Mitotic SUMOylation: from the mechanism to functions

BY

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Dedicated to my mentor Dr. Azuma Y and my parents

ABSTRACT

Protein modification by conjugation of SUMO molecules to target proteins is an essential process for both genomic stability and cell viability. In vertebrates, the SUMOylation process involves three SUMO paralogues, SUMO 1, 2, and 3. During cell division, certain chromosome-associated protein are modified by SUMO2/3; however, the regulatory mechanisms that monitor and control the SUMOylation process are poorly defined.

Previous studies have revealed that DNA Topoisomerase IIα (TopoIIα) is a mitotic target of SUMO2/3, and that defects in TopoIIα SUMOylation are linked to chromosomal missegregation, suggesting a relationship between TopoIIα SUMOylation and the regulation of mitotic progression. Using an *in vitro* SUMOylation and decatenation assay with recombinant TopoIIα protein, we demonstrated that SUMO conjugation inhibits the intrinsic activity of TopoIIα. By mass spectrometry and biochemical analysis, we identified Lys 660 of TopoIIα as a SUMOylation site in both *Xenopus* egg extracts (XEE) and *in vitro* assays. Lys 660 is located within the catalytic domain of TopoIIα and elimination of Lys 660 SUMOylation by mutation abolished the SUMOylation-mediated inhibition of TopoIIα activity that is normally observed in wildtype (WT), suggesting that Lys 660 SUMOylation is responsible for regulating TopoIIα activity. In addition, biochemical analysis has shown that SUMO conjugation of Lys 660 requires the

presence of DNA, suggesting that catalytically active TopoIIα is a target of SUMO conjugation during mitosis.

Subsequent investigations of mitotic SUMOylation, lead to the isolation of SUMOylated forms of another protein from mitotic chromosomes. Using mass spectrometry, we identified this protein, poly (ADP-ribose) polymerase 1 (PARP1) as another SUMO2/3 target during mitosis. Similar to the conjugation pattern seen with TopoIIα, SUMO conjugation of PARP1 first appears during prometaphase, shows the strongest intensity during metaphase, and disappears with the onset of anaphase. Interruption of SUMOylation increased PARP1-dependant PARylation, implicating SUMO2/3 conjugation in the regulation of mitotic PARylation.

We also investigated the role of PIASy, a SUMO E3 enzyme of the SIZ/PIAS family that is essential for the completion of SUMO2/3 modification during mitosis. Analysis of PIAS family proteins has shown that only PIASy is able to bind to chromosomes during mitosis. Domain analysis has suggested that the N-terminus of PIASy is responsible for chromosome binding. In our studies, we analyzed the functional importance of the N-terminus of PIASy using recombinant PIAS N-terminal truncations. We demonstrated that the most N-terminal region of PIASy is not involved in either substrate binding or in SUMO modification; however, it is required for governing chromosome interaction. Furthermore, an ~130 amino acid polypeptide located at the N-terminus of PIASy was capable of accumulating at the centromere, the site where most mitotic SUMO2/3 conjugation takes place. Mass spectrometry following pull-down

assays have shown that Rod/Zw10, a critical component of spindle checkpoints, specifically interacts with the N-terminus of PIASy, but not with other PIAS family members. We demonstrated that elimination of Rod proteins by immunodepletion in XEE causes mislocalization of PIASy on mitotic chromosomes followed by abnormal SUMOylation.

In summary, we have investigated how SUMOylation is regulated in a spatial and temporal manner by the SUMO E3 ligase, PIASy. We have also analyzed the function of SUMO conjugation during mitosis using two SUMO target proteins, TopoIIα and PARP1. Our *in vitro* reconstitution assay has enabled us to closely examine the nature of mitotic SUMOylation and has demonstrated potential roles for SUMOylation during mitosis. Together, these results contribute to the understanding of mitotic SUMO conjugations and raise specific questions for future studies.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Yoshiaki Azuma. I have been 100% financially supported by him since I joined his lab in May, 2007, and will be until my graduation in December, 2010. Dr. Azuma has encouraged me to overcome difficult moments and stood by me whenever I needed help. All the time spent in discussion with my mentor was so precious, and I learned how to hypothesize, interpret data, and build the next part of the story of my projects. He gave me wonderful projects that I couldn't have imagined before. Then he helped me to be the owner of my projects and taught me to enjoy them. Before this, I believed that I liked science. But, that was just my imagination and admiration of science as an amateur. Thanks to my mentor, I had the chance to realize what science is, what I wanted to do, what I am doing, and what I want to do in the future. I am glad to officially show how much I appreciate him. I am honored to dedicate my doctoral degree to my mentor.

Together with my mentor, Dr. Mizuki Azuma has been my supporter. She has taken care of my health, and has been a good friend. Because of them, I passed day by day with happiness and smiles.

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like to say to my parents, “I am happy, and I don’t want you to be worried anymore. I thank you so much for supporting me no matter what.”

I also would like to thank the committee members for helping me to become a better scientist. In particular, I thank Dr. Robert Cohen and Dr. Scott Hefty who gave me valuable comments on my dissertation. I thank Lisa Embree so much for careful proofreading and helpful comments on my dissertation.

Lastly, I want to mention three more sentences. I love my projects that have followed me with belief. I personally thank my projects that have made me so delighted and thrilled. I will miss you and already miss you.

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LIST OF ABBREVIATIONS

APC/C	Anaphase promoting complex/Cyclosome
CSF	Cytostatic factor
ICR	Inner centromeric region
ID	Immuno-depletion
IP	Immuno-precipitation
K-fiber	Kinetochore fiber
NPC	Nuclear pore complex
PARP1	Poly (ADP-ribose) polymerase 1
PARylation	Poly (ADP-ribosyl)ation
Pc2	Polycomb group
PIAS	Protein inhibitor of activated STAT
RanBP2	RanGTPase binding protein 2
RanGAP1	RanGTPase activating protein 1
Rod	Rough deal
SAP	Scaffold attachment factor A/B/acinus/PIAS
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIM	SUMO interacting motif
SMC	Structural maintenance of chromosomes
SUMO	Small ubiquitin-related modifier
SUMOylation	SUMO modification of proteins

TopoIIα	DNA Topoisomerase IIα
WT	Wild type
XEE	Xenopus egg extracts
Zw10	Zeste whigt 10

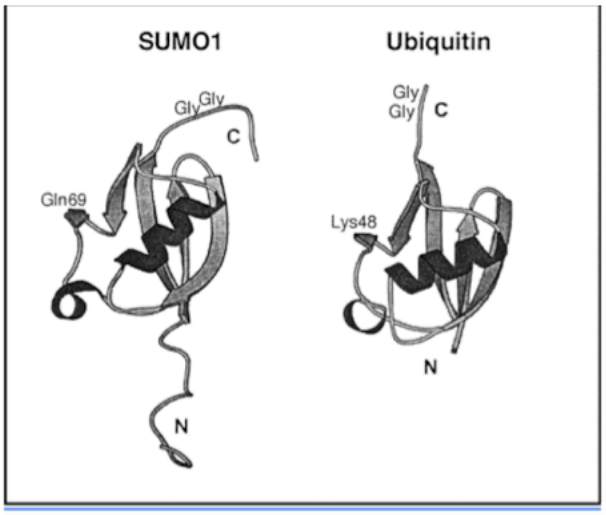
CHAPTER 1

INTRODUCTION: SUMOylation, MITOSIS, The ROLES of SUMOylation, and SUMO E3 LIGASE

Part I. SUMOylation

SUMOylation is a post-translational process in which a SUMO (**s**mall **u**biquitin-related **mo**difier) protein (~100 amino acids) is conjugated to targets (1) (Figure 1.1). SUMOylation is highly conserved in eukaryotes (1). Lower eukaryotes, including yeast, worm, and fruit fly, utilize a single SUMO (SUMO1) (2,3). Higher eukaryotes have developed more sophisticated mechanisms, alternatively utilizing different SUMO paralogues depending on environmental states within the cell (2,4). In vertebrates, there are three SUMO parologues: SUMO1, 2, and 3 (Table 1.1).

