SUMOylation in centromere organization: Regulation of a putative chromatin remodeler, Polo-like kinase 1 interacting checkpoint helicase (PICH) by mitotic SUMOylation

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SUMOylation in centromere organization: Regulation of a putative chromatin remodeler, Polo-like kinase 1 interacting checkpoint helicase (PICH) by mitotic SUMOylation

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ABSTRACT

SUMOylation is a posttranslational modification that can mediate diverse cellular processes such as DNA replication, transcription, DNA repair, cell cycle regulation, signal transduction and cell death. Similar to ubiquitination, SUMOylation requires a cascade of enzymes in its conjugation pathway: a SUMO activating E1 enzyme, a SUMO conjugating E2 enzyme and a SUMO E3 ligase. This process of SUMO modification of proteins is reversible and SUMO can be cleaved from its substrate by deSUMOylating enzymes. Mitotic SUMOylation has an essential role in faithful chromosome segregation in eukaryotes, although its molecular consequences are not yet fully understood. Previously, we have identified two mitotic SUMO substrates: Topoisomerase IIα and Poly (ADP-ribose) polymerase 1, PARP1 using *Xenopus* egg extract. In *Xenopus* egg extract assays, we showed that poly (ADP-ribose) polymerase 1 (PARP1) is modified by SUMO2/3 at mitotic centromeres and that SUMOylation could regulate its enzymatic activity, most likely by mediating substrate specificity. To determine the molecular consequence of mitotic SUMOylation, we analyzed SUMOylated PARP1-specific binding proteins. We identified Polo-like kinase 1- interacting checkpoint helicase (PICH) as an interaction partner of SUMOylated PARP1 in *Xenopus* egg extract. Interestingly, PICH also bound to SUMOylated Topoisomerase IIα (TopoIIα), a major centromeric SUMO substrate. Purified recombinant human PICH interacted with SUMOylated substrates, indicating that PICH directly interacts with SUMO, and this interaction is conserved among species. Polo-like kinase 1 (Plk1)-interacting checkpoint helicase (PICH) localizes at the centromere and is critical for proper chromosome segregation during mitosis. However, the precise molecular mechanism of PICH's centromeric localization and function at the centromere is not yet fully understood. We found that PICH is modified by SUMO2/3 on mitotic chromosomes prepared by *Xenopus* egg

extract and in cultured cells. PICH SUMOylation is highly dependent on PIASy, consistent with other mitotic chromosomal SUMO substrates. Finally, the SUMOylation of PICH significantly reduced its DNA-binding capability, implicating SUMOylation in regulation of PICH DNA-dependent ATPase activity. To further determine the molecular consequence of PICH/SUMO interaction on PICH function, we identified three SUMO-interacting motifs (SIMs) on PICH and generated a SIM-deficient PICH mutant. Mutation of SIMs on PICH drastically reduced PICH binding to SUMOylated substrate. To understand how SIM on PICH is important during mitosis, we established TET-ON mediated conditional ectopic expression of PICH in HeLa cells. Using the conditional expression of PICH in cells, we found distinct roles of PICH SIMs during mitosis. Although all SIMs are dispensable for PICH's localization on ultrafine anaphase DNA bridges, SIM3 is critical for its centromeric localization. Intriguingly, the other two SIMs function in chromatin bridge prevention. Currently, using the combination of siRNA and inducible expression of different PICH mutant constructs, we are examining how SUMOylation is spatially and temporally regulating PICH's localization and elucidating its role on centromeric nucleosomes of mitotic chromosomes. With these results, we propose a novel SUMO-dependent regulation of PICH's function on mitotic centromeres. In parallel, we observed SUMO-dependent localization defects on mitotic centromere markers/histones (HP1γ and H2A.Z). Collectively, our findings suggest SUMOylation dependent centromere organization and a novel SUMO-mediated regulation of PICH's function at mitotic centromeres, which are required for faithful chromosome segregation in mitosis.

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LIST OF ABBREVIATIONS

PICH – Polo-like kinase 1 interacting checkpoint helicase

SUMO- Small ubiquitin-like modifier

SIM- SUMO-interacting motif

SENP- SUMO-proteases

BLM- Bloom's syndrome helicase

TopoIIα- Topoisomerase IIα

PARP1- Poly (ADP-ribose) polymerase 1

CENPA- Centromeric protein A

UFB- Ultrafine bridges

XEE- *Xenopus* egg extract

SETDB1- SET domain, bifurcated 1

HP1- Heterochromatin protein 1

SENP2-CD- SUMO protease 2 catalytic domain

CHAPTER I

INTRODUCTION

Biological question leading to the work in this dissertation

Accurate cell division and transmission of the genome to the next generation daughter cell is a requirement for proper growth, development and viability of cells and organisms. To attain proper chromosome segregation, chromosomes depend on a specialized region known as the centromere. Centromeres are the specialized regions of chromosomes that serve as docking sites for the formation of kinetochores. The Kinetochore is a macromolecular structure made of multiple proteins and serves as an attachment site for microtubules from mitotic and meiotic spindles that pull sister chromatids apart. Centromeres also ensure proper cohesion during mitosis with the help of a domain named as the pericentric heterochromatin domain surrounding the kinetochore (1). Together, centromeres and kinetochores ensure proper chromosome segregation and the smallest of defects in the functionality of these structures can lead to major developmental disorders and diseases (2). Despite more than one hundred years of research on this complex mitotic structure, understanding the molecular organization and regulation of the centromere/kinetochore to ensure accurate chromosome segregation is an ongoing challenge.

The long-term goal of this research is to better understand how the formation of a centromere is regulated at the molecular level to ensure its function to promote accurate chromosome segregation. Studies of centromeres from several organisms have highlighted the importance of epigenetics in the definition of centromeres. Two major contributing epigenetic changes at the centromere are nucleosome positioning and histone modifications (elaborated more in the next section), without which, a functional centromere is not formed (3). Centromeres are associated with a specialized nucleosome that contains histone H3 variant, centromere protein A (CENPA)

(4). Additionally, a few chromatin modifications serve as an epigenetic mark for the centromere along with the underlying DNA sequence. These epigenetic alterations at the centromere are mostly achieved by posttranslational modifications and nucleosome remodeling (5-8). One of the posttranslational modifications at the centromere, SUMOylation, is essential for proper chromosome segregation during mitosis (explained later in the chapter) (9). The work in this dissertation started with an aim to understand the molecular mechanism of SUMO regulation of epigenetics at the centromere during mitosis. I wanted to investigate the functional significance of one of the two previously identified SUMO substrates by my research group – Poly (ADP-ribose) polymerase 1 (PARP1) (10). I observed that SUMOylation of PARP1 does not alter its enzymatic activity but changes the pattern of PARylation (explained in detail in chapter two) (11). This led to the hypothesis that SUMOylation of PARP1 mediates novel protein-protein interactions. In order to identify novel proteins that interact with SUMOylated PARP1, we performed a large-scale pull-down assay *in vitro*. Of all the identified proteins, there was one protein around 250 KDa that was prominently binding to SUMOylated PARP1. We identified the protein by LC-MS/MS (Liquid chromatography-Mass spectrometry) as Polo-like kinase 1 interacting checkpoint helicase (PICH). PICH is a putative mitosis-specific chromatin remodeler. Our work, described here, delineates the beginning of understanding the consortium and complexity of posttranslational modification (SUMOylation) and putative chromatin remodeler, PICH at the centromere, potentially to regulate centromere epigenetics in order to ensure proper chromosome segregation. This work will significantly add to our understanding of the complex regulation of centromere structure and function.

Epigenetics at the centromere

As mentioned in the beginning of this chapter, the centromere is the specialized locus on chromosomes where a kinetochore is formed and ensures proper segregation during mitosis. The centromere region is defined by distinct chromatin organization and epigenetic factors, defined and controlled by nucleosome remodeling, centromere associated proteins, specific histone modifications and histone variants. The centromere is comprised of a multi-subunit complex throughout the cell cycle termed the constitutive centromere-associated network (CCAN) that contains centromere-associated proteins (CENPs C, H, I, K-U, W and X) (12,13). CENPs are well conserved across organisms. One of the major CENPs, evolutionarily conserved and involved in the formation of centromeres, is the histone H3 variant centromeric protein A (CENPA). CENPA has been previously shown to be essential for the localization of kinetochore components using gene targeting in human cells and fission yeast (14-16). The centromere-targeting domain (CATD) of CENPA is initially used to assemble chromatin and subsequently the N- or C- terminal tail of CENPA assists in the recruitment of inner kinetochore proteins. Additionally, multiple factors are implicated in the control of epigenetics at the pericentric heterochromatin. A Histone H2A variant, H2A.Z, known to play a role during transcription, has been shown to be present at pericentric heterochromatin regions of budding yeast (17). Over the years, the role of H2A.Z during mitosis is being elucidated. Studies have shown that H2A.Z plays a role in the organization of centromere structure and centromere silencing/chromosome segregation (18,19). Histone modifications concentrated at the pericentric heterochromatin from different organisms include, H3K9me2 in *Drosophila* (20) and H3K9me3 and H4K20me3 in mammals (21,22). In fission yeast, H3K9me2 serves as a binding site for Heterochromatin

protein (HP1), which further binds to Cohesin subunits (23,24). Heterochromatin protein 1 is localized with the pericentric heterochromatin known for its gene silencing function.

CENPA has a key role at the centromere and the epigenetics of CENPA is controlled by nucleosome remodeling enzymes and histone chaperones that are also involved in chromatin regulation including at regions other than the centromere (gene regulation) (25). At pericentric heterochromatin, epigenetic control is mediated mainly by nucleosome remodeling enzymes and histone modifiers (25). A lot is still unknown about the physical structure of centromeric chromatin and its contribution towards proper chromosome segregation. There are various hypotheses proposed to explain how a centromere is defined in the cell. Some of these include the presence of CENPA, chromosome remodeling at the centromere and tension at the centromere. Described more in the section below, our work on the putative chromatin remodeler, PICH most likely will be helpful in understanding centromere-chromosome regulation since unlike other nucleosome remodeling enzymes, PICH is mitosis-specific and most likely doesn't play a role during the other stages of the cell cycle.

Chromatin remodeling and its significance during mitosis

Chromatin remodeling refers to the dynamic process of altering the packaging state of chromatin. Remodelers utilizes energy from ATP hydrolysis to move, eject and reconstruct nucleosomes in the process of changing the chromatin packaging state (26). Chromatin remodelers along with other factors play a regulatory role in many processes such as, replication, transcription, DNA repair and mitosis (26). They are required for packaging of chromatin and regulating the accessibility of DNA inside the packaged chromatin.

There are four families of chromatin remodelers: the SWI/SNF family, the ISWI family, the CHD family and the INO80 family (26). All the families utilize energy from ATP hydrolysis to alter DNA-histone contacts. PICH belongs to the SWI/SNF family of chromatin remodelers.

1.1

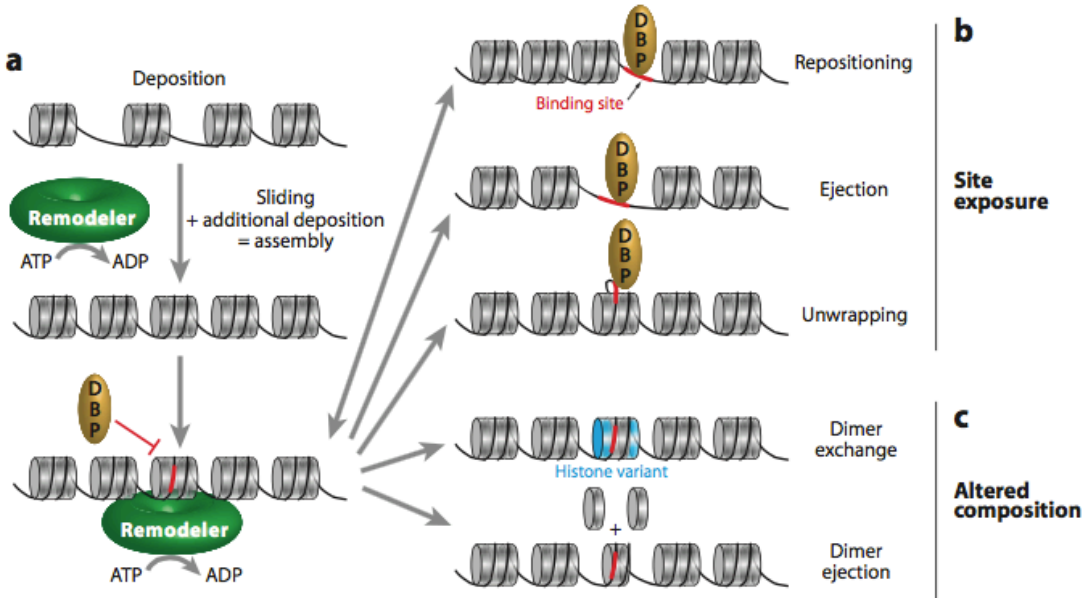


Figure 1.1. Some of the possible outcomes of chromatin remodeling. These are some of the possible results due to the activity of chromatin remodelers. Figure adapted from Clapier and Cairns, 2009 (26).

SWI/SNF stands for switching defective/sucrose non-fermenting. The first SWI/SNF complex (~ 2 MDa) was initially purified from *Saccharomyces cerevisiae*. SWI/SNF genes were initially identified as mutants that activate a few promoters under repressive conditions (27). The SNF2-like family is further divided into subfamilies that include SNF2, SNF2L, ERCC6, MOT1, RAD54 and other subfamilies (28). They have a conserved ATP-binding motif that is required for ATPase activity and are known to play a role in different biological functions, including transcription, repair, recombination and chromosome segregation (28).

1.2

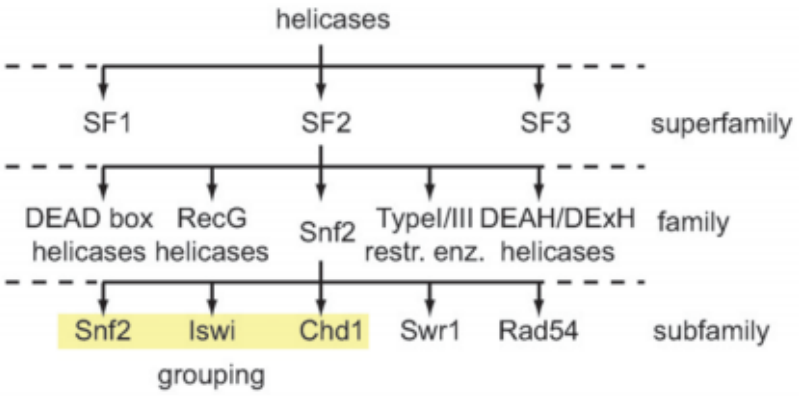


Figure 1.2. Snf2 family tree. Schematic illustration of the Snf2 superfamily, family and subfamily. (Adapted from Flaus et al., 2006) (29).

As mentioned in the first section of this chapter, there are very few examples of chromatin remodelers, whose importance to ensure proper chromosome segregation during mitosis is known (5-7). Most of these chromatin remodelers cause chromosome segregation defects. Some of the examples studied have shown potential roles during mitosis, but most of them are important during interphase and the segregation defects may be a result of their dysfunction during interphase. During mitosis, chromatin remodelers most likely will be required to specialize certain chromatin regions (centromere?) and to provide DNA accessibility to packaged regions and our current knowledge of such chromatin remodelers during mitosis is very limited. **The putative chromatin remodeler studied in this dissertation is a highly attractive candidate for chromatin remodeling during mitosis for the following reasons:** 1) PICH associates with chromosomes (centromeres) only during mitosis and is localized in the cytoplasm during interphase, making it a putative mitosis-only chromatin remodeler 2) Discovery of PICH led to the visualization of unique DNA threads called Ultrafine bridges (UFBs) during mitosis (explained more in detail below). UFBs properties and functions are not well understood and PICH is one of the very few proteins that can be used to visualize/study UFBs 3) PICH is known to interact with some important mitotic proteins, (Topoisomerase IIα (Topo IIα) and Bloom

syndrome helicase (BLM), which play critical roles during chromosome segregation during mitosis 4) The function/role of this protein during mitosis is not well understood though it is known that deletion of PICH results in chromosome segregation defects. The next section describes some of the key observations regarding PICH made since its discovery almost a decade ago.

Polo-like kinase 1 interacting checkpoint helicase (PICH)

As the name suggests, PICH was identified, in 2007, as a binding partner and substrate of the mitotic kinase, Polo-like kinase 1 (Plk1) (30). Baumann et al. performed a far western ligand-binding assay with Plk1-PBD (Polo-box domain that mediates binding of Plk1 to other proteins) to identify mitotic targets of Plk1 and they identified PICH by mass spectrometry (30). PICH belongs to the family of SNF2 family of helicases due to the presence of predicted HELICc and DEXH domains (required for conventional helicase activity) with walker A and walker B motifs (known to bind and hydrolyze ATP) (29,31). PICH also has two predicted tetratricopeptide repeats (TPRs) that are known to mediate protein-protein interactions (32). Additionally, BLAST sequence analysis revealed a conserved domain that was present only in PICH orthologs that Baumann et al. named as PICH family domain (PFD). Based on the presence of PFD, we know that PICH is present in vertebrates and plants but not in yeast or invertebrates, such as *Drosophila* and *C. elegans* (30).

1.3

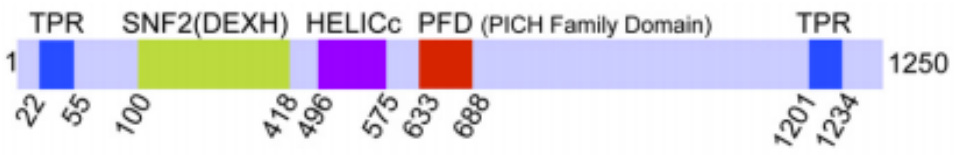


Figure 1.3. Schematic representation of primary sequence of PICH. Amino acid numbers marked and the conserved domains highlighted. Image adapted from Baumann et al. (30). TPR – tetratricopeptide repeats that can mediate protein-protein interactions. DEXH contains walker A and walker B motifs that enables binding and hydrolyzing ATP. HELICc enables translocase/helicase activity. PFD-conserved motif among PICH orthologs.

Baumann et al. made some key observations about PICH. They showed that PICH associates with centromere/kinetochore regions of the chromosomes only during mitosis and is cytoplasmic during interphase. Plk1 depletion resulted in arm localization of PICH suggesting that Plk1 is critical to remove PICH from chromosome arms. Interestingly, Erich Nigg's group saw that PICH decorates DNA threads (mostly during anaphase) in both, transformed and non-transformed cells. These special DNA threads were not detected earlier as the usual DNA staining dyes (DAPI) could not bind to these threads. The DNA threads were named ultrafine DNA bridges (UFBs). Another interesting observation by Baumann et al. was an increase in the number of PICH positive UFBs when Topo IIα activity was inhibited. In this study they showed that inhibition of PICH by siRNA resulted in a spindle assembly checkpoint defect but later the group showed that the siRNA they used in the study had an off-target (Mad2) effect (30,33). Around the same time when the Baumann et al. study was published, Chan et al. identified UFBs while studying a protein involved in Bloom's syndrome, BLM (34). Subsequently, Wang et al. showed that Topo IIα activity is required for both the resolution of PICH-positive UFBs and sister chromatid disjunction (35).

Multiple studies showed that depletion of PICH via siRNA or microinjection of PICH antibody resulted in chromatin bridges and micronuclei formation (36-38). Biebricher et al. did an extensive *in vitro* study of PICH-DNA binding (39). They showed the ATPase activity and DNA-dependent translocase activity of PICH on dsDNA. Additionally, they demonstrated that

PICH binding to DNA was enhanced when the DNA was under tension. Recently, Nielsen et al. analyzed and reported the characteristics of PICH-/- cells, especially in the presence of a Topo II inhibitor. Unlike the previous studies, they analyzed interphase morphology post mitosis in PICH depleted cells. They observed that most of the PICH-/- cells showed binucleation irrespective of chromatin bridge phenotypes in the previous anaphase (40). Importantly, they showed that unresolved UFBs can lead to binucleation and polyploidy. They also showed that the ATPase activity of PICH is required for the resolution of UFBs. Overall, their study suggested that PICH-/- cells are hypersensitive to Topo IIα inhibitor.

In the last 8 years since the discovery of PICH, certain properties of the protein have been unraveled. But there is a still a huge gap in the precise understanding of the role of PICH. We currently don't know the precise molecular mechanism of PICH's localization at the centromere and UFBs. We don't clearly understand why PICH needs to be at the centromere and UFBs. We know that PICH cooperates with Topo IIα to ensure proper segregation but the mechanism isn't well understood. We know that inhibition of Topo IIα activity by Topo II inhibitors increases SUMOylation of Topo IIα during mitosis (41). Agostinho et al. showed by *in vitro* biochemical assays and cytological assays that Topo IIα conjugation to SUMO is increased when Topo II activity is inhibited. Altogether, these studies bolster the hypothesis connecting PICH, SUMO and Topo IIα functional regulation during mitosis. The work in this dissertation started with the big goal of understanding the molecular mechanism of SUMOylation during mitosis and lead to identification of PICH as a novel SUMO substrate and SUMO-binding protein.

Introduction to SUMOylation

Post-translational modification of proteins is one of the ways to diversify their structure and function. It generally refers to covalent addition of a functional group to protein. Post-translational modification of a protein can regulate activity, localization, stability and interaction with other cellular molecules. Some of the most common types of post-translational modifications are phosphorylation, glycosylation, methylation, acetylation and ubiquitylation. Ubiquitylation involves addition of a 76-residue protein called Ubiquitin (Ub) to proteins and was initially found to target the substrate protein for degradation (42,43). After the discovery of Ubiquitin, several small ubiquitin-like proteins (Ubls) that can also covalently attach to proteins have been discovered and they have a wide range of functional consequences. One of the most studied Ubls is the member of the Small Ubiquitin-like Modifier (SUMO) family.

Discovery of SUMO

SUMO was discovered in a in a series of studies that spanned two years (1995-1997). The first SUMO gene identified was in *Saccharomyces cerevisiae* (named as SMT3, suppressor of Mif two protein 3) in a genetic screen for suppressors of Mif2, a human centromeric CENP-C protein homolog (44). Three groups independently identified SUMO-1 as a binding protein in yeast two hybrid assays. Shen et al. found SUMO-1 interacting with human RAD51/RAD52 proteins (double-strand break repair proteins) and named it Ubiquitin- like 1 (UBL1) since it was found to be a distant homolog of Ubiquitin (45). Okura et al. identified SUMO-1 as a novel interacting partner of cell-death mediating proteins, Fas/APO-1 and TNF receptor 1 (46). They named the protein Sentrin, based on its guardian (sentry) function against cell-death signaling. Boddy et al. identified SUMO-1 as a PML interacting protein and named it PIC1 (PML-interacting clone 1) (47). Finally, two different groups identified SUMO-1 covalently bound to

Ran GTPase-activating protein (RanGAP1) (48,49). Matunis et al. called it GAP modifying protein 1 (GMP1) and showed that the modification is reversible and can alter localization of a protein. Mahajan et al. named it as Small Ubiquitin-like MOdifier 1 (SUMO-1) and demonstrated that this modification can lead to novel protein-protein interactions.

SUMO and Ubiquitin

SUMO-1 was determined to have similarity to Ubiquitin and a group also named it Ubiquitin-like protein. Bayer et al., in 1998 solved the structure of SUMO-1 by NMR (50) and found the three-dimensional structure of SUMO-1 is very similar to Ubiquitin despite sharing only 18% amino acid sequence identity. SUMO-1 has the ββαββαβ fold of the Ubiquitin protein family and the C-terminal Glycine residues important for isopeptide bond formation is conserved between the two proteins.

1.4

SUMO-1 Ubiquitin

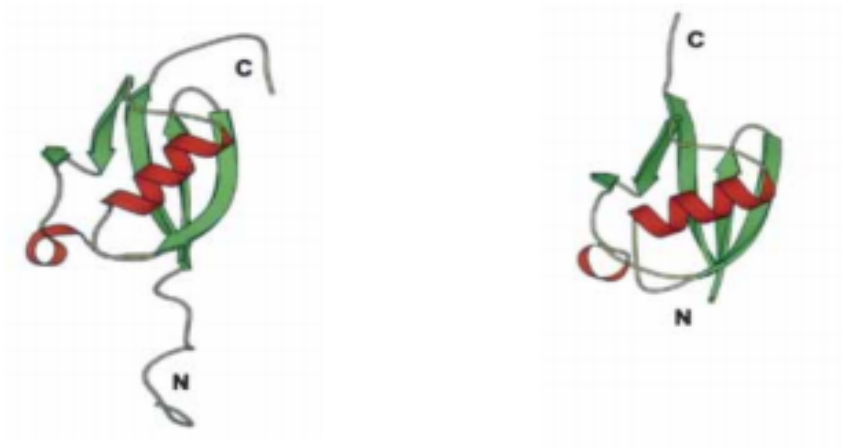


Figure 1.4. Ribbon representation of SUMO-1 and Ubiquitin structures. SUMO-1 and Ubiquitin share the same ββαββαβ fold despite having only 18% sequence identity. Figure adapted from Gill G (51).

One prominent difference is the long and flexible N-terminus that is absent in Ubiquitin. Despite having similar folds, the overall charge distribution of the SUMO-1 surface is significantly different from that of Ubiquitin. These differences indicated the potential for SUMO specific conjugation enzymes and target proteins.

SUMO Paralogs

Though invertebrates have a single form of SUMO, mammals have four isoforms of SUMO: SUMO-1, -2, -3 and -4. SUMO-1, -2 and -3 share approximately 50% sequence identity with the yeast SUMO homolog, Smt3. SUMO-2 and -3 are 96% identical to each other whereas SUMO-1 shares 46% identity with SUMO -2 and -3. Finally, SUMO-4 was identified to be 86% identical to SUMO-2, although SUMO-4 expression was detected only in a few tissue types: kidneys, lymph nodes and spleen, in contrast to SUMO-2/3, which is highly expressed in most of the tissues examined (52). As a result, most of the studies in the SUMO field have been limited to SUMO -1, 2 and 3. Saitoh et al. in 2000 studied the differences between SUMO-1 and SUMO-2/3 using an antibody that detects SUMO-2/3 but not SUMO-1 (53). They reported that SUMO-2/3 modification of total cellular proteins was greater than SUMO-1. Additionally, they demonstrated SUMO-2/3 modified RanGAP1 poorly, which is known to be a major SUMO-1 substrate. RanGAP1 is a part of the RanGTPase cycle, which plays a key role in nucleocytoplasmic transport. In vertebrates, SUMO modification of RanGAP1 is involved in directing RanGAP1 to the nuclear pore complex (NPC) (49). Saitoh et al. study reported that

targets of SUMO-1 and SUMO-2/3 are distinct and SUMO-2/3 modification is predominant in the cellular responses to environmental stresses (53).

