for cell viability [35,36]. Using Smt3p affinity chromatography, one group has identified yeast Ubc9p as the second enzyme involved in SUMOylation pathway [4]. This study showed UBc9p specifically forms a thioester bond with Smt3p, not with ubiquitin. Further, *in vitro*

results also indicated that Ubc9p is required to conjugate Smt3p to other proteins [37]. Later, using *Xenopus* egg extracts (XEEs) another group reported that Ubc9 is required for SUMO-1 modification of RanGAP1 [38].

SUMO E3 ligase enzyme

SUMO is transferred from the SUMO-Ubc9 complex to the substrate and conjugated on a lysine residue with the help of third class of enzymes known as SUMO E3 ligase. After the discovery of E1 and E2 enzymes scientists had observed inefficient SUMOylation in XEEs and speculated that there might be a third class of enzyme involved in this modification [33]. While, *in vitro* SUMOylation can be governed without E3 ligases, under physiological conditions E3 ligase is important for SUMOylation [9].

The SUMO E3 ligase was first discovered in *Saccharomyces cerevisiae* as Siz1 and required for Septin SUMOylation [39]. In a Siz1 yeast mutant, Septin SUMOylation was completely abolished. Further, another study had provided evidence that Siz1 directly interacts with Ubc9p and Smt3p [40]. Later, another *E3*-like protein Siz2 was discovered that also promotes Septin-SUMOylation [41]. Septins are GTP- binding cytoskeletal proteins in yeast and are required for cytokinesis. Interestingly, both Siz1 and Siz2 were reported to have a conserved signature Zn-binding RING domain similar to ubiquitin E3 ligases. Therefore, Siz1 and Siz2 are classified as SUMO E3 ligase enzymes.

Post discovery of Siz1 and Siz2, PIAS (protein inhibitor of activated STAT) proteins were identified as SUMO E3 ligases in mammalian cells [42,43]. There are four PIAS family proteins in mammals that are encoded by different genes: PIAS1, PIAS2α/xα or PIAS2β/xβ, PIAS3, and PIAS4/y. Initially, PIAS proteins were identified as transcription regulators because of their interaction with STATs (signal transducer and activator of

transcription). PIAS1 and PIAS3 interact with transcription factor STAT1 and STAT2 respectively [42,44]. Later, these proteins were reported to have several other functions including SUMO E3 ligase activity. All PIAS family proteins have a significant sequence homology at the N -terminus (first 60 amino acids) and cysteine rich central region. PIAS proteins have five important structural domains. The N -terminal SAP (scaffold attachment factor-A/B Acinus and PIAS) domain is involved in interaction with DNA and other proteins such as transcription factors. The PINIT domain is important for subcellular localization and SP-RING (Siz/PIAS RING finger zinc binding) domain is required for E3 ligase activity. The SIM (SUMO-interacting motif) interacts with SUMO. The last domain is a variable serine/threonine rich C-terminal domain [45].

Chapter 3 and 4 of this dissertation are focused on the PIAS functions and describes all PIAS family proteins in more detail.

SUMO proteases

SUMOylation is a reversible post-translational modification and SUMO moieties are de-conjugated from the substrate during de-SUMOylation. De-SUMOylation enzymes or SUMO proteases are required for this process. SUMO proteases are also essential for the initial activation of SUMO to generate a mature SUMO isoform with a C-terminal di-glycine. SUMO proteases were first discovered in yeast and named ubiquitin-like proteases (Ulp). Yeast has two different SUMO proteases Ulp1p and Ulp2p/Smt4 [46,47]. Ulp1p is normally localized to the nuclear envelope and involved in Smt3p maturation [47]. Whereas, Ulp2p is localized in the nucleoplasm and essentially important for chromosome segregation, meiotic development, and recovery from cell-cycle checkpoint arrest [46]. Mammals have six SUMO proteases (SENPs): SENP1, SENP2, SENP3, SENP5, SENP6,

and SENP7 [48]. Ulps and SENPs have a conserved cysteine protease domain at their C-terminus. The N-terminus is known to regulate its localization and substrate specificity. Mammalian SENP1, 3, and 5 are more similar to Ulp1p whereas SENP6 and SENP7 are similar to Ulp2p [49]. Like Ulps, SENPs also have distinct localization in the cell; SENP1 and SENP2 primarily localize to the nuclear envelope [48]. SENP3 and SENP5 localize to nucleoli whereas SENP6 and SENP7 localize within the nucleoplasm [50–52]. All SENPs perform SUMO processing and de-SUMOylation activity for SUMO1 and SUMO2/3.

Biological functions of SUMOylation

SUMO shares a significant structure similarity with ubiquitin. Like ubiquitination, SUMOylation can modulate various cellular processes **(Fig 1.3)**. SUMOylation can regulate a protein's cellular localization, mediate protein interactions, enzymatic activity, and ubiquitin-mediated protein degradation [53]. Depending upon the localization of a target protein, SUMOylation can occur either in the cytoplasm or nucleus and can regulate the subcellular localization of the modified protein. For example, RanGAP1 is predominantly present in the cytoplasm and modification with SUMO directs RanGAP1 to the nuclear pore complex (NPC) via interaction with RanBP2/ Nup358 [19,20]. SUMO modification of RanGAP1 creates a binding interface for the interaction with RanBP2/Nup358 and results in its mobilization to the NPC.

Non-covalent interaction with SUMO through SUMO-interacting motif

A SUMOylated substrate can mediate non-covalent protein interactions through a SUMO-interacting motif (SIM). The SUMO-SIM interactions could result in either simple or a large multiprotein complex assembly.

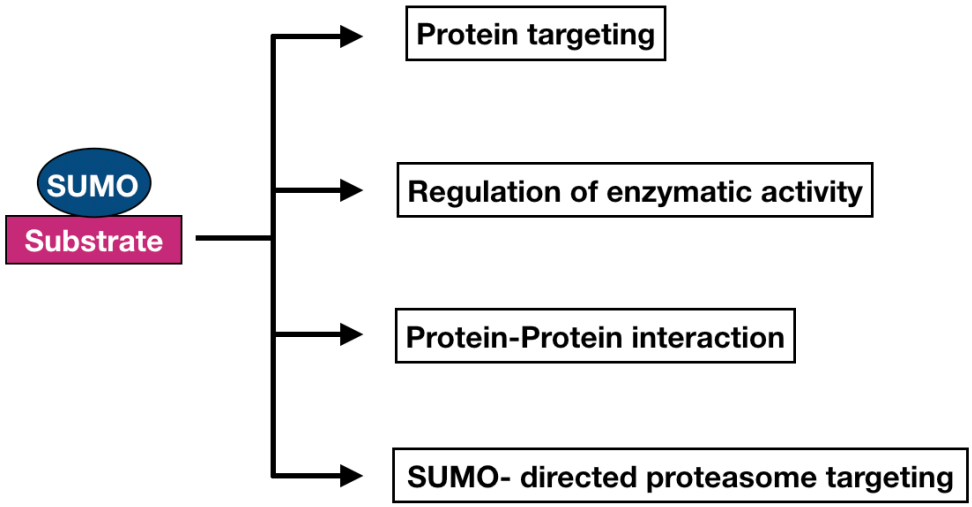

Figure 1.3 Molecular functions of SUMOylation: SUMOylation regulates variety of biological processes. For details, refer to text.

Using a yeast two-hybrid assay three different studies have shown non-covalent interaction of SUMO with human RAD51 and RAD52 [18,54,55]. Another group also reported that specific proteins can interact with SUMO using a conserved motif in a non-covalent manner [56]. Again, using a yeast two-hybrid approach they have also shown various proteins interact with SUMOylated p73 (a p53 family protein). After analyzing this motif they found a common Ser-X-Ser (SXS) sequence in all these interacting proteins [56]. In this sequence X represents any amino acid. Generally, the SXS sequence is flanked by hydrophobic amino acid residues on one side and acidic amino acids on the other side. This group had also emphasized that two serines are required for the interaction with SUMO. However, another group had reported that SUMO interaction through SIMs does not

require serine residues [57]. They did agree with the importance of flanked hydrophobic core in SUMO binding and characterized SIM as a consensus site Val/Ile-X-Val/Ile-Val/Ile (V/I-X-V/I-V/I). Since then, there are various proteins identified that contain SIMs including the SUMO E1 activating enzyme (in Uba2 subunit), PIAS E3 ligase and RanBP2/Nup358. PML nuclear bodies (PML-NBs) are a unique example of SUMO-mediated non-covalent interaction. PML contains SUMOylation sites and SIMs that allow non-covalent interaction between two PML molecules and result in PML-network formation. Further, SUMO moieties on PML-network provide a binding surface for other proteins containing SIMs [58,59].

SUMOylation can also regulate the enzymatic activity of a protein. SUMOylation of *Xenopus* Topo IIα at lysine 660 inhibits its decatenation activity [15]. Lysine 660 SUMOylation is important to maintain cohesion between sister chromatids during mitosis. Similarly, SUMO modification of BRCA1 (breast cancer type 1 susceptibility protein) increases its ubiquitin ligase activity in cells [60]. Since both SUMO and ubiquitin can be conjugated to lysines, SUMOylation can influence protein stability by promoting or blocking ubiquitination. While, ubiquitination is an important modification for the degradation of proteins, targeted proteins may be modified with SUMO first. SUMOylation of these proteins allow the binding of StUbl (SUMO-targeted ubiquitin ligases) enzymes to ubiquitinate the target protein for the degradation by proteasome. In a similar manner, SUMO conjugation to lysine can inhibit ubiquitination by masking the lysine residue. PCNA SUMOylation at a conserved K164 prevents its ubiquitination at the same lysine [14]. Lastly, SUMOylation can regulate transcription by modifying various transcription factors. For example, apoptotic pathway protein Daxx contains two SIMs and

is known to interact with different SUMO - modified transcription factors [61,62]. Daxx interaction with SUMOylated transcription factors such as c-Jun, ETS-1, NFκB and p53 downregulates their transcription activity [63].

PART-II Mitosis

During the cell cycle, cells undergo various biological events including cell growth, genome duplication, and subsequent division into two daughter cells. In a eukaryotic cell, the cell cycle has two major phases: interphase and mitosis. During interphase, cells grow, genomic DNA is replicated, and a cell prepares itself to go into mitosis by synthesizing required proteins and checking for potential DNA damage. Next, in mitosis, a cell divides and replicated DNA is evenly distributed into two daughter cells. Chromosomes undergo various morphological changes through five mitotic stages. First, in prophase, with nuclear envelope breakdown replicated chromosomes start to condense and mitotic spindles are formed with microtubules. In prometaphase, the nuclear membrane disappears completely and elongated microtubules are attached to condensed chromosomes at specialized region called kinetochores. Next, in metaphase, all chromosomes are aligned at the metaphase plate using the bipolar mitotic spindle. Correct kinetochore-microtubule attachment and alignment of sister chromatids at the metaphase plate are crucial to allow faithful chromosome segregation. The spindle assembly and tension checkpoints play important roles to make sure that these events occur without an error. The catenation in centromeric DNA is critical to maintain the cohesion between the sister chromatids. For error-free chromosome segregation, centromere DNA must be fully decatenated. During anaphase, sister chromatids are moved towards opposite spindle poles of an elongated cell and segregate. Two new daughter cells will have a complete set of genetic content. Lastly, at

telophase nuclear membrane is formed around each nuclei and the cell proceeds to cytokinesis. Mitosis is a dynamic process and requires various mitotic proteins to be post-translationally modified.

SUMOylation during mitosis

SUMO modification of various mitotic proteins is essential for proper progression through mitosis. Many studies in yeast and mammalian cells have indicated that disruption of SUMOylation during mitosis causes chromosome segregation defects. In budding yeast, degradation of M-phase cyclins by Ubc9 is required to exit from mitosis. The repression of Ubc9 arrests the cell cycle at G2 or early M phase with severe defects such as large budded cells containing a single nucleus and shorter spindles [35]. A study in *Saccharomyces cerevisiae* also showed that the SMT3 gene is essential for sister chromatid separation and mutation of SMT3 causes a chromosome segregation defect [64]. Similarly, Azuma et.al discovered that SUMOylation is critical for faithful chromosome segregation in cell-free model system XEEs [65]. In *Drosophila melanogaster,* a mutation in the *Su (var)2-10* locus which encodes a protein of the PIAS family causes chromosome condensation defects [66]. Additionally, another group had reported that Ubc9-deficient mice are embryonic lethal. The loss of Ubc9 function causes chromosome condensation and segregation defects in mouse embryos [67]. In budding yeast, cells lacking Ulp2 (a de-SUMOylation enzyme) showed abnormal cell morphology and reduced chromosomal stability [46]. Similarly, in mammalian cells, de-SUMOylation enzymes SENP1 and SENP2 are vital for chromosome segregation during mitosis. Knocking down SENP1 delays chromosome disjunction at metaphase [68]. Together, these findings indicated that

not only SUMOylation, but also de-SUMOylation, is important for proper cell cycle progression.

SUMOylation is one of the essential modifications to regulate centromere and kinetochore function during mitosis. Several centromere and kinetochore proteins are modified by SUMO and important for chromosome segregation [69]. DNA Topoisomerase IIα (Topo IIα) is one of the conserved mitotic substrates for SUMOylation.

DNA topoisomerase II

DNA topoisomerases are essential enzymes that resolve topological problems in DNA during different cellular processes such as DNA replication, recombination, and chromosome segregation. These enzymes are classified into two categories: type I and type II. Type I and type II enzymes have distinct catalytic mechanisms to alter DNA topology. Mao et.al showed that both type I and type II enzymes are modified with SUMO [70,71]. Type I topoisomerase is a monomeric enzyme which resolves tangled DNA by introducing a transient single strand break in the DNA [72,73]. However, type II topoisomerase is a homodimer and resolves topological issues by creating a transient double strand break in the DNA. Yeast and Drosophila contain only one Topo II isoform whereas mammals contain two different isoforms: α and β encoded by two separate genes [74]. Topo II in yeast and Drosophila is more similar to the α isoform of mammals. Topo IIα and Topo IIβ share around 70 % sequence identity with a significant difference in the C-terminal domains [75–78]. Despite having similar structure, Topo IIα and Topo IIβ have different cellular functions.

The α isoform is essential for cell viability and is important for cell cycle progression. Topo IIα is important for DNA replication, chromosome segregation, and recombination. Topo

IIα activity is crucial to resolve catenated centromeric DNA for error-free chromosome segregation. [79,80]. Additionally, a study in Drosophila S2 cells indicated that Topo II has a role in chromosome condensation during mitosis. They depleted Topo II using RNAi and mitotic chromosomes showed abnormal morphology due to the reduced chromosome compaction [80]. Compared to the α isoform, β is less explored and is mainly involved in transcription [81,82]. The mitotic function of Topo IIα cannot be substituted by Topo IIβ, suggesting that both isoforms have specific biological functions in the cell cycle [79]. This dissertation is primarily focused on the α isoform and its post-translational modification with SUMO.

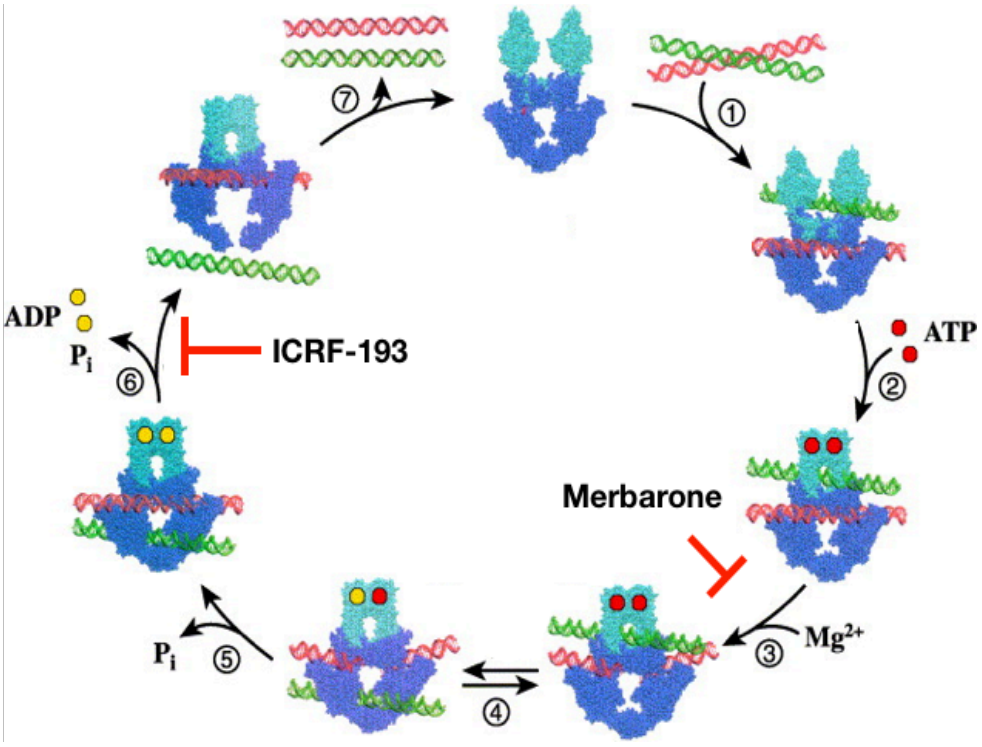

Figure 1.4 DNA Topoisomerase II catalytic cycle strand passage reaction (SPR): Figure adapted and modified from Larsen A.K et al, 2003 [83]. Topo II enzyme undergoes various conformational changes during SPR. The major steps of SPR are numbered through 1 to 7. For details, refer below the text. Topo II activity can be disrupted using different Topo II catalytic inhibitors. The Topo II catalytic inhibitors ICRF-193 and Merbarone have a distinct mode of action to block Topo II activity during SPR. Merbarone specifically blocks the DNA scission activity of Topo II enzyme at the early stage of SPR. ICRF-193 blocks ATP hydrolysis and traps Topo II in closed-clamp structure along with DNA*.

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Topo II performs its catalytic function via a unique Strand Passage Reaction (SPR). The eukaryotic Topo II structure possesses three major domains termed the N gate, DNA gate, and C gate. Topo II has a disordered C- terminal domain that is not included in the above figure because its structure is not defined yet. Topo II undergoes different conformational changes during SPR that makes Topo II as an important target for various anticancer drugs **(Fig 1.4)** [84]. The SPR activity of Topo II begins with two initial steps. First, a DNA strand **(G-segment, red)** binds to the N gate of the homodimer **(Step1)**. Second, one ATP molecule binds to each ATPase domain of the enzyme. ATP binding results in N gate closure and capturing the second DNA strand **(T - segment, green) (step 2)**. N gate closure also promotes a transient double strand break in the G segment **(step 3)**. ATP hydrolysis causes a conformational change in the Topo II enzyme that results in the opening of the DNA gate. Once the DNA gate opens, the T- segment is transported through the cleaved G -segment **(step 4)**. Next, the double strand break in the G-segment is religated by Topo II enzyme **(step 5)**. Lastly, a second ATP hydrolysis induces a conformational change that allows the opening of the C gate **(step 6)**. The T-segment is released through the opened C gate and Topo II initiates a second round of the SPR cycle **(step 7)** [85,86].

Topo IIα SUMOylation during mitosis

Topo IIα was initially discovered as a major mitotic substrate for SUMOylation in XEEs [65,87]. SUMOylated Topo IIα mostly accumulates at the mitotic centromere and appears to be important for chromosome segregation. A previous study in *Saccharomyces cerevisiae* showed that Topo II SUMOylation regulates chromosome cohesion at the centromere region [87]. In mammalian cells, Topo IIα SUMOylation by RanBP-2 (a nucleoporin with SUMO E3 ligase activity) is essential for the resolution of sister centromeres at anaphase onset [88]. This study also showed that SUMO modification of Topo IIα is required for its localization to the inner centromere. SUMOylated Topo IIα decatenates the centromeric DNA and prevents anaphase bridge formation. Furthermore, in mice, a reduced level of RanBP-2 results in increased sensitivity for tumor formation [88]. In XEEs, Ryu.et al. discovered that Lysine 660 (Lys 660) SUMOylation of Topo IIα greatly inhibits the decatenation activity of the enzyme [15]. *Xenopus* Topo IIα Lys 660 SUMOylation regulates the decatenation of centromeric DNA and appears to be important for chromosome segregation. Later, Ryu.et al. also identified three major SUMOylation sites in the Topo IIα C-terminal domain that mediate protein-protein interactions. SUMOylated Topo IIα CTD interacts with Claspin (a DNA damage checkpoint protein) at the mitotic centromere [89]. Yoshida et.al reported that SUMOylated Topo IIα CTD also interacts with histone H3 Kinase Haspin and regulates threonine phosphorylation of histone H3 (H3T3) at the mitotic centromere [90]. The Haspin Kinase-H3T3P pathway is crucial for the recruitment of chromosome passenger complex (CPC) at the mitotic centromere. Altogether, these findings indicate that Topo IIα SUMOylation at the centromere is pivotal for proper mitosis progression. How Topo IIα SUMOylation regulates chromosome

segregation during mitosis remains poorly understood. The results discussed in chapter 2 using XEEs and mammalian cells describe a novel role of Topo IIα CTD SUMOylation in mitosis progression.

DNA topoisomerase II inhibitors and SUMOylation

Topo II is required for the proliferation of cancer cells and there are many anti-cancer drugs available that target Topo II catalytic cycle. The drugs that block Topo II activity are primarily divided into two major classes based on their mode of action: poisons and catalytic inhibitors. Topo II poisons stabilize the DNA cleavage complex by inhibiting the ligation activity of the enzyme whereas Topo II catalytic inhibitors reduce the catalytic activity by targeting the other stages of the cycle. Etoposide (VP-16) is one of the most clinically used poisons that cause apoptosis by inducing double strand DNA breaks in cancer cells. However, treating patients with Etoposide is reported to cause several side effects such as acute myeloid leukemia or acute promyelocytic leukemia [91–93]. Agostinho et.al reported that cells treated with Etoposide upregulate Topo IIα SUMOylation during interphase [94].

Catalytic inhibitors can attenuate Topo II activity by trapping different catalytic intermediates during the SPR. Currently; there are many Topo II inhibitors available for clinical use. I have utilized Merbarone and ICRF-193 in this dissertation to block Topo II activity at specific stages of the SPR. Merbarone specifically inhibits DNA scission activity of the Topo II enzyme and eventually blocks the initiation of the SPR cycle [95]. ICRF-193 impedes the second ATP hydrolysis during SPR and traps Topo II in a closed-clamp conformation with DNA. Agostinho et.al showed that ICRF-187 (similar to ICRF-193) treatment upregulates Topo IIα SUMOylation during mitosis [94]. Yet, how Topo IIα

SUMOylation is increased with Topo II inhibitor treatment and biological function of Topo IIα SUMOylation remains unclear. Chapter two is primarily focused on inhibitor-mediated Topo IIα SUMOylation and its significance in mitotic progression.

Model system to study SUMOylation

SUMOylation has always been challenging to study because of its transient and reversible nature. As mentioned earlier, only a small amount (approximately 2%) of total protein is SUMOylated within a cell. SUMOylation is a transient post-translational modification because SUMO proteases rapidly perform the de-SUMOylation within the cell. These proteases are highly active and maintain a balance between SUMOylation and de-SUMOylation. Researchers have used different approaches to study SUMOylation including *in vitro* SUMOylation. An *in vitro* SUMOylation assay utilizes recombinant SUMOylation enzymes and substrates. Scientists have also utilized different genetic model organisms to study the SUMOylation *in vivo* such as mouse models, mammalian cell culture, and *Xenopus* egg extracts. Each model system has its advantages and can be used depending upon the research question to be addressed. In this dissertation, two different model systems are utilized to study SUMOylation: *Xenopus* egg extracts (XEEs) and mammalian cell culture. The rationale for each model system is provided in the next section.

Xenopus Egg Extracts (XEEs) - A cell free model system

Cell biologists have utilized XEEs to study the cell cycle for more than 30 years. XEE is an excellent system to recapitulate key cellular processes such as DNA replication and mitosis *in vitro*. Lokha and Masui prepared the first egg extracts in 1983 and then used demembraned sperm nuclei from South African clawed frog *Xenopus laevis*. Using this egg extract they analyzed DNA replication, chromosome condensation, decondensation, and nuclear envelope formation [96,97]. *Xenopus* eggs have several advantages over those from other amphibians and are chosen for the extract preparation. Normally, a single *Xenopus laevis* frog can lay more than 3,000 eggs and each egg has approximately 1µl of the cytoplasm. Therefore, one or two frogs provide enough cytoplasmic extracts for many biochemical experiments. *Xenopus* eggs are highly concentrated with maternal proteins and RNA to progress through interphase and mitosis. Therefore, post fertilization, *Xenopus* eggs can undergo 11 rounds of synchronous cell division without transcription [98,99]. Also, XEEs have a great advantage in functional analysis of proteins because depleting a protein and adding back a recombinant wild type or mutant protein is efficient compared to other model systems such as RNAi. We can analyze the consequences of protein depletion and the function of a mutant protein in a single cell cycle.

XEEs have been exploited to study the mitotic or interphase SUMOylation and different substrates were identified using this system. Using XEEs, we have identified three important mitotic chromosomal proteins as SUMOylation substrates; Poly-ADP ribose polymerase 1 (PARP1), Topoisomerase IIα (Topo IIα), and Polo-like kinase 1-interacting checkpoint helicase (PICH) [8,10,100]. This dissertation is mainly focused on Topo IIα SUMOylation. To prepare the XEEs, female *Xenopus laevis* frogs are injected with human

chorionic gonadotropin (HCG) hormone to induce ovulation. Fifteen hours post HCG injection, eggs are collected, and the jelly coat is removed using a dejellying solution. Further, eggs are packed by centrifuging at 170 ×g for 30 seconds. The packed eggs are centrifuged at 10,000 ×g for 15 minutes to allow the separation of cytoplasmic extract from maternal chromosomes, lipid content, and pigment granules [101]. The crude cytoplasmic extract contains all soluble components required for nuclear envelope assembly. The *Xenopus* eggs are arrested at the metaphase of the meiosis II due to cytostatic factor (CSF) activity, therefore the cytoplasmic extract is called CSF extract. Addition of Ca^{2+} releases CSF arrest and initiates interphase that allows formation of the interphase nuclei **(Fig 1.5 A&B)**. Further, addition of fresh CSF extract will induce mitosis because of high levels of Cyclin B. During mitosis, fully replicated chromosomes will be condensed and the bipolar spindles will be formed. To study mitosis-specific SUMOylation we add a dominant negative mutant of the E2 SUMO-conjugating enzyme (dnUbC9) right before adding the CSF extract to induce mitosis. The dnUbc9 enzyme is a catalytically inactive SUMO-conjugating enzyme that harbors the mutations (C93S and L97S) in the catalytic domain. The addition of dnUbC9 to extract impairs the SUMO conjugation to the substrate by sequestering the endogenous E3 ligases. This way we can specifically modulate the mitotic SUMOylation without affecting SUMOylation in interphase.