A



B

*** Sequence alignment ***

```
            Identities = 10/44 (23%), Positives = 24/44 (55%)

Ubiquitin   KEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG
            ++G+P +  R +F G+++ D  T  +  +++E  + +    GG
SUMO1       RQGVPMNSLRFLFEGQRIADNNTPKELGMEEEDVIEVYQEQTGG
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Figure 1. 1

Structural comparison of SUMO1 and Ubiquitin. A and B. SUMO1 shows high structural similarity to ubiquitin even though SUMO1 and ubiquitin share only ~20% sequence identity (Adapted from Melchior, 2000 (1)).

SUMO1 is ~50% identical in sequence to both SUMO2 and SUMO3 while SUMO2 and SUMO3 are ~95% identical in sequence (Table 1.1). Because of the inability to distinguish SUMO2 and SUMO3 by antibodies, SUMO2 and SUMO3 are collectively grouped as SUMO2/3.

	Smt3p (S. cerevisiae)	SUMO1	SUMO2
SUMO1 (human; Q93068)	52%	--	--
SUMO2 (Human; NP_008868)	45%	47%	--
SUMO3 (Human; NP_008867)	51%	51%	95%

Table 1. 1

Sequence comparison of SUMO paralogues. A single SUMO (Smt3p) in S. cerevisiae shows roughly 50% similarity to each SUMO in vertebrates. Vertebrate SUMO1 displays ~50% identity to both SUMO2 and SUMO3, while SUMO2 and SUMO3 are highly similar in sequence and cannot be distinguished by antibodies *in vivo*.

SUMO is covalently attached to its target substrates through an enzymatic conjugation cascade similar to the biochemical process of ubiquitination (5). However, the SUMOylation process utilizes unique components: SUMO E1 activating enzyme, E2 conjugating enzyme Ubc9, and several E3 ligating enzymes (Figure 1.2) (5). SUMO E1 is composed of Aos1 and Uba2 enzymes to form an Aos1/Uba2 heterodimer, and initiates SUMO attachment by activating the formation of a thioester bond between the mature form of SUMO and E1 Uba2. Subsequently, SUMO E2, Ubc9, takes the activated SUMO and transfers it to the ε-amino group of the substrate with the aid of an E3 ligase. The region of the target substrate that is directly bound by Ubc9 is called the "SUMOylation concensus sequence" and is composed of the amino acid sequence ΨKXE, where Ψ is a hydrophobic branched amino acid, K is the lysine residue that will be SUMOylated, X is any amino acid, and E is glutamic acid (1,6,7).

Although there are reports that Ubc9 recognizes its target proteins without the aid of SUMO E3, accumulating data suggests that E3 ligases are necessary for recognition of targets *in vivo* (7-10). Evidence of this includes the yeast strain (ΔSiz) that is lacking a major E3 ligase and has lost most of its capacity for SUMOylation (11,12). Also, immunodepletion of the E3 ligase PIASy, completely eliminates SUMOylation during mitosis in *Xenopus* egg extracts (XEE) (8). Lastly, SUMOylation is reversed by SUMO specific proteases (Fig 1.2) (13,14). There are six SUMO specific proteases in vertebrates called SENP (**Sen**trin specific **p**roteases) 1, 2, 3, 5, 6, and 7 (14). Accumulating reports have shown that distinct subcellular localization of these SUMO proteases allows the deconjugation of specific SUMOylated target proteins (13).

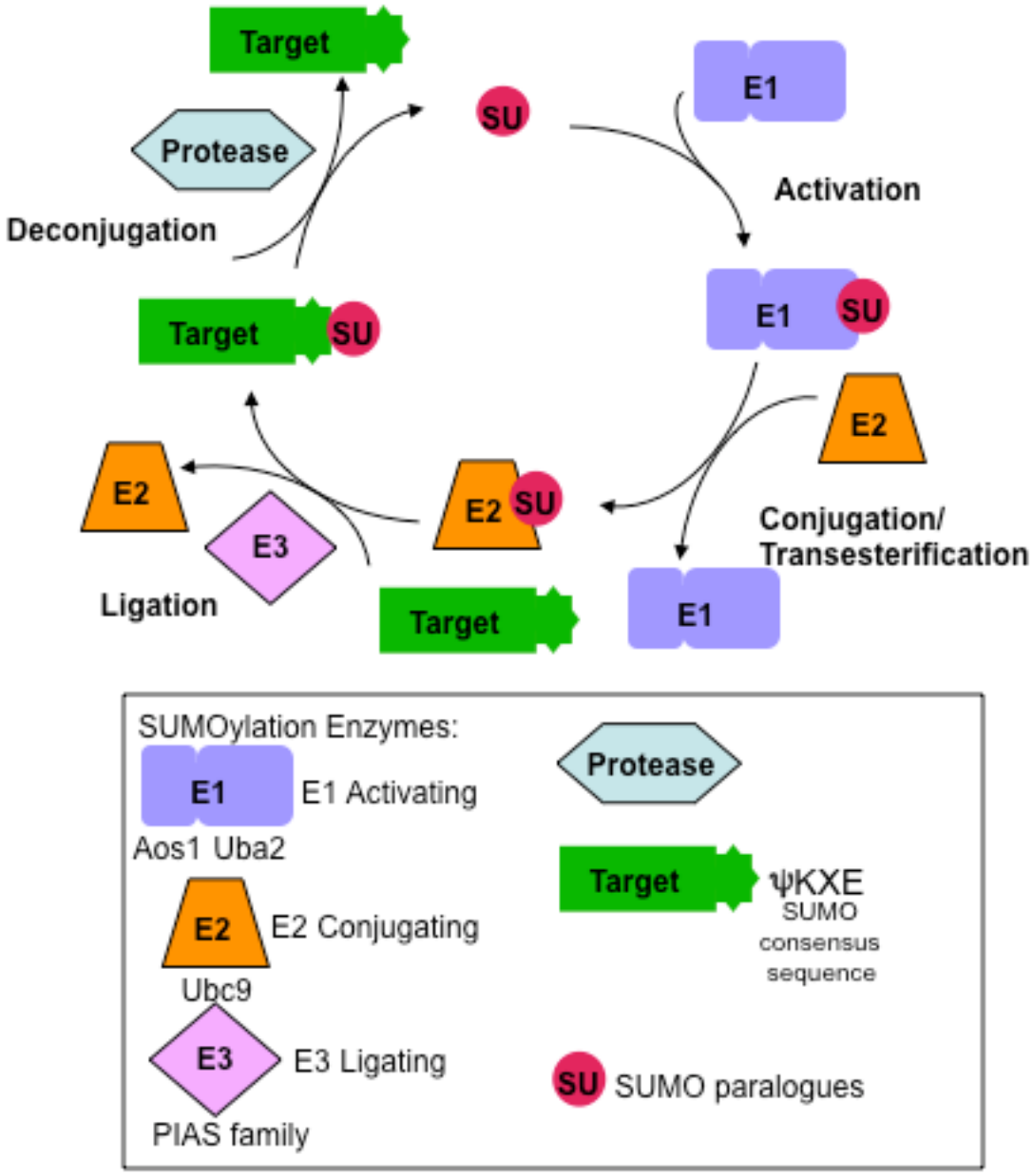

Figure 1. 2

The SUMO conjugation pathway. Using unique E1, E2, and E3 enzymes, SUMO is conjugated to various targets *in vivo* and *in vitro*, in order to modify target function. SUMO conjugation is highly dynamic, and the process is reversible by the action of SUMO proteases that cleave SUMO from the target proteins. Su stands for SUMO paralogues, E1 for E1 activating enzyme, E2 for conjugating enzyme, E3 for ligase protein. (Adapted from Hay, 2005 (2)).

Part II. Mitosis and chromosome segregation

The eukaryotic cell undergoes two biochemically discrete phases in order to proliferate: interphase and mitosis. Interphase is the period in which the cell prepares for division of its genomic material. During this phase, the cell expresses proteins required for chromsome condensation and cell division, replicates its DNA, and subsequently inspects for DNA damage before entry into mitosis.

Mitosis is a highly transient and dynamic stage that is subdivided into five phases: prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, chromatin starts to condense and two centrosomes are positioned on opposite sides of the nucleus. The cell also begins to lose its nuclear envelope during prophase. In prometaphase, growing spindles search for chromosomes and arrange these chromosomes near the metaphase plate. At metaphase, all the chromosomes are aligned at the metaphase plate, located between the two spindle poles. After both the spindle assembly checkpoint and the tension checkpoint are completed, sister chromatids move towards opposite spindle poles, and are physically divided into two sets of chromosomes during anaphase. In telophase, the nuclear envelope reforms, followed by completion of cytokinesis (Figure 1.3) (15).

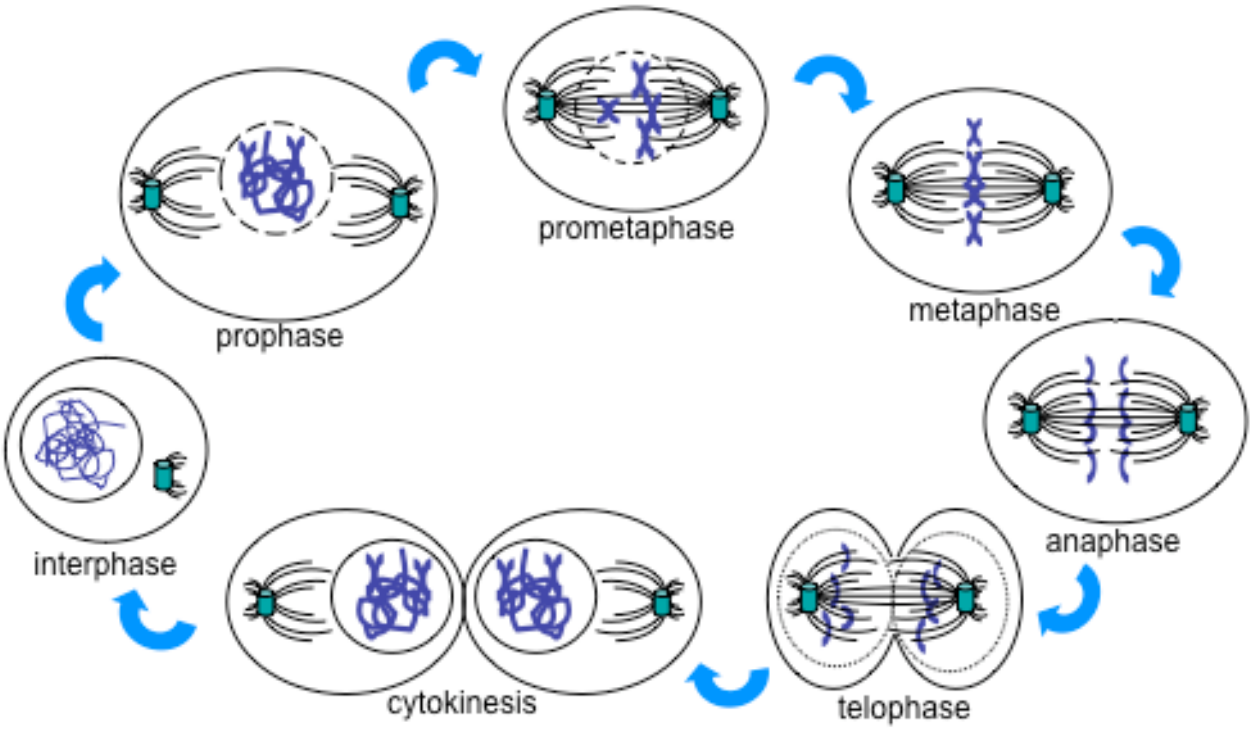

Figure 1.3

Schematic diagram of the cell division cycle. Cells go through a series of events, each of which is characterized by specific features. Mitosis is the process whereby the cell attempts to divide into two daughter cells, each of which inherits the same quantity of genomic material. Mitosis is comprised of five phases: prophase, prometaphase, metaphase, anaphase, and telophase. (Adapted from Salaün et al., 2008 (15)).

In order to pass on genomic material with high fidelity, there is a specialized region of the cell called the kinetochore that is dedicated to chromosome segregation (16). The kinetochore is composed of multiple protein complexes built on the centromere (Figure 1.4) (16). All of the kinetochore proteins identified to date are required for either K-fiber formation (microtubule-kinetochore interaction), mitotic checkpoints, or chromosome segregation (16-19).

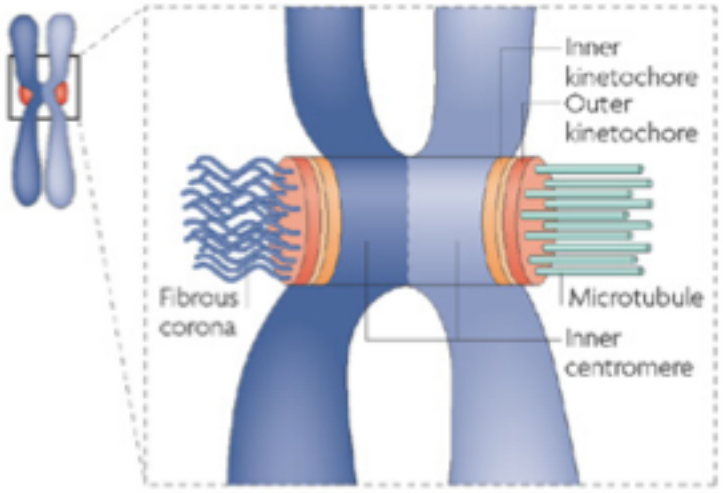

Figure 1. 4

Schematic of a kinetochore on the chromosome. The kinetochore is composed of three distinct structures: the inner kinetochore, outer kinetochore, and fibrous corona. (Adapted from Cheezeman and Desai, 2008 (20)).

Rough deal and Zeste white 10

Rod (**Ro**ugh **d**eal) and Zw10 (**Z**este **w**hite 10) are outer kinetochore proteins (21). Together with Zwilch protein, they form the RZZ complex (21). Rod and Zw10 are responsible for the recruitment of the mitotic checkpoint proteins, Mad1 and Mad2, which sequester Cdc20 (21,22). Cdc20 is an activator of the anaphase promoting complex (APC/C) that is involved in the ubiquitination and proteasome-associated degradation of Securin and CyclinB (23,24). Therefore, Rod and Zw10 are required for the activation of mitotic checkpoint, and mutations in Rod and Zw10 cause premature sister chromatid segregation in the cell (25,26).

DNA topoisomerase IIα and poly(ADP-ribose) polymerase I

DNA topoisomerase IIα (TopoIIα), is a crucial enzyme which, together with the kinetochore proteins, is required for proper chromosome segregation during mitosis (27,28). TopoIIα supports the rod-shaped chromosome structure, and therefore is known as a scaffold protein (Figure 1.5) (29). Numerous studies have implicated the catalytic activity of TopoIIα in chromosome condensation and segregation (27,30,31).

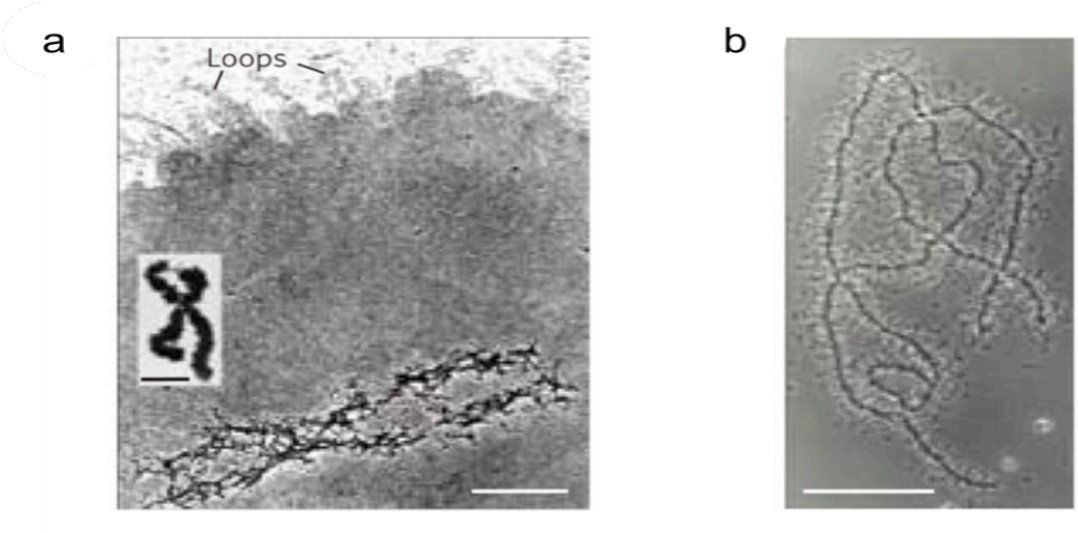

Figure 1. 5

Micrographic images of chromosome scaffold in metaphase. A. A metaphase chromosome consists of ~1/3 DNA, ~1/3 histone proteins, and ~1/3 non-histone proteins. After harsh extraction such as treatment with high salt concentrations, non-histone proteins remain on the metaphase chromosome and maintain the shape of the chromosome. TopoIIα and SMC (**S**tructural **m**aintenance of **c**hromosomes) family proteins provide the skeletal framework for the sister chromatids. The inset shows an intact chromosome before harsh extraction. B. Light microscopic image of a single meiotic chromosome from an oocyte of Triburus viridescens. (Adapted from Bloom and Joglekar, 2010 (29)).

TopoIIα resolves DNA tangles by cleaving one DNA duplex, passing a second duplex through the cleaved first duplex, and religating the break, in an

ATP-dependent manner (Figure 1.6) (32). The catalytic function of TopoIIα is tightly regulated although the mechanism for regulation has not been identified. TopoIIα activity is not observed in the fractions located on the chromosome arms; it is only observed at the centromeric region of chromosomes during mitosis (33). The molecular mechanism and role of TopoIIα at the centromere during mitosis are poorly understood.

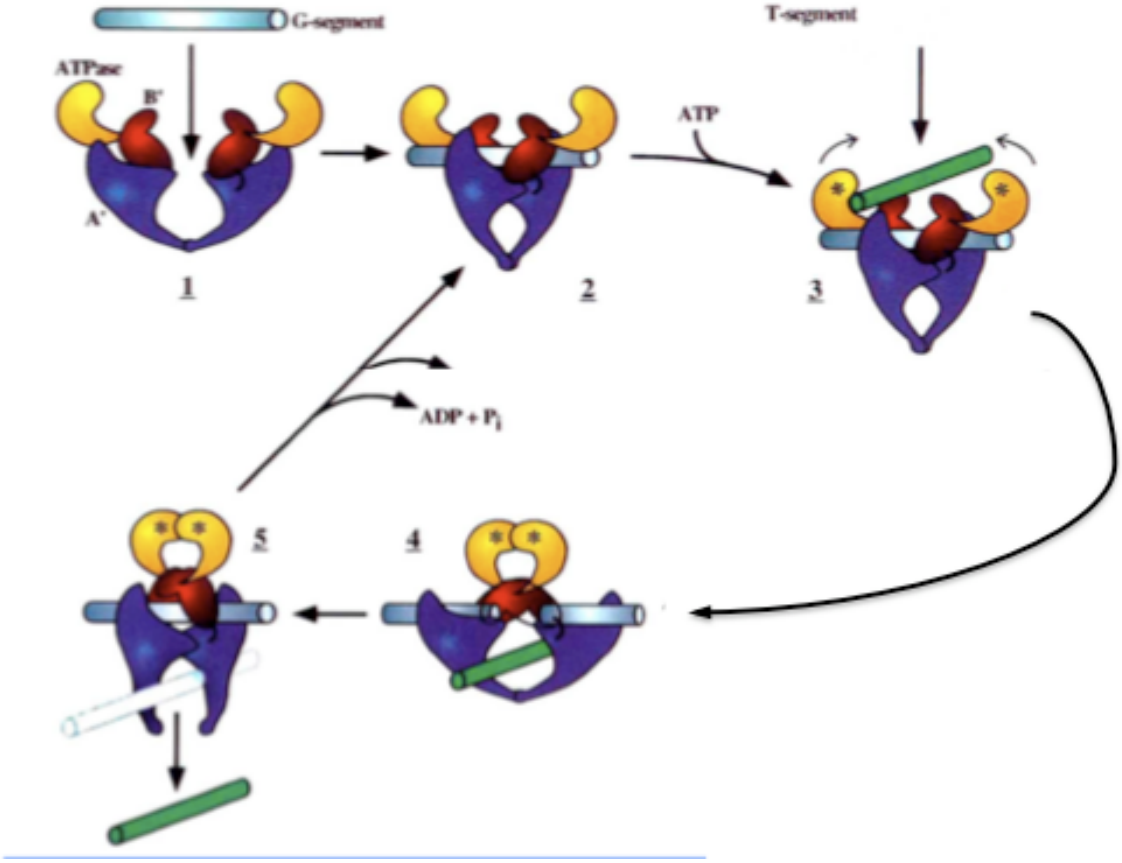

Figure 1.6

Schematic diagram of the catalytic reactions of DNA Topoisomerase IIα. The DNA gate segment is labeled G-segment; transported DNA segment as T-segment. TopoIIα binds the G-segment and changes its structure (1 and 2). ATP is placed in the ATPase domain (in yellow) followed by binding of the T-segment (3). Further conformational changes in TopoIIα allow the cleavage of the G-segment and transport of the T-segment (3 and 4). The G-segment is resealed and the T-segment is released as a final step (5). (Adapted from Berger et al., 1996 (32))

Poly(ADP-ribose) polymerase I (PARP1) is a nuclear enzyme activated by DNA strand breaks (34). Once activated, PARP1 attaches ADP-ribose moieties onto certain glutamate and lysine residues of target proteins (35). The process of PARP1-mediated polymerization using ADP-ribose onto target proteins is called PARylation. Studies of PARP1 have implicated PARP1 in DNA damage repair systems (35,36). It is likely that the presence of large, negatively charged ADP-ribose polymers on substrate targets recruits proteins involved in DNA damage repair, such as XRCC1 (37). XRCC1 is an adaptor protein that activates the DNA base excision repair machinery (37). In addition, it was recently shown that PARP1-mediated PARylation regulates the activity of Aurora B kinase, which is responsible for the initiation of chromosome condensation, as well as segregation, at the onset of anaphase (38). However, the detailed mechanism of this regulation remains poorly understood.

Part III. The functions of SUMOylation

SUMOylation is an essential biological event that is implicated in diverse cellular processes. SUMO modifications have been shown to affect the transcription, localization, interaction, and regulation of activity of a variety of proteins, including those involved in chromosome morphology, and cell cycle progression (39-42).

SUMO modifications *in vivo* are either constitutive or cell cycle-specific. SUMO1 conjugations tend to be more stable than SUMO2/3 conjugations, and SUMO1 targets are immediately conjugated to SUMO1 once the targets are

expressed (43). An example of this is the SUMOylation of RanGTPase activating protein 1 (RanGAP1). RanGAP1 is constituitively conjugated to SUMO1, and the conjugated form of RanGAP1 is then able to interact with the nuclear pore complex (NPC) (43). The localization of RanGAP1 on the cytoplasmic side of the NPC is known to be involved in regulation of nuclear cytoplasmic-trafficking (44).

Cell cycle-specific SUMOylation can be further divided into interphase- and mitosis-specific modifications. Accumulating reports have shown that, during interphase, several transcription factors are targeted for SUMO conjugation, resulting in repression of transcription (42). An example of this involves AR- and Smad4-mediated transcription (45,46). Daxx, a transcription cofactor, binds to the SUMOylated forms of AR and Smad4, and this interaction recruits histone deacetylase (HDAC) which represses AR- and Smad4-mediated transcription (26,45-48). It has been demonstrated that mutagenized substrates (AR or Smad4) that have been rendered non-SUMOylatable are no longer inhibited by Daxx (47,48).

The importance of mitosis-specific SUMOylation is evidenced by the mitotic defects manifested in SUMOylation-deficient organisms (41). The SUMO knockout strain (Δsmt3) in budding yeast exhibits early mitotic arrest, and the SUMO-deficient fission yeast strain (Δpmt3) exhibits aberrant chromosome structure and missegregation (49,50). Mice lacking Ubc9 are embryonic lethal and exhibit severe mitotic defects (40).

Recently, proteomic screening in yeast has found over 300 potential targets for SUMOylation, the majority of which are centromere-resident proteins

(Figure 1.7) (51). Examples of these include condensin- and cohesin-complex proteins, such as SMC (**S**tructural **m**aintenance of **c**hromosomes) 1, 3, and 4 (Figure 1.7) (51). Pds5 is an inner-centromeric protein that mediates cohesin interaction with chromosomes in a SUMOylation-dependant manner (52). As described in CHAPTER 3, TopoIIα activity is also regulated in a SUMO2/3 modification-dependent manner. A detailed mechanism for SUMOylation-mediated regulation of mitotic progression has been poorly resolved owing to the complexity of mitosis. However, the evidence suggests that successful chromosome segregation and proper mitotic progression are dependent on a myriad of complex events involving SUMOylation (41).

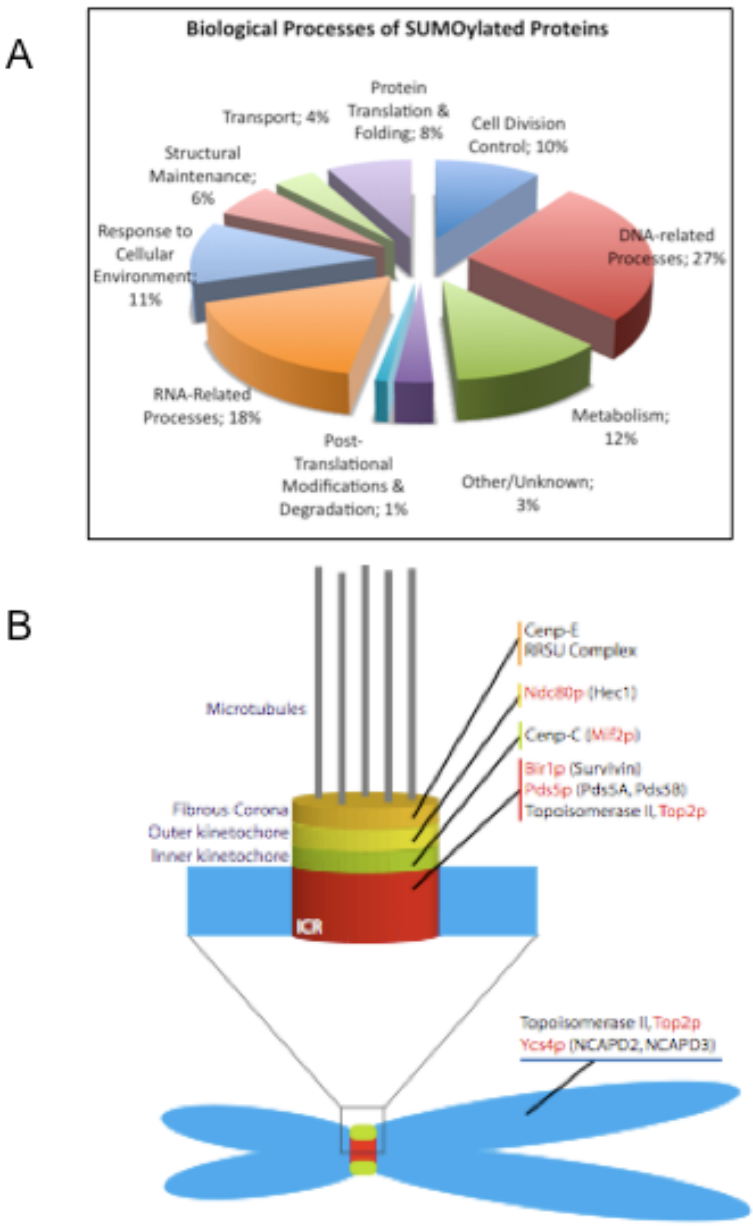

Figure 1. 7

The distribution of SUMO targets on mitotic chromosomes. A. Biological processes of SUMO-modified proteins. B. The approximate localization of selected SUMOylation substrates are schematically displayed. Only target proteins verified by proteomic analysis are represented in this figure. Black indicates vertebrate proteins, and red indicates homologues in yeast. ICR: inner centromere region. (Adapted from Denison et al., 2005 and M. Dasso, 2008 (41,51)).

Part IV. PIASy, a SUMO E3 ligase of the SIZ/PIAS family

There are two types of SUMO E3 ligases: RING-domain dependent ligases, (the SIZ/PIAS family), and RING-domain independent ligases, (Pc2 and RanBP2, (also known as Nup358) enzymes) (11,53,54). RanBP2 and Pc2 are specialized enzymes that have been identified in vertebrates and whose counterparts have not yet been found in yeast (54). The structure of these enzymes is not the typical HECT- or RING-type ligase structure (55).

The RING-domain dependent E3 ligases are evolutionarily conserved from yeast to vertebrate (SIZ in yeast and PIAS in vertebrate) (56). The vertebrate PIAS (**p**rotein **i**nhibitor of **a**ctivated **S**TAT) family consists of PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy (Figure 1.8) (56,57). Initially discovered during the process of identifying proteins that inhibit transcription of STAT proteins, several PIAS members are also well-known transcription repressors (57). As shown in Figure 1.8, the PIAS family of E3 ligases share several motifs in common: SAP (**s**caffold **a**ttachment factor A/B/acinus/**P**IAS), PINIT, SP-RING (SIZ/PIAS RING), and SIM (**S**UMO **i**nteracting **m**otif) (57). The SAP domain binds AT-rich regions of DNA (58). PINIT is a conserved amino acid sequence involved in substrate interaction and localization (59). SP-RING is a RING-like catalytic domain that interacts with the SUMO E2 conjugating enzyme, Ubc9 (8). The SIM region interacts with SUMO paralogues (60). Domain analyses have indicated that all domains of PIAS are necessary for proper SUMO conjugation *in vivo (8)*.

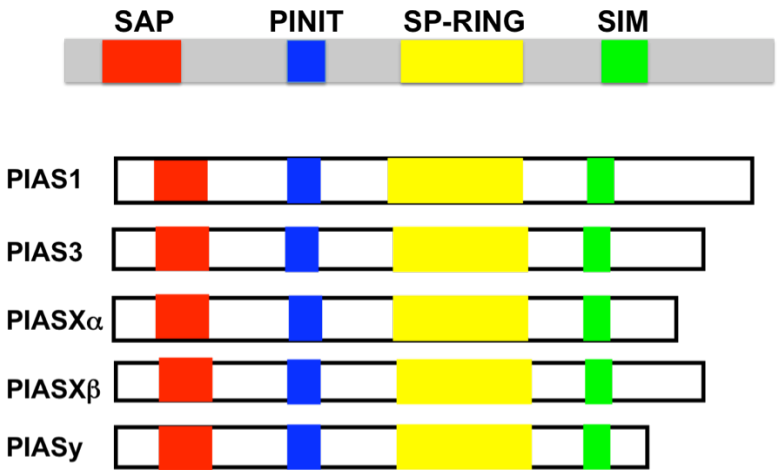

Figure 1. 8

The primary structure of the PIAS family of SUMO E3 ligases. The vertebrate PIAS family consists of PIAS1, 3, Xα, Xβ, and PIASy. PIAS proteins contain a SAP domain at the N terminus, followed by PINIT, SP-RING, SIM, and finally S/DE domains at the C-terminus. Domain analyses have identified functions for each domain in PIAS proteins, as described in the text.

Although PIAS family members display extensive sequence similarity with one another, the PIAS proteins have distinctive binding partners (57). The SAP domains of PIAS proteins share more than 95% sequence identity among family members, however, several investigations have demonstrated that the SAP domain is actively involved in binding to unique transcription factors and regulators: PIAS1 binds to p73, PIAS3 binds to p300/CBP, and PIAS4 binds to p53; all of these interactions involve the SAP domain (57,61,62). The mechanism of specific partner recognition in PIAS family members *in vivo* remains to be elucidated.

Part V. *Xenopus* egg extract (XEE) cell free system

Xenopus egg extract (XEE) is a powerful system to study cell cycle specific features (27,63). This is because chromosomes are arrested at

metaphase due to a cytostatic factor (CSF) that prevents anaphase entry (Fig. 1.9). By addition of calcium cloride solution, the metaphase-arrested extract is induced to interphase. SUMO modification of certain target proteins such as TopoIIα and PARP1 occurs in a mitosis-specific manner (8,10). Therefore, by using XEE cell free system, we can examine mitotic SUMOylation while constitutive or interphase-specific SUMO modifications are not affected.

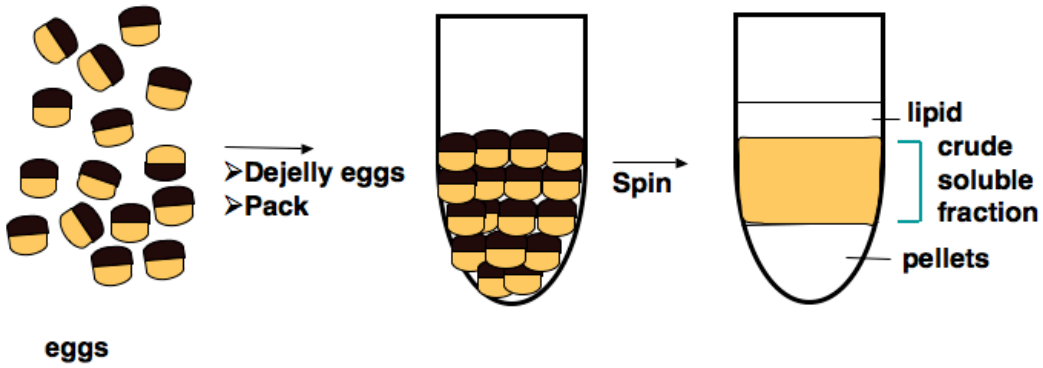

Figure 1.9

Preparation of *Xenopus* egg extracts (XEE). Eggs are collected from *Xenopus laevis* which were induced using pregnant mare's serum gonadotropin and human chorionic gonadotropin. Collected eggs are dejellyed and subjected to centrifugation in order to obtain the crude soluble fraction. This fraction contains the cytostatic factor that allows chromosomes at metaphase.

Part V. Summary

Genetic analysis has shown that cell cycle-specific SUMO conjugation is a vital process in the cell, demonstrated by the numerous errors in mitotic progression manifested in SUMOylation-deficient organisms (41). The aim of this study was to generate a detailed description of the mechanism of SUMOylation during mitosis using both *in vitro* reconstitution assays and *in vivo* XEE cell-free systems. We demonstrated that PIASy, a SUMO E3 ligase, functions through N-terminal interaction with the centromeric region of chromosomes (Chapter 4). We also determined that the Rod/Zw10 complex is

necessary for the proper localization of PIASy and for SUMO modification of centromeric proteins (Chapter 4). Furthermore, we have shown that SUMO conjugation negatively modulates the intrinsic activity of TopoIIα *in vitro*, implicating a role for TopoIIα SUMOylation in chromosome segregation (Chapter 3). We found that Lys 660 is a SUMOylation site of TopoIIα and may be completely responsible for the SUMOylation-dependent regulation of TopoIIα activity (Chapter 3). We also determined that PARP1 is a target for SUMO2/3 conjugation during mitosis and is SUMOylated in a PIASy-dependent manner (Chapter 2). Finally, we have shown that SUMOylation affects PARP1-dependant PARylation, demonstrating the multiple roles for SUMOylation during mitotic progession (Chapter 2).

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CHAPTER 2

PIASy mediates SUMO-2/3 conjugation of poly (ADP-ribose) polymerase1 (PARP1) on mitotic chromosomes in vertebrates.

Abstract

PIASy is a SUMO ligase that modifies chromosomal proteins in mitotic *Xenopus* egg extracts, and plays an essential role in mitotic chromosome segregation. We have isolated a novel SUMO-2/3 modified mitotic chromosomal protein, and identified it as poly (ADP-ribose) polymerase 1 (PARP1). PARP1 was robustly conjugated to SUMO-2/3 on mitotic chromosomes, but not on interphase chromatin. PIASy promotes SUMOylation of PARP1 both in egg extracts and *in vitro* reconstituted SUMOylation assays. Through tandem mass spectrometry analysis of mitotically SUMOylated PARP1, we identified a residue within the BRCA1 C-terminal (BRCT) domain of PARP1 (lysine 482) as its primary SUMOylation site. Mutation of this residue significantly reduced PARP1 SUMOylation in egg extracts, and enhanced the accumulation of species derived from modification of secondary lysine residues in assays using purified components. SUMOylation of PARP1 did not alter in vitro PARP1 enzyme activity, poly-ADP-ribosylation (PARylation), nor did inhibition of SUMOylation of PARP1 alter the accumulation of PARP1 on mitotic chromosomes, suggesting that SUMOylation regulates neither the intrinsic activity of PARP1 nor its localization. However, loss of SUMOylation increased PARP1-dependent PARylation on isolated chromo-

somes, indicating SUMOylation controls the capacity of PARP1 to modify other chromatin-associated proteins.

Introduction

Small Ubiquitin-related Modifiers (SUMOs) are proteins found in all eukaryotes that become covalently conjugated to other cellular proteins in a manner similar to ubiquitination (5,64). This modification (SUMOylation) regulates many aspects of interphase nuclear function and structure (64-66), as well as events during mitosis (41). Consistent with the important and broad roles of the SUMOylation pathway, hundreds of SUMOylation targets have been identified in proteomic screens (51,67-69). Three major SUMO family proteins, called SUMO-1, SUMO-2 and SUMO-3, are expressed in vertebrates. SUMO-2 and SUMO-3 are closely related in their primary sequence (95% identical), whereas SUMO-1 is around 45% identical to either of the other two paralogues. In contexts where SUMO-2 cannot be functionally distinguished from SUMO-3, we will refer to these paralogues collectively as SUMO-2/3. All vertebrate paralogues are around 50% identical to the single SUMO protein found in budding yeast, Smt3p (5). SUMO conjugation to cellular substrates is carried out in a manner similar to Ubiquitin. The conjugation process requires three sequential enzymes: an activating (E1) enzyme, a conjugating (E2) enzyme and usually a SUMO ligase (E3 enzyme). All SUMOylation utilizes the same E1 and E2 enzymes, which are called Uba2/Aos1 and Ubc9, respectively. However, there are a number of differ-

ent E3 enzymes that show a high degree of specificity for particular conjugation targets (64).

All eukaryotes possess E3 enyzmes with variant RING-finger like domains (SP-RINGs) (64). These proteins are called Siz (SAP and miz-finger domain) proteins in yeast and PIAS (protein inhibitor of activated STAT) proteins in vertebrates. The five vertebrate PIAS proteins (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy) are involved in a wide variety of processes, including signal transduction, gene expression and genome maintenance (70). We have previously shown that PIASy-mediated conjugation of SUMO-2/3 to mitotic chromosomal proteins is essential for proper chromosomal segregation at anaphase in *Xenopus* egg extract (XEE) cell free assays (8). One of the major targets for PIASy-directed SUMOylation is DNA topoisomerase IIα (TopoIIα) (71). PIASy deficient mice are viable (72), suggesting PIASy-catalyzed SUMOylation is not essential during anaphase in mice. However, siRNA-mediated PIASy knock down causes mitotic arrest with aberrant chromosome cohesion in HeLa cells (73). Moreover, the yeast Siz1p and Siz2p proteins are essential for accurate segregation of chromosomes in yeast (28). It thus appears that PIASy-mediated SUMOylation plays an essential role in regulating mitotic chromosomal structure and progression of mitosis in many cellular contexts.

Poly-ADP-ribosylation (PARylation) of proteins is another major post-translational modification of many nuclear proteins, catalyzed by Poly-ADP-ribosyl Polymerases (PARPs) (34,74,75). All PARPs share a conserved catalytic domain that interacts with NAD^+, which is the donor molecule of poly(ADP-

ribose) (76). The importance of PARylation during mitosis has been demonstrated: Inhibition of PARylation compromises spindle formation in XEEs (77). PARylation mediated by the enzyme Tankyrase-1 has been particularly implicated the correct assembly of spindle poles (78). Another PARylation enzyme, PARP1, has been shown to interact with centromeres and could mediate PARylation of centromeric proteins (79,80). PARP1 interacts with the mitotic chromosomal protein kinase, Aurora B, and could mediate PARylation of Aurora B to regulate its activity (38). Although the mitotic function of PARP1 has not been clearly demonstrated, the increasing evidence that PARP1 plays a role in chromatin structure suggests the potential relevance of PARP1 activity to chromosomal structure and function in mitosis (75,81).