Localizations of SUMO-1 and SUMO-2/3 are distinct and vary during cell cycle progression (54). SUMO-1 was distributed to the nucleolus and nuclear envelope since its major target, RanGAP1 is localized there (as mentioned above), whereas SUMO-2/3 was mostly concentrated throughout the nucleoplasm (54). SUMO-2/3 is capable of forming polymeric SUMO chains on lysine 11 of SUMO2/3 that has the consensus SUMOylation motif, whereas SUMO-1 cannot (55).

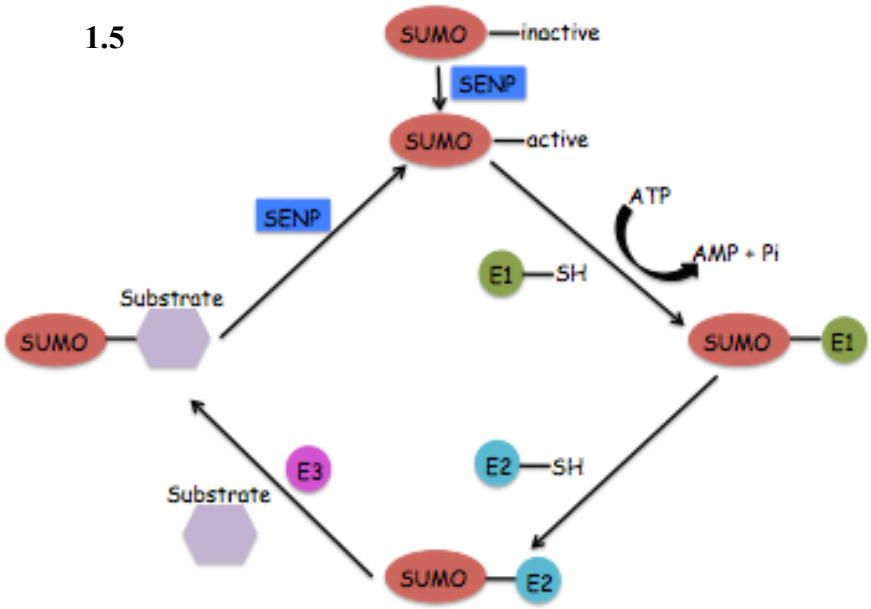
The process of SUMO modification (SUMOylation)

SUMOs three-dimensional structures are very similar to that of Ubiquitin, but the overall surface charge distribution and amino acid sequences are considerably different. This difference leads to a distinct set of modification pathway enzymes (50,56). Similar to ubiquitylation, SUMO attaches to a preferred lysine on the substrate. The modified lysine is often a part of the consensus sequence: ψKxE (where ψ is a hydrophobhic amino acid, K is the modified lysine, x is any amino acid followed by E, glutamate) (57,58). However, not all SUMOylated lysines are part of this consensus sequence, leading to what may be called as a non-consensus sequence site (59). Also, not every lysine that is part of this consensus sequence is SUMOylated.

The SUMO pathway is similar to that of ubiquitin but as mentioned before, cells have distinct E1 (activating enzyme), E2 (conjugating enzyme) and E3 (ligases) for SUMOylation. Each SUMO isoforms need to be in its mature form in order to be conjugated, just like ubiquitin. The SUMO precursor is first cleaved in its C-terminal region by SUMO proteases to generate a C-terminal di-glycine. This SUMO first forms a SUMO-adenylate conjugate with the help of the E1 activating enzyme (Aos1/Uba2) (identified in yeast) and ATP, as an intermediate in the

formation of a thioester bond with the C-terminus glycine and Cys 173 on Uba2 (yeast). The E1 activating enzyme promotes three different interactions via its domains: the catalytic domain for the formation of thioester bond with SUMO, the adenylation domain that possesses the ATP-binding site and the ubiquitin-fold domain (UFD) that interacts with Ubc9 (E2 enzyme) (60,61). The next step in SUMO conjugation is the transfer of SUMO from Uba2 to the E2 conjugating enzyme, Ubc9. The C-terminal glycine of SUMO forms a thioester bond with cysteine 93 of Ubc9. Ubc9 can also form a non-covalent interaction with SUMO that potentially helps during polymeric SUMO chain formation (62,63). Finally, the SUMO E3 ligase acts as an adaptor protein in the transfer of SUMO from Ubc9 to ε-amino group of lysine on the substrate. SP-RING domain of the ligase interacts with Ubc9 (64) and the N-terminal SAP and PINIT domains interact with the substrates (60).

Figure 1.5. The SUMO conjugation pathway. SUMO is conjugated to a lysine on a substrate with the help of three enzymes (E1, E2 and E3) and this modification is reversible.



SUMO activating enzyme E1

The SUMO activating enzyme E1 is a heterodimer of proteins named Aos1 (activation of Smt3p) and Uba2 (ubiquitin activating enzyme). Uba2 (71 kDa), first identified in budding yeast (65), was initially thought to be a component of the Ub system based on its sequence similarity to Ub E1 activating enzyme (Uba1, 114 kDa) in yeast and other organisms. Dohmen et al. showed that Uba2p is essential for cell viability. Additionally, Uba2p could not functionally complement the essential functions of Uba1p and did not display Ub E1 activity. Two years later, Johnson et al. identified Uba2p as a Smt3p activating enzyme and showed that it interacts with Aos1p (40 kDa) to be fully active in an ATP dependent manner (66). Aos1p shared sequence similarity with the N-terminus of Uba1p. The group also showed that Gly98 (C-terminal residue) of the mature Smt3p is required for its conjugation to substrates. Sequentially, the E1 enzyme for SUMO-1 conjugation in humans, similar to Aos1p (38 kDa) and Uba2p (72 kDa), was identified and named SAE1/SAE2 (SUMO-1-activating enzyme) (67). Most organisms contain only one SUMO activating enzyme that is responsible for SUMO conjugation to all substrates.

SUMO conjugating enzyme E2

The SUMO conjugating enzyme, Ubc9 was discovered in *Saccharomyces cerevisiae* before SUMO as a putative ubiquitin conjugation enzyme (Ubc9) (68). Ubc9 shared sequence similarity (~35% identity) to known ubiquitin conjugating enzymes. Surprisingly, unlike most known *UBC* genes, Seufert et al. showed that Ubc9 is essential for cell viability and mediates cyclin B degradation in yeast. Parallel to Seufert et al. discovery of Ubc9, Khodairy et al. identified Ubc9 in *Schizosaccharomyces pombe* in a screen to identify new elements in controlling cell cycle

checkpoints (69). The *Pombe* homolog of Ubc9 was named Hus5 based on its sensitivity to a DNA synthesis inhibitor (hydroxyurea).

In 1997, after SUMO-1 in humans and Smt3p in yeast were identified, three groups independently showed that Ubc9 is a SUMO conjugating enzyme. Johnson and Blobel purified Ubc9p by covalent affinity chromatography. An ATP containing yeast protein mixture was incubated with an affinity column coupled with Smt3p and pre-bound to E1 (Aos1p/Uba2p) (70). They showed that Ubc9p forms a thioester bond with Smt3p and not Ub and is essential for Smt3p conjugation. Gong et al. performed a yeast two-hybrid assay with sentrin (SUMO) as bait and identified Ubc9 as an interacting protein (71). They showed sentrin and Ubc9 conjugate *in vitro*. Finally, the Dasso group showed that the *Xenopus* homolog of Ubc9 acted as an E2 conjugating enzyme in SUMO-1 conjugation to its first identified substrate, RanGAP1 (72).

Most likely, Ubc9 is the only SUMO conjugating enzyme present in yeast and vertebrates unlike the ubiquitin pathway, where multiple E2s have been identified. Ubc9 has been shown to be essential in most organisms in which it has been determined (68,73). Although Ubc9 has sequence similarity with other identified ubiquitin E2s, the overall charge distribution of Ubc9 is distinct and this feature allows for specific interaction of Ubc9 with SUMO and not ubiquitin (74). Unlike Ub conjugation, SUMO E2 enzymes can be sufficient to recognize and SUMOylate substrates. Structural studies have identified the regions of Ubc9 that are critical for binding directly to the substrate and to SUMO, assisting in transfer of SUMO from the E1 (75-77).

SUMO Ligases E3

Ubiquitin conjugation to its substrates requires a third class of enzymes called E3 Ligases and studies after the initial discovery of SUMO showed that SUMO conjugation can happen as long

as SUMO E1 and E2 enzymes are present. How SUMOylation is regulated spatially and temporally was a big question. Ubiquitin has multiple E2 conjugating enzymes and hundreds of E3 ligases that confer specificity in contrast to SUMO that has a single E1 and a single E2. Additionally, SUMOylation in the absence of HeLa cell extract or *Xenopus* egg extract was inefficient with only SUMO E1 and E2 (78).

In 2001, two independent groups discovered potential SUMO E3 ligases in *Saccharomyces cerevisiae*. The Yoshiko Kikuchi group identified a novel protein, Siz1 that potentially acted as an E3 ligase for septin-SUMOylation (79). Siz1 was reported to belong to a new family of proteins containing a conserved domain (zinc-binding RING-domain), similar to ubiquitin ligases. This group showed the absence of septin SUMOylation in *siz1* mutant yeast and showed physical interaction of Siz1 with Ubc9 and septin components. Further the Takahashi and Kikuchi groups showed that the RING domain of Siz1 directly interacted with Ubc9 with septin component acting as an adapter (80). Johnson and Gupta, in order to identify other proteins involved in septin SUMOylation, examined proteins that interact with septin and identified Siz2 (81). They found Siz2 to have a RING domain and identified Siz1 to have the same domain. They showed that deletion of both Siz1 and Siz2 reduces septin SUMOylation drastically (81).

In parallel to the discovery of Siz1 and Siz2 as SUMO E3 ligases in yeast, PIAS (protein inhibitor of activated STAT1) proteins were shown to act as SUMO E3 ligases in mammalian cells (64,82). Yasuda's group identified PIAS1 as a SUMO-1 binding protein. They showed a physical interaction between PIAS1 and Ubc9, dependent on the RING finger-like domain of PIAS1. Additionally, they demonstrated a PIAS1-dependent SUMOylation of p53 in U2OS cells and *in vitro*. Sachdev et al. identified PIASy as a novel interacting partner of LEF1 (Wnt-

responsive transcription factor). They showed that PIASy mediated SUMOylation of LEF1 and other proteins.

PIAS proteins were discovered around the same time as SUMO (83,84). PIAS1 and PIAS3 were discovered as inhibitors of transcription factors, STAT1 and STAT3 respectively. Currently, the PIAS family of proteins consists of five members: PIAS1, PIASxα, PIASxβ, PIASy and PIAS3. PIAS proteins do not act only as SUMO E3 ligases but, rather, have multiple functions based on the various domains. PIAS proteins have high sequence homology at the N-terminus. They consists of five different domains: An N-terminal SAP (scaffold attachment factor-A/B, acinus and PIAS) domain, a PINIT motif, a RING finger zinc-binding domain, a SUMO-interacting motif (SIM) and a C-terminal region rich in serine and threonine (85).

SUMO proteases

SUMOylation is a reversible post-translational modification. SUMO specific proteases cleave SUMO from its substrate and are also involved in the initial activation of SUMO by cleaving C-terminal residues to expose the di-glycine motif for attachment to substrates. SUMO proteases were first identified in yeast as ubiquitin-like proteins (Ulp1 and Ulp2). Li and Hochstrasser identified a SUMO-1/Smt3 specific protease and named it Ubl-specific protease (Ulp1) (86). Around the same time, SUMO specific proteases were identified in mammalian cells (87).

1.6

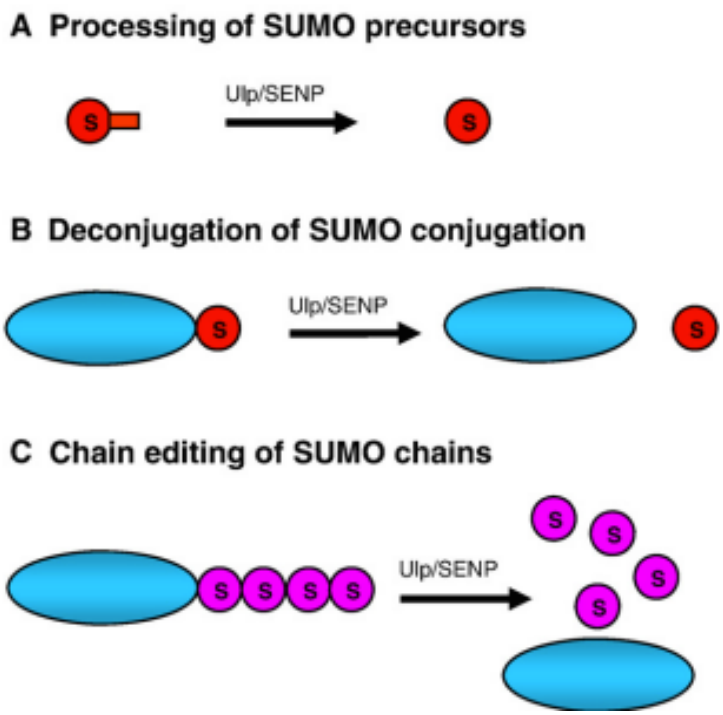


Figure 1.6. Different catalytic functions of SUMO-specific proteases (SENPs). Figure adapted from Kim et al. (88).

Six different SENPs have been identified in humans: SENP1, 2, 3, 5, 6 and 7. SENP1, 2, 3 and 5's sequences are related to Ulp1 whereas SENP6 and 7 sequences relate to Ulp2 (89). These SENPs are highly specific towards SUMO paralogs (90). Multiple groups have now shown that the different SENP localization and the phenotypes of their deletion mutants differ, suggesting distinct roles for each SENP (89). It is proposed that the N-terminal region of SENP determines its localization and substrate specificity. Below is a table showing different SENP localizations and catalytic activities.

Table 1.1. SUMO-specific proteases (SENPs) in humans. Information in the table is adapted from Mukhopadhyay et al. (89) and Kim et al. (88).

SUMO Proteases	Subcellular localization	Processing	Deconjugation	Chain editing	Reference
SENP 1	Nuclear speckled foci, Nuclear pore	SUMO 1>2>3	SUMO 1/2/3	No	(86,91-93)
SENP 2	Nuclear Pore	SUMO 2>1>3	SUMO 1/2/3	No	(94-97)
SENP 3	Nucleolus	Unknown	SUMO 2/3	No	(98,99)
SENP 5	Nucleolus	SUMO 3	SUMO 2/3	NO	(99,100)
SENP 6	Nucleoplasm	No (Very low)	SUMO 1	SUMO 2/3	(88,89,101)
SENP 7	Nucleoplasm	No (Very low)	No (Very low)	SUMO 2/3	(88,89,101)

The introduction to SUMOylation above describes the SUMOylation pathway and the key enzymes that play a role in the dynamics of SUMOylation. Similar to Ubiquitin, SUMO can modulate a protein by either covalently conjugating to its substrate or mediating non-covalent interactions with other proteins via SUMO-interacting motifs (SIMs). Additionally, there are a few proteins that can be SUMOylated as well as can non-covalently interact with SUMO substrates. In this study, we identified PICH as one such protein that can potentially be regulated by SUMO in both ways (102,103).

Physiological roles of SUMOylation

Covalent modification by SUMO

RanGAP1 was the first covalently modified SUMO substrate identified (49). Since its discovery, many substrates of SUMO have been identified. Over the years, SUMO modification of many proteins has been demonstrated to play a role in multiple different functions. Physiological functions of covalent modification by SUMO are diverse and include, change in subcellular localization (48,49,104,105), regulation of gene expression (106-114), DNA damage response and repair (115-119), DNA replication (120), signal transduction (108,121-124), cell division and chromosome segregation (9,59,125).

Non-covalent interactions with SUMO (SUMO-interacting motif, SIM)

Soon after the discovery of covalent modification by SUMO, multiple groups also discovered that SUMO could mediate novel non-covalent interactions with other proteins. Three studies between 1996 and 2000, using yeast two-hybrid assays identified Rad51 and Rad52 as SUMO-interacting proteins (45,126,127). Finally, Minty et al. in 2000 showed specific proteins that can interact with SUMO and have a conserved SUMO-interacting motif (SIM) (128). They performed a two-hybrid assay and identified certain proteins that interacted with SUMOylated p73. After comparison of the sequences of the identified interacting proteins, Minty and group came up with a common Ser-X-Ser (SXS) sequence (128). X is any amino acid in the sequence and they also noticed that the SXS sequence was flanked by hydrophobic amino acids on one side and acidic amino acids on the other side. Four years later, Song et al. showed that SIM sequence does not require these serine residues (129). Song and colleagues used NMR to study the interaction between SUMO-1 and peptides from proteins that have been previously shown to

bind to SUMO. They characterized the SIM site as the hydrophobic core along with flanking acidic regions, Val/Ile-X-Val/Ile-Val/Ile (V/I-X-V/I-V/I). Since then multiple studies have demonstrated that the hydrophobic core is indeed essential.

The hydrophobic core of the SIM forms a β-strand that interacts with the β2-strand and the α-helix of SUMO (130,131). This interaction results in intermolecular β-sheet formation. A cluster of acidic residues that juxtaposes the hydrophobic core promotes electrostatic interactions in SIM-SUMO associations. Sometimes, serines and threonines can be found instead of the acidic residues in SIMs. Ser/Thr residues can be phosphorylated, introducing negative charges for the SIM-SUMO interaction (130). These types of SIMs are categorized as phosphor-SIM. Major residues involved in the SIM interaction are conserved among the SUMOs. However, a crystallographic study has shown that arrangement of hydrophobic core and acidic residues in SIMs might control binding to specific SUMO isoforms (132). The affinity of SUMO to SIM interaction is in the micromolar range and stronger affinities requires multiple SUMO-SIM binding.

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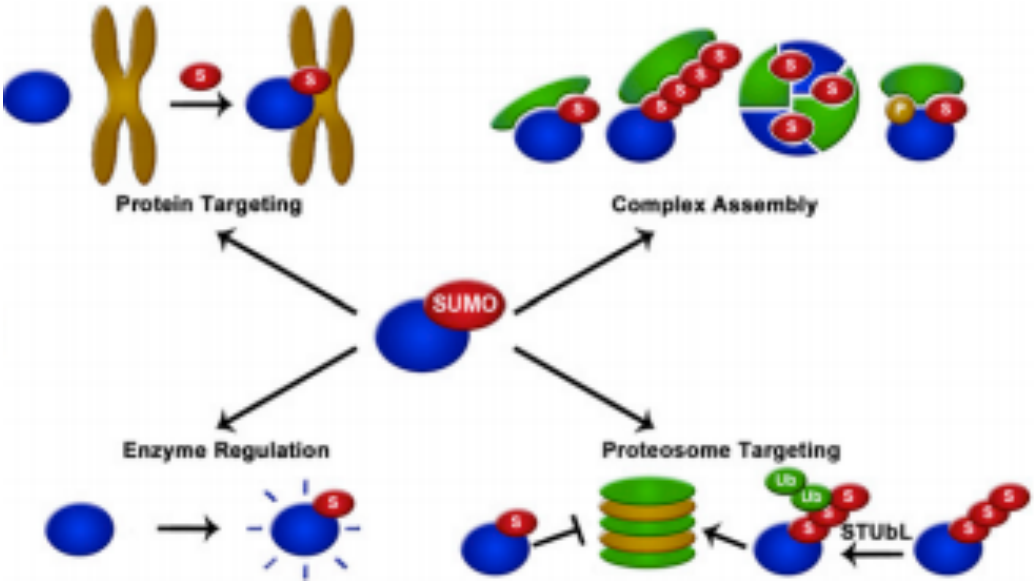

Figure 1.7. Molecular roles of SUMOylation. Figure adapted from Cubenas-Potts et al. (133)

I initially identified PICH as a promiscuous SUMO-binding protein (an example of SUMO-mediated non-covalent interaction) and later showed PICH to be a novel SUMO substrate (an example of covalent SUMO modification). PICH is not the first mitotic protein identified to be regulated by SUMOylation. SUMO research in the past two decades has established a strong base for the significance of SUMOylation during mitosis.

SUMOylation in cell division and chromosome segregation

The link between mitosis and SUMO was established early in SUMO history when Seufert et al. showed in yeast that Ubc9 is required for the degradation of M-phase cyclins, a key for anaphase onset (68). Additionally, SMT3 (SUMO) was identified in yeast screens for mutants defective in segregation of chromosomes (134). Wan and Zhang in a review have discussed SUMOylation's importance during proper chromosome segregation (9). Multiple genetic studies inhibiting different SUMO pathway enzymes in yeast and mammalian cells have indicated a role of SUMO in mitosis. In order to understand the molecular mechanism of the SUMO regulated proteins during mitosis, it is critical to identify the mitotic SUMO targets. Multiple mitotic SUMO targets have been identified in the last two decades but one of the biggest challenges in understanding the role of SUMOylation in mitosis is the absence of extensive studies on the regulation of those identified mitotic SUMO target. Nevertheless, identification of these targets indicated the potential importance of SUMOylation during different stages/aspects of mitosis.

1.8

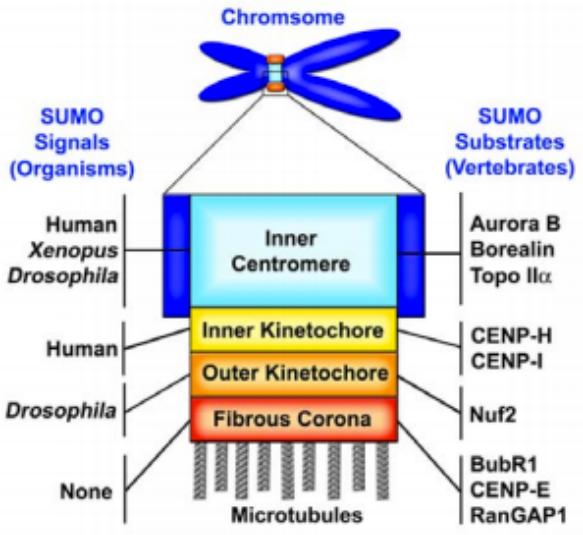

Figure 1.8. SUMO signals and known SUMO substrates from invertebrates to mammals at the centromere and kinetochore during mitosis (until metaphase). Figure adapted from Wan et al. (9).

Topoisomerase IIα

Topoisomerases are enzymes that have the capability to relax negatively supercoiled DNA. They also possess strand passaging activity that enables disentanglement of linear DNA (135). Multiple evidence suggests that Topoisomerase II is crucial for both DNA condensation and chromosome segregation (136-138). Topo IIα was one of the first and major mitotic SUMO targets identified in both budding yeast and vertebrates (139,140). Bachant et al. showed that yeast strains without the SUMO-deconjugating enzyme (Ulp2 or SENP) lost their centromeric cohesion and this loss was restored by the overexpression of Topo II (140). They showed that Topo II itself is SUMOylated and SUMOylation-deficient Topo II mutants were not able to resume the cohesion loss. Soon after, Azuma et al. published their work on Topo IIα SUMOylation in *Xenopus* egg extract (139). They showed defects in chromosome segregation when SUMOylation was inhibited, suggesting a possible role for Topo IIα downstream of

SUMOylation. Following this study, Ryu et al. showed that SUMOylated Topo IIα can inhibit the decatenation activity of Topo IIα *in vitro,* suggesting Topoisomerase IIα SUMOylation is critical for accurate chromosome segregation (59). SUMOylation of Lysine 660 of *Xenopus laevis* Topo IIα was critical for its *in vitro* enzymatic activity regulation. Additionally, Ryu et al. also showed that SUMOylation of the C-terminus domain (CTD) of Topoisomerase IIα mediates protein-protein interactions at the centromere during mitosis, suggesting more than one role for Topo IIα SUMOylation (141). Yoshida et al. showed the importance of Topo IIα SUMOylation in Histone H3 threonine3 phosphorylation (H3T3P) that is required for chromosome passenger complex (CPC) localization at the centromere via protein-protein interaction (Yoshida et al. 2016, Journal of Cell Biology, in press). The above two studies emphasize the importance of Topo IIα CTD SUMOylation in recruitment of proteins at the centromere, albeit, how SUMOylation of Topo IIα regulates its enzymatic activity is not well understood.

Since the discovery of PICH, multiple studies have emphasized the co-operative roles of PICH and Topo IIα in Topo IIα's decatenation activity at the centromere (as explained in detail in the PICH section of this chapter) (35,40). Is SUMOylation of Topo IIα critical for its co-operative activity with PICH? My results in chapter two and three give a novel insight into potential regulation of Topo IIα activity via SUMOylation and PICH (discussed in chapter five).

Other important mitotic SUMO targets

Condensin and Cohesin complexes are important for the structural maintenance of chromosomes. Both complexes are comprised of multiple subunits. Some of the subunits of Condensin are identified as SUMO targets though functional roles of SUMOylation of those subunits are not well understood (142,143). A protein named Pds5 is known to interact with the

Cohesin complex and increase the sister chromatin cohesion. Pds5 is identified to be SUMOylated and SUMOylation of Pds5 disrupts its interaction with the Cohesin complex and facilitates its release during anaphase (144). Proteomic analysis in budding yeast by mass spectrometry revealed multiple SUMOylation targets associated with the centromere/kinetochore region (142,143). Some of the identified targets were confirmed by additional experimental studies (145). Another major regulator of mitosis, Aurora B was recently shown to be SUMOylated, and SUMOylation regulates its activation (125). Additionally, the Matunis group also showed that overexpression of deSUMOylating enzyme, SENP2, results in chromosome segregation defects (146,147). Our research group has previously identified Poly (ADP-ribose) polymerase 1 (PARP1) as a mitotic SUMO substrate in *Xenopus* egg extracts but a role for PARP1 SUMOylation is not well understood (10).

The above-mentioned examples briefly describe studies that have contributed to the establishment of a connection between SUMOylation and mitosis. Though some of these targets are identified to be SUMOylated under physiological conditions, not every target identified has been tested under physiological conditions. More importantly, our current understanding of the role of these mitotic SUMOylated proteins at a molecular level is minimal. Additionally, we don't know if and how these mitotic SUMOylated proteins act together in the spatial and temporal regulation of SUMOylation. Our research group is working to better understand the role of SUMOylation with importance to the above-mentioned points to ensure proper segregation of chromosomes during mitosis.

Methods of studying SUMO modification and functions

The SUMO field has advanced immensely since the discovery of SUMO almost two decades ago. Many SUMO substrates have been identified and the importance of their SUMO

modification has been somewhat characterized for a few of them. Nevertheless, many substrates are yet to be identified and much of the identified substrate's functions need to be characterized.