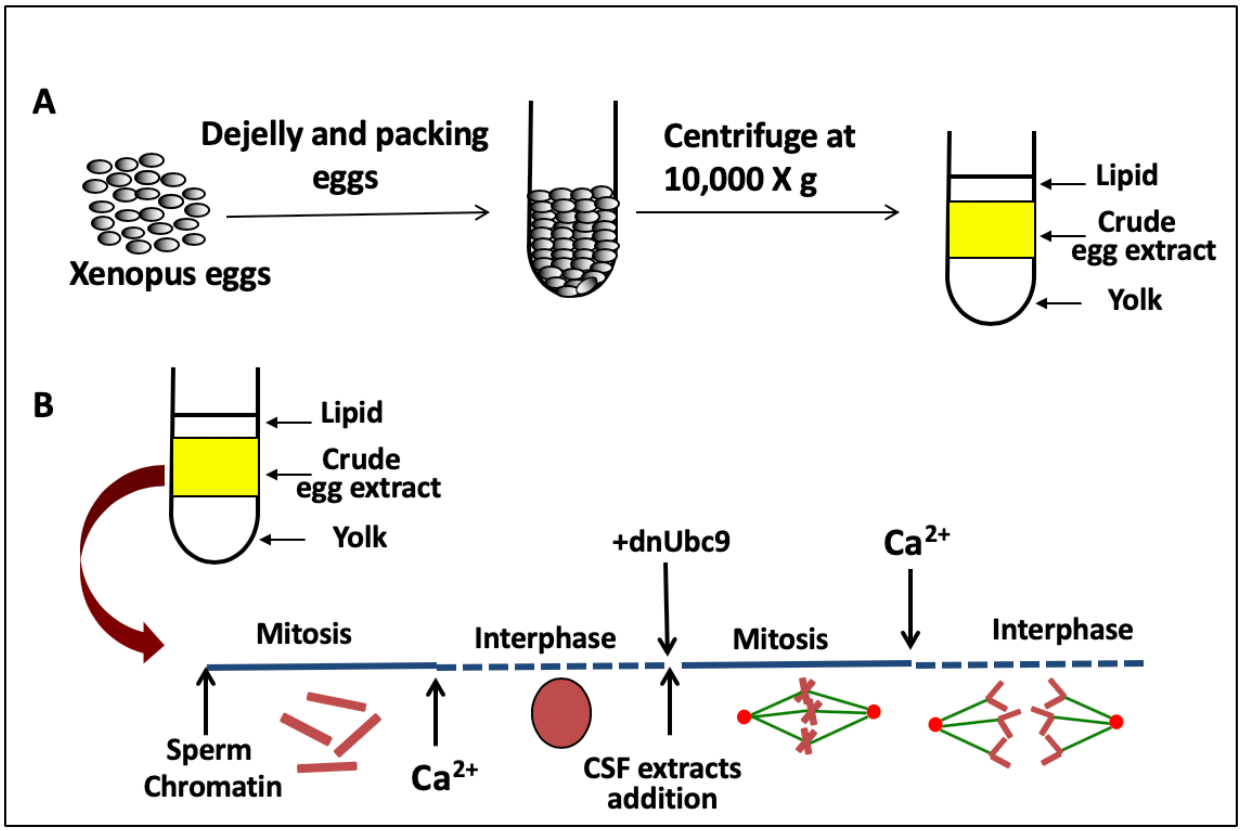

Figure 1.5 Xenopus egg extract preparation and cell cycle progression: (A) Xenopus eggs are collected, washed with dejellying solution to remove the jelly coat around the eggs. Eggs are subjected to low-speed centrifuge for further packing and removal of residual buffer. Centrifugation at 10,000 ×g in a swing rotor (Beckman/Coulter JS13.1 or Sorval HB-6) allows the crude cytoplasmic extract separation from the yolk and lipid. **(B)** Addition of sperm chromatin into CSF extracts results in chromosome condensation. Next, addition of Ca^{2+} releases the metaphase II arrest and induces interphase. Chromosomes are decondensed and interphase nuclei are formed. Further addition of CSF extract causes mitosis induction and replicated chromosomes are attached with bipolar mitotic spindles. Further, addition of Ca^{2+} drives exit from mitosis.

CRISPR-Cas9 edited Tet-ON inducible mammalian cell culture

XEE is an excellent system to analyze SUMOylation biochemically but has a limitation to perform phenotypic studies. To address this issue, we can utilize mammalian cell culture as an alternative approach. Modulating SUMOylation in cells is one of the key limiting factors with mammalian cell system. In the past, researchers used cells to study SUMOylation *ex vivo* [102,103]. In this dissertation, I have utilized genetically edited

human cells to study SUMOylation. One approach to modulate SUMOylation in cells is targeting the SUMOylation enzymes. Using this strategy, I have targeted SUMO E3 ligases to regulate SUMOylation in cells. DLD-1 (Colon adenocarcinoma cell lines) cells are used for the cell-based SUMOylation analysis and described in chapter 3 and 4. DLD-1 cells are epithelial adherent cells chosen because they have relatively stable genomes and have near diploid chromosome numbers compared to other cancer-derived cell lines. To establish the transgenic stable cell lines for the ectopic expression of PIAS E3 ligases, genes were stably integrated at the *AAVS1* safe harbor locus using a CRISPR-Cas9 editing method [104]. The expression of a transgene at *AAVS1* is inducible, under the control of a Tet-ON promoter **(Fig 1.6)**. Positive clones were screened with Puromycin selection marker for 10 days. Using genomic PCR and western blotting, we have confirmed the positive clones.

In this section of the dissertation, I have established transgenic stable cell lines for the ectopic expression all four human PIAS SUMO E3 ligases: PIAS1, PIASxα, PIAS3, and PIASy.

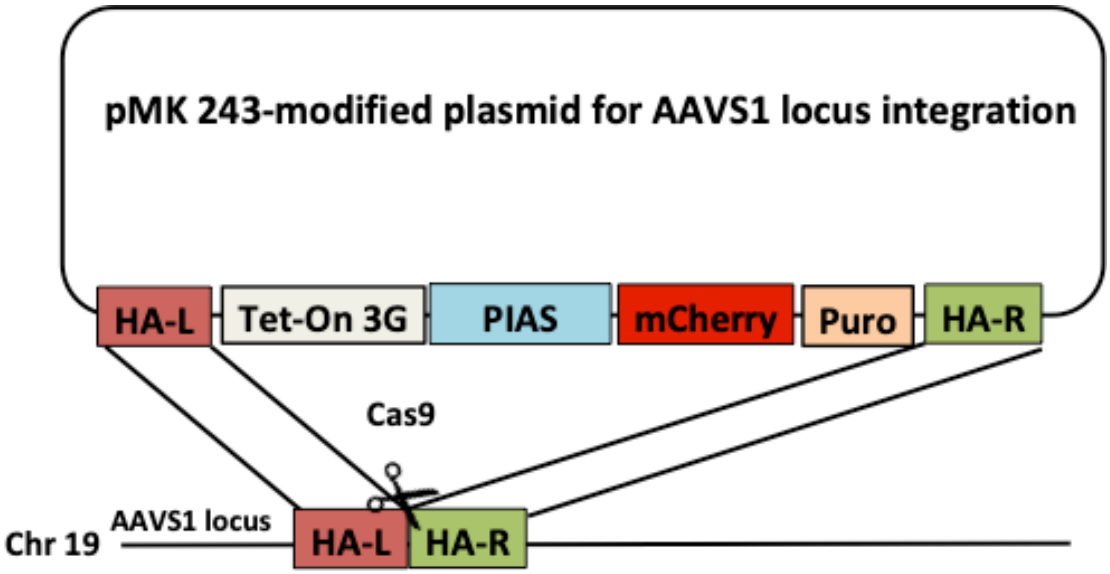

Figure 1.6 Schematic representations of the Tet-ON inducible stable cell line generation using CRISPR-Cas9 gene editing: PIAS genes were introduced into the *AAVS1* locus using pMK-243 plasmid (obtained from Addgene). Puromycin selection was used and positive clones were selected. The Tet-On 3G promoter is upstream to the PIAS gene and can induce protein expression in the presence of doxycycline.

Research Question leading to this dissertation work

Faithful chromosome segregation allows equal distribution of the genome to daughter cells. Chromosome segregation defects could result in various devastating consequences such as birth defects or aneuploidy. SUMOylation is conserved among different species and regulates the chromosome segregation process. Topo IIα is a conserved mitotic protein modified with SUMO. SUMOylated Topo IIα is concentrated at the centromere from early mitosis until the onset of anaphase. Topo IIα SUMOylation at the centromere regulates chromosome segregation at the onset of anaphase. Lys 660 SUMOylation in the catalytic core of Topo IIα greatly reduces the decatenation activity of the enzyme [15]. The potential function of Lys 660 SUMOylation is maintaining the cohesion between sister chromatids and not allowing their disjunction before anaphase starts. However, CTD SUMOylation

does not affect the decatenation activity of the enzyme but is important for some non-catalytic function of Topo IIα. Earlier studies have demonstrated that the CTD is dispensable for the SPR activity of the enzyme [105,106]. We have identified that the SUMOylated Topo IIα CTD has two novel binding partners in XEEs: Claspin and Haspin kinases [89,90]. In human cells, Claspin is known to interact with Chk1 kinase that activates Aurora B at the centromeres via Aurora B S331 phosphorylation [107]. Whereas in XEEs and yeast, SUMOylated CTD-Haspin interaction promotes Aurora B recruitment to the centromeres via phosphorylating the threonine 3 residue of histone H3 (H3T3P) [90,108]. Altogether, Topo IIα CTD SUMOylation regulates a key mitotic kinase Aurora B function and localization at the centromeres. Two independent studies in yeast and mammalian cells have shown that disruption of Topo IIα activity at the strand passage step causes a delay in anaphase [109,110]. Interestingly, another study in mammalian cells also showed that the catalytic disruption of Topo II activity also resulted in increased Topo IIα SUMOylation [94]. In this dissertation, I have asked two independent questions to understand in more detail how SUMOylation regulates the chromosome segregation. **Does Topo IIα CTD SUMOylation contribute to anaphase delay when Topo II activity is perturbed?** Since SUMOylated Topo IIα CTD binds with known Aurora B regulators Claspin and Haspin kinases we hypothesize that Topo IIα CTD SUMOylation could be involved in checkpoint signaling. Yeast cells expressing a Topo II mutant deficient in ATP hydrolysis delay anaphase and display metaphase arrest [109]. To further understand that how impaired Topo IIα activity induces this anaphase delay we have utilized catalytic inhibitors to disrupt the Topo IIα activity in XEEs and human cells. The catalytic inhibitor ICRF-193 specifically blocks Topo IIα SPR activity and mimics the yeast ATP-hydrolysis

deficient mutant. In chapter 2, using XEEs and human cells we have shown that ICRF treatment causes a delay in anaphase that is Topo II CTD SUMOylation dependent.

After more than two decades of research in SUMOylation, there is still not much known about the role of PIASy E3 SUMO ligase in mitotic SUMOylation of human cells. Azuma et.al discovered that PIASy is an essential SUMO E3 ligase for the mitotic SUMOylation in XEEs and modifies different substrates including Topo IIα and PARP-1 [8,10]. Immunodepletion of PIASy from the XEE eliminated mitotic chromosomal SUMOylation and addition of other PIASs could not restore it [8]. PIASy E3 ligase contains two SIMs that allow SUMO conjugation to the different substrates including Topo IIα and PARP1. In a collaborative study, we have shown that both SIMs are required for the Topo IIα and PARP1 SUMOylation *in vitro* [111]. However, the role of these SIMs to SUMOylation in the cells has not yet been studied. In the second part of this dissertation, **we investigate the role of PIASy E3 ligase in mitotic SUMOylation using human cells.** To study PIASy-directed SUMOylation during mitosis, I have created transgenic cell lines that will express PIASy with mutant SIMs. The ectopic expression of human PIASy with SIM mutants is Tet inducible. Using these PIASy SIM mutant cell lines, we can modulate PIASy-directed SUMOylation in human cells. I have observed that a PIASy double SIM mutant greatly reduced the mitotic SUMOylation in cells. Until now, we have only studied PIASy-directed SUMOylation in XEEs but not in human cells. Results from these genetically modified PIASy transgenic cell lines will expand our understanding of mitotic SUMOylation in human cells. Chapter 3 describes in detail some of the key observations using these cell lines. Altogether, this dissertation work increases our understanding of SUMOylation and its complex function during mitosis.

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CHAPTER -2 Topoisomerase II SUMOylation activates a metaphase checkpoint via Haspin and Aurora B kinases

Abstract

Topoisomerase II (Topo II) is essential for mitosis since it resolves catenations between sister chromatids. Topo II dysfunction promotes aneuploidy and can drive cancer. To protect from aneuploidy, cells possess a mechanism to delay anaphase onset when Topo II is perturbed, providing additional time for decatenation. Molecular insight into this checkpoint is lacking. Here we present evidence that catalytic inhibition of Topo II, which activates the checkpoint, leads to SUMOylation of the Topo II C-terminal domain (CTD). This modification triggers mobilization of Aurora B kinase from inner centromeres to kinetochores and the chromosome arms. Aurora B recruitment accompanies histone H3 threonine-3 phosphorylation and requires Haspin kinase. Strikingly, activation of the checkpoint depends both on Haspin and Aurora B. Moreover, mutation of the conserved CTD SUMOylation sites perturbs Aurora B recruitment and checkpoint activation. The data indicate SUMOylated Topo II recruits Aurora B to ectopic sites, constituting the molecular trigger of the metaphase checkpoint when Topo II is catalytically inhibited.

Introduction

Type II DNA Topoisomerases are universal enzymes that play crucial roles in mitosis due to their unique Strand Passage Reaction (SPR). The SPR is a multistep action involving large step-wise conformational changes and utilizing energy from hydrolysis of two ATP molecules [1,2]. First, the dimeric Topo II holoenzyme introduces a transient double-strand

break into a bound DNA helix. Next, a second, intact DNA helix is passed through the cleaved DNA, which is then re-ligated. Lastly, the transported DNA is released. It is not clearly understood how Topo II enzyme recognizes the catenated DNA to begin SPR cycle. This catalytic cycle has been well studied because widely-used anti-microbial and anti-cancer drugs target the SPR in their modes of action [3]. Previous studies have shown that during mitosis, catalytic inhibition of Topo II activates a metaphase checkpoint which results in delayed anaphase onset [4–6]. In yeast, Topo II mutants with defects in the SPR result in metaphase checkpoint activation [7,8]. In particular, yeast cells expressing mutant Topo II that has a low rate of ATP hydrolysis activate the metaphase checkpoint, while yeast expressing Topo II mutants defective at the initiation step of the SPR do not. This suggests the checkpoint is only activated when the SPR is impaired at specific stages, requiring ATP hydrolysis, and not due to a defect in SPR initiation.

The catalytic Topo II inhibitor ICRF-193 acts at the step of ATP hydrolysis and thus chemically mimics the genetic effects of the yeast mutants with a slow rate of ATP hydrolysis [3]. Human cells treated with ICRF-193 also activate a metaphase checkpoint [4–6]. However, it remains unclear how disruption of the Topo II SPR, particularly as late as the ATP hydrolysis stage, can induce a metaphase checkpoint. Recent studies provided a hint toward the molecular mechanism of this mitotic delay. HeLa cells treated with ICRF-187 (which inhibits Topo II activity using the same mechanism as ICRF-193), upregulate Small Ubiquitin-like Modifier 2/3 (SUMO2/3) modification of Topo IIα on mitotic chromosomes [9]. Another Topo II inhibitor, Merbarone, that blocks an early step of the SPR, did not upregulate SUMO2/3 modification. SUMOylation is important for error-free chromosome segregation in many eukaryotic organisms[10–14]. The α-isoform of Topo II

is modified predominantly by SUMO2/3 during mitosis in *Xenopus* laevis and human cells [9,15]. These observations indicate that catalytic inhibition of Topo IIα at the ATP hydrolysis step leads to SUMO2/3-modification of Topo IIα and that this biochemical event may play a role in metaphase checkpoint activation.

Supporting this notion, we reported that Topo IIα CTD SUMOylation regulates Aurora B at mitotic centromeres [16,17]. Aurora B is the kinase component of the Chromosome Passenger Complex (CPC) that controls the metaphase-to-anaphase transition. In *Xenopus* egg extracts (XEEs), SUMOylated Topo IIα CTD interacts with Claspin [18] which binds to Chk1 kinase; Chk1 can activate Aurora B via phosphorylation of S331 in human cells [19]. Further, SUMOylated Topo IIα CTD binds to Haspin kinase and promotes Aurora B recruitment to inner centromeres via phosphorylation of H3T3P [20–24]. This Topo II SUMOylation-dependent mechanism of Aurora B recruitment to mitotic centromeres is conserved in yeast and XEEs [16,17]. Here, we provide evidence that the metaphase checkpoint accompanies SUMOylation-dependent activation of Aurora B kinase in XEE and cultured cells. Checkpoint activation requires Aurora B and Haspin kinase, both of which are recruited to novel chromosomal positions upon Topo II catalytic inhibition. Aurora B and H3T3P become depleted from their normal residence at inner centromeres: ectopic phosphorylation of H3T3 is induced at kinetochores and chromosome arms; Aurora B is recruited to those same locales. We propose that upon detection of a stalled SPR, SUMOylation of the Topo II CTD triggers Aurora B activation to then induce a metaphase delay. The data have implications for cancer therapies under development that use Aurora B and Topo II inhibitors.

Results

Topo II catalytic inhibition upregulates Topo IIα SUMOylation on mitotic chromosomes assembled in XEE

SPR defects at the step of ATP hydrolysis activate a metaphase checkpoint in yeast and human cells [4,25]. We determined that Topo IIα SUMOylation stimulates Aurora B recruitment to centromeres in yeast and XEE [16,17] and Aurora B is known to regulate the progression from metaphase to anaphase. Thus, we postulated that SPR stalling at the step of ATP hydrolysis leads to SUMOylation of Topo II that recruits Aurora B to mitotic centromeres. We first asked if ICRF-193, which inhibits ATP hydrolysis by Topo II, induces Topo II SUMOylation, as was observed in somatic cells using the related inhibitor ICRF-187 [9]. In the well-defined XEE cell-free system, we observed chromosome condensation under the microscope, then immediately added either ICRF-193 or Merbarone (a Topo II inhibitor that does not act at the step of ATP hydrolysis). Adding the inhibitors after condensation eliminates indirect effects due to disruption of Topo IIα activity required for chromosome condensation **(Fig 2.1A)**. After 10-minute incubation with the inhibitors, chromosomes were isolated and subjected to western blot. Both inhibitors increased chromosomal mitotic SUMOylation but ICRF-193 had a much greater effect **(Fig 2.1B and C)** than control. Though, the observed effect of inhibitors on mitotic SUMOylation was non-significant. The increased mitotic SUMOylation was observed prominently for large molecular weight proteins, consistent with the molecular weight of SUMOylated Topo IIα. Indeed, anti-Topo IIα antibodies revealed ICRF-193-treated chromosomes had more SUMOylated Topo IIα than the control and Merbarone-treated samples **(Fig 2.1B and D)**. Quantification revealed a significant increase in SUMOylated

Topo IIα in ICRF-193 treated extracts, approximately 1.2 times more than the DMSO control, whereas Merbarone treated extracts showed only a slight increase. Increased SUMOylation of PARP1, another major SUMOylated protein on mitotic chromosomes in XEE [26], was not observed **(Fig 2.1B and E)**. Notably, increased chromosomal SUMOylation with ICRF-193 was clearly reduced in the presence of a dominant negative E2 SUMO-conjugating enzyme, UbC9 (dnUbC9), indicating that the normal SUMOylation machinery was utilized for ICRF-193-mediated SUMOylation of Topo IIα **(Fig 2.1B)**. Because we observed a large increase in overall chromosomal SUMO2/3 modification, we examined if the SUMOylation machinery itself is affected by ICRF-193. Addition of ICRF-193 to in vitro SUMOylation assays using recombinant Topo IIα-CTD as a substrate did not have a measurable effect on SUMOylation efficiency of the Topo IIα-CTD **(Fig 2.2B and C)**. Thus, the SUMOylation machinery was not targeted directly by ICRF-193. This is consistent with a specific effect of ICRF-193 on Topo IIα SUMOylation, not on PARP1 SUMOylation, on chromosomes in XEE. The results demonstrate that the Topo II inhibitor ICRF-193, which inhibits ATP hydrolysis by Topo II, specifically increases Topo IIα SUMOylation and Merbarone, which blocks initiation of the SPR, has a much weaker effect. Thus, Topo IIα at a specific stage in the SPR enzyme cycle could be especially susceptible to SUMOylation, perhaps due to changes in the accessibility of the modified lysine residues.

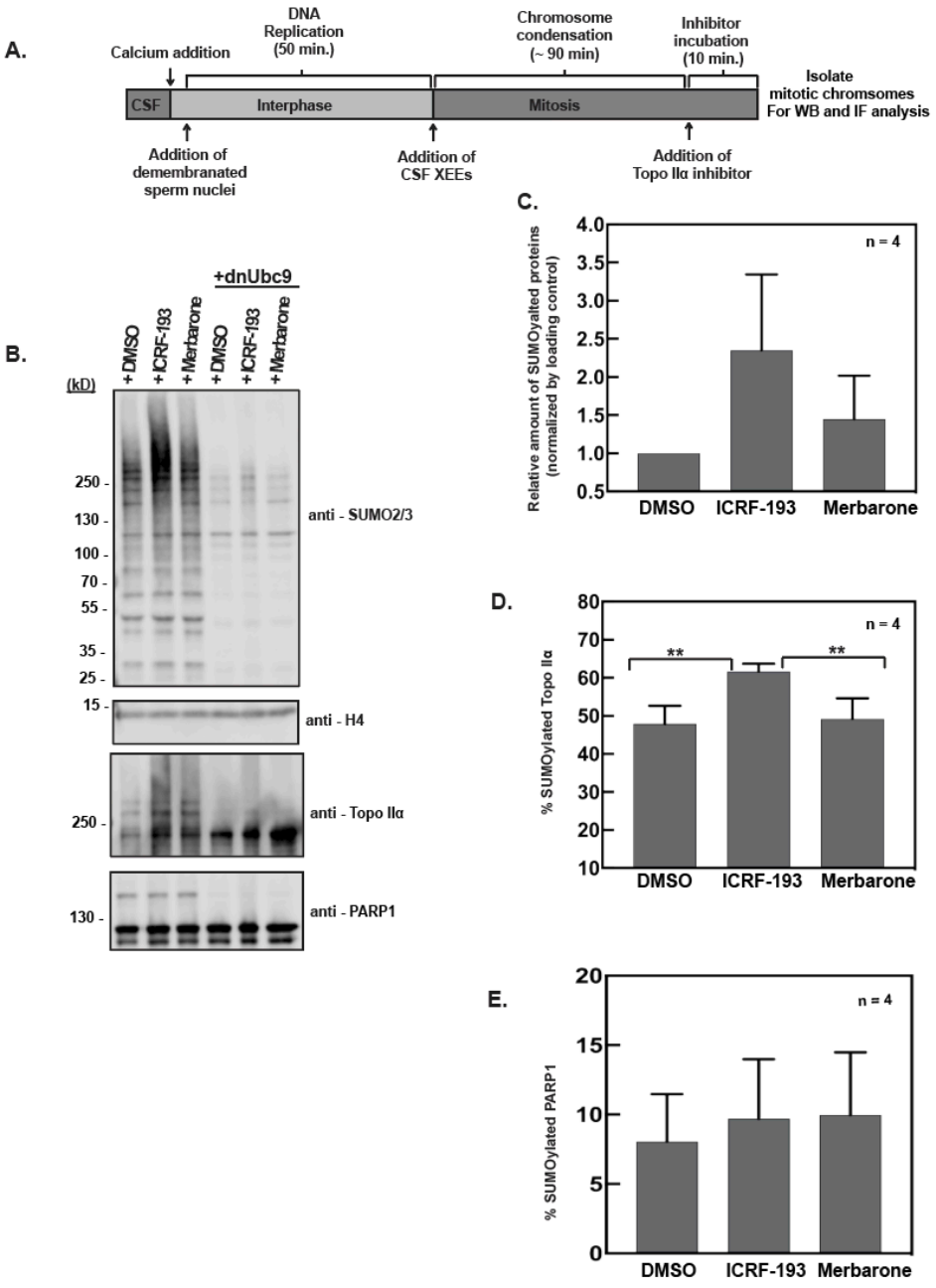

Figure 2.1 Topo II inhibitor addition increases mitotic SUMOylation on mitotic chromosomes in XEE. (A) A schematic representation for the preparation of mitotic replicated chromosomes treated with inhibitors from XEEs. (B) Inhibitor treated mitotic chromosomes were isolated from XEEs as shown in A with (+dnUbc9) and without (Control) dnUbc9 and subjected to western blotting. Histone H4 was probed as a loading control for the mitotic chromosomes. (C-E) Quantification of mitotic SUMOylation on the inhibitor treated mitotic chromosomes relative to DMSO treated chromosomes, % SUMOylation of Topo IIα and PARP1 as seen in B, from four independent experiments (n = 4). Error bar represents standard deviation. *, Probability (P) value from a student's t test. ** Indicates statistically significant difference, P≤0.01.