We identified PARP1 as a SUMO-2/3 modified substrate associated with mitotic chromosomes. PARP1 was robustly conjugated to SUMO-2/3 on mitotic chromatin, but not on interphase chromatin. PIASy promoted PARP1 SUMOylation both in XEEs and *in vitro* SUMOylation assays using purified, recombinant proteins. Through tandem mass spectrometry analysis of mitotically SUMOylated PARP1, we identified a residue within the BRCA1 C-terminal (BRCT) domain of PARP1 (lysine 482) as its primary SUMOylation site. Mutation of this residue significantly reduced PARP1 SUMOylation in XEEs, and enhanced the accumulation of species derived from modification of secondary lysine residues in assays using purified components. SUMOylation of PARP1 did not alter PARP1 auto-PARylation activity *in vitro*, nor did inhibition of SUMOylation of PARP1 alter the accumulation of PARP1 on mitotic chromosomes in XEEs, suggesting that SU-

MOylation regulates neither the intrinsic activity of PARP1 nor its localization. However, loss of SUMOylation increased PARP1-dependent PARylation on isolated chromosomes, indicating SUMOylation controls the capacity of PARP1 to modify other chromatin-associated proteins.

Methods

Recombinant protein expression, Cloning, site directed mutagenesis, and antibodies.

cDNAs of *Xenopus* PARP1 were cloned from *Xenopus* tadpole cDNA (kindly provided by T. Amano and Y.B. Shi, NICHD) using PCR amplification with addition of the BglII recognition sequence at the 5'end and SalI recognition sequence at the 3'end. Amplified cDNA was subcloned into pEGFP-C1 plasmids using BglII and SalI sites and was verified by DNA sequencing. The full-length cDNAs were subcloned into pET28 using BamH1 and XhoI restriction sites.
cDNA fragments encoding *Xenopus* PAPR1 (amino acids 1-150 ; $PARP1^{xl}$-N and 500-650; $PARP1^{xl}$-M) were amplified from a pEGFP-PARP1construct and were subcloned into pGEX4T-1(GE-Healthcare) and pET28a (Novagen) using BamHI and XhoI restriction sites. The recombinant proteins encoded by these cDNAs were expressed in *E. coli* and purified by tag-based purification according to the manufacture's protocol, followed by conventional ion-exchange chromatography. The Lysine to Argenine substitution of PARP1 was produced by site-directed mutagenesis (Quick change, Stratagene) according to the manufacture's protocol.

Polyclonal antibodies against PARP1 and TopoIIα were generated in rabbits by injection with recombinant fragments of His6-T7-PAPR1^{xl}-N, His6-T7-PAPR1^{xl}-M, and His6-T7-TopoIIα^{xl} C-terminal (amino acids 1359-1579) respectively. The rabbit antisera were subjected to affinity purification on NHS-Sepharose (GE-Healthcare) columns with their covalently bound antigens. The anti-SUMO-2/3 polyclonal Guinea Pig antibody was raised and purified similar to previous polyclonal rabbit antibody (71). The anti-PAR monoclonal antibody and anti-T7 tag antibody conjugated with HRP were purchased from Trevigen and EMD bioscience, respectively. Unless otherwise specified, other reagents were obtained from Sigma-Aldrich Chemical Company.

Protein purification and in vitro SUMOylation assay.

The recombinant E1 complex (Aos1/Uba2 heterodimer) was purified by co-expression His6-Aos1 and Uba2 (kindly provided by Dr. F. Melchior) in *E. coli* as described previously (54). The bacterially expressed E1 complex was purified with Talon affinity resin (Clontech) purification followed by SUMO affinity and conventional chromatography as previously described (82). Purification of recombinant SUMO-2, wild type Ubc9, dnUbc9 and PIASy were as previously described (8,71).

Recombinant PARP1wt and PARP1K482R proteins were expressed in BL21(DE3) at 15 °C in the presence of 5% Glycerol, 2.5% Ethanol and 0.1mM $ZnCl_2$. The expressed proteins were purified from the soluble fraction of the bacterial lysate using Talon affinity resin (Clonetech), followed by Sephacyryl-300 gel

filtration and SP-Sepharose column chromatography (GE Healthcare). Purified PARP1 proteins were concentrated up to 2~3 mg/ml by centrifugal concentrator (Amicon Ultra-4, 30kDa, Millipore) then snap frozen by liquid N2 in small aliquots.

In vitro SUMOylation assay was performed with 15nM E1, 5mM SUMO-2, 2.5mM ATP, and 500nM PARP1 in reaction buffer (20mM Tris (pH7.5), 50mM NaCl, 10mM $MgCl_2$, 0.05% Tween 20 and 0.5mM DTT) together with the indicated Ubc9 (E2) and PIASy (E3) concentrations. Reactions were incubated at 25 C° for the indicated periods.

Xenopus egg extracts.

Sperm chromatin and low-speed extracts of *Xenopus* eggs arrested in metaphase by CSF were prepared as described (63). Where required, CSF extracts were driven into interphase by the addition of 0.6 mM $CaCl_2$. For mitotic chromosome assembly or interphase nuclear assembly, demembranated sperm chromatin were added to CSF or interphase extracts, and incubated for 40~60 minutes at room temperature. Chromatin isolation was performed as previously described (71). Unless otherwise specified, all reactions contained 5,000 sperm nuclei per µl.

Immunodepletion and immunofluorescence.

The immunodepletion experiment with XEE was performed as previously described (8) with an affinity purified anti-PARP1 antibody. For add-back experiments, recombinant PARP1 proteins were added at an endogenous concentration at 4 µg/ml.

The immunofluorescence analysis in Figure 5C was demonstrated as previously described (8). In brief, 100 µl of CSF extracts were released into interphase by 0.6 mM $CaCl_2$ addition. 200 sperm/µl were added 5 minutes after the $CaCl_2$, and the extracts were incubated at 23˚C for 55 minutes to complete DNA replication. Re-entry into mitosis was induced by the addition 50 µl of fresh CSF extract. In the case of inhibition of mitotic SUMOylation, dnUbc9 (150 ng/µl final concentration) was also added at this point. The reactions were incubated for 40 minutes after the addition of fresh CSF extract to reach metaphase. Then 50 µl were removed from each reaction, diluted with 200 µl of buffer A (0.8x CSF-XB buffer, containing 10 mM β-Glycerolphosphate and 250 mM sucrose), and incubated for 5 min at 23˚C. 300 µl of buffer A containing 4% paraformaldehyde was added to this sample and incubated for an additional 10 minutes. Samples were spun onto cover slips through a 35% glycerol cushion. Chromosomal samples on cover slips were post-fixed by 1.6% paraformaldehyde and processed for immunostaining with anti-PARP1 (rabbit polyclonal), -SUMO-2/3 (guinea pig polyclonal), and -CENP-A (chicken polyclonal). Anti-rabbit Alexa 568, anti-guinea pig Alexa 684 and anti-chicken Alexa 488 were used as secondary antibodies respectively. Specimens were observed by Nikon TE2000-U microscope with Plan Apo 100x/1.40 objective. Images were taken with Retiga SRV CCD camera (Qimageing) operated by Volocity software (Improvision).

PARylation assay

To measure PARylation activity of *in vitro* SUMOylated PARP1, reactions of *in vitro* SUMOylation were incubated for 60 minutes and then the reactions were diluted twice with the reaction buffer (same as *in vitro* SUMOylation reaction buffer) containing 2mM NAD^+. The reactions were incubated further at 25°C for indicated periods. Termination of reaction was done by addition of SDS-PAGE sample buffer to the reaction mixture. For analysis of PARylation of chromosomal proteins, the mitotic chromosomes, assembled in XEE reaction, were suspended into 30 ul of the reaction buffer containing 1mM NAD^+ and incubated at room temperature for 10 minutes. The reactions were centrifuged and chromosomal pellets were boiled in SDS-PAGE sample buffer. PARylation of proteins in SDS-PAGE samples were determined by immunoblotting with anti-PAR monoclonal or polyclonal antibodies (Trevigen).

Results

PIASy-mediates SUMO-2/3 conjugation of PARP1 on mitotic chromosomes.

PIASy-mediated SUMO-2/3 modification of chromosomal proteins is essential for faithful anaphase chromosome segregation in the *Xenopus* egg extract (XEE) *in vitro* system (8). There are a number of readily detectable SUMOylated proteins on mitotic chromosomes in XEEs whose modification requires PIASy, including Topoisomerase-IIα (TopoIIα) (8). To identify the full spectrum of mitotic SUMOylation substrates for PIASy in XEEs, we added Hexa-Histidine (His_6)-tagged SUMO-2 protein to XEEs arrested in meiotic M phase through the action of cytostatic factor (CSF XEEs) in the presence of demembranated sperm chro-

matin. We purified a chromosomal fraction from these reactions and isolated the SUMOylated proteins by Co^{2+} affinity chromatography under denaturing conditions (Figure 2.2), in a manner analogous to earlier studies (71). We observed a prominent SUMOylated species, with a molecular weight around 140kDa, and subjected it to LS-MS/MS analysis for protein identification at the Harvard Microchemistry & Proteomics Facility (Cambridge, MA). The results indicated that this species was SUMO-2-conjugated poly (ADP-ribose) polymerase1 (PARP1). To confirm this identification, we cloned *Xenopus laevis* PARP1, and prepared antibodies against fragments of the PARP1 protein (Figure 2.1D). Immunoblotting analysis of mitotic chromosomes prepared from CSF XEEs clearly showed more slowly migrating forms of PARP1 protein, which were abolished when a dominant negative form of Ubc9 (dnUbc9 (8)) was added to the reactions, or increased in abundance upon addition of exogenous PIASy (Figure 2.1A).

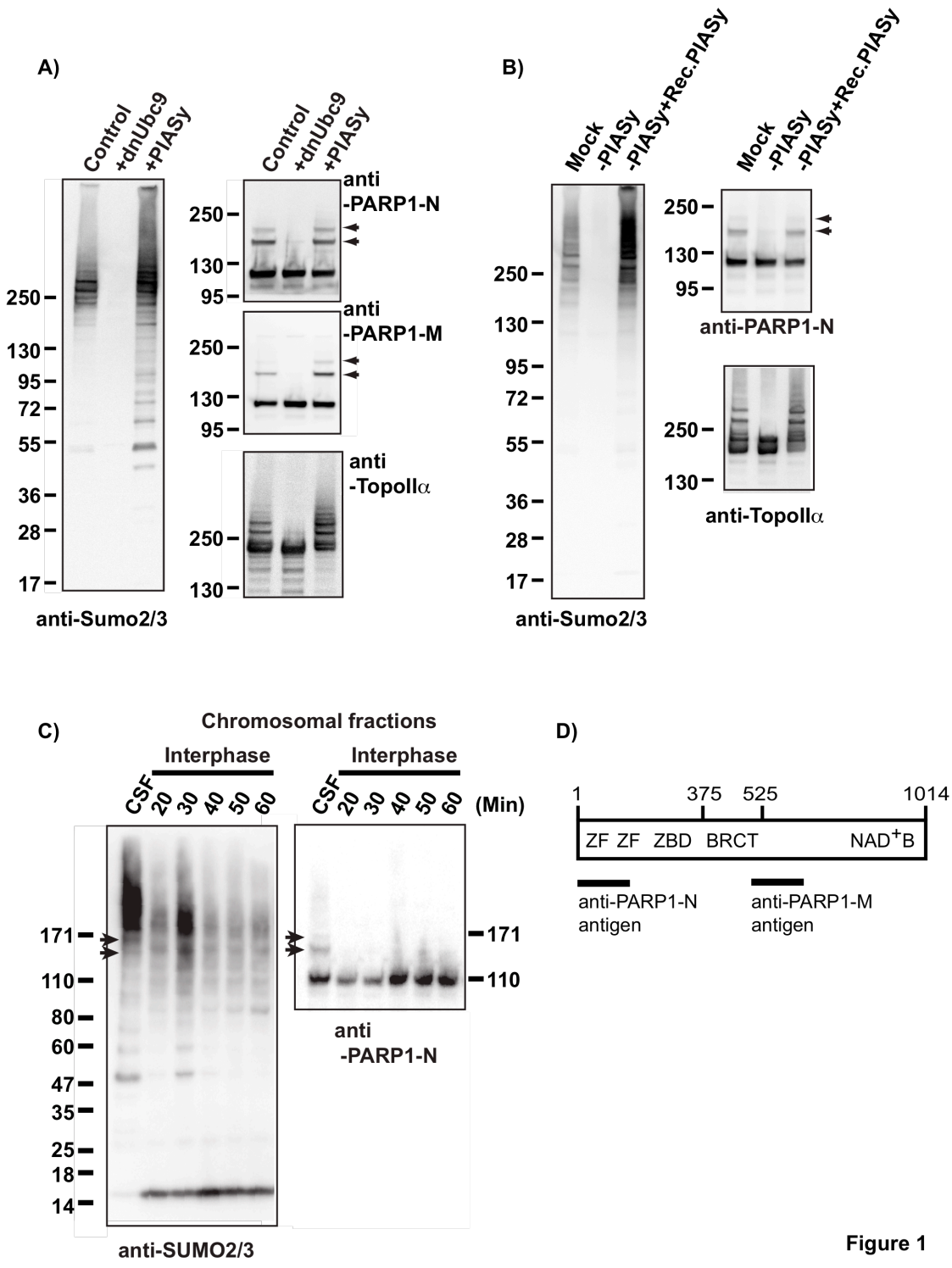

Figure 1

Figure 2. 1
PARP1 is a PIASy-mediated SUMOylated protein on mitotic chromosomes.
A) PARP1 is SUMOylated on mitotic chromosomes. Chromosomal fractions were prepared in CSF extracts in the presence of either dnUbc9 or PIASy as described in Materials and Methods. Isolated chromosomes were analyzed by im-

munoblotting with indicated antibodies. Arrows indicate the position of SUMOylated PARP1, which were diminished when SUMOylation was inhibited by dnUbc9. **B) PIASy is required for PARP1 SUMOylation.** CSF extracts were subjected to immunodepletion with anti-PIASy antibody (-PIASy), and then a purified recombinant PIASy was supplemented to PIASy-depleted extracts (-PIASy+Rec.PIASy). Chromosomal fractions were prepared in the indicated conditions of CSF extracts, then the SUMOylation status of isolated chromosomes was analyzed with the indicated antibodies. Arrows indicate the position of SUMOylated PARP1. **C) PARP1 SUMOylation is specific in mitosis.** CSF extracts were released into interphase by addition of $CaCl_2$. Sperm nuclei were added to either CSF or interphase extracts. After incubation of the indicated periods with interphase extracts or 40 minutes with CSF extracts, the chromatin fractions were isolated as described in Materials and Methods. Isolated chromatin fractions were subjected to immunoblotting with the indicated antibodies. Arrows indicate SUMOylated PARP1. **D) Representation of functional domains of PARP1** (adjusted from (75)). Lines indicate the fragments of PARP1 that were used as antigens.

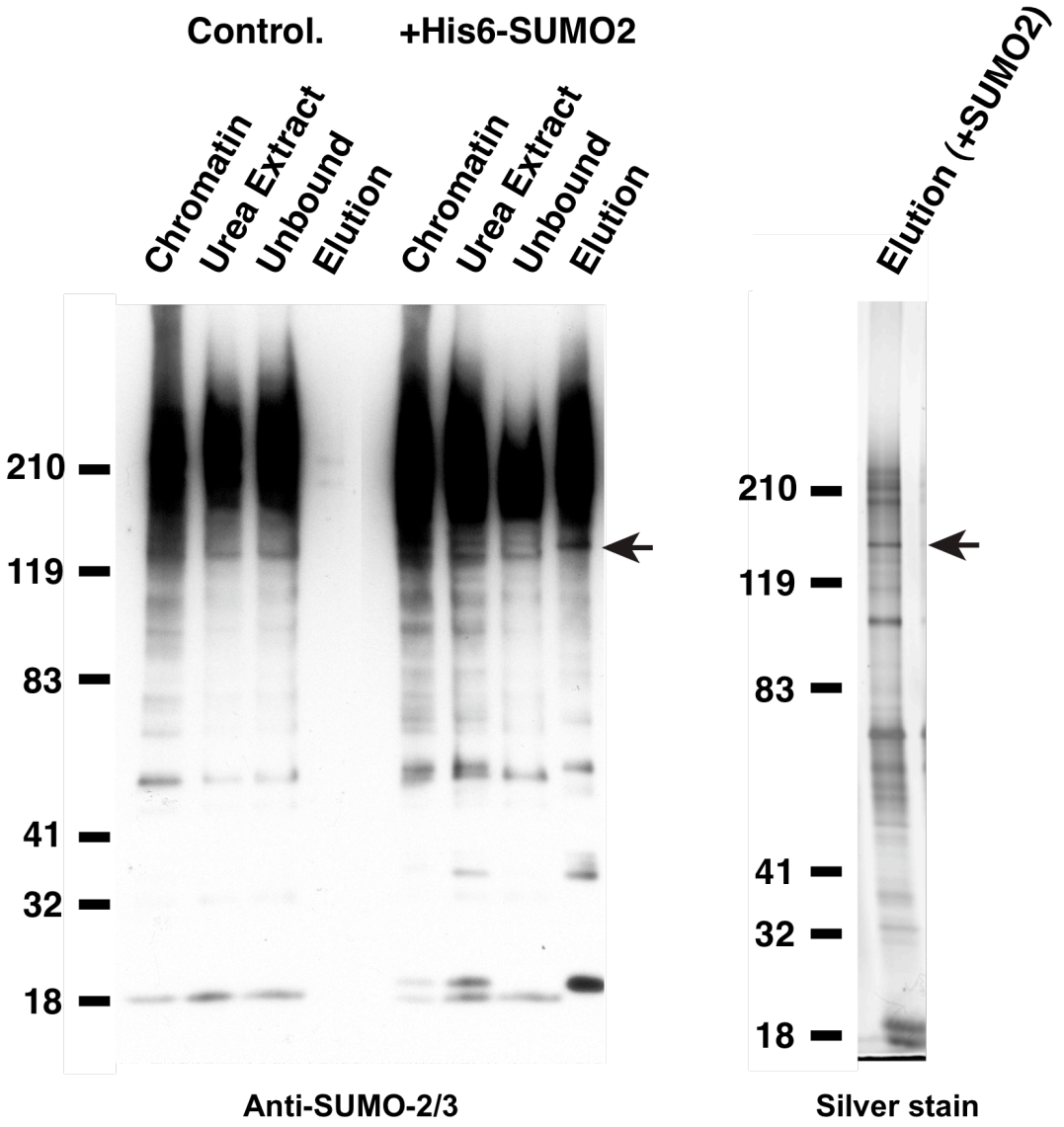

Figure 2. 2
Purification of novel SUMO-2 modified proteins from mitotic chromosomes. Mitotic chromosomes were prepared with the CSF extract with or without His6-SUMO-2. After sixty minutes of incubation, the mitotic chromosomes were separated by centrifugation (Chromatin). Chromosomal proteins were extracted in a buffer containing 8.5M urea (Urea Extract), and the recovered fractions were subjected to further purification using metal affinity chromatography. Aliquots from unbound fractions and eluted fractions were analyzed by immunoblotting with an anti-SUMO-2/3 antibody together with Chromatin and Urea extract fractions (Left panel). Proteins in the elution from the His6-SUMO-2 containing sample were analyzed by silver staining (Right panel). Arrows indicate the isolated His6-SUMO-2 modified protein, which was subjected to identification by LS-MS/MS analysis.

To test directly whether the SUMOylation of PARP1 requires PIASy, we depleted PIASy from CSF XEEs (Figure 2.1B); PIASy depletion eliminated the slowly migrating SUMOylated forms of PARP1. These forms of PARP1 were restored upon the addition of a recombinant PIASy to the depleted CSF XEEs, confirming that they arise in a PIASy-dependent manner. To test whether SUMOylation of PARP1 was sensitive to the cell cycle status of the XEE, we compared chromatin fractions isolated from CSF XEEs to chromatin fractions from interphase XEEs (Figure 2.1C). Immunoblotting analysis with anti-PARP1 and anti-SUMO-2/3 antibodies showed robust SUMOylation in the CSF XEEs (indicated with arrows), as expected, but no SUMOylated PARP1 could be detected in the interphase chromatin fractions. Together, these results show that PARP1 is a target of mitotic, PIASy-mediated SUMO-2/3 conjugation that is associated with mitotic chromosomes in XEEs. Notably, this pattern was closely similar to the SUMOylation of TopoIIα, an established PIASy substrate in this system (71).

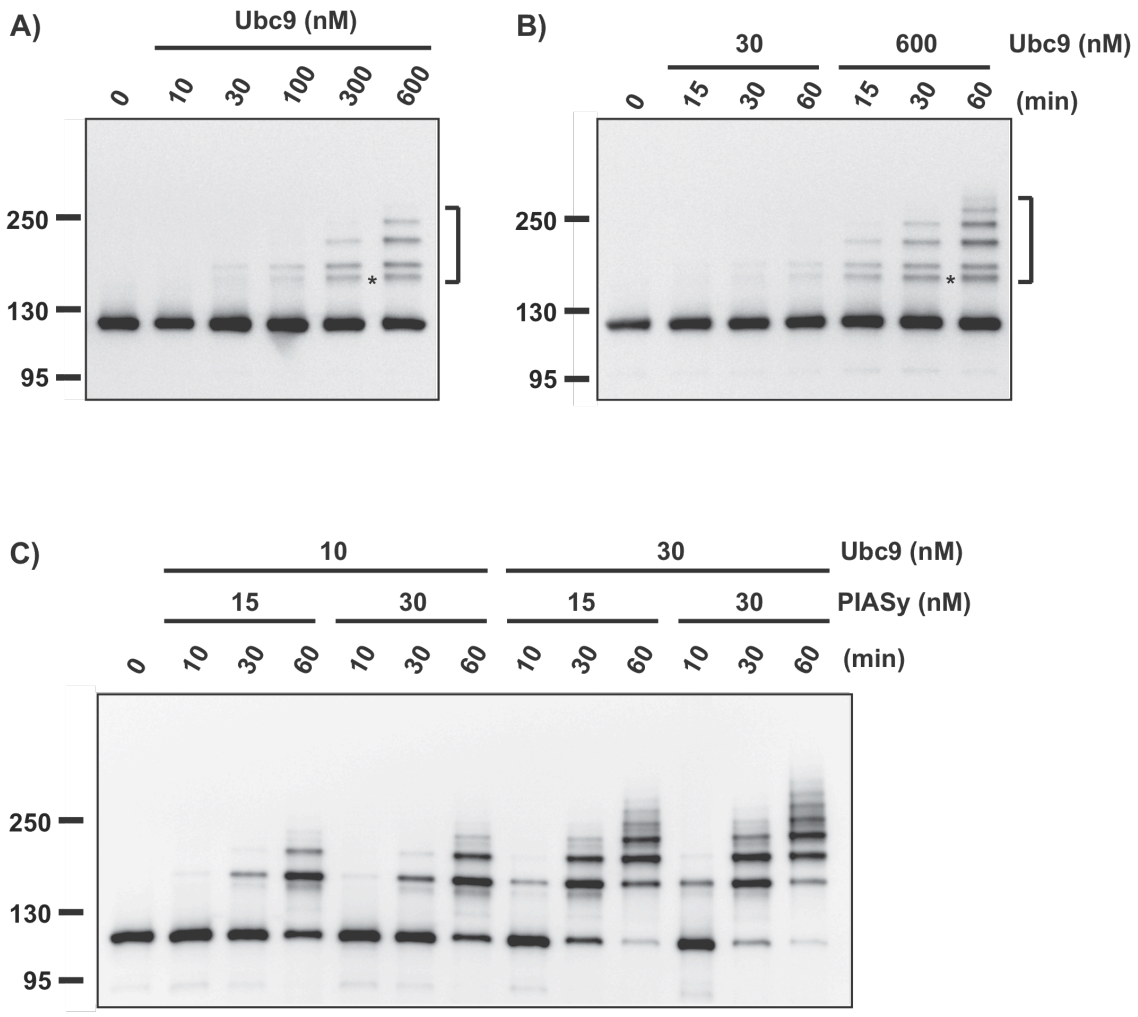

Figure 2. 3
PIASy stimulate PARP1 modification by SUMO-2/3 in reconstituted *in vitro* SUMOylation reaction.
All recombinant proteins, E1, E2, E3, PARP1 and SUMO, were expressed in *E. coli* and purified as described in Materials and Methods. *In vitro* SUMOylation reaction was performed with 15nM E1, 500nM PARP1 and 6μM of SUMO at 25°C. Reactions were terminated at the indicated times by the addition of SDS-PAGE sample buffer. SUMOylation of PARP1 was analyzed by immunoblotting with HRP-conjugated anti-T7 antibody (Novagen). The bracket indicates the position of SUMOylated PARP1. **A) Ubc9 dose dependency of PARP1 SUMOylation without E3. B) Kinetics of Ubc9 dependent PIASy SUMOylation.** The SUMOylation reaction was performed with the indicated concentration of Ubc9 without PIASy. The reaction mixtures were incubated for 60 minutes in A) and for the indicated time periods in B). **C) PIASy dependent SUMOylation of PARP1.** The reaction mixtures containing the indicated concentration of Ubc9 and PIASy were incubated for the indicated times.

***In vitro* reconstitution of PARP1 SUMOylation by PIASy.**

To examine PARP1 SUMOylation by PIASy further, we reconstituted this modification in an *in vitro* system using entirely purified components, which were expressed in bacteria and prepared as described in the Experimental Procedures section. We found that PARP1 could be modified by SUMO-2/3 in the absence of PIASy in reactions containing a very high concentration of Ubc9 (600 nM) (Figure 2.3A indicated with brackets). These concentrations significantly exceeded the physiological concentration of Ubc9 in XEEs, which was determined to be around 30 nM through quantitative immunoblotting of XEE in comparison to purified re-combinant Ubc9 (data not shown). In reactions containing 30 nM Ubc9, SUMOy-lated PARP1 was hardly detected (Figure 2.3B), even with prolonged incubation. Notably, strong SUMOylation of PARP1 was observed when PIASy was added to reactions containing near-physiological concentrations of Ubc9 (Figure 2.3C). Modified forms of PARP1 were evident in reactions containing 10 nM Ubc9 and 15 nM PIASy. Such SUMOylation was enhanced in reactions containing 30 nM PIASy. Increasing the concentration of Ubc9 to 30 nM provided a robust boost in PARP1 SUMOylation at both concentrations of Ubc9.

Our results collectively showed that PIASy stimulates SUMOylation of PARP1 at physiological concentrations of Ubc9. A subtler but equally important finding from these *in vitro* reactions was that the pattern of SUMOylated PARP1 bands differed between the reactions with and without PIASy. Without PIASy, we observed a SUMOylated form of PARP1 with an aberrant migration on SDS-PAGE that did not correspond to the bands observed in CSF XEEs (indicated

with asterisk in Figure 2.3A and B). This form was dramatically decreased in PIASy-dependent reactions (compare Figure 2.3B and C). In other words, the pattern of SUMOylated PARP1 species found in the PIASy-dependent *in vitro* reactions (Figure 2.3C) was much closer to the pattern observed within the chromatin fractions of complete CSF XEEs.

Lysine 482 is a primary SUMOylation site of PARP1.

To determine the function of PARP1 SUMOylation, we mapped its SUMOylation site(s). We isolated SUMOylated PARP1 by immunoprecipititation with anti-PARP1 antibodies under denaturing conditions from a mitotic chromatin fraction made from CSF XEEs (Figure 2.4A) (83). Both SUMOylated and non-SUMOylated PARP1 were subjected to MS/MS analysis for mapping of SUMOylation site(s). The SUMOylation site was identified by mass spectrometry following a double digestion with trypsin and chymotrypsin. Chymotrypsin generated a signature QQQTGG tag on the modified lysine of PARP1. With over 70% of sequence coverage of PARP1, this analysis indicated that Lysine 482 was a candidate SUMOylation site on PARP1. Moreover, alignment of *Xenopus laevis* PARP1 primary sequence to *Homo sapiens* PARP1 (Figure 2.4B) indicated that Lysine 482 is conserved to a recently reported SUMOylation site of human PARP1, which was found by mutational analysis (84).

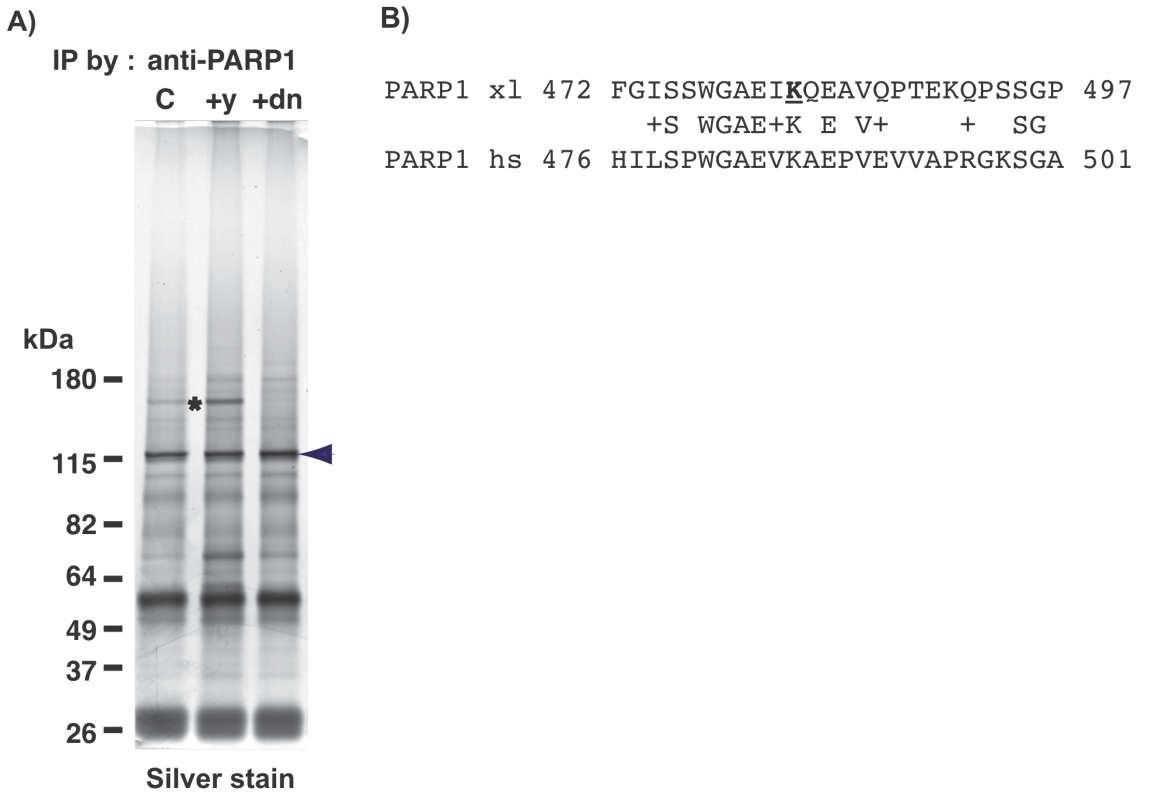

Figure 2. 4
Mapping of SUMOylation site of PARP1 by MS/MS.
Chromosomal proteins were isolated by denaturing buffer containing SDS from mitotic chromosomes that were assembled in the indicated condition of CSF extracts. After renaturation with buffer containing thesit, extracted fractions were subjected to immunoprecipitation with affinity purified anti PARP1 antibody. Precipitated fractions were separated by SDS-PAGE. Both SUMOylated (asterisk) and non-SUMOylated PARP1 (arrow head) were subjected to MS/MS analysis for mapping SUMOylation site. The SUMOylation site was identified by mass spectrometry following a double digestion with trypsin and chymotrypsin. The Chymotrypsin is responsible for generating the QQQTGG signature tag on the modified lysine which was observed for PARP1. MS/MS analysis indicates the Lysine at 482 as a candidate site of SUMOylation with over 70% of sequence coverage of PARP1. B) The diagram shows the comparison of primary sequences of *Xenopus laevis* PARP1 (PARP1 xl) and *Homo sapience* PARP1 (PARP1 hs) around the identified SUMOylation sites. The bold and underlined letter indicates lysine 482, the mapped candidates site of SUMOylation.

To confirm this mapping result, we introduced a mutation at Lysine 482 to encode an Arginine at this position (PARP1-K482R). We compared the SUMOy-

lation patterns of wild type PARP1 (PARP1-wt) and PARP1-K482R within SUMOylation assays using recombinant proteins. In the absence of PIASy, PARP1-wt and PARP1-K482R became SUMO-2/3 conjugated to similar extents, and showed similar dependence upon the concentration of Ubc9 (Figure 2.5A). However, PARP1-K482R showed a much greater accumulation of the aberrant SUMOylation product that was preferentially observed in PIASy-independent reactions (Figure 2.5A, indicated with arrow. Compare to Figure 2.3).

In reactions containing PIASy, there were two important differences between PARP1-K482R and PARP1-wt (Figure 2.5B). First, PARP1-K482R showed a significantly lower efficiency of SUMOylation than PARP1-wt. For instance, more than 50% of PARP1-wt became SUMOylated within 60 minutes at the lowest Ubc9 and PIASy concentrations (10 nM and 15 nM, respectively), whereas the extent of PARP1-K482R SUMOylation was much less under the same conditions. In the presence of 30 nM Ubc9 and 30 nM PIASy, the decreased efficiency of PARP1-K482R SUMOylation was even more obvious; PARP1-wt was almost 100% SUMOylated within 30 minutes, while PARP1-K482R was only about 50% SUMOylated in the same interval. Second, aberrantly migrating SUMOylated species were more abundant in reactions containing PARP1-K482R (Figure 2.5B. Indicated by arrow). As before, these forms were largely suppressed by the presence of PIASy in reactions containing PARP1-wt (Figure 2.5B, Figure 2.3). Collectively, these results indicate that Lysine 482 is a primary SUMOylation site of PARP1 for PIASy-mediated SUMO-2/3 modification *in vitro*. Mutation of this residue is accompanied by the enhanced

modification of other lysines within PARP1, leading to the formation of species with electrophoretic motilities that differ from the primary SUMOylation products observed in intact CSF XEEs.

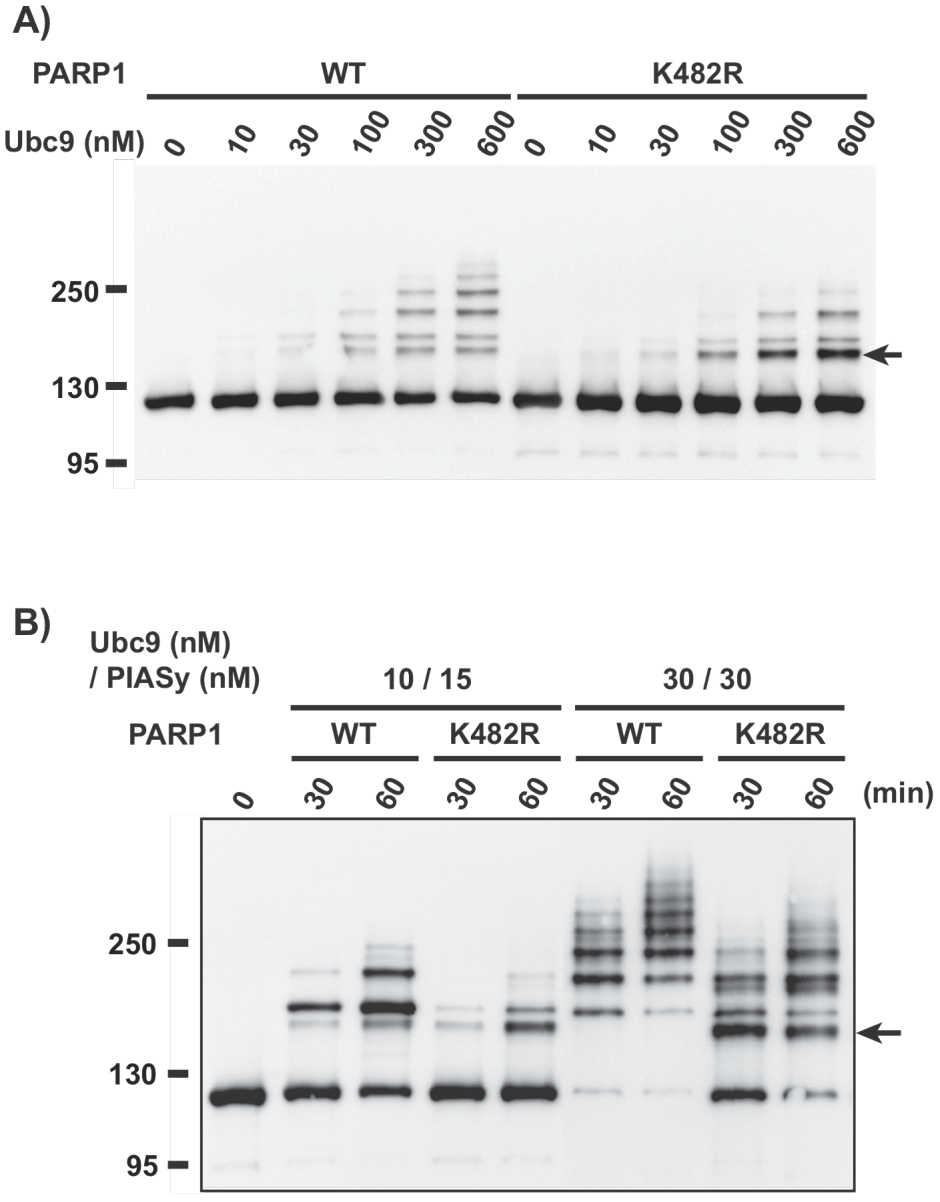

Figure 2. 5

The substitution of Lysine 482 to Arginine causes an alteration in the efficiency and specificity of the SUMOylation of PARP1 in *in vitro* SUMOylation assay. We have introduced a mutation at lysine 482 to arginine (PARP1 K482R) to confirm the mapping result. Purified PARP1 proteins were subjected to *in vitro* SUMOylation assay as in Figure 2.3. **A) Ubc9 dose dependent reaction without PIASy.** The SUMOylation reaction was performed with the indicated concentration of Ubc9 without PIASy. The reaction mixtures were incubated for 60 minutes. **B) PIASy-dependent reaction.** The reaction mixtures containing the indicated concentration of Ubc9 and PIASy were incubated for the indicated times. The arrow indicates the aberrant molecular weight shifted form that is more abundant in K482R mutant than the wild type.

PARP1-K482R shows deficient SUMOylation in XEE assays

We wished to compare the SUMOylation of PARP1-K482R to PARP1-wt under more physiological conditions in XEE CSF extracts. We added recombinant, T7-tagged PARP1-K482R and PARP1-wt to chromatin-containing CSF XEEs that had been immunodepleted of endogenous PARP1 using anti-PARP1 antibodies (-PARP1) (Figure 2.6). The efficiency of depletion was measured by immunoblotting the extracts with an anti-PARP1 antibody (Left panel, indicated as Extracts). More than 99% of PARP1 was eliminated from CSF XEEs without detectable changes in the concentration of another PIASy substrate, TopoIIα. After a 60 minute incubation, mitotic chromosomes were prepared from these reactions and the chromosomal proteins were isolated (Figure 2.6, right panels). As expected, chromosomes prepared from the PARP1-depleted extract did not contain detectable levels of PARP1 (Figure 2.6, -PARP1 lane on right panels). Reactions reconstituted through the addition of recombinant PARP1-wt to depleted CSF XEEs showed levels of SUMOylation similar to endogenous PARP1 modification in non-depleted CSF XEEs (right panels, arrowheads), and this modification was similarly sensitive to dnUbc9. On the other hand, PARP1-K482R was

SUMOylated to a lesser extent, and the resultant species showed greater electrophoretic mobility than SUMOylated forms of the endogenous protein (Figure 2.6, indicated with asterisk), as we had previously observed the assays with fully purified components (compare Figure 2.5 and 2.6). Notably, the formation of these species remained sensitive to dnUbc9. Our findings would be consistent with the utilization of an alternative or secondary SUMOylation site(s) in PARP1 that lacks lysine 482.

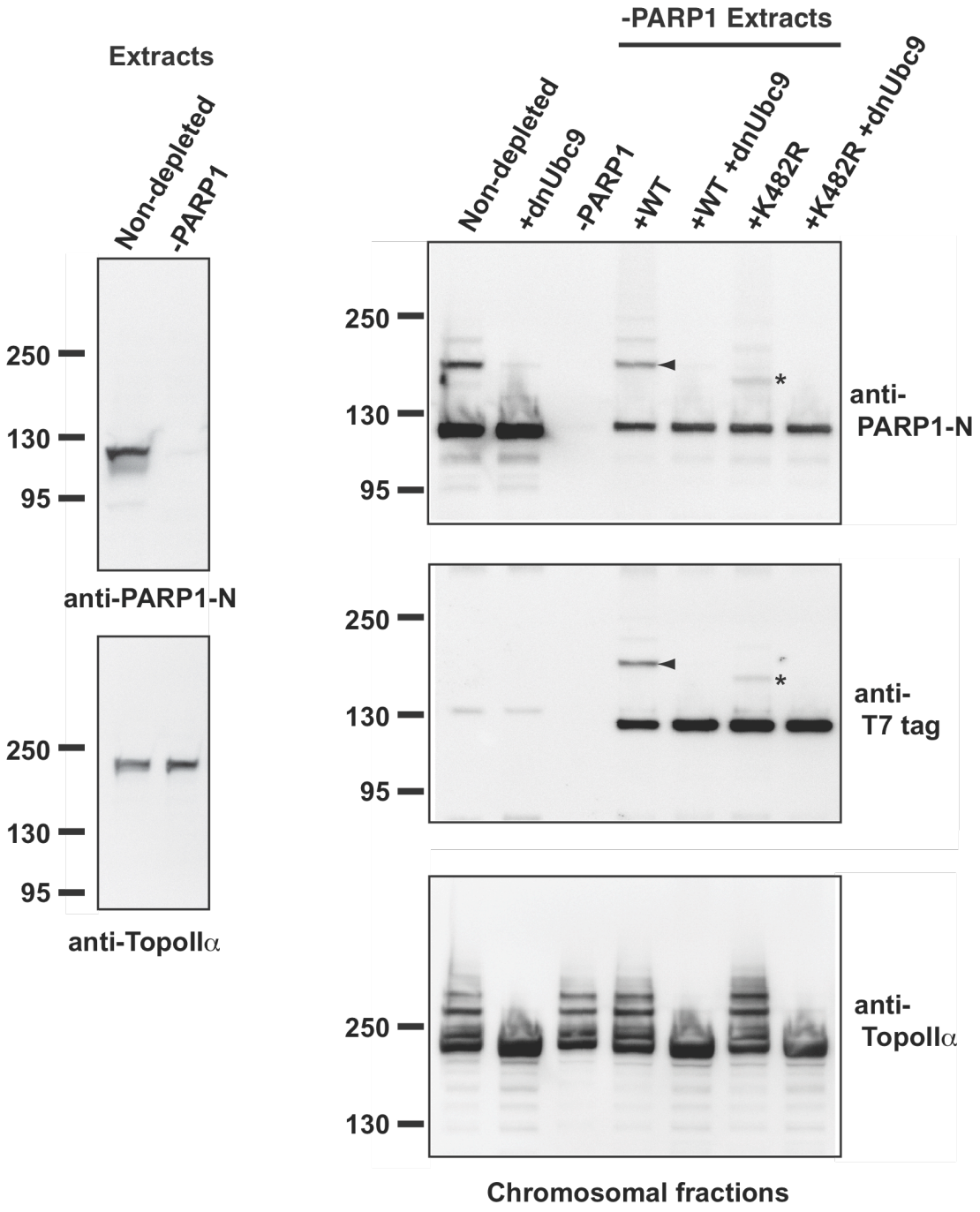

Figure 2. 6
Lysine 482 of PARP1 is a primary acceptor site of SUMO-2/3 on mitotic chromosomes.
CSF extracts were immunodepleted with an anti-PARP1-M antibody. Either wild type (wt) or K482R mutant PARP1 (K482R) was added to the PARP1-depleted extracts. Mitotic chromosomes were assembled with these altered CSF extracts

and the chromosomal fractions were isolated. The chromosomal proteins were analyzed by immunoblotting with the indicated antibodies. Exogenous PARP1 is detected with anti-T7 tag antibody (middle right panel). Arrowhead indicates SUMOylated PARP1 corresponding to the endogenous SUMOylated PARP1. Asterisk indicates a shifted PARP1 that does not correspond to the endogenous SUMOylated PARP1 and was observed only with K482R.

SUMOylation regulates PARP1 activity on chromosomes.

Lysine 482 is located within the BRCT domain of PARP1. This domain is predicted to bind to other proteins, as well as to mediate auto-PARylation. Therefore, we hypothesized that SUMOylation of K482 may either affect auto-PARylation activity by modulating the conformation of the catalytic domain or affect the PARylation of other substrates by modulating PARP1's protein-protein interactions (75,76).

To test the first possibility, we examined whether SUMOylation of PARP1 altered auto-PARylation activity in an *in vitro* reaction. An *in vitro* SUMOylation reaction was performed in the presence of PIASy as in Figure 2.5B, using either unconjugatable SUMO-2 that lacked a C-terminal diglycine motif (SUMO-2-G) or conjugatable SUMO-2 (SUMO-2-GG). As expected, there was no SUMOylated PARP1 detected in the SUMOylation reaction with SUMO-2-G, while the bulk of PARP1 was modified in the reaction with conjugatable SUMO-2-GG (Figure 2.7A, lower panel at t = 0 min) (82). After PARP1 had become SUMOylated, NAD^+ was added to the reaction, and further incubation allowed the formation of poly(ADP-ribosyl) chains on PARP1. Poly(ADP-ribosyl) chain formation was analyzed by immunoblotting with a monoclonal anti-PAR antibody (Figure 2.7A upper