There are challenges associated with studying SUMO modification. First, this modification is highly transient inside the cell. At any given point, only a small fraction (1-5%) of the total protein is SUMOylated in most cases with the exception of RanGAP1 (which is 50% - >90% SUMOylated) (48,49,148). This transient nature of the modification is mainly due to two things. First, SUMO proteases are highly active inside the cell. These enzymes are not easily inhibited and result in rapid deSUMOylation upon cell lysis or within the cell. Second, SUMOylation is dependent on the substrate localization at a given time. For instance, it is possible that a protein gets SUMOylated only if it is associated with chromatin or is interacting with another protein. Moreover, SUMOylation can also alter a protein's subcellular localization. Second, SUMOylation is dependent on the concentration of SUMO pathway enzymes at a particular time inside the cell. Our group has previously shown that SUMO E3 ligases localization differs inside the cell (149). *In vitro*, we can SUMOylate a known substrate in the absence of SUMO E3 ligase whereas *in vivo* SUMO modification is dependent on the presence of a SUMO E3 ligase, specific to the substrate. This makes SUMOylation a highly complex spatial and temporal post-translational modification. Finally, knockout studies of SUMO isoforms and SUMO E3 ligases show redundancy. Most of the model organisms with SUMO-1, SUMO-2/3 or PIAS knockouts are viable, albeit with defects (150-152). SUMO E1 and E2 enzymes knockouts are embryonically lethal.

With these challenges, the SUMO field has developed effective ways to identify SUMO targets and study their functions. Some common methods that have been used in the past to identify SUMOylated proteins include immunoprecipitation-using SUMO antibodies from cell

extracts, *in vitro* SUMOylation assays with recombinant proteins, model organisms (mouse model), proteomic analysis using mass spectrometry, and *Xenopus* egg extracts (139,153-155). Further, to study the functional significance of the identified SUMO target, methods that can be deployed are: 1) *in vitro* assays based on the protein of interest and its function 2) inhibition of SUMOylation by overexpression of deSUMOylating enzymes and looking for phenotypic changes 3) identification of SUMOylation sites (covalently modified lysine or SUMO-interacting motif dependent non-covalent interaction) and generation of a SUMO deficient mutant. Many systems can be used to study SUMOylation as mentioned above, including mammalian cell cultures, *Xenopus* egg extracts, and genetic model organisms. Each system has its strengths and limitations. One of the best systems to study SUMOylation during mitosis is *Xenopus* egg extracts for the reasons explained in the next section.

***Xenopus* Egg Extract (XEE)**

Over the years, cell cycle research has made numerous discoveries using cell-free extracts from the eggs of *Xenopus* (South African clawed toad). Since their first use a little more than 30 years ago (156,157), *Xenopus* cell-free extracts have been used as an established model system in a diverse cell cycle field for studying apoptosis, cell cycle progression, chromatin structure, dynamics of spindle microtubules, DNA replication etc. *Xenopus* eggs are arrested in metaphase of meiosis II. Details for how this soluble egg extract works are explained in the review by Gillespie et al. (158). Most of the proteins needed for the progression of cell cycle are pre-formed in these eggs (11 rounds of cell division).

One of the major strengths of the *Xenopus* egg extract system is its ability to recapitulate eukaryotic cell cycle events *in vitro* under the same conditions that exist *in vivo*. XEE has the capability to assemble chromatin and make interphase nuclei. Multiple cycles of mitosis and

interphase can be controlled manually *in vitro*. After interphase, replicated DNA can be induced back to mitosis to undergo chromosome segregation with the help of the mitotic spindle. All these nuclear events of the eukaryotic cell cycle *in vitro* enable the study of mechanisms and dynamics of cell-cycle dependent processes at a biochemical level. Additionally, using XEE, we can perform depletion/addback experiments as described in some of the research papers from our laboratory (10,59). Unlike mammalian cells, where we inhibit a gene of interest using siRNA, in XEE we can achieve close to 100% depletion of most proteins and control the amount of recombinant protein we add back. This unique feature of XEE allows the study of functions/roles of SUMO/SIM mutants in different proteins efficiently.

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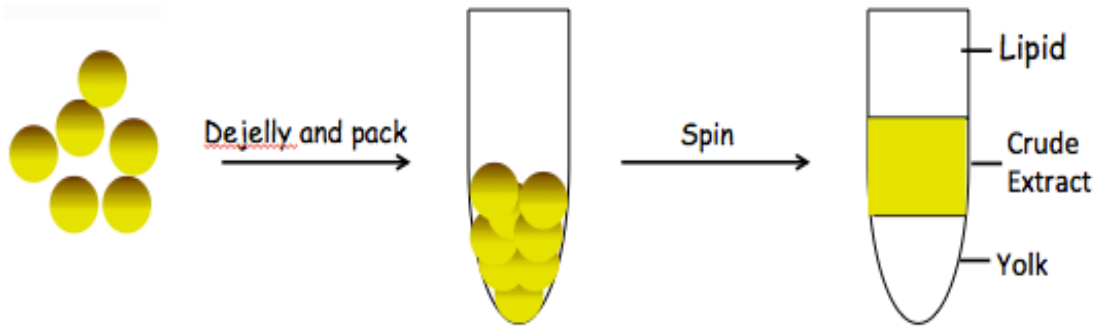

Figure 1.9. Schematic diagram of *Xenopus* egg extract preparation from *Xenopus* eggs.

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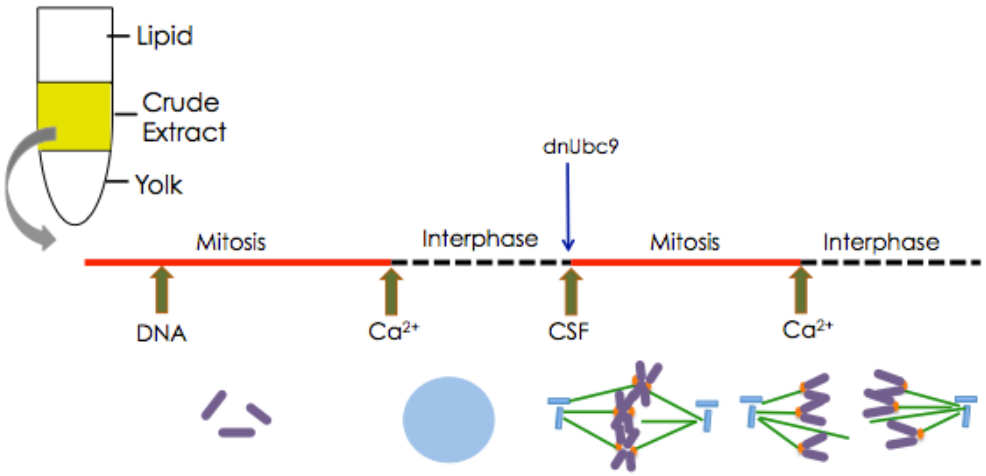

Figure 1.10. Schematic diagram of the how *Xenopus* egg extract can be cycled between mitosis and interphase.

The XEE has been previously used to identify SUMO substrates and understand SUMO-dependent mechanisms (10,11,139,141). This system can be used to isolate mitotic chromosomes in a nearly *in vivo* state and this has led to the identification of two important mitotic SUMO substrates by our and Dr. Dasso's research groups: Poly-ADP ribose polymerase I (PARPI) and Topoisomerase IIα (10,139). Work in this dissertation identifies another mitotic SUMO substrate, PICH (11). Additionally, since XEE has a plethora of proteins that are involved in the cell cycle, this extract can be used to perform pull-down assays to identify protein-protein interactions. We have the capacity to SUMOylate proteins *in vitro* and incubate the SUMOylated protein with *Xenopus* egg extract to identify proteins with SIMs that can interact with SUMO (11,141). Thus, this system can be successfully and efficiently used to identify novel SUMO substrates and SUMO-binding proteins (11,141,159). The second chapter of this dissertation will

have more details on the use of XEE for pull-down assays and identification of novel SUMO-interacting proteins.

Summary

The work in this dissertation is aimed to understand the molecular mechanism of SUMO regulation during mitosis. We wanted to investigate the functional significance of one of the two previously identified SUMO substrates by our research group – Poly (ADP-ribose) polymerase 1 (PARP1) in protein-protein interactions (10). We observed that SUMOylation of PARP1 does not alter its enzymatic activity but changes the pattern of PARylation (explained more in detail in chapter two) (11). We identified the protein Polo-like kinase 1 interacting checkpoint helicase (PICH) as a SUMOylated PARP1 binding protein by LC-MS/MS. In chapter two of this dissertation, we show PICH to be a promiscuous SUMO-binding protein as well as a novel SUMO substrate. PICH is a DNA-dependent ATPase/translocase that belongs to the SWI-SNF family of remodelers. We also showed that SUMOylation of PICH can significantly reduce DNA binding *in vitro*. We wanted to further understand the functional importance of SUMO-interacting motifs (SIMs) on PICH. In chapter three of this dissertation, we identified three SIMs in PICH, which, when mutated, lessen its ability to efficiently bind to SUMOylated substrates. We next developed conditional expression of PICH in cells and observed that PICH without SIMs has chromosome localization defects during mitosis. Additionally, we saw that the absence of SIMs in PICH also resulted in chromatin bridges. Surprisingly, we observed very interesting differences in the centromere localization pattern and corresponding chromatin bridge formation between the different SIMs. This led us to hypothesize that PICH SIMs have dual roles during mitosis: localization/retention on chromosomes and activity to ensure proper chromosome

segregation. In chapter four of this dissertation, parallel to PICH SUMO observations, we saw defects in some of the centromere specific markers (H2A.Z and HP1 gamma) in the absence of SUMOylation. Chapter five of this dissertation briefly summarizes the results of each chapter and sets forth future perspectives in PICH-SUMO research along with more global SUMO-centromere-mitosis regulation.

Research and discoveries described in this dissertation have opened novel avenues to understand the role of PICH during mitosis. The work delineates a new potential function of Topoisomerase IIα during mitosis. Overall, this work contributes to solving the jigsaw puzzle of mitosis-centromere-SUMO.

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CHAPTER 2

SUMOylation regulates Polo-like kinase 1-interacting checkpoint helicase (PICH) during mitosis

INTROUCTION

SUMOylation is a posttranslational modification with diverse biological consequences. The addition of the small ubiquitin-like modifier (SUMO) affects target proteins by altering their subcellular localization, enzymatic activity and/or protein-protein/protein-DNA interactions (1-5). Similar to ubiquitylation, SUMO conjugation to its substrates requires a cascade of three enzymes: an activating E1 enzyme, a conjugating E2 enzyme, and typically a SUMO E3 ligase enzyme. All SUMO modifications utilize the same E1 (Uba2/Aos1) and E2 (Ubc9) enzymes, but different E3 enzymes have been identified that show specificity to certain targets (6). Multiple lines of evidence show that SUMOylation is a vital posttranslational modification to ensure the proper segregation of chromosomes during mitosis (7,8). Consistent with observed segregation defects, many mitotic proteins have been identified as SUMO targets (7).

On mitotic chromosomes, a major SUMO signal is observed at the centromeric region in both somatic cells and *Xenopus* egg extract (XEE) cell-free assay (9,10). Using the XEE assays, our group have previously identified two major PIASy-dependent mitotic chromosomal SUMO2/3 substrates: DNA topoisomerase IIα (TopoIIα) and poly(ADP-ribose) polymerase 1 (PARP1) (11,12). TopoIIα was one of the first mitotic SUMOylated substrates identified in budding yeast and vertebrates (11,13) and is pivotal for DNA decatenation to separate sister chromatids during chromosome segregation. Accumulating evidence indicates that SUMOylation is important for

the regulation of TopoIIα activity (14,15). Another robust mitotic SUMOylation substrate, PARP1 (12), is a member of the PARP family that catalyzes the formation of poly (ADP-ribose) on target proteins, leading to multi-faceted biological consequences (16). Although we have previously shown potential PARP1 activity regulation by SUMOylation on mitotic chromosomes (12), the comprehensive mitotic role of PARP1 and how SUMO modification affects the function of PARP1 during mitosis has not yet been determined.

SUMO modification often provides a new site for protein-protein interactions (17-19), and non-covalent interactions between SUMO-interaction motif (SIM)-containing proteins and SUMOylated proteins have been shown to produce multiple critical functional consequences (20-22). To extend our understanding of the downstream effects of SUMOylation at mitotic centromeres, we intended to identify SUMOylation-dependent binding protein(s) using PARP1 as bait. We identified Polo-like kinase 1 (Plk1)-interacting checkpoint helicase (PICH), which is also known as ERCC6-like protein and belongs to the SNF2 family of ATPases, as a novel SUMO-interacting partner. Prior studies have shown that PICH is essential for the proper segregation of chromosomes during mitosis (23-25). In this study, we have detected PICH as a novel SUMO substrate on mitotic chromosomes. *In vitro* SUMOylated PICH showed reduced DNA binding capability, implicating the SUMO-dependent regulation of PICH activity. Altogether, we propose a novel regulation of PICH function at mitotic centromeres by SUMOylation.

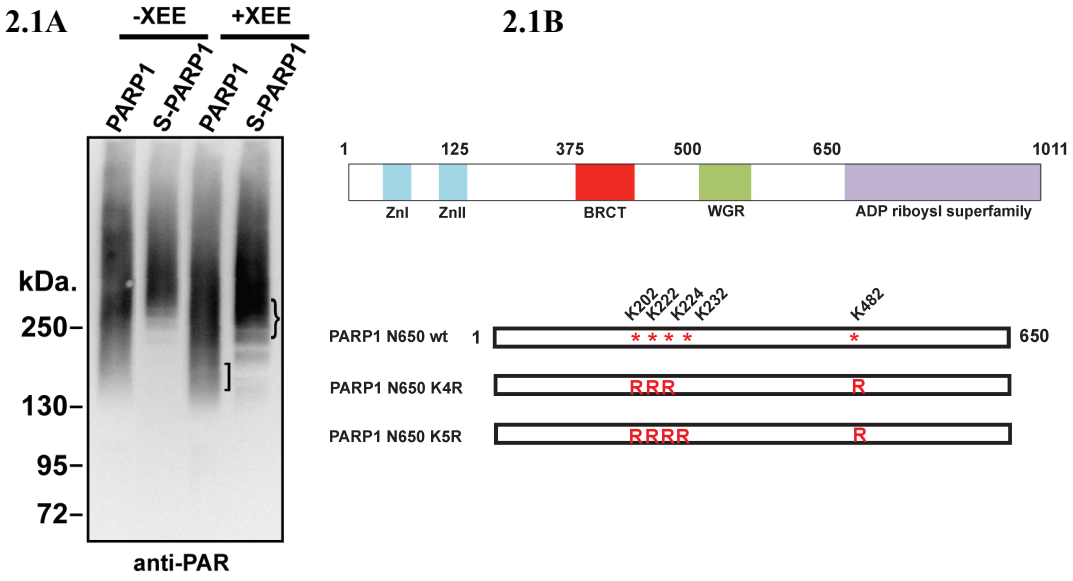
RESULTS

SUMOylation of PARP1 mediates protein- protein interactions.

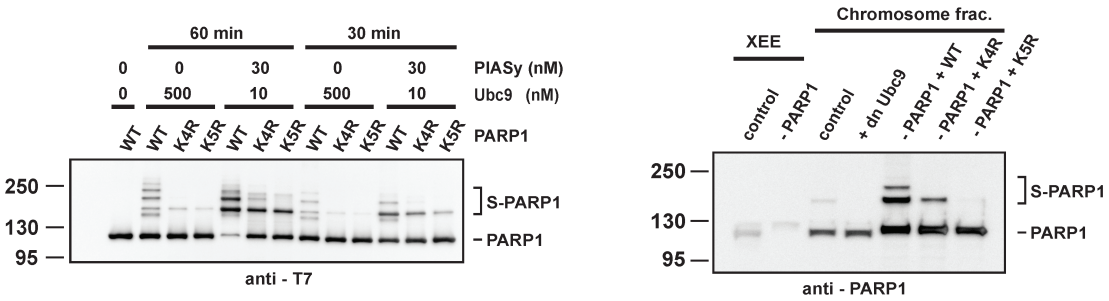
We previously showed that PARP1 SUMOylation does not alter its enzymatic activity but

does result in subtle differences in the PARylation profile of mitotic chromosomal proteins (12). To examine whether the SUMOylation of PARP1 could affect its substrate specificity, we performed a PARylation reaction with a PARP1 pull-down fraction using *Xenopus* egg extract (XEE). Either SUMOylated or non-SUMOylated PARP1 was captured on agarose beads, and the beads were then incubated with XEE to isolate binding proteins. The beads were incubated in a reaction buffer containing NAD^+ to initiate PARylation by PARP1. Consistent with our previous finding, PARP1 showed robust self-PARylation activity regardless of SUMOylation (Lanes 1 and 2 in Figure 2.1A); however, we observed a difference in the PARylation profile when we compared PARylation on beads between samples without XEE and samples after pull-down with XEE using PARP1 and SUMOylated PARP1 (Lanes 3 and 4 in Figure 2.1A). In the pull-down samples incubated with XEE, there was an increase in the PARylation of approximately 150 kDa for non-SUMOylated PARP1, whereas more PARylation, an apparent mass of approximately 250 kDa, was observed for SUMOylated PARP1 (Figure 2.1A). This finding suggests that PARP1 promotes the PARylation of its interacting proteins and that the SUMOylation of PARP1 could alter its substrate specificity in XEE. Conjugation of SUMO to cellular proteins is known to create new docking sites for protein-protein interactions through a SUMO-interacting motif (SIM) (26). Our results are consistent with this notion because SUMOylation potentially regulates the interacting proteins of PARP1. To examine this further, we sought to identify the binding proteins for PARP1 and SUMOylated PARP1. We utilized a PARP1 N-terminal 650 amino acid fragment (PARP1- N650) that contains most of the SUMOylation sites that we identified by *in vitro* SUMOylation with a series of mutants (Figure 2.1B&C). Because the expected binding proteins will be larger than 100 kDa, the use of PARP1-N650, the molecular weight of which is approximately 85 kDa with the affinity tag, will be beneficial for

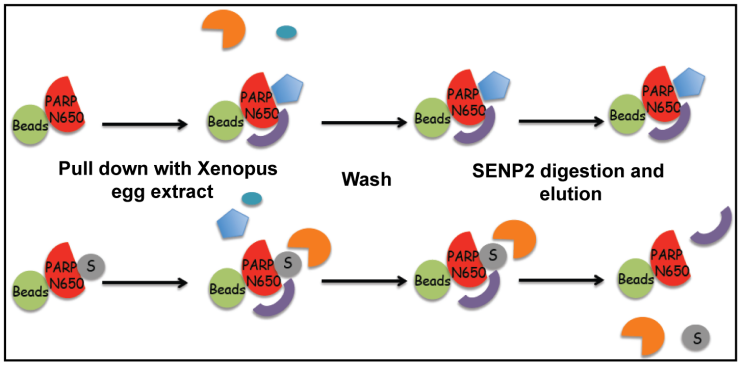
distinguishing the bands of the bound proteins by SDS-PAGE. In addition, we introduced SENP2 digestion after pull-down to eliminate the bands of SUMOylated bait for better visibility of the bound proteins (Figure 2.1D). As shown in Figure 2.1E, PARP1 bound to many proteins in XEE, and several proteins were specific for SUMOylated PARP1, suggesting that PARP1 SUMOylation mediated specific protein-protein interactions.



2.1C


60 min
30 min
0
0
30
0
30
PIASy (nM)
0
500
10
500
10
Ubc9 (nM)
WT
WT
K4R
K5R
WT
K4R
K5R
WT
K4R
K5R
WT
K4R
K5R
PARP1
250
130
95
S-PARP1
PARP1
anti - T7
Chromosome frac.
XEE
control
- PARP1
control
+ dn Ubc9
- PARP1 + WT
- PARP1 + K4R
- PARP1 + K5R
250
130
95
S-PARP1
PARP1
anti - PARP1

2.1D


PARP N650
Beads
S
Pull down with Xenopus egg extract
Wash
SENP2 digestion and elution

2.1E

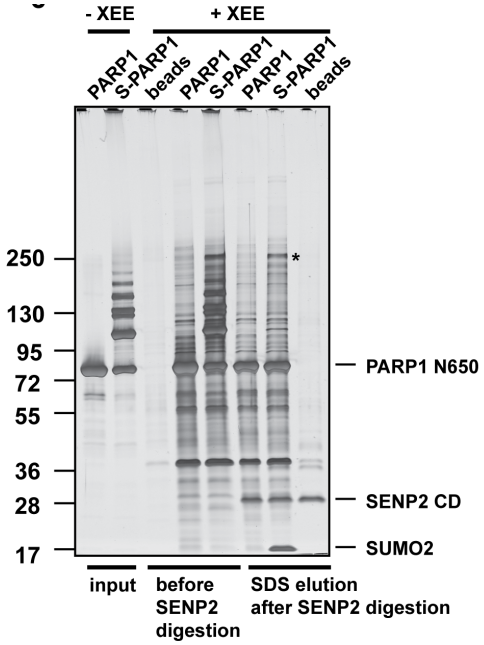
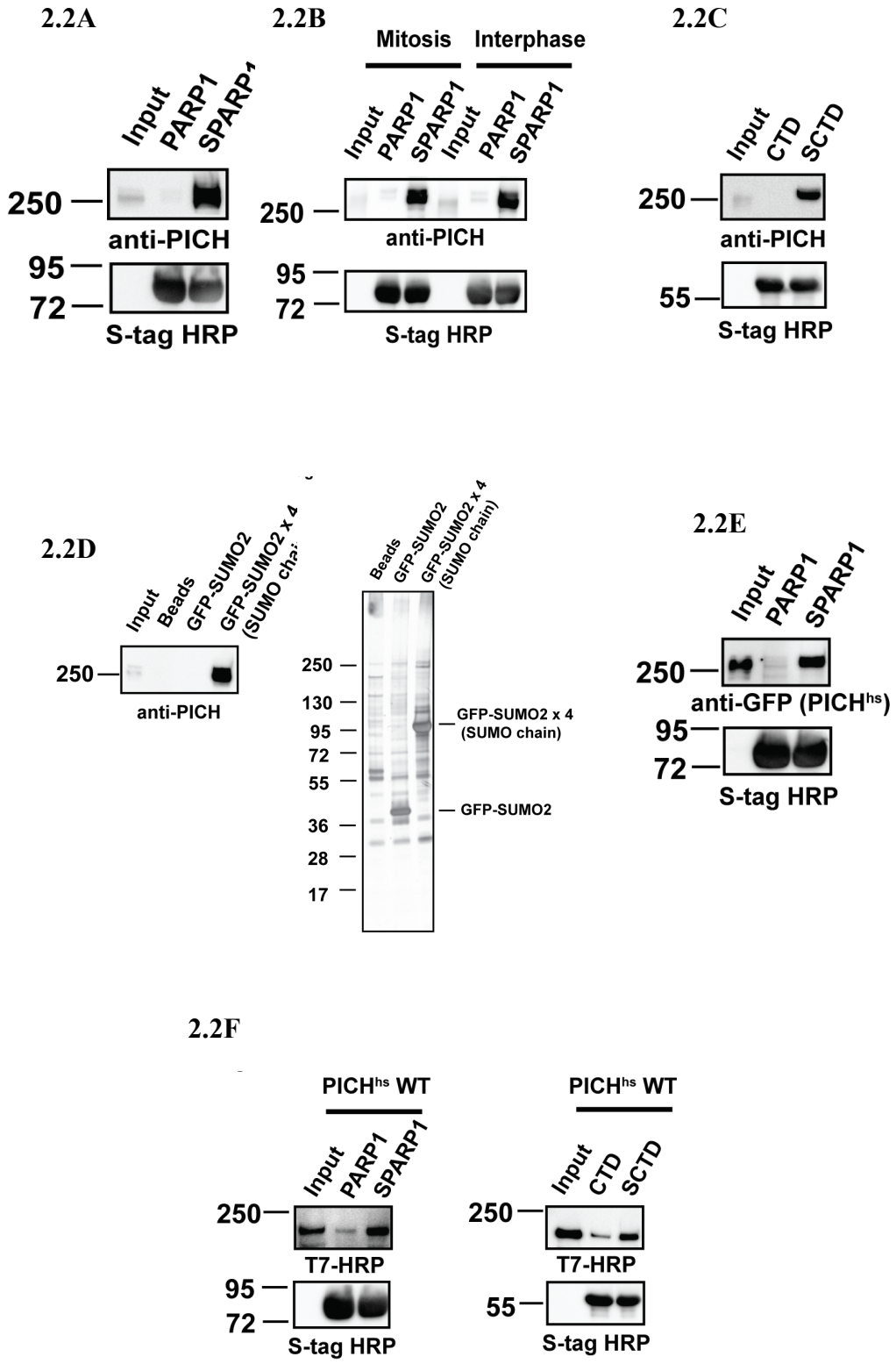

- XEE
+ XEE
PARP1
S-PARP1
beads
PARP1
S-PARP1
PARP1
S-PARP1
beads
250
130
95
72
55
36
28
17
*
PARP1 N650
SENP2 CD
SUMO2
input
before SENP2 digestion
SDS elution after SENP2 digestion

FIGURE 2.1. SUMOylation of PARP1 mediates protein- protein interactions. A) Differences in the PARylation profiles of PARP1 (PARP1) and SUMOylated PARP1 (S-PARP1) pulled down samples from XEE. The PARylation profile of pulled down samples was analyzed as described under "Materials and Methods." The baits are indicated on each lane, and lanes 1 and 2 were not incubated with XEE, showing the self-PARylation of PARP1 and S-PARP1, respectively. **B)** Schematic diagram of PARP1 with identified SUMOylated lysines. **C)** SUMOylation on recombinant PARP1 K5R mutant was not detected on mitotic chromosomes and *in vitro*. The substitution of five identified SUMOylated lysines to arginines in PARP1 reduces PARP1 SUMOylation in vitro. Purified full length PARP1 WT and mutants (K4R and K5R) were subjected to in vitro SUMOylation with the indicated concentrations of Ubc9 and PIASy for the indicated times. Recombinant PARP1 was detected by immunoblot using anti-T7 tag antibody. Endogenous PARP1 was immunodepleted from XEE and recombinant PARP1 (WT, K4R or K5R) was added to the immunodepleted extract. Mitotic chromosomes were assembled in endogenous PARP1 replaced extracts and isolated. Recombinant PARP1 on mitotic chromosomes was detected by immunoblot using PARP1 antibody. **D)** Schematic diagram of the pulldown assay. PARP1/TopoII-CTD and SUMOylated PARP1/TopoII-CTD (SCTD) were bound to S-protein-agarose beads. After incubation with XEE and washes, the bound proteins were incubated with the catalytic domain of SENP2. SENP2 assisted in the elimination of SUMOylated PARP1/TopoII-CTD bands during the analysis by deconjugating SUMO. **E)** Isolation of SUMOylated PARP1-specific binding proteins. A pulldown assay was performed with the non-SUMOylated (PARP1) and SUMOylated PARP1-N650 (S-PARP1) fragment, which were bound to S-agarose beads. Bound proteins were visualized by silver staining. SENP2 digestion eliminated the SUMOylated PARP1-N650 bands. A 250-kDa protein that specifically interacted with S-PARP1 (indicated by an asterisk) was identified as PICH by LC-MS/MS.

Polo-like kinase 1-interacting checkpoint helicase (PICH) interacts with multiple SUMOylated substrates and SUMO chain.