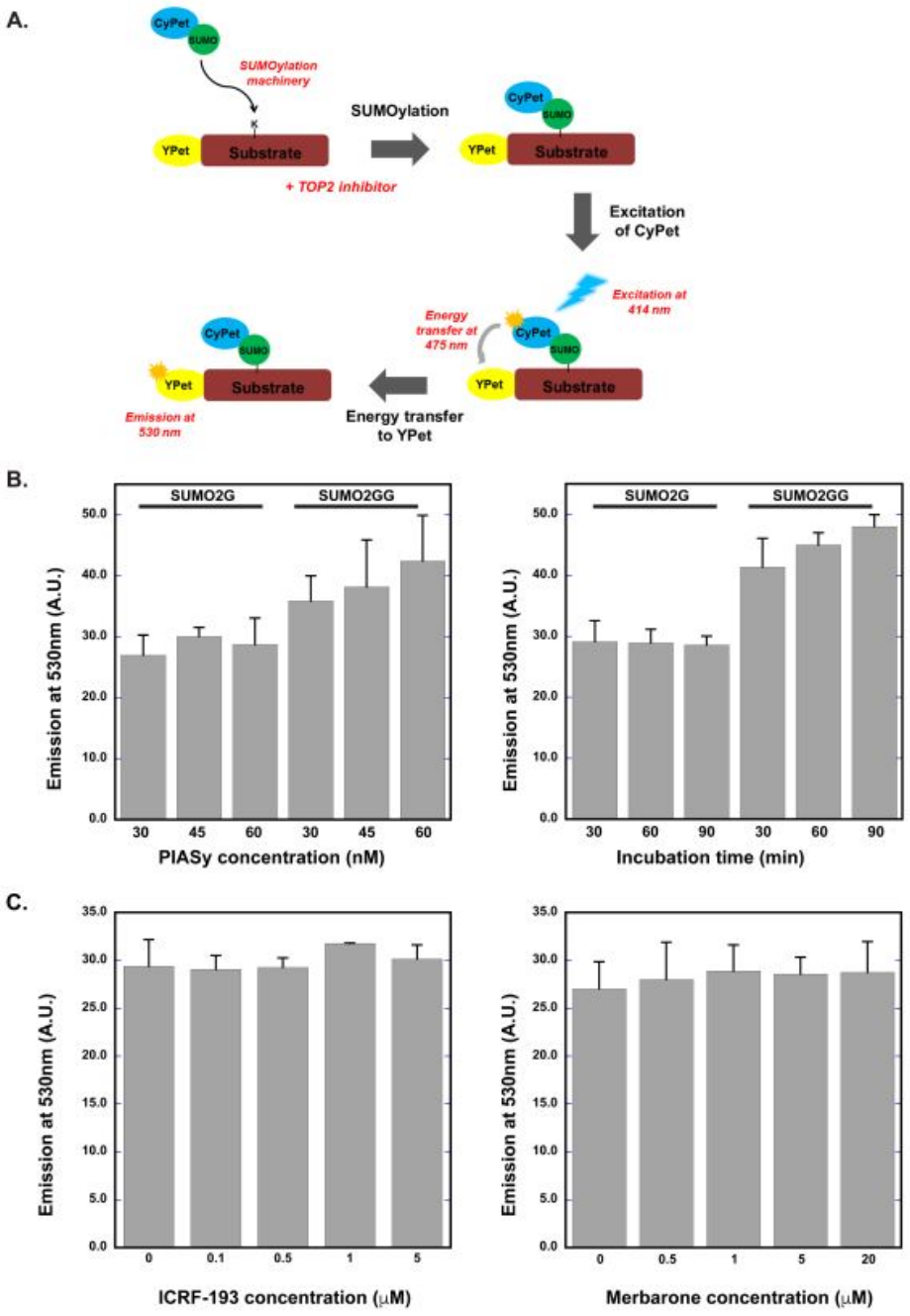

Figure 2.2 Topo II inhibitor treatment does not affect SUMOylation machinery. (A) A schematic representation of FRET based in vitro SUMOylation assay. YPet tagged Topo IIα CTD was used as a substrate along with CyPet tagged SUMO2G and SUMO2 GG isoforms in the reaction. (B) In vitro SUMOylation was performed with different PIASy concentrations and at different time points. (C) In vitro SUMOylation was performed for 30 min with different ICRF-193 and Merbarone concentrations. The emission spectrum was recorded at 530 nm from three different experiments (n=3) and error bars represent standard deviation.

Topo II catalytic inhibition induces SUMOylation of the physiologically relevant CTD residues

Next, we asked if ICRF-193 induces Topo IIα SUMOylation at the physiologically relevant residues. We previously identified all SUMO acceptor lysines in *Xenopus* Topo IIα, which is exclusively modified with SUMO2/3 during mitosis [18]. Three sites are located in the C-terminal domain (CTD) and one is in the DNA gate domain. To ask if ICRF-193 mediated Topo IIα SUMOylation occurs at the native lysine residues in the CTD, we prepared mitotic chromosomes after immuno-depletion of endogenous Topo IIα from XEEs and added recombinant T7-tagged WT Topo IIα or the 3KR mutant where all three lysine residues in the CTD are mutated to arginine **(Fig 2.3A and B)**. Since Topo IIα depletion prevents proper chromosome formation in replicated chromatin, we used un-replicated chromosomes for this analysis, which have less SUMOylated Topo IIα than replicated mitotic chromosomes [15]. Nevertheless, the mitotic chromosomes with recombinant WT Topo IIα exhibited increased SUMOylation with ICRF-193, though the SUMOylation increase was not as clear as in replicated chromosomes (as shown in Fig 2.1B). Importantly, the mitotic chromosomes with the recombinant Topo IIα 3KR mutant added back, did not show Topo IIα SUMOylation even in the presence of ICRF-193 **(Fig 2.3B)**. These findings indicate that the Topo II inhibitor ICRF-193 caused upregulation of Topo IIα SUMOylation on the native SUMO acceptor lysine residues in the CTD.

Previous studies revealed that SUMOylated proteins are mainly confined to mitotic centromeres during mitosis in XEE. Thus, we examined if increased SUMOylation with ICRF-193 occurs on mitotic chromosomes. Immunofluorescence staining of mitotic chromosomes from ICRF-193-treated XEEs showed increased SUMO2/3 staining on the chromosomes **(Fig 2.3C and D)**. Consistent with the western blot analysis, SUMOylation

on chromosome arms in the presence of ICRF-193 was diminished by the addition of dnUbC9 **(Fig 2.3C)**. The ICRF-193 mediated upregulation of SUMO2/3 on chromosome arms was observed in 97% of the treated chromosomes **(Fig 2.3D)**. Interestingly, we also observed spreading of the inner-kinetochore protein CENP-A after ICRF-193 treatment, which did not alter the overall chromosome morphology but could affect recruitment of centromeric components **(Fig 2.3C)**. In contrast, the localization of Topo IIα throughout the chromosome axis and with enrichment at mitotic centromere was not affected by ICRF-193 or Merbarone treatment **(Fig 2.4A and B)**. Together with the biochemical analysis, the data indicate that ICRF-193 induces increased mitotic SUMOylation (primarily at the CTD lysine residues) of Topo IIα located on chromosome arms and at centromeres.

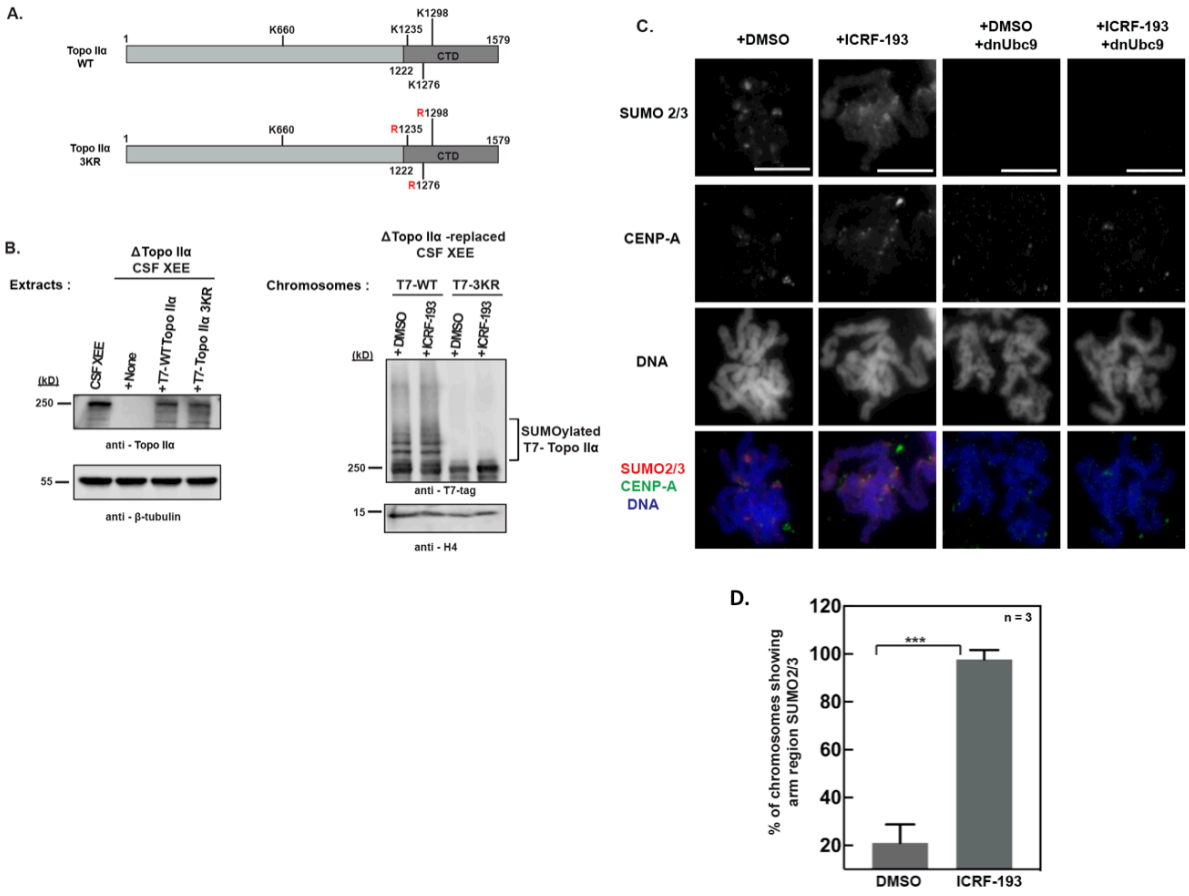

Fig 2.3 Topo II inhibitor ICRF-193 increases Topo IIα SUMOylation at C-terminal domain (CTD) lysine residues and upregulates SUMOylation on mitotic centromeres and chromosome arms in XEE. (A) A schematic representation for the primary structure of WT laevis Topo IIα and Topo IIα 3KR mutant. The three lysine residues indicated in the CTD were mutated to arginine which inhibits SUMO 2/3 conjugation of the CTD. (B) Endogenous Topo IIα in CSF XEEs was depleted using affinity-purified anti Topo IIα antibody and replaced with recombinant full-length WT T7-tagged Topo IIα or Topo IIα 3KR (left). β-Tubulin was probed as loading control for Topo IIα levels in CSF XEEs. The inhibitor treated mitotic chromosomes were isolated from Topo IIα replaced CSF XEEs and probed for Topo IIα SUMOylation using T7 antibody by western blotting (right). Histone H4 was probed as a loading control for the mitotic chromosomes. (C) DMSO and ICRF-193 treated mitotic replicated chromosomes were isolated from XEEs as shown in Fig. 2.1A with or without dnUbc9 (control). The mitotic chromosomes were subjected to immunofluorescence staining using antibodies indicated and DNA stained with Hoechst 33342. Bars, 10 µm. (D) Quantification of mitotic chromosomes showing arm region SUMO2/3 signals. The mitotic chromosomes with arm region SUMO2/3 signal were counted for 30 chromosomes each from three independent experiments (n=3). Error bars, standard deviation. *, Probability (P) value from a student's t test. *** Indicates statistically significant difference, P≤0.001.

ICRF-193-induced Topo IIα SUMOylation recruits Aurora B to mitotic chromosomes

Previously, we demonstrated a role for Topo IIα CTD SUMOylation in mediating protein interactions in XEEs [17,18]. Mass spectrometry identified Claspin and Haspin as SUMOylated CTD binding proteins and both were recruited to mitotic centromeres dependent on CTD SUMOylation. Since here we observed ICRF-193-mediated upregulation of Topo IIα SUMOylation, we asked if Topo IIα SUMOylation-dependent binding proteins are recruited to chromosomes in the context of Topo II inhibition. First, we examined Claspin localization on mitotic chromosomes isolated from ICRF-193-treated XEEs. With ICRF-193 treatment Claspin was enriched at mitotic centromeres and co-localized with Topo IIα foci **(Fig 2.4A),** but the pattern of Claspin localization did not change after ICRF-193 treatment. Next we examined Aurora B, which is recruited to centromeres in part by SUMOylated Topo IIα via Haspin-mediated H3T3P [16,17]. In contrast to Claspin, Aurora B kinase became more abundant at centromeres after ICRF-193 treatment, and was also recruited to chromosome arms **(Fig 2.4B)**. The chromosome arm foci were striking because Aurora B normally remains restricted to inner centromeres in metaphase. In contrast to the ICRF-193-induced recruitment, Aurora B kinase remained localized at centromeres and did not move to chromosome arms after Merbarone treatment **(Fig 2.4B)**. Also consistent with Topo II SUMOylation inducing the recruitment of Aurora B to these novel chromosome sites, addition of dnUbc9 eliminated the Aurora B signals on chromosome arms with ICRF-193 treatment **(Fig 2.4C)**. The ICRF-193-induced and SUMOylation-dependent chromosome arm localization of Aurora B was observed in 45% of mitotic chromosomes and addition of dnUbc9 reduced (by about half, ~ 22.6%) the

number of chromosomes showing arm region Aurora B signals. However, after Merbarone treatment, the number of mitotic chromosomes with arm region Aurora B signals was less than the DMSO control and was not affected by dnUbc9 **(Fig 2.4D)**. Aurora B foci on chromosome arms were not colocalized with CENP-A foci on chromosome arms after ICRF-193 treatment, suggesting that SUMOylation-dependent CENP-A mis-localization does not contribute to Aurora B recruitment. Altogether, the results suggest that increased Topo IIα SUMOylation on chromosome arms induced by ICRF-193 triggers recruitment of Aurora B kinase. On the other hand, Claspin remained restricted to the mitotic centromeres, suggesting that the chromatin association of Claspin is not solely dependent on the SUMO/SIM interaction between Claspin and the SUMOylated Topo II CTD.

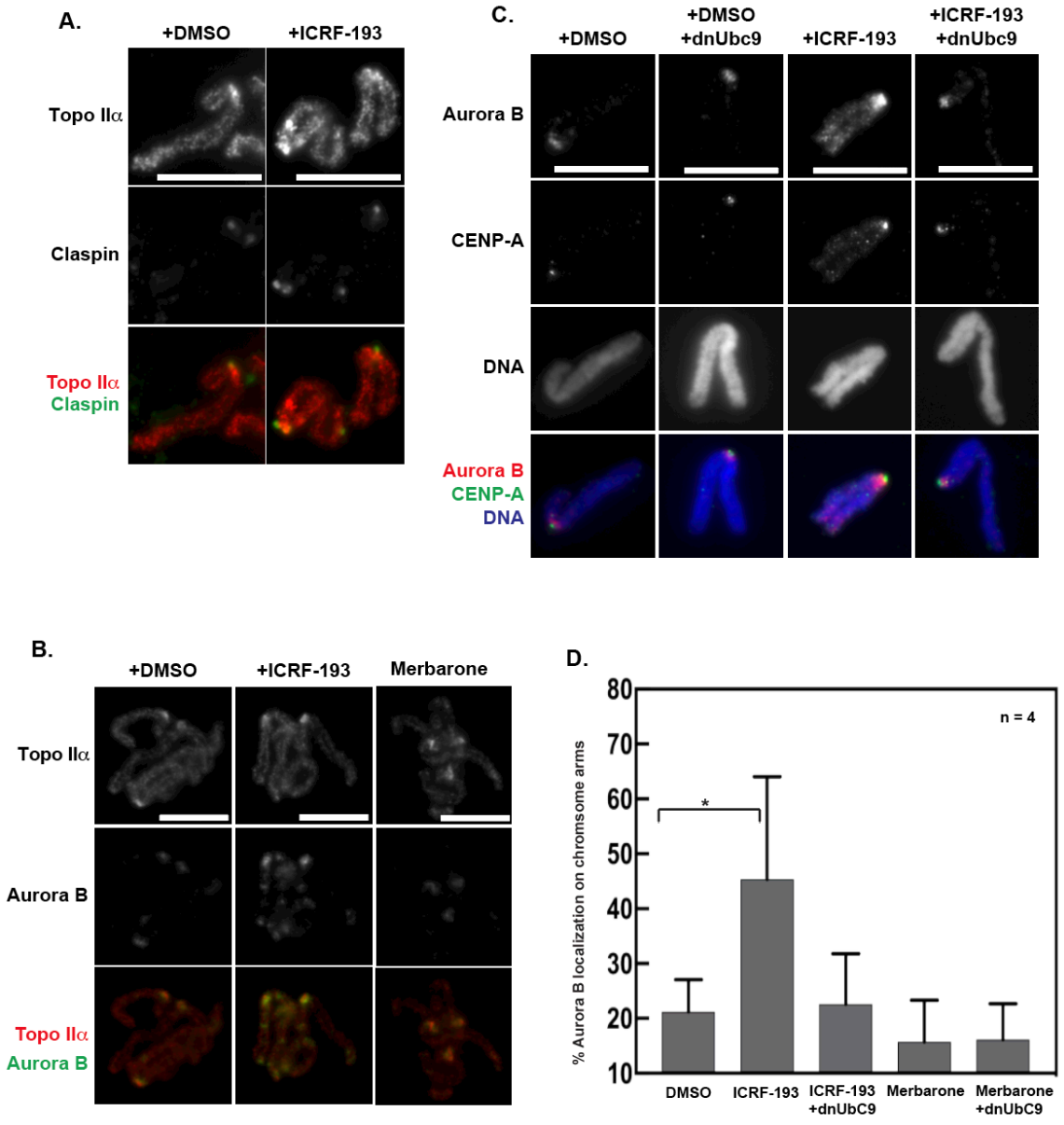

Fig 2.4 Aurora B is recruited to chromosome arms in response to ICRF-193 addition in XEE. (A&B) Mitotic replicated chromosomes were isolated from XEEs as in Fig. 2.1A with DMSO, ICRF-193, or Merbarone addition and immuno-stained using indicated antibodies and DNA stained with Hoechst 33342. (C) Immunostaining of a single pair of sister chromatids showed Aurora B recruitment to chromosome arms with ICRF-193 addition and inhibition of SUMOylation by dnUbc9 reduced Aurora B signals at chromosome arms. Bars indicate 10 μm. (D) Quantification for the mitotic chromosomes showing arm region Aurora B signals. The mitotic chromosomes showing arm region Aurora B foci were counted for more than 25 chromosomes each from four independent experiments (n=4). Error bar represents standard deviation. *, Probability (P) value from a student's t test. *, P≤0.05.

Aurora B recruitment to chromosome arms is conserved in human cells upon Topo II catalytic inhibition

To ask if Aurora B recruitment is conserved, we treated mammalian cells with nocodazole to render a pure population of pseudo-metaphases with condensed chromosomes, and then samples were split and incubated a further 45 min. +/- ICRF-193. As in XEE, we aimed to avoid effects that might arise due to Topo II inhibition during chromosome condensation. We defined the positions of the inner kinetochores (iKs) by immuno-staining with anti-CENP-A antibodies and CS1058 CREST serum **(Fig 2.5)**. The iKs are distinct from the inner centromeres of a chromatid pair, lying more peripherally at the base of the kinetochores [27,28]. In control and ICRF-193-treated HeLa cells, chromosomes were indistinguishable in terms of these co-localizing iK epitopes **(Fig 2.5B and C)**. The distribution of iK-to-iK distances was similar **(Fig 2.5D)**. Thus, ICRF-193 treatment did not measurably alter the overall centromere architecture. Surprisingly, we did not see observe the CENP-A spreading in HeLa cells upon ICRF-193 treatment as we see in XEEs. This CENP-A difference between two model systems can be addressed with sample preparation that can affect the CENP-A localization on chromosomes.

Having established that the ICRF-193 treatment did not grossly affect the morphology of condensed mammalian chromosomes, we immuno-stained cells with anti-Aurora B antibodies. As observed previously in control cells, Aurora B localized to a discrete focus at the inner centromere of each chromatid pair, observed here between the CREST-reactive iK epitopes **(Fig 2.6A)**. In stark contrast, ICRF-193 treatment induced recruitment of Aurora B to chromosome arms, similar to the observations in XEE. In most cases, Aurora B was diminished at inner centromeres and instead concentrated more peripherally near

the iKs **(Fig 2.6A-C)**. We have observed increased mitotic SUMOylation and Topo IIα SUMOylation with ICRF-193 treatment in cells similar to XEE **(Fig 2.7)**.

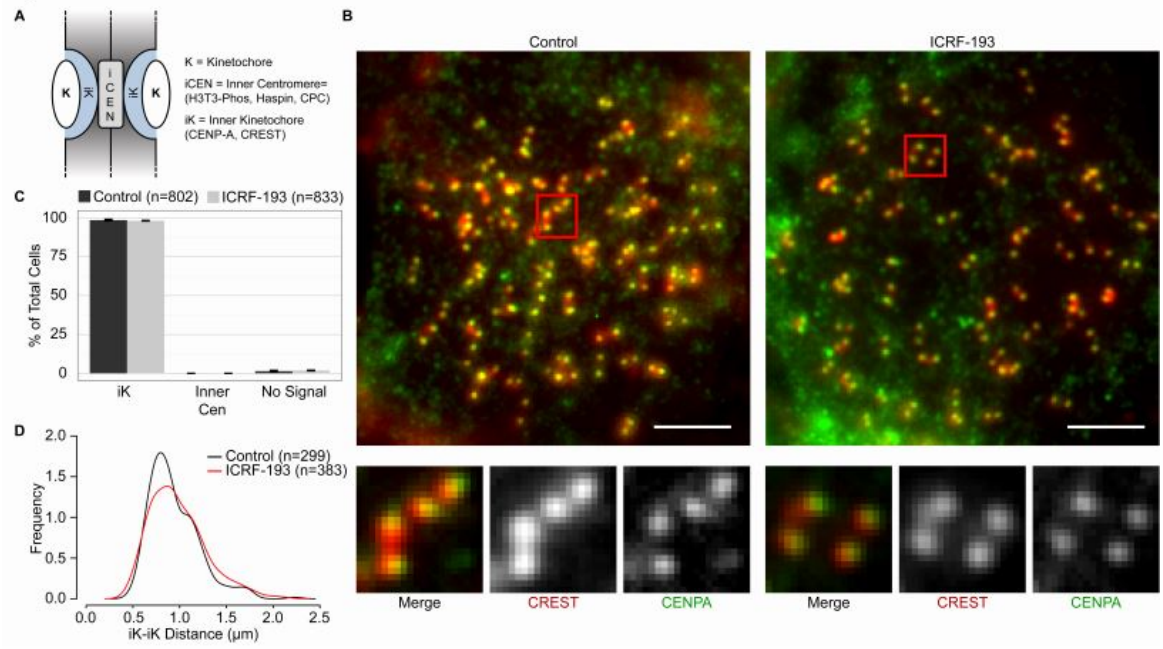

Fig 2.5 CENP-A and CREST co-localize regardless of ICRF-193 treatment in HeLa cells. (A) Cartoon depicting centromere/kinetochore regions. (B) Representative immunofluorescent stained images of pseudo-metaphase HeLa cells (nocodazole arrested) +/- ICRF-193 treatment for 45 minutes. CREST; red. CENP-A; green. (C) Quantification of immunofluorescent staining at centromeres. (D) Distribution plot of iK to iK distances (iK; inner kinetochore). Bar, 10μm. Error bars, standard deviation. Data were collected from at least three independent experiments.

Aurora B is required for metaphase arrest upon Topo II catalytic inhibition

In control metaphases, Aurora B is largely restricted to inner centromeres. However, previous studies experimentally tethered Aurora B to the kinetochore by fusing the INCENP subunit of the CPC to the DNA binding domain of CENP-B, inducing metaphase arrest [29]. Since Topo II catalytic inhibition recruited Aurora B to sites peripheral to inner centromeres, we asked if Aurora B is required for the metaphase arrest induced by ICRF-193. In a first approach, we collected pseudo-metaphases via nocodazole synchrony then

after washing, we seeded them into medium +/- ICRF-193 and +/- the Aurora B inhibitor ZM447439 and collected cells at intervals for preparing chromosome spreads. We used a modified spreading technique that retains chromosome positions on the mitotic spindle, allowing accurate assessment of anaphase onset [30]. Control cells initiated anaphase after 45-90 minutes, presumably the time needed to assemble mitotic spindles and biorient all the chromosomes **(Fig 2.8A).** ICRF-193 treated cells delayed in metaphase as expected, but strikingly inhibition of Aurora B (using an Aurora B inhibitor, ZM447439) completely abolished this cell cycle response. However, cells seeded into medium with ZM447439 and nocodazole (microtubule polymerization inhibitor), Aurora B inhibition was not able to immediately bypass the metaphase checkpoint, as reported previously [31]. This suggests the metaphase arrest by nocodazole and ICRF-193 is distinct and utilizes different mechanism. Thus, Aurora B is required for the metaphase arrest upon ICRF-193 treatment. In a second approach, we employed live single-cell analysis. HeLa cells were grown in normal culture conditions in an environmental chamber housed around an inverted microscope. ICRF-193 was added at time zero and images recorded at 5 min. intervals to provide enough temporal resolution for accurate estimates of metaphase duration. We only analyzed cells that were in metaphase at the time of ICRF-193 and/or ZM447439 addition, to exclude effects of Topo II and Aurora B inhibition prior to metaphase. Lastly, we included two alternative inhibitors of Topo II: Merbarone, which did not induce Topo II SUMOylation in the XEE experiments, and 6-Hydroxydaidzein (6HD), which is a naturally occurring catalytic inhibitor abundant in plants. In controls, the interval from metaphase to anaphase was on average 39.2 min **(Fig 2.8B**). ICRF-193 addition in metaphase substantially delayed the onset of anaphase and most cells remained arrested in metaphase

for the duration of the experiments. Consistent with the lack of Topo II SUMOylation after Merbarone treatment, this inhibitor did not arrest cells in metaphase **(Fig 2.8C)**. 6HD did induce metaphase arrest, though more weakly than ICRF-193 **(Fig 2.8D)**. In the cases of both ICRF-193 and 6HD treatment, inhibition of Aurora B completely abolished the metaphase arrest **(Fig 2.8B and D).**

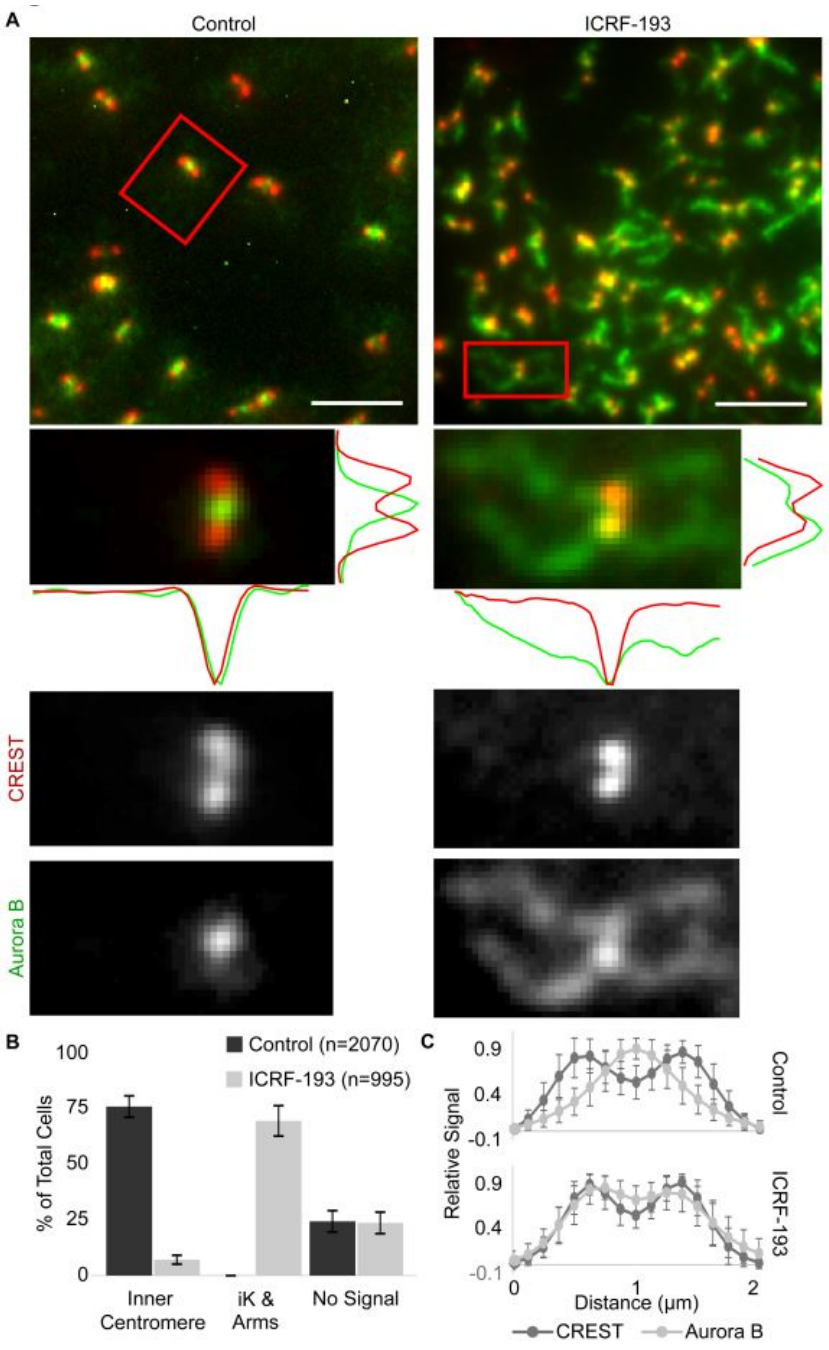

Fig 2.6 Aurora B is recruited to the iK and chromosome arms during ICRF-193 treatment in HeLa cells. (A) Representative immunofluorescent stained images of pseudo-metaphase HeLa cells (nocodazole arrested) +/- ICRF-193 treatment for 45 minutes. CREST; red. Aurora B; green. Line scans were done in the X and Y axis. (B) Quantification of immunofluorescent staining at centromeres/chromosome arms. (C) Averaged plots of Aurora B and CREST signal intensities in line scans across centromeres in cells treated as in *A.* n=25 for each sample and each scan was normalized to the highest value. Bars, 10µm. Error bars, standard deviation. Data were collected from at least three independent experiments.