panel). There was a similar amount of PARylated PARP1 in both SUMO-2-G- and SUMO-2-GG- containing reactions. These results indicate that SUMOylated PARP1 retains robust auto-PARylation activity. Our findings suggest that SUMOylation of PARP1 has neither an inhibitory nor an enhancing effect on the auto-PARylation of PARP1.

To test the second possibility, we examined whether inhibition of SUMOylation in XEE assay affects a PARylation on chromosomal proteins in CSF XEEs (Figure 2.7B). We prepared mitotic chromosomes under conditions where SUMOylation occurred normally, and under conditions where it was blocked by dnUbc9. We then induced PARylation of chromosomal proteins by incubating the isolated chromosomes with NAD^+ as described in experimental procedures. The PARylation of chromosomal proteins was analyzed by immunoblotting with anti-PAR polyclonal antibody. As shown in Figure 2.7B, the chromosomes from the reaction where SUMOylation was inhibited (+dnUbc9 lane) showed more PARylation, and the pattern of PARylated proteins was clearly different from the control reaction (control lane). To confirm that this PARylation was dependent upon PARP1, we performed the same experiment using CSF XEEs that were depleted of PARP1. The PARP1-depleted chromosomes showed little PARylation, indicating that PARP1 was the primary enzyme that mediated chromosomal PARylation in this assay.

Finally, we investigated whether SUMOylation may control PARylation by modulating PARP1 localization on mitotic chromosomes. Sperm chromatin was allowed to undergo a single round of DNA replication in interphase XEEs. The

XEEs were then returned to mitosis through addition of a fresh aliquot of CSF XEE, allowing the formation of mitotic chromosomes. Individual chromosomes were isolated on cover slips and stained using antibodies against SUMO-2/3, PARP1 and centromeric histone variant CENP-A (Figure 2.7C). Consistent with earlier experiments (8), the anti-SUMO2/3 antibody stained the inner centromere region (ICR) of the chromosomes and this signal was completely abolished in the presence of dnUbc9 (Figure 2.7C right panels). PARP1 associated broadly on chromosome arms, with slight accumulation on ICR, partially co-localizing with the region of highest SUMO-2/3 accumulation (Figure 2.7C upper/left panel). As shown in the magnified insets, red (PARP1) and green (SUMO-2/3) merged to yellow at the edge of SUMO-2/3 signals although majority of SUMO-2/3 signals did not overlap with PARP1. This pattern was not obviously changed by the addition of dnUbc9, arguing against the possibility that SUMO-dependent changes in PARylation by PARP1 are due to its mislocalization on chromosomes. Together, our findings favor the possibility that SUMOylation primarily controls the capacity of PARP1 to act on PARylation substrates, without substantially altering its intrinsic enzymatic activity or subcellular localization.

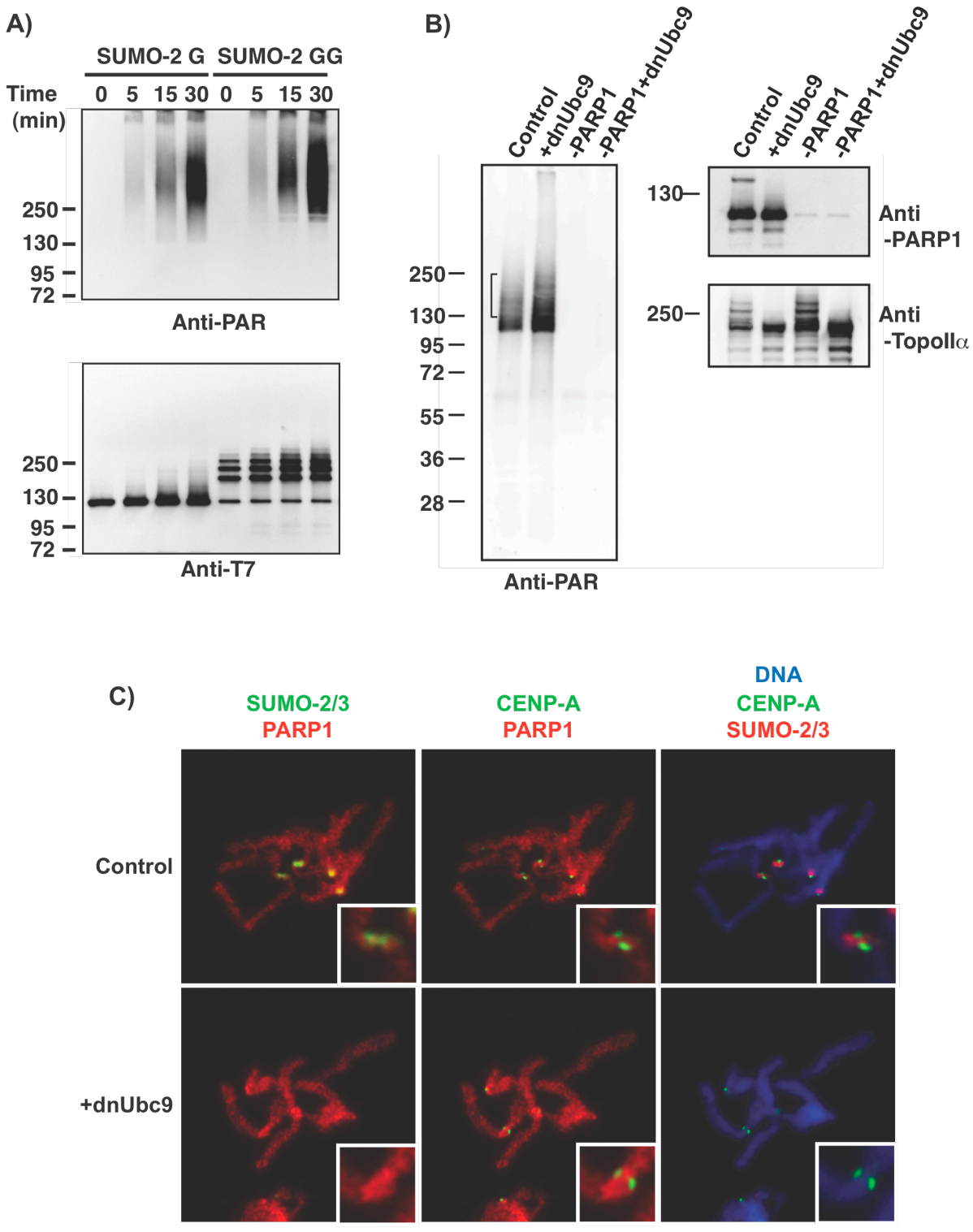

Figure 2. 7
Inhibition of SUMOylation on mitotic chromosomes alters chromosomal PARylation.

A) SUMOylated PARP1 retains robust enzymatic activity of poly(ADP-ribosyl)ation *in vitro*. An *in vitro* SUMOylation reaction was performed in the presence of the indicated SUMO-2 proteins as in Figure 2.3. After the SUMOylation reaction, NAD^+ was added to the reaction mixture and then the reaction mixture was incubated for the indicated periods. Poly(ADP-ribosyl) chain formation was analyzed by a monoclonal anti-PAR antibody (Trevigen). PARP1 was detected with an anti-T7 antibody.
B) SUMOylation affect PARylation on chromosomes prepared from XEE. Mitotic chromosomes were isolated from the extracts that were manipulated as indicated. Isolated chromosomal fractions were incubated with NAD^+ containing buffer to promote PARylation on chromosomal proteins, and then the reactions were terminated with the addition of SDS-PAGE sample buffer. The obtained chromosomal samples were analyzed by immunoblotting with the indicated antibodies. The bracket indicates the area of PARylation signals affected by the inhibition of SUMOylation.
C) Inhibition of SUMOylation does not affect in the localization of PARP1 on mitotic chromosomes significantly.
CSF extracts were released into interphase by $CaCl_2$ addition. 200 sperm/μl were added 5 minutes after $CaCl_2$ and the extract was incubated at 23˚C for 55 minutes. Re-entry into mitosis was induced by addition of fresh CSF extract (50% of reaction volume) with or without dnUbc9 (150 ng/μl final concentration). Chromosomes from each reaction were spun onto cover slips and analyzed by immunofluorescence with antibodies against SUMO-2/3, PARP1, and CENP-A as described in material and methods. Each signal was pseudo colored as indicated at the top of the panels. DNA was visualized with Hoechst 33342 staining. Insets are magnified images around the centromere region.

Discussion

We have identified PARP1 as a novel mitotic SUMO-2/3-modified chromosomal substrate in M-phase *Xenopus laevis* egg extracts (CSF XEEs). In a manner similar to a previously identified SUMO-2/3 conjugation target, DNA topoisomerase IIα, PARP1 modification in CSF XEEs required PIASy, the presence of chromatin and the mitotic phase of the cell cycle (8,71). We have mapped the SUMOylated residues of PARP1 that was purified from mitotic chromosomes, and found that Lysine at 482 was a primary SUMOylation site.

We have reconstituted SUMOylation of PARP1 using purified components, and shown that PIASy plays two important roles in its conjugation: It enhances both the efficiency of SUMOylation and the accuracy of site selection. At high concentrations of Ubc9, PARP1 could be SUMOylated in the absence of PIASy. At more physiological concentrations of Ubc9, however, PARP1 SUMOylation became almost entirely PIASy-dependent. The aberrant migration of the modified species in reactions lacking PIASy suggested that secondary or tertiary SUMOylation site(s) on PARP1 were being used at a greater frequency than when it was present. Similar species were observed even in the presence of PIASy when the reactions were performed with a PARP1 mutant, PARP1-K482R, which lacked the major SUMOylation site that we had mapped.

Control of chromosomal PARylation by SUMOylation

We did not find that PARP1 activity in auto-PARylation assays was substantially dependent upon its SUMOylation status (Figure 2.7A), arguing against the idea the SUMOylation of PARP1 changes its intrinsic enzymatic activity. On the other hand, we observed that PARP1 was the major PARylation enzyme associated with mitotic chromosomes in XEEs, since this modification was essentially abolished in its absence, and that its capacity to mediate PARylation of other chromosomal proteins was enhanced under conditions where SUMOylation was suppressed (Figure 2.7B).

These data are consistent with the idea that SUMOylation of PARP1 substantially decreases its capacity to recognize or modify other proteins. Since Ly-

sine 482 is located within the BRCT domain of PARP1, we speculate that SUMOylation of this residue may substantially change PARP1 association with other proteins (85). Such interactions did not appear to be important for targeting of PARP1 onto mitotic chromosomes (Figure 2.7C), but may disrupt its capacity to PARylate its chromatin-bound substrates. An alternative explanation for our findings is that SUMOylation inhibits PARylation through modification of PARP1 target proteins. The dnUbc9 would thus increase PARylation by relieving such inhibition. It has not been possible to restore chromosomal PARylation to PARP1-depleted XEEs with recombinant PARP1, possibly because immunodepletion of PARP1 co-precipitates other factors necessary for its activity (data not shown), so it has not been possible to rigorously test these two alternatives; notably they are not mutually exclusive, so that SUMOylation of both PARP1 and its targets could modulate the level of chromosomal PARylation.

Further investigation will be required to identify the substrates of PARP1-mediated PARylation in XEEs and the role of this modification in altering their function. Consistent to our finding that a portion of PARP1 localizes on ICR with XEE assay (Figure 2.7C), PARP1 is localized to the centromeres of mammalian cells (79,86). It has been shown to be involved in the regulation of chromatin structure (87) and to promote the PARylation of several centromeric proteins (80). One attractive possibility is that these aspects of PARP1 function might be regulated through SUMOylation. In this context, the Aurora B kinase is a particularly intriguing potential target for PARP1; it interacts with the BRCT domain of PARP1 and can be PARylated by PARP1 (38). Moreover, the fact that PARP1

associated with the ICR might be consistent with this possibility (Figure 2.7C). However, it was not possible for us to validate this notion because we did not find evidence that Aurora B becomes PARylated in XEEs, nor that such a modification could be modulated by changes in SUMOylation (data not shown). It also remains possible that PARP1 itself is a major PARylation substrate on mitotic chromosomes. In this case, the capacity of chromosome-bound PARP1 to catalyze auto-PARylation may be more regulated through SUMOylation than isolated PARP1 protein in our purified assays (Figure 2.7A).

Role of PIASy in PARP1 SUMOylation.

SUMOylation sites of many targets lie within a preferred consensus motif, ΨKX(D/E) (Ψ is an aliphatic branched amino acid; X is any amino acid). Ubc9 can directly bind this motif, and conjugate the lysine residue within it in an E3-independent manner (88). Lysine 482 of PARP1 lies within such a consensus motif (Figure 2.4) and Ubc9 interacts PARP1 in yeast two-hybrid assays (89). Consistent with these facts, we found that PARP1 could be SUMOylated in the absence of PIASy (Figure 2.3). We note, however, that Ubc9 was also able to recognize other lysine residues of PARP1 in these E3-independent reactions, since we continued to observe SUMOylation in reactions with a mutant form of PARP1 that lacked this residue (PARP1-K482R). Notably, direct recognition by Ubc9 is relatively inefficient, and we observed little PARP1 SUMOylation at physiological concentrations of Ubc9, even for PARP1 possessing a wild-type lysine at residue 482.

Recent structural analysis on a fragment of Siz1p, a SIZ/PIAS family SUMO ligase from budding yeast, showed that it binds the thioester-linked Ubc9~Smt3p complex in such a way as to properly configure it for catalysis and to promote correct interactions between the conjugation target and Ubc9's active site (90). PIASy dramatically increased the extent of PARP1 SUMOylation at physiological concentration of Ubc9 (Figure 2.3C). This increased SUMOylation is likely to reflect the capacity of PIASy to recognize both Ubc9~SUMO-2 and PARP1, analogous to the interactions formed by Siz1p. This binding would bring them into a single complex, elevating the effective concentration of Ubc9~SUMO-2, as well as increasing the efficiency of SUMO-2 transfer. Notably, Siz1p also directs the transfer of Smt3p to the appropriate lysine residue of the target protein (90). We find that PIASy can similarly bias conjugation toward a preferred residue, Lysine 482. The preference for this residue is not absolute, as we find that alternative residues can be utilized when it is mutated, either in purified assays (Figure 2.5B) or in XEEs (Figure 2.6), albeit inefficiently.

In summary, we have shown that PARP1 is a substrate of PIASy-mediated SUMOylation on mitotic chromosomes in XEEs. This modification regulated its activity in this context, perhaps through its capacity to recognize or modify other chromosomal proteins. PIASy facilitates the modification of PARP1 by increasing both the extent of SUMOylation and by directing the modification to a preferred site on PARP1. In combination with earlier observations showing that PIASy modifies TopoIIα in XEEs (8,71), our findings suggest that PIASy is re-

sponsible for a series of coordinated changes in the activity of chromatin-associated enzymes that contribute to chromosome segregation in this system.

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Contributions

Dr. Yoshiaki Azuma contributed to Figure 1, 2 and 4. Katelyn Deckert and Gada AlAni contributed to Figure 7A and 7B, respectively. All other figures are my work.

CHAPTER 3

PIASy-dependent SUMOylation regulates DNA Topoisomerase IIα activity

Abstract

DNA Topoisomerase IIα (TopoIIα) is an essential chromosome-associated enzyme with activity implicated in the resolution of tangled DNA at centromeres prior to anaphase onset. However, the regulatory mechanism of TopoIIα activity is not understood. Here we show that PIASy-mediated SUMO2/3 modification of TopoIIα strongly inhibits TopoIIα decatenation activity. Using mass spectrometry and biochemical analysis we demonstrate that TopoIIα is SUMOylated at lysine 660 (Lys660), a residue located in the DNA-gate domain where both DNA cleavage and re-ligation take place. Remarkably, loss of SUMOylation on Lys660 eliminates SUMOylation-dependent inhibition of TopoIIα, indicating that Lys660 SUMOylation is critical for PIASy-mediated inhibition of TopoIIα activity. Together, our findings provide evidence for the regulation of TopoIIα activity on mitotic chromosomes by SUMOylation. Therefore, we propose a novel mechanism for regulation of centromeric DNA catenation during mitosis by PIASy-mediated SUMOylation of TopoIIα.

Introduction

Resolution of sister chromatid cohesion is a fundamental process required for faithful chromosome segregation. Together with cohesin-mediated cohesion, DNA catenation maintains chromosome cohesion in the early stages of mitosis (91,92). By the onset of anaphase, however, the cohesin complex is removed by separase through APC/C-dependent ubiquitination and degradation of securin, and the catenation of centromeric DNA is resolved by the action of a specialized enzyme called DNA Topoisomerase IIα (TopoIIα) (93,94). A number of early reports showed that TopoIIα relocalizes from chromosome arms to the centromere during mitosis (95-97), and further studies using self-primed *in situ* labeling revealed that catalytically active TopoIIα accumulates primarily at the centromere (33). In addition, recent studies have shown that ultrafine bridges originating from tangled DNA in metaphase chromosomes were resolved by TopoIIα activity following removal of the cohesin complex (98), indicating a role for TopoIIα activity in mitosis. These evidences strongly suggest tight regulation of TopoIIα activity in space and time. Although extensive biochemical studies have elucidated the molecular mechanism of TopoII family proteins' enzymatic reactions (99), how the catalytic activity of TopoIIα is regulated at the centromere in such a specific manner is unknown.

Studies examining the relationship between TopoIIα activity and post-translational modification (PTM) have not clearly demonstrated that TopoIIα activity is regulated by PTM (100,101). Yet, one PTM of TopoIIα, SUMOylation, has

been suggested as a potential regulator of TopoIIα activity given that TopoIIα SUMOylation is mitosis-specific and occurs near centromeres (102). SUMO, a small ubiquitin-like modifier, is a conserved ubiquitin family protein in eukaryotes (5,64). Vertebrates typically express three SUMO paralogues designated as SUMO1, 2, and 3. SUMO2 and 3 are ~95% identical while SUMO1 has ~45% identity with both SUMO2 and 3. (In this report, we refer to SUMO2 and 3 as SUMO2/3 when they are indistinguishable.) SUMO proteins contain a C-terminal di-glycine motif that is exposed by a hydrolase prior to a SUMOylation reaction of target proteins. The biochemical process of SUMOylation requires unique components but is somewhat similar to the ubiquitination pathway. First, SUMO proteins are activated by the E1 enzyme (Aos1/Uba2 heterodimer); then, they are transferred to the E2 enzyme (Ubc9), and finally conjugated to cellular substrates via an E3 ligase enzyme. Defects in the SUMOylation pathway have been reported to cause faulty mitosis (41,103), typically represented in most organisms by failure of proper chromosome segregation (40,104,105).

Siz1p and Siz2p, conserved eukaryotic SUMO E3 ligases, are responsible for the SUMOylation of TopoII in budding yeast, and the loss of Siz-mediated TopoII SUMOylation decreases chromosome transmission fidelity (28). Using a *Xenopus* Egg Extract (XEE) cell-free assay, we previously showed that PIASy, a member of the PIAS/Siz family of SUMO ligases, is an essential chromosomal component for promoting TopoIIα SUMO2/3 modification in vertebrates, and suggested a role for PIASy in chromosome segregation (8). Moreover, reports using HeLa cells revealed that PIASy is required for faithful chromosomal sepa-

ration, which is not dependent on centromeric cohesin but is related to TopoIIα localization at the centromere (73). Together, this evidence indicates that the PIAS/Siz family of E3 ligases has a conserved role in chromosome segregation in eukaryotes through regulation of TopoII SUMOylation. In contrast, studies using lysates from mouse embryonic fibroblasts (MEF) that were deficient in RanBP2/Nup358, a SUMO E3 ligase that is also a component of the nuclear pore complex in vertebrates (54), provided evidence that RanBP2 facilitates SUMOylation of TopoIIα through SUMO1 conjugation (106). The discrepancy between these models remains to be examined.

For this report, we established an *in vitro* SUMOylation assay using recombinant TopoIIα as substrate, in order to elucidate the biochemical consequence of PIASy-mediated SUMO2/3 modification on TopoIIα activity. Consistent with our previous results using XEE assays, we demonstrate that PIASy robustly facilitates SUMOylation of TopoIIα, and this modification is SUMO2/3-specific *in vitro*. We also observed that SUMOylated TopoIIα exhibits much less DNA decatenation activity, indicating a potential mechanism for inhibition of TopoIIα activity by SUMOylation. Using mass spectrometric analysis of TopoIIα isolated from mitotic chromosomes, we identified lysine 660 (Lys660) as a novel SUMOylation site of TopoIIα. Further biochemical studies demonstrated that elimination of SUMOylation at Lys660 suppressed SUMOylation-dependent inhibition of TopoIIα activity, independent of other SUMOylation sites. Finally, we show that SUMOylation of Lys660 is stimulated by DNA binding of TopoIIα, sug-

gesting that the enzymatically active TopoIIα might be a primary target of Lys660 SUMOylation.

Our findings strongly suggest that TopoIIα SUMOylation regulates decatenation of centromeric DNA. The co-localization of TopoIIα and PIASy at centromeres, and the stimulation of inhibitory SUMOylation at Lys660 by DNA binding of TopoIIα further suggest that this novel regulation of TopoIIα activity is controlled spatiotemporally during mitosis. Therefore, we propose that the SUMO ligase PIASy catalyzes SUMO2/3 modification of TopoIIα that regulates TopoIIα activity during mitosis.

Methods

DNA subcloning, site-directed mutagenesis, recombinant protein expression and purification, and antibodies. The cDNA of TopoIIα was cloned from *Xenopus laevis* tadpole cDNA (kindly provided by T. Amano and Y.B. Shi, NICHD) using PCR amplification. The *X. laevis* TopoIIα coding sequence was subcloned into a pPIC 3.5Kb vector in which either CBP-ZZ, CBP-T7, or T7-ZZ tag sequences were inserted. (The CBP and ZZ TAP-tag plasmids were kindly provided by H. Yoon and K. Gould.) The Lysine to Arginine substitution of TopoIIα was generated by site-directed mutagenesis using a QuikChange kit (Agilent) according to manufacturer's protocol. All constructs were verified by DNA sequencing.

For preparation of recombinant TopoIIα proteins, the plasmids were transformed into the GS115 strain of *Pichia pastoris* yeast and expressed according to the manufacturer's instructions (Invitrogen). Protein purification with the CBP and ZZ tags was performed using a modified TAP protocol (EMBL Heidelberg). Briefly, yeast cells expressing TopoIIα were frozen and ground with dry ice in a coffee mill, and then mixed with lysis buffer (50mM Tris-HCl (pH7.5), 150mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 0.1% Triton-X100, 5% Glycerol, 1mM DTT and 10mM PMSF). Samples were then centrifuged at 25,000xg for 40 minutes. To capture the CBP tagged TopoIIα, the supernatant was mixed with Calmodulin-Sepharose resin (GE Healthcare) for 90 minutes at 4°C. The resin was then washed with lysis buffer, and TopoIIα was eluted with buffer containing 10 mM EGTA. For ZZ-tagged TopoIIα, proteins were captured on IgG-Sepharose (GE Healthcare) and elution was performed by cleaving with PreScission Protease (GE Healthcare) according to the manufacturer's protocol. The elution was further purified by Mono Q anion-exchange chromatography (GE Healthcare). The E1 complex (Aos1/Uba2 heterodimer), PIASy, Ubc9, dominant negative form of Ubc9 (dnUbc9), and SUMO paralogues were expressed in BL21(DE3) or Rosetta(DE3) bacteria and purified as previously described.

Anti-TopoIIα/β monoclonal antibody was purchased from MBL. Anti-SUMO2/3 polyclonal antibody was generated in guinea pigs, (71)and polyclonal antibody against TopoIIα, C-terminus (aa 1358-1579) was prepared in rabbits by injection with a recombinant His-T7 fused fragment, and affinity purified (8). The anti-PIASy antibody used in this study was reported previously (8).

***Xenopus* egg extracts, Immunodepletion-addback assays.** Sperm nuclei and low-speed extracts of *X. laevis* eggs arrested in metaphase by cytostatic factor (CSF) were prepared following standard protocols (63,107) . Immunodepletion was performed as previously reported with protein A–conjugated magnetic beads (Dynal) (108). For addback experiments, the purified recombinant TopoIIα proteins were added to the immunodepleted extracts at a final concentration of ~300 nM, which is comparable to the concentration of endogenous TopoIIα measured by quantitative Western blotting. Chromosome isolation and analysis of SUMOylation were performed as previously described (107).

Immunofluorescence. The mitotic chromosomes for immunofluorescence analysis were prepared as previously described (108). Briefly, CSF extracts were driven into interphase by 0.6 mM $CaCl_2$. 500 sperm/μl were incubated with the interphase extracts for ~60 minutes at 23°C to allow complete DNA replication. Then, a volume of fresh CSF extract equal to half of the original volume was added to induce mitosis. For inhibition of mitotic SUMOylation, a dominant negative form of Ubc9 (dnUbc9) was added just before induction of mitosis, at a final concentration of 150 ng/μl. After a 30 minute incubation, the reactions were diluted three fold and fixed with *para*formaldehyde (PFA) at a final concentration of 2%. The samples were spun onto cover slips through a 35% glycerol cushion, post-fixed in 1.6% PFA, and analyzed by immunostaining using antibodies against TopoIIα (either mouse monoclonal or rabbit polyclonal), SUMO2/3

(guinea pig polyclonal), PIASy (rabbit polyclonal), and Aurora B (rabbit polyclonal). Anti-rabbit Alexa 568, anti-guinea pig Alexa 684, and anti-mouse Alexa 488 were used as secondary antibodies. Specimens were observed using Volocity Imaging Software (Improvision) on a Nikon TE2000-U microscope with Plan Apo 100x/1.40 objective, and images were taken with a Retiga SRV CCD camera (QImaging).

Purification of SUMOylated TopoIIα from mitotic chromosomes. After mitotic chromosomes were assembled and isolated from CSF extracts, chromosomal proteins were subsequently extracted by boiling the chromosome fractions with SDS-PAGE sample buffer. Immunoprecipitation was performed on extracted denatured proteins as described (83) using affinity-purified anti-TopoIIα antibody that had been covalently cross-linked to Protein-A Dynabeads (Invitrogen) by Dimethyl pimelimidate 2 HCl (Pierce Chemical Co.). Isolated proteins were separated on 8-16% Tris-HCl gradient gels (Invitrogen) by SDS-PAGE, and visualized by silver staining (Owl kit, Daiichi). For LS-MS/MS analysis, the isolated proteins were stained with CBB-R250.