The prominent 250kDa band present only in the SUMOylated PARP1-N650 pull-down fraction (Figure 2.1E, asterisk) was subjected to LC-MS/MS analysis and identified as ERCC6-like protein, also known as Polo-like kinase1-interacting checkpoint helicase (PICH). To confirm the LC- MS/MS result, we prepared an anti-PICH antibody with *Xenopus laevis* PICH fragments. Full-length *Xenopus laevis* PICH cDNA information is not available in databases, but we found EST clones that show homology to human PICH. Thus, we isolated these clones from *Xenopus* cDNA and then prepared proteins for antigens. Thus far, we have been unable to obtain a full-length *Xenopus laevis* PICH cDNA using PCR-based cloning. Immunoblotting of the pull-down fractions with a PICH-specific antibody confirmed that PICH specifically interacts with SUMOylated PARP1- N650 (Fig 2.2A). To further characterize the PICH/SUMO interaction, we examined whether the interaction could be regulated by cell cycle stages as well as its specificity for SUMOylated proteins. Pull-down assays performed with mitotic and interphase extracts indicated that PICH interacted with SUMOylated PARP1-N650, irrespective of the cell cycle stage (Fig 2.2B). Additionally, PICH bound to a SUMOylated TopoIIα fragment, another major SUMOylated protein at mitotic chromosomes (Fig 2.2C) (11,13). PICH also bound to SUMO chain and not to a single SUMO moiety, suggesting the presence of potentially multiple SIMs in PICH (Fig 2.2D). These results suggest that PICH interacts with the SUMO moiety, regardless of the cell cycle stage and species of protein that is SUMOylated. To confirm whether the PICH/SUMO interaction is conserved for human PICH ($PICH^{hs}$), we performed a pull-down assay with XEE expressing $PICH^{hs}$. Consistent with endogenous PICH, $PICH^{hs}$ specifically bound to SUMOylated PARP1, indicating that the PICH/SUMO interaction is conserved

between *Xenopus* and humans (Fig 2.2E). Because PICH forms a complex with other proteins (27-29), it is possible that PICH interacts with SUMOylated PARP1 through its binding partner(s). To examine whether PICH directly binds to SUMOylated proteins, we prepared a recombinant PICH^{hs} protein by expressing it in yeast, *P. pastoris*. Pull-down assays were performed using PARP1-N650 and the TopoIIα fragment as baits. As shown in Figure 2.2F, recombinant PICH^{hs} clearly bound more highly to the SUMOylated baits than to the non-SUMOylated baits, indicating that PICH could directly bind to SUMOylated substrates and, thus, could have a SUMO-interacting motif (SIM) in its primary sequence. Supporting this observation, computational prediction indicated multiple potential SIMs in the PICH^{hs} primary sequence.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PARP1
SPARP1
250
anti-PICH
95
72
S-tag HRP
2.2B
Mitosis
Interphase
Input
PARP1
SPARP1
Input
PARP1
SPARP1
250
anti-PICH
95
72
S-tag HRP
2.2C
Input
CTD
SCTD
250
anti-PICH
55
S-tag HRP
2.2D
Input
Beads
GFP-SUMO2
GFP-SUMO2 x 4
(SUMO chain)
250
anti-PICH
Beads
GFP-SUMO2
GFP-SUMO2 x 4
(SUMO chain)
250
130
95
72
55
36
28
17
GFP-SUMO2 x 4
(SUMO chain)
GFP-SUMO2
2.2E
Input
PARP1
SPARP1
250
anti-GFP (PICHhs)
95
72
S-tag HRP
2.2F
PICHhs WT
Input
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SPARP1
250
T7-HRP
95
72
S-tag HRP
PICHhs WT
Input
CTD
SCTD
250
T7-HRP
55
S-tag HRP

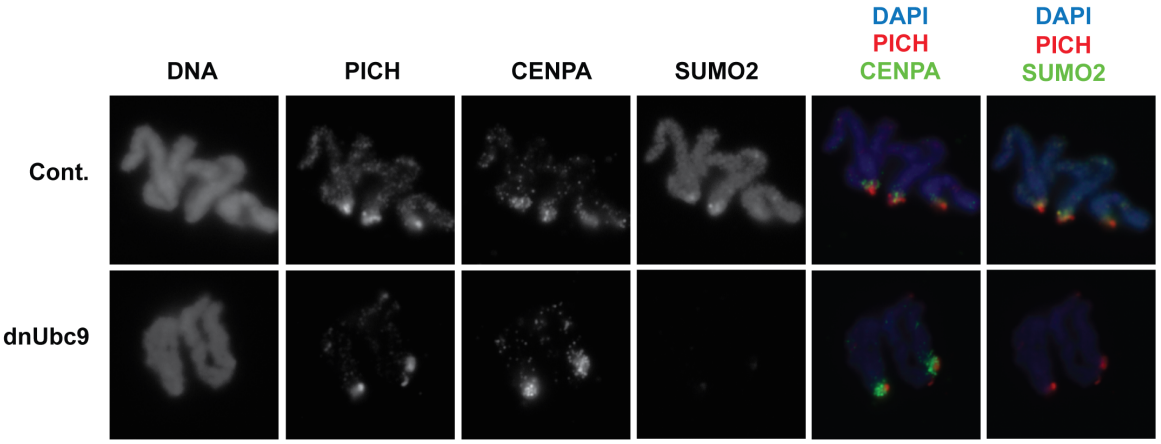
FIGURE 2.2. PICH (also known as ERCC6-like) interacts with SUMOylated substrates. A) Specific interaction of PICH with SUMOylated PARP1-N650. Pulldown fractions digested by SENP2 as in c were subjected to immunoblotting using an affinity-purified anti-PICH antibody. The CSF input lane on the membrane was 3% of the XEE used for the assay. The amount of bait (PARP1-N650) was analyzed by immunoblotting using S-tag HRP. **B)** Cell cycle-independent PICH interaction with SUMOylated substrates. PICH interaction with SUMOylated PARP1-N650 was analyzed by pulldown assays using mitotic or interphase XEE. S-tag HRP was used to detect the amount of bait. **C)** PICH interacts with multiple SUMOylated substrates. Pulldown assays were performed with non-SUMOylated and SUMOylated TopoII CTD (SCTD). SENP2 eluted fractions were analyzed by immunoblotting using an anti-PICH antibody. S-tag HRP was used as the bait loading control after SENP2 digestion. **D)** PICH interacts with SUMO chain. A pulldown assay was performed with GFP-SUMO and GFP-SUMO chain. The pulled down fractions were analyzed by silver staining and immunoblotting using an anti-PICH antibody. **E)** PICH-SUMO interaction is conserved in humans. XEE expressing EGFP-tagged PICHhs was subjected to pulldown assays. PICHhs in pulldown fractions was analyzed by immunoblotting with an anti-GFP antibody. S-tag HRP depicted the amount of bait in each sample. **F)** PICHhs directly interacts with SUMOylated PARP1-N650 and CTD. Purified recombinant PICHhs fused to a T7 tag was incubated with non-SUMOylated and SUMOylated PARP1-N650 or TopoII-CTD. The bound fraction was analyzed by immunoblotting using an anti-T7 tag-HRP antibody.

PICH is enriched at the centromere of mitotic chromosomes and its localization is not dependent on SUMOylation.

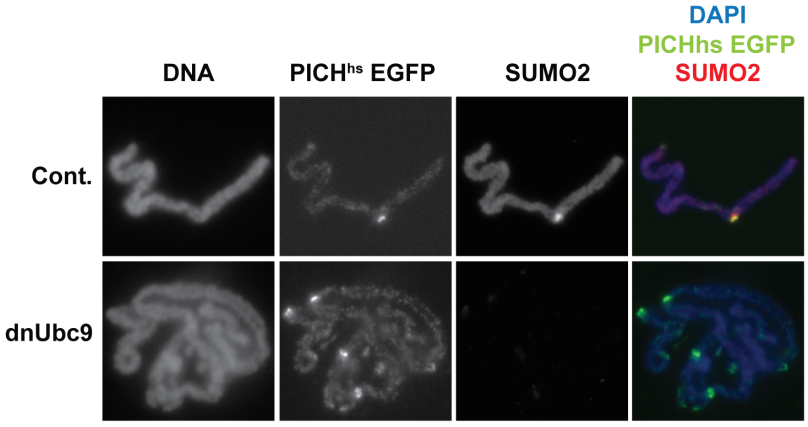
Mitotic chromosomal SUMOylation is mainly detected at the centromere. We have recently observed that some centromeric proteins not only interact with SUMO-modified TopoIIα but also require mitotic SUMOylation for their proper localization (30). PICH was shown to localize

at mitotic centromeres in mammalian cells (23,31), implicating that its localization might be dependent on SUMOylation. To test this possibility, condensed replicated chromosomes were isolated in the presence and absence of mitotic SUMOylation and were subjected to immunofluorescence analysis using an anti-PICH antibody. Endogenous PICH was enriched at the centromere of replicated mitotic chromosomes, co- localizing with CENPA, a centromere-specific H3 variant, and SUMO2/3. This enrichment was not altered when SUMOylation was inhibited by the addition of a dominant-negative form of Ubc9 (Fig 2.3A). The localization of EGFP tagged $PICH^{hs}$ was similar to endogenous PICH and was not altered upon the inhibition of SUMOylation (Fig 2.3B). To bolster our observation that SUMOylation does not alter PICH localization on mitotic chromosomes, the amount of endogenous PICH was analyzed by immunoblotting. Mitotic chromosomes were isolated from XEE, and the amount of PICH on chromosomes with and without SUMOylation was evaluated using an anti-PICH antibody. Consistent with the immunofluorescence data, the overall amount of PICH was not altered, but surprisingly, we observed a shift above the expected molecular weight of PICH. The shift was absent on the chromosomes isolated under SUMOylation inhibition by dnUbc9 (Fig 2.3C). This finding suggests that PICH is most likely posttranslationally modified in a SUMO- dependent manner at mitotic chromosomes. To test whether the posttranslational modification was SUMOylation itself, we added EGFP (~ 25 KDa) tagged SUMO2GG to the mitotic extract and assessed super-shifted PICH on mitotic chromosomes. A fraction of PICH indeed was super-shifted with the addition of SUMO2GG- EGFP, similar to a previously identified SUMO substrate, PARP1. This shift was not observed on chromosomes isolated from extracts with dnUbc9 (Fig 2.3D). Altogether, we concluded that PICH is a SUMO substrate on mitotic chromosomes.

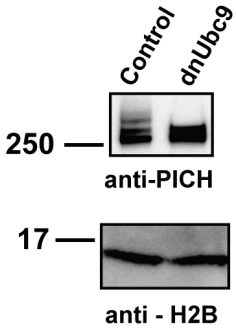
2.3A



2.3B



2.3C



2.3D

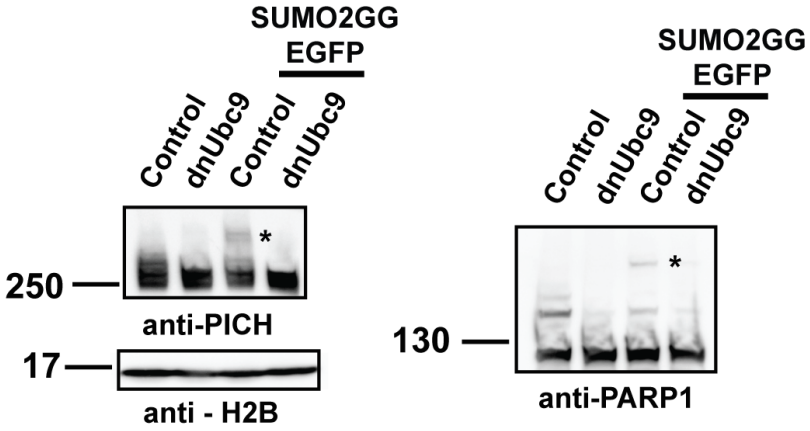


FIGURE 2.3. PICH is enriched at the centromere independently of mitotic SUMOylation and is SUMOylated on mitotic chromosomes. **A)** PICH is enriched at the centromere, co-localizing with CENPA and SUMO2/3, and the inhibition of SUMOylation by dnUbc9 does not alter PICH localization at the mitotic chromosomes. Replicated mitotic chromosomes were isolated from XEE and subjected to immunofluorescence staining with the indicated antibodies. Cont., control. **B)** PICHhs was enriched at the centromere, similar to endogenous PICH on mitotic chromosomes isolated from XEE. PICHhs-EGFP mRNA was added to XEE with and without the addition of dnUbc9 and was visualized on mitotic chromosomes along with CENPA and SUMO2/3. **C)** PICH is posttranslationally modified on mitotic chromosomes in XEE. Non-replicated mitotic chromosomes were isolated from XEE and analyzed by immunoblotting with an anti-PICH antibody. SUMOylation was inhibited by the addition of dnUbc9. **D)** PICH is SUMOylated on mitotic chromosomes in XEE. SUMO2GG-EGFP was added to the XEE, and non-replicated mitotic chromosomes were isolated and analyzed by immunoblotting. PARP1, a known SUMO substrate, served as a positive control for the PARP1-SUMO-EGFP supershift (indicated with asterisks).

PICH^{hs} is a strong PIASy-dependent SUMOylation substrate *in vitro*.

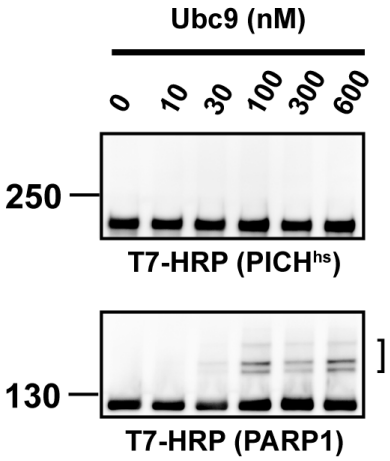
To examine whether PICH can be SUMOylated *in vitro*, similar to our previously identified substrates, TopoIIα and PARP1 (12,14), we performed an *in vitro* SUMOylation assay with recombinant PICH^{hs} as a substrate. In contrast to PARP1, PICH^{hs} could not be SUMOylated at the highest Ubc9 concentration (600 nM) tested, at which PARP1 could be modified by SUMO2 (Fig 2.4A) (12). PICH modification by SUMO2, however, was detected in the reaction with lowest concentration of PIASy (10 nM), a major mitotic SUMO E3 ligase (Fig 2.4B). Most SUMO substrates, including TopoIIα and PARP1, can be SUMOylated in the absence of the E3

ligase in an *in vitro* assay, albeit the efficiency of the modification is much lower than the reaction with E3 ligases. Within this context, PICH is a unique SUMO substrate that eminently requires an E3 ligase for its modification *in vitro*.

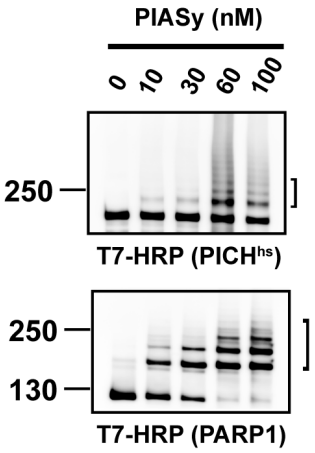
SUMOylation of PICH^{hs} alters its ability to bind to DNA *in vitro*.

Because PICH is suggested to play a role in centromeric nucleosome eviction during mitosis, we sought to examine whether PICH SUMOylation has any impact on its enzymatic activity (27). Because PICH has been previously characterized as a DNA-dependent ATPase (31), we examined the effect of PICH SUMOylation on the PICH/DNA interaction. PICH^{hs} was SUMOylated *in vitro* (Fig 2.4C), and its ability to bind DNA was observed by an electrophoretic mobility shift assay by adding DNA to the *in vitro* SUMOylation reaction. The mobility of the DNA was significantly reduced when PICH^{hs} was SUMOylated in comparison to non-SUMOylated PICH^{hs} (Fig 2.4C). We still observed a modest shift in the DNA in the lane where PICH^{hs} was subjected to SUMOylation (Figure 3c); however, this result was expected because we could not obtain more than 50% SUMOylated PICH^{hs} in the reactions (Fig 2.4C). Nevertheless, the quantification of the remaining DNA with or without SUMOylation from three independent experiments indicated that the DNA/PICH^{hs} interaction is negatively affected by more than 20% when PICH^{hs} is SUMOylated (Fig 2.4D). This result suggests that the SUMOylation of PICH might impair its DNA-dependent ATPase activity. Collectively, our findings suggest that the SUMOylation of PICH at mitotic centromeres could regulate its activity during mitosis.

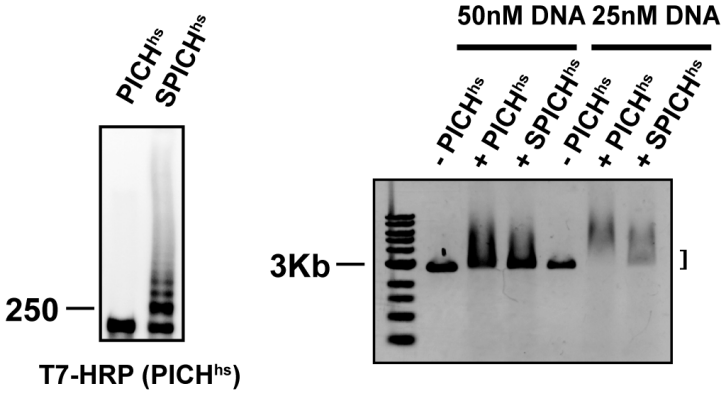
2.4A



2.4B



2.4C



2.4D

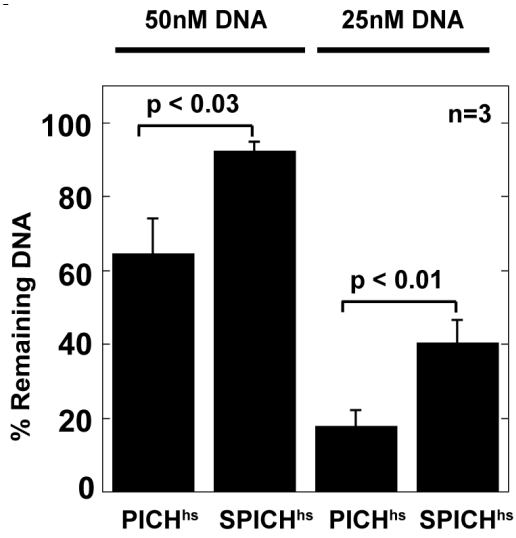


FIGURE 2.4. PICHhs is SUMOylated in vitro in a PIASy-dependent manner, and PICHhs SUMOylation reduces DNA binding *in vitro*. A and B) *in vitro* SUMOylation reactions were performed as described previously (12, 14) with 40 nM E1, 80 nM Ubc9, 40 nM PIASy, 0.5 M PICHhs or PAPR1, and 10 M SUMO2, unless otherwise indicated, and were analyzed by immunoblotting using an anti-T7 antibody conjugated to HRP. PIASy is required for robust PICH SUMOylation. SUMOylation reactions with increasing concentrations of Ubc9 in the absence of PIASy (A) and the reaction with increasing PIASy are shown (B). Brackets indicate SUMOylated species. **C)** Effect of PICH SUMOylation on PICH/DNA interaction. PICHhs was SUMOylated (SPICHhs) in vitro and incubated with linear pBluescript plasmid. PICHhs-DNA interactions were observed using agarose gels, followed by EtBr staining. The bracket indicates the non-shifted DNA position. **D)** Quantification of the percentage of non-shifted DNA in the EMSA. The p values and S.D. were calculated from three independent experiments.

DISCUSSION

Our results imply two mechanisms of the SUMOylation-dependent regulation of PICH function at the mitotic centromere: 1) the SUMOylation of PICH, which could inhibit the PICH/DNA interaction, and 2) the interaction of SUMOylated centromeric proteins (PARP1 and TopoIIα) through the SIM of PICH. This novel regulation of PICH function by mitotic SUMOylation might affect the regulation of centromeric nucleosomes by PICH, which has been shown to be important for resolving sister chromatids during anaphase.

The inhibition of DNA binding by the SUMOylation of PICH could be the result of 1) a direct effect on the DNA binding site of PICH by SUMOylation or 2) a conformational change by intra-molecular interactions of SUMO and SIM. In the first case, we expect that the SUMOylation sites of PICH could be close to its DNA binding site, which is currently not

defined. Because PICH SUMOylation could not be observed in the Ubc9- dependent reaction, the SUMOylation sites might not be a canonical SUMOylation sequence that can be mediated by direct interaction with the Ubc9-SUMO adduct (26,32). The identification of the SUMOylation sites and the SIM of PICH will further help us uncover these questions.

We initially identified PICH as one of the binding proteins of SUMOylated PARP1 in XEE, but further analyses showed that PICH also bound to SUMOylated TopoIIα CTD and SUMO chain alone (Figure 1d, f and g). This finding suggests that PICH can bind non-specifically to SUMOylated proteins. BLM, a known PICH binding protein, is SUMOylated in cells (33). Thus, it is possible that the SUMOylation of BLM could modulate BLM/PICH complex function via an interaction between the SUMO moiety on BLM and the SIM of PICH. Another piece of intriguing evidence could be in relation to the SUMOylation of TopoIIα. The inhibition of TopoIIα activity by ICRF-193 treatment has been shown to increase PICH-loaded ultrafine DNA bridges (UFBs) (34). Interestingly, the same inhibitor is known to increase TopoIIα SUMOylation in mitosis (35). Thus, it is possible that the hyper-SUMOylation of TopoIIα by ICRF-193 contributes to the retention of PICH at UFBs during anaphase, in addition to the maintenance of catenated DNA via the inhibition of TopoIIα activity. The possibility remains that our initial hypothesis of PICH being a potential PARP1 substrate for the PARylation of SUMOylated PARP1 is true. To date, we have not been able to detect the PARylation of PICH in an *in vitro* assay, although this hypothesis could hold true for mitotic chromosomes.

PICH's ATPase activity is potentially required for centromeric nucleosome eviction (27); therefore, the SUMOylation of PICH could attenuate PICH- dependent centromeric nucleosome eviction by inhibiting the DNA-dependent ATPase activity of PICH. Within this context, because the inhibition of PICH SUMOylation could increase the activity of PICH at mitotic

centromeres, fewer centromeric histones are expected when PICH SUMOylation is inhibited. Supporting that notion, a previous report indicated that the elimination of PICH in cells increases centromeric histones (27). Recent *in vitro* data with PICH did not show the efficient remodeling activity of nucleosomes with canonical histones or octamers composed of the centromeric histone variant CENPA (36), indicating an unidentified histone specificity for PICH nucleosome eviction function at centromeres. Combining PICH's capability of interacting with SUMO and considering the potential SUMOylation of histones at heterochromatin loci (37), it is possible that PICH's nucleosome remodeling activity is more effective toward SUMOylated histones. Future studies using potential SUMOylation-deficient mutants and SUMO-interaction deficient mutants in XEE and somatic cells will provide insight into the precise role of the SUMOylation-dependent regulation by PICH of chromatin organization at mitotic centromeres.

MATERIALS AND METHODS

Plasmids and antibody preparation.

Human PICH ($PICH^{hs}$) cDNA was amplified from a plasmid obtained from Addgene (Plasmid 41163: Nigg CB62) (23) and subcloned into pPIC3.5K fused to calmodulin-binding protein and with a T7 tag (14). $PICH^{hs}$ cDNA for mRNA expression was cloned into the pTGFC70 plasmid, a generous gift from Dr. Funabiki, and utilized for mRNA expression as previously described (38). Partial cDNAs for *Xenopus laevis* PICH was obtained by PCR amplification from *Xenopus laevis* cDNA based on EST clone sequences that are homologous to $PICH^{hs}$. The obtained partial $PICH^{xl}$ cDNAs were subcloned into pET28a and pMalc5x for recombinant protein expression. A polyclonal antibody against $PICH^{xl}$ was generated in rabbits by injecting His6-

tagged recombinant PICH^{xl} fragments (Pacific Immunology, Ramona, CA, USA), and the specific antibody was purified via MBP-tagged PICH^{xl} affinity column chromatography (11). A guinea pig anti-SUMO2/3 antibody and chicken anti-CENPA antibody were prepared as previously described (12). Commercial antibodies used in this study are S-protein-HRP and anti-T7-HRP (EMD Millipore, Billerica, MA, USA), monoclonal anti- GFP (JL-8) (Clontech, Mountain view, CA, USA), monoclonal anti-Histone 2B (Abcam, Cambridge, MA, USA), monoclonal anti-PAR (Trevigen, Gaithersburg, MD, USA) and fluorescently labeled secondary antibodies (Life Technologies, Carlsbad, CA, USA).

***Xenopus* egg extract immunofluorescence and immunoblotting.**

Low-speed extracts arrested in metaphase by cytostatic factor (CSF) from *X. laevis* egg and sperm nuclei were prepared using standard protocols (39). An interphase extract was obtained by releasing CSF upon the addition of $CaCl_2$ to the CSF extracts (39). The mitotic chromosomes used for the immunofluorescence analysis were prepared as previously described (9). Mitotic SUMOylation was inhibited by the addition of a dominant-negative form of Ubc9 (dnUbc9) at a concentration of 150 ng/μl before the induction of mitosis. The localization of human PICH on mitotic chromosomes was observed by GFP signals from exogenously expressed human PICH-EGFP mRNA in the extract (38). The DNA was stained with Hoechst 33342 dye (EMD Millipore, Billerica, MA, USA), and the samples were mounted using Vectashield (Vector Laboratory). The specimens were analyzed using a Nikon TE2000-U microscope with a Plan Apo 100X/1.40 objective, and the images were taken with a Retiga SRV CCD camera (QImaging) using the volocity imaging software (Improvision).

Chromatin isolation for immunoblotting was performed as previously described (11).

Recombinant SUMO2GG-EGFP protein (100 ng/µl extract) was added for the super-shift assay shown in Figure 2d. The samples were subjected to immunoblotting with the indicated antibodies.

Pull-down assay.

Pull-down assays were performed as previously described, with a few modifications (28). Non-SUMOylated and SUMOylated bait proteins were bound to either anti-T7 agarose or S-protein agarose (EMD Millipore), depending on the affinity tag fused to the bait protein. XEE was diluted three times with IP buffer (20 mM NaPi pH 7.8, 18 mM β-glycerol phosphate pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM $CaCl_2$ and 10 µM $ZnCl_2$) and clarified by centrifugation at 25,000 x *g* for 30 minutes at 4°C. The soluble fraction was further diluted with an equal volume of ChIP buffer (IP buffer containing 0.2% Triton X-100 and 0.2% Tween 20) and then incubated with protein-bound beads for 1 hour at 4°C. SENP2-CD digestion was performed by incubating approximately 50 ng/µl SENP2-CD with beads in ChIP buffer for 30 min at 25°C. Half of the volume of 3X SDS-PAGE sample buffer was added to the SENP2-CD-digested samples, resolved by SDS-PAGE on 8-16% gradient gels (Life Technologies) and analyzed by silver staining and/or immunoblotting, as indicated. For the pull-down analysis of $PICH^{hs}$, either the egg extract expressing full-length $PICH^{hs}$-EGFP or 15 µg of purified $PICH^{hs}$ in CHIP buffer containing 5% gelatin was incubated with the bait. For SUMO2 pull down, GFP-SUMO2 and GFP- SUMO2x4 (GFP-SUMO chain) were incubated with GFP trap magnetic beads (Bulldog Bio Inc.). The pulled-down samples were analyzed as described above using silver staining and immunoblotting, as indicated.