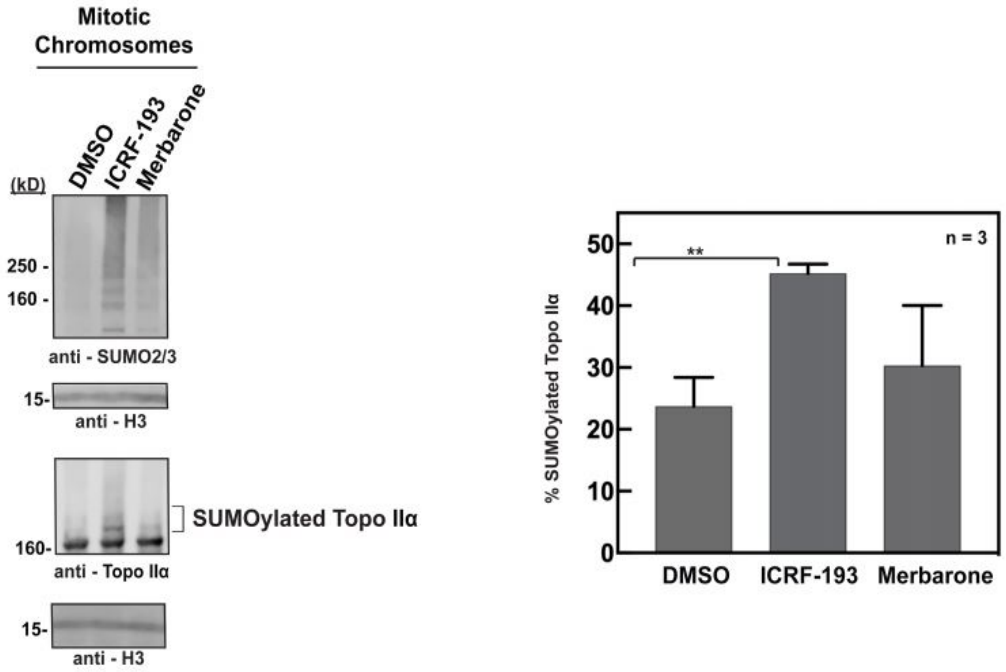

Figure 2.7 Topo II inhibitor ICRF-193 upregulates Topo IIα SUMOylation in HeLa cells. DMSO, ICRF-193 and Merbarone treated mitotic chromosomes were isolated from HeLa cells and subjected to western blotting. The mitotic SUMOylation and Topo SUMOylation were probed using indicated antibodies. Histone H3 was probed as a loading control for the mitotic chromosomes. % SUMOylation of Topo IIα was calculated from three independent experiments (n = 3). Error bars, standard deviation. *, Probability (P) value from a student's t test. ** Indicates statistically significant difference, $P \leq 0.01$.

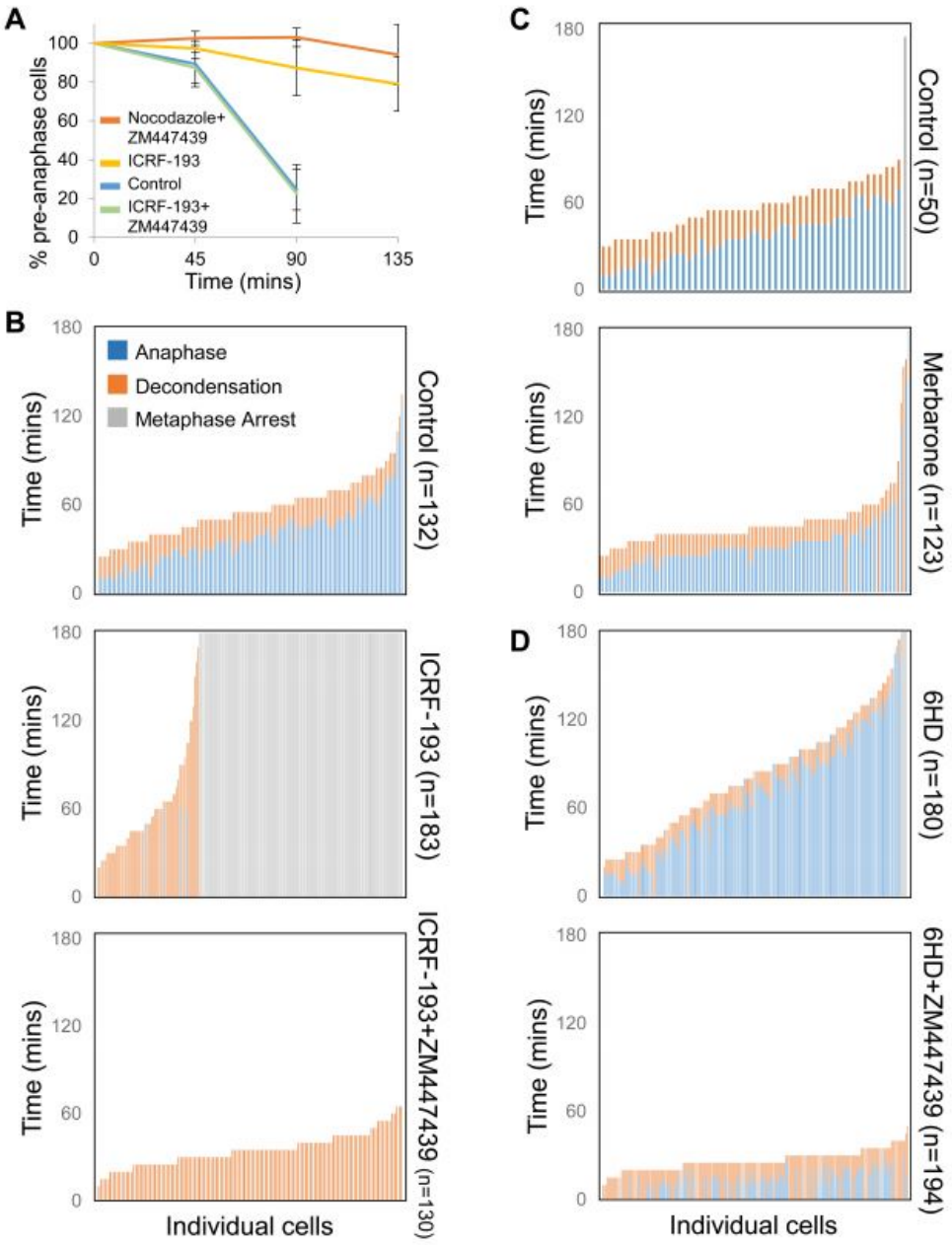

Fig 2.8 Aurora B inhibition bypasses the metaphase checkpoint induced by Topo II catalytic inhibitors
(A) Quantification of % pre-anaphase cells following nocodazole arrest and release. Cells were treated with various combinations of drugs after release from nocodazole arrest and pre-anaphase cells were counted at each time point to assess mitotic progression. (B) Live single-cell analysis of mitotic progression. Quantitation of time to anaphase and decondensation during drug treatments. Each vertical bar represents one cell, with each cell being scored for time to anaphase and decondensation from the start of the time course. Only cells that were in metaphase at the beginning of the time course were scored. Error bars, standard deviation. Data were collected from at least three independent experiments.

Ectopic histone H3 threonine 3 phosphorylation recruits Aurora B to the kinetochore and chromosome arms

Aurora B was recruited to the iKs and chromosome arms upon Topo II catalytic inhibition and is required for the coincident cell cycle response that arrests cells in metaphase. To gain insight into the mechanism of Aurora B recruitment, we asked if the enzyme is recruited together with the INCENP subunit of the CPC to which Aurora B binds directly. In control pseudo-metaphase cells, INCENP localized to inner centromeres, as expected **(Fig 2.9A**). However, similar to Aurora B, INCENP was recruited to iKs and chromosome arms after a 45' ICRF-193 treatment. This indicates Aurora B is mobilized as a component of the CPC, where Aurora B binds to INCENP and bridging molecules tether INCENP to chromatin. One such bridging molecule is Survivin, which binds to INCENP and interacts with H3T3P via its BIR domain [22,23,32]. In metaphase, H3T3P is a localized histone mark, largely restricted to nucleosomes at inner centromeres and which, in part, accounts for the specificity of CPC localization to inner centromeres [28]. Given that INCENP and Aurora B adopted new chromosomal positions upon Topo II catalytic inhibition, we asked if de novo phosphorylation of H3T3 might account for their recruitment. As expected, we detected H3T3P in control pseudo-metaphase cells with a restricted localization between the iK foci of sister chromatids; that is, at inner centromeres **(Fig 2.9C**). Strikingly, upon ICRF-193 treatment, H3T3P localized to ectopic sites corresponding to kinetochores and chromosome arms, similar to that observed for Aurora B and INCENP.

An explanation for these localization patterns is that Topo II catalytic inhibition induced H3T3P within nucleosomes at iKs and chromosome arms, leading to recruitment of the CPC. To test this, we asked if Haspin, which phosphorylates H3T3 [20,23], is required for recruitment of Aurora B to iKs and chromosome arms. To achieve this, we combined

ICRF-193 treatment with Haspin inhibitor, CHR-6494 **(Fig 2.10A)**. In pseudo-metaphases treated with ICRF-193 and the Haspin inhibitors there was a qualitative difference in Aurora B localization. Compared to ICRF-193 treatment alone, Aurora B localization appeared more diffusely dispersed on chromatin, as has been previously observed after Haspin inhibition [23,24,33,34]. Categorization of cells based on this phenotype revealed a clear difference between ICRF-193 alone and ICRF-193 plus Haspin inhibitor treated cells **(Fig 2.10B)**. To gain quantitative information, we analyzed chromosomes based on two features that were observed to distinguish the samples. First we measured the distribution of Aurora B across the width of chromosome arms, because inhibition of Haspin appeared to spread the Aurora B laterally. An averaged plot of Aurora B intensity indeed revealed a small difference in distribution **(Fig 2.10C)**. Second, we measured the abundance of Aurora B on the chromosome arms. This revealed a significant reduction in Aurora B recruitment in the presence of CHR-6494 **(Fig 2.10D)**. Together, the data are consistent with Haspin inhibition restricting the ICRF-193-induced recruitment of Aurora B to chromosomes. Thus, H3T3P likely contributes to Aurora B recruitment upon Topo II catalytic inhibition.

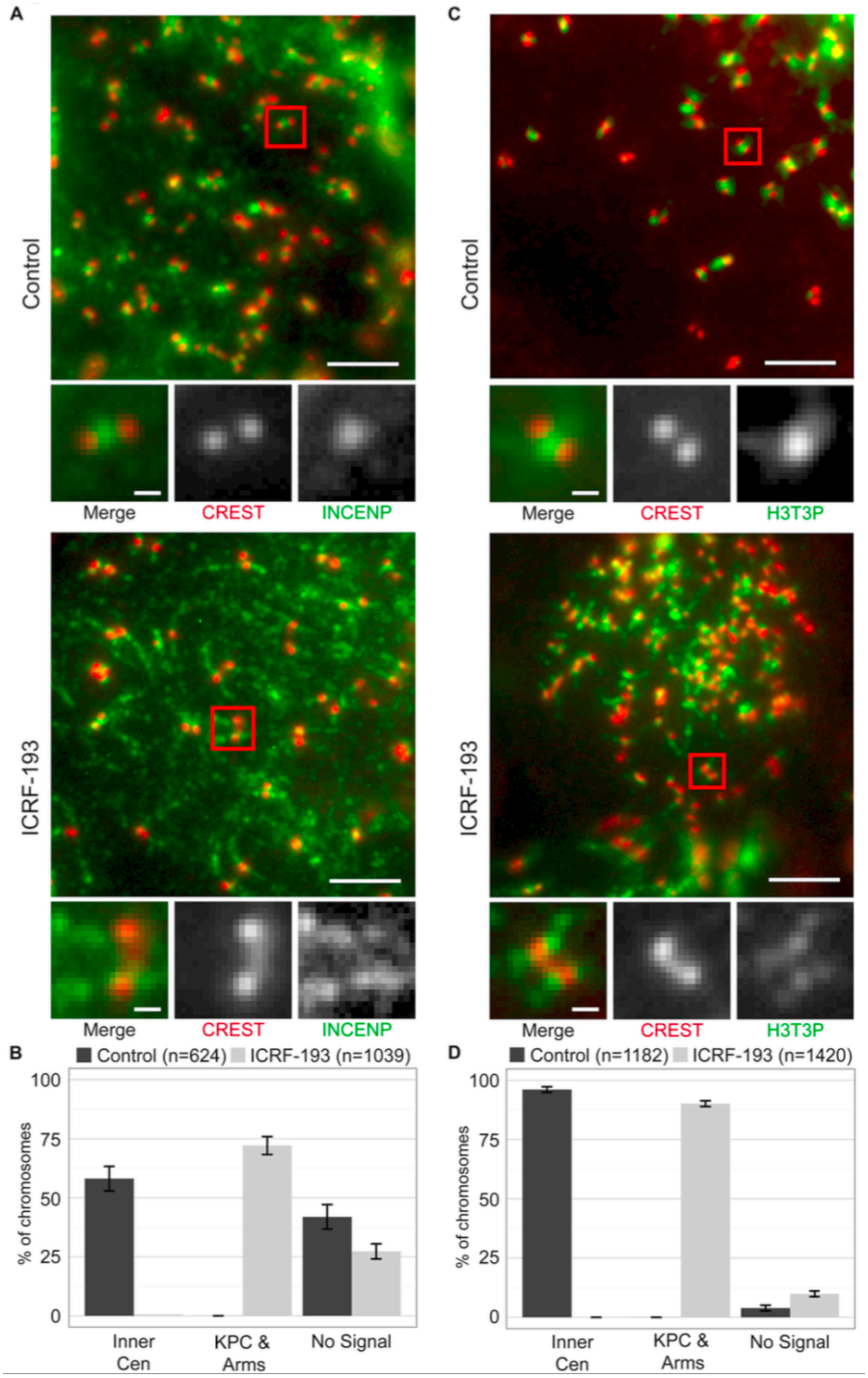

Fig 2.9 INCENP and H3T3 Phosphorylation at iKs and chromosome arms after ICRF-193 treatment in mitosis (A,C) Representative immunofluorescent stained images of pseudo-metaphase HeLa cells (nocodazole arrested) +/- ICRF-193 treatment for 45 minutes. (A) CREST; red. INCENP; green. (B,D) Quantification of immunofluorescent staining at centromeres/chromosome arms. Bar, 10µm. Error bars, standard deviation. Data were collected from at least three independent experiments. (C) CREST; red. H3T3-Phos; green.

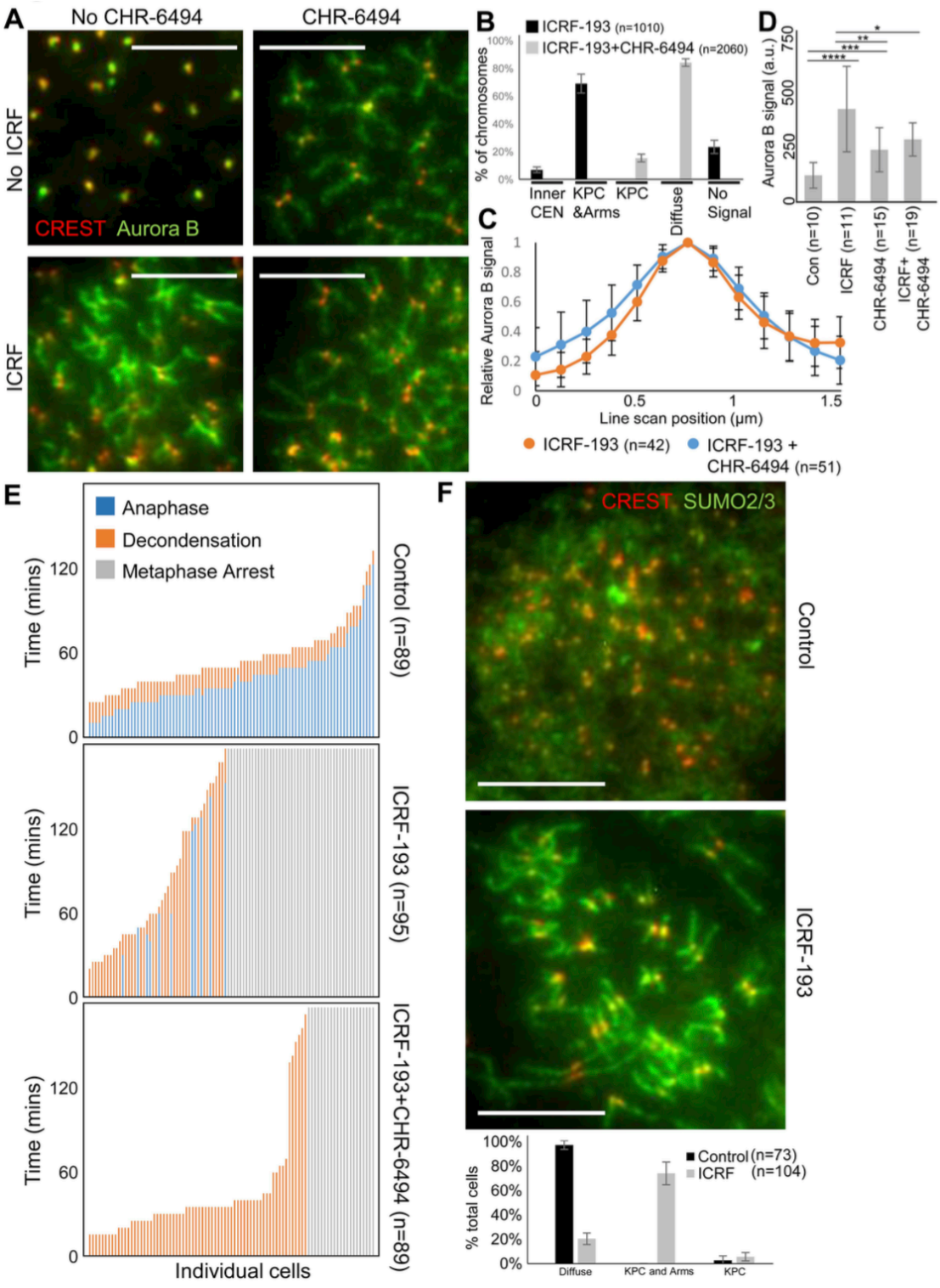

Fig 2.10 Haspin promotes Aurora B localization to iK and chromosome arms during ICRF-193 treatment in HeLa cells. (A) Representative immunofluorescent stained images of pseudo-metaphase HeLa

cells (nocodazole arrested) +/- ICRF-193 +/- CHR-6494 treatment for 45 minutes. CREST; red. Aurora B; green. (B) Classification of Aurora B staining pattern. (C) Quantification of average Aurora B signal intensity spanning chromosome arms. Each scan was normalized to the highest value. (D) Quantification of Aurora B signal intensity on chromatin. Probability (P) values from a student's t test. *, P=0.04; **P=0.01; ***P=0.001; ****P=0.0004. (E) Live single-cell analysis of mitotic progression. Quantitation of time to anaphase and decondensation during drug treatments. Each vertical bar represents one cell, with each cell being scored for time to anaphase and decondensation from the start of the time course. Only cells that were in metaphase at the beginning of the time course were scored. (F) Representative immunofluorescent stained images of pseudo-metaphase HeLa cells (nocodazole arrested) +/- ICRF-193 for 45 minutes. CREST; red. SUMO2/3; green. Plot shows classification of SUMO2/3 staining pattern. Bars, 10μm. Error bars, standard deviation. Data were collected from at least three independent experiments.

Haspin kinase activity is required for ICRF-193-induced metaphase arrest

If the mechanism of metaphase arrest upon Topo II catalytic inhibition involves activation of Haspin at iKs and chromosome arms, which mobilizes Aurora B to these sites, then Haspin ought to be required for the cell cycle arrest in metaphase. To test this, we used the live single-cell analysis method, adding CHR-6494 at the same time as ICRF-193. Metaphase cells treated with CHR-6494 alone behaved similar to controls, spending on average 42.5 min. in metaphase (data not shown). However, in the presence of ICRF-193, CHR-6494 bypassed the metaphase arrest in most of the cells **(Fig 2.10E)**. Together, the data indicate that Haspin is required for the metaphase arrest, presumably due to its ability to generate H3T3P at iKs and chromosome arms for the CPC to bind to.

Chromosome arms become enriched with SUMO2/3 upon Topo II catalytic inhibition

In XEE and yeast cells, Topo II SUMOylation promotes Aurora B recruitment to centromeres in mitosis [16,17]. The single-cell data provided evidence that metaphase arrest upon Topo II catalytic inhibition is mediated by Haspin and Aurora B kinases.

Concomitantly, Aurora B was recruited, likely via H3T3P, to kinetochores and chromosome arms. Mutations in yeast that disrupt Aurora B recruitment include mutation of the conserved Topo II SUMOylation sites in the CTD [16]. To ask if Topo II SUMOylation in human cells might induce Aurora B recruitment to chromosome arms, we characterized SUMO2/3 localization on mitotic chromosomes after ICRF-193 treatment. In control nocodazole-arrested HeLa cells, weak diffuse signals were observed in the nucleoplasm after staining with anti-SUMO2/3 antibodies. Strikingly, after ICRF-193 treatment, SUMO2/3 became prominently localized to chromosome arms in almost all cells, similar to the pattern of the recruited Aurora B **(Fig 2.10F)**. This provides circumstantial evidence for a link between Topo IIα SUMOylation and Aurora B recruitment upon Topo II catalytic inhibition.

Direct evidence that Topo II CTD SUMOylation induces recruitment of Aurora B to kinetochores and chromosome arms and promotes metaphase checkpoint activation

The immunostaining results, though suggestive, could not provide a definitive link between Topo IIα CTD SUMOylation, Aurora B recruitment to chromosomes, and metaphase checkpoint activation. To directly test these relationships we exchanged endogenous human Topo IIα with a mutant lacking the three conserved SUMOylation sites in the CTD. We used a system previously characterized in detail to insert a single copy of a doxycycline (Dox) inducible mCherry-Topo IIα or a mCherry-3KR-Topo IIα mutant allele into the 5q31.3 locus using Flp recombinase [35]. We achieved efficient depletion of endogenous Topo IIα using siRNAs targeting the 3' UTR and we simultaneously induced the mCherry-Topo IIα proteins with Dox **(Fig 2.11A**). We then synchronized the cells with nocodazole

and after mitotic shake-off incubated for a further 45 min with +/- ICRF-193. Without ICRF-193 treatment, immuno-staining revealed that mCherry-Topo IIα or mCherry-3KR Topo IIα cells had similar patterns of Aurora B localization, largely restricted to inner-centromeres **(Fig 2.11 B&C)**. After ICRF-193 treatment, the mCherry-Topo IIα cells efficiently recruited Aurora B to chromosome arms. However, there was a statistically significant defect (about a seven-fold decrease; $P \leq 0.03$) in the ability of the mCherry-3KR Topo IIα cells to recruit Aurora B. This suggests that Topo IIα CTD SUMOylation at these conserved residues promotes Aurora B recruitment.