***In Vitro* SUMOylation assays.** SUMOylation reactions were incubated at 25°C for 1 hour unless otherwise indicated. SUMO2 was used in most of reactions except where noted. The reactions contained 15 nM E1, 5 μM SUMO2-GG, 500 nM T7-tagged TopoIIα, 2.5 mM ATP, and various concentrations of Ubc9 and PIASy as indicated in Fig. 5. Reaction buffers were composed of 20 mM HEPES (pH

7.8), 100 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween20, 5% glycerol, 1mM AEBSF and 1 mM DTT. The reactions were stopped with half volumes of 3× SDS-PAGE sample buffer, and the samples were resolved on 8-16% Tris-HCl gradient gels by SDS-PAGE, and analyzed by Western blot with HRP conjugated anti-T7 monoclonal antibody (EMD Biosciences).

***In vitro* SUMOylation-Decatenation coupled assays**. These assays were performed with 60 nM Ubc9 and 30 nM PIASy together with other protein components as described above. A control was performed under the same conditions except that SUMO2-G was used instead of SUMO2-GG. After 1 hour incubation at 25°C, the non-SUMOylated (Cont.) and SUMOylated samples were further incubated with 6.2 ng/ul of kDNA (TopoGEN, Inc.) in decatenation buffer at 25°C for the indicated time periods. Decatenation buffer consisted of 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT, 30 μg BSA/ml, and 2 mM ATP. The reactions were stopped by adding one third volume of 6X DNA dye (30% glycerol, 0.1% SDS, 10 mM EDTA, and 0.2 μg/ul bromophenol blue), and samples were loaded on a 1% agarose gel and electrophoresed at 100V in TAE buffer until the samples reached the middle of the gel. The amount of kDNA remaining in the wells was measured using an Image Station 4000R (Kodak) and standard error was calculated using Kaleidagraph (Synergy Software).

Electrophoretic Mobility Shift Assay. The assay was performed as described previously (109) with slight modifications. In brief, various amounts of TopoIIα

WT and K660R (as indicated in Figure 5b) were incubated with 90ng of pBS KS(+) DNA for 5 min at 25°C. *In vitro* SUMOylation reaction buffer was used as the reaction buffer in these assays in order to keep protein-DNA binding conditions similar to those used in *in vitro* SUMOylation reactions. The reactions were stop by adding one half volume of loading buffer (50% glycerol, 10mM EDTA, and 0.2% Bromophenol Blue) and the samples were subjected to electrophoresis at 40V for 2 hours at 4°C in a 1% agarose gel containing ethidium bromide. The DNA was documented using an Image Station 4000R.

Results

PIASy promotes SUMO2/3 conjugation of TopoIIα in *Xenopus* egg extracts.

We previously demonstrated using a *Xenopus* Egg Extract (XEE) cell-free assay, that TopoIIα is modified by SUMO2/3 in a PIASy-dependent manner (8). More recently, studies in MEFs suggested that RanBP2 promotes SUMOylation of TopoIIα through SUMO1 conjugation (106). To investigate whether RanBP2 has a role in SUMOylation of TopoIIα in XEE assays, we immunodepleted XEE of specific E3 enzymes (RanBP2, PIASy, or RanBP2/PIASy) and examined TopoIIα for alterations in SUMOylation. Following immunodepletion of RanBP2 from XEE, SUMO2/3 modification was still intact in the chromosomal fractions (indicated by the bracket in Fig. 3.1a). In contrast, no detectable TopoIIα SUMOylation was observed in the absence of PIASy (Fig. 3.1a). The lack of SUMO1-modified proteins associated with mitotic chromosomes indicates that TopoIIα on mitotic chromosomes is exclusively modified by SUMO2/3 (Fig. 3.1a).

We further analyzed PIASy-dependent SUMO2/3 modification of TopoIIα on mitotic chromosomes by immunofluorescence microscopy. Using EGFP-fused SUMO2 in XEE assays, we previously showed that SUMO2/3-modified proteins are localized on inner centromeric regions (8). For the current study, we prepared an antibody to enable visualization of endogenous SUMO2/3 on chromosomes. Mitotic chromosomes prepared from replicated chromatin in XEE were fixed and immunostained using antibodies specific for TopoIIα, PIASy, and SUMO2/3 (Fig. 3.1b). We observed that PIASy localizes to distinct foci on mitotic

chromosomes and these foci overlap with SUMO2/3 localization (Fig. 3.1b). Because endogenous SUMO2/3 also co-localizes with an inner centromeric marker, Aurora B (Figure 3.2), we conclude that PIASy displays centromeric localization. Finally, we verified the localization of TopoIIα using two different antibodies. Both antibodies revealed that TopoIIα is located throughout the chromosome axis with clear accumulation at the centromeres of mitotic chromosomes as previously shown in other species (Fig. 3.1b and 3.2) (96,110). Overall, the co-localization of TopoIIα and SUMO2/3 was clearly apparent at the centromeric regions, but not in other parts of the chromosome, suggesting that SUMOylation of TopoIIα mainly occurs at the centromere and in proximity to PIASy (Fig. 3.1b). The staining pattern of TopoIIα did not change following addition of dominant negative Ubc9, (dnUbc9), which prevents SUMOylation, indicating that localization of TopoIIα was not altered by the perturbation of SUMOylation (Fig. 3.1b and 3.2). Since we found no evidence for RanBP2 involvement in SUMO2/3 modification of TopoIIα in our assay system, we focused our subsequent studies on PIASy-mediated TopoIIα SUMOylation.

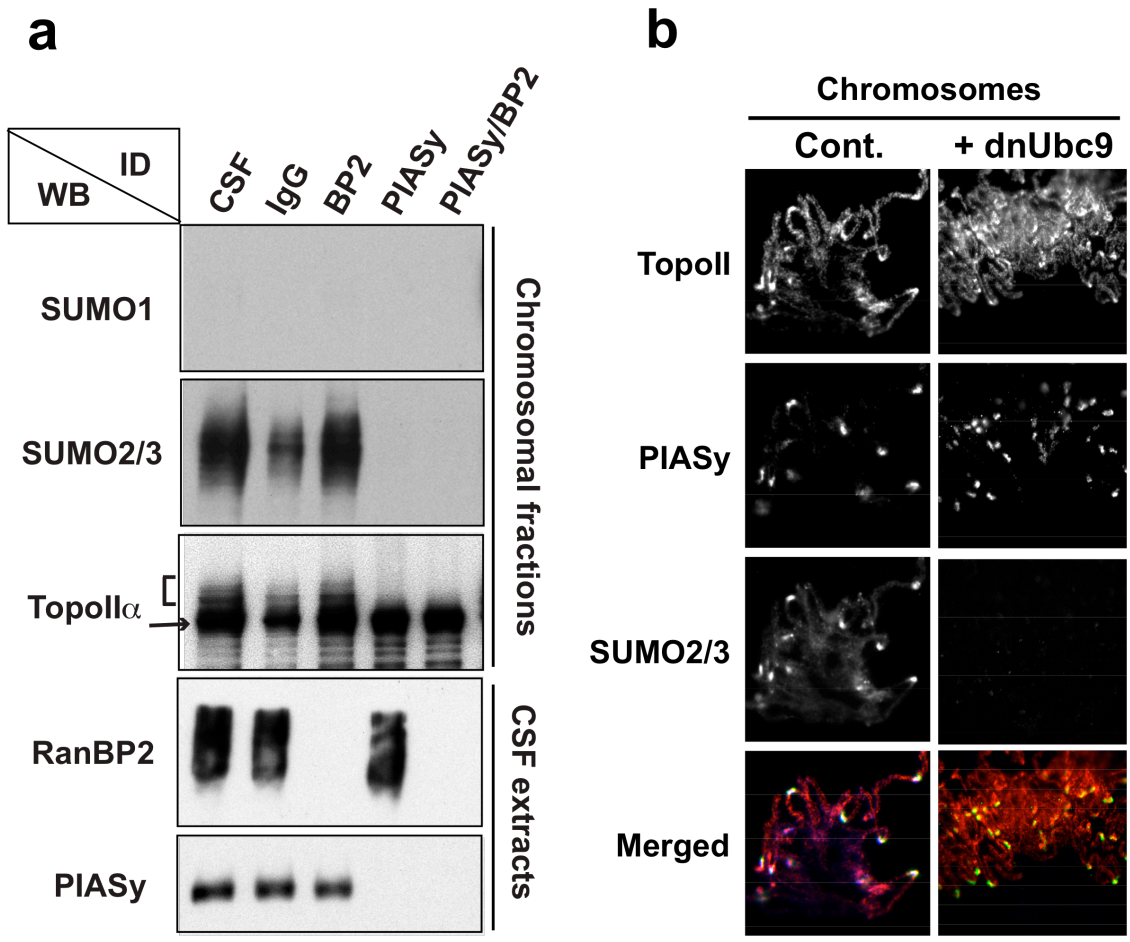


Figure 3. 1
PIASy but not RanBP2 is required for SUMO2/3 conjugation of TopoIIα in XEE. (**a**) XEE were immunodepleted using antibodies against RanBP2, PIASy, or RanBP2/PIASy. The depleted extracts were incubated with 10,000 sperm nuclei/μl for 1 hour at 25°C. Non- or mock (IgG)-depleted extracts were also subjected to the same procedure. Isolated chromosomes from each reaction were analyzed by Western blot (WB) for the indicated protein. Immunodepletion (ID) of RanBP2 had no effect on the SUMOylation of TopoIIα whereas PIASy ID eliminated TopoIIα SUMOylation. (**b**) The mitotic chromosomes were prepared as in *METHODS* and were analyzed by immunostaining with the indicated antibodies: TopoIIα in red, PIASy in green, and SUMO2/3 in blue in merged panel. TopoIIα co-localized with PIASy and SUMO2/3 at the centromeres. Addition of dnUbc9 eliminated SUMO2/3 modification but did not alter the localization of TopoIIα at the centromeres of mitotic chromosomes.

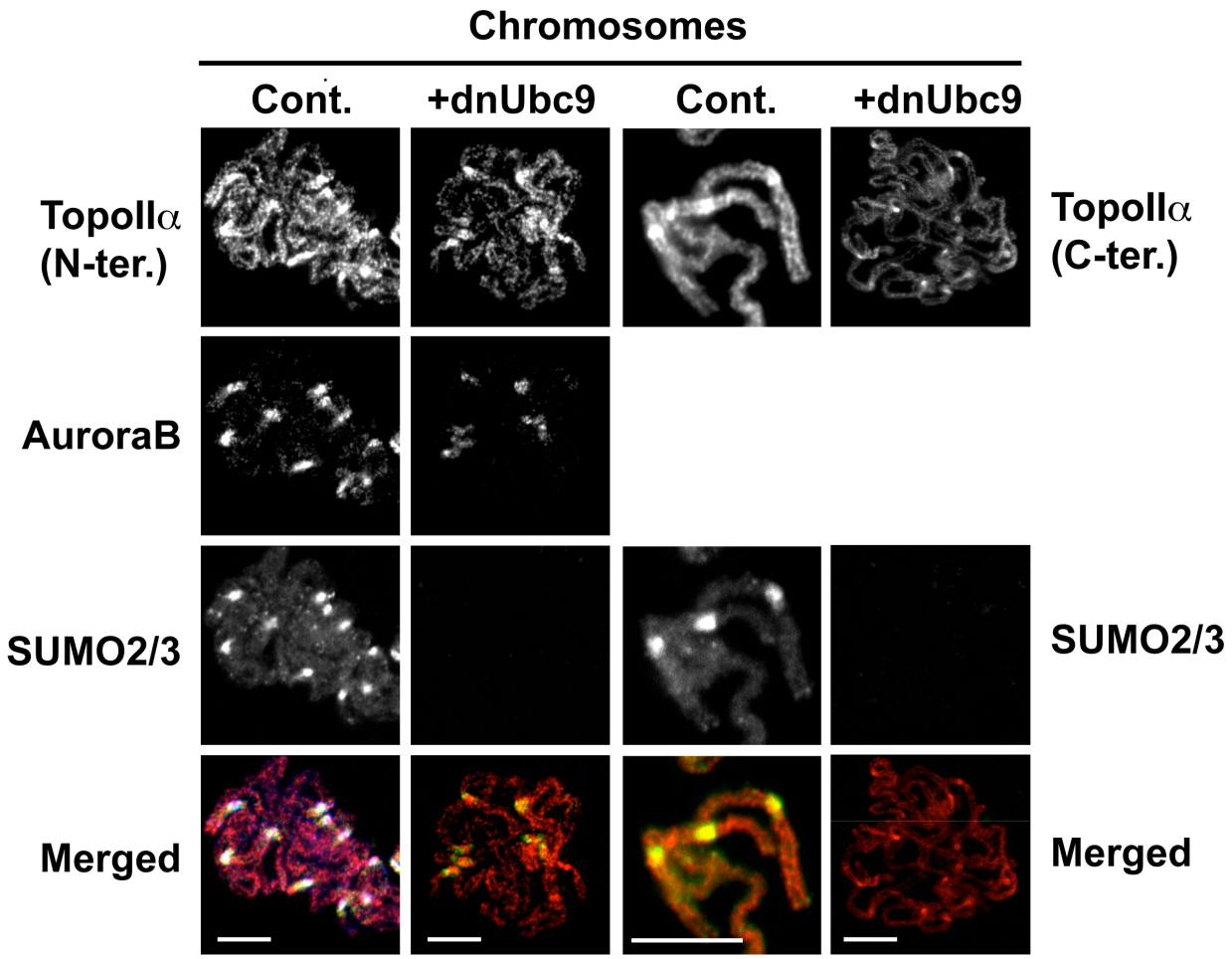


Figure 3. 2
TopoIIα colocalizes with AuroraB on the centromere of mitotic chromosomes. The mitotic chromosomes were prepared as in Figure 1 and samples were analyzed by immunostaining as indicated. (**Left panels**) monoclonal TopoIIα (N-ter.) in red, AuroraB in green, and SUMO2/3 in blue in merged panel. (**Right panels**) polyclonal TopoIIα (C-ter.) in red and SUMO2/3 in green. Both monoclonal and polyclonal TopoIIα antibodies showed indistinguishable staining. Bars : 10μm

PIASy promotes SUMO2/3 conjugation of TopoIIα in reconstituted *in vitro* SUMOylation assays.

We previously demonstrated the requirement of PIASy activity for SUMOylation of chromosomal TopoIIα in XEE assays (71). However, due to the extreme

instability of the SUMOylation reaction, experimentally managing the state of SUMOylation *in vivo* was difficult, limiting the use of XEE assays for further investigations of TopoIIα SUMOylation. Therefore, we next sought to examine the PIASy-dependent SUMOylation of TopoIIα in an *in vitro* assay. For these studies, all of the protein components required for SUMOylation were prepared as described in *METHODS*. For most *in vitro* SUMOylation reactions, we used 500nM T7-taged TopoIIα, 5uM SUMO and 15nM E1 (Aos1/Ubc2), and the SUMOylation was detected by western blot using the T7 tag.

Ubc9, a SUMO E2 conjugating enzyme, directly binds to its consensus sequence on substrates without E3 ligase (64,88) and, in fact, major SUMOylation sites of budding yeast TopoII were identified based on this consensus sequence (102). Hence, we sought to identify whether SUMOylation of *Xenopus* TopoIIα is dependent on Ubc9. Purified *Xenopus* TopoIIα was incubated with various concentrations of Ubc9 and analyzed by western blot. Our results indicated that Ubc9 could promote SUMOylation of TopoIIα in a dose-dependent manner (Fig. 3.3a) and 300 nM Ubc9 was sufficient to promote SUMOylation of more than 50% of the TopoIIα. However, when the concentration of Ubc9 was close to the endogenous levels found in XEE, 30nM, the efficiency of TopoIIα SUMOylation dropped to barely detectable levels (Fig. 3.3a, b).

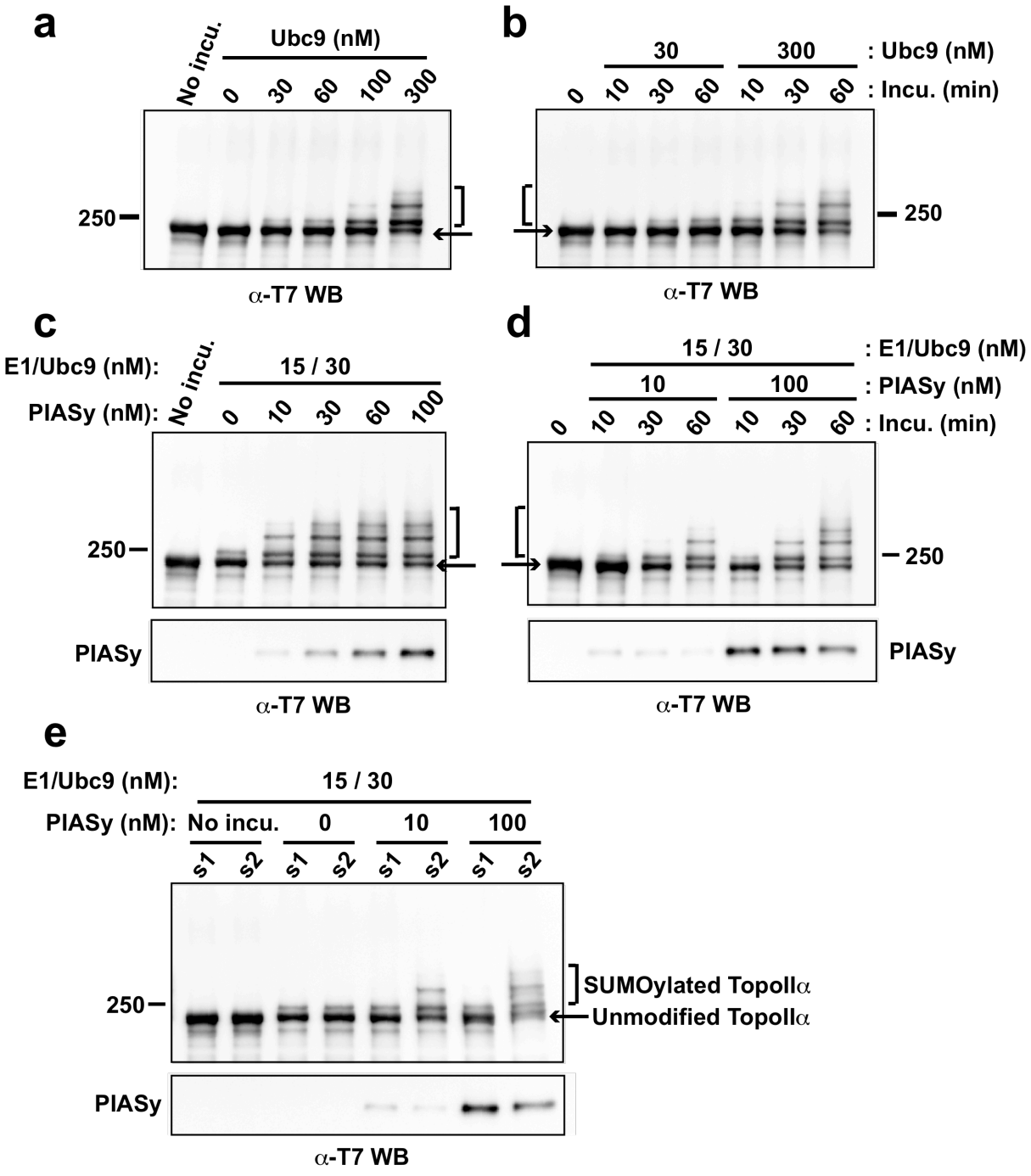


Figure 3. 3

PIASy is required for the efficient SUMOylation of TopoIIα and for the selection of SUMO paralogues. (**a**) Ubc9 dosage dependent SUMOylation. T7 tagged-TopoIIα was incubated in a reaction containing various concentrations of Ubc9 (0-300nM) in the presence of SUMO2. The amount of SUMO2-conjugated TopoIIα was similar to that seen in XEE only when 300nM Ubc9 was added. (**b**) Time course experiment with physiological (30nM) and higher (300nM) concen-

tration of Ubc9. (**c**) PIASy dosage-dependent SUMOylation. T7-TopoIIα was incubated as in (a**)** except with various concentrations of PIASy (0-100nM) and with the physiological concentration of Ubc9 (30nM). PIASy efficiently facilitated SUMOylation of TopoIIα under conditions using 30nM Ubc9, where SUMOylation had barely appeared in the absence of PIASy. SUMOylation was saturated using more than 60nM PIASy. (**d**) Time course experiment of PIASy-dependent SUMOylation. The reactions were performed with physiological (10nM) or higher (100nM) concentrations of PIASy in the presence of 30nM Ubc9. (**e**) T7-TopoIIα was incubated with either SUMO1 (s1) or SUMO2 (s2) in the presence of PIASy as indicated. PIASy showed a preference for SUMO2 over SUMO1.

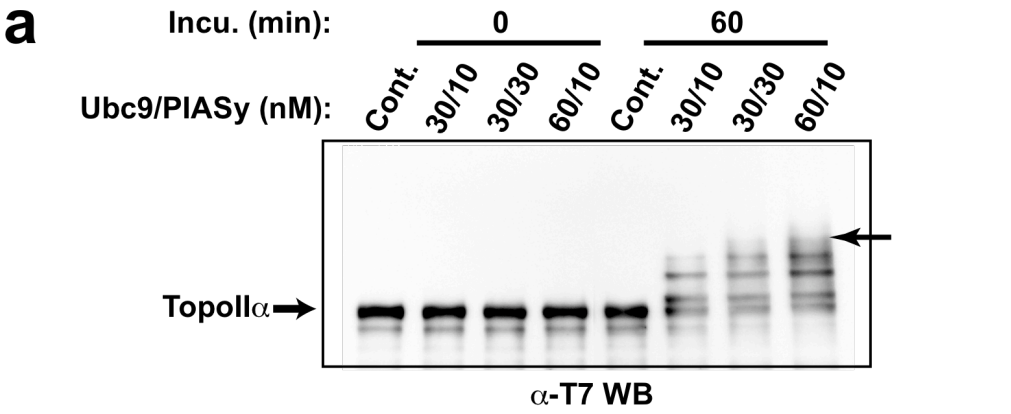
Next, to assess the contribution of PIASy to SUMOylation, TopoIIα was incubated with various concentrations of PIASy using a physiologically relevant concentration of Ubc9 (30nM). Under such conditions, TopoIIα was efficiently SUMOylated in the presence of PIASy and could be SUMOylated even when PIASy concentration was 10nM, which is near the endogenous level in XEE (Fig. 3.3c, d).

The observation that TopoIIα is specifically modified by SUMO2/3 in XEE led us to hypothesize that PIASy may select the SUMO paralogue for the conjugation. To test this hypothesis, TopoIIα was incubated with either SUMO1 or SUMO2 in the presence of PIASy and physiological concentrations of Ubc9. This assay revealed that PIASy mediates modification of TopoIIα by SUMO2 but not by SUMO1 (Fig. 3.3e), even though SUMO1 was highly active with RanGAP1 as the substrate (data not shown) as previously shown (82). This indicates that PIASy plays a role in the selection of SUMO paralogues. We also confirmed that PIASy mediates modification of TopoIIα with SUMO3 in similar level to with SUMO2 (data not shown). Together, we conclude that PIASy is an essential element for SUMO2/3 modification of TopoIIα under physiological conditions.

PIASy-mediated SUMOylation inhibits the decatenation activity of TopoIIα.

Previous studies in HeLa cells have suggested that PIASy is required for regulation of cohesin-independent cohesion of centromeres (73). We speculated that PIASy might play a role in localizing TopoIIα to the centromere by regulating its SUMOylation; however, we found that inhibition of SUMOylation did not alter TopoIIα localization in XEE assays (Fig. 3.1b). Therefore, we examined whether PIASy-dependent SUMOylation alters TopoIIα activity, which is required for proper chromosome segregation. To this end, TopoIIα was incubated in an *in vitro* SUMOylation reaction with either processed form of SUMO2 (SUMO2-GG), or a truncated form of SUMO2 (SUMO2-G) that cannot be conjugated to substrates due to a lack of one C-terminal glycine. SUMOylated and non-SUMOylated TopoIIα were then analyzed for decatenation activity using kinetoplast DNA (kDNA) as substrate. Once decatenated by TopoIIα, the interlocking chain of circular kDNA releases to form minicircles. As shown in Figure 3.4a, TopoIIα was differentially SUMOylated in 60 min *in vitro* using various concentrations of Ubc9 and PIASy in the presence of SUMO2-GG. A control reaction that contained Ubc9 and PIASy in the presence of SUMO2-G showed no SUMOylation. Western blot analysis revealed that the intensity of TopoIIα SUMOylation could be controlled by the *in vitro* reaction conditions and that more than 50% of the TopoIIα was modified under each condition. Subsequent assays indicated that the decatenation activity of TopoIIα is markedly inhibited by SUMO conjugation (Fig. 3.4b, c). One interesting aspect of this analysis is that, despite subtle differ-

ences in TopoIIα SUMOylation among the series of reactions, the presence of the highest molecular weight band (indicated by the arrow in Fig. 3.4a) appeared to correlate with strong inhibition of TopoIIα decatenation activity. Together, our results indicate that PIASy-mediated SUMO modification of TopoIIα inhibits TopoIIα decatenation activity.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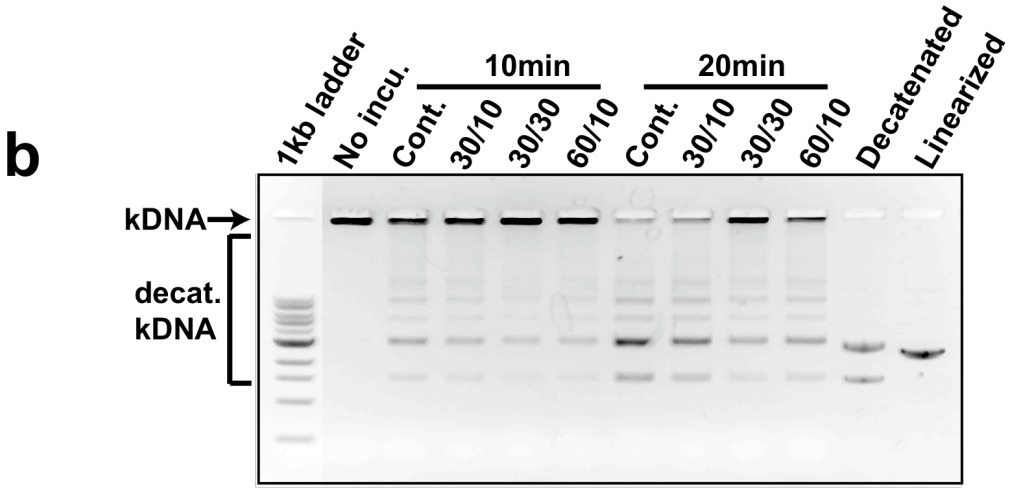
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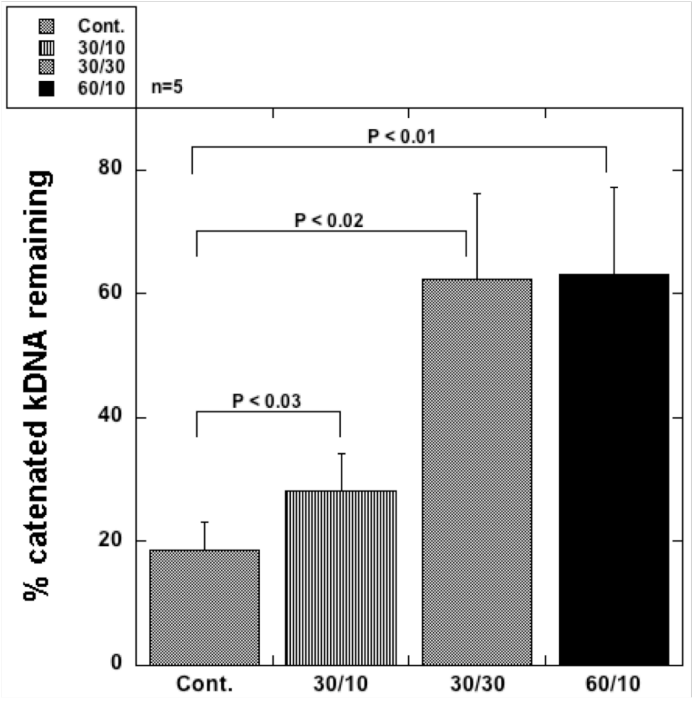

c
Cont.
30/10
30/30
60/10
n=5
P < 0.01
P < 0.02
P < 0.03
% catenated kDNA remaining
80
60
40
20
0
Cont.
30/10
30/30
60/10

Figure 3. 4
SUMO modification affects the decatenation activity of TopoIIα. (**a**) T7-TopoIIα was incubated with various combinations of Ubc9/PIASy as indicated to obtain a series of SUMOylation profiles. All control reactions (Cont.) were performed with 60nM Ubc9/10nM PIASy and SUMO2-G which could not be conjugated. The samples were analyzed by Western blot for T7 tag. Arrow indicates maximal SUMO modification of TopoIIα (seen in 30/30 and 60/10). (**b**) Representative data of decatenation assay. Samples in (a) were further incubated with decatenation buffer that contained kinetoplast DNA (kDNA) for 10 or 20 minutes and the products were resolved in an agarose gel. Decatenated and linearized markers are designated. (**c**) Band intensity data from five independent experiments performed as in **b** are presented as % catenated kDNA remaining after 20 min incubation with standard error and probability value from Student-t test. SUMO2 modification of TopoIIα decreased its decatenation activity.

Lysine at 660 is one SUMOylation site of TopoIIα in XEE.

To better understand the molecular basis of TopoIIα inhibition by SUMOylation, we sought to identify SUMOylation sites of TopoIIα. To this end, a SUMOylated form of endogenous TopoIIα was purified from mitotic chromosomes prepared in XEE (Figure 3.5). Isolated bands were double-digested with trypsin and chymotrypsin followed by mass spectrometric analysis using the same method as used to identify the SUMOylation site of poly (ADP-ribose) polymerase I (PARP1), another mitotic chromosomal substrate (10). Double digestion with trypsin and chymotrypsin generates a remnant of the SUMO sequence (QQQTGG, with a mass of 599.2663 Da) on the lysine of a SUMO modified peptide. With approximately 50% sequence coverage, lysine residue 660 (Lys660, K*EWLTNFMQDR, where * refers to QQQTGG) was shown to have the SUMO signature in the digested pool of SUMO2/3-TopoIIα (Figure 3.6). This result was unexpected given that 1) the sequences surrounding Lys660 do not match the canonical or non-canonical consensus sequences predicted using a SUMO pre-

diction program (111), and 2) the genetically determined SUMOylation sites in budding yeast were all located in the C-terminal domain (28,102) while Lys660 is located in the core active domain (DNA-gate) of TopoIIα (32,112) (Fig. 3.7a). Also, it was striking that the sequences including and surrounding Lys660 are highly conserved, from yeast to human (Fig. 3.7a).

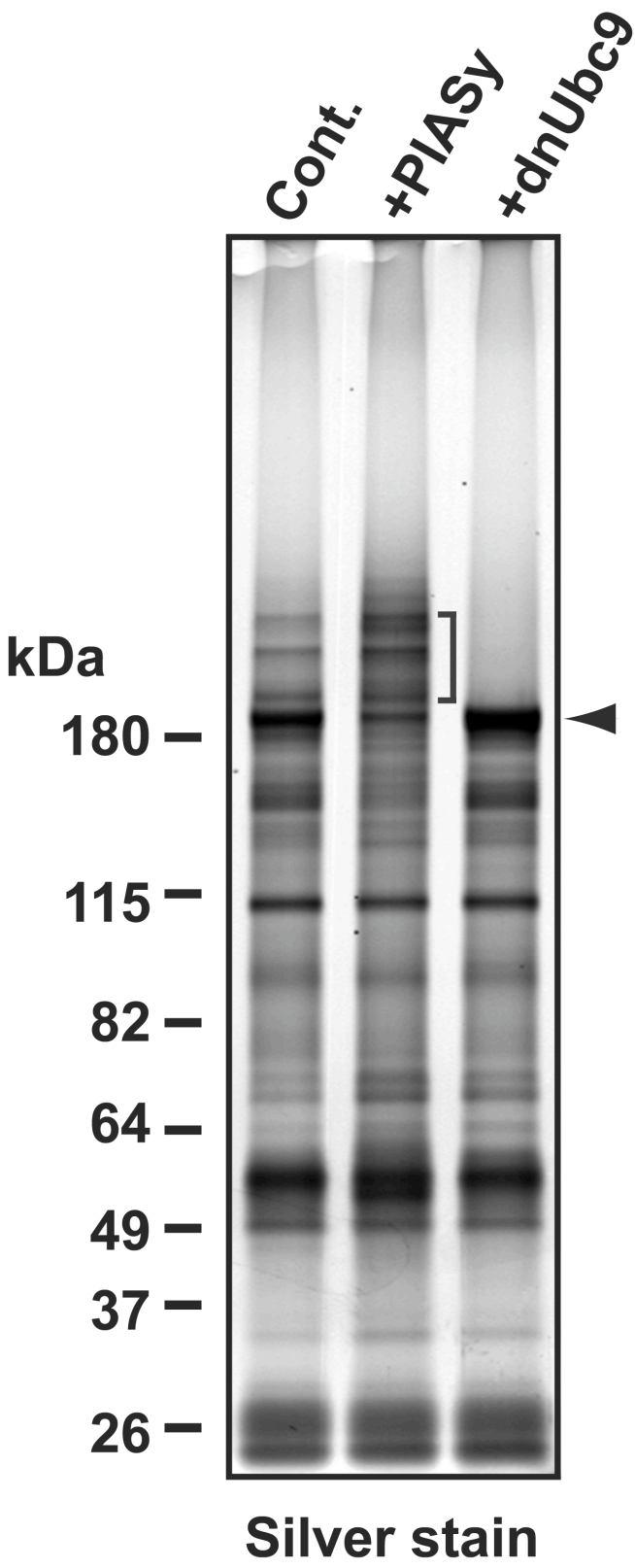
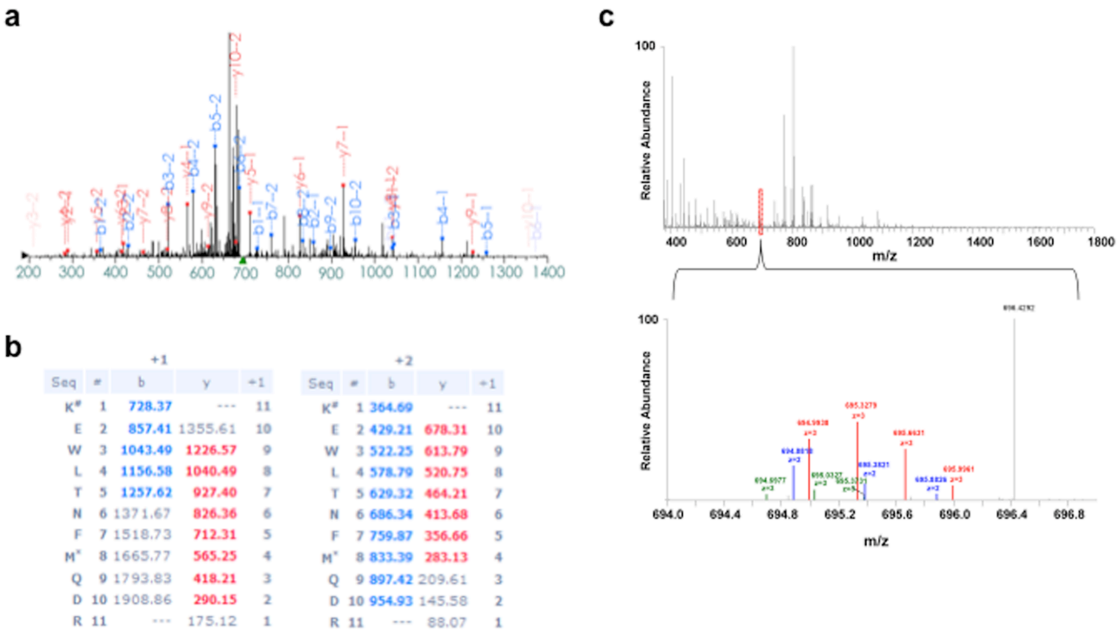

IP by α-TopoIIα antibody
Cont.
+PIASy
+dnUbc9
kDa
180
115
82
64
49
37
26
Silver stain

Figure 3. 5
Isolation of SUMOylated TopoIIα for mapping SUMOylation site(s) by Mass spectrometry. Chromosomal proteins were isolated from mitotic chromosomes that were assembled in CSF extracts (Cont.), CSF extracts with exogenous PIASy (+PIASy) or dnUbc9 (+dnUbc9) using SDS-PAGE sample buffer. After samples were renatured with buffer containing thesit, the extracted fractions were immunoprecipitated with affinity purified anti-TopoIIα antibody. Immuno-precipitated fractions were further separated by SDS-PAGE and silver stained (Owl/Daiichi). Both SUMOylated (bracket) and non-SUMOylated TopoIIα (arrow head) were subjected to MS/MS analysis: in brief, the samples were double digested with trypsin and chymotrypsin. The Chymotrypsin digestion provides the QQQTGG signature tag on the modified lysine. The MS/MS analysis, with around 50% TopoIIα sequence coverage, indicated lysine 660 as a candidate site for SUMOylation (detailed LC-MS/MS analysis data is shown in Figure 3.6).



		+1			+2			
Seq	#	b	y	+1	b	y	+1	
K#	1	728.37	---	11	364.69	---	11	K#
E	2	857.41	1355.61	10	429.21	678.31	10	E
W	3	1043.49	1226.57	9	522.25	613.79	9	W
L	4	1156.58	1040.49	8	578.79	520.75	8	L
T	5	1257.62	927.40	7	629.32	464.21	7	T
N	6	1371.67	826.36	6	686.34	413.68	6	N
F	7	1518.73	712.31	5	759.87	356.66	5	F
M*	8	1665.77	565.25	4	833.39	283.13	4	M*
Q	9	1793.83	418.21	3	897.42	209.61	3	Q
D	10	1908.86	290.15	2	954.93	145.58	2	D
R	11	---	175.12	1	---	88.07	1	R

Figure 3. 6
SUMOylation of TopoIIα at K660 was identified by LC-MS/MS following trypsin/chymotrypsin double digest. a-b) Precursor of 695.33 m/z was isolated and fragmented, with a series of b- and y- ions matching the sequence K#EWLTNFM*QDR. Asterisk refers to oxidized methionine. The SUMO2/3 sig-

nature of QQQTGG is denoted by #. The measured m/z of the monoisotopic precursor ion (694.9938) matched the expected within 0.3 ppm. c) Multiple ion species, denoted by interlaced isotopic envelopes (blue, green) were found co-eluting within the isolation window of the SUMOylated TopoIIα peptide (red).

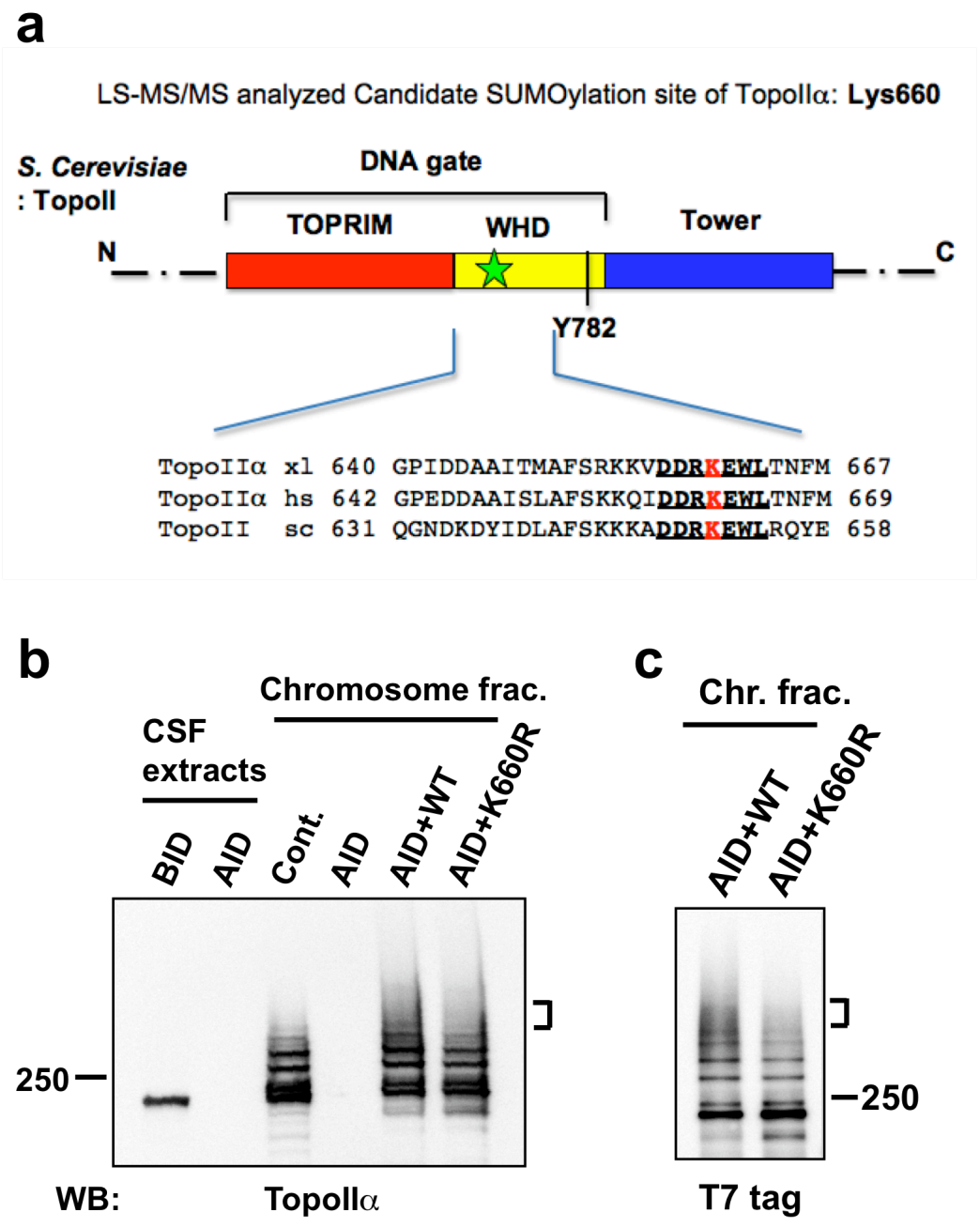


Figure 3. 7

TopoIIα K660R, a candidate SUMOylation mutant, shows incomplete SUMOylation in XEE. (**a**) Schematic diagram of *S. cerevisiae* TopoIIα primary structure. Domains are denoted by color. (Modified from (99)). TOPRIM: Topoisomerase-primase fold domain, WHD: Winged-helix domain, Tower: Adjacent domain to WHD, black bar indicates the catalytic tyrosine (Y782) for DNA cleavage in the WHD domain. Lys660 in *X. laevis* TopoIIα was designated as a potential SUMOylation site by mass spectrometric analysis. The approximate position of the candidate lysine is shown by a green star in the DNA-gate domain of TopoIIα. The sequences near TopoIIα Lys660 from *X. laevis* (xl), *homo sapiens* (hs), and *S. cerevisiae* (sc) are conserved. (**b**) XEE were immunodepleted using non-specific IgG (Cont.) or an anti-TopoIIα antibody (-Topo). Efficiency of TopoIIα depletion was confirmed by comparison of mock-depleted (Cont.) to TopoIIα-depleted (-Topo) CSF extracts (left two lanes, labeled CSF extracts). Wild type non-tagged TopoIIα (WT) or mutant TopoIIα, with substitution of arginine for Lys660 (K660R) was added to the TopoIIα-depleted extracts (-Topo). After 1hour incubation at 25°C, mitotic chromosomes were isolated and analyzed by anti-TopoIIα Western blot. Analyzed chromosome samples were from mock-depleted (Cont.), TopoII-depleted (-Topo) and recombinant TopoIIα added-back extracts (-Topo+WT or –Topo+K660R). (**c**) Same examination as in (b) except that the recombinant TopoIIα proteins had a T7-tag at the N-terminus. Both non-tagged and T7-tagged K660R mutant showed subtle but reproducible reduction in higher shifted bands (indicated by bracket) of SUMOylation compared to WT.


TopoIIα
WT
K660R
250
130
95
72
55
36
28
17

Figure 3. 8
The purity of TopoIIα WT and K660R proteins. Recombinant proteins of TopoIIα was expressed and purified as described in *METHODS*, and 200ng of each protein was resolved on a 8-16% gradient gel and coomassie blue stained to determine purity of the proteins. WT and K660R show comparable quality.

To confirm the mapping result, recombinant wild type TopoIIα (WT) or TopoIIα with an arginine substitution for Lys660 (K660R) were prepared as described in *METHODS,* and the purity of these recombinant proteins was confirmed, as shown in Figure 3.8. Add-back experiments of the recombinant proteins to TopoIIα -immunodepleted XEE indicated that the K660R mutant exhibited a slight reduction in higher shifted species of SUMOylated TopoIIα compared to that of WT. Although major SUMOylations still occurred (Fig. 3.7b and c), using both an untagged (Fig. 3.7b) and T7-tagged (Fig. 3.7c) recombinant TopoIIα, K660R showed a reproducible deficiency in generating SUMOylation represented by higher shifted bands. In summary, Lys660 is a SUMOylation site of TopoIIα associated with mitotic chromosomes in XEE, and other SUMOylation sites remain to be identified.

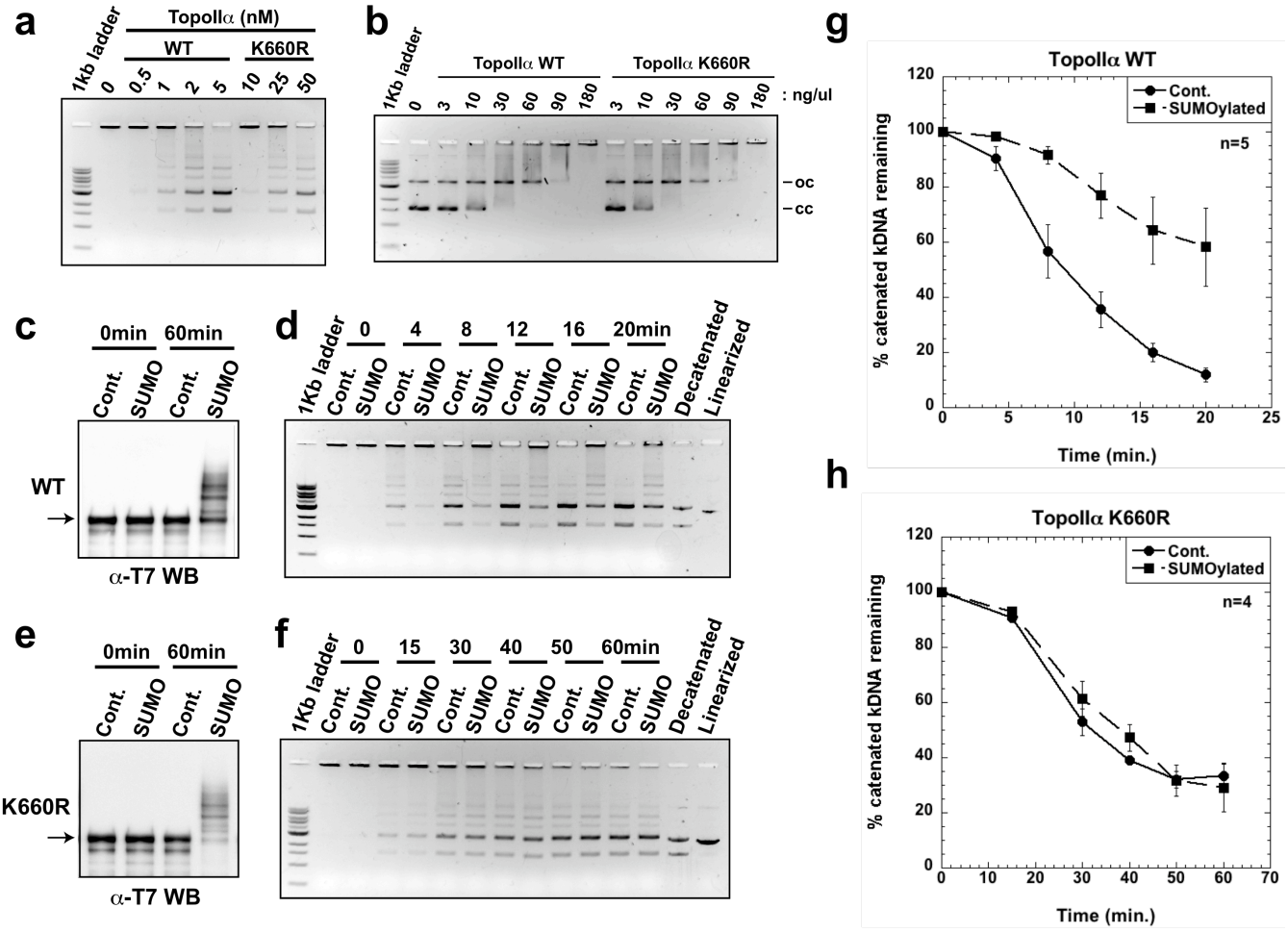


Figure 3. 9

The elimination of TopoIIα SUMOylation at Lys660 blocks SUMOylation-dependent inhibition of TopoIIα activity. (**a**) unmodified TopoIIα WT and K660R proteins were incubated with kDNA to determine relative activity. K660R had approximately 20 times less activity than WT. (**b**) Electrophoretic mobility shift assay. Unmodified TopoIIα WT and K660R were incubated with plasmid DNA to determine relative DNA binding affinity. Both WT and K660R displayed similar binding affinity to DNA. Oc and cc stand for open and closed circle, repectively. (**c, e**) The TopoIIα WT and K660R were SUMO2-modified *in vitro* with 60nM of Ubc9 and 30nM of PIASy. Control reactions (Cont.) using the same condition except for SUMO2-G were also performed. Non- or SUMOylated TopoIIα samples were assayed for decatenation activity. (**d, f**). Representative results of decatenation activity assays with TopoIIα WT (**c**) and K660R (**f**) are shown. The average decatenation activity from five independent experiments with TopoIIα WT (**g**) and four independent experiments with TopoIIα K660R (**h**) are displayed as % catenated kDNA remaining, with standard error. The strong inhibition of TopoIIα decatenation activity by SUMOylation was abolished in reactions using TopoIIα K660R.

Lack of TopoIIα SUMOylation at Lys660 abolishes SUMOylation-dependent inhibition of TopoIIα activity.

Available X-ray crystal structure information for TopoIIα indicates that Lys660 faces DNA (113). Therefore, we predicted that alteration of Lys660 would affect TopoIIα activity. Indeed, when we compared the decatenation activity of recombinant, unSUMOylated WT and K660R TopoIIα, K660R was approximately 20 times less active than WT (Fig. 3.9a). Yet, gel mobility shift assays showed that both WT and K660R bind to DNA with similar affinity (Fig. 3.9b), suggesting lower decatenation activity of K660R is not simply due to the deficiency of DNA binding. Given that the relatively minor alteration, substituting Lys660 with arginine, reduces TopoIIα decatenation activity, we speculated that SUMO conjugation of Lys660 might have a significant impact on the activity of TopoIIα.

To test this, TopoIIα WT and K660R were applied to the *in vitro* SUMOylation-decatenation coupled assay. For SUMOylation reactions (Fig. 3.9c, e), we used 60/30 nM of Ubc9/PIASy to better observe the inhibition of TopoIIα WT decatenation activity by SUMOylation and the potential alteration in the SUMOylation-dependent regulation of TopoIIα activity for the K660R mutant. Consistent with our earlier results, the decatenation activity of TopoIIα WT was efficiently inhibited by SUMO modification (Fig. 3.9d, g). On the other hand, it was striking that the decatenation activity of TopoIIα K660R was no longer inhibited by SUMO modification (Fig. 3.9f, h) despite the fact that both TopoIIα WT (Fig. 3.9c) and K660R mutant (Fig. 3.9e) were robustly modified by SUMO2. Similar results were

obtained using different tagged TopoIIα WT and K660R for the analysis (Figure 3.10). These results suggest that PIASy-mediated SUMOylation on Lys660 have a crucial role in the regulation of TopoIIα activity.

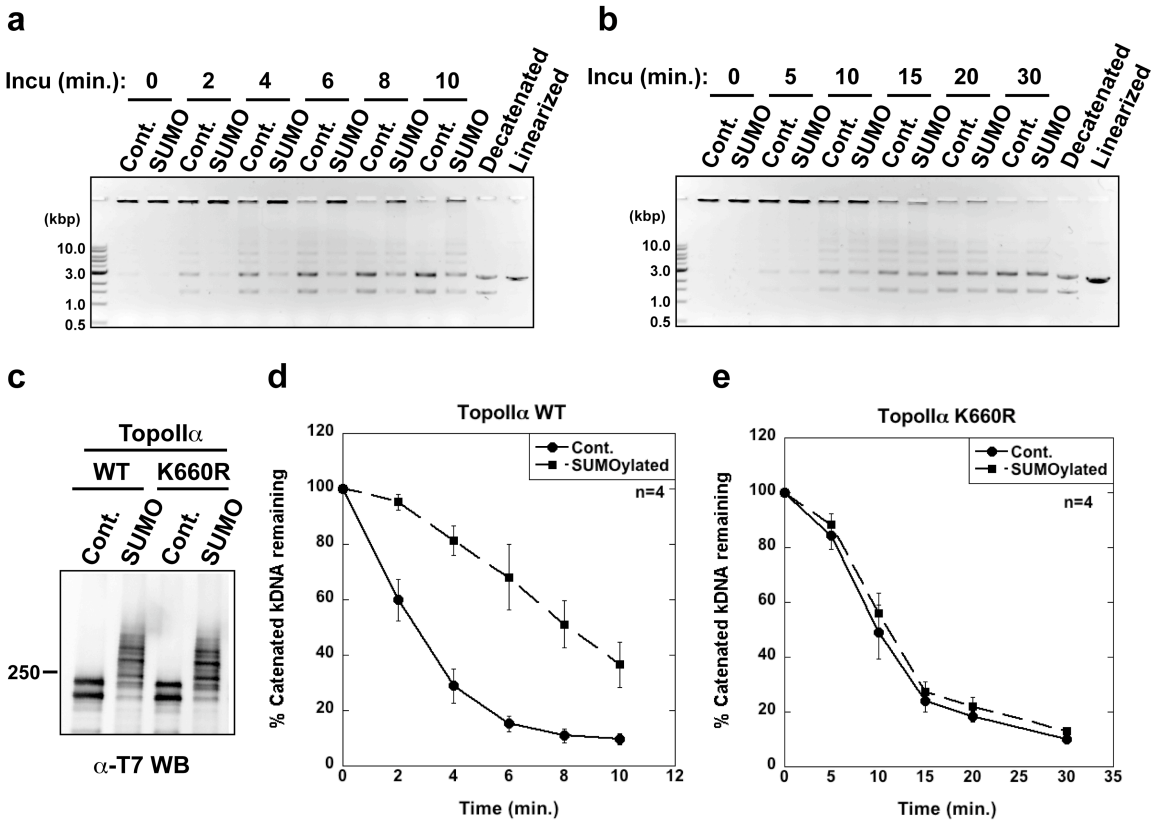
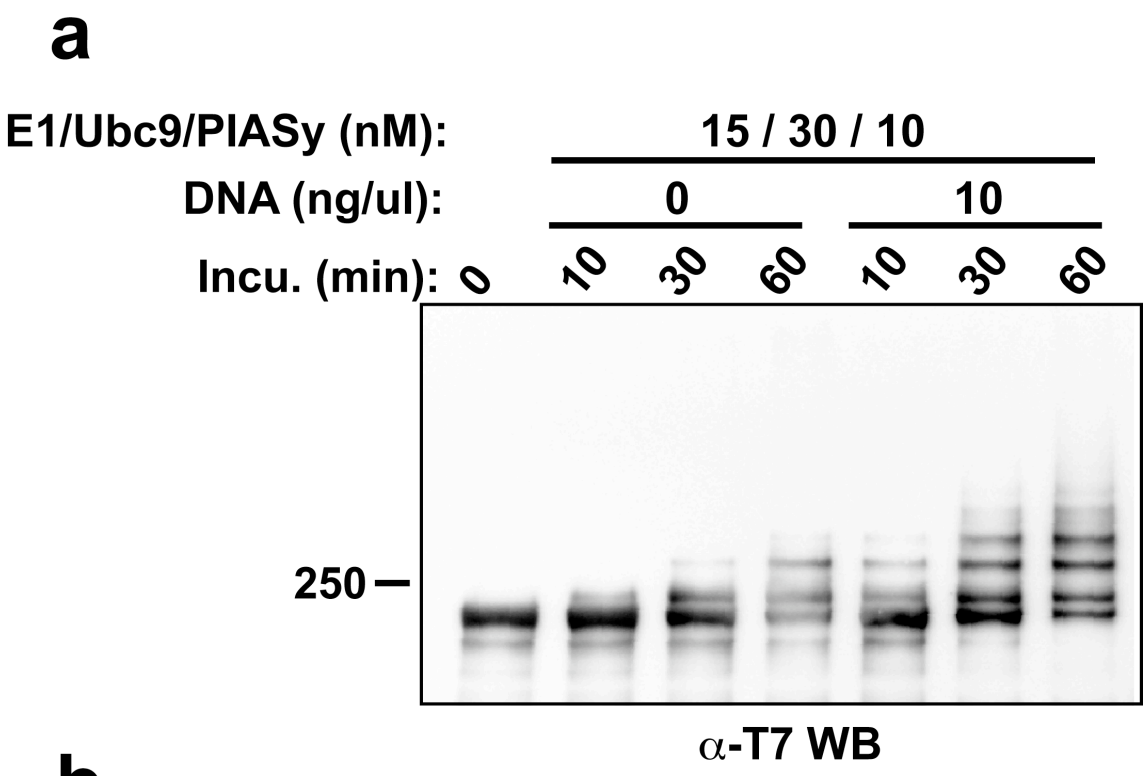