Protein purification and *in vitro* SUMOylation assays.

Recombinant $PICH^{hs}$ with CBP and T7 tags was purified from a GS115 strain of *Pichia pastoris* as previously described (14). The E1 complex (Aos1/Uba2 heterodimer), Ubc9, dnUbc9, PIASy and SUMO2 were purified as previously described (12,14). *In vitro* SUMOylation assays were performed with 40 nM E1, 80 nM Ubc9, 40 nM PIASy, 3 μM PARP1 N650 and SUMO2 (6-10X substrate concentration), unless otherwise indicated, for approximately 2 hours at 25°C. SUMOylated PICH and PARP1 were analyzed by immunoblotting using a T7-HRP antibody.

Electrophoretic mobility shift assay.

Non- SUMOylated PICH was prepared via *in vitro* SUMOylation with SUMO2-G, a non-conjugatable form of SUMO2. Linearized pBluescript DNA (approximately 25 nM or 50 nM) was incubated in a 10 μl *in vitro* SUMOylation reaction containing $PICH^{hs}$ (approximately 1 μM) for 20 min on ice. The entire reaction was separated on a 0.8% agarose gel in pre-chilled buffer at 25 V for 3 hours. The DNA was visualized via ethidium bromide staining (post run). Quantification of the non- shifted DNA was performed using ImageJ and KaleidaGraph software.

PARylation assay.

The pulled-down fractions with non-SUMOylated and SUMOylated PARP1 were used for a PARylation assay, as previously described (12). The beads were incubated with SUMOylation buffer containing NAD^{+}, and the samples were subjected to an immunoblotting analysis of PARylation with an anti-PAR antibody.

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The work in this chapter is accepted with minor revisions in Cell Cycle

CHAPTER 3

SUMO-interacting motifs (SIMs) in Polo-like kinase 1 interacting checkpoint helicase (PICH) ensure proper chromosome segregation during mitosis

INTROUCTION

Proper sister chromatid disjunction during mitosis is a highly regulated phenomenon that ensures genome stability. This complex regulation involves multiple families of proteins including kinases, phosphatases, histone modifiers and chromatin remodelers, coordinating their functions spatially and temporally (1-4). Of these proteins, Polo-like kinase 1 (Plk1) interacting checkpoint helicase (PICH), a SNF2 family member of chromatin remodelers was identified as a binding partner and substrate of mitotic kinase, Plk1 (5). PICH localizes at the centromere/kinetochore region of chromosomes only during mitosis. Additionally, PICH localizes to ultrafine anaphase DNA bridges (UFBs), which are, specialized mitotic DNA structures that cannot be visualized with conventional DNA staining dyes (5,6). The kinase activity of Plk1 is critical for the dynamic localization of PICH as depletion of Plk1 results in mislocalization of PICH on chromosomal arm regions (5,7). Furthermore, mutant PICH without its ATPase activity localizes on chromosome arms even in the presence of Plk1 (8). Therefore in addition to Plk1, DNA-dependent ATPase activity of PICH is also required for the elimination of PICH from the arm regions of chromosomes. Although a few mechanisms for the removal of PICH from chromosomal arm regions have been proposed, the specific mechanism for the positive recruitment of PICH at the centromere/kinetochore region of chromosomes is not clear. The elimination of PICH by RNAi or genome editing results in chromatin bridge formation in mitosis and micro-nucleation after cytokinesis (8,9). A PICH ATPase-deficient mutant could not

rescue this phenotype, suggesting that DNA-dependent translocase activity of PICH is required for resolving chromatin bridges in mitosis (8,9). While PICH is known to be required for proper completion of chromosome segregation, questions about how PICH localizes to the centromere and its precise function at the centromere and UFBs at a molecular level are still under investigation.

Recently, we reported that PICH can covalently bind to SUMO through SUMOylation and also interact non-covalently (10). SUMOylation is a posttranslational modification that regulates diverse cellular processes. SUMO modification of a protein can mediate novel protein-protein interaction and alter the protein's localization, activity, and stability (11-14). SUMO promotes novel binding via SUMO-interacting motifs (SIMs) on the binding protein (14-16). Proteins with SIM have been shown to regulate multiple biological processes including DNA replication, PML-body assembly and function, and mitosis (14,17-19). Several genetic studies in model organisms and cell biological studies show that SUMOylation is a critical modification in dictating proper chromosome segregation during mitosis (20). For instance, SUMO protease, SENP2 overexpression has been demonstrated to cause mitotic defects by induction of prometaphase arrest (21,22). Additionally, human cells lacking SUMO E3 ligase PIASy results in a prolonged metaphase (23). However, comprehensive roles of mitotic SUMOylation on chromosome segregation at the molecular level are not fully understood yet.

In this study, we identified a novel mechanism of localization of PICH on centromeres likely through PICH/SUMO interaction. Additionally, we uncovered the distinct function of each SIM on PICH in the regulation of PICH at mitotic centromeres. One of the PICH SIMs was important for robust PICH localization at the centromere and others were critical to PICH's function for resolving chromatin bridges during anaphase. Altogether, our results suggest a

complex spatial and temporal regulation of PICH by its SUMO interaction and we propose a novel molecular mechanism for PICH's dynamic localization and activity during mitosis involving PICH/SUMO interaction.

RESULTS

Multiple SIM domains in PICH mediate interaction with SUMOylated substrate *in vitro*.

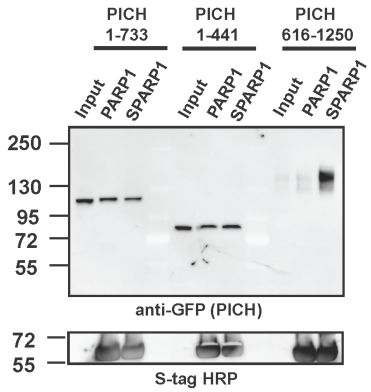
We identified PICH to be a promiscuous SUMO-binding protein by pull-down assay using *Xenopus* egg extract (XEE) (10). To investigate the functional significance of the PICH/SUMO interaction, we aimed to ascertain the functions of potential SIM sequences on PICH. In order to identify the SUMO-interacting regions on PICH, we made PICH truncations. Three truncation mutants covering and overlapping the N- and C-terminus portions of PICH were utilized for *in vitro* pull-down assays using SUMOylated-PARP1 (1-650 a.a.) with XEE expressing the mutants by mRNA addition to the XEE (10). The C-terminal portion of PICH containing amino acids 616-1250 was highly enriched, specifically on SUMOylated-PARP1 (1-650 a.a.) beads (Fig 3.1A). The SIM prediction program (24) suggested four potential SIM sequences within PICH 616-1250 a.a. Two were overlapping SIMs at amino acid 912-917, one at 1013-1016 and one at 1236-1239 (Fig 3.1B) (henceforth referred to as SIM1, SIM2, and SIM3, respectively). We mutated the SIM sequences individually by changing the hydrophobic amino acids to alanine (Fig 3.1B) and analyzed their interactions with SUMOylated-PARP1. Individual SIM mutants within the context of truncated PICH (616-1250 a.a.) were expressed in XEE and a pull-down assay was performed. None of the individual SIM mutations showed a clear reduction of the SUMO-PICH interaction *in vitro* (Fig 3.1C). Therefore, we mutated all the SIMs in full-length PICH (PICH d3SIM) and expressed it in XEE for pull-down assay. Immunoblotting

analysis of the pulled-down samples showed a drastic reduction of PICH d3SIM binding to the SUMOylated-PARP1 (Fig 3.1D). Because the SUMO/SIM interactions are relatively weak with dissociation constants between 60 and 90 μM (16), we surmise that all SIMs together contribute to the robust interaction of PICH to SUMOylated proteins in the pull-down assay.

PICH d3SIM is enzymatically active *in vitro*

Since multiple amino acids were mutated to obtain a SUMO-interacting deficient PICH, we wanted to ensure that mutations do not affect the activity of PICH and thereby justify the use of PICH d3SIM in our future studies. PICH is a DNA-dependent ATPase and an ATP-dependent translocase (25). For the verification of PICH activity, we tested the ability of PICH d3SIM to translocate on dsDNA by measuring the displacement of a fluorescently tagged oligo from a DNA duplex *in vitro* (DNA-triplex assay) (25). PICH d3SIM was enzymatically active and displaced the triplex-forming oligo similarly to PICH WT in a time- and concentration-dependent manner, whereas PICH K128A (ATPase-deficient mutant) had no activity, consistent with a previous study (Fig 3.1E) (25). These results suggest that mutations of SIMs do not affect DNA binding or the enzymatic activity of PICH.

3.1A



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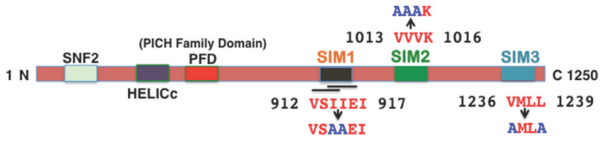

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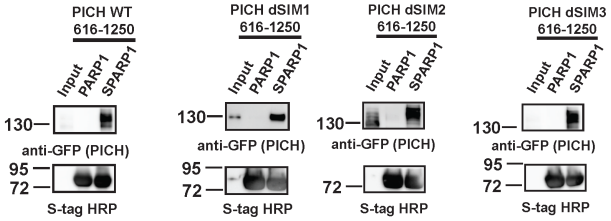

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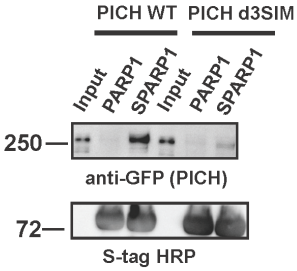


3.1E

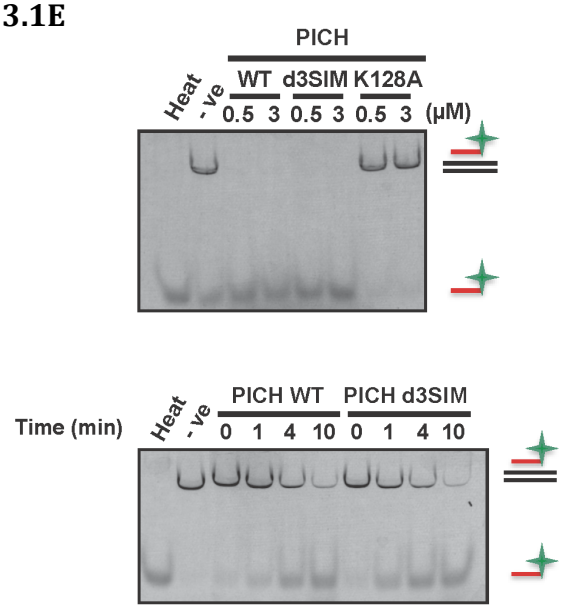


Figure 3.1. Polo-like kinase 1 interacting checkpoint helicase (PICH) has multiple SUMO-interacting motifs (SIMs).

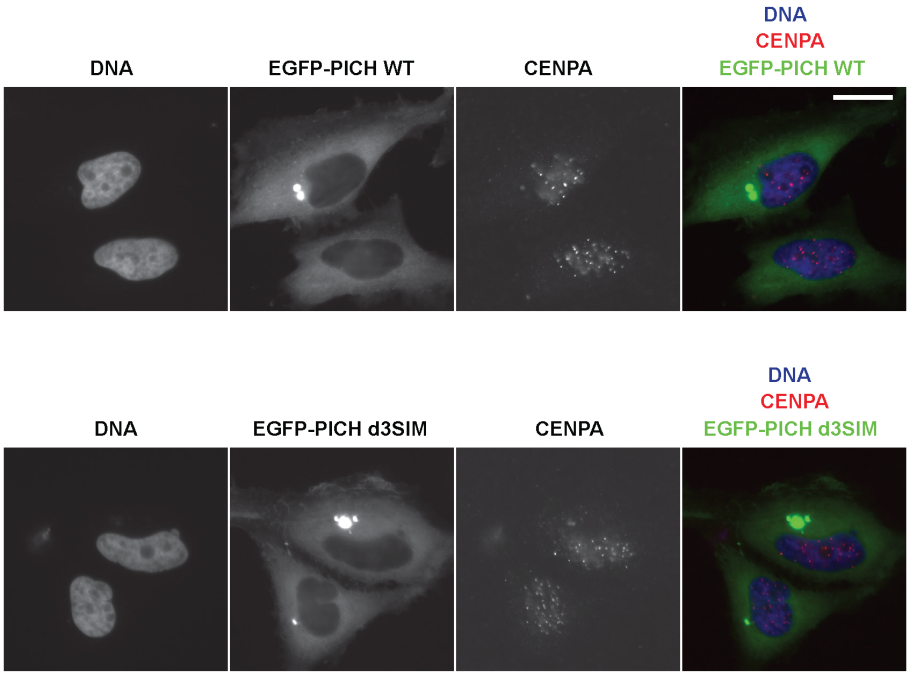
A) The C-terminal portion of human PICH (616-1250 a.a.) interacts robustly with SUMOylated-PARP1 (SPARP1) of *Xenoups laevis*. Pull-down assays with PICH truncations were performed and the pulled-down samples were analyzed by immunoblotting using anti-GFP antibody. S-tag HRP depicted the amount of bait in the pull-down samples. **B)** Schematic diagram of PICH with SIM sequences at a.a. 912-917 (VSIIEI), 1013-1016 (VVVK) and 1236-1239 (VMLL). Point mutations in each SIM are indicated in blue. **C)** Individual PICH dSIM1, dSIM2 and dSIM3 mutants robustly bind to SUMOylated-PARP1. XEE expressing GFP-tagged PICH WT and SIM mutant fragments (616-1250 a.a.) were used for pull-down assays. Pulled-down samples were analyzed by immunoblotting using anti-GFP antibody. S-tag HRP was used to detect the bait amount in each sample. **D)** Mutations in all SIMs in PICH (PICH d3SIM) drastically reduced PICH binding to SUMOylated-PARP1 (SPARP1). XEE expressing GFP-tagged PICH WT and d3SIM were subjected to pull-down assays. Pulled-down samples were analyzed by immunoblotting using GFP antibody. S-protein HRP was used to detect the amount of bait in the samples. **E)** PICH d3SIM is enzymatically active *in vitro*. Concentration- and time-dependent DNA translocase activity of PICH WT and d3SIM was tested *in vitro* by triplex assay. Alexa 488 tagged oligo was detected by Typhoon Imager. Positions of DNA triplex and released oligo are indicated at right side.

PICH SIMs are critical for mitotic centromere localization of PICH but not for localization on UFBs

Localization of PICH on chromosomes has been shown to be highly dynamic. PICH localizes in the cytoplasm during interphase and is enriched at the centromere/kinetochore (KT) region during mitosis (5). Additionally, PICH localizes to the ultra-fine DNA bridges (UFBs) during anaphase (5). To test if SIM sequences are important for PICH's localization on the UFBs during anaphase, we generated tetracycline-inducible constructs of EGFP-fused PICH WT and PICH

d3SIM to express exogenous PICH in cells (26,27). We tested and confirmed the cytoplasmic localization of both EGFP-PICH WT and d3SIM during interphase, as previously reported (Fig 3.2A)(5). Anaphase cells expressing exogenous EGFP-fused PICH WT and PICH d3SIM were visualized. We observed that both PICH WT and PICH d3SIM associated with UFBs without distinguishable differences during anaphase (Fig 3.2B). Interestingly, PICH d3SIM did not show chromosomal foci in contrast to WT, indicating a defect in the d3SIM mutant's association with the centromere/KT region. To test if SIM sequences are important for PICH's localization on the mitotic centromeres, we isolated different stages of mitotic cells and examined PICH centromere/KT region association. In mitotic cells, WT was enriched at the centromere/KT region as previously shown (Fig 3.2C) (5). On the other hand, PICH d3SIM's localization at the centromere/KT region was clearly reduced (Fig 3.2D). These results suggest that PICH SIM interaction with SUMO is likely essential for PICH localization and retention at the mitotic centromeres/KT region and the mechanism of PICH localization at the centromere/KT region is different from that at UFBs.

3.2A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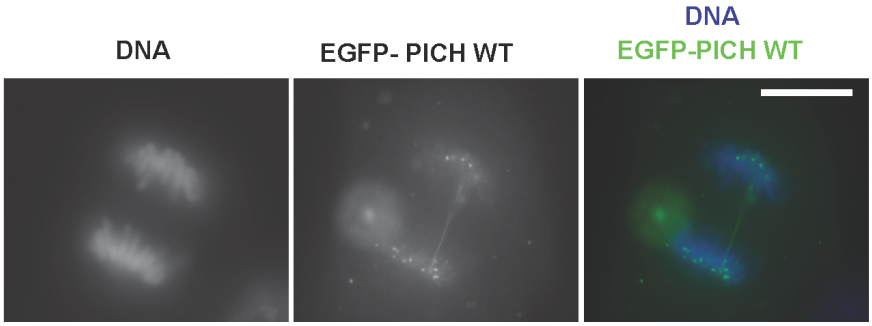
CENPA
DNA
CENPA
EGFP-PICH WT
DNA
EGFP-PICH d3SIM
CENPA
DNA
CENPA
EGFP-PICH d3SIM

3.2B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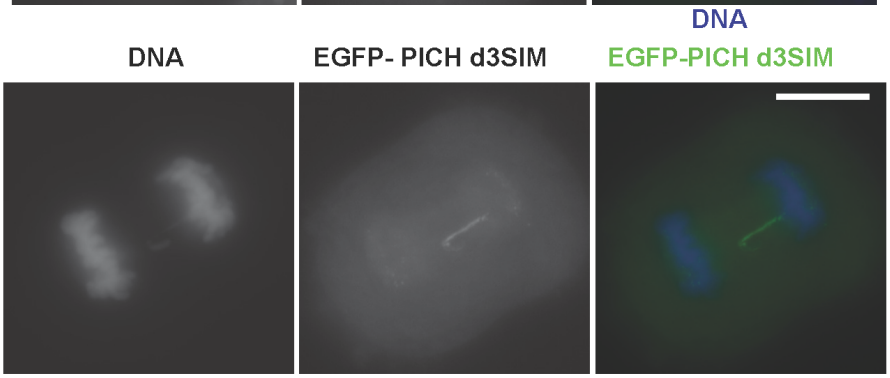
DNA
EGFP-PICH WT

3.2C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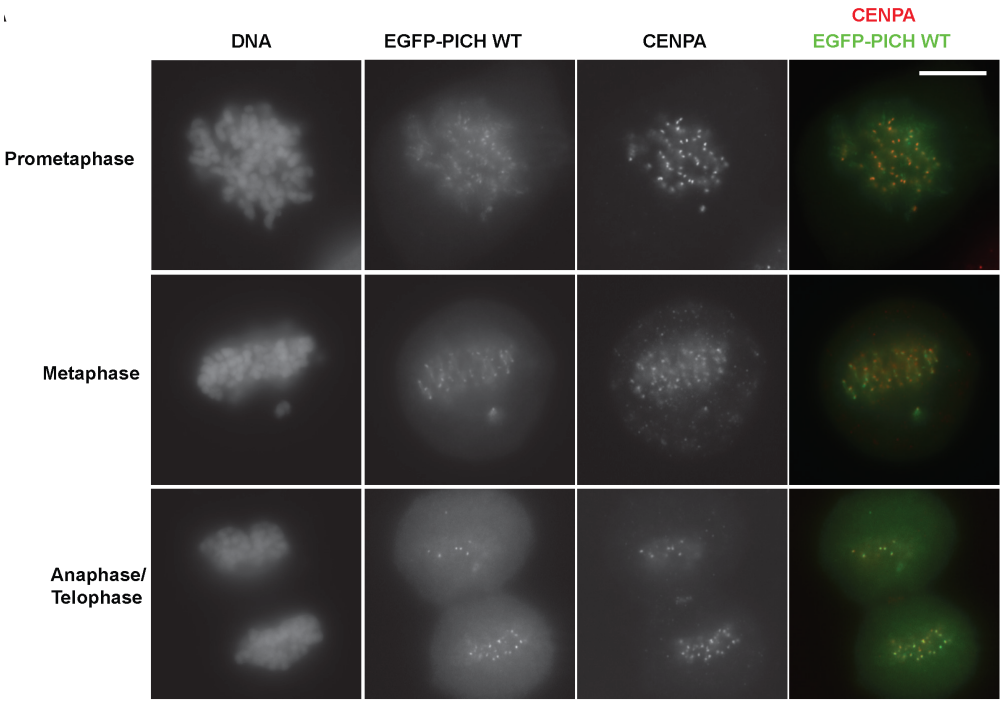
EGFP- PICH d3SIM
DNA
EGFP-PICH d3SIM

3.2D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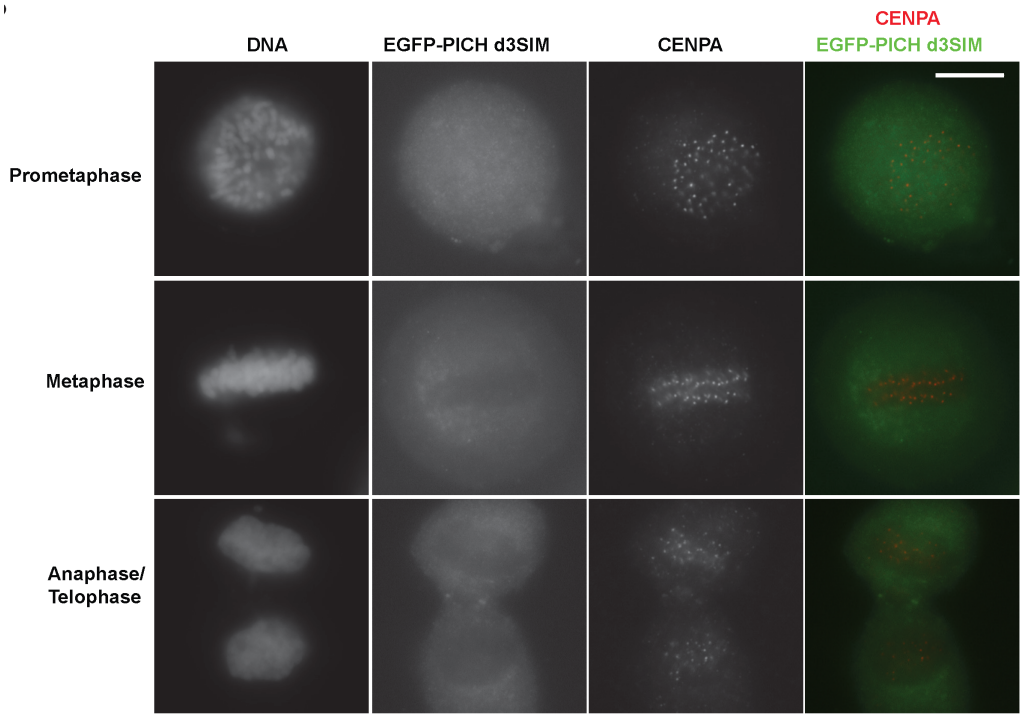
CENPA
CENPA
EGFP-PICH WT
Prometaphase
Metaphase
Anaphase/
Telophase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EGFP-PICH d3SIM
CENPA
CENPA
EGFP-PICH d3SIM
Prometaphase
Metaphase
Anaphase/
Telophase

Figure 3.2. SIMs in PICH regulates centromere localization of PICH.

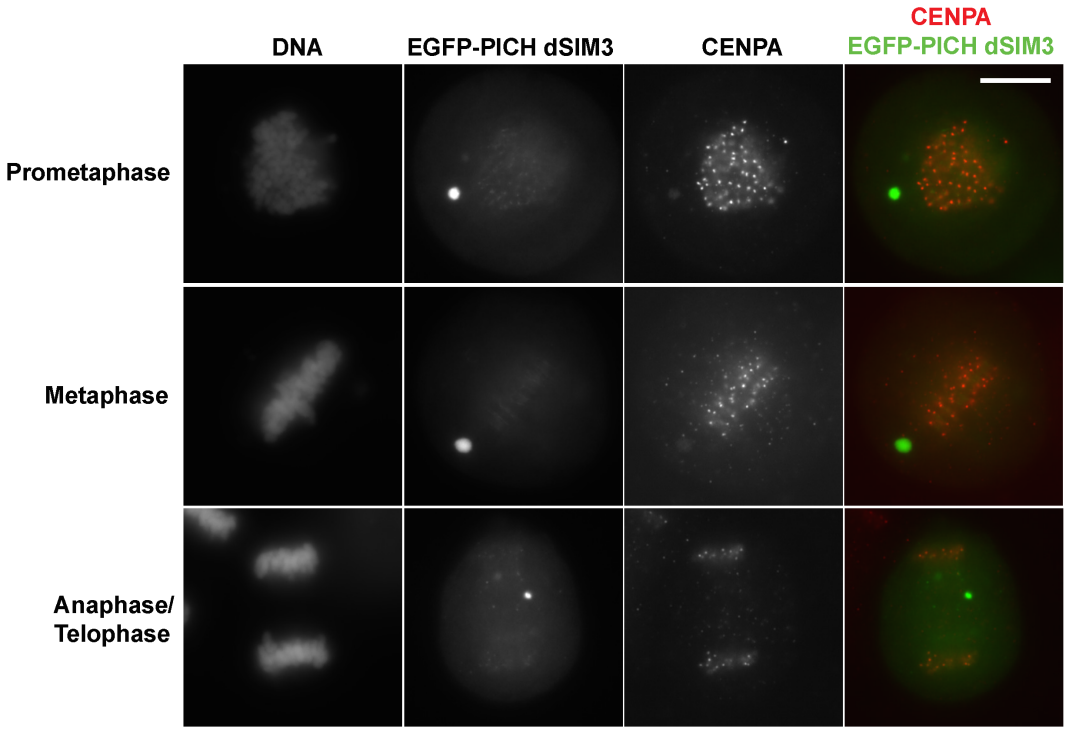
A) PICH d3SIM is restricted to cytoplasm during interphase similar to PICH WT. Ectopically induced EGFP-tagged PICH WT and d3SIM were fixed and visualized along with CENPA using immunofluorescence staining. White bar indicates 10μm. **B)** SIMs in PICH does not alter PICH's association with UFBs. Ectopically expressed EGFP-tagged PICH WT and PICH d3SIM induced in HeLa Tet-ON cells were detected by fluorescence microscopy. White bar indicates 10μm. **C)** PICH WT localizes at the centromere/KT region during mitosis as previously shown. Ectopically expressed EGFP-PICH WT induced by doxycycline visualized in different mitotic stages of HeLa Tet-ON cells. Cells were subjected to immunofluorescence staining for CENPA antibody as a centromere marker. Right panel is the merged images with PICH in green and CENPA in red. White bar indicates 10mm. **D)** Localization of PICH d3SIM is drastically reduced at the centromere during mitosis. Similar to PICH WT, EGFP-tagged PICH d3SIM expression was induced and detected along with CENPA by immunofluorescence. White bar indicates 10μm.