Next, we used the same strategy to test if the 3KR mutant had a functional checkpoint. After depletion of endogenous Topo IIα and induction of the exogenous Topo IIα alleles, cells in metaphase were analyzed immediately following addition of the drug treatments. Mitotic progression in these single cells using time-lapse microscopy was followed by directly observing the exogenous mCherry-Topo IIα or mCherry-3KR Topo IIα proteins. Thus, we could quantify the level of the exogenous Topo IIα proteins in the same cohort of cells analyzed to determine metaphase duration. Most single cells did not vary more than two-fold in their levels of exogenous Topo IIα expression, and the distributions were similar **(Fig 2.11D)**. However, exogenous mCherry-Topo IIα expressing cells had a substantially more proficient checkpoint response in the presence of ICRF-193 than did the mCherry-3KR-Topo IIα expressing cells **(Fig 2.11E)**. These experiments therefore provide direct support to the hypothesis that SUMOylation of the conserved Topo IIα CTD acceptor lysine residues constitutes a molecular signal that recruits Aurora B to kinetochores and the chromosome axial core and triggers activation of the metaphase checkpoint.

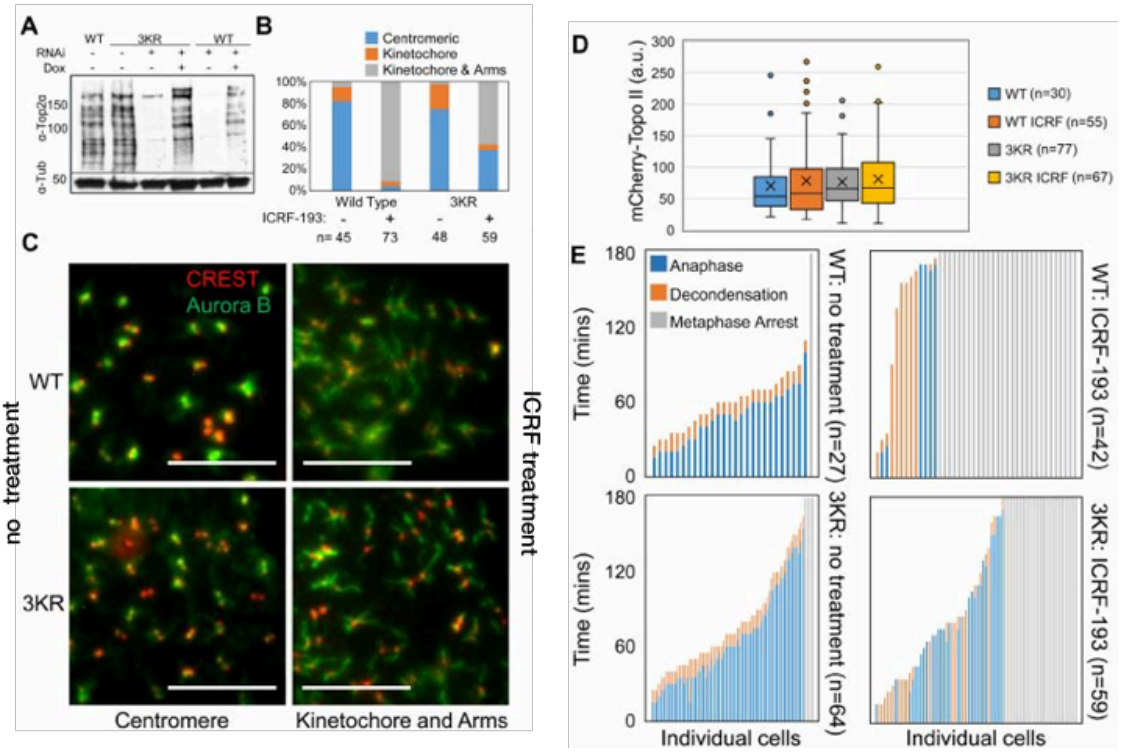

Fig 2.11 Topo II CTD SUMOylation promotes Aurora B recruitment and metaphase checkpoint activation. (A) Western blot showing endogenous Topo II depletion achieved after RNAi in HeLa EM2-11ht cell lines and induction (Dox) of exogenous mCherry-Topo II and mCherry-TopoII-3KR. (B) Classification of Aurora B localization in cells treated as in *A*, with endogenous Topo II depleted and exogenous Topo II expressed. (C) Representative examples of Aurora B localization categories as in B, (left panel is without ICRF-193 and the right panel is with ICRF-193 treatment). (D, E) Analysis of metaphase duration in live HeLa cell lines in *A*, after depletion of endogenous Topo II and expression of exogenous alleles. (D) Quantification of the mCherry-Topo II signal at the first time point captured for each of the cells analyzed in *E*. Bars, 10µm. Error bars, standard deviation. Data were collected from at least three independent experiments.

Discussion

The SPR has been studied for decades because it has extensive roles in cell division and is the target of major classes of anti-cancer drugs. A yeast *top2* mutant, *top2-B44*, which hydrolyzes ATP slowly triggers activation of the metaphase checkpoint [7,8]. Thus, progression through mitosis and Topo II activity are coupled. However, molecular insight

into the mechanism of activation of the metaphase checkpoint when Topo II is perturbed is lacking.

Studies in mammalian cells revealed that this checkpoint may be conserved [4,5]. Analogous to the *top2-B44* mutant, catalytic Topo II inhibitors (e.g. ICRF-193) inhibit ATP hydrolysis and activate a metaphase checkpoint in human cells [4–6]. In both cases, impaired ATP hydrolysis delays the enzyme in a particular structural conformation, within the SPR. This suggests cells might detect the persistence of that Topo II conformer. Here we have provided evidence that SUMOylation of the Topo II CTD triggers the checkpoint, perhaps because the conformation of the enzyme is specifically recognized by SUMO ligases when ATP hydrolysis is slow.

In yeast, the checkpoint is transduced by the CTD of the enzyme, which possesses conserved SUMOylation sites. [36]. Inhibition of Topo II with ICRF-193 in mitotic XEE induced Topo II CTD SUMOylation on these physiologically relevant sites (Fig 2.1, 2.3). This SUMOylation was conserved in mitotic HeLa cells treated with ICRF-193, which activated the metaphase checkpoint, but was not observed after treatment with Merbarone, which did not activate the checkpoint (Fig 2.6, 2.8). How might Topo II CTD SUMOylation activate the downstream checkpoint factors? The data presented here are congruent with studies revealing a conserved mechanism of Aurora B regulation controlled by Topo II CTD SUMOylation [16,17]. In both budding yeast and XEE, Topo II CTD SUMOylation regulates Haspin kinase activity to promote H3T3 phosphorylation that recruits the CPC to mitotic centromeres. This mechanism relies on binding of Haspin to the SUMO2/3 moieties on the Topo II CTD which in turn recruits Aurora B. The results

presented here provide evidence that this Topo II SUMOylation-Haspin-Aurora B pathway is conserved in human cells.

When we mutated the three conserved SUMOylated sites in the Topo II CTD, activation of the checkpoint and recruitment of Aurora B was abrogated but not abolished (Fig 2.11). One interpretation of this result is that additional SUMOylated CTD lysine residues contribute to Aurora B activation in human cells. A recent study provided important insight into as many as eleven lysine residues that can be SUMOylated as well as their functions in Topo II localization in mitosis [37]. It is clear CTD SUMOylation serves at least two mitotic functions: Topo II localization and recruitment of Aurora B. These functions could be mediated by different SUMOylation sites or the same SUMOylated residues may be capable of providing the protein-protein interactions needed for both functions. Dissecting these requirements will be challenging, though important.

In XEE, ICRF-193 simultaneously induced CTD SUMOylation and mobilization of Aurora B. This was conserved in human cells, where Aurora B was recruited away from inner centromeres to the iK and chromosome arms and the metaphase checkpoint was activated. Aurora B has been experimentally targeted to the iK using several approaches, including expression of a fusion between INCENP and the DNA binding domain of CENP-B [38]. This leads to forced activation of the metaphase checkpoint. Moreover, a pool of Aurora B is revealed at kinetochores after inhibition of Haspin which causes Aurora B to be dispersed from chromatin [33]. Thus, when Topo II is catalytically inhibited, it is possible that cells similarly induce recruitment of Aurora B to the iK in order to activate the checkpoint. However, we also observed that Aurora B is strongly recruited to chromosome arms. It will be interesting to test if Aurora B recruitment to either arms or

iKs is sufficient for checkpoint activation. This will require understanding the mechanism of interaction of Topo II with chromosome cores and iKs.

A body of work has revealed that restriction of the CPC to inner centromeres in mitosis is based on feedback cycles [28]. Factors that recruit Aurora B are themselves regulated by Aurora B, e.g. phosphorylation by Aurora B enhances Haspin kinase activity [39,40]. Conversely, phosphorylation of a chromosome bound protein phosphatase 1 (PP1γ) Repo-Man by Aurora B protects the centromeric H3T3P from PP1γ-Repo-Man [41,42]. Clustering of Aurora B promotes its maximum activity by inducing auto-phosphorylation [43]. It is perhaps striking then that catalytic Topo II inhibition is able to break this cycle, release Aurora B from inner centromeres and allow recruitment to iKs and chromosome arms. It will be interesting to establish if H3T3P is sufficient for Aurora B recruitment to iK and chromosome arms. Previous work has established that Pds5 (a sister chromatid cohesion protein) functions to recruit Aurora B by facilitating localization of Haspin to inner centromeres [44,45]. It will be important to test if Pds5 is recruited to iK and chromosome arms upon Topo II catalytic inhibition.

The ICRF-193 mediated metaphase checkpoint requires Aurora B activity, suggesting upregulation of Aurora B activity at the iK and/or chromosome arms induces mitotic arrest. What molecules are phosphorylated by Aurora B to activate this checkpoint? If Aurora B at the iK is responsible for checkpoint activation, we anticipate the down-stream targets are kinetochore microtubule network proteins such as Ndc80/Hec1, Dsn1 and CENP-E [46–48] and in that case checkpoint activation likely occurs via microtubule detachment from kinetochores. If recruitment to the chromosome arms is required for checkpoint activation, Aurora B may have novel substrates.

In summary, we propose a model where Topo II catalytic inhibition activates a metaphase checkpoint via Aurora B. Checkpoint activation involves Topo II CTD SUMOylation as an initial signaling step, which recruits Haspin kinase as a down-stream mediator of Aurora B recruitment via H3T3P. We anticipate this mechanism contributes to the timing of mitosis so that cells can monitor catenations and stalled SPR cycles. Studies revealed that Topo II catalytic inhibition in G2 cells delays entry into mitosis [49,50], but that this cell cycle response is frequently diminished in cancer cells [51–53]. Intriguingly, a deficiency in the G2 checkpoint may render the ability to delay in metaphase critical for survival of cancer cells [53,54]. In conclusion, understanding the molecular mechanism of Topo II checkpoint upon Topo II catalytic inhibition may differentially kill cancer cells.

The cell-based experiments were performed by Dr. Daniel Keifenheim and Dr. Duncan J. Clarke at the college of biological sciences, University Minnesota.

Material and Methods

Plasmid construction, site-directed mutagenesis, recombinant protein expression and purification, XEE antibodies and inhibitors.

For full-length recombinant WT Topo IIα and 3KR Topo IIα, the coding sequence was subcloned into a modified pPIC3.5Kb vector, which had a Calmodulin-binding protein (CBP) -T7 tag sequence for N-terminal fusion [17,18]. The recombinant proteins were expressed in GS 115 strain of *Pichia pastoris* yeast and purified as previously described [17]. In brief, frozen yeast cells expressing these recombinant proteins were ground in a coffee grinder with dry ice and lysed by adding lysis buffer (150mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 25mM HEPES, 0.1 % Triton X-100, 5% Glycerol, 1mM DTT and 1mM PMSF). Topo IIα protein in soluble fractions was isolated by calmodulin-sepharose resin (GE Healthcare) then further purified by anion exchange chromatography (GE Healthcare). For 3KR Topo IIα, lysine to arginine substitution was done by site-directed mutagenesis using QuikChange II kit (Agilent) according to the manufacturer's protocol.

Affinity purified antibodies used for immunoblotting; polyclonal rabbit anti-SUMO2/3, anti- Topo IIα antibody (against C-terminus region aa 1358-1579) and rat anti-PARP1 (against N-terminus region aa 1-150) were prepared as previously described [26]. Affinity purified polyclonal anti-rabbit human Topo IIα antibody (against C-terminus region aa 1359-1580). Commercial antibodies include mouse monoclonal anti-histone H4-HRP (#197517, 1:1,000, Abcam), mouse monoclonal anti-β tubulin (#T4026-2ML, 1:1,000, Sigma Aldrich), mouse monoclonal anti-histone H3 (#IB1B2, 1:2000, Cell Signaling) and rabbit polyclonal anti -T7 tag (#ab 19291, 1:1,000, Abcam). Secondary antibodies include Licor specific goat anti- rabbit IRDye 680RD (#925-68071, 1:20,000), goat anti- rabbit

IRDye 800CW (#925-32211, 1:20,000), goat anti- mouse IRDye 800CW (# 925-32210, 1: 20,000) and goat anti- rat IRDye 680 RD (#925-68076, 1:20,000).

For immunofluorescence staining, anti-SUMO2/3 guinea pig polyclonal antibody (1:500) and anti *X. laevis* CENP-A chicken polyclonal (1:500) were prepared as previously reported [26,55]. Anti-Aurora B rabbit polyclonal antibody (1:500) was prepared using full-length *X. laevis* Aurora B kinase as an antigen and anti- Claspin rabbit polyclonal antibody was prepared using a His6-T7- tagged N-terminus region of Claspin (aa1-271). For Topo IIα staining a commercial anti- Topoisomerase IIα/β mouse monoclonal antibody (#M052-3, 1:500, MBL International) was used. The fluorescence labeled secondary antibodies goat anti- mouse IgG Alexa Fluor 568 (#A11031, 1:500, Invitrogen), goat anti-rabbit IgG Alexa Fluor 568 (#A11036, 1:500, Invitrogen), goat anti- rabbit IgG Alexa Fluor 488 (#A11034, 1:500, Invitrogen), goat anti- guinea pig IgG Alexa Fluor 647 (#A21450, 1:500, Invitrogen) and goat anti- chicken IgG Alexa Fluor 488 (#A11039, 1:750, Invitrogen) were used for visualization in experiments using XEE.

The Topoisomerase II inhibitors used in this study, ICRF-193 (#BML-GR332, Enzo life sciences), Merbarone (#445800, Calbiochem) and 6-Hydroxydaidzein (4',6,7-Trihydroxyisoflavone; CAS#17817-31-1; Carbosynth, San Diego USA) were dissolved in DMSO and stored at -20°C.

XEEs assays

Xenopus demembraned sperm nuclei and low speed cytostatic factor (CSF) arrested *Xenopus* egg extracts (XEEs) were prepared according to standard protocols. For mitotic replicated chromosome isolation CSF extracts were driven into interphase with the addition of 0.6mM $CaCl_2$. Demembraned sperm nuclei were added to interphase extract at either

4000 sperm nuclei/µL (for western blotting analysis) or 800 sperm nuclei/µl (for immunofluorescence analysis), then incubated for ~ 45 min. Further, equal amounts of CSF XEEs were used to induce mitosis. To inhibit the mitotic SUMOylation dnUbC9 was added to XEEs at 150ng/µl at the onset of mitosis-induction by addition of CFS XEE. After incubating 60~90 min, followed by microscopic analysis of condensed mitotic chromosomes, Topo II inhibitors (ICRF-193 1µM and Merbarone 10 µM) were added into XEEs for 10 min.

For western blotting analysis, chromosomes were isolated using 40 % glycerol cushion as previously described [17] then isolated mitotic chromosomes were boiled in SDS-PAGE sample buffer. Samples were resolved on 8-16% gradient gels and subjected to immunoblotting with indicated antibodies. Signals were acquired using LI-COR Odyssey^{Fc} digital imager and the quantification was performed using Image Studio Lite software.

For the immunofluorescence staining, XEEs were diluted with three times of their volume in IF dilution buffer (0.5X CSF-XB containing 18mM β-glycerophosphate and 250 mM sucrose) and then fixed with equal volume of fixation buffer (IF dilution buffer containing 4 % p-formaldehyde) for 6 min incubation at RT. Fixed chromosome samples were loaded onto 8 ml of 40 % glycerol cushion with coverslips at bottom and spun for 6000 g for 20 min at RT. Precipitated chromosomes on coverslips were post- fixed with 1.6 % p-formaldehyde in PBS and blocked with PBS containing 5 % BSA and processed for immunostaining with indicated antibodies. For DNA staining, Hoechst 33342 dye (EMD Millipore) and for mounting the specimen Vectashield H-1000 medium (Vector Laboratories) were used. Images were acquired using the Plan Apo 100x/1.4 objective lens on Nikon TE2000-U microscope with a Retiga SRV CCD camera (QImaging) operated by

Velocity imaging software (PerkinElmer). Adobe Photoshop (CS6) software was used to process the images for signal intensities and size according to journal policy. For arm region Aurora B quantification, ImageJ software was used. The distinct Aurora B foci at arm regions for more than 25 chromosomes of four independent experiments were counted. For immunodepletion experiments, Topo IIα antibodies (1mg/ml) were captured on Protein A- conjugated magnetic beads (Thermo Fisher Scientific; Arnaoutov and Dasso, 2003) with 1:1 ratio. The immunodepletion/add back experiment and chromosome isolation was performed exactly as previously described [17]. Further, endogenous Topo IIα depletion from XEEs was confirmed by immunoblotting.

HeLa cell Topo IIα SUMOylation assay

For the analysis of mitotic chromosomes, HeLa cells were synchronized with 2mM Thymidine for 18 hours. Cells were released from Thymidine into fresh media and 6 hour later 0.1 μg/mL nocodazole added to cells. Four hours after nocodazole addition mitotic cells were collected by mitotic shake off and released with 7μM ICRF-193 containing fresh media for 20 minutes. For mitotic chromosomes isolation, cells were lysed with lysis buffer (250mM Sucrose, 20mm HEPES, 100mM NaCl, 1.5mM MgCl2,1mM EDTA, 1mM EGTA, 0.2%TritonX-100, 1:2000 LPC, 15mM IAA) and lysed cells were layered on 40% glycerol cushion the same as for chromosome isolation from XEEs. Later, isolated mitotic chromosomes were boiled in SDS-PAGE sample buffer, resolved on 8-16% gradient gels and subjected to immunoblotting with indicated antibodies. Signals were acquired using LI-COR Odyssey Fc machine.

In vitro SUMOylation FRET assay

The CyPet- tagged SUMO2G, CyPet- SUMO2GG and YPet-tagged Topo IIα CTD were subcloned in pET28a with His6-tag. CyPet and YPet coding sequence was amplified by PCR from pH2B-CyPet-FHA2-AuroraB Substrate-YPet-IRES-puro2b obtained through Addgene (Wurzenberger et al., 2012). Recombinant proteins were expressed in Rosseta2(DE3) strain then purified using Cobalt affinity beads (Talon Beads; Takara Bio Inc). The imidazole eluted His6-tag proteins were further purified using ion-exchange columns. The enzymes used for in vitro SUMOylation FRET assays - E1 complex (Aos1/Uba2 heterodimer), PIASy, Ubc9, and dominant-negative form of Ubc9 (dnUbc9-C93S/L97S) - were expressed in BL21 (DE3) or Rosetta2 (DE3) bacteria and purified as previously described [15,17,56].

In vitro SUMOylation FRET reactions were performed at 25°C for 30 min using 30nM E1 Uba2/Aos1, 60nM E2 Ubc9, 60nM PIASy, 2.5μM Cypet-tagged SUMO2G and SUMO2GG, 0.5μM Ypet-tagged Topo IIα, 2.5mM ATP, 1 μM ICRF-193 and 10 μM Merbarone. The reactions were stopped by addition of 10mM EDTA. There were different concentrations for PIASy, inhibitors and different time points used for time course experiments as indicated in Fig 2.2. Reaction buffer consisted of 20mM HEPES (pH 7.8), 100mM NaCl, 5mM $MgCl_2$, 5% glycerol, 0.05% Tween 20 and 1mM DTT. Emission spectrum at 530nM was measured using fluorescence spectrophotometer with Excitation at 414nM (Varian Cary Eclipse fluorescence spectrophotometer).

Cell Fixation and Staining

Mammalian cells were grown to ~70-80% confluency on a 10cm dish and arrested for 3 hours in 100 ng/mL nocodazole. Cells were then treated with 14μM ICRF-193 and/or 2μM

CHR-6494 for 45 minutes before isolating mitotic cells by mitotic shake off into 500 μL of PBS. Cells were treated with 1 mL of water to hypotonic shock the cells for better chromosome spreading, for 5 minutes. 500 μL of the hypotonic mixture was added to a cytology funnel attached to a glass slide with a Cytospin (Thermo Fisher Scientific) clip. The assembly was spun for 5 minutes at 2000 rpm with max acceleration. Working quickly so the cells did not fully dry out, glass slides were removed from the clip and a ring drawn around the deposited cells with a Super-PAP pen to form a hydrophobic barrier to help keep the fixing and staining reagents on the cells. There is a fine balance between letting the Super-Pap solution dry (about 30-60 seconds) and not letting the cells dry out. When the Super-PAP dried sufficiently, the cells were fixed with 3.8% paraformaldehyde for 5 minutes. Cells were permeated with 0.05% Trition X-100 in PBS for 5 minutes. Cells were then treated with 50mM Ammonium chloride in PBS for 2 minutes to quench paraformaldehyde. Cells were washed with PBS-0.01% Trition X-100 and then blocked with 0.1% Casein in PBS-0.01% Triton X-100. Cells were stained overnight with primary antibodies. Following primary antibody staining, cells were washed three times with PBS-0.01% Triton X-100 and stained with secondary antibody for 1 hour. Following secondary antibody staining, cells were washed twice with PBS-0.01% Triton X-100 and once with PBS-DAPI and mounted with ProLong Gold (Invitrogen). The following primary antibodies were used for staining: CREST antibody (#CS1058, 1:200; Cortex Biochem), anti-AIM-1 (Aurora B) antibody (#611083, 1:200; BD Transduction), anti-CENP-A antibody (#2186S, 1:200; Cell Signaling Technology), anti-INCENP antibody (#I5283, 1:2000; Millipore Sigma), anti-H3T3-Phos antibody (#ab78351, 1:1000; Abcam). Secondary antibodies used were Goat anti-Human (#A11013, 1:200; Thermo Fisher

Scientific), Goat anti-Rabbit (#A11011, 1:200; Thermo Fisher Scientific), Goat anti-Mouse (#A11004, 1:200; Thermo Fisher Scientific).

Cell imaging

Cells were imaged using a Delta Vision microscope system (Applied Precision) based on an Olympus IX-71 inverted microscope and either an Olympus UPLSAPO 100x, 1.40 NA oil objective for stained images or an Olympus LUCPLFLN 20x, 0.45 NA air objective for live cell imaging. A CoolSNAP HQ2 camera (Photometrics) was used to capture images. Soft Worx (version 6.1.3, Applied Precision) software was used to acquire images. Images were cropped and contrast enhanced using ImageJ (Fiji) and Photoshop (Adobe). All imaging was done at 37°C.

Quantification of metaphase checkpoint duration

For Nocodazole arrest and release time courses, Hela cells were grown to ~80% confluency in a 15 cm dish. Cells were then arrested in100 ng/mL Nocodazole for 3 hours. After arrest, cells were washed once with warm media and then mitotic cells were shaken off and aliquoted into 14 - 3 cm dishes and treated with various drugs. At each 45-minute time point, cells were trypsinized and then fixed for preparation of chromosome spreads (Gimenez-Abian and Clarke, 2009; Gimenez-Abian et al., 2005). Briefly, cells were washed with PBS and then resuspended in 2 mL of PBS in a 15 mL conical tube. To hypotonic shock the cells, 3 mL of water was added for 5 minutes. To the hypotonic mixture, 10 mL of freshly made Carnoy's fixation solution (25% glacial acetic acid, 75% Methanol) was added to cells and the mixture was spun at 1000g for 5 minutes. After the spin the supernatant was carefully aspirated off the pellet, taking care not to disturb the delicate pellet. Cells were then washed 3 times with 15 mL Carnoy's solution, taking care

to gently but thoroughly resuspend cells during each wash. After the three washes, the cells were incubated overnight at room temperature in 15m mL Carnoy's solution. The next day the cells were spun down and resuspended in 0.2 mL Carnoy's solution and dropped onto a glass microscope slide. After the slides dried, they were stained with 5% Giemsa solution for 7 minutes and then mounted with Entellan (Merck).

For the live cell imaging time courses, Hela cells with H2B-GFP were plated on a four chamber 3 cm glass-bottom plate and grown overnight to a confluency of ~80%. Media was replaced with CO_2 independent media. Cells were then treated with various drugs and quickly transferred to the heated Delta Vision microscope chamber. Cells were imaged for 3 hours and cells that were in metaphase at the beginning of the time course were scored for time to anaphase and chromosome decondensation. ZM447439 (8μM), ICRF-193 (14μM), Merbarone (400μM), 6HD (120μg/ml).

Endogenous Topo IIα knockdown and mutant expression

Wild-type mCherry-Topo IIα or 3KR mCherry-Topo IIα (K1240R, K1267R, K1287R) were inserted into the self-inactivating viral vector S2F-IMCg-F-mCherry/luc-F3 and inserted into the Flp site at the 5q31.3 locus of the HeLa EM2-11ht cells (Lane et al., 2013). The inserted Topo IIα was induced with 250ng/mL Doxycycline for 24 hours prior to experiments. Endogenous Topo IIα was knocked-down using the hs.Ri.TOP2A.13.1 (IDT) oligo (5'GGUGUUUUUAGUACAAGAUUUGAUGUCUUGUACUAA3') targeting the 3' UTR. Cells were grown to ~40% confluence in a 3cm dish to account for cells doubling during the RNAi treatment. The oligo was prepared for transfection with 50 pmol of oligo, 2 μL Lipofectamine RNAiMAX Reagent (Life Technologies), and 150 μL OptiMEM

Medium as carrier for the oligo and RNAiMAX per manufacture protocol. Cells were treated for 24 hours prior to experiments.