Figure 3. 10

The elimination of the SUMOylation at K660 abolishes the SUMOylation-mediated inhibition of TopoIIα activity. TopoIIα proteins that were fused to T7-tag and ZZ-tag at the N- and C-terminus, respectively, were purified by ZZ-affinity using IgG sepharose column (GE healthcare). The ZZ-tag was removed by Pre-Scission protease (GE healthcare). Further purified T7-TopoIIα was applied to the SUMOylation-decatenation coupled assay as in Figure 5. (**a**) Representative result of SUMOylation-decatenation coupled assay with TopoIIα WT. (**b**) Representative result of SUMOylation-decatenation coupled assay with TopoIIα K660R. (**c**) Representative samples of *in vitro* SUMOylated TopoIIα WT and K660R. *In vitro* samples were analyzed by western blot for T7 tag. Control (Cont.) contained SUMO2-G instead of SUMO2-GG. (**d, e**) Results from four independent experiments performed as in **a** are presented as average of % catenated DNA remaining with standard error. The different preparations of TopoIIα showed the same results as in Fig. 3.9.

SUMOylation of TopoIIα Lys660 is regulated by DNA.

Based on X-ray crystalographic structure analysis, TopoIIα Lys660 lies near the DNA backbone and appears not easily accessible for SUMOylation (113). We speculated that this limited accessibility might correlate with our inability to see a dramatic difference in the *in vitro* SUMOylation profile of WT and K660R TopoIIα, even though we observed subtle but reproducible reduction of K660R TopoIIα SUMOylation in XEE assays (Fig. 3.7b,c). We suspected that when TopoIIα binds to DNA it changes conformation, making it susceptible to SUMOylation of Lys660 in the XEE assay. To examine this hypothesis, we performed *in vitro* SUMOylation assays with WT TopoIIα in the presence or absence of DNA. As shown in Figure 3.11a, WT TopoIIα was extremely susceptible to SUMOylation in the presence of DNA. Addition of DNA to the *in vitro* reaction increased the amount of TopoIIα SUMOylation as well as the rate of modification, such that the amount of SUMOylated WT TopoIIα after one hour incubation without DNA was comparable to that formed after 10 minutes with DNA (Fig. 3.11a).


a
E1/Ubc9/PIASy (nM):
15 / 30 / 10
DNA (ng/ul):
0
10
Incu. (min):
0
10
30
60
10
30
60
250
α-T7 WB

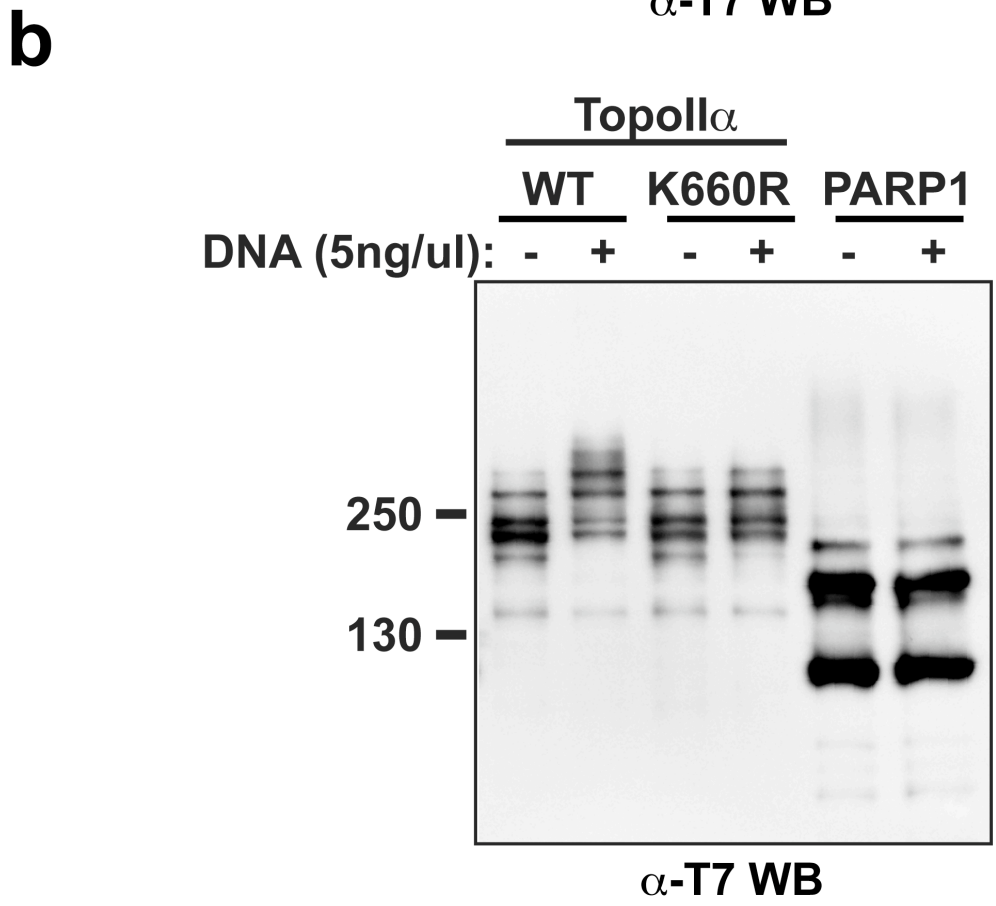

b
TopoIIα
WT
K660R
PARP1
DNA (5ng/ul):
-
+
-
+
-
+
250
130
α-T7 WB

Figure 3. 11
DNA binding of TopoIIα increases susceptibility of SUMOylation at Lys660. (**a**) TopoIIα WT *in vitro* SUMOylation reactions were performed with or without DNA. The samples were analyzed with anti-T7 tag antibody Western blots. The presence of DNA in the SUMOylation reactions significantly stimulates TopoIIα WT SUMOylation. (**b**) TopoIIα WT and TopoIIα K660R were subjected to *in vitro* reactions under the same condition as in (a) except for using 5ng/μl of DNA. PARP1, a mitotic chromosomal SUMO2/3 substrate, was used as control. A deficiency of TopoIIα K660R SUMOylation is observed in the presence of DNA compared to TopoIIα WT.

Since both TopoIIα and PIASy can bind DNA, we also considered the possibility that DNA acts as an adaptor to increase the binding affinity of TopoIIα and PIASy, leading to acceleration of SUMOylation. However, because the SUMOylation of PARP1, another chromosomal substrate of SUMO2/3 found in XEE (10), is barely affected by the addition of an equivalent amount of DNA (Fig. 3.11b), this possibility seems unlikely, supporting the idea that DNA-dependent enhancement of TopoIIα SUMOylation results from exposure of a SUMOylation site(s) by DNA binding. Remarkably, we found that the K660R mutant displayed a reduction of SUMOylation in the presence of DNA, albeit there was no significant difference in the SUMOylation profile between WT and K660R in the absence of DNA (Fig. 3.11b). Considering the comparable DNA binding affinity of both proteins (Fig. 3.9b), therefore, it is reasonable to conclude that the alteration of TopoIIα conformation by DNA binding (32,113,114) increases the efficiency of TopoIIα SUMOylation. Together, our results suggest that there is a SUMOylation site(s) of TopoIIα whose availability for SUMOylation depends on the conformational change of TopoIIα resulting from DNA binding and that the SUMOylation of

Lys660, which plays a key role in SUMOylation-dependent TopoIIα inhibition, is one of those sites.

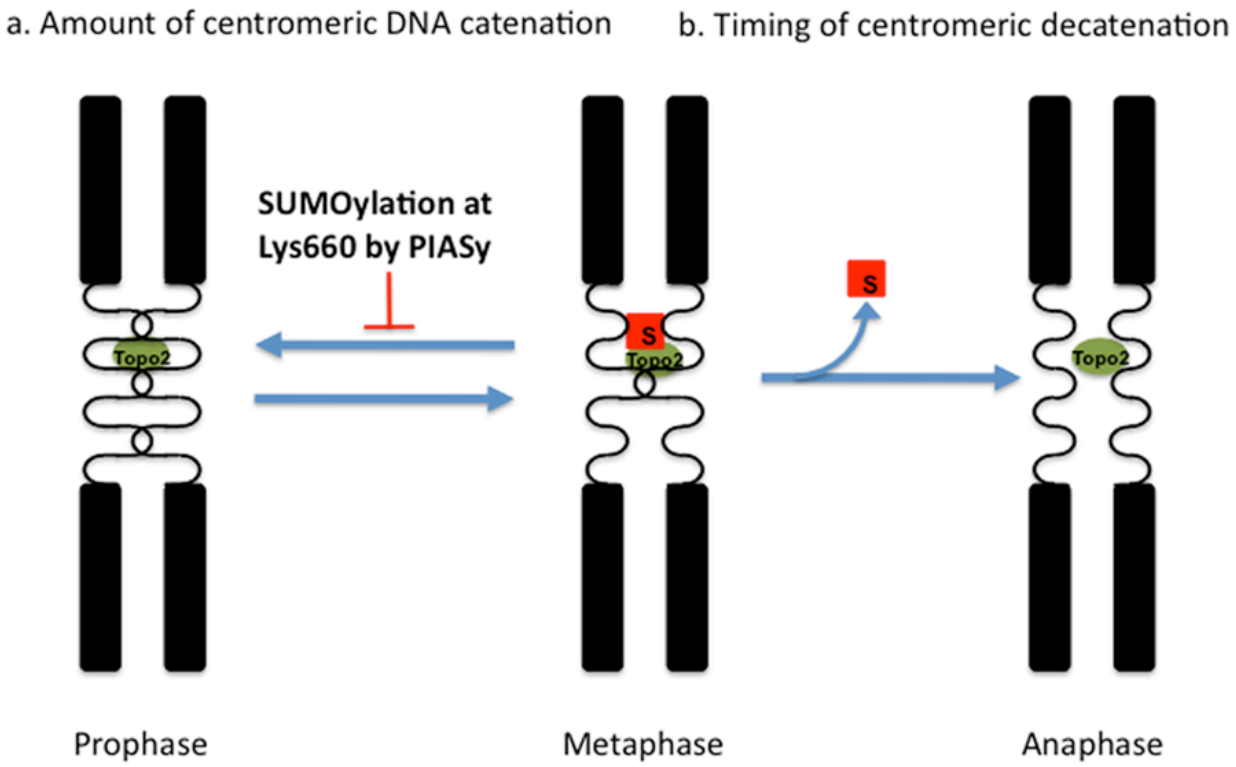


Figure 3. 12
Implications of SUMOylation in regulating the resolution of centromeric DNA. (**a**) Regulating amount of catenated centromeric DNA. Active TopoIIα resolves catenated DNA at the centromere and SUMOylation reduces the activity of TopoIIα which has completed the decatenation of DNA. Without SUMOylation, overly active TopoIIα could recatenate DNA at the centromere. (**b**) Regulation of timing of decatenation. TopoIIα SUMOylation keeps centromeric TopoIIα temporally inert until anaphase, when decatenation of centromeric DNA must take place. Without proper deSUMOylation of TopoIIα, decatenation of centromeric DNA will be compromised.

Discussion

The central achievement of this paper is finding the SUMOylation-dependent regulation of TopoIIα activity. We previously reported that SUMOylation of TopoIIα did not significantly alter its enzymatic activity when using TopoIIα-containing fractions from XEE (71). However, we anticipated a potential role for SUMOylation in the regulation of TopoIIα activity, based on the difficulty separating SUMOylated TopoIIα associated with centromeres from unSUMOylated TopoIIα associated with other chromosome regions. The *in vitro* SUMOylation assay established for the current study allowed us to overcome the time and space obstacles of SUMOylation of TopoIIα, and thus to reexamine the possible role of SUMOylation in regulating TopoIIα activity during mitosis. Our assay clearly reveals that SUMOylation significantly inhibits the decatenation activity of TopoIIα and that this inhibition is correlated with the TopoIIα SUMOylation profile. That is, the existence of the highest shifted species of SUMOylated TopoIIα, which appear when relatively high concentration of enzymes (Fig. 3.4a) or DNA are added to the reactions (Fig. 3.11a), correlates with strong inhibition of TopoIIα activity.

The identification of Lys660 as a SUMOylation site further supports the premise that TopoIIα activity is SUMOylation dependent. Lys660 is located within the DNA-gate, which plays a key role in manipulation of double helical DNA strands (99,113). Considering the importance of this region for TopoIIα function, it was predictable that even slight alteration of Lys660 would impact the activity of

TopoIIα. Supporting this idea, replacement of Lys660 with arginine resulted in a substantial reduction of the TopoIIα decatenation activity (Fig. 3.9a), although no loss of DNA binding is observed (Fig. 3.9b). Notably, the abolition of SUMO modification at Lys660 eliminated the SUMOylation-dependent inhibition of TopoIIα decatenation activity. The interpretation of this result is obviously limited by the lower catalytic activity of the Lys660 mutant, which might render partial inhibition due to SUMOylation of other sites of TopoIIα imperceptible. Therefore, detailed kinetic analyses of TopoIIα reactions, combined with analysis of other currently unidentified SUMOylation site(s) will be necessary to clarify the function of each TopoIIα SUMOylation site.

We further observed that Lys660 SUMOylation is enhanced in the presence of DNA, and this is likely due to exposure of the Lys660 site during the catalytic action of TopoIIα (Fig. 3.11b). The hypothesis that an active TopoIIα conformation makes Lys660 more susceptible to SUMOylation is supported by an earlier report that etoposide (or VP16) treatment of human cells induces hyperSUMOylation of TopoIIα at the centromere (115). Etoposide immobilizes TopoIIα in an intermediate structure with cleaved DNA (116), thus potentially exposing a SUMOylation site(s) as shown in Figure 11. Taken together, our results strongly suggest that SUMOylation of Lys660 is responsible for the control of decatenation activity. However, it is possible that other TopoIIα SUMOylation sites contribute to changes in TopoIIα activity.

The IR-domain of RanBP2 possesses SUMO E3 ligase activity *in vitro*, and studies in RanBP2-deficient mouse embryonic fibroblasts (MEFs) have impli-

cated RanBP2 in SUMO1 conjugation of TopoIIα (106). We found that the IR-domain of RanBP2 could enhance TopoIIα SUMOylation *in vitro* (data not shown), but paralogue specificity and SUMOylation site selection under these conditions were different from TopoIIα SUMOylation in XEEs. There are a number of other findings that argue against a role of RanBP2 as the primary E3 enzyme for TopoIIα in mitosis: First, the addition of Nocodazole, which disrupts the localization of RanBP2 from the centromere (117), does not eliminate SUMOylation of TopoIIα (107). Second, while Dawlaty et al. (2008) found that RanBP2 promotes SUMO1 conjugation of TopoIIα, TopoIIα is exclusively conjugated to SUMO2/3 in XEEs unless ectopic SUMO1 is supplied (71). Finally, we observed that SUMO2/3 modification of TopoIIα on mitotic chromosomes was intact in RanBP2-immunodepleted XEEs unless PIASy was co-depleted (Fig. 3.1a), strongly suggesting that RanBP2 is dispensable. Additionally, endogenous TopoIIα, PIASy and SUMO2/3 co-localized at the centromeres of mitotic chromosomes (Fig. 3.1b), consistent with the notion that PIASy is the E3 enzyme for TopoIIα SUMOylation at this site. By contrast, RanBP2 localizes to the outer kinetochore (117). It is possible that the discrepancy between findings in XEEs and MEFs simply reflects the difference in experimental systems, and that different SUMO ligases mediate TopoIIα conjugation in mice and frogs. Alternatively, we have recently shown that centromeric SUMOylation results from precise localization of PIASy and its substrates (118). RanBP2 might indirectly affect the SUMOylation of TopoIIα by regulating the localization of TopoIIα centromeres in MEFs through a mechanism that is not used in XEEs. The failure to localize

TopoIIα could thus impair its subsequent PIASy-dependent SUMO2/3 modification.

Our findings that 1) PIASy colocalizes with TopoIIα at the centromere where enzymatically active TopoIIα is thought to accumulate (33), 2) the DNA-bound form of TopoIIα is more susceptible to SUMOylation on Lys660, and 3) the SUMOylation of Lys660 inhibits TopoIIα activity, lead us to propose that PIASy-dependent SUMOylation of TopoIIα regulates centromeric catenation. In this model, when SUMOylation is depressed, overly active TopoIIα leads to DNA re-catenation at the centromeres where sister chromatids are highly compact and close to each other. With proper SUMOylation, active TopoIIα is rendered temporarily inert to prevent re-catenation (Fig. 3.12a), and so only the proper amount of catenated DNA remains avoiding early disjunction of sister chromatid before anaphase. This model explains why the perturbation of SUMOylation by either the elimination of PIASy or addition of dnUbc9 causes abnormal chromosomal segregation as represented by anaphase bridges, which could be the result of hypercatenation of centromeric DNA (8,71). The model also explain why depletion of PIASy in HeLa cells produces cohesin-independent sister chromatids cohesion (73).

There are several pieces of evidence that support a requirement of TopoIIα activity for proper anaphase execution. According to a recent study of PICH-positive (Plk1-interacting checkpoint helicase) DNA threads, centromeric DNA catenation was resolved at the onset of anaphase (119). Wang *et al*. also demonstrated that TopoIIα decatenates centromeric DNA after removal of the cohe-

sin complex (98). Lastly, TopoIIα SUMOylation is highly dynamic, with TopoIIα heavily modified by SUMO during metaphase and the rapid disappearance of modified TopoIIα at the onset of anaphase (71). In this context, it is possible that deSUMOylation of TopoIIα regulates the timing of resolution of the catenated DNA at the centromere at the onset of anaphase. At anaphase, when the centromeres of sister chromatids are distal enough, deSUMOyation of TopoIIα allows the preferential decatenation of the last tangled sister chromatids. As such, we further propose that deSUMOylation of TopoIIα is critical to control the timing of the final decatenation at anaphase (Fig. 3.12b). Extensive analysis using specific deSUMOylation enzymes of TopoIIα must be performed to directly test this hypothesis.

In summary, our finding that TopoIIα activity is inhibited by PIASy-mediated SUMOylation allows us to answer a long-standing question of how the catalytic activity of TopoIIα is tightly regulated in a space- and time-dependent manner. Future studies using somatic cells to observe consequential phenotypes caused by SUMOylation-deficient TopoIIα and an examination of specific deSUMOylation mechanisms of TopoIIα will strengthen the functional significance of SUMO-modified TopoIIα .

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Contributions

Dr. Maiko Furuta contributed to Figure 3.1A. Dr. Yoshiaki Azuma contributed to Figure 3.5. Dr. Donald Kirkpatrick contributed to Figure 3.6. All other figures are my work.

CHAPTER 4

Rod/Zw10 complex is required for PIASy-dependent centromeric SUMOylation.

Abstract

SUMO conjugation of cellular proteins is essential for proper progression of mitosis. PIASy, a SUMO E3 ligase, is required for mitotic SUMOylation of chromosomal proteins, yet the regulatory mechanism behind the PIASy-dependent SUMOylation during mitosis has not been determined. Using a series of truncated PIASy proteins, we have found that the N-terminus of PIASy is not required for SUMO modification *in vitro* but is essential for mitotic SUMOylation in *Xenopus* egg extracts. We demonstrate that swapping the N-terminus of PIASy protein with the corresponding region of other PIAS family members abolishes chromosomal binding and mitotic SUMOylation. We further show that the N-terminal domain of PIASy is sufficient for centromeric localization. We identified that the N-terminal domain of PIASy interacts with the Rod/Zw10 complex, and immunofluorescence further reveals that PIASy colocalizes with Rod/Zw10 in the centromeric region. We show that the Rod/Zw10 complex interacts with the first 47 residues of PIASy which were particularly important for mitotic SUMOylation. Finally, we show that depletion of Rod compromises the centromeric localization of PIASy and SUMO2/3 in mitosis. Together, we demonstrate a fundamental

mechanism of PIASy to localize in the centromeric region of chromosome to execute centromeric SUMOylation during mitosis.

Introduction

SUMOylation is a protein modification process conserved from yeast to vertebrates (5). The consequences of SUMO (**s**mall **u**biquitin like **mo**difier) modification that have been elucidated over the past decade include modulation of gene transcription, DNA repair, protein translocation, protein/protein interaction, chromosomal organization and sister chromatid segregation (2,6,41). Vertebrates have three SUMO isoforms and all three display roughly 50% identity with the single SUMO found in yeast (5). SUMO is conjugated to cellular substrates by an analogous pathway to that of ubiquitin. It has been reported that SUMOylation is mediated without E3 ligases *in vitro* (88,120), but under physiological conditions, SUMO E3 ligases are essential to execute SUMOylation of cellular substrates (8,10,11,106). There are mainly two types of SUMO E3 ligases in vertebrates, RanBP2 (Nup358) and Siz/PIAS. RanBP2 has no homolog in yeast and its ligase function is independent of either HECT or Ring finger type ubiquitin E3 ligases (55). Siz/PIAS, on the other hand, initially identified in budding yeast, functions similar to Ring finger ubiquitin ligases (11). Vertebrates have four PIAS proteins (PIAS1, PIAS3, PIASx, and PIASy) that share important conserved functional domains (70). The SAP (scaffold attachment factor-A/B, acinus and PIAS) domain is positioned at the N-terminus, and directly binds AT-rich regions of DNA (58,62,121). The SP-Ring domain is related to that of ubiquitin E3 ligase and is

responsible for Ubc9 recruitment (90). The SUMO-interacting motif (SIM) is situated after the SP-Ring and redirects the Ubc9~SUMO complex on substrate proteins, potentially contributing to SUMO paralogue specificity (60,122). Domain analysis of Siz protein *in vitro* and *in vivo* suggests each domain contributes to SUMOylation with distinct functions. The N-terminal domain of Siz1 is involved in substrate recognition and the C-terminal domain in cell-cycle dependent localization, which is critical for septin SUMOylation (123). Whether vertebrate PIAS proteins are organized in a similar manner is unknown.

Among PIAS family members, we have identified PIASy as crucial for SUMO2/3 modification of chromosome-associated proteins in mitosis (8). For example, DNA Topoisomerase IIα (TopoIIα) and Poly [ADP-ribose] polymerase I (PARP1) are each modified by SUMO2/3 in a PIASy-dependent manner during mitosis (10,71). Immunodepletion of PIASy completely abolishes mitotic chromosomal SUMOylation in *Xenopus* egg extracts (XEE) and other PIAS family proteins fail to restore this defect, indicating a unique role of PIASy in mitotic chromosomal SUMOylation in XEE (8). Our initial domain analysis with mutated PIASy suggested that the N-terminal domain of PIASy is required for its association with mitotic chromosomes (8). The SUMO2/3 modification of chromosomal proteins is restricted not only to the early stages of mitosis but also to the centromeric regions, raising the question of how PIASy regulates mitotic SUMOylation in a temporal and regional manner (8,10). Recent immunostaining has elucidated that PIASy is exclusively localized to centromeric regions and colocalizes with TopoIIα during mitosis, suggesting that the centromeric localization of PIASy is

critical for the spatiotemporal regulation of mitotic SUMOylation (Ryu et al. JCB In print). However, the molecular mechanism underlying this localization has remained unidentified.

Centromeres are specified regions of DNA where kinetochores are assembled to capture growing microtubules from spindle poles in mitosis (124). Kinetochores include multiple proteins whose functions are involved in mitotic checkpoints directly or indirectly, and each component is absolutely required for the accurate progression of the cell cycle including proper chromosome segregation (16). Rod (**Ro**ugh **D**eal) and Zw10 (**Z**este **W**hite 10) are kinetochore proteins in higher eukaryotes (21). As a stable complex called RZZ (**R**od/**Z**w10/**Z**wilch), Rod and Zw10 are localized to the kinetochore until anaphase commences (125). Rod and Zw10 are involved in mitotic checkpoint by recruiting Mad1/Mad2 and dynein/dynactin onto unattached kinetochores (21,25,126). Mutation of Rod, Zw10 or both Rod and Zw10 result in improper chromosome alignment and sister chromatid missegregation in *Drosophila* (127-129). Somatic mutations in Rod and Zw10 genes have been found in human colorectal cancers, implicating the complex in the progression of cancer (130).

To determine how PIASy distinctively executes mitotic SUMOylation in a spatiotemporal manner, we have identified the mechanism of PIASy recruitment onto mitotic chromosomes. Using a series of purified PIASy truncations, we found that the N-terminal region of PIASy is vital for chromosome localization and for consequent mitotic SUMOylation but is dispensable for the catalytic activity *in vitro*. Additionally, we demonstrated the ability of a PIASy N-terminal peptide to

interact with chromosomes. Chimeric PIASy containing the N-terminal domain of other PIAS family proteins is defective in chromosome interaction. A PIASy N-terminal peptide fused with mCherry is localized to the centromeric region. Immunofluorescence microscopy combined with biochemical analysis and mass spectrometry revealed that Rod and Zw10 are unique binding proteins of PIASy N-terminus among PIAS family proteins. Finally, we show that depletion of Rod proteins from XEE causes mis-localization of PIASy as well as loss of SUMO2/3 foci on the chromosomes. Taken together, these data reveal that PIASy is recruited onto the centromeric region of chromosomes through interaction of the PIASy N-terminus with the Rod/Zw10 kinetochore complex and this results in specific SUMOylation of the centromeric region.

Methods

Plasmids construction.

The C-terminal portion, encoding amino acids 970~1060, of Rod DNA was cloned from *Xenopus laevis* tadpole cDNA (kindly provided by Drs. A. Arnaoutov and M. Dasso) using PCR amplification. For recombinant protein production, the C-terminal fragment of Rod cDNA was subcloned into pET28a and pGEX4T-1 with BamHI/XhoI restriction enzyme sites. PIASy truncations, shown in Figure 1 and 5 were produced by PCR and subcloned into the pET28a vector using EcoRI/XhoI restriction sites. N-terminal fragments of PIAS family DNAs were amplified from original full-length cDNA of each PIAS gene (8) with additional EcoRI sites at 5' end and HinDIII sites at 3'end, respectively, and were subcloned

into the pET30a vector for recombinant protein production. For N-terminal swapped chimeric constructs of PIASy, a DNA fragment of *Xenopus* laevis PIASy without the N-terminal domain (amino acids 142~501) was amplified by PCR creating HinDIII site at 5' end and XhoI site at 3' end, respectively, and subcloned into pET28a. Subsequently, the N-terminal fragments of each PIAS equivalent of 1~141 amino acids of PIASy was ligated to the PIASy C-terminal construct. In the case of the C-terminal PIAS swapped chimeric constructs (y-1, y-3 and y-x in Figure 2), C-terminal fragment of PIAS DNAs equivalent to 410-501 amino acid of PIASy were amplified with additional BglII site at 5' end and XhoI sites at 3'end, respectively. A DNA fragment of *Xenopus* laevis PIASy lacking the C-terminal domain (1- 410 amino acids) was obtained by digesting full-length PIASy in pET28a with EcoRI and BamHI. The C-terminal fragments of PIAS proteins and the PIASy (1-410) fragment were ligated into pET28a EcoRI/XhoI sites. For mCherry fusion of N-terminal fragments of PIASx and PIASy, PCR amplified mCherry cDNA (kindly provided by Dr. B. Oakley) with NotI and XhoI sites at 5' and 3' ends respectively was inserted into pET30a N-terminal PIAS constructs described above. cDNA of TopoIIα was subcloned into a pPIC 3.5K vector in which CBP-T7 Tag sequences were inserted (The CBP and ZZ TAP-tag plasmid was kindly provided from Dr. K. Gould). All constructs were verified by DNA sequencing.

Recombinant protein expression and purification, and preparation of antibodies.

For preparation of recombinant TopoIIα proteins, the plasmids were transformed into a GS115 strain of *Pichia pastoris* yeast and expressed according to the manufacturer's instructions (Invitrogen). Protein purification with CBP was performed according to TAP protocol (EMBL Heidelberg) with a slight modification of our needs. Briefly, frozen TopoIIα expressed in yeast cells were grinded with dried ice in a coffee mill and then mixed with lysis buffer (50mM Tris-HCl (pH7.5), 150mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 0.1% Triton-X100, 5% Glycerol, 1mMDTT and 10mM PMSF). After centrifugation at 25,000xg for 40 minutes, the supernatant was mixed with Calmodulin-sepharose resin (GE healthcare) for 90 minutes at 4°C to capture CBP-tagged TopoIIα. The resin was washed with lysis buffer, and TopoIIα was eluted with buffer containing 10 mM EGTA. The elution was further purified by Mono-Q anion-exchange chromatography (GE-healthcare).

For preparation of the antigen for anti-Rod antibody, Both GST fused and His-6 tagged C- terminal fragment of Rod was expressed in BL21 (DE3) and His6-tagged C-terminal Rod polypeptide was purified from inclusion bodies under denaturing conditions (6M Urea) following the manufacture's protocol (Clonethech). GST fused C-terminal Rod polypeptide was solubilized by Urea and directly conjugated to NHS-sepharose beads. All N-terminal fragments of PIAS family and mCherry fusion proteins were expressed in BL21 (DE3) or Rossetta2 (DE3) (EMD Biosciences) and purified with Talone metal affinity resin (Clonthech) followed by ion-exchange chromatography. Preparation of E1 com-