PICH SIM3 is more critical for centromere localization of PICH than SIM1&2

Although results from *in vitro* pull-down assays suggested that all SIMs on PICH contribute to effective SUMO binding, it is plausible that the outcome may be different for the *in vivo* centromeric PICH localization. To test this, we made individual SIM mutations in inducible PICH constructs (dSIM1, dSIM2 and dSIM3) and analyzed mitotic HeLa cells by immunostaining. Surprisingly, PICH dSIM3 mutation by itself drastically reduced PICH's association with mitotic centromeres in contrast to the effects of dSIM1&2 combined (Fig 3.3A & B). Combinations of other SIM sequence mutants were also tested. Mutations in SIM1&3 and SIM2&3 were observed to reduce PICH's association with mitotic chromosomes when compared to dSIM1&2. (Fig 3.3C&D & 3.3B). We quantified the centromeric PICH signal of

the mutants (d3SIM, dSIM3 and dSIM1&2) relative to PICH WT by normalizing with CENPA levels. Centromeres from different mitotic stages (prometaphase, metaphase and anaphase) were analyzed in each set. Regardless of the mitotic stage, the result indicated that the amount of centromere-associated PICH when SIM3 was mutated (d3SIM and dSIM3) was significantly lower than the amount of PICH when the other two SIMs were mutated (dSIM1&2) (Fig 3.3E). These observations shed light on the different role(s) of each SIM on PICH and suggest that SIM3 plays a critical role in the robust centromeric localization of PICH.

3.3A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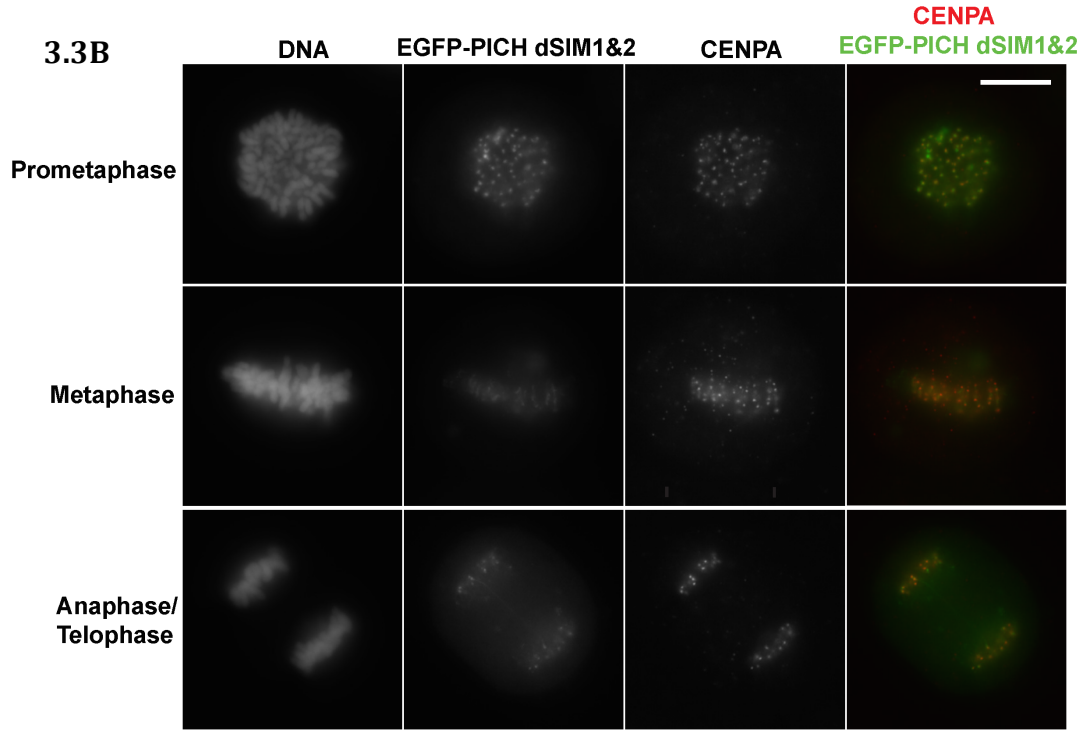
CENPA
CENPA
EGFP-PICH dSIM3
Prometaphase
Metaphase
Anaphase/
Telophase

3.3B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CENPA
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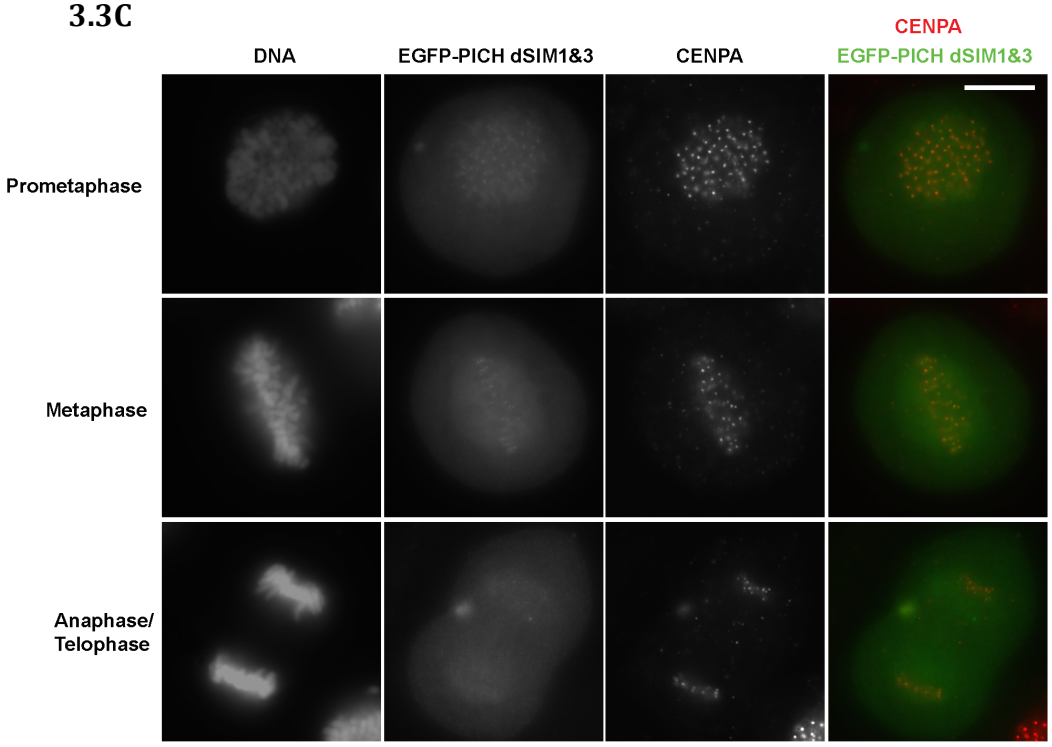
Prometaphase
Metaphase
Anaphase/
Telophase

3.3C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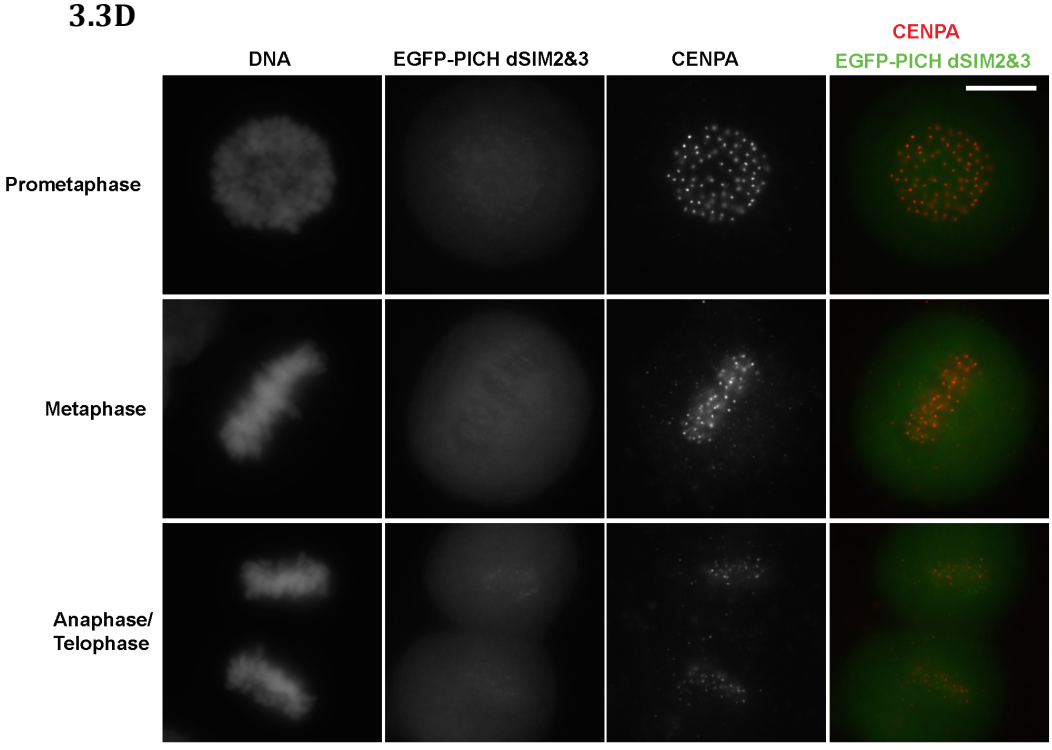
Anaphase/
Telophase

3.3D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Metaphase
Anaphase/
Telophase

3.3E

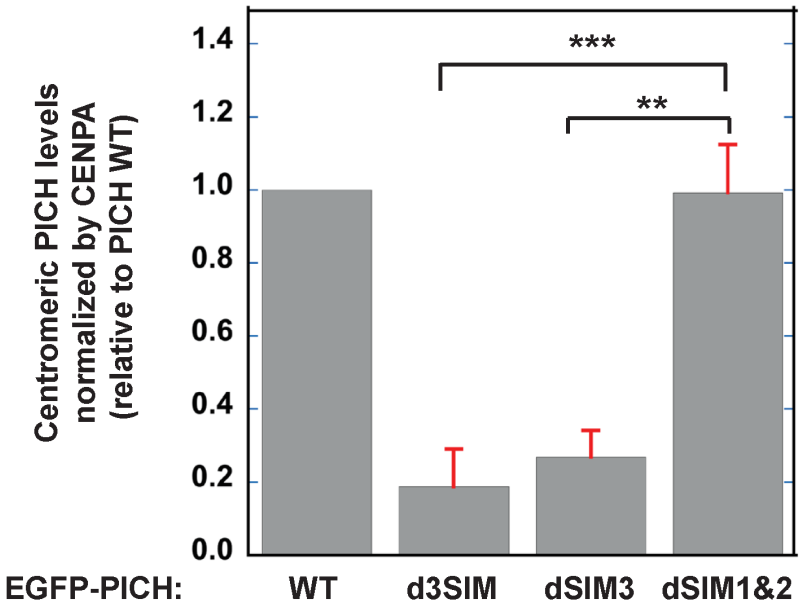


Figure 3.3. SIM3 is critical for centromeric localization of PICH.

A) Mutation of SIM3 of PICH drastically reduces centromeric association of PICH. EGFP-tagged PICH dSIM3 transiently expressed and induced in HeLa Tet-ON cells. Different stages of mitotic cells isolated and EGFP-PICH dSIM3 visualized along with centromere marker, CENPA by immunofluorescence staining. White bar indicates 10μm. **B)** Mutation of SIM1 and SIM2 of PICH does not affect the centromere localization of PICH in HeLa Tet-ON cells. Similar to PICH dSIM3, PICH dSIM1&2 was ectopically induced and expressed in HeLa Tet-ON cells. PICH mutant was visualized by EGFP along with CENPA by immunostaining analysis. White bar indicates 10μm. **C)** PICH dSIM1&3 loses robust binding at the centromere during mitosis. EGFP-tagged PICH dSIM1&3 expressing mitotic HeLa Tet-ON cells were analyzed for EGFP signal along with CENPA by immunostaining. White bar indicates 10μm. **D)** Similar to PICH dSIM1&3 in A, EGFP-PICH dSIM2&3 localization at the centromere was analyzed. White bar indicates 10μm. **E)** PICH signals of the mutants (d3SIM, dSIM3, and dSIM1&2) at the centromeres were normalized to CENPA levels and quantified relative to PICH WT. Values represent the average of at least 30 different centromeres analyzed in at least three different cells in each set (n, n=3).

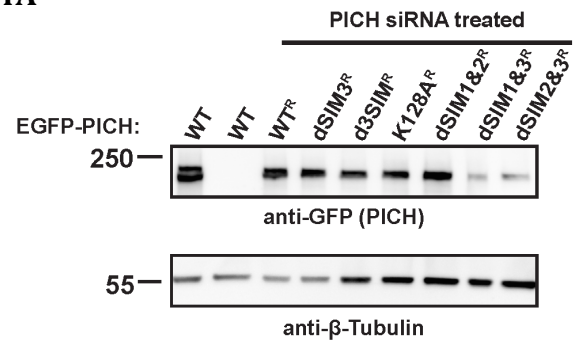
Error bars indicate standard deviation and statistical analysis by paired student t-test is indicated as **p<0.01, ***p<0.001.

Robust centromeric localization of PICH is not essential to prevent chromatin bridges in anaphase

Depletion of PICH is known to cause chromosome segregation defects, such as the formation of chromatin bridges and micronuclei (8,26). We wanted to test if SUMO-interaction of PICH is critical to prevent this chromatin bridge phenotype. To test this, we replaced endogenous PICH with mutant forms using PICH depletion with siRNA and expression of siRNA resistant EGFP-PICH. We confirmed the resistance of exogenous PICH to siRNA by immunoblotting analysis (Fig 3.4A). For measuring chromatin bridges, we established stable inducible EGFP-PICH cell lines with HeLa Tet-ON cells that express different mutant. Expression of EGFP-PICH WT and mutants in stable cell lines was confirmed by immunoblotting analysis (Fig 3.4B). Three independent clones were isolated from each mutant. Isolated cell lines were synchronized as shown in Figure 3.4C with endogenous PICH-replacement by siRNA followed by tetracycline induction. Anaphase cells were enriched for the analysis of the chromatin bridge phenotype by mitotic shake off and cells with chromatin bridges were counted as shown in Figure 4B. Anaphase cells with chromatin bridges and the total number of anaphase cells in a set were counted to calculate the percentage of anaphase cells with chromatin bridges in each sample. Cells expressing EGFP-PICH WT with endogenous PICH depleted were able to rescue the chromatin bridge phenotype in comparison to cells expressing just EGFP. A PICH mutant with the loss of ATPase activity (PICH K128A) was included as a negative control because it was previously shown to not rescue the chromatin bridge phenotype (8) (Fig 3.4D). Surprisingly, PICH dSIM3 mutant that was not robustly associated to

centromere/KT region was able to rescue the chromatin bridge phenotype, similar to PICH WT. More interestingly, PICH dSIM1&2 mutant that did not show defective localization at the centromere/KT region was unable to rescue the bridge phenotype. All clones isolated for each mutant showed a consistent phenotype. The results suggest that SIM1&2 functions differently from SIM3, and SIM1&2 is required for PICH activity at mitotic centromeres to resolve chromatin bridges.

3.4A



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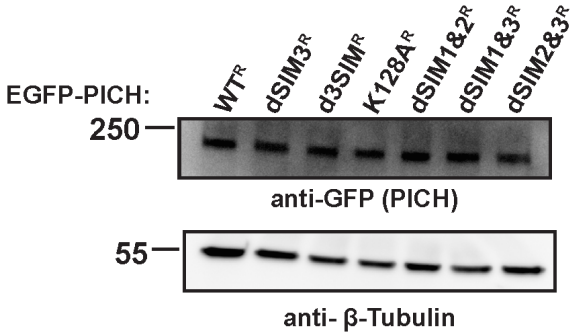
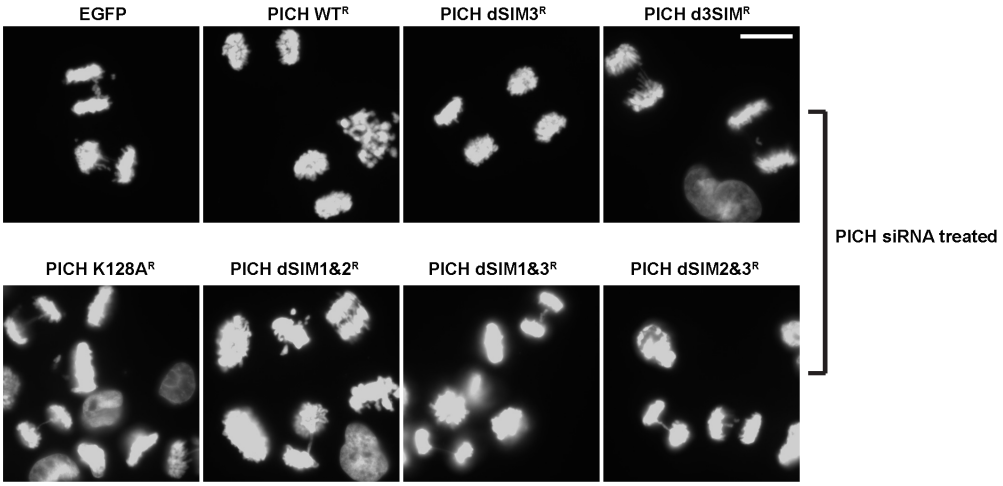


3.4C

3.4D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PICH K128A^R
PICH dSIM1&2^R
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PICH dSIM2&3^R

3.4E

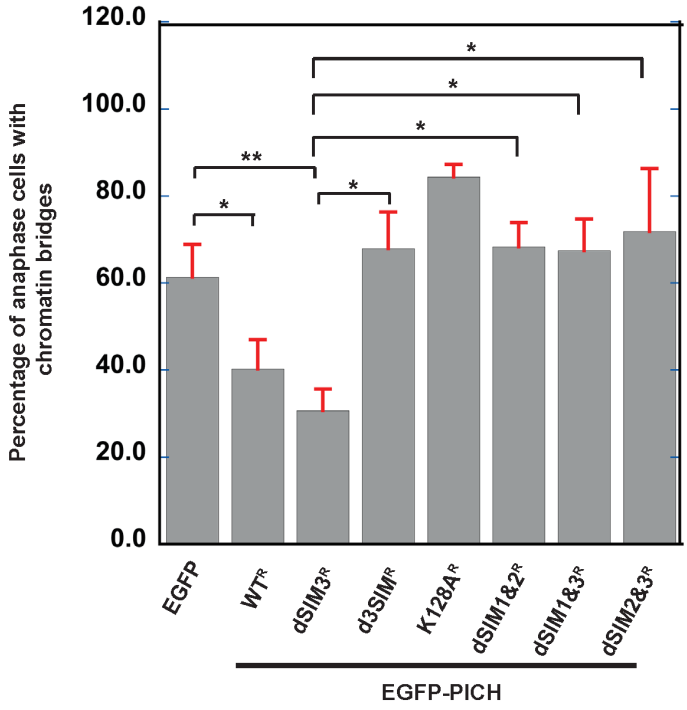
Percentage of anaphase cells with chromatin bridges
120.0
100.0
80.0
60.0
40.0
20.0
0.0
*
**
*
*
*
*
EGFP
WT^R
dSIM3^R
d3SIM^R
K128A^R
dSIM1&2^R
dSIM1&3^R
dSIM2&3^R
EGFP-PICH

Figure 3.4. PICH's SIMs role in localization is different from its role in chromatin bridge resolution

A) PICH siRNA resistant constructs were made and validated for expression in the presence of siRNA by immunoblotting analysis of the transiently transfected HeLa Tet-ON cells using anti-GFP antibody. β-Tubulin was used as a loading control. **B)** The stable cells lines made were validated for EGFP-PICH expression by immunoblotting analysis using anti-GFP antibody. β-Tubulin was used as a loading control. **C)** Schematic representation of the synchronization method used in the chromatin bridge analysis with thymidine (Thy), doxycycline (Dox), and nocodazole (Noc) addition. **D)** Robust defective centromere localization of PICH dSIM3 does not result in chromatin bridge phenotype whereas mutations in SIM1&2 (PICH dSIM1&2) causes chromatin bridge phenotype. Stable HeLa Tet-ON cell lines expressing different PICH constructs were made. Endogenous PICH was depleted by siRNA and nocodazole-arrested cells were released to analyze for anaphase cells. Representative images of different PICH constructs cell lines are shown. White bar indicates 10μm. **E)** Percentage of anaphase cells with chromatin bridges per total anaphase cells were counted and graphed. At least 30 anaphase cells to a maximum of 360 anaphase cells were counted in each experiment (n=3). Error bars indicate standard deviation and statistical analysis by paired student t-test is indicated as *$p<0.05$, **$p<0.005$.

Taken together, we propose that PICH SIMs have two separate functions in the regulation of PICH at mitotic centromeres (Fig 3.5). SIM3 is important for stable PICH localization at the centromere whereas PICH SIM1&2 is most likely required for the activity of PICH at centromeres for resolving chromatin bridges. We expect this difference to be due to distinct interacting proteins for each SIM, which most likely are SUMOylated on mitotic chromosomes.

3.5

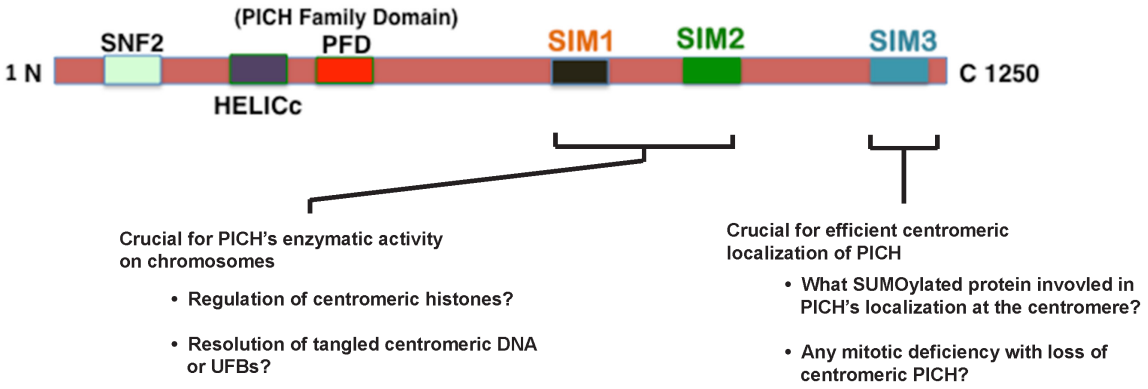

Figure 3.5. Schematic representation of PICH with proposed functions of SIMs.

Proposed functions of the different SIMs on PICH and remaining questions to be answered about each SIM. SIM3 is critical for the centromeric localization of PICH whereas SIM1&2 is critical to for PICH's function in the resolution of chromatin bridges during mitosis through binding to distinct SUMOylated chromosomal proteins.

DISCUSSION

PICH was found to be associated with centromere/KT region and UFBs during mitosis (5). Multiple studies since PICH's discovery have identified different factors that are critical to prevent chromosomal arm localization of PICH including the Plk1-dependent phosphorylation (5) and the ATPase activity of PICH (8). However, the precise molecular mechanism for how PICH localizes to the centromere/KT region is not known. Our results implicate a novel mechanism of centromeric localization of PICH via SIMs (Fig 3.2A & B). This localization could be due to PICH's direct interaction with a SUMOylated centromeric protein. Histone SUMOylation, including CENPA SUMOylation, at the heterochromatin loci has been shown

previously (28,29). PICH could potentially be interacting with SUMOylated CENPA at the centromere. Additionally, DNA topoisomerase IIα (TopoIIα) is known to be SUMOylated at the centromere (30,31) and multiple studies have linked TopoIIα's function with PICH (9,32,33). It is plausible that TopoIIα SUMOylation assists in the recruitment and retention of PICH at the centromere.

Interestingly, our results also suggest that SIM3 is more important for centromeric recruitment and retention whereas SIM1 and SIM2 are critical to resolve chromatin bridges during anaphase (Fig 3.2 & 3.4). This difference could be because different SUMOylated proteins interact with different SIMs. While SIM3 could be interacting with SUMOylated histones or TopoIIα at the centromere as discussed above, SIM1 and SIM2 could be interacting with a different SUMOylated protein to resolve chromatin bridges. One candidate SUMOylated protein is the DNA helicase BLM mutated in Bloom's syndrome (34), and inhibition of BLM has been previously shown to cause chromatin bridges (26,35). Additionally, PICH is a SUMO substrate itself (10) and it may be possible that SUMOylation can cause conformational changes through intra-molecular interaction between SUMOylated PICH and SIM1&2 (Fig 3.5). Further studies to identify specific SUMOylated binding proteins of PICH to SIM3 that mediate its centromeric localization and to SIM1&2 for its activity, will provide further insight into the molecular mechanism controlling PICH localization and function at the centromere.

MATERIALS AND METHODS

DNA constructs, cell transfection, and antibodies

PICH cDNA was obtained from pEGFP PICH construct from Addgene (plasmid 41163: Nigg CB62) and cloned into pTGFC70 plasmid for mRNA expression in XEE as previously described

using AclI and SpeI restriction sites (10,36). PICH N- and C-terminus truncation mutants (PICH 1-733 a.a., PICH 1-414 a.a. and PICH 616-1250 a.a.) were amplified and cloned into pTGFC70 as the full length PICH. EGFP-PICH was cloned into pTRE3G Tet-ON vector (Clonetech Laboratories, Mountain view, CA) using SalI and EagI restriction sites. PICH SIM mutants were generated using the QuikChange II XL Site-Directed Mutagenesis kit from Agilent Technologies (Santa Clara, CA). PICH siRNA target sequence was purchased from Life Technologies (Carlsbad, CA) (5'-GCUUGCUACUUUAUCGAGATT-3') and cells were transfected with 40 pmols of siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). PICH RNAi resistant mutant was generated by introducing 4 silent mutations in PICH DNA that disrupt siRNA binding using Quikchange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). HeLa Tet-ON cells were grown in McCoy's medium (Mediatech Inc., Manassas, VA) supplemented with 10% FBS and transfected using Clonetech's Xfect protein transfection reagent as per manufacturer's instructions. PICH WT and mutant expression was induced by the addition of doxycycline (100μg/ml) for 10-12 hours for transient transfection.