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Chapter 3 - The role of SUMO interacting motifs (SIMs) in PIASy mediated SUMOylation

Introduction

The **s**mall **u**biquitin-like **mo**difier (SUMO) conjugation to the substrate can regulate a protein's activity, cellular localization, and stability [1]. Currently, there are five distinct SUMO paralogues identified in vertebrates: SUMO-1, SUMO-2, SUMO-3, SUMO-4, and SUMO-5. Among these five isoforms SUMO-1, SUMO-2, and SUMO-3 are ubiquitous and most studied. SUMO proteins are conjugated at lysine residue of the substrate with the aid of SUMO E3 ligase. PIAS/Siz family proteins are conserved SUMO E3 ligase in yeast and vertebrates that perform SUMO conjugation to a variety of the substrates. In human, four different PIAS family members are found; PIAS1, PIAS2 (or PIASxα), PIAS3, and PIAS4 (or PIASy). All four PIAS proteins share a significant sequence homology at the N-terminus and possess five conserved domain or motifs: an N-terminus SAP domain (scaffold attachment factor-A/B Acinus and PIAS), a PINIT motif, an SP-RING, a SIM (SUMO interacting motif), and a variable C-terminus S/T (serine/threonine-rich) region. The SAP domain interacts with the A-T rich chromosomal region [2]. The PINIT motif is required for subcellular localization and substrate interactions [3]. The SP-RING domain (similar to Zinc-finger domain of ubiquitin E3 ligases) interacts with Ubc9 (SUMO E2 conjugating enzyme) and is required for SUMO ligase activity. The S/T region of PIAS proteins is an unstructured domain and its biological function is not well defined. All PIAS E3 ligases possess a SIM that can interact with SUMO in a non-covalent manner. The SIM

is important for the E3 ligase activity of PIAS proteins and plays a critical role in SUMO conjugation to the target protein. Different structural studies have indicated that SIM is required for proper positioning of donor SUMO while it is transferred from the E2-SUMO complex to the substrate [3–5]. Recently, using NMR studies we have discovered that PIAS4 or PIASy E3 ligase has two SIMs unlike other PIAS family proteins that have only one [6].

Furthermore, we have previously reported that PIASy is an essential E3 ligase for mitotic SUMOylation in XEEs [7,8]. In cell-free assay XEEs, Topo IIα and PARP-1 are major SUMO2/3 modified chromosomal substrates for PIASy. PIASy directed SUMO 2/3 modified substrates primarily accumulate at the mitotic centromere including Topo IIα and PARP-1. Immunodepletion of PIASy in XEEs completely abolished the observed SUMOylation at the mitotic centromere [7], and replacement of PIASy with other PIAS family proteins could not restore SUMOylation. Additionally, disruption of PIASy function causes chromosome segregation defects in XEEs [7]. Together, these findings suggest that PIASy is a critical SUMO E3 ligase in XEEs, plausibly has many other substrates, and regulates centromeric function to allow faithful chromosomes segregation. With almost a decade of research, we came to know that PIASy has a crucial role in proper mitosis progression. But, we still do not know how PIASy E3 ligase activity is regulated. Why PIASy has many more substrates for the SUMO2/3 modification as compared to other PIAS family members? How does PIASy identify specific substrates during mitosis? We hypothesize that one potential approach to regulate PIASy E3 ligase activity and substrate specificity could be through its SUMO interacting motifs (SIMs). In this chapter, we have highlighted some of the roles of SIMs in PIASy-directed SUMOylation. To address these

questions, we have utilized *in vitro* SUMOylation assays; cell-free *Xenopus* egg extracts, and mammalian cells.

Results

Two SIMs in PIASy are crucial for in-vitro E3 ligase activity

Unlike other PIASs that have only one SIM, PIASy contains two SIMs near the C-terminus. To determine the significance of SIMs in PIASy-mediated SUMOylation we have created three distinct *Xenopus* PIASy SIM mutants.

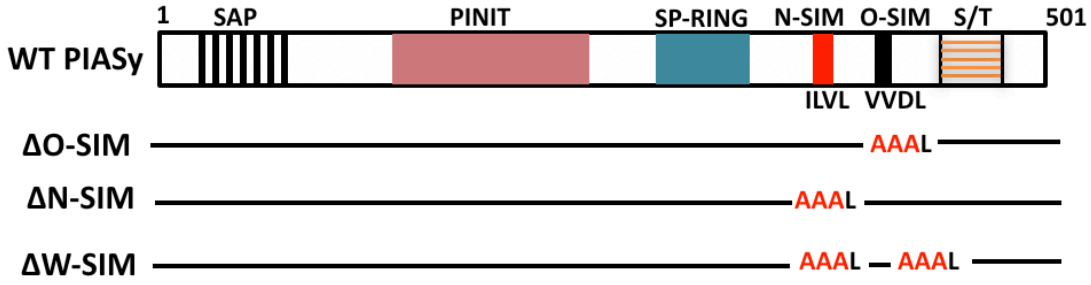


Figure 3.1 A schematic for WT PIASy and SIM mutants illustrating the different mutations introduced in SIM sites.

The three important amino acid residues of these SIMs are mutated into alanines. The full-length PIASy constructs have mutations in the original SIM (referred to as ΔO-SIM), the new SIM (referred to as ΔN-SIM), and both SIMs (referred to as ΔW-SIM) **(Fig 3.1)**. The wild type PIASy and SIM mutants were analyzed in an *in vitro* SUMOylation assay to examine their E3 ligase activity. We have used recombinant Topo IIα and PARP-1 as a substrate for *in vitro* reactions. The mutation in original SIM (ΔO-SIM) did not significantly reduce PIASy E3 ligase activity for Topo IIα and PARP-1, and displayed SUMOylation efficiency close to WT **(Fig 3.2 A&C)**. The mutation in new SIM (ΔN-SIM) exhibited two different outcomes for Topo IIα and PARP-1 SUMOylation. There was no

significant difference seen in Topo IIα SUMOylation with ΔN-SIM **(Fig 3.2 B)**. Whereas, PARP-1 SUMOylation was significantly decreased with ΔN-SIM compared to WT **(Fig 3.2 D)**. Altogether, mutation of both SIMs (ΔW-SIM) displayed a significant reduction in Topo IIα and PARP-1 SUMOylation *in-vitro* **(Fig 3.2 B&D)**. Though, the effect of ΔN-SIM PIASy for PARP-1 SUMOylation was more prominent than Topo IIα. Altogether, this data suggests that ΔN-SIM and ΔO-SIM are important for E3 ligase activity to direct the PIASy-specific SUMOylation *in vitro*. However, we did observe Topo IIα SUMOylation with ΔW-SIM (~40%) suggesting PIASy could have another SIM.

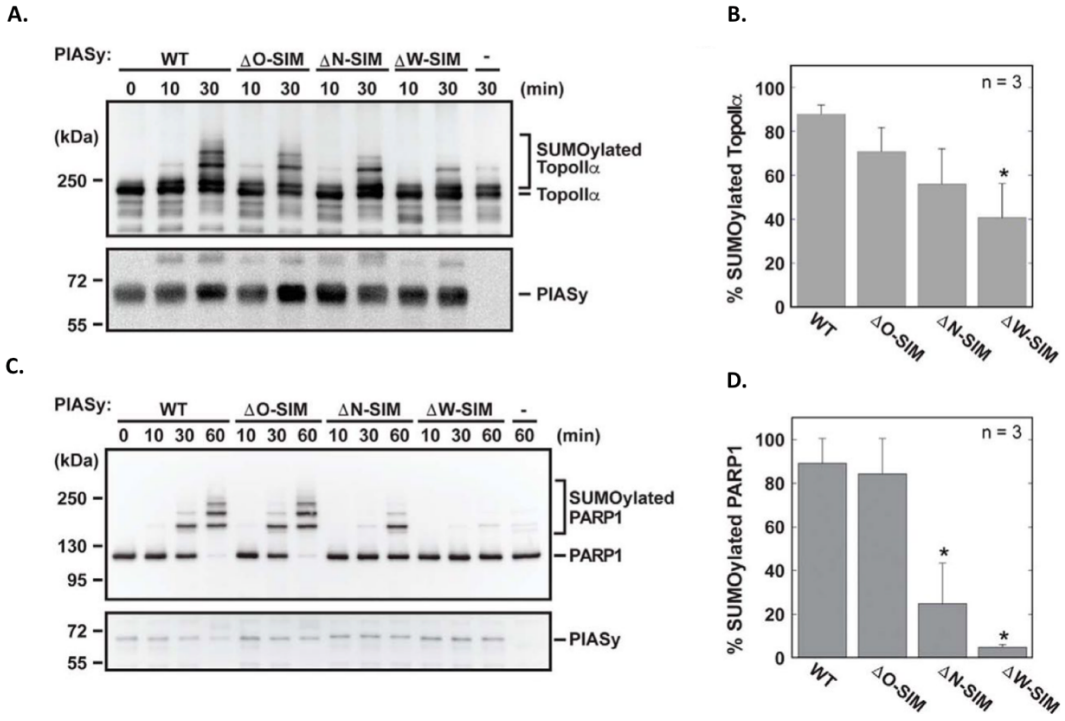


Figure 3.2. The contribution of two SIMs in *in-vitro* SUMOylation reactions. (A & C) Time course in-vitro SUMOylation assay for Topo IIα and PARP-1. PIASy levels are probed as input for each time point. **(B & D)** Quantification analysis for Topo IIα and PARP-1 SUMO2/3 modification. p values were calculated by student t test from three independent experiments, error bars represent standard deviation and asterisk indicates statistically significant difference than WT PIASy*. Figure adapted from Kaur et. al 2017 [6].

* Performed *in-vitro* SUMOylation for Topo IIα and contributed as a co-author in Kaur K. et.al 2017, JBC.

Two SIMs in PIASy E3 ligase are important to mediate mitotic SUMOylation in cell free *Xenopus* egg extracts.

PIASy is a primary E3 ligase for mitotic SUMOylation that modifies different mitotic chromosomal proteins in egg extracts. To further examine the contribution of SIMs to PIASy-specific chromosomal SUMOylation we have isolated the mitotic chromatin associated with PIASy SIM mutants from the egg extracts. Endogenous PIASy was immunodepleted from the egg extracts as described in the materials and methods section. PIASy depletion was confirmed by western blotting **(Fig 3.3A)**. Recombinant WT and PIASy SIM mutants were added back into extracts nearly to the endogenous concentrations. Sperm chromatin was allowed to assemble into mitotic chromosomes from the extracts containing recombinant PIASy mutants. Isolated chromosome samples were subjected to western blotting and probed for SUMO2/3 modification. Interestingly, recombinant WT PIASy showed a similar SUMOylation profile on the mitotic chromosomes as previously reported in control **(Fig 3.3B)**. This suggests recombinant WT PIASy addition to extract can restore PIASy-specific mitotic chromosomal SUMOylation. Surprisingly, ΔO-SIM PIASy had exhibited a drastic reduction in SUMOylation whereas; ΔN-SIM PIASy addition did not show any notable difference in chromosomal SUMOylation. ΔN-SIM PIASy associated chromosomes mostly showed a similar SUMOylation pattern as WT PIASy. Furthermore, the addition of a double SIM mutant PIASy (ΔW-SIM) to extract had almost eliminated the global mitotic SUMOylation. Though, our *in vitro* results did not complement with results from XEEs and will be analyzed in depth. Plausibly, the presence of other PIAS enzymes in XEEs could hinder with SIM mutant activity.

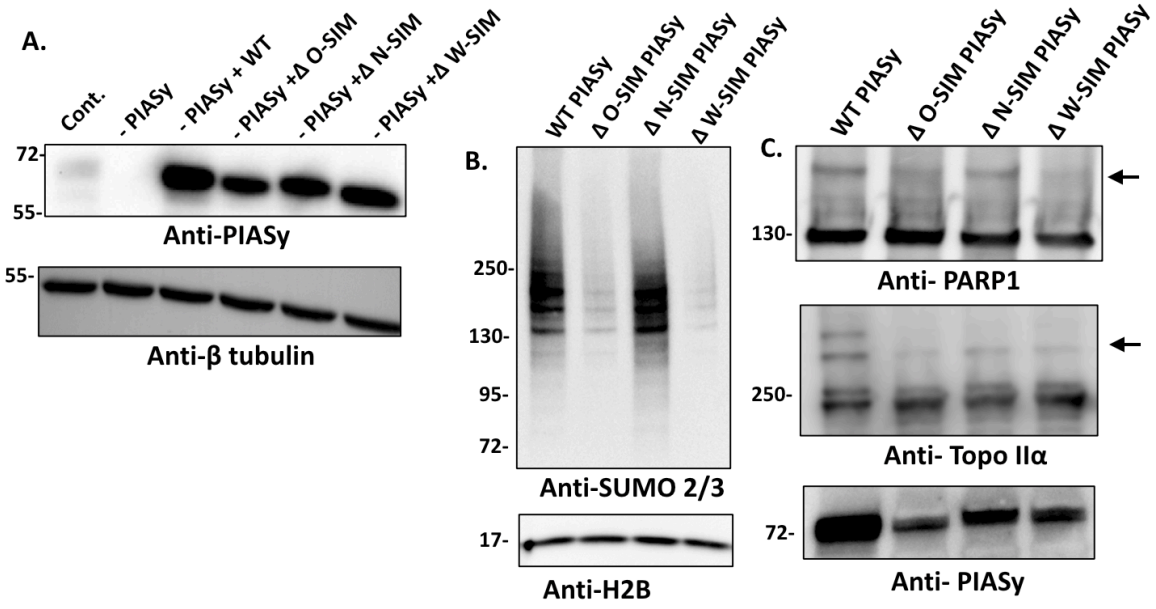


Figure 3.3 PIASy mediated mitotic SUMOylation in *Xenopus* egg extracts is regulated by both SIMs. **(A)** CSF egg extracts were confirmed for immunodepletion of PIASy using anti-PIASy antibody. WT PIASy and SIM mutant proteins were added to extracts. The β tubulin level was probed as a loading control. **(B)** Mitotic chromosomes were subjected to western blotting to analyze global SUMO2/3 modification. H2B was probed as a loading control. **(C)** Topo IIα and PARP-1 SUMOylation was probed with chromosome samples using indicated antibodies. The arrow indicates SUMOylated band for PARP-1 and Topo IIα distinct from endogenous non-SUMOylated form.

Next, we examined PARP-1 SUMOylation on the mitotic chromosomes. Consistent with global chromosomal SUMOylation for ΔO-SIM PIASy we did not observe a strong SUMOylated band for PARP-1 similar to WT PIASy **(Fig 3.3 C,** marked by an arrowhead**).** On the contrary, ΔN-SIM PIASy sample did show a SUMOylated band for PARP-1. Moreover, ΔW-SIM PIASy associated chromosome samples lack a strong SUMOylated band for PARP-1. Also, we analyzed Topo IIα SUMOylation on the mitotic chromosomes with PIASy SIM mutations. Interestingly, we have observed a similar pattern for Topo IIα SUMOylation. The mitotic chromosomes associated with ΔO-SIM PIASy and ΔN-SIM PIASy showed a reduction in SUMOylated Topo IIα. We did not observe a second band

for SUMOylated Topo IIα similar to WT PIASy. Altogether with ΔW-SIM Topo IIα SUMO2/3 modification is reduced as compared to WT PIASy **(Fig 3.3 C,** marked by an arrowhead**)**. Our results from XEEs suggest that both SIMs are important to mediate PIASy-specific mitotic SUMOylation. Though, each SIM in PIASy showed different SUMOylation efficiency, plausibly because of their preferences for specific substrate on the mitotic chromosomes.

Human PIASy E3 ligase utilizes two SIMs to induce mitotic SUMOylation

PIASy-specific mitotic SUMOylation in human cells is not been studied before. To study the role of SIMs in human cells we have established transgenic stable PIASy-mCherry cell lines harboring the ΔO, ΔN, and ΔW mutations in SIMs. The mutations were introduced similar to *Xenopus* PIASy as described earlier. Using CRISPR-Cas9 gene editing approach, WT human PIASy and SIM mutants were ectopically expressed at the safe harbor locus *AAVS1* with Tet-On inducible system **(Fig 3.4 A, B, and C)**. In these transgenic cells endogenous PIASy was expressed in background without impairing the transgene expression. WT PIASy-mCherry and SIM mutant cells were synchronized in mitosis and mitotic chromosomes were isolated to analyze the SUMOylation status on the chromosomes **(Fig 3.5A)**. Western blotting analysis indicated that chromosomal SUMOylation was upregulated in WT PIASy-mCherry expressing transgenic cells **(Fig 3.5B)**. Quantification analysis also confirmed that mitotic SUMOylation is significantly increased by approximately 3.5 fold in WT PIASy cells **(Fig 3.5C)**. The ectopic expression of ΔO-SIM and ΔN-SIM PIASy-mCherry showed significant reduction in chromosomal SUMOylation. However, we did observe a difference in SUMOylation pattern for ΔO-SIM

and ΔN-SIM PIASy expression and that needs to be explored in more detail. We think this difference in chromosomal SUMOylation could occur because each of the SIMs may have specificity towards different substrates. Furthermore, ΔW-SIM PIASy-mCherry cells displayed a significant reduction of SUMOylation **(Fig 3.5B and C)**. Surprisingly, in this result we observed more SUMOylation for ΔWSIM -Dox than +Dox sample and that will be re-examined. Additionally, we think inefficient transgene expression at the *AAVS1* locus caused inconsistent mCherry levels in cells. A recent article had supported our notion that inserted transgene at the *AAVS1* locus can be silenced due to DNA methylation [9]. Next, we examined Topo IIα SUMOylation in these transgenic cells. We observed increased SUMO2/3 modified Topo IIα with WT PIASy cells. Western blotting analysis suggests Topo IIα SUMOylation is reduced with ectopic expression of ΔW-SIM PIASy. However, ΔO-SIM PIASy-mCherry cells display less efficient Topo IIα SUMOylation compared to ΔN-SIM expressing cells. Together, these results suggest that two SIMs are important for E3 ligase activity of PIASy in human cells **(Fig 3.5B)**. Immunofluorescence analysis of mitotic cells with ectopic expression WT PIASy-mCherry (+Dox) showed increased mitotic SUMOylation. Whereas with ΔW-SIM PIASy-mCherry (+Dox) expression mitotic SUMOylation seems reduced **(Fig 3.5 D)**. Notably, we did observe a mosaic expression among cells and less mCherry expression in ΔW-SIM transgenic cells (+Dox) than WT PIASy cells. We think this is due to unstable transgene expression and we will address this concern by choosing the other safe harbor loci such as *CCR5* and *H11* for the stable transgene integration. Moreover, Immunostaining analysis provided similar evidence that PIASy E3 ligase directs mitotic SUMOylation with the aid of both SIMs.

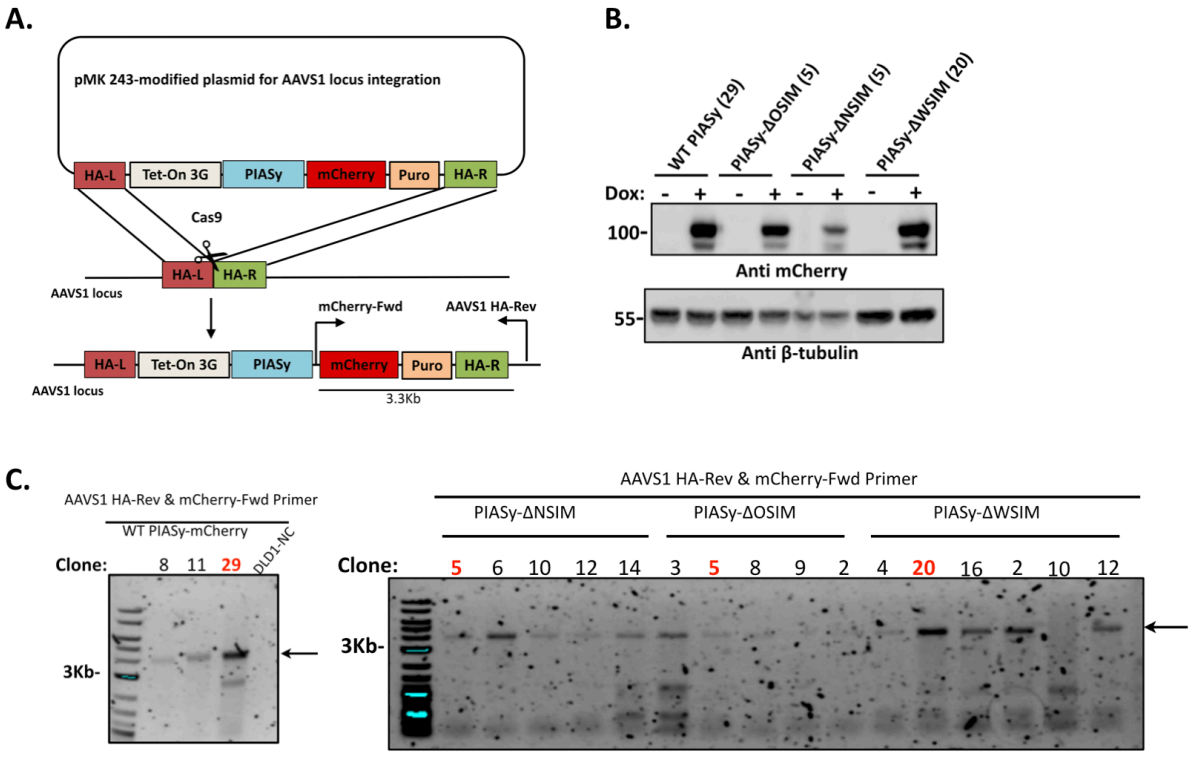
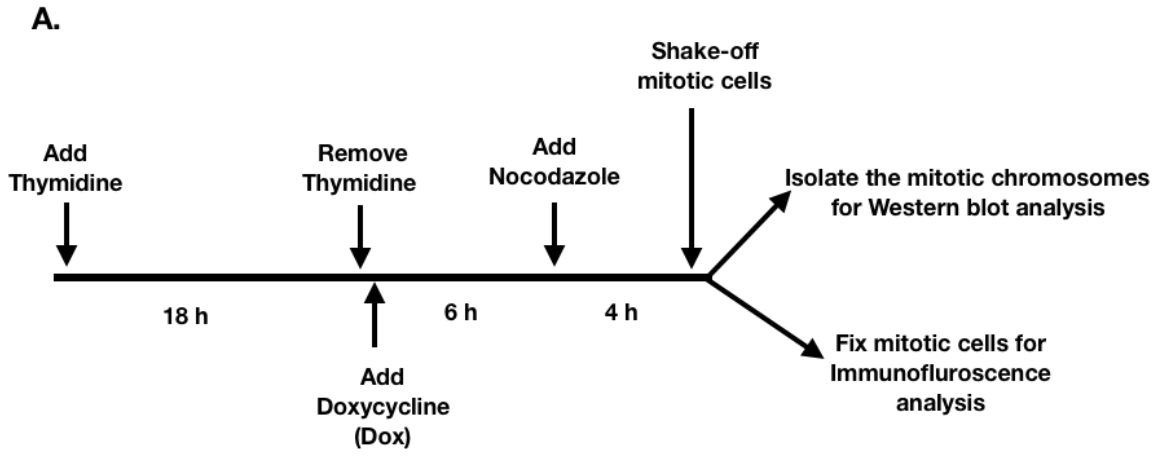


Figure 3.4 Construction of WT PIASy-mCherry and SIM mutant cell lines. (A) Schematic representation to introduce WT PIASy and SIM mutants at AAVS1 locus. Cells were transfected with modified PMK243 plasmid (obtained from Addgene) to introduce the WT PIASy, PIASy-ΔNSIM, PIASy-ΔOSIM, and PIASy-ΔWSIM genes at AAVS1 locus. **(B)** Whole cell lysates were probed for the transgene expression by western blotting. Anti-mCherry antibody was used to probe the expression level in -Dox and +Dox samples. β-tubulin was used as a loading control. **(C.)** After Puromycin selection clones were amplified to confirm the integration at AAVS1 locus using AAVS1 HA-reverse and mCherry forward primers.



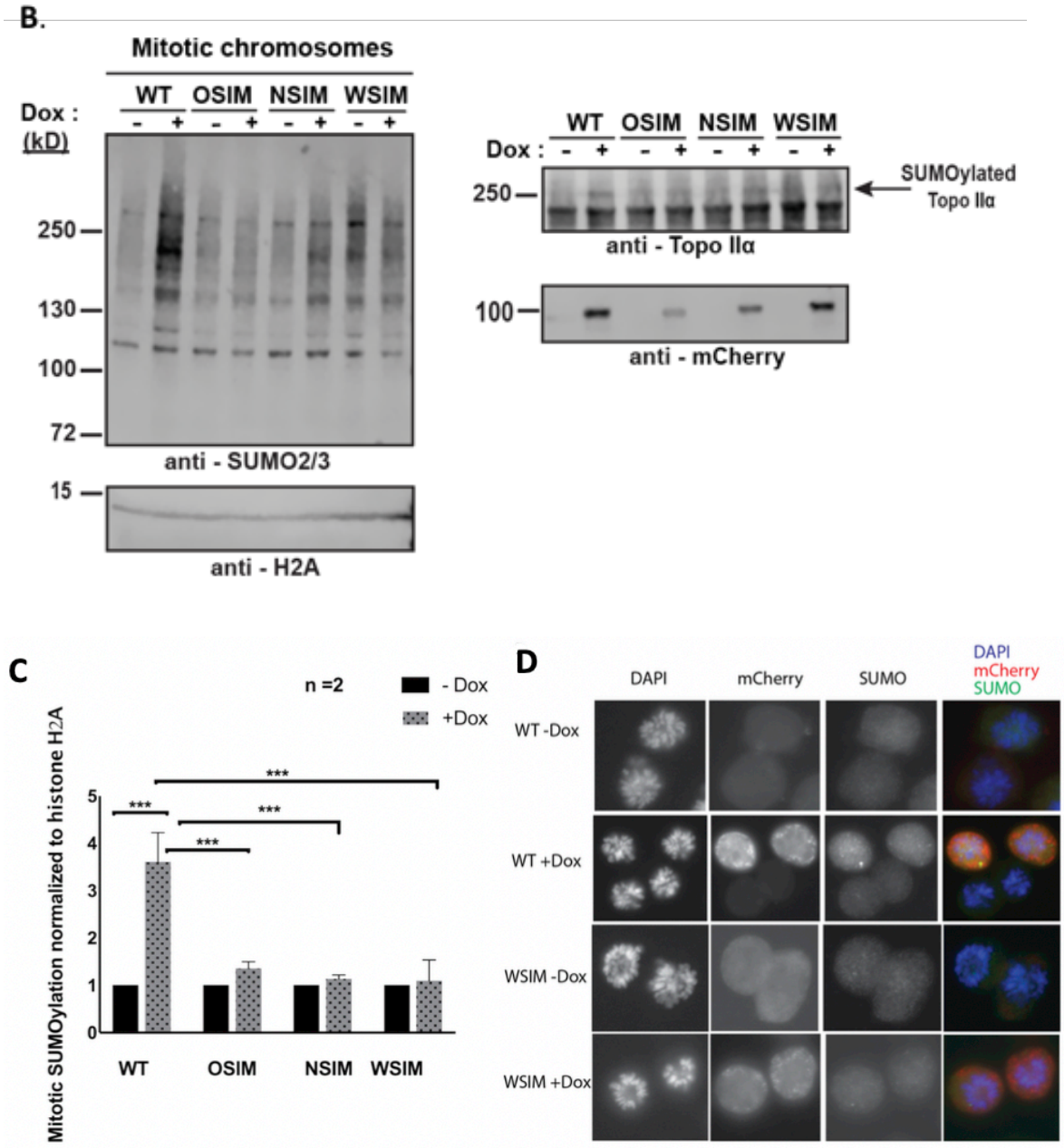


Figure 3.5 Both SIMs in PIASy are required for mitotic SUMOylation in human cells. (A) A schematic for the cell synchronization and sample preparation. **(B)** Chromosome samples were subjected to western blotting and probed using the indicated antibodies. **(C)** Total mitotic SUMOylation were measured using image studio software and normalized to histone H2A. p values were calculated using a one-way ANOVA analysis of variance with Tukey multi-comparison correction from two independent experiments. *** $p < 0.001$ **(D)** Mitotic cells were stained with indicated antibodies. mCherry (Red), SUMO2/3 (Green) and DNA stained with DAPI.