plex (Aos1/Uba2 heterodimer) (54), PIASy (8), Ubc9, and SUMO2 (82) were previously described.

Polyclonal antibodies against TopoIIα C-terminus (1358-1579) were prepared in rabbits by injection with recombinant His-T7 fused fragments, and affinity-purified with antigens as described previously (71). Anti-PARP1, Anti-PIASy and anti-SUMO2/3 antibodies used this study were reported previously (8,10). Anti-Aurora B antibody was kindly provided from A. Arnaoutov and M. Dasso. Anti-Zw10 monoclonal antibody was purchased from AbCam. HRP conjugated anti-T7 tag antibody and S protein were purchased from EMD Biosciences.

In vitro SUMOylation assay.

The reactions were performed as previously described (10). In brief, the reaction contained 15 nM E1 Uba2/Aos1 heterodimer, 40 nM E2 Ubc9, 20 nM PIASy, 5 mM SUMO2-GG, 500 nM T7-tagged TopoIIα or PARP1 and 2.5 mM ATP. Reaction buffer consisted of 20 mM HEPES (pH7.8), 100 mM NaCl, 5 mM $MgCl_2$, 0.05% Tween20, 5% glycerol, 1mM AEBSF and 1 mM DTT. The reactions were incubated at 25°C for 1 hour and stopped by addition of one half volume of 3× SDS-PAGE sample buffer. Samples were resolved on 8-16% Tris-HCl gradient gels (Invitrogen) by SDS-PAGE, and analyzed by Westernblot with HRP conjugated anti-T7 monoclonal antibody (EMD Biosciences) or other antibodies as indicated.

Xenopus egg extracts assays, pull-down, and immunoprecipitation.

Xenopus sperm chromatin and low speed cytostatic factor (CSF)-arrested XEE (CSF-XEE) or interphase XEE were prepared according to methods described by Kornbluth et al. (63) with slight modification (107). In order to obtain mitotic chromosomes for Westernblot analysis, 5000 sperm chromatin/ml was incubated with freshly prepared CSF-XEE for 50 min at room temperature. Chromosomes were isolated as previously described (107).

For pull-down assays from XEE, S-protein agarose beads (Novagen) or T7 antibody-conjugated agarose beads (Novagen) were incubated with S-tagged PIAS N-terminal proteins or T7-tagged PIASy truncations, respectively, as specified in figures 4 and 5 overnight at 4°C. Next day, protein-bound beads or non-protein-bound beads were blocked with 5% gelatin for 1 hour. CSF extracts were diluted with two volumes of IP extract buffer (20 mM NaPi pH7.8, 18 mM b-glycerol phosphate pH7.5, 5 mM $MgCl_2$, 50 mM NaCl, 5% Glycerol) followed by centrifugation at 25,000 xg for 20min at 4°C. Supernatants were mixed with an equal volume of ChIP buffer (20 mM NaPi, 18 mM b-glycerol phosphate, 50 mM NaCl, 5 mM $MgCl_2$, 5% Glycerol, 0.2% Tween 20, and 0.2% Triton X-100) and incubated with protein-bound or non-protein-bound beads for 2 hours at 25°C. Collected beads were washed thoroughly with ChIP buffer and PBS-T, and eluted in 1x SDS PAGE buffer. Samples were, then, resolved in 8-16% gradient gels (Invitrogen) by SDS-PAGE, and analyzed by Westernblot for the proteins indicated in figures 4 and 5.

Immunoprecipitation experiments were performed in a similar way to that described above. Briefly, protein A Dyna beads (Invitrogen) were incubated with

either IgG (Cont.) or antibody against PIASy C-terminus or Rod overnight at 4°C according to the manufacture's manual. Antibody-bound protein A beads were washed with PBS-T and blocked by 5% gelatin. CSF-XEE prepared as in the pull-down assay was incubated with the antibody-bound beads for 2 hours at 25°C. Collected beads were washed with ChIP buffer and PBS-T, and eluted in 1x SDS PAGE sample buffer. Samples were resolved in 8-16% gradient gels (Invitrogen) by SDS-PAGE, and analyzed by Westernblot for the proteins in figure 4.

Immunodepletion was performed in a similar way to that previously described (8). Briefly, antibody-bound Protein-A magnetic beads as above were blocked with CSF-XB buffer containing 5% BSA followed by mixing with freshly prepared CSF-XEE and depleted specified proteins described in Figures. For addback experiments, recombinant PIASy proteins or reticulocyte lysates expressing chimeric PIAS proteins were added to the PIASy-depleted CSF-XEE at a final concentration of 50 nM or 10% of the final reaction volume, respectively.

*Immunofluorescence analysis of chromosomes***.**

Immunofluorescence experiments were performed as previously described (8,10). In brief, CSF XEE was induced to undergo interphase by the addition of 0.6 mM $CaCl_2$. 500 sperm/ml were incubated to replicate chromatins for ~1 hour at 25°C. After checking the morphology of interphase nuclei, subsequently, mitosis was re-induced by the addition of one half volume of fresh CSF-XEE. For figure 4.3, mCherry-fused PIAS N-terminal proteins were supplemented

immediately before inducing mitosis at a final concentration of 50 nM. After additional incubation for 40 min at 25°C, reactions were diluted three times with dilution buffer and chromosomes were fixed by addition of an equal volume of 4% p-formaldehyde in dilution buffer. Samples were incubated for 5 min followed by spinning onto coverslips through a 35% glycerol cushion. After post-fixation with 1.6% paraformaldehyde, chromosome samples were subjected to immunostaining with indicated antibodies.

For immunofluorescence study with immunodepleted XEE (Figure 4.6), anti-Rod antibody was crosslinked to protein A-Dyna beads with DMP (Dimethyl Pimelimidate·2HCl) according to company's instructions (Pierce), then CSF-XEE was depleted with the crosslinked anti-Rod antibody-bound bead as described above. Immunofluorescence experiments were performed as described above. Anti-rabbit Alexa 568, anti-guinea pig Alexa 684, anti-mouse Alexa 488 and anti-chicken Alexa 488 (Invitrogen) were used as secondary antibodies to visualize primary antibodies and DNA was visualized with Hoechst 33342 (1mg/ml). Specimens were observed with Nikon TE2000-U microscope equipped with Plan Apo 100x/1.40 objective. Images were taken with a Retiga SRV CCD camera (Qimageing) operated by Volocity software (Improvision).

Results

The N-terminus of PIASy is dispensable for reconstituted SUMOylation assay *in vitro* but crucial for mitotic SUMOylation in *Xenopus* egg extract assays.

We have previously shown that PIASy is required for mitotic SUMOylation (8). Because mitotic SUMOylation is not fully restored by other PIAS proteins in PIASy-depleted XEE, it is clear that PIASy has a distinct role as an E3 ligase during mitosis among PIAS family members (8). Domain analysis further suggested that this specificity is likely due to the unique ability of the N-terminal domain of PIASy to mediate mitotic chromosome binding (8). However, how the N-terminal domain facilitates the chromosome interaction of PIASy was not determined. One of our hypotheses was that PIASy might locate to chromosomes by the interaction of its N-terminus with chromosomal substrates given that major substrates of PIASy, TopoIIα and PARP1, are chromosome-resident proteins (10,71). To address this question, we sought to examine whether N-terminal deletions of PIASy decrease SUMOylation activity in reconstituted SUMOylation assay *in vitro*. We constructed systematic truncations that lack a series of N-terminal region of PIASy (Figure 4.1A). Purified truncated proteins were applied to *in vitro* reactions for SUMO2 modification of TopoIIα or PARP1. We observed that PIASy displayed robust activity to SUMOylate each substrate until deleted for amino acids 1-127 (127C) and the activity was reduced by further truncation (287C), indicating that the interaction between PIASy and each substrate was not dependent on the N-terminal 127 amino acids of PIASy but on the PINIT domain (Figure 4.1B). This result also indicates that the N-terminal 127 amino acids, including the SAP

domain, is dispensable for PIASy activity *in vitro*. Interestingly, PIASy that lacks the first 47 amino acids reproducibly exhibited stronger SUMOylation activity than full length PIASy on TopoIIα but not on PARP1 (Figure 4.1B). Further investigation is required for a clear explanation of this phenomenon.

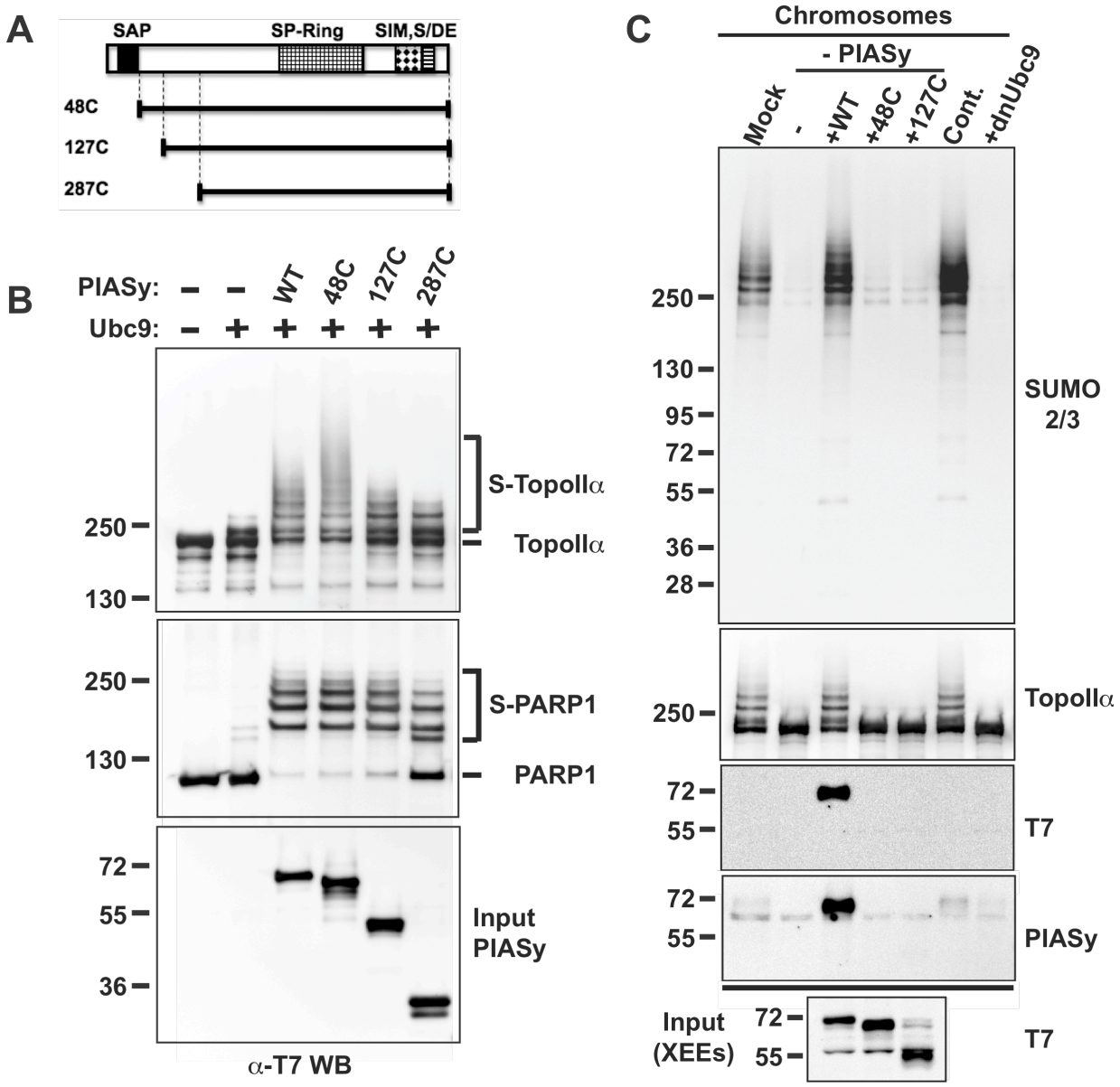

Figure 4. 1
N-terminus of PIASy is dispensable *in vitro* but essential for mitotic SUMOylation in *Xenopus* egg extract assays.
(A) Schematic diagram of PIASy truncations used in experiments in B and C. N-terminal deletions of PIASy are drawn to approximate scale and compared

to full length PIASy shown on the top. All truncation constructs and full length PIASy contain a T7-tag at the N-terminus. Conserved domains among PIAS family are indicated as follows: SAP (scaffold attachment factor-A/B, acinus and PIAS) in solid black, PINIT in vertical lines, SP-Ring (Siz/PIAS-Ring) in grid, SIM (SUMO-interacting motif) in diamond and S/DE motif in horizontal lines. **(B*) In vitro* SUMOylation assay using N-terminal truncated PIASy series.** All constructs in A were expressed and purified as in Experimental procedure. Either TopoIIa or PARP1 was incubated with PIASy and Ubc9 as indicated in the presence of E1 and ATP for 1hour at 25°C and samples were analyzed by Westernblotting with anti-T7 tag antibody, which detect recombinant TopoIIa and PARP1. SUMOylated and unmodified forms of proteins are specified by bracket and bar, respectively. The PIASy input is shown in the bottom panel. (**C) PIASy immunodepletion and addback experiments in *Xenopus* egg extracts (XEE).** CSF-XEE was immunodepleted using IgG (Mock) or anti-PIASy antibody. Purified PIASy truncation proteins were added to the depleted XEE and 5,000 sperm chromatin/ml was incubated in the reactions at 25°C for ~1hour. The reactions without (Cont.) and with dnUbc9 were also prepared for positive and negative control of chromosomal SUMOylation. Isolated chromosome fractions were resolved on SDS-PAGE gel and analyzed by Westernblot for indicated proteins. Input of PIASy proteins were analyzed in the bottom panel.

The function of the N-terminal domain of PIASy under physiological conditions was examined in a XEE cell free assay system. Cytostatic factor-arrested XEE (CSF-XEE) was immuno-depleted for PIASy and recombinant truncated PIASy proteins were used to supplement the extract in the presence of sperm chromatin. Elimination of over 95% of PIASy resulted in near abolition of mitotic SUMOylation, and the addition of recombinant PIASy wild type fully restored the SUMOylation (Figure 4.1C, lane 1, 2, 3 and 6). Consistent with our previous studies using reticulocyte lysate (8), truncated PIASy that lacks either 47 (48C) or 127 (127C) amino acids from the N-terminus completely failed to re-establish SUMOylation of mitotic chromosomal proteins (Figure 4.1C, lane 4 and 5). Only full-length PIASy but not PIASy with N-terminal truncations was able to interact with chromosomes (Figure 4.1C, T7 and PIASy Westernblot), verifying that the N-

terminus is involved in the recruitment of PIASy onto mitotic chromosomes. Together, these data indicate that N-terminal domain of PIASy is not engaged in the interaction with its substrates and not important for SUMOylation *in vitro*, but facilitates chromosomal interaction and is essential for mitotic SUMOylation.

Functional comparison of PIAS N-terminal domain in XEE assay.

PIAS family proteins are categorized by several conserved domains: a SAP (scaffold attachment factor-A/B, acinus and PIAS) domain resides at the N-terminus, a SP-Ring (Siz/PIAS-Ring) domain is in the middle, followed by a SIM (SUMO-interacting motif) (70). The SAP domains of mammalian PIAS family members (PIAS1, 3, $x_{\alpha/\beta}$, and y) are ~60% identical and ~90% similar in amino acid sequence. The SAP domain has been determined to bind scaffold or matrix attachment DNA (58,62) and, in fact, all PIAS proteins have been shown to bind interphase chromatin (8). Yet, only PIASy and a minor fraction of PIASx are recruited to chromosomes during mitosis in XEE assays, suggesting there are unique features of the PIASy N-terminus that direct mitotic chromosome binding. To test this hypothesis, we constructed chimeras of PIAS family proteins. The first group of constructs was composed of SAP and SP-Ring domains from *Xenopus laevis* PIASy and the C-terminal SIM, S/DE domains from other PIAS proteins (y-1, y-3, y-x and y-yhs). The second group of constructs contained the N-terminal ~140 amino acids of either PIAS1, 3, or x protein linked with the remaining domains of *Xenopus leavis* PIASy (1-y, 3-y and x-y) (Figure 4.2A). The chimera constructs were transcribed and translated in rabbit recticulocyte lysates

(Promega) and their expression levels were verified by Westernblot with anti-T7 tag and anti-PIASy antibodies. The PIAS3-PIASy chimeric protein (3-y) was barely detectable with anti-T7 tag antibody, probably due to the degradation of the T-7tag, but Westernblot with anti-PIASy (C-terminus) antibody indicated a similar level of expression of all proteins (Figure 4.2B lower panel). These reticulocyte lysates were supplemented into the PIASy-immunodepleted CSF-XEE and incubated with sperm chromatin to assemble mitotic chromosomes. Westernblot analysis of isolated chromosomes indicated that each chimeric protein containing the N-terminus of PIASy was recruited to mitotic chromosomes (Figure 4.2C lower two panels), suggesting N-terminus of PIASy can uniquely facilitate chromosome binding. In contrast, constructs containing the N-terminus of either PIAS1, 3, or x failed to bind to chromosomes and execute TopoIIα SUMOylation, confirming that the N-terminus of other PIAS family members cannot mediate chromosome interaction. Taken together, these results indicate that the N-terminus of PIASy is a key element to achieve the unique role of PIASy for mitotic chromosomal SUMOylation by allowing interaction of PIASy to mitotic chromosomes.

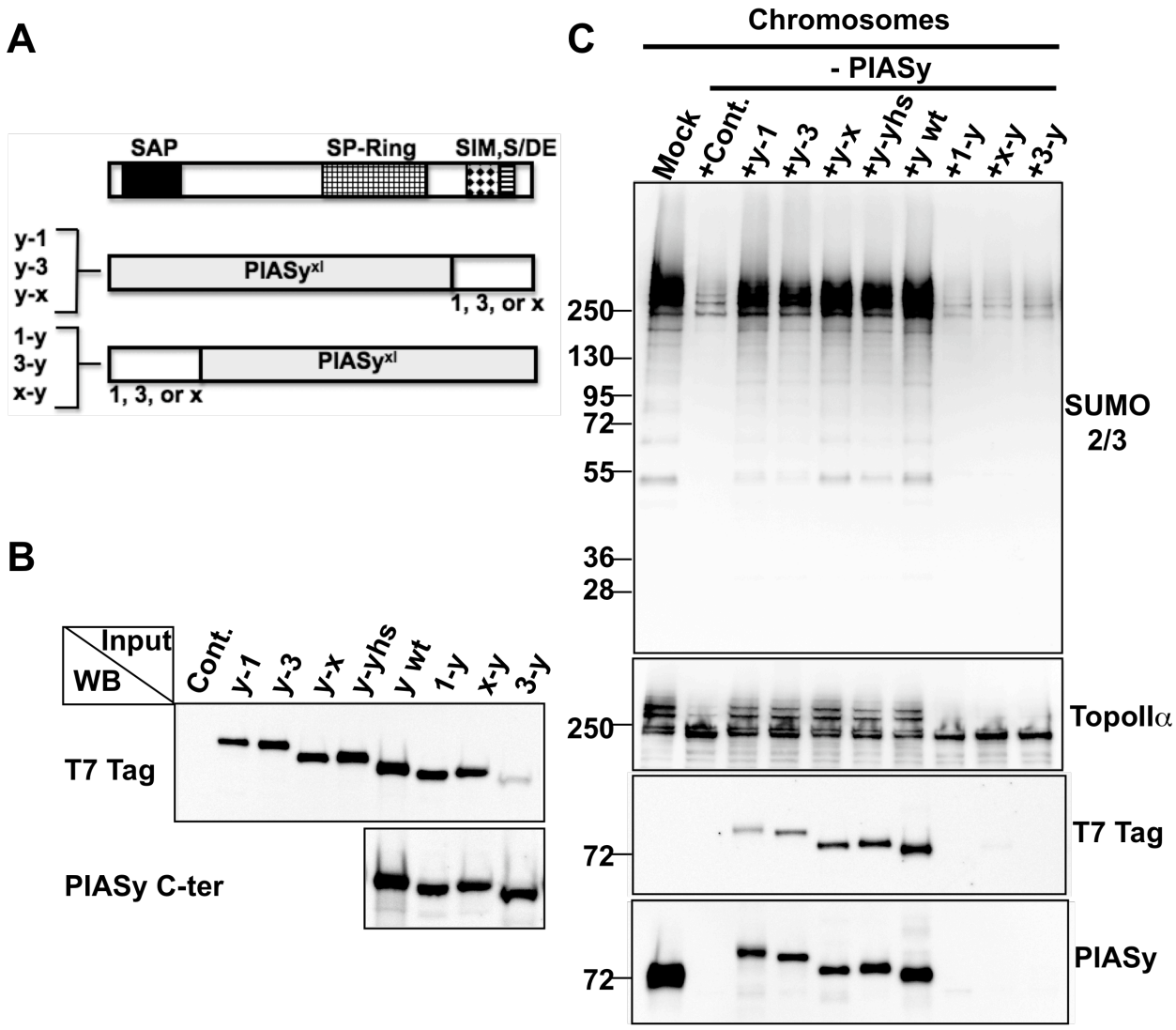

Figure 4. 2
Comparison of function of PIAS N-terminal domains in XEE assay.
(A) Schematic diagram of PIAS chimera proteins. The chimeric PIAS proteins with T7-tag at N-terminus were obtained by swapping conserved domains between PIAS family members as described in the Experimental procedures. Diagram indicates the positions of swapped domain between PIAS family proteins. **(B) Expression of the chimeric PIAS proteins in reticulocyte lysates.** The chimera proteins constructed in A were expressed in rabbit reticulocyte lysates and the expression level was analyzed by Westernblot for T7 tag or anti-C-terminus of PIASy antibody. Reticulocyte lysate containing empty vector was prepared for the negative control (Cont.). **(C) Immunodepletion and chimeric PIAS addback experiments in XEEs.** CSF-XEE was immunodepleted by IgG (Mock) or anti-PIASy antibody. Lysates prepared in B were added to the PIASy-depleted CSF extracts, and the extracts were incubated in the presence of 5,000

sperm chromatin/ml for ~1hour at 25°C. Isolated chromosomes were analyzed by Westernblot for indicated proteins.

The N-terminus of PIASy is sufficient for localization to the centromeric region.

Because removal of the N terminus PIASy or replacement with the N-terminus of other PIAS members resulted in failure to bind chromosomes, we sought to identify whether the N-terminal domain of PIASy simply is sufficient for chromosomal localization in XEE assay. To test this, fluorescent protein, mCherry, was fused to the N-terminal peptide of PIASx (PIASx N) or PIASy (PIASy N) and expressed in bacteria. PIASx was previously shown to partially restore mitotic SUMOylation in the absence of PIASy but not to the same extent as PIASy (8). Purified proteins were added when DNA replication of sperm chromatin was completed in interphase XEE, then mitosis was induced by addition of CSF-XEE to obtain chromosomes. The amounts of PIASx N and PIASy N added to the extracts were shown to be equivalent by Westernblot analysis (Fig 4.3D). In Figure 4.3A, Immunostaining indicated that PIASy N not only binds chromosomes but also accumulates at centromeric regions, resulting in colocalization with both SUMO2/3 and Aurora B, markers of inner-centromere, meanwhile PIASx N was sparsely distributed and barely detectable. In particular, PIASy N resembles the behavior of full length PIASy on chromosomes localizing at the centromeric region during mitosis (Ryu et al, JCB in print, Figure 4.3B and Figure

4.4D), indicating that the N-terminal ~140 amino acids of PIASy is sufficient to direct the localization of PIASy to the centromeric region (Figure 4.3B and C).

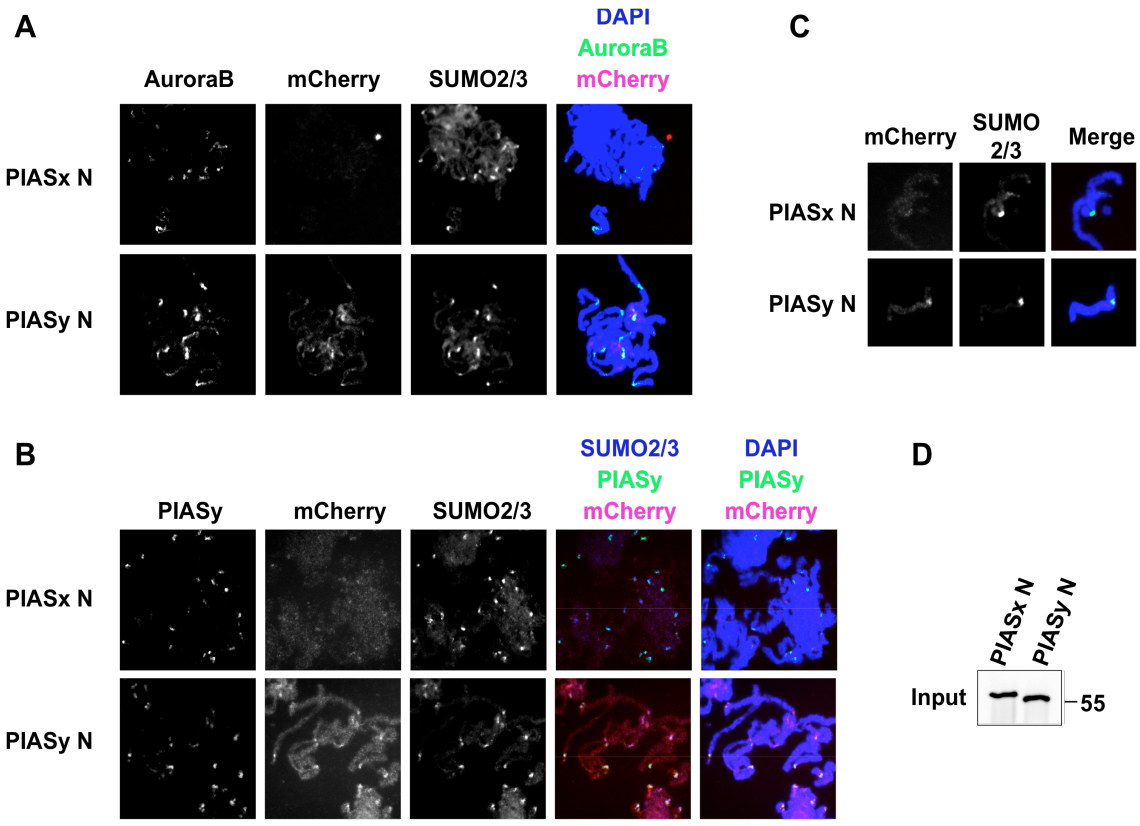

Figure 4. 3
N-terminal region of PIASy locates to centromere independent of its remaining residues.
(A and B) Localization of mCherry-tagged N-terminal peptides of PIASx and PIASy. Replicated chromosomes were obtained by incubating 500 sperm nuclei/μl in interphase extract followed by re-entry into mitosis by CSF-XEE. N-terminal peptides of PIASx (PIASx N) or PIASy (PIASy N) that have mCherry fused at the C-terminus were added before re-entry into mitosis. After re-induction into mitosis by fresh CSF-XEE, chromosomes were spun down on coverslips by centrifugation. Samples were immunostained for indicated proteins. **(C) Centromeric localization of mCherry-fused N-terminal peptide of PIASy.** Single sister chromatids are enlarged for the comparison of localization between PIASx N and PIASy N. **(D) Input of PIASx N and PIASy N** The amount of PIASx N and PIASy N input was analyzed by Westernblot with S-protein HRP (EMD Bioscience), which detects the S tag at the N-terminus on both PIASx N and PIASy N peptides.

Rod and Zw10 bind to PIASy N-terminus.

It has been shown that N-terminal domains of PIAS proteins are involved in protein interactions for multiple purposes (57,59). Therefore, we speculated that the N-terminal region of PIASy might interact with specific protein(s) for centromeric localization. In order to identify protein(s) that specifically interact with the N-terminus of PIASy but not with other PIAS family members, we performed pull-down assay with N-terminal fragments of PIAS family proteins. The 1-139 amino acid fragment of PIASy and the equivalent residues from other PIAS proteins were fused to an S-tag at the N-terminus and expressed in *E*. coli. Purified proteins were captured on S-tag affinity beads and the beads were mixed with XEE to isolate binding proteins. Associated proteins were separated by SDS-PAGE and analyzed by silver staining. As shown in Figure 4.4A, several bands (indicated with asterisks) were reproducibly observed to associate with the PIASy N-terminal peptide but not with the N-termini of any other PIAS protein. Bands of ~250 and 90 kDa were further identified as Rod and Zw10, respectively, by tandem mass spectrometry. To confirm the interaction of PIASy with Rod and Zw10, we prepared a polyclonal antibody against Rod and obtained commercially available anti-Zw10 antibody. Westernblot analysis of samples from pull-down assays as shown in Figure 4.4A revealed that Rod and Zw10 specifically interact with the PIASy N-terminal region and not with other PIAS proteins (Figure 4.4B). We next investigated whether the interaction of PIASy and the identified proteins takes place under physiological conditions. To test this, endogenous PIASy proteins were immunoprecipitated from CSF-XEE with antibody against C-terminus of PI-

ASy, and co-precipitants were analyzed with Westernblot. We observed that PI-ASy co-precipitated with Rod, confirming that they interact in XEE *bona fide*. Yet, only a minor fraction of Rod interacts with PIASy, indicating that limited portion of PIASy interact to Rod or their interaction is highly transient in XEE (Figure 4.5A left). In a reciprocal immunoprecipation performed with an antibody against Rod, Rod was able to precipitate a small but detectable amount of PIASy reproducibly (Figure 4.5A right).

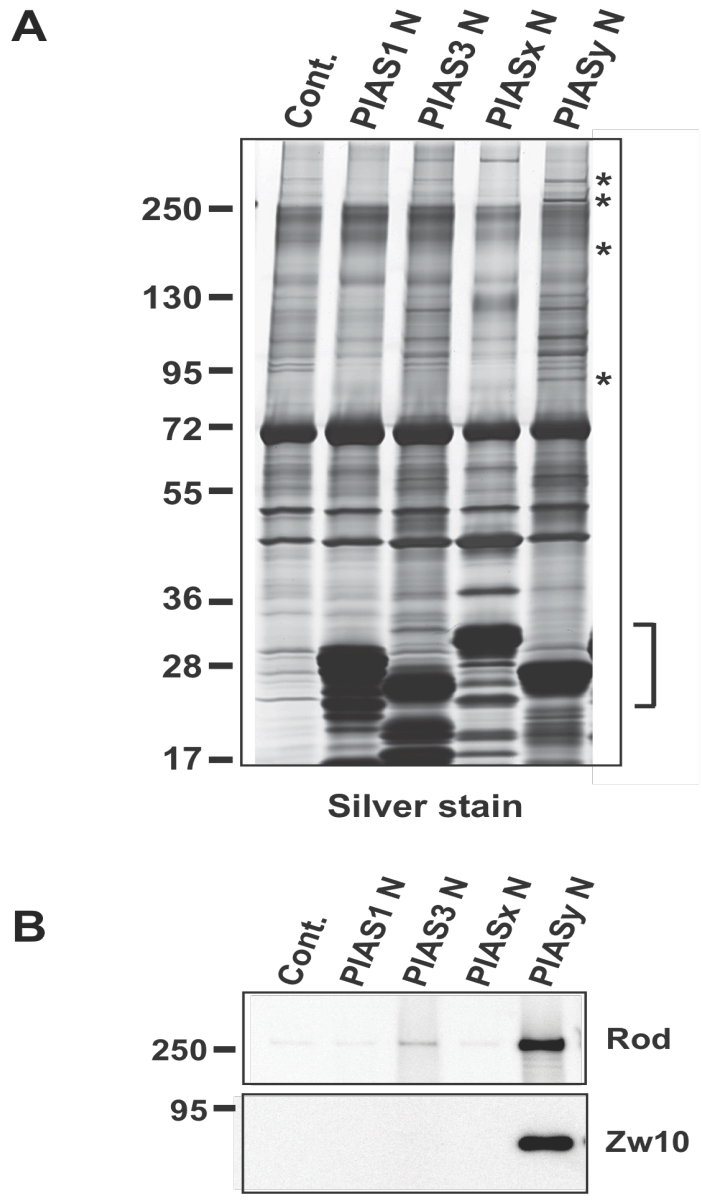

Figure 4. 4
Identification of N-terminal binding proteins of PIASy.
(A) N-terminus of PIASy has distinctive binding proteins. S-tagged N terminal domains of PIAS family proteins were bound to S-agarose beads. Either S-agarose beads (Cont.) or PIAS N-terminal protein-bound bead preparations were incubated in CSF XEE. Precipitated proteins together with beads were resolved by SDS-PAGE followed by silverstaining. Specific binding proteins for PIASy N-terminal domain are indicated with an asterisk. S-tagged PIAS N terminal peptides used as bait are indicated with bracket. **(B) Confirmation of Rod and Zw10 binding to PIASy N-terminus.** Pull-down samples as in (A) were analyzed by Westernblot for Rod and Zw10. The antibody against Rod, prepared as

described in *Experimental procedures*, or commercially available anti-Zw10 antibody (AbCam) were used to detect them in pull-down samples.

Rod and Zw10 are components of a stable complex called RZZ (Rod/Zw10/Zwilch) (126,131). The RZZ complex is localized at kinetochores from pro/metaphase to metaphase in somatic cells (125). PIASy was also observed near centromeres in XEE assays (Figure 4.3B, Ryu et al. JCB in print). Thus, we sought to determine whether Rod and Zw10 interact with PIASy on mitotic chromosomes using chromatin immuno-precipitation and immuno-fluorescence approaches. Chromatin immuno-precipitation of mitotic chromosomes prepared from XEE showed that PIASy co-precipitates Rod and Zw10 (Figure 4.5B left). Reciprocal analysis with an anti-Rod antibody also showed co-precipitation of both PIASy and Zw10 (Figure 4.5B right). Moreover, Immunofluorescence data indicated that Rod and Zw10 colocalize with PIASy at centromeric regions together with SUMO2/3 as expected (Figure 4.5C), supporting the idea that PIASy binds to the Rod/Zw10 complex for the execution of mitotic SUMOylation. It is also possible that PIASy directs the Rod/Zw10 complex to chromosomes. Immunodepletion of PIASy in CSF-XEE, however, did not eliminate chromosomal association of Rod/Zw10 (Figure 4.5D), arguing against PIASy-dependent chromosomal localization of the RZZ complex. Taken together, we conclude that Rod and Zw10 bind to the N-terminus of PIASy at the kinetochore, and their colocalization with PIASy and immunodepletion analysis suggest that Rod/Zw10 might direct the centromeric localization of PIASy.

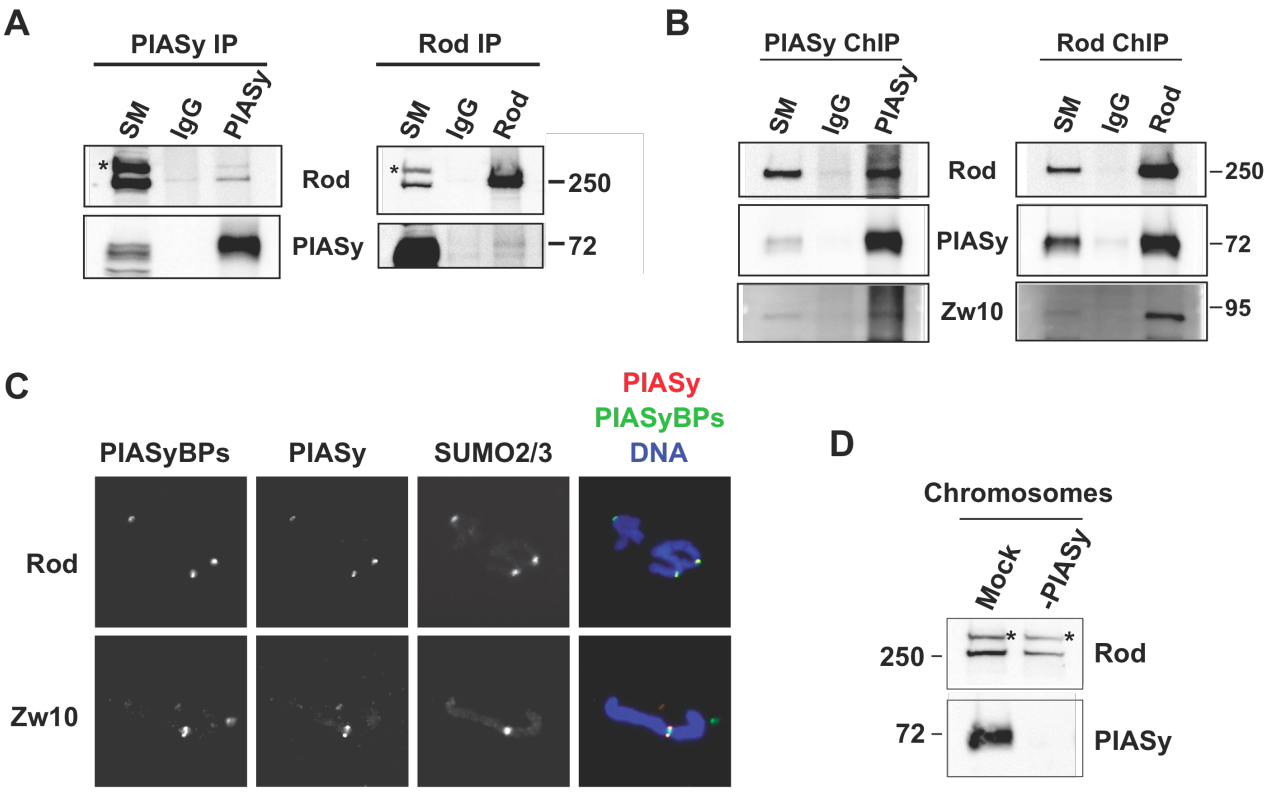

Figure 4. 5

PIASy interacts with Rod/Zw10 complex on mitotic chromosomes. (A) Interaction of Rod and Zw10 proteins with endogenous PIASy in CSF-XEE. (Left) IgG or anti-PIASy antibody crosslinked protein A beads were incubated with CSF-XEE. Immunoprecipitated proteins were resolved in SDS-PAGE and analyzed by Westernblot for indicated proteins. (Right) IgG or anti-Rod antibody crosslinked protein A beads were incubated in CSF-XEE in the same manner. The precipitants were resolved by SDS-PAGE followed by Westernblot. SM stands for starting material. Band labeled with asterisk is a non-specific protein cross-reacting with anti-Rod antibody in CSF-XEE. IP: Immunoprecipitation. **(B) Interaction of PIASy and Rod/Zw10 complex on chromosomes.** Mitotic chromosomes prepared in CSF-XEE were subjected to Chromatin immunoprecipitation (ChIP) as described in *Experimental procedures* , and prepcitated samples were analyzed as above. **(C) Localization of endogenous PIASy and binding proteins, Rod and Zw10.** Replicated mitotic chromosome samples were prepared as in Figure 4.3 followed by immunostaining for the indicated proteins. PIASyBPs: PIASy binding proteins. **(D) Depletion of PIASy does not eliminate the interaction of Rod/Zw10 complex to mitotic chromosomes.** Mock-depleted by IgG (Mock) or PIASy-depleted CSF-XEE (-PIASy) were incubated in the presence of 5,000 sperm nuclei/ml for 1 hour at 25°C. Isolated chromosomes were resolved by SDS-PAGE and analyzed by Westernblot. The antibody cross-reacting band is indicated with asterisk.

Depletion of Rod causes mislocalization of PIASy during mitosis.