The following antibodies were used in this study. For immunoblotting, antibodies used were mouse monoclonal anti-GFP (JL-8) (Clonetech; 1:1000), S-protein-HRP (EMD Millipore, Billerica, MA; 1:1000), and mouse monoclonal anti-β-tubulin (Sigma-Aldrich; 1:1000). For immunofluorescence analysis, antibodies used were mouse anti-CENPA (MBL, Japan; 1:200), and goat anti-mouse Alexa 568 (Life Technologies, Carlsbad, CA; 1:1000)

Recombinant protein purification and *in vitro* SUMOylation assay

Recombinant human PICH cDNA was cloned into yeast protein expression vector pPIC3.5 that had T7 and calmodulin-binding protein (CBP) tags. PICH expressed in GS115 strain of *Pichia pastoris* was extracted using the following buffer (150mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$,

50mM Tris (pH 7.8), 5% glycerol) and affinity purified by calmodulin sepharose (GE healthcare) as previously described (37). PICH mutant genes used in this study were cloned and purified the same way as WT. Recombinant *Xenopus laevis* PARP1 (1-650 a.a.), used for *in vitro* pull-down assays, was expressed at 15 °C in the presence of 2.5% ethanol and 0.1mM $ZnCl_2$ and affinity purified using Talon cobalt affinity resin (Clonetech) followed by SP-Sepharose column chromatography (GE healthcare) as previously described (37). Recombinant proteins used for SUMOylation assay: E1 complex (Aos1 and Uba2) subcloned into pRSF Duet vector with His-6 tag, Ubc9 subcloned into pT7-7, SUMO2-GG subcloned into pGEX4T-1 with a cleavable N-terminal GST tag, PIASy and SENP2 catalytic domain (SENP2 CD) (363-589 a.a.) subcloned into pET28a vectors with a N-terminal His-6 tag. All proteins were expressed either at 15°C (E1 complex, PIASy and SENP2 CD) or 30°C (Ubc9 and SUMO2-GG). His-6 tagged proteins (PIASy and SENP2 CD) were purified using Cobalt affinity beads (Clonetech) followed by ion-exchange chromatography. Ubc9 was purified by passing the soluble fraction onto anion exchange column followed by cation exchange column and sephacryl S-100 gel filtration. E1 complex was purified using cobalt affinity beads (Clonetech) and elutions were loaded onto a NHS-sepharose conjugated GST-SUMO1-GG affinity column in the presence of ATP. Bound E1 complex was eluted by 10mM DTT and further purified with an anion exchange column. GST-SUMO2-GG was purified using Glutathione-sepharose beads (GE healthcare) and the GST-tag was cleaved with thrombin. Untagged SUMO2-GG was purified by anion exchange chromatography followed by sephacryl S-100 gel filtration. All proteins were concentrated and stored by snap freeze with liquid nitrogen in storage buffer (100mM NaCl, 1mM $MgCl_2$, 20mM HEPES (pH 7.8), 5% glycerol, and 0.5mM TCEP).

In vitro SUMOylation assay was performed with 40nM E1, 40nM PIASy, 80nM Ubc9, 3μM

PARP1 (1-650 a.a.) and 30μM SUMO2-GG in SUMOylation buffer (20mM HEPES (pH 7.8), 5% glycerol, 100mM NaCl, 0.05% Tween 20 and 5mM $MgCl_2$) for 2 hours at 25 °C.

Construction of a DNA triplex and Translocation assay

Construction of DNA triplex and translocation assay were performed as described previously, with a few modifications (25). Two oligonucleotides with sequences (5'-CGCAAGAAAAGAAAGAAGAAAGAAACCGAGCT-3' and 5'-CGGTTTCTTTCTTCTTTCTTTTCTTGCGGTAC-3') were annealed and the duplex was cloned into pBluescript KS+ vector. A 400 bp fragment containing the duplex sequence was PCR amplified. A triplex forming oligonucleotide (10pmol) (5'-TTCTTTTCTTTCTTCTTTCTTT-3') was end-labeled with Alexa 488, and was then annealed to an equimolar amount of the 400 bp fragment in buffer containing 10mM $MgCl_2$, 50mM NaCl and 40mM MES, pH 5.5. The reaction was placed on a heat block (80 °C) for 2 minutes and allowed to cool to room temperature overnight.

For the translocation assay, 25nM of the triplex substrate and 0.5μM or 3μM PICH were used per reaction. Reactions were performed at room temperature for 25 min, or when specified time, in buffer containing 20mM Tris-HCL, pH 7.0, 100mM NaCl, 2mM $MgCl_2$, 1mM DTT, 2mM ATP and 0.1mg/ml BSA. Reactions were stopped by the stop buffer (containing 0.5% SDS, 0.04mg/ml proteinase K, 10% glycerol, and 20mM MES, pH 5.5) and incubated at 37 °C for 15 min. The reactions were loaded onto a 10% native polyacrylamide gel and run at 100V for 2 hours in pre-chilled TAM buffer (40mM Tris/HAc, pH 5.5, 1mM $MgCl_2$). The gel was imaged using a Typhoon Imager (GE Healthcare).

Pull-down Assays

Pull-down (PD) assays were performed as previously described (10). GFP-tagged PICH (WT, mutants or truncations) mRNA was generated using mMESSAGE mMACHINE SP6 transcription kit (Life Technologies, Carlsbad, CA) and was expressed in *Xenopus* egg extract for pull-down assay (36). Non-SUMOylated and SUMOylated bait (*Xenopus laevis* PARP1 (1-650 a.a.) fused to S-tag) were bound to S-protein-agarose beads (EMD Millipore, Billerica, MA). XEE was first diluted three times with the PD buffer (20mM NaPi, pH 7.8, 18mM β-glycerol phosphate, pH 7.5, 5mM MgCl2, 100mM NaCl, 1mM CaCl2, and 10μM $ZnCl_2$) and clarified by centrifugation at 25,000 x g for 30 min at 4 °C. The soluble fraction was diluted with equal volume of PD buffer supplemented with 0.2% Tween 20 and 0.2% Triton X-100 followed by centrifugation. Diluted and clarified XEE was incubated with bait bound S-protein beads for an hour at room temperature. Bound proteins samples were digested by SENP2-CD (50ng/μl) for 30min at room temperature and resolved by SDS-PAGE on 8-16% gradient gels (Life Technologies, Carlsbad, CA) for immunoblotting analysis.

Immunofluorescence analysis

Transfected cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X 100. Cells were blocked in 5% BSA and 2.5% fish gelatin in PBS-T (1X PBS with 0.1% tween 20) for 30 minutes. Cells were stained with primary antibody (1:200 dilution in blocking buffer) followed by Alexa dye-tagged secondary antibody (1:1000) (Life Technologies) for an hour each at room temperature. DNA was stained with Hoechst 33342 (EMD Millipore) and the cells were mounted using Vectashield mounting medium (Vector laboratories, Burlingame, CA). The specimens were analyzed using a Nikon TE2000-U microscope with a Plan Apo 100/1.40 and 60X objective, and the images were taken with a Retiga SRV CCD camera (QImaging) using

Volocity imaging software (Improvision), room temperature. Obtained images were processed to intensities according to JCB policy using Photoshop CS6 and Illustrator software (Adobe). Quantification of the PICH signal was through Adobe Photoshop CS6 software. Intensity around PICH was measured and was normalized with the CENPA signal. Averages of the PICH intensities from at least 30 centromeres from different mitotic stages cells (prometaphase, metaphase and anaphase) per set were calculated. The average and standard deviation of three sets was determined for each PICH construct. Graph and paired student t-test were plotted using KaleidaGraph software.

Stable Cell line generation and synchronization for chromatin bridge analysis

Inducible siRNA-resistant EGFP-PICH DNA constructs were co-transfected into HeLa Tet-ON cells along with a linear selection marker, hygromycin resistance, using Clonetech's Xfect transfection reagent as per the manufacturer's instructions. Stable clones were isolated using cloning cylinders and screened for expression. PICH in stable cell lines was induced by 500μg/ml doxycycline for 24 hours. HeLa Tet-ON cells stably expressing different PICH constructs were treated with PICH siRNA (40pmols). Five hours later, cells were synchronized with 2mM thymidine. 500μg/ml doxycycline was added along with the thymidine. Approximately 19 hours later, cells were released from thymidine into fresh media with doxycycline. Six hours post release from thymidine, 100ng/ml nocodazole added to cells. Four hours after nocodazole addition, cells collected by mitotic shake off and released for 45 minutes before fixation. Anaphase cells with chromatin bridges were counted manually. The average and standard deviation from three independent experiments was determined. Graph and paired student t-test were plotted with KaleidaGraph software.

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CHAPTER 4

Role of mitotic SUMOylation on organization of centromeric chromatin and higher order centromeric structures

INTRODUCTION

Proper formation of kinetochores based on centromere organization is critical for accurate segregation of chromosomes to prevent aneuploidy. Centromeres are a specialized region of chromosomes with distinct histones and centromere-associated proteins. Centromeres in most organisms contain repetitive DNA sequences, suggesting critical role for these sequences in the function of centromere (1-3). Albeit studies have shown that some centromeres with repetitive DNA sequences do not nucleate the formation of kinetochores (4,5) and functional neocentromeres can form without the repeat DNA sequences (6). Centromere protein A (CENPA) was the first centromere protein identified, co-purify with core histones and determined to be a variant of core Histone H3 (7). Since the identification of CENPA, numerous studies have shown that CENPA is required to build a functional kinetochore and loss of CENPA is lethal in all the organisms tested (8-10). Other than CENPA, many centromere-associated proteins (CENPs) were discovered that are highly conserved across organisms (11). Despite all the advances made in unraveling the CENPA contribution to accurate chromosome segregation, regulation of centromere structural organization is not well understood.

Over the last few decades, the importance of centromere epigenetics for its function has become evident (12-14). Along with the Histone H3 variant, CENPA, the centromere also has other unique histone modifications and variants spanning the centromere and heterochromatin regions adjacent to the centromere (pericentric chromatin) that are critical for proper chromosome segregation. For instance, histone H3 and H4 methylation and histone H3 phosphorylation at the centromere (15-18). Histone H2A variant, H2A.Z have been shown to

play multiple roles during mitosis (19-21). Precise mechanisms of spatiotemporal regulation of these histone modifications and variants are not well understood.

In addition to phosphorylation and methylation, SUMOylation is another post-translational modification, whose importance during mitosis has become apparent in the last decade (22). Over the years, multiple mitotic proteins have been identified as SUMO substrates that are critical in the formation of microtubules (23), of centromeres/kinetochores or are involved in mitotic chromosome structure (24,25). We have identified three SUMO substrates using *Xenopus* egg extracts (XEE): Poly (ADP)-ribose polymerase 1 (PARP1), Topoisomerase IIα (Topo IIα), and Polo-like kinase 1 interacting checkpoint helicase (PICH) as indicated in previous chapters. One of the downstream consequences of SUMOylation is the establishment of novel protein-protein interactions (26). Our group found that SUMOylation of Topoisomerase IIα (TopoIIα) helps in the recruitment of proteins to the centromere (27) (Yoshida et al., in press, JCB). Comprehensive identification of SUMOylated TopoIIα and SUMOylated PARP1 binding proteins by pull-down assays followed by mass spectrometry analysis led to the identification of multiple proteins binding specifically to SUMOylated TopoIIα and PARP1 (27). Multiple identified proteins were previously characterized as chromatin regulators. Therefore, we wanted to test if mitotic SUMOylation was involved in the structural organization of mitotic centromeres via regulation of the identified chromatin regulator proteins. Some of the identified candidate proteins were tested for their SUMO-specific localization in *Xenopus* egg extract. Results discussed in this chapter led us to potential novel SUMO-dependent centromeric localization of proteins that are known to be involved in centromere organization and architecture.

RESULTS

Identification of SUMOylated PARP1 and Topoisomerase IIα CTD binding proteins by mass spectrometry

PARP1 and Topo IIα were previously identified as SUMO substrates in *Xenopus* egg extract (XEE) (28,29). We wanted to test if SUMOylation of PARP1 and Topo IIα mediates novel protein-protein interactions. To identify potential SUMO-specific PARP1/Topo IIα binding proteins, we performed a pull-down assay using XEE. We SUMOylated PARP1/ Topo IIα *in vitro*, bound the SUMOylated proteins to S-agarose beads and incubated the beads with XEE (27). Bound proteins were eluted and subjected to LC-MS/MS analysis. The LC-MS/MS analysis identified numerous binding proteins specific to SUMOylated Topo IIα and PARP1. Some of the proteins were identified to bind to both when SUMOylated. Table 1 and 2 lists the proteins identified by mass spectrometry that bound specifically to SUMOylated Topo IIα and PARP1 respectively. One of the known SUMO-interacting proteins that were pulled down with both SUMOylated PARP1 and Topo IIα was SET domain, bifurcated 1, SETDB1 (30).

SUMOylated substrate favorably interacts with SETDB1 but does not alter its mitotic localization

SETDB1 is a histone methyltransferase that is known to methylate lysine 9 on histone H3 (H3K9) (31). H3K9 methylation is linked to heterochromatin protein 1 (HP1)-mediated gene silencing and heterochromatin formation (32,33). SETDB1 was previously shown to interact with SUMO and therefore, served as a good candidate to test the efficiency of our mass spectrometry results (34). We wanted to test if SETDB1 specifically interacted with SUMOylated Topo IIα and PARP1. To do that we performed a pull-down assay with *Xenopus*

egg extract (XEE) *in vitro* as previously described in chapter 2 and 3 and analyzed the pulled-down samples by immunoblotting analysis. SETDB1 interacted with both SUMOylated Topo IIα and PARP1 *in vitro* by pull-down assay (data not shown). Some of the chromatin-associated proteins are listed below along with their SUMO-dependency and specificity. (Table 4.1).

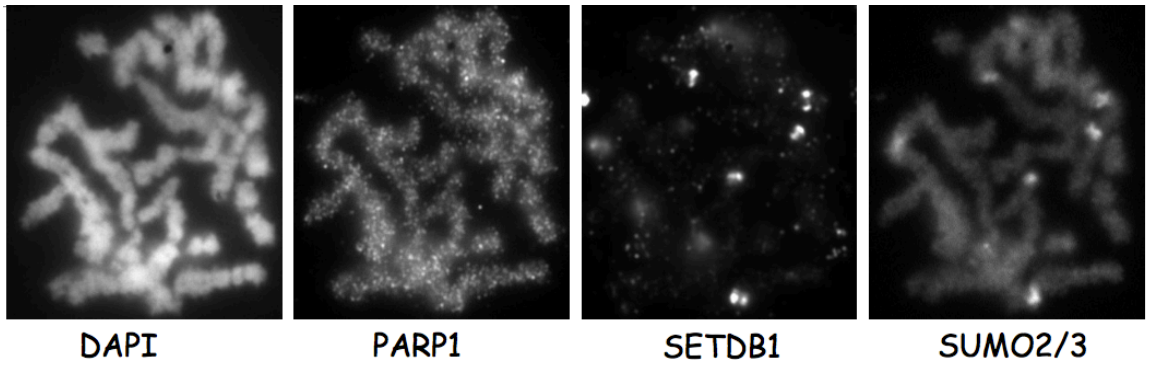
Table 4.1. List of some of the SUMO-binding Chromatin-associated proteins identified by Mass spectrometry. A pull-down assay was performed with non-SUMOylated and SUMOylated Topo IIα and PARP1 using XEE. Pulled-down samples were sent to LC-MS/MS analysis and a list of putative SUMO-binding proteins identified. Table lists some of the proteins that have predicted chromatin remodeling or histone modifying functions. N.E. is not examined.

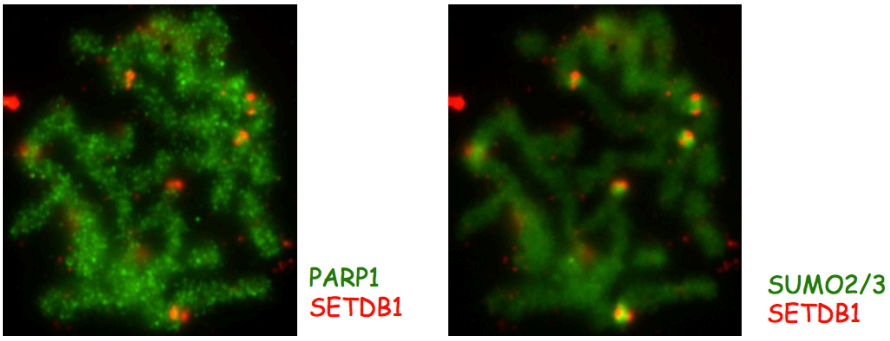
SDBPs	Protein family	Specificity (MS result)	Specificity (Experimental confirmation)	SUMO-dependency (Experimental confirmation)
ISWI	Chromatin remodeler	TopoIIα/ PARP1	TopoIIα/PARP1	++
PICH	Chromatin remodeler	TopoIIα/ PARP1	PARP1/TopoIIα	+++
Mi-2/ Nurd	Chromatin remodeler	PARP1	?	+++
Rad54B	Helicase	TopoIIα	N.E.	N.E.
ACF1	Chromatin remodeler	TopoIIα	N.E.	N.E.
HELLS	Chromatin remodeler	TopoIIα	N.E.	N.E.
Brg1	Transcriptional regulator	TopoIIα	N.E.	N.E
Spt16 (Duf140)	Chromatin factor	TopoIIα	TopoIIα/PARP1	N.E
SETDB1	Histone modifier	TopoIIα/ PARP1	TopoIIα/PARP1 (?)	+++
Ruv-like2	ATP/Helicase	PARP1	N.E.	N.E.
RBBP4-B	Histone binding protein	PARP1	N.E.	N.E.

Next, we wanted to test if this SUMO-mediated interaction of SETDB1 with Topo IIα and PARP1 is critical for its localization on mitotic chromosomes. Chromosomes were isolated from XEE and SUMOylation was inhibited by the addition of dominant negative Ubc9 (dnUbc9) as described in chapter 2. By immunostaining analysis of SETDB1, we observed that SETDB1 localized to the centromere region in a SUMO-independent manner (Fig 4.1B & 4.1C).

4.1A

Control replicated mitotic chromosomes





4.1B

+dnUbc9 treated-replicated mitotic chromosomes

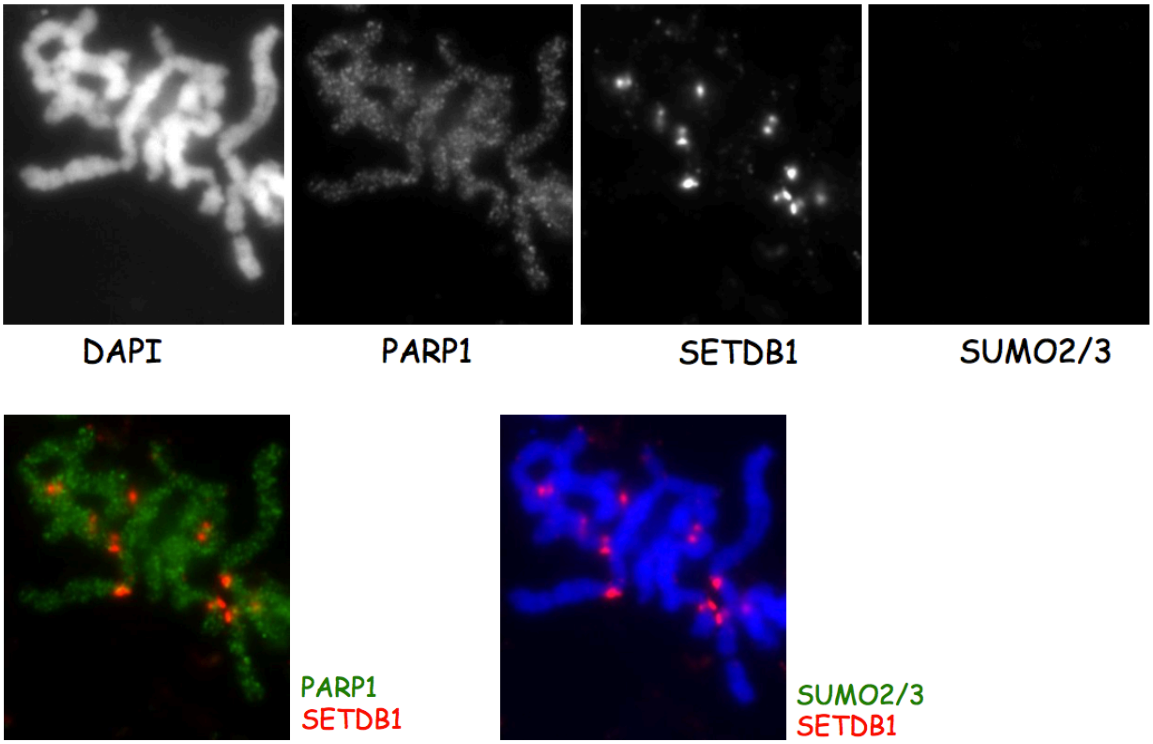


Figure 4.1. SETDB1 localizes to the centromere in a SUMO-independent manner. (A) Replicated mitotic chromosomes isolated from XEE were subjected to immunofluorescence analysis by indicated antibodies. **(B)** SUMOylation was inhibited by the addition of dnUbc9 and replicated mitotic chromosomes were analyzed for SETDB1 localization by immunofluorescence analysis.

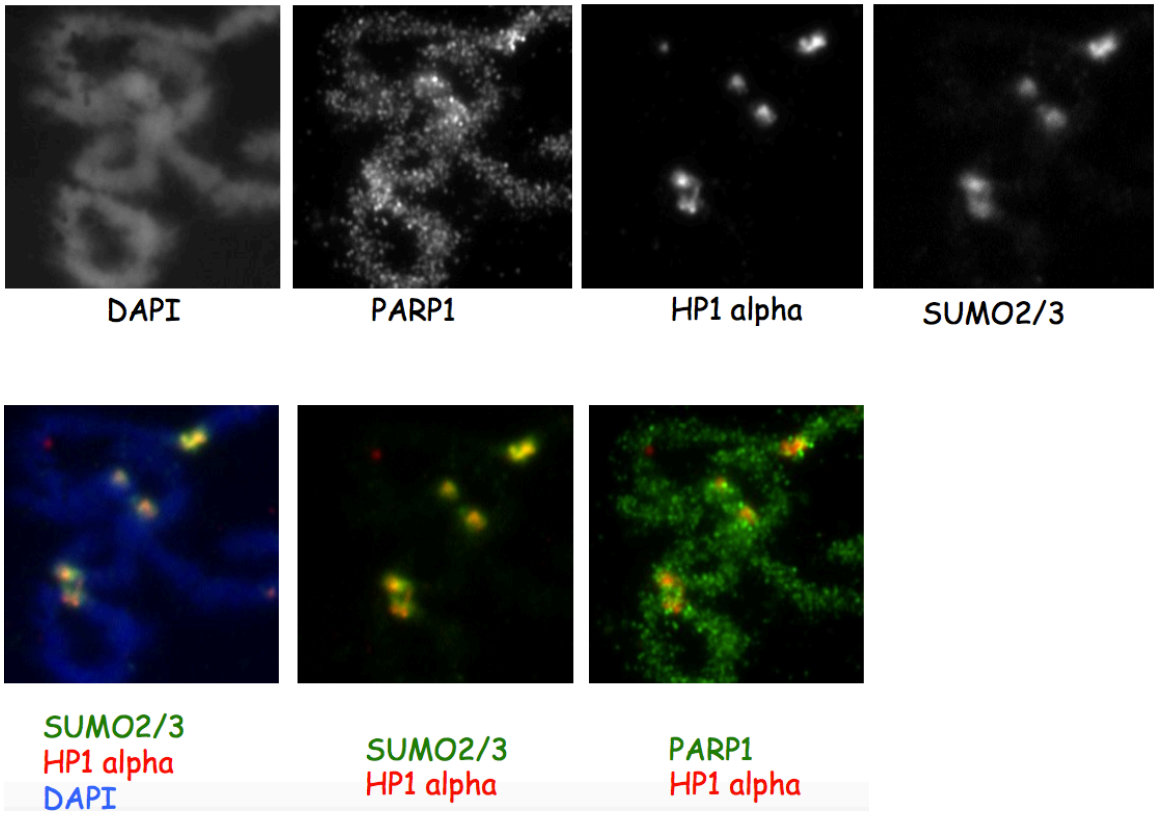
SUMO regulates localization of heterochromatin protein 1α and γ (HP1α and γ) differently

The histone methyltransferase, SETDB1 methylates histone H3 lysine 9 (H3K9), a known epigenetic marker for heterochromatin protein 1 (HP1) localization to mediate heterochromatin

formation (31-33). Though SUMOylation did not affect the localization of SETDB1, it could still negatively regulate the methyltransferase activity of SETDB1, resulting in defective H3K9me3 and HP1 localization. Isoforms of HP1 (HP1α, HP1β, and HP1γ) have distinct localization pattern in the mitotic and interphase nuclei (35,36). Though HP1α has been shown to be predominant at mitotic centromeres, the precise mechanism of HP1's association with mitotic chromosomes is not well understood (35,37). Since *Xenopus* has only two of the three isoforms of HP1 (HP1α and HP1γ), we wanted to test if HP1 localization is affected in a SUMO-dependent manner. Replicated mitotic chromosomes with and without the addition of dnUBc9 were isolated from XEE and HP1 isoforms localization was visualized. Expectedly, HP1α was enriched at the centromeric region of the chromosome and the enrichment was not affected by the inhibition of SUMOylation (Fig 4.2A&B).