Discussion

Previous studies have indicated that PIASy is an important SUMO E3 ligase in XEEs that mediates robust SUMO2/3 modification on chromosomes during mitosis [7,8,10]. SUMO interacting motif (SIM) is an important structural region in E3 ligase that transfers SUMO moiety to the target protein during modification. We are the first group which has reported that unlike other PIAS E3 ligases *Xenopus* PIASy possess two SIMs and *in-vitro* results indicated E3 ligase activity can be significantly reduced with a mutation in both SIMs [6]. The OSIM and NSIM showed distinct substrate specificity in *In -vitro* reactions as NSIM seem more important for PARP1 than Topo IIα SUMOylation **(Fig 3.2C&D)**.

Using XEEs, PIASy is discovered as a primary E3 ligase that has many targets for SUMO2/3 conjugation. But, we still do not know about the role of PIASy to mitotic SUMOylation in human cells and how E3 ligase activity is regulated for different substrates? A study had reported that PIASy is required for faithful chromosome segregation in human cells [11].

Our results implicate that PIASy is a conserved E3 ligase in human cells that performs mitotic chromosomal SUMOylation similar to egg extracts. Though, the inducible expression of PIASy-mCherry is uneven in cells and make cell-based analysis difficult. Therefore, to overcome this issue we would like to have transgenic cells with a stable expression of transgene in the future. Additionally, using XEEs cell-free assay and human cells we showed the role of two SIMs in PIASy-specific SUMOylation is conserved in vertebrates. With obtained results we hypothesize SIMs in PIASy potentially govern the substrate specificity during SUMOylation. We have identified Topo IIα is one of the conserved mitotic protein for PIASy-specific SUMOylation in human cells. It is possible

that PIASy has other conserved SUMOylation substrates in human cells like egg extracts. Another potential PIASy substrate in human cells could be PARP1. A future study to identify the PIASy substrates will provide further insight into the biological significance of PIASy in SUMOylation research. PIASy is predominantly accumulated at the mitotic centromere and modifies different centromeric proteins with SUMO2/3 [7,8]. Depletion of PIASy eliminates centromeric SUMOylation and blocks the chromosome segregation. This suggests PIASy-specific SUMOylation probably regulates the centromere function during mitosis. It will be interesting to investigate the role of these two SIMs and their specific substrates in the regulation of centromere function and anaphase progression. To summarize, we have identified PIASy is an important conserved E3 ligase that mediates mitotic SUMOylation in human cells. Our data also indicated that two SIMs are important to facilitate mitotic SUMOylation in XEEs and human cells.

Materials and methods

In-vitro SUMOylation assay

In-vitro SUMOylation reactions were performed with recombinant purified proteins. 5μM SUMO-2, 2mM ATP, 20nM E1, 30nM E2, 20nM PIASy, and 500nM T7-tagged *Xenopus* Topo IIα or PARP-1 was used in reaction mixtures. Reaction buffer contained 20mM HEPES, pH7.8, 100mM NaCl, 5mM $MgCl_2$, 0.05% Tween-20, 5% glycerol, and 0.5mM DTT. All the ingredients were incubated in reaction buffer for the 30 min or 60 min (depending upon the substrate) at 25° C. Reactions were stopped at various time points with the addition of SDS-PAGE loading buffer. Samples were boiled and subjected to western blotting using anti-T7 HRP conjugated monoclonal antibody.

Xenopus egg extract preparation, immunodepletion/add-back, and mitotic chromosome isolation

Low-speed cytostatic factor (CSF) arrested *Xenopus* egg extracts (XEEs) and demembraned sperm nuclei were prepared according to standard protocols [12,13]. To isolate the replicated mitotic chromosomes CSF extracts were induced into interphase with the addition of 0.6mM $CaCl_2$ and demembraned sperm nuclei (4000sperm nuclei/µl) were incubated for ~ 45 min. Further, to induce mitosis equal amount of CSF XEEs was added. After incubating around 90 min, condensed replicated mitotic chromosomes were isolated using 40 % glycerol cushion as previously described [14]. Later, isolated mitotic chromosomes were boiled in SDS-PAGE sample buffer, resolved on 8-16% gradient gels and subjected to immunoblotting with indicated antibodies.

For immunodepletion experiment, anti-PIASy antibodies (1mg/ml) were captured on Protein A-conjugated magnetic beads in 1:1 ratio overnight at 4° C. PIASy antibody bound beads were blocked with 5% BSA in CSF-XB buffer (10mM HEPES- KOH pH 7.7, 100mM KCl, 2mM $MgCl_2$, 5mM EGTA, and 50 mM sucrose). XEEs were mixed with antibody-beads and incubated for 15 min on room temperature and followed by 15 min on the ice twice. PIASy depleted extracts were used to the experiments. For add back, recombinant WT PIASy and SIM mutants were mixed with depleted extracts at final concentration ~ 100nM. Further, endogenous PIASy depletion from XEEs was confirmed by immunoblotting.

Mitotic chromosomes were isolated exactly as previously described in this section and subjected to western blotting with indicated antibodies.

Cell culture, transfection, and stable cell line generation

For the preparation of stable cell lines, the exogenous sequence was targeted to AAVS1 safe harbor locus using donor plasmids by CRISPR-CAs9 system and homologous recombination. DLD-1 cells were transfected with donor and guide plasmid using ViaFectTM (#E4981, Promega) on 3.5cm dishes. 48 hours post-transfection cell were split and re-plated into 10 cm dishes at ~ 20% confluency with Puromycin (1μg/ml) containing media for the selection. Selection media was changed after every two days after 10-14 days colonies were isolated and grown in 24 well plates. When 24 well plates were confluent these clones were split to prepare SDS and DNA samples to verify the sequence insertion by western blotting and genomic PCR. For western blotting, cells were pelleted and boiled/vortexed with 1X SDS PAGE buffer. SDS samples were resolved on an 8-16 % gradient gel and probed with indicated antibodies.

Cell synchronization for mitotic cell and chromosome isolation

DLD-1 cells were grown in McCoy's 5A 1x L-glutamine 7.5% Tet-ON FBS media. To analyze mitotic chromosomes mitotic cells were enriched with single thymidine and nocodazole synchronization protocol. Cells were arrested in S-phase with 2mM thymidine for 17 hours. Next, cells were released from thymidine block by three washes with non-FBS containing McCoy's 5A 1x L-glutamine media. Further, cells were released for 6 hours with 500ng/ml doxycycline and 7.5% FBS containing fresh media. After releasing for 6 hours 0.1ug/mL nocodazole was added to the cells for 4 hours. Four hours after nocodazole addition, a shake-off was performed to collect the mitotic cells. Mitotic cells were washed twice with McCoy's non-FBS containing media to release from nocodazole. Cells were re-suspended in 7.5% FBS containing fresh media and released for 20 minutes.

After 20 minutes, mitotic cells were plated on fibronectin coated coverslips for immunostaining. Additionally, mitotic chromosomes were isolated for western blot analysis. Cells were lysed with lysis buffer (250mM sucrose, 20mM HEPES, 100mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 0.2% TritonX-100, 1:2000 LPC (Leupeptin, Pepstatin, Chymostatin, 20mg each/ml in DMSO; Sigma-Aldrich), and 20mM IAA) for 5 minutes on ice. Lysed cells were added on top of the 40% glycerol containing 0.025% Triton-X-100 cushion and spun at 10,000xg for 5 minutes twice. Mitotic chromosomes were boiled with SDS-PAGE sample buffer, resolved on an 8-16% gradient gel, and subjected to immunoblotting with indicated antibodies.

Cell fixation and immunostaining

For immunostaining, mitotic cells were plated onto fibronectin coated cover slips (NEUVITRO, #GG-12-1.5-Fibronectin) after the mitotic shake-off and incubated for 20 minutes. For cell fixation, cells were incubated with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were washed with 1X PBS containing 10mM Tris-HCL three times to quench PFA. Further, Cells were permeabilized with ice cold 100% methanol for 10 minutes and briefly washed with 1X PBS. Post-permeabilization cells were blocked with 2.5% hydrolyzed gelatin for 30 minutes at room temperature. Cells were stained with primary antibody for one hour and washed with PBS-T (1X PBS containing 0.1% Tween-20) thrice. Next, cells were incubated with secondary antibody for one hour at room temperature followed by three PBS-T washes. Cells were mounted onto glass slide using VECTASHIELD® Antifade Mounting Medium with DAPI (#H-1200, Vector laboratory).

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(This chapter is primarily focused on generating tools that can be utilized to study SUMOylation in human cells)

Chapter 4 - Establishment of ectopically expressing PIAS transgenic cell lines to study SUMOylation in human cells

Introduction

PIASs (protein inhibitor of activated STAT (signal transducer and activator of transcription)) were previously discovered because of their role in transcription. Initially, two PIAS family proteins PIAS1 and PIAS3 were identified as inhibitors of STAT because of their interactions with STAT1 and STAT3 [1,2]. Now, it has become more clear that PIAS function is not only specific to STATs but they have a variety of roles in transcriptional and cellular regulation [3]. One of the known functions of PIAS protein is to promote SUMOylation because of their SUMO E3 ligase ability. All PIAS proteins possess a putative SP-RING finger domain essential for the E3 ligase activity. PIAS proteins are much investigated for their role in transcription, immune system, and cytokine signaling [4,5]. Their roles as SUMO E3 ligases are not well studied. Human PIAS proteins are encoded by four different genes; PIAS1, PIAS2 (PIASxα), PIAS3, and PIAS4 (PIASy) and have conserved structural domains and motifs **(Fig 4.1)**. The specific function of each domain and motif is previously described in chapters 1 and 3.

PIAS proteins can enhance SUMOylation of various substrates in a cell cycle-dependent manner. For example, PIASy is predominantly known to upregulate SUMO2/3 modification during mitosis [6–9]. One of the important questions in SUMOylation biology is how these PIAS proteins determine substrates for modification and their selectivity for SUMO isoforms. Interestingly, all PIAS proteins have a preference for SUMO2/3 over SUMO1. PIAS proteins can efficiently form a polymer chain of SUMO2/3 but not SUMO1

[10]. SUMO interacting motifs (SIMs) in PIAS proteins do not participate in the selection of SUMO isoforms. To study these PIAS proteins in more detail I have developed tools for the ectopic expression of human PIAS2 (PIASxα), PIAS3, and PIAS4 (PIASy) that can be further utilized to study SUMOylation in human cells.

This chapter mainly summarizes the establishment of these transgenic cell lines along with some initial analysis and future directions for this study.

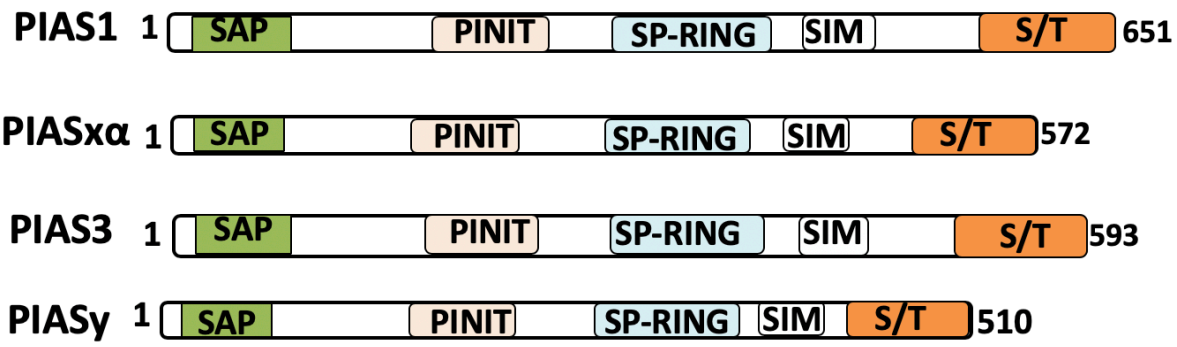

Figure 4.1 Schematic structures of PIAS proteins with different domains. All PIAS proteins share five conserved structural domains. These domains have specific functions. The SAP domain is involved in interaction with DNA and other transcriptional factors. The PINIT domain is important for subcellular localization. The SP-RING domain is essential for the E3 ligase activity and SIM is important for the interaction with SUMO. The S/T rich region at the C-terminus is highly variable among all PIASs and its function is not defined yet.

Experimental design to establish the stable cell lines for the ectopic expression of PIASs.

To understand the specific role of PIAS proteins as SUMO E3 ligases we have developed transgenic stable cell lines for the ectopic expression of PIASxα, PIAS3, and PIASy with C-terminal mCherry. Using CRISPR-Cas9 gene editing and homologues recombination, PIAS E3 ligase genes are integrated at safe harbor locus *AAVS1* with Tet-On inducible system. In these transgenic cells endogenous PIAS E3 ligases are expressed in background without impairing the transgene expression. The doxycycline addition will allow the

ectopic expression of the transgene. Earlier PIAS proteins were studied in HeLa cells with transient expression [11]. Analyzing these transient PIAS expressing cells could be challenging in terms of the expression level of a transgene and cell cycle specific analysis. Therefore, to overcome these challenges we have developed transgenic cell lines. In chapter 3, **Figure 3.4** demonstrates a schematic for the integration of PIAS genes in the genome and inducible expression using the Tet-ON system. PIAS genes are inserted downstream of the Tet-ON promoter to induce the expression of PIAS-mCherry when Doxycycline is added. Using these cell lines, we can ectopically express PIAS proteins in cells and SUMOylation can be modulated (**Fig 4.2 A and B)**. Furthermore, we aim to study SUMOylation in more detail using these transgenic human cells.

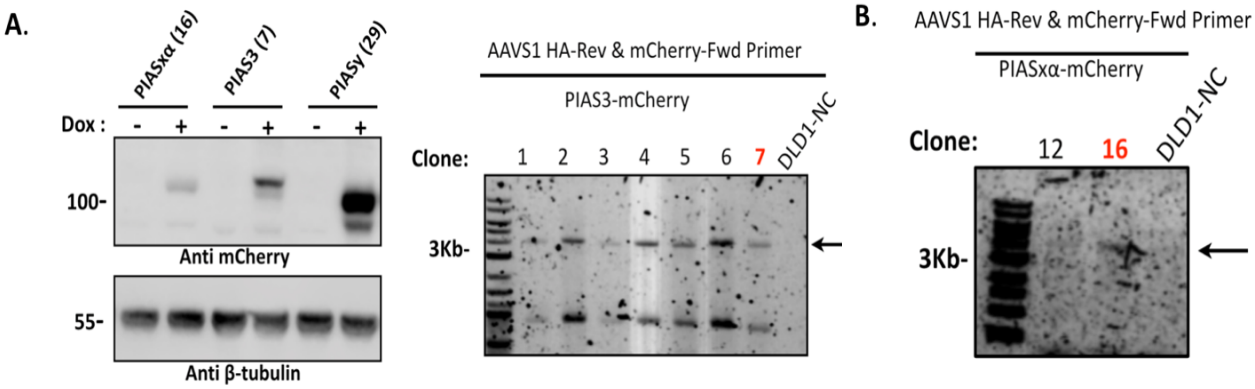

Figure 4.2 Construction of transgenic PIASs-mCherry cell lines. (A) Whole cell lysates were probed for the transgene expression by western blotting. Anti-mCherry antibody was used to probe the expression level in -Dox and +Dox samples. β-tubulin was used as a loading control. **(B.)** After Puromycin selection clones were amplified to confirm the integration at AAVS1 locus using AAVS1 HA-reverse and mCherry forward primers.

Alternative approach to modulate SUMOylation at the centromere during mitosis

As explained in chapter 3, in XEEs we have shown PIASy E3 ligase is primarily accumulated at the centromere during mitosis and modifies many centromeric proteins with SUMO2/3. The disruption of PIASy compromises centromeric SUMOylation and eventually centromere function. Our previous finding has indicated that PIASy-directed SUMOylation at the centromere is essential for faithful chromosome disjunction. All these studies have been performed primarily in the cell-free model system XEE. Modulating SUMOylation in mammalian cells has always been challenging because of the dynamic nature of this modification and lack of tools. To overcome this, we developed a novel approach that consists of a fusion gene expression. The N-terminal region of human PIASy is required for its localization to mitotic centromeres through its specific binding with the RZZ (Rod/Zw10/Zwilch) complex at the kinetochore [12]. The catalytic domain of SENP2 protease is essential for its de-SUMOylation activity. We have fused the N-terminal region of PIASy with the catalytic domain of SENP2 (referred to as Py-S2 WT). This construct is expected to abolish the SUMOylation at the centromere. Additionally, we have also created a construct where a cysteine at position 548 of SENP2 is substituted for an alanine that attenuates the SENP2 de-SUMOylation activity (referred to as Py-S2 Mut) (**Fig 4.3A)** [13,14]. These fusion proteins were expressed in human cells using a tetracycline inducible system. Now, using this novel approach we can modulate SUMOylation in human cells.

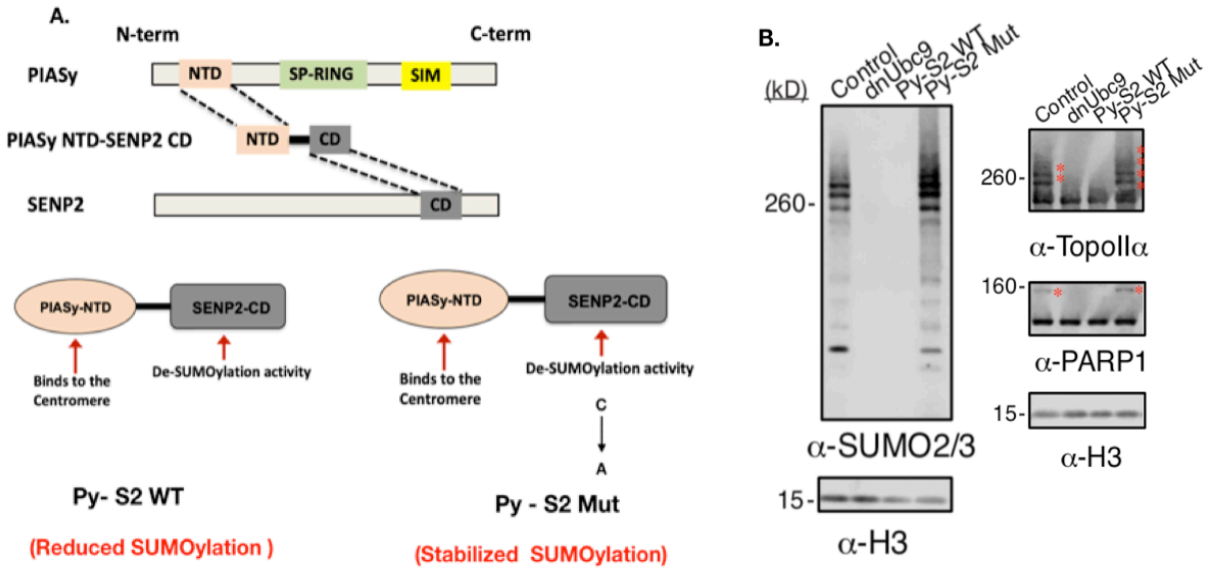

Figure 4.3 A novel fusion gene approach to modulate SUMOylation in human cells. (A) A schematic for the establishment of constructs to express PIASy -SENP2 fusion genes. (B) Recombinant fusion proteins were added to the egg extracts and tested for their activity for mitotic SUMOylation. Further, the mitotic chromosomes isolated from XEEs with these fusion proteins and probed for chromosomal SUMOylation using SUMO2/3 antibodies. Topo IIα and PARP1 SUMOylation were probed with Topo IIα and PARP1 antibodies*.

We have already verified this experimental approach by testing the activity of these fusion proteins in XEEs **(Fig 4.3B)**. The addition of Py-S2 WT fusion protein in XEEs eliminates the mitotic SUMOylation on the chromosomes. Whereas, Py-S2 Mut further stabilizes the SUMOylation and behaves as a negative control. Topo IIα and PARP1 SUMOylation were analyzed in XEEs with this fusion protein addition. SUMOylation for these substrates disappeared with Py-S2 WT protein activity whereas Py-S2 Mut further restored the Topo IIα and PARP1 SUMOylation. The red asterisks indicated SUMOylated bands for Topo II IIα and PARP1*.

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* The result shown in figure 4.3B is a courtesy from Dr. Yoshiaki Azuma.

Results

PIASs ectopic expression does not increase global SUMOylation in unsynchronized cells

To analyze the roles of PIAS family SUMO ligases in global SUMOylation, transgenic stable cell lines were treated with or without doxycycline to induce the ectopic expression of proteins. After 24 hours, cells were harvested for whole cell lysate and chromatin isolation as described in materials and methods. Whole cell lysate and chromosomal fractions were subjected to western blotting to analyze the SUMOylation status. For whole cell lysate, PIAS ectopic expression did not show a significant increase in global SUMOylation **(Fig 4.4A and B)**. Similarly, we also analyzed the chromosomal SUMOylation for these cells. PIASxα, PIAS3, and PIASy ectopic expression did not cause an increase in overall chromosomal SUMOylation **(Fig 4.4C and D).** Though we observed a different SUMOylation pattern for each E3 ligase suggesting that each PIAS protein have a distinct set of chromosomal substrates for SUMOylation.

Further, we investigated the localization of different PIASs and SUMO2/3 staining in these cells. Immunostaining showed the mosaic expression of PIAS proteins, plausibly due to unstable transgene expression at *AAVS1*, therefore we primarily analyzed the mCherry positive cells (**Fig 4.5A**). Different PIAS E3 ligases show distinct nuclear distribution in cells. The nuclear distribution for PIAS proteins is consistent with our previous observation [11]. Additionally, we observed a distinct pattern for SUMO2/3 staining for PIASxα, PIAS3, and PIASy. PIASxα-mCherry expression did not show an apparent increase in SUMO2/3 signal but interestingly enriched nuclear SUMO2/3 foci were seen. Unlike to PIASxα, SUMO2/3 signal was increased with PIAS3-mCherry expression. For PIASy-

mCherry expressing cells, we made two distinct observations first, the SUMO2/3 signal was increased secondly strong SUMO2/3 foci were seen like PIASxα-mCherry **(Fig 4.5A)**. The distinct nuclear distribution of each PIASs and pattern for SUMO2/3 staining indicated that each protein might have a unique set of nuclear substrates. The quantification analysis did not show a significant increase for SUMO2/3 when PIASxα and PIASy were ectopically expressed. The SUMO2/3 signal was significantly increased with PIAS3 expression **(Fig 4.5B)**. Further, we would like to redo these experiments with mCherry positive cells a using a cell sorter to eliminate the effect of mosaic expression.

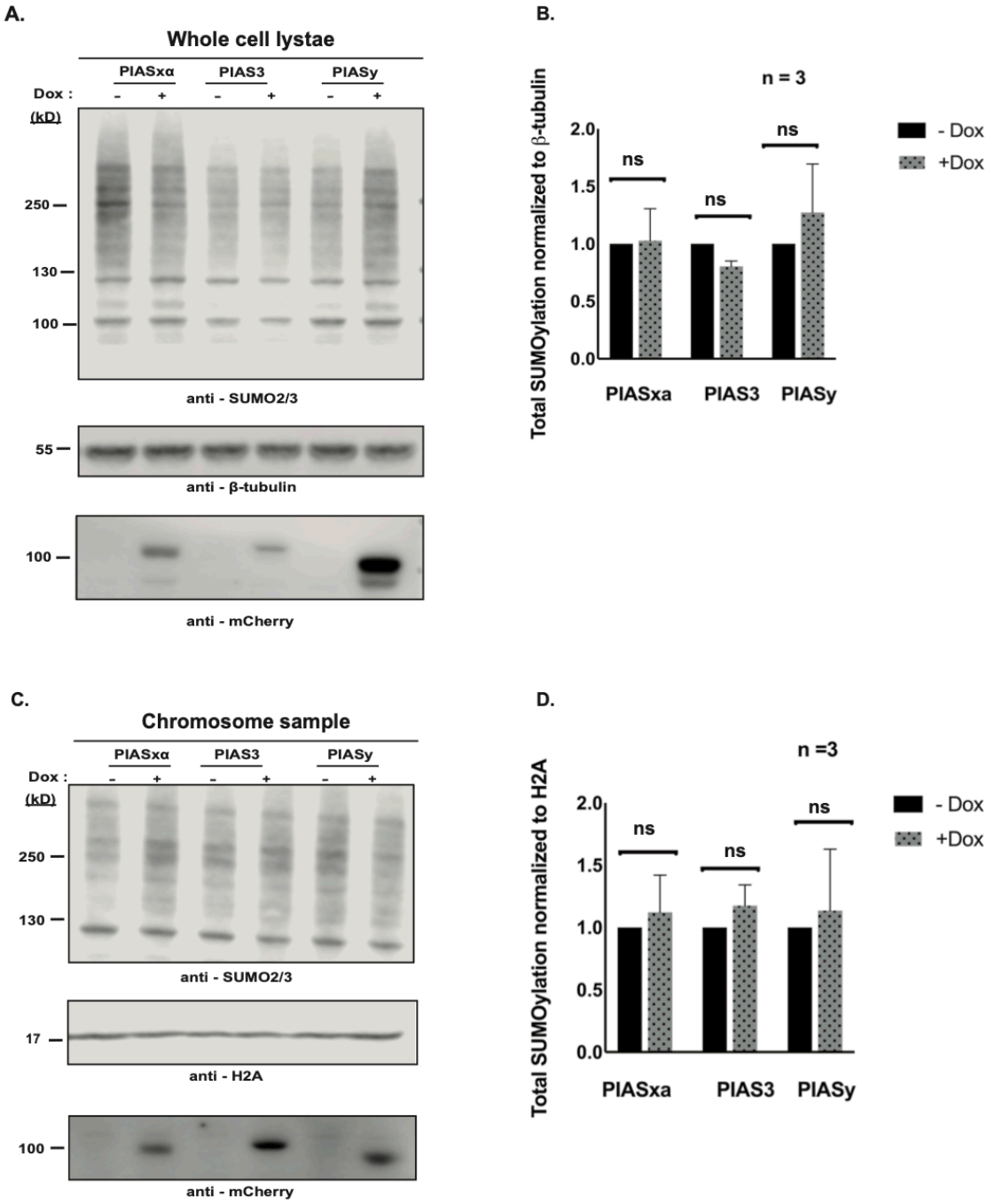

Figure 4.4 The global SUMOylation analysis for unsynchronized cell population. (A) Whole cell lysate of PIAS -mCherry expressing cells were subjected to western blotting and probed with indicated antibodies. **(B)** Total SUMOylation were measured using image studio software and normalized to β-tubulin. **(C)** Chromatin samples were from these cells were isolated and subjected to western blotting and probed with indicated antibodies. **(D)** Total chromosomal SUMOylation were measured using image studio software and normalized to histone H2A.