Deletion of amino acids 1-47 of PIASy (48C) eliminates its chromosome binding (Figure 4.1C), suggesting that a protein interacting with the first 47 residues is critical for chromosome localization. Thus, we sought to further characterize where Rod and Zw10 interact within the N-terminal 140 amino acid region of PIASy, which we used as bait in pull-down assay (Figure 4.4). To this end, serial truncation constructs of PIASy that contain T7 tag at the N-terminus were prepared (Figure 4.6A). Purified truncation proteins were bound to anti-T7 tag antibody-conjugated beads followed by incubation in CSF-XEE. Westernblot analysis of pull-downed proteins by the beads indicated that Rod and Zw10 interact with full length of PIASy similar to data shown in Figure 4.4. Strikingly, those interactions were no longer observed with PIASy that 1-47 amino acids were deleted (48C) as well as with further truncations (Figure 4.6B). Consistent with pull-down result in Figure 4.4, PIASy (N325) with a C-terminal deletion retained the interaction with Rod and Zw10, confirming that the Rod/Zw10 complex binds to the N-terminus, but not the C-terminus of PIASy. This result reveals that the interaction of the Rod/Zw10 complex with PIASy is restricted to 1-47 amino acids of the N-terminus, the region critical for chromosome localization of PIASy (Figure 4.1C).

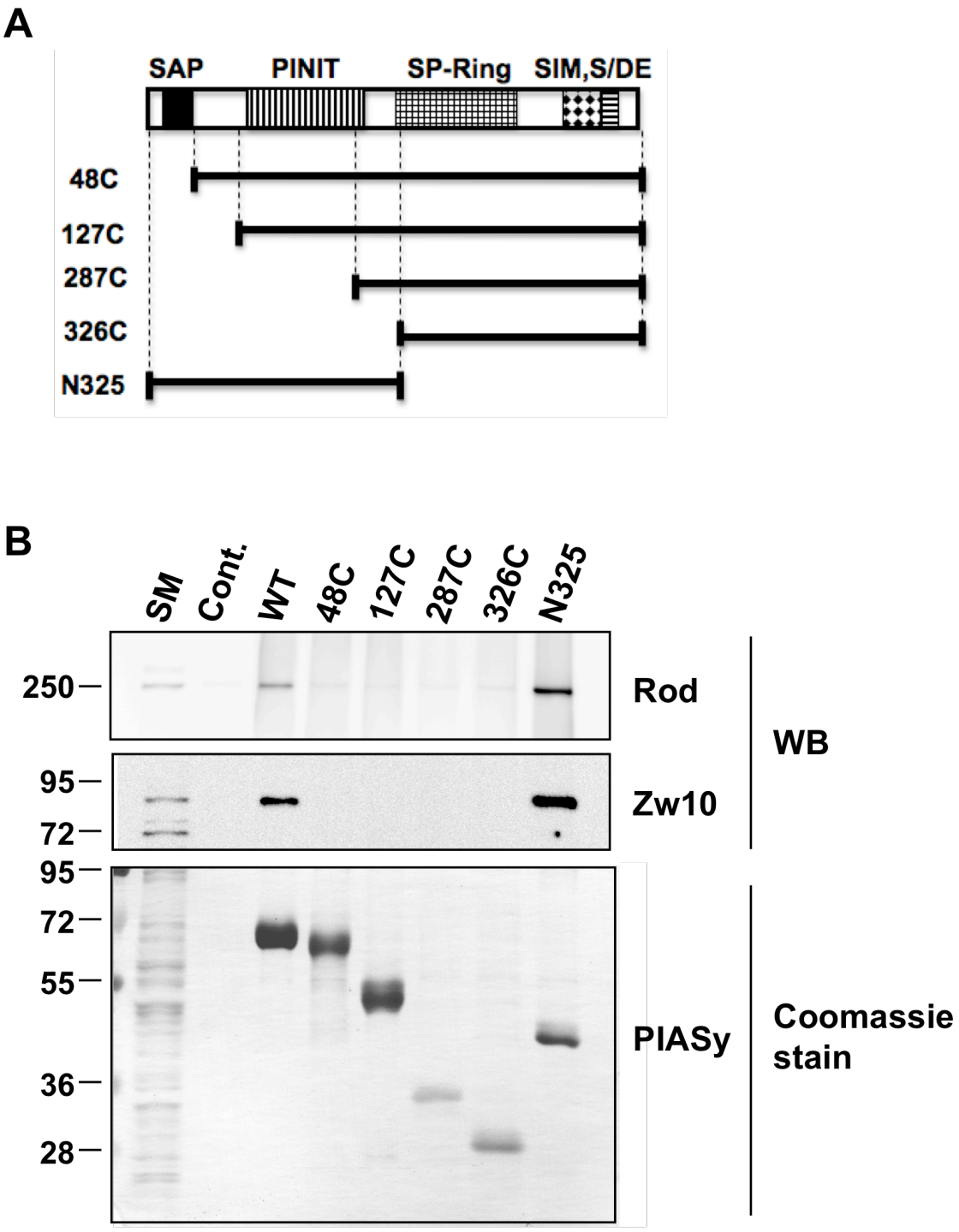

Figure 4. 6
Rod/Zw10 complex interacts with the first 47 reside of PIASy protein.
(A) Schematic diagram of PIASy and its truncations used in B. PIASy and its truncations contain T7-tag at the N-terminus. **(B) Further identification of**

Rod/Zw10-interacting area on PIASy protein. Purified T7-tagged PIASy truncation proteins as well as full length (WT) were bound to T7 antibody-conjugated agarose beads. Protein bound beads or beads alone (Cont.) were incubated with CSF-XEE. The precipitated proteins together with the T7-antibody beads were resolved by SDS-PAGE and analyzed by Westernblot for the indicated proteins or Coomassie staining. SM: starting material.

It has been shown that PIASy-dependent SUMOylation is confined to the centromeric region of chromosomes in mitosis (8,10). Given that the Rod/Zw10 complex is localized at the kinetochore, we hypothesized that the Rod/Zw10 complex is required for PIASy to localize to the centromeric region. To address this question, we immuno-depleted endogenous Rod and examined the localization of PIASy and of SUMOylation. The efficiency of Rod depletion was ~80% (Figure 4.7C) and a Rod signal was detected by immunofluorescence on some chromosomes prepared from Rod-depleted XEE (-Rod), albeit its signal was much weaker than the one in Mock-depleted samples (-IgG). Nonetheless, it was evident that the centromeric localization of Rod was completely linked to that of PIASy: chromosomes containing Rod showed the centromeric localization of PIASy without exception while ones without Rod had mis-localized PIASy on the chromosomes (Figure 4.7A, compare chromosomes indicated with arrows and squares). Rod-deficient chromosomes never showed clear PIASy foci, rather they showed dispersed and weak signals of PIASy. Chromosome samples were also stained with another anti-PIASy antibody for further confirmation. We consistently observed mislocalized PIASy on the Rod-depleted chromosomes, but rarely on Mock-depleted chromosomes (Figure 4.7B). It was notable that Rod-deficient chromosomes showed loss of intensive SUMO2/3 foci presumed to lo-

cate near centromeres, following mislocalization of PIASy (Figure 4.7A, B and 4.8). All together, our data indicate that the Rod/Zw10 complex is required for the centromeric localization of PIASy and spatial regulation of SUMO2/3 modification near the centromere.

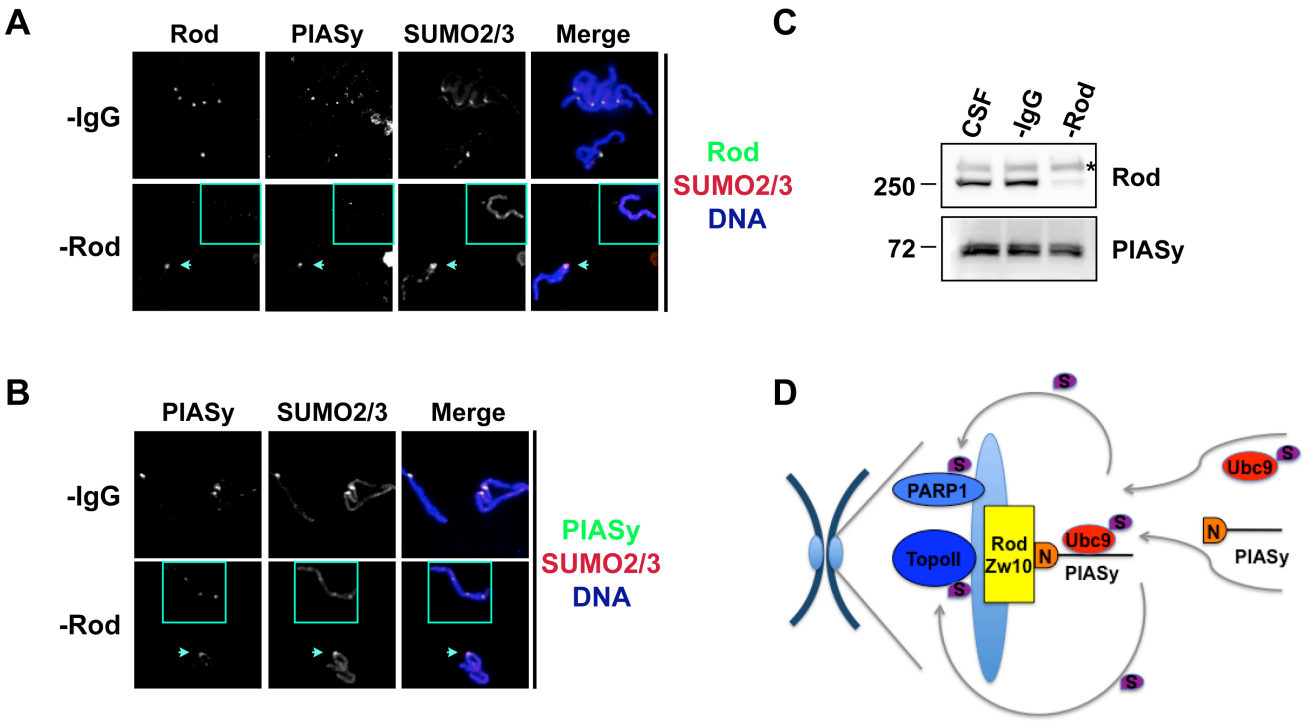

Figure 4. 7
Rod/Zw10 complex is required for localization of PIASy on centromeric region as well as SUMO2/3 during mitosis.
(A and B) Immunodepletion of Rod causes mis-localization of PIASy on mitotic chromosomes. (A) CSF-XEE was immunodepleted for Rod and released into interphase by the addition of $CaCl_2$. Sperm chromatin was incubated with the interphase extracts, and sister chromatids were obtained from the replicated chromatin by the addition of Rod-depleted CSF-XEE (-Rod). CSF-XEE was also mock-depleted using non specific IgG and processed as above (-IgG). Isolated chromosome samples were subjected to immunostaining for proteins as indicated. Affinity purified anti-PIASy chicken IgY and anti-SUMO2/3 Guinea Pig IgG were used to visualize PIASy and SUMO2/3, respectively. (B) Samples in A were immunostained with affinity purified anti-PIASy antibody obtained from Rabbit. The chromosome lacking Rod protein is shown in squares. The chromosome that still contains Rod in Rod-depleted samples is indicated with arrows. The centromere-specific localization of both PIASy and SUMO modification was compromised if Rod was completely eliminated. **(C) Confirmation of Rod depletion.** CSF-XEE before and after immunodepletion with anti-IgG or Rod antibody were

analyzed for the indicated proteins. Asterisk shows background signals from antibody cross-reacting in CSF extracts. **(D) Model for molecular mechanism of PIASy-dependent centromeric SUMOylation during mitosis.** PIASy interacts with the Rod/Zw10 complex at the kinetochore through its N-terminal region (designated as N). Consequently, Ubc9-SUMO2/3 adduct is recruited onto centromeric regions via binding to PIASy and facilitates SUMO2/3 modification of substrates such as TopoIIa and PARP1 in a spatially regulated manner

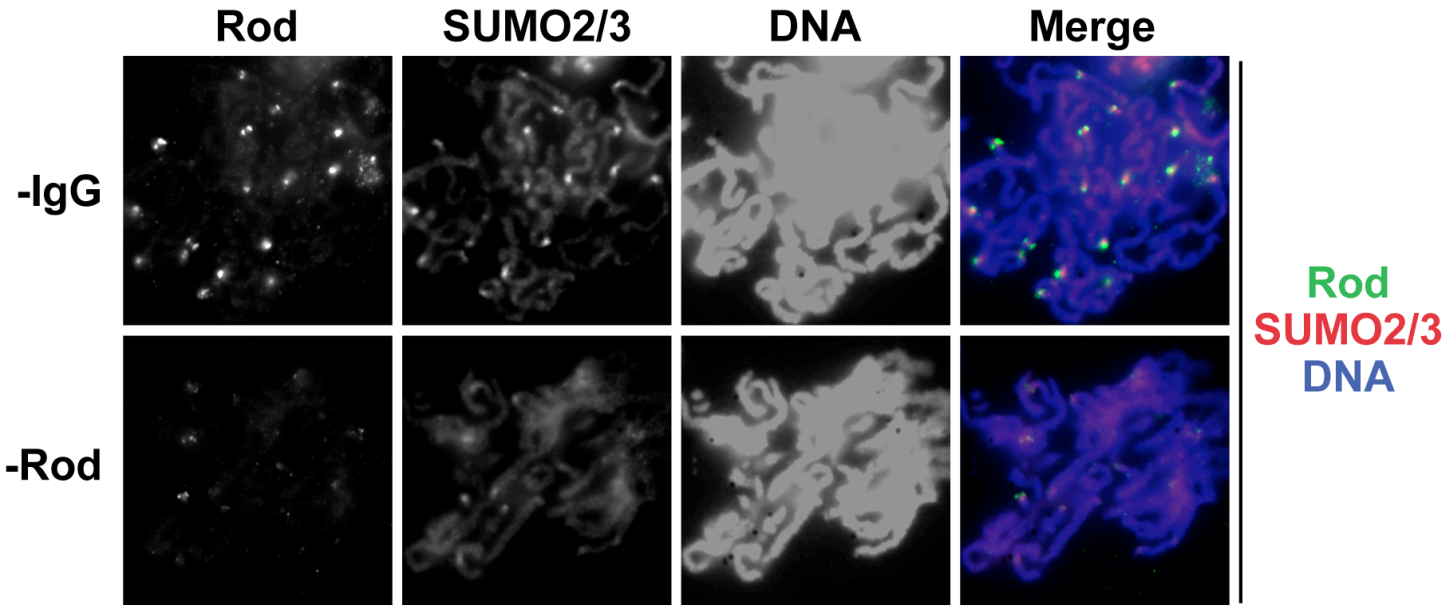

Figure 4. 8
Loss of Rod/Zw10 complex causes mis-localization of SUMO2/3 on mitotic chromosomes. CSF-XEE was immunodepleted for Rod and released into interphase by the addition of $CaCl_2$. Sperm chromatin was incubated in the interphase extracts for ~ 1 hour, and sister chromatids were obtained from the replicated chromatin by the addition of Rod-depleted CSF-XEE (-Rod). CSF-XEE was also mock-depleted using non-specific IgG and processed as above (-IgG). Isolated chromosome samples were subjected to immunostaining for proteins as indicated. Affinity purified anti-Rod rabbit antibody and anti-SUMO2/3 Guinea Pig antibody were used to visualize Rod and SUMO2/3, respectively. DNA was visualized with Hoechst33342.

DISCUSSION

Biological significance of the PIASy N-terminal domain

We have thoroughly characterized PIASy, an essential SUMO E3 ligase of mitotic substrates, using both systematic *in vitro* domain analysis and *Xenopus* egg extract cell free assays. It was previously demonstrated that the PIASy N-terminus, mostly composed of a SAP domain, plays a key role in chromosome interaction (8). Recent studies have shown that Siz1, a Siz/PIAS SUMO E3 ligase in budding yeast, interacts with substrates via its N-terminal domain (90,123), suggesting that the N-terminal domain of Siz/PIAS is responsible for substrate binding. Given that major substrates of PIASy are chromosome-resident proteins in mitosis, we hypothesized that the N-terminus of PIASy achieves chromosome binding through the interaction with its substrates. *In vitro* analysis using purified N-terminal PIASy truncations revealed that the first 127 amino acids of PIASy are dispensable for SUMOylation *in vitro*, with further deletion [including PINIT domain (59,90)] starting to affect SUMOylation (Figure 4.1B). This finding suggests that N-terminus anterior to the PINIT domain is not involved in substrate binding. Conversely, XEE assays have shown that the first 47 amino acids of PIASy, including the SAP domain, is absolutely required for mitotic SUMOylation (Figure 4.1C), strongly suggesting that the PIASy N-terminus binds specific molecule(s) to mediate chromosomal localization, and that the interaction between PIASy and these partners is critical for mitotic SUMOylation.

A number of studies have described a discrepancy between *in vivo* and *in vitro* substrate specificity of Siz/PIAS proteins of SUMO E3 ligases (12,123,132). It has been suggested that subcellular localization of SUMO E3 ligases is crucial for substrate specificity *in vivo* (12,123,132). Our XEE assays have shown that chimera PIASy proteins containing the N-terminus of PIAS1, 3, or x fail to interact with chromosomes and fail to facilitate mitotic SUMOylation (Figure 4.2). We have also shown that the N-terminus of PIASy is sufficient to direct localization to the centromeric region (Figure 4.3), arguing that PIAS proteins display substrate specificity by means of subcellular localization. The SAP domain was characterized as a putative A/T rich DNA binding domain, yet proteins such as p53, Msx1, and Oct-4 interact with specific PIAS proteins through their SAP domain (62,133,134). We have identified that the N-terminus of PIASy containing the SAP domain interacts with kinetochore-associated proteins, Rod and Zw10 (Figure 4.4 and 4.6), raising the possibility that the SAP domain contributes to protein interactions for regulating subcellular localization of PIAS proteins. It will be of interest to investigate whether the SAP domain-mediated localization is a common mechanism to regulate the localization of other PIAS family proteins in cells. This information will provide insight into the *in vivo* substrate specificity of mammalian PIAS family proteins.

Molecular mechanism of PIASy recruitment to the kinetochore

PIASy-dependent SUMOylation becomes detectable in prometaphase, peaks in metaphase and disappears with the onset of anaphase (71). This mitotic

phase-specific SUMOylation is further restricted to the inner centromere (8). Here we show that PIASy binds to kinetochore proteins, Rod and Zw10; components of the RZZ complex, through its N-terminus (Figure 4.4, 4.5 and 4.6) and that elimination of Rod compromises PIASy localization to kinetochores (Figure 4.7). These results suggest that the Rod/Zw10 complex has a critical role in the centromeric recruitment of PIASy and, thus, in the SUMOylation of chromosomal substrates near the centromere. Previous studies in *Drosophila* embryos revealed that GFP-Rod enters the nucleus by the time that nuclear envelope breaks down, accumulates on kinetochores, and disperses as sister chromatids are separated (125). This aspect of Rod and Zw10 distribution during mitosis highly resembles PIASy distribution, leading to an explanation for how PIASy executes centromeric SUMOylation during mitosis. We propose that PIASy accumulates on kinetochores by docking with Rod/Zw10 complex that is concentrated on kinetochores when the nuclear envelope disassemble and then PIASy facilitates SUMO2/3 modification of its substrates (TopoIIα and PARP1) near the centromere (Figure 4.7D). As Rod and Zw10 are shed from kinetochores, PIASy falls off and SUMO modification decreases. One puzzling observation is that PIASy is mislocalized but does not fail to interact with chromosomes deficient in Rod/Zw10 complex. This does not fit with the observation that PIASy lacking first 47 residues failed to interact with chromosomes (Figure 4.1B), implying that there might be another protein(s) delivering PIASy close to chromosomes. Further analysis of PIASy movement dynamics in mitosis may elucidate this question.

The RZZ complex delivers dynein/dynactin onto unattached kinetochores (135). The complex also functions in activating spindle assembly checkpoint signaling by bringing two checkpoint components, Mad1 and Mad2 (22,25). In *Drosophila* and *C. elegans*, the phenotypes of mutation in Rod, Zw10, or both cause as premature chromosome segregation with anaphase bridges (128,131). Components of the SUMO pathway are clearly connected to mitosis. Mutations in SUMO (smt3, pmt3), Aos1, and Ubc9 show defects in mitotic progression in budding and fission yeast (50,105,136,137). Defects in SUMO modification also show chromosomal missegregation in XEE (8,71). Given that the Rod/Zw10 complex is required for proper centromeric localization of PIASy and SUMO modification (Figure 4.7 and 4.8), it is reasonable to consider that the RZZ complex could regulate two completely different pathways: metaphase checkpoint and centromeric SUMOylation. If so, the abnormal chromosome segregation shown in Rod/Zw10 mutants might be the dual outcome from defects in both the mitotic checkpoint and the SUMO pathway.

In summary, we have demonstrated the molecular mechanism of PIASy-dependent SUMOylation in mitosis by showing that PIASy interacts with the Rod/Zw10 complex, a kinetochore component, through its SAP domain in the N-terminus. Our results suggest that the SAP domain of PIAS family proteins could show binding specificity that plays a critical role in determination of substrate specificity *in vivo*. Together, our studies reveal the likely molecular mechanism of PIASy-dependent spatiotemporal regulation of SUMO modification and a novel role of the RZZ kinetochore complex on chromosomal SUMOylation.

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Contributions

All Figures are my work.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

Since the discovery of SUMOylation in 1996, mounting information has revealed that SUMO conjugation regulates many aspects of cellular function at various stages of the cell cycle (41,42,64). During interphase, numerous transcription factors are targeted for SUMOylation and, once modified, change their binding patterns, leading to alterations in transcription levels (42). During mitosis, various centromeric proteins are modified by SUMO, and the combination of these modifications ultimately contributes to proper cell division (41). One of these centromeric resident proteins, DNA topoisomerase IIα (TopoIIα) was first determined to be a SUMO substrate in yeast (102). Yeast TopoIIα mutants incapable of SUMO modification displayed abnormal chromosome segregation, suggesting that SUMO modification is involved in proper mitotic progression (102).

We utilized *Xenopus* egg extracts (XEE) and an *in vitro* reconstitution assay to elucidate how SUMOylation regulates TopoIIα during mitosis (71). The advantages of our approach are twofold. First, mitosis-dependent SUMOylation can be completely isolated from interphase-dependent SUMOylation in XEE. Secondly, the spatiotemporal limits of studying SUMOylation in XEE (namely, that SUMOylation occurs at the centromeric region during mitosis) can be overcome by using an *in vitro* reconstitution assay. By isolating chromosome fractions

from XEE, we also identified PARP1, (an enzyme involved in the repair of single strand DNA breaks), as a novel mitosis-specific substrate for SUMO2/3 conjugation (10). We demonstrated that Lys 482 on PARP1 is a major site for SUMO2/3 conjugation, in a PIASy-dependent manner (10). Finally, the SUMO E3 ligase PIASy was extensively examined to determine the molecular dynamics of mitotic SUMOylation (118).

In the following paragraphs, the effects of SUMOylation on mitotic progression, and how PIASy contributes to cell cycle-specific SUMO conjugation, will be discussed. The section will conclude with a discussion of future directions for this work.

Functional analysis of mitotic SUMOylation

PARP1 SUMOylation in mitosis

PARP1 is an enzyme that assembles a chain of ADP-ribose moieties on PARP1 itself and on its substrates, using NAD+ as an ADP-ribose source (35). PARP1-mediated PARylation plays a role in DNA damage repair during interphase, and the interaction of PARP1 with BRCA1/2 has lead to the identification of PARP1 as a potential anti-cancer target (138). In addition, it has been shown that upon detection of DNA damage, PARP1-mediated PARylation prevents progression of mitosis by inhibiting the activity of aurora kinase B, a molecule implicated in spindle checkpoint control, thus underlining the importance of mitotic PARP1 activity in cell cycle progression (38).

We identified PARP1 as a target for SUMO2/3 conjugation in a cell cycle-

specific manner (10). SUMO conjugation of PARP1 first appears when the cell cycle is near metaphase, and disappears, with the onset of anaphase. We have also shown that PARP1-mediated PARylation is negatively affected by SUMO2/3 conjugation. Elimination of SUMOylation increases PARylation in mitosis. Because of the inability to restore immunodepleted PARP1 by adding back recombinant PARP1 proteins in XEE, in future studies, siRNA and mutant constructs of nonSUMOylatable PARP1 will be utilized in cell lines in order to fully understand the role of PARP1 SUMOylation in mitosis. To this end, the identification of all of the SUMOylation sites of PARP1 will be necessary. Also, identifying the potential substrates and binding proteins of PARP1 during mitosis, and determining how SUMOylation affects these will add to our understanding of this regulatory mechanism.

TopoIIα SUMOylation in mitosis

DNA topoisomerase IIα (TopoIIα) is responsible for proper chromosome segregation during mitosis (8,93). This is likely because of the catalytic activity (catenation and decatenation) of TopoIIα being able to resolve DNA topology. Catenation and decatenation of DNA are identical biochemical reactions, and the ionic strength determines either reaction in certain circumstance (139,140). Our research revealed that SUMO conjugation regulates the catalytic activity of TopoIIα *in vitro* (141). Using a reconstitution assay, we were able to generate SUMO-conjugated forms of TopoIIα. After this modification process, SUMOylated TopoIIα and non-SUMOylated control were applied in a decatenation assay

in which TopoIIα activity can be assessed. Our results indicated that TopoIIα decatenation activity is strongly inhibited by SUMO2/3 conjugation. Tandem mass spectrometric analysis in XEE and *in vitro* confirmed that Lys 660 is an endogenously SUMOylated site on TopoIIα. Using a mutated molecule that replaces Lys 660 with Arg, we showed that the K660R mutant does not allow SUMOylation-dependent inhibition of TopoIIα activity, leading to the conclusion that SUMOylation negatively regulated TopoIIα activity through Lys 660 conjugation. A remaining concern is that, because Lys 660 is located within the catalytic domain of TopoIIα, the K660R mutant protein has ~20 times less activity than wild-type, although the DNA binding capacity of the mutant is not affected. Thus, it is not clear whether abolition of SUMOylation-dependant inhibition of TopoIIα activity is affected by the lower catalytic activity of the mutant. The remaining SUMOylation sites of TopoIIα will have to be identified in order to answer this question.

In summary, cell cycle-specific SUMO2/3 conjugation during mitosis appears to negatively affect the intrinsic activity of its target proteins, based on the results with PARP1 and TopoIIα. It is likely that the temporal inhibition of TopoIIα activity directs the accurate progression of the mitotic phases: inhibition of TopoIIα by SUMOylation prevents both recatenation and premature chromosome segregation that may occur when the catalytic activity of TopoIIα persists. Further experiments using the K660R or equivalent mutants in the *in vivo* system are necessary in order to understand the function of TopoIIα SUMOylation under physiological conditions.

Molecular mechanism of centromeric SUMOylation

SUMO conjugation of mitotic substrates has been observed mainly within the centromeric region of chromosomes. It was previously reported that PIASy is required for centromeric SUMOylation (8). However, the details of this molecular modification and its effect on mitosis are unknown.

The PIAS family of SUMO E3 ligases is comprised of PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy (57). All have the same basic structural elements, with an N-terminal, conserved SAP motif, an internal SP-RING domain, and variations in their C-terminal sequences (57). However, only PIASy is able to bind to chromosomes during mitosis (8). Because of the unique mitotic SUMOylation capacity of PIASy, we analyzed the subdomains of the PIASy protein. Using recombinant truncated PIASy proteins, we determined that the sequence responsible for chromosome binding was the N-terminal region, including the SAP domain (118). This was surprising, given the small size (~130 amino acids) and sequence similarity to other PIAS proteins. We found that the 130 amino acid polypeptide was sufficient not only to bind to chromosomes, but also to locate to the centromeric area (118). This localization is consistent with that of full length PIASy protein (which is restricted to the centromeric region) and with the fact that SUMO modification occurs in the vicinity of the centromere on chromosomes (71).

We subsequently analyzed the binding partners of the PIASy N-terminal domain. Mass spectrometry indicated that both Rod (Rough deal) and Zw10 (Zeste

white 10) are binding partners of PIASy. Rod forms a complex with Zw10 and Zwilch in vertebrates (21). The complex then recruits the checkpoint proteins Mad1 and Mad2 to the kinetochore and thus is required for spindle checkpoint activation (22,126,127). We demonstrated that Rod and Zw10 bind only to the PIASy protein, and not to other PIAS family members. Immunodepletion of PIASy does not affect Rod/Zw10 localization, but immunodepletion of Rod/Zw10 eradicates the centromeric localization of PIASy, indicating that the Rod/Zw10 complex recruits PIASy to the centromeric region. We also found that binding of Rod/Zw10 occurs within the first 49 amino acids of PIASy, in the region composed of the SAP domain. The DNA binding function of SAP has been previously described (58), however, this result suggests the novel concept that PIAS proteins utilize their SAP domain for proper cellular localization as well.

Combining the pieces to develop a picture of mitotic SUMOylation

Based on our results, we can trace the process of SUMOylation that occurs during mitosis as follows: Once the nuclear envelope breaks down, the Rod/Zw10 complex gains access to the kinetochore on the chromosomes. PIASy is then able to locate to the centromeric region by binding to Rod/Zw10. PIASy recruits the activated SUMO2/3-Ubc9 complex and facilitates the SUMOylation of target proteins near the centromere. TopoIIα, one of these SUMO target proteins, is immediately made inert by SUMOylation, thereby, blocking any further entanglement or disentanglement of centromeric DNA. SUMO conjugation of another target, PARP1, halts PARylation. When mitotic checkpoints are com-

pleted, the Rod/Zw10 complex begins to fall off the kinetochores, thus weakening the interaction between PIASy and the centromeric region. As PIASy disappears, SUMO modification is reversed by nearby SUMO proteases, leading to the reactivation of TopoIIα (Figure 5.1).

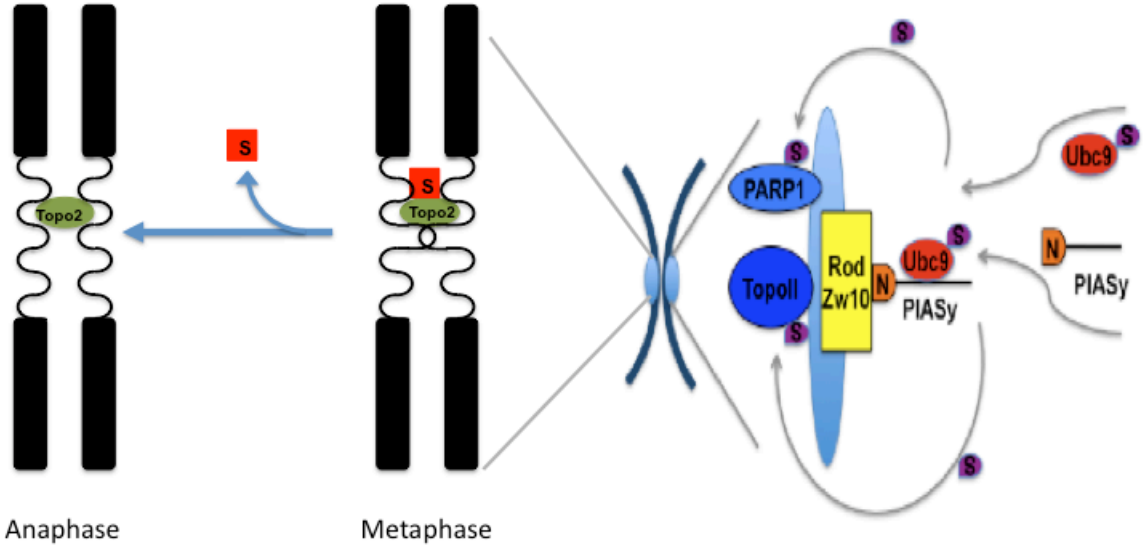

Figure 5. 1.

A molecular model for the PIASy-dependent mechanism of mitotic SUMOylation. Once the nuclear envelope breaks down, the Rod/Zw10 complex localizes to the centromeric region. Subsequently, PIASy recognizes the Rod/Zw10 complex through its N-terminus and is recruited to the centromeric region. Centromere-resident SUMO target proteins such as TopoIIα and PARP1 are modified by SUMO2/3 in a PIASy-dependent manner. The catalytic activity of TopoIIα is regulated by SUMO modification during mitosis.

In summary, we endeavored to examine the field of mitotic SUMOylation in order to address the following questions:

1) What is the role of SUMO conjugation of TopoIIα during mitosis?

2) Are there other targets for SUMOylation in mitosis?

3) How does centromeric SUMOylation take place in such a well-defined, spatio-temporal manner?

In answer to these questions, we found that:

1) SUMO2/3 conjugation inhibits the intrinsic activity of TopoIIα through modification of Lys 660 *in vitro*.

2) PARP1 is a novel SUMO2/3 substrate during mitosis in *Xenopus* egg extracts. Lys 482 is a major SUMOylation site in PARP1 during mitosis.

3) PIASy mediates centromeric SUMOylation through its N-terminal binding to chromosomes. PIASy exclusively localizes at the centromeric region during mitosis, and this centromeric localization is executed through binding of Rod/Zw10 complex proteins.

In future work, the identification of the remaining SUMOylation sites of TopoIIα will clarify the role of Lys 660 SUMOylation in the regulation of TopoIIα activity. Identification of the SUMOylation sites on TopoIIα will further elucidate other potential roles for SUMOylation that, at present, remain unidentified.

It is also of great interest to identify why PARylation increases when PARP1 is not SUMOylated.

Lastly, PIASy was shown to be able to interact with chromosomes without the presence of the Rod/Zw10 complex, suggesting that there are other ways to recruit PIASy to chromosomes. Therefore, identifying this route is another future

goal, in order to completely understand the molecular mechanism of mitotic SUMOylation.

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