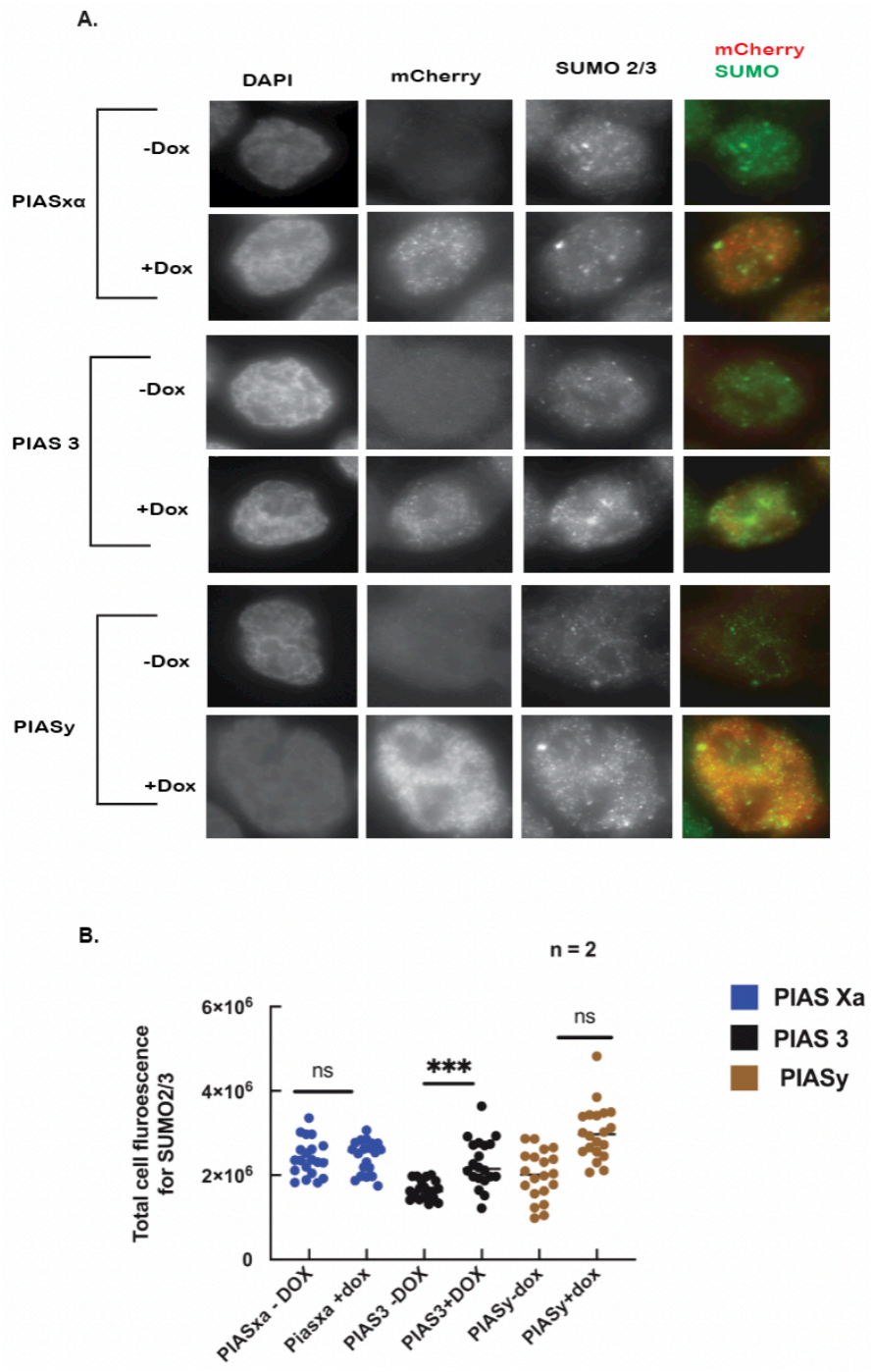

Figure 4.5 Differential nuclear localization of PIAS SUMO E3 ligases in human cells. **(A)** PIASs-mCherry cells were immunostained after 24-hour inducible expression with doxycycline, mCherry (red) and SUMO2/3 (green). **(B)** Nuclear SUMO2/3 signal were measured using Fiji software. p values were calculated using a one-way ANOVA analysis of variance with Tukey multi-comparison correction from two independent experiments. *** $p < 0.001$

PIASy is a conserved SUMO E3 ligase in human cells for mitotic SUMOylation

We have reported earlier that PIASy E3 ligase is required for robust mitotic chromosomal SUMOylation in XEEs [15]. To examine if PIASy has a similar role in human cells, cells were synchronized in mitosis and mitotic chromosomes were isolated as explained in materials and methods section. Western blotting of the chromosomal fraction showed SUMO3/3 modification was drastically increased when PIASy was ectopically expressed **(Fig 4.6A)**. Quantification analysis also confirmed that PIASy directed mitotic SUMOylation is significantly increased **(Fig 4.6B)**. However, we did not observe any apparent difference in mitotic SUMOylation for PIASxα and PIAS3 **(Fig 4.6A)**.

This result indicates that compared to other PIASs, PIASy is a primary E3 ligase and regulates mitotic SUMOylation in cells. Topo IIα is one of the important mitotic SUMOylation substrates in XEEs. Therefore, we wanted to determine Topo IIα SUMOylation for these chromosomal fractions (**Fig 4.6A**). The obtained result suggests that PIASy expression upregulates Topo IIα SUMOylation in cells. On the contrary, PIASxα and PIAS3 expression did not show an apparent increase in Topo IIα SUMOylation. This result suggests that Topo IIα is one of the conserved substrates for PIASy directed SUMOylation in human cells similar to XEEs. Our initial analysis using these transgenic cell lines suggests that among all PIASs, PIASy is a primary E3 ligase that regulates mitotic chromosomal SUMOylation in human cells. This observation is consistent with our previous finding in XEEs.

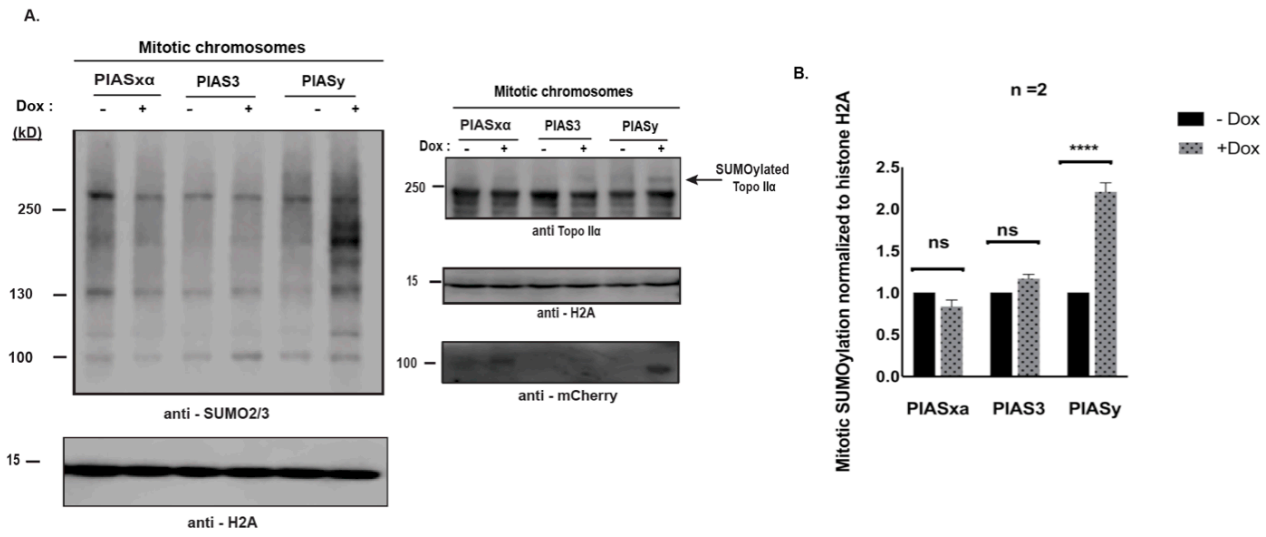

Figure 4.6 PIASy is a conserved SUMO E3 ligase in human cells for mitotic chromosomal SUMOylation (A) Mitotic Chromosomes were isolated from the cells as described in materials and methods section. Samples were subjected to western blotting and probed with indicated antibodies. An arrowhead indicated the SUMOylated Topo IIα. **(B)** Total mitotic SUMOylation were measured using image studio software and normalized to histone H2A. The p values were calculated using a one-way ANOVA analysis of variance with Tukey multi-comparison correction from two independent experiments. *** $p < 0.001$

Future directions

With these established transgenic tools, we will examine the role of human PIAS family proteins in SUMOylation. Among all PIAS proteins PIASy is well studied and characterized as a primary E3 ligase for mitotic SUMOylation using *in vitro* assay and XEEs [9,12,15,16]. Our group had discovered Topo IIα and PARP-1 as major mitotic SUMOylation substrates for PIASy. Other PIAS proteins are not explored much for their role in SUMOylation. Mostly we know that these PIAS proteins are critical regulators of transcription because they can act as repressors and activators. Some previous studies have suggested that subcellular localization of PIAS proteins is an important factor for substrate specificity *in vivo*. It will be of great interest to identify the other PIAS-specific substrates for SUMOylation in cells. Additionally, in the future we would like to analyze if other

PIAS proteins can substitute for the PIASy-specific mitotic SUMOylation via knocking out the endogenous PIASy and replacing with other PIASs in cells. To address this question we will utilize the Auxin inducible degron (AID) system to deplete PIASy from cells and Tet-On replacement system to induce the other PIASs expressions. We would like to analyze what SUMO isoforms are preferentially used by these PIAS E3 ligases during SUMOylation. Additionally, we sought to address a long-standing question how do PIAS family members determine the specificity towards SUMO-1 vs. SUMO2/3?

Different studies in budding yeast have shown that Siz1 (a PIAS E3 ligase paralogue) interacts with its substrates through its N-terminus [17,18]. Our group has also determined that the PIASy N- terminus is not required for *in vitro* SUMOylation but is required for mitotic SUMOylation in XEEs [12]. Furthermore, the first 47 residues of PIASy are also known to direct PIASy to the centromere where it colocalizes with Topo IIα, suggesting its role in centromeric SUMOylation [12]. The SAP domain at the N-terminus interacts with a stable Rod/Zw10 complex at the kinetochore and this interaction is important to recruits PIASy at the centromere region. Since all PIAS proteins share a significant sequence similarity we sought to investigate whether the role of the SAP domain is conserved in their localization in human cells. In-depth analysis of the subcellular localization of these PIAS proteins will be useful to identify the PIAS-specific substrates. In order to address these questions, we have also created the other PIAS N-terminus fusion constructs with SENP-2 catalytic domain similar to PIASy as explained earlier. This approach will allow us to understand the contribution of other PIAS family proteins to centromeric SUMOylation and its function.

Lastly, for this part of the dissertation, I have designed and developed different experimental tools to study the PIAS family proteins for their role in SUMOylation. We hope the obtained information using these tools will provide insight into the unexplored role of PIASs E3 ligase in SUMOylation biology.

Materials and methods

CRISPR -Cas9 DNA constructs and antibody preparation

DNA constructs for the ectopic expression of different PIAS family proteins were created by using modified pMK243 (AAVS1-tetOsTIR1-PURO-AAVS1) plasmid [19]. The donor pMK243 was used to target OsTIR1 to AAVS1 locus as it harbors the homology arms for AAVS1 [19]. Original pMK243 (Addgene # 72835) was purchased from Addgene and OsTIR1 sequence was removed using BglII and MluI restriction digestion followed by a multi-cloning site insertion. For all constructs, MluI and BglII sites were used to insert PIAS sequence with C-terminus mCherry in the modified pMK243. For gene integration at AAVS1 locus a guide plasmid AAVS1 T2 CRIPR in pX330 was obtained from Addgene (Plasmid #72833) [19]. Primary antibodies for SUMO2/3 were prepared as described previously ((Azuma et al., 2003& 2005). Human Topo IIα primary antibodies were prepared by targeting the C-terminus of Topo IIα (amino acids 1359~1589). The c-terminus sequence was amplified from Topo II Iα cDNA. Further, the amplified fragment was subcloned into pET28a and pGEX-4T vectors (GE Healthcare). The recombinant protein was expressed in Rossetta2 (DE3) E.coli. The expressed C-terminus fragment was purified using His6-tag and GST-tag by Talon-resin (Clontech/Takara) or Glutathione-sepharose (GE healthcare) following the manufacture's protocol. Further, the purified fragments were separated using cation-exchange column. Purified His6-tagged Topo IIα

fragment was used as an antigen to immunize the rabbits. For antibody purification, antigen affinity columns were prepared by conjugating purified antigens GST-tagged Topo IIα C-terminus fragment to the NHS-Sepharose resin as per manufacturer's instructions (GE healthcare). The antibodies were purified from the rabbit antisera using antigen affinity columns.

Cell culture, transfection and stable cell line generation

For the preparation of stable cell lines, exogenous sequence was targeted to AAVS1 safe harbor locus using donor plasmids by CRISPR-CAs9 system and homologous recombination. DLD-1 cells were transfected with donor and guide plasmid using ViaFectTM (#E4981, Promega) on 3.5cm dishes. 48 hours post-transfection cell were split and re-plated into 10 cm dishes at ~ 20% confluency with puromycin (1μg/ml) containing media for the selection. Selection media was changed after every two days and 10-14 days later colonies were isolated and grown in 24 well plates. When 24 well plates were confluent these clones were split to prepare SDS and DNA samples to verify the sequence insertion by western blotting and genomic PCR. For western blotting, cells were pelleted and boiled/vortexed with 1x SDS PAGE buffer. SDS samples were resolved on an 8-16 % gradient gel and probed with indicated antibodies. Signals were acquired using LI-COR Odyssey Fc imager. For genomic PCR, DNA was extracted from cells using lysis buffer 100mM Tris-HCl pH 8.0, 200mM NaCl, 5mM EDTA, 1% SDS, and 0.6mg/mL proteinase K (#P8107S, NEB). Further, DNA was precipitated with isopropanol and spun to a pellet. DNA pellet was washed with ethanol and re-suspended with TE buffer containing 50ug/mL RNase A (#EN0531, Thermo Fisher).

Cell synchronization for mitotic cell and chromosome isolation

DLD-1 cells were grown in McCoy's 5A 1x L-glutamine 7.5% Tet-ON FBS media. To analyze mitotic chromosomes mitotic cells were enriched with single thymidine and nocodazole synchronization protocol. Cells were arrested in S-phase with 2mM thymidine for 17 hours. Cells were released from thymidine block by three washes with non-FBS containing McCoy's 5A 1x L-glutamine media. Further, cells were released for 6 hours with 500ng/ml doxycycline and 7.5% FBS containing fresh media. After releasing for 6 hours 0.1ug/mL nocodazole was added to the cells for 4 hours. Four hours after nocodazole addition, mitotic shake-off was performed to collect the mitotic cells. Mitotic cells were washed twice with McCoy's non-FBS containing media to release from nocodazole. Cells were re-suspended in 7.5% FBS containing fresh media and released for 20 minutes. After 20 minutes, mitotic cells were plated on fibronectin coated coverslips for immunostaining. Additionally, mitotic chromosomes were isolated for western blot analysis. Cells were lysed with lysis buffer (250mM sucrose, 20mM HEPES, 100mM NaCl, 1.5mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 0.2% TritonX-100, 1:2000 LPC (leupeptin, pepstatin, chymostatin, 20mg /ml each in DMSO; Sigma-Aldrich), and 20mM IAA) for 5 minutes on ice. Lysed cells were added on top of 40% glycerol containing 0.025% Triton-X-100 cushion and spun at 10,000xg for 5 minutes twice. Mitotic chromosomes were boiled with SDS-PAGE sample buffer, resolved on an 8-16% gradient gel, and subjected to immunoblotting with indicated antibodies. Signals were acquired using the LI-COR Odyssey Fc machine.

To analyze the asynchronous cell population, cells were grown in McCoy's 5A media with 24-hour 500ng/ml doxycycline addition. Chromosomes were isolated as described above using 20% glycerol cushion.

For western blotting following primary antibodies were used: anti Topo IIα (1:20,000), anti SUMO2/3 (1:1,000) as described previously in [20] [15], anti-Histone H2A (1:3,000) (#18255, Abcam), anti-β-tubulin (1:3,000) (#M4884V, Sigma-Aldrich), anti mCherry (1:500) (#167453, Abcam).

Cell fixation and immunostaining

For immunostaining, mitotic cells were plated onto fibronectin coated cover slips (NEUVITRO, #GG-12-1.5-Fibronectin) after the mitotic shake-off and incubated for 20 minutes. For cell fixation, cells were incubated with 4% paraformaldehyde for 10 minutes at room temperature. Cells were washed with 1X PBS containing 10mM Tris-HCL three times to quench PFA. Further, cells were permeabilized with ice cold 100% methanol for 10 minutes and briefly washed with 1X PBS. Post-permeabilization cells were blocked with 2.5% hydrolyzed gelatin for 30 minutes at room temperature. Cells were stained with primary antibody for one hour and washed with PBS-T (1X PBS containing 0.1% Tween-20) three times. Next, cells were incubated with secondary antibody for one hour at room temperature followed by three PBS-T washes. Cells were mounted onto glass slide using VECTASHIELD® Antifade Mounting Medium with DAPI (#H-1200, Vector laboratory). Fiji software was used to calculate the total cell fluorescence for SUMO2/3. For immunostaining, following primary antibodies were used: anti-SUMO (1:500), anti-Topo IIα (1:300) (# 189342, Abcam), anti-RFP (1:1000) (GMA3H9, Bulldog).

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Chapter 5 - Current understanding and future directions

Introduction

With more than two decades of research in the SUMOylation field, scientists have studied the many aspects of SUMOylation and its different roles in cell cycle progression. Many studies in different model systems have indicated that disruption of SUMOylation results in mitotic defects. SUMOylation during mitosis is highly dynamic in nature and regulates various mitotic processes. Using XEEs we have found DNA Topoisomerase IIα (Topo IIα) is one of the major mitotic substrates for SUMOylation. SUMOylated Topo IIα is found most concentrated at the centromere and plausibly has a role in chromosome segregation. Lys 660 in the catalytic domain of Topo IIα was one of the first SUMOylation sites reported that regulate Topo IIα enzyme activity [1]. Later, our group also identified three other SUMOylation sites in C-terminus of the enzyme. Though CTD SUMOylation does not have a role in Topo IIα enzyme activity [2]. However, CTD SUMOylation mediates protein-protein interactions at the mitotic centromeres as explained in chapter 1. Using mass spectrometry analysis, two SUMO binding proteins were identified that interact with SUMOylated CTD: Claspin and Haspin kinase. We further determined that CTD SUMOylation regulates chromosome segregation because Haspin is involved in Aurora B kinase recruitment at the mitotic centromeres in a SUMOylation dependent manner [3,4]. The catalytic disruption of Topo II enzyme during mitosis causes a delay in anaphase onset [5] and activates a checkpoint. Though, the molecular mechanism behind this Topo II checkpoint was not well understood. The studies presented in chapter 2 show a novel mechanism to activate the Topo II checkpoint and CTD SUMOylation is required as an initial signaling step.

Topo IIα CTD SUMOylation and its role in mitosis

Though the structure of the Topo II C-terminus is not solved yet, many studies have revealed that the CTD is crucial for error-free chromosome segregation during mitosis in yeast, human cells and XEEs [6–9]. These findings gave a piece of evidence that the CTD has some conserved function during mitosis that is independent of the catalytic cycle (SPR) of the enzyme. Topo IIα CTD comprises many sites for different post-translational modification such as phosphorylation and SUMOylation. Further, this led to a hypothesis that the CTD might be playing a role in genome transmission via these modifications. Our recent findings in XEEs have provided a better understanding of the role of SUMOylated CTD in mitosis progression [2,3]. Topo II inhibitors are reported to increase Topo IIα SUMOylation [10]. However, the SUMOylation sites in Topo IIα and downstream effects of inhibitor-mediated increased Topo IIα SUMOylation remained unknown. In chapter 2, using XEEs and human cells we have confirmed that three C-terminal lysine residues are primary SUMO acceptor sites when treated with Topo II inhibitor (ICRF-193). Additionally, we have shown that CTD SUMOylation acts as a signal transducer upon Topo II catalytic inhibition with ICRF-193 and causes a delay in anaphase onset.

Haspin localization at the mitotic centromeres is regulated by Topo II CTD SUMOylation and recruits Aurora B kinase, which is important for faithful chromosome segregation. Topo IIα catalytic disruption by ICRF-193 causes stalled Topo IIα on chromosomes in a closed clamp conformation and induces upregulated SUMOylation Topo IIα CTD. Spatial and temporal regulation of Topo II SUMOylation is important for proper mitosis progression. Therefore, when Topo IIα CTD SUMOylation is misregulated it causes aberrant localization of Aurora B on mitotic chromosomes. With ICRF-193 treatment,

Aurora B showed a conserved phenotype in XEEs and human cells. Aurora B levels are enriched at the mitotic centromeres and are mobilized to chromosome arms (Fig 2.4B, C, and 2.6A). The centromeric localization of Aurora B is essentially important for timely metaphase to anaphase progression transition. Further, it will be a great interest of study to examine the downstream targets for Aurora B kinase on the kinetochore and chromosome arms. Aurora B is known to phosphorylate different substrates at kinetochores such as Ndc80/hec1, CENP-E, and Dsn1 [11–13]. Further studies to analyze these substrates or any other novel substrates may shed more light to understand the molecular signaling for Topo II checkpoint activation.

Another SUMO binding protein Claspin is known to interact with SUMOylated CTD at the mitotic centromere. Claspin is a DNA damage checkpoint protein and known to interact with Chk1 kinase that phosphorylates Aurora B at S331 for its activation [14]. However, the mitotic role of Claspin is still not clear. Though ICRF-193-induced increased Topo II SUMOylation did not cause Claspin mislocalization on the chromosomes (Fig 2.4A) similar to Aurora B, Claspin levels are increased on the mitotic centromeres. This suggests that Claspin could have an important mitotic function and might be regulating Aurora B activation in a SUMOylation-dependent manner. Studying the Claspin-Chk1 pathway in more detail will help us to understand the role of SUMOylated Topo IIα CTD in mitosis. Further analysis of SUMOylation-dependent Claspin-chk1 and Haspin-H3T3P pathway in Aurora B regulation will expand our understanding of the role of Topo II SUMOylation in mitosis.

A previous study had shown that ICRF-193 treatment also increases PIASy (an important SUMO E3 ligase) on mitotic chromosomes [10] further suggesting that upregulation of E3

ligase increases Topo II SUMOylation. Our results from XEEs also show that addition of ICRF-193 caused increased binding of PIASy with chromosomes and mislocalization to chromosome arms (data not shown). Normally, PIASy primarily accumulates on mitotic centromeres as reported previously [15]. These finding gave us a hint that plausibly ICRF-mediated upregulation of PIASy results in increased Topo II CTD SUMOylation and causes a mitotic delay. Further analysis of mitotic progression using the PIASy overexpression transgenic cell line will tell us if PIASy overexpression can also activate the Topo II checkpoint. Therefore, to address this hypothesis it will be necessary to study Aurora B localization and H3T3P localization on mitotic chromosomes with PIASy overexpression.

Metaphase Topo II checkpoint: future prospective

Disrupting the Topo II strand passage reaction causes a delay in anaphase and activates a Topo II checkpoint in budding yeast [5]. A yeast Top2-B44 mutant is deficient in ATP hydrolysis and has a six times slower rate of decatenation. Top2-B44 completes the strand passage reaction with a much slower rate and does not completely arrest. This yeast mutant showed a delay in anaphase onset and activated Topo II checkpoint. On the other hand, yeast mutants having defects at other stages of SPR did not show a delay in anaphase onset. This finding suggested that cells could monitor Topo II strand passage reaction or remaining catenations in DNA. Additionally, this study also reported that the Topo II CTD is required for Topo II checkpoint activation [5]. However, the major question remains how cells sense defects in strand passage via CTD and what molecular signaling induces this checkpoint activation.

Results shown in this dissertation demonstrate that Topo II catalytic inhibition using ICRF-

193 (an inhibitor mimic of Top2-B44) result in increased Topo IIα CTD SUMOylation. It might be possible that CTD SUMOylation sites are more accessible to SUMOylation machinery when Topo IIα is stalled on the chromosomes with ICRF-193 treatment. What causes upregulation in CTD SUMOylation upon Topo II catalytic inhibition is a question that needs to be addressed in the future.

Now it is important to understand how stalled Topo IIα is removed from the chromosomes to allow faithful chromosome segregation. Topo II inhibitor ICRF-193 induces mitotic defects and results in increased Ultra-fine DNA bridge (UFBs) formation [16,17]. A SUMO-binding protein PICH (Polo-like interacting checkpoint helicase) is known to bind with UFBs and plays an important role in chromosome disjunction. Recently, we have identified PICH as a promiscuous SUMO binding protein that interacts with SUMOylated proteins through its SIMs [18]. PICH is known to interact with Topo IIα and can affect its activity [17]. To resolve chromosomes, PICH can preferentially interact with stalled SUMOylated Topo IIα using SIMs and can remove Topo IIα by its DNA translocase activity. In the case of the Topo II checkpoint, we think ICRF-193-mediated upregulated CTD SUMOylation could induce strong interaction with PICH and somehow does not allow the removal of stalled Topo IIα. A comprehensive analysis using a non-SUMOylatable CTD mutant, PICH SIM mutants, and ATPase mutant can determine the role of PICH in Topo II checkpoint activation. Lastly, it is interesting to analyze cells that bypass the Topo II checkpoint upon Aurora B kinase inhibition for chromosome missegregation defects such as aneuploidy.

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