4.2A

Control replicated mitotic chromosomes



4.2B

+dnUbc9 treated-replicated mitotic chromosomes

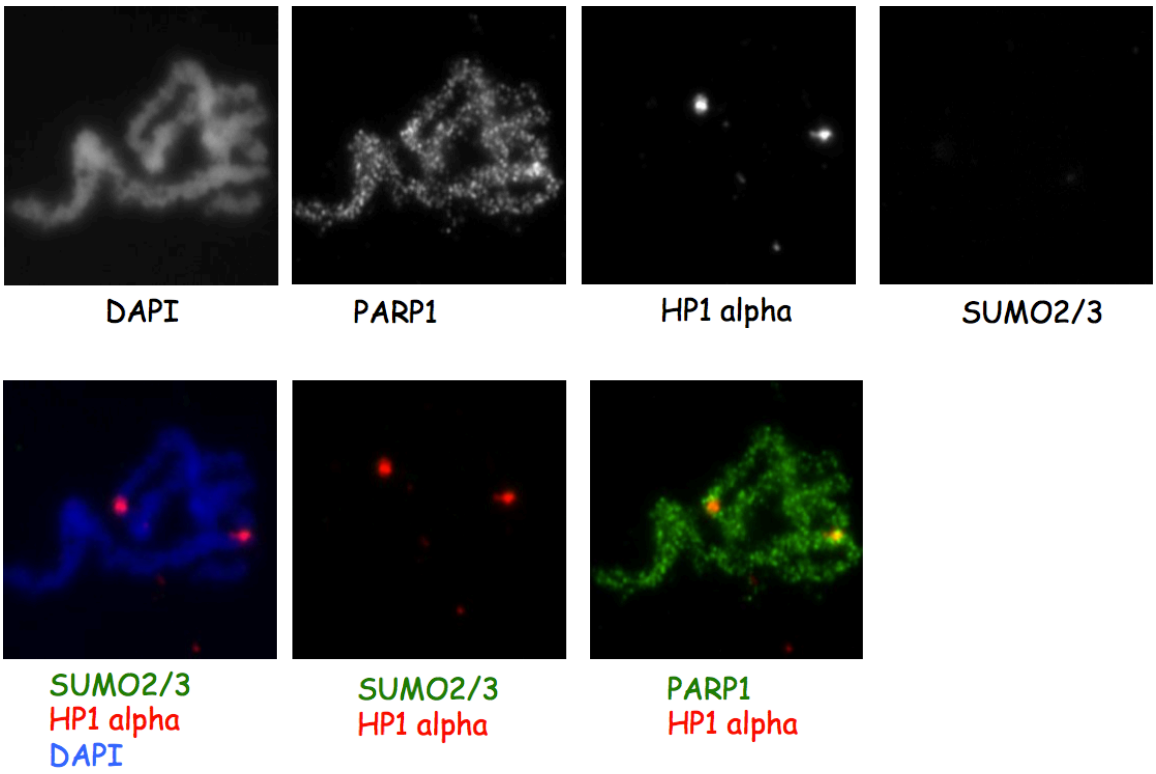


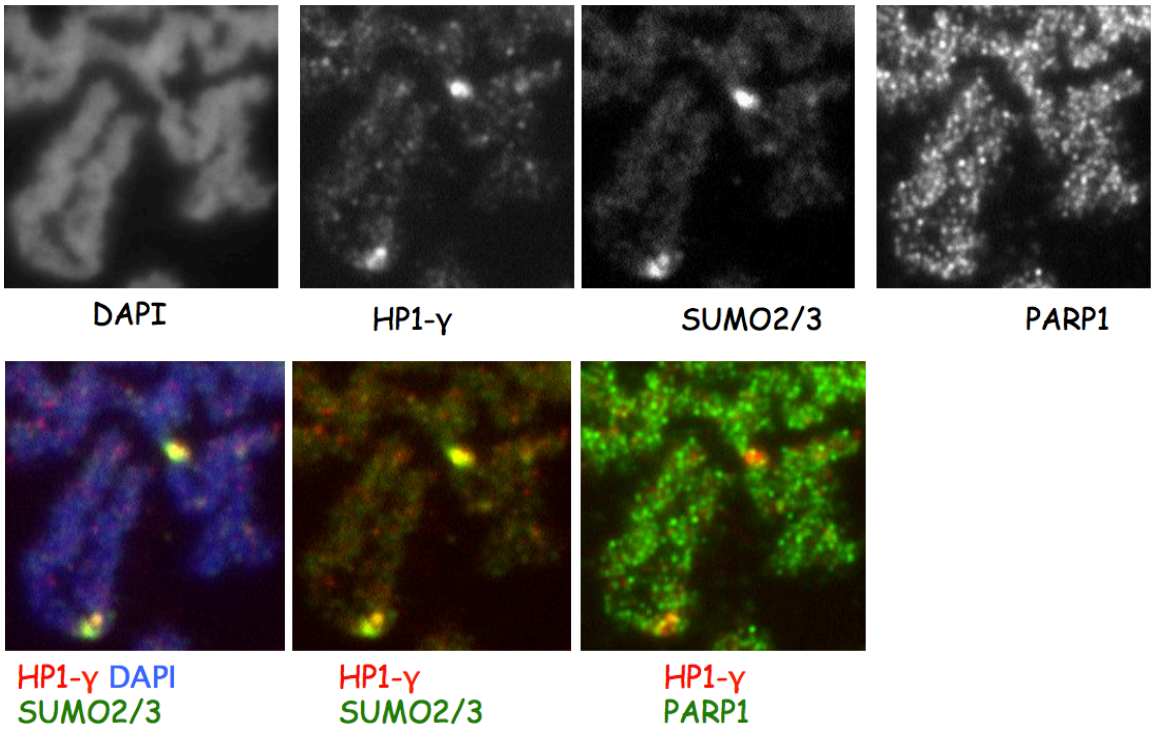
Figure 4.2. Heterochromatin protein 1α (HP1α) is enriched at the centromere in a non-SUMO dependent manner. (A) Replicated mitotic chromosomes isolated from XEE were subjected to immunofluorescence analysis by indicated antibodies. **(B)** SUMOylation was inhibited by the addition of dnUbc9 and replicated mitotic chromosomes were analyzed for HP1α localization by immunofluorescence analysis.

HP1γ is linked more with euchromatin in interphase nuclei and its localization at the centromere is not very clear (35,36). We observed centromeric association of HP1γ on mitotic chromosomes isolated from XEE. Surprisingly, HP1γ was enriched at the centromere and the

centromeric association was disrupted when SUMOylation was inhibited by the addition of dnUbc9 (Fig 4.3A&B).

4.3A

Control replicated mitotic chromosomes



4.3B

+dnUbc9 treated-replicated mitotic chromosomes

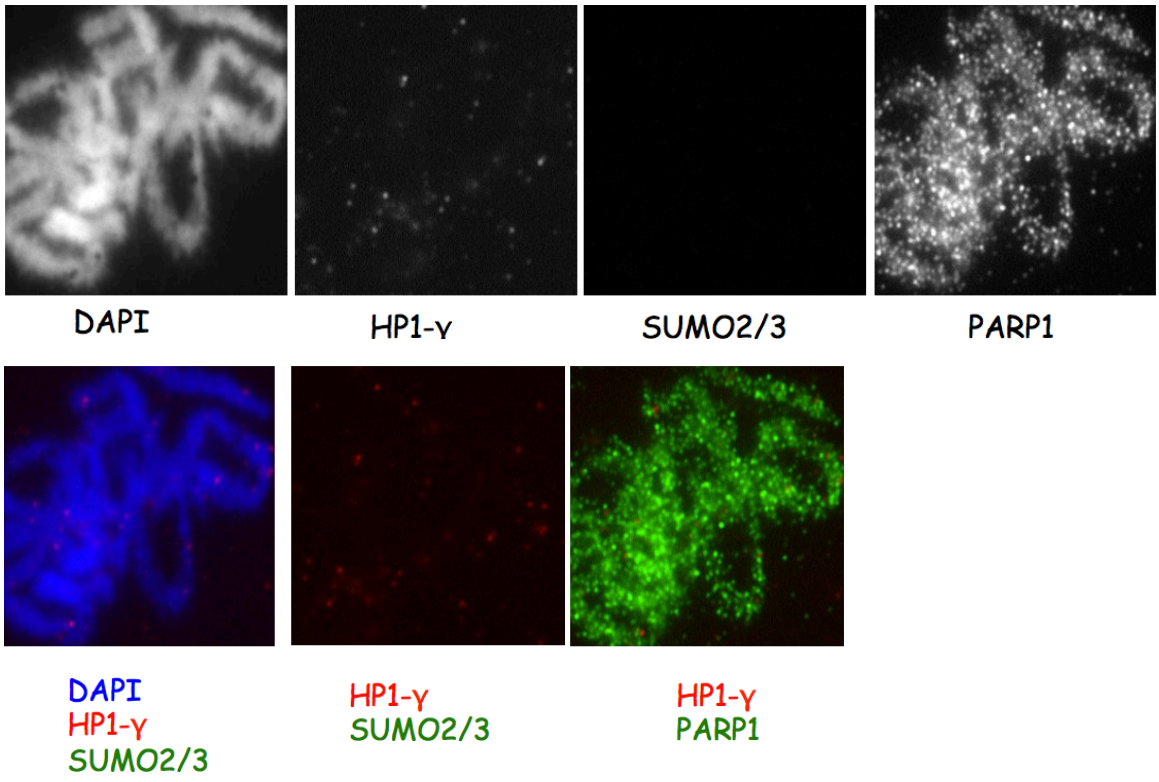


Figure 4.3. Heterochromatin protein 1γ (HP1γ) is enriched at the centromere in a SUMO dependent manner. (A) Replicated mitotic chromosomes isolated from XEE were subjected to immunofluorescence analysis by indicated antibodies. **(B)** SUMOylation was inhibited by the addition of dnUbc9 and replicated mitotic chromosomes were analyzed for HP1γ localization by immunofluorescence analysis.

These data suggest that HP1α and HP1γ are differently regulated for their centromeric localization on mitotic chromosomes isolated from XEE and mitotic SUMOylation is critical for centromeric localization of HP1γ.

SUMOylation does not alter histone H3 lysine9 (H3K9) methylation on mitotic chromosomes

Though SETDB1 localization is not affected by inhibition of mitotic SUMOylation on chromosomes isolated from XEE, we observed a difference in the localization pattern of HP1α and HP1γ. Therefore, we wanted to test if the H3K9me3 localization pattern is different when SUMOylation is inhibited on mitotic chromosomes. Replicated mitotic chromosomes were isolated from XEE and immunostained for H3K9me3. Intriguingly, H3K9me3 was observed throughout the chromosomes with some enriched foci on the chromosome arm region. H3K9me3 localization was not detectably changed when SUMOylation was inhibited by the addition of dnUbc9 (Fig 4.4). This suggests that H3K9me3 is not the only epigenetic marker that directs HP1 localization on the centromere.

4.4

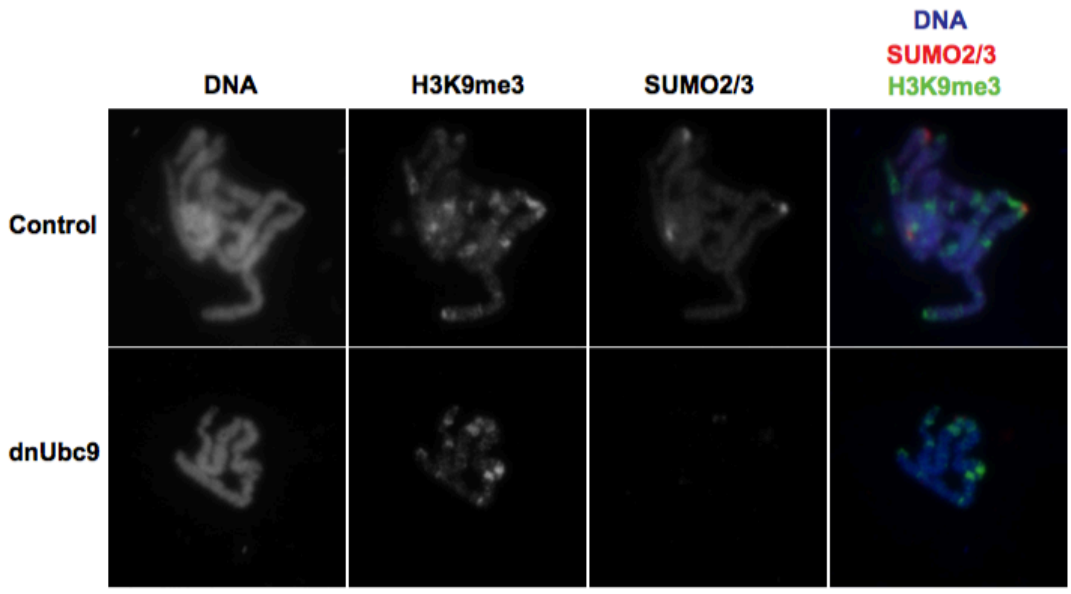


Figure 4.4. Histone H3 lysine9 (H3K9) methylation on mitotic chromosome is not dependent on SUMOylation. Replicated mitotic chromosomes were isolated from XEE and immunostained with the indicated antibodies. SUMOylation was inhibited by the addition of dnUbc9.

SUMOylation is essential for robust localization of H2A.Z but not CENPA at the centromere

The above results along with observations from our research group suggest that SUMOylation can alter epigenetics at the centromere. The putative role of the histone H2A variant, H2A.Z, during mitosis has recently emerged from studies by multiple groups (19,20). What controls the localization of H2A.Z during mitosis is not well understood yet. We wanted to test if SUMOylation controls H2A.Z's localization on mitotic chromosomes. To test that, we isolated replicated mitotic chromosomes from XEE with and without the inhibition of SUMOylation. Analysis of H2A.Z localization was done by immunofluorescence staining. We observed that H2A.Z had an enriched focus at the centromere that was dependent on SUMOylation (Fig 4.5). H2A.Z's centromeric association was drastically reduced when replicated mitotic chromosomes were isolated from dnUbc9 treated XEE (Fig 4.5).

4.5

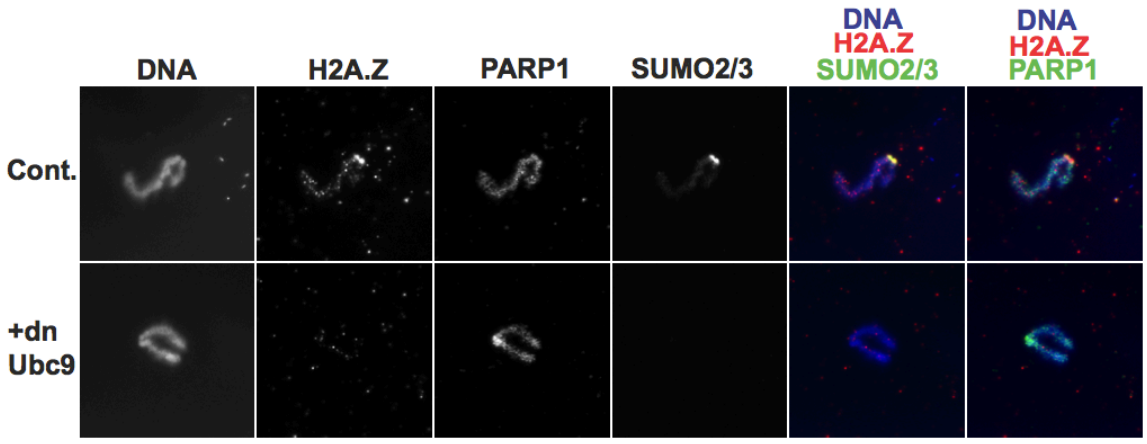
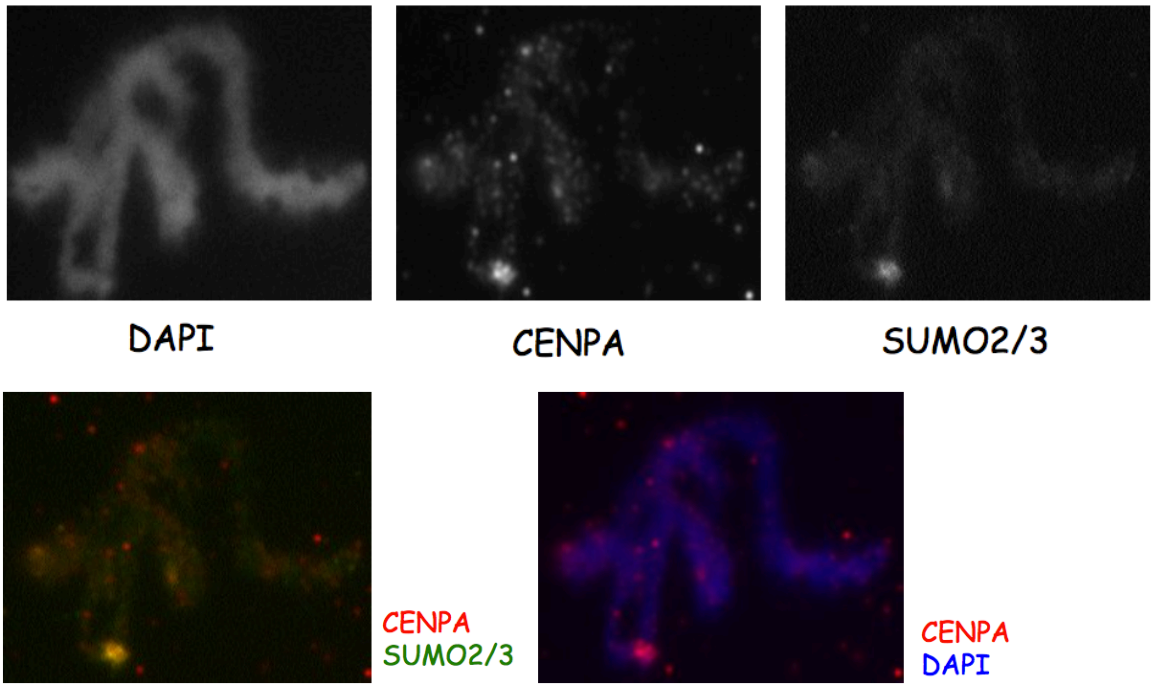


Figure 4.5. H2A.Z localization on mitotic centromere is dependent on SUMOylation. Replicated mitotic chromosomes were isolated from XEE with and without the addition of dnUbc9 and immunofluorescence analysis was performed with the indicated antibodies.

CENPA was the first centromere protein identified and we wanted to test if CENPA localization was SUMO-dependent, like H2A.Z. Replicated mitotic chromosomes were isolated and visualized by immunofluorescence analysis. Unlike H2A.Z, CENPA localization at the centromere was not dependent on SUMOylation (Fig 4.6A&B).

4.6A

Control replicated mitotic chromosomes



4.6B

+dnUbc9 treated-replicated mitotic chromosomes

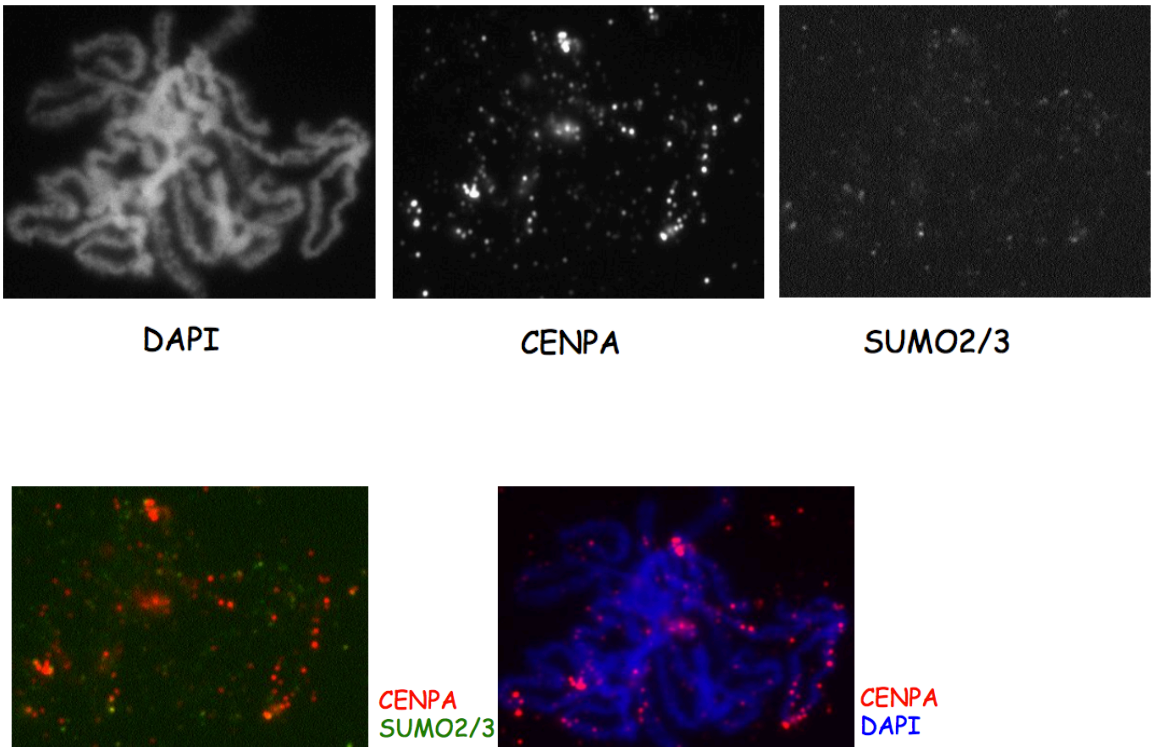


Figure 4.6. CENPA localization on mitotic centromere is not SUMO-dependent. (A) Replicated mitotic chromosomes isolated from XEE were subjected to immunofluorescence analysis by indicated antibodies. **(B)** SUMOylation was inhibited by the addition of dnUbc9 and replicated mitotic chromosomes were analyzed for CENPA localization by immunofluorescence analysis.

Overall, our results suggest that SUMOylation can differentially affect the association of HP1 isoforms on the mitotic centromeres and H3K9me3 most likely is not the histone marker for HP1γ centromere localization. Further investigation of the SUMO-regulated proteins will give more insight into the precise SUMO-regulated mitotic epigenetic regulation. In the previous two

chapters, we showed that a putative chromatin remodeler, PICH is regulated by SUMOylation. It would be an interesting hypothesis to test if PICH plays a key role in the SUMO-dependent localization of H2A.Z at the centromere.

DISCUSSION

The complex process of kinetochore formation at the centromere and of accurate chromosome segregation is under investigation. Despite decades of research unraveling the molecules and mechanisms involved in this complex process ensuring genome stability and maintenance many key molecular mechanisms are not well understood (38). SUMOylation is one of the posttranslational modifications that plays a significant role in ensuring proper chromosome segregation during mitosis (39). In order to understand the molecular mechanism of SUMOylation during segregation, we identified proteins that interact non-covalently with two SUMO substrates (Topo IIα and PARP1), only when these two proteins are SUMOylated. Of all the identified proteins listed in table 4.1, some were characterized previously as histone modifying enzymes and chromatin remodelers.

SET domain, bifurcated 1 (SETDB1), histone methyltransferase is one such histone modifier identified to interact with both SUMOylated Topo IIα and PARP1. Our results imply that SUMOylation does not regulate localization of SETDB1 or SETDB1 mediated H3K9me3 on mitotic chromosomes. HP1 isoforms in *Xenopus* showed differential localization pattern. This suggests that HP1 isoforms most likely recognize different epigenetic markers on mitotic chromosomes and are not restricted to just H3K9me3. Some of the mitotic histone markers studied so far include H3K4ac, H3T3ph, H3S10ph, H3K27me3 (40) and future studies will require investigation of SUMO dependency of these modifications along with their connection with HP1 proteins. HP1 isoforms have two major domains, chromo domain and chromoshadow

domain (41). It will be interesting to see if one of these domains is responsible for directing HP1 localization at the centromere by making a chimera of HP1α and γ and testing their SUMO-dependency for centromeric localization.

In addition to HP1 isoforms, the H2A variant, H2A.Z localized to the centromere in a SUMO-dependent manner. Over the years, the importance of H2A.Z during mitosis has been studied (19-21). Greaves et al. highlighted the potential importance of H2A.Z in the organization of the centromere structure (20). Hou et al. showed a potential role for H2A.Z in centromere silencing and chromosome segregation (21), although, the precise mechanism of its localization and mitotic role is not fully understood yet. We identified a few chromatin remodelers in the mass spectrometry analysis of SUMOylated Topo IIα and PARP1 proteins. Topo IIα or PARP1 SUMOylation mediated regulation of one or more of the identified chromatin remodelers may result in SUMO-dependent localization of H2A.Z. Polo-like kinase 1 interacting checkpoint helicase (PICH), discussed in chapter 2 and 3 may be one of the candidates regulating H2A.Z. Further studies with chromatin immunoprecipitation using H2A.Z antibody with and without SUMOylation will give more mechanistic insight.

MATERIALS AND METHODS

Recombinant protein purification and *in vitro* SUMOylation assay

Recombinant *Xenopus laevis* PARP1 (1-650 a.a.), used for *in vitro* pull-down assays, was expressed at 15 °C in the presence of 2.5% ethanol and 0.1mM $ZnCl_2$ and affinity purified using Talon cobalt affinity resin (Clonetech) followed by SP-Sepharose column chromatography (GE healthcare) as previously described (28). Recombinant proteins used for SUMOylation assays: E1 complex (Aos1 and Uba2) subcloned into pRSF Duet vector with His-6 tag, Ubc9 subcloned

into pT7-7, SUMO2-GG subcloned into pGEX4T-1 with a cleavable N-terminal GST tag, PIASy, Topo IIα CTD (1,220-1,579 a.a.) and SENP2 catalytic domain (SENP2 CD) (363-589 a.a.) subcloned into pET28a vectors with a N-terminal His-6 tag. All proteins were expressed either at 15°C (E1 complex, Topo IIα, PIASy and SENP2 CD) or 30°C (Ubc9 and SUMO2-GG). His-6 tagged proteins (PIASy and SENP2 CD) were purified using Cobalt affinity beads (Clonetech) followed by ion-exchange chromatography. Ubc9 was purified by passing the soluble fraction onto anion exchange column followed by cation exchange column and sephacryl S-100 gel filtration. E1 complex was purified using cobalt affinity beads (Clonetech) and elutions were loaded onto a NHS-sepharose conjugated GST-SUMO1-GG affinity column in the presence of ATP. Bound E1 complex was eluted by 10mM DTT and further purified by anion exchange column. GST-SUMO2-GG was purified using Glutathione-sepharose beads (GE healthcare) and the GST-tag was cleaved with thrombin. Untagged SUMO2-GG was purified by anion exchange chromatography followed by sephacryl S-100 gel filtration. All proteins were concentrated and stored by snap freezing with liquid nitrogen in storage buffer (100mM NaCl, 1mM $MgCl_2$, 20mM HEPES (pH 7.8), 5% glycerol, and 0.5mM TCEP).

In vitro SUMOylation assays were performed with 40nM E1, 40nM PIASy, 80nM Ubc9, 3μM PARP1 (1-650 a.a.) and 30μM SUMO2-GG in SUMOylation buffer (20mM HEPES (pH 7.8), 5% glycerol, 100mM NaCl, 0.05% Tween 20 and 5mM $MgCl_2$) for 2 hours at 25 °C.

Pull-down Assays

Pull-down assays were performed as previously described (42). GFP-tagged PICH (WT, mutants or truncations) mRNA was generated using the mMESSAGE mMACHINE SP6 transcription kit (Life Technologies, Carlsbad, CA) and was expressed in *Xenopus* egg extract for pull-down assays (43). Non-SUMOylated and SUMOylated baits (*Xenopus laevis* PARP1 (1-650 a.a.) fused

to S-tag) were bound to S-protein-agarose beads (EMD Millipore, Billerica, MA). XEE was first diluted three times with pull-down buffer (20mM NaPi, pH 7.8, 18mM β-glycerol phosphate, pH 7.5, 5mM MgCl2, 100mM NaCl, 1mM CaCl2, and 10μM $ZnCl_2$) and clarified by centrifugation at 25,000 x g for 30 min at 4 °C. The soluble fraction was diluted with an equal volume of Pull-down buffer supplemented with 0.2% Tween 20 and 0.2% Triton X-100 followed by centrifugation. Diluted and clarified XEE was incubated with bait bound S-protein beads for an hour at room temperature. Bound proteins samples were digested by SENP2-CD (50ng/μl) for 30min at room temperature and resolved by SDS-PAGE on 8-16% gradient gels (Life Technologies, Carlsbad, CA) for immunoblotting analysis.

***Xenopus* egg extract immunofluorescence and immunoblotting.** Low-speed extracts arrested in metaphase by cytostatic factor (CSF) from *X. laevis* egg and sperm nuclei were prepared using standard protocols (44). An interphase extract was obtained by releasing CSF upon $CaCl_2$ addition to CSF extracts (44). The mitotic chromosomes used for the immunofluorescence analysis were prepared as previously described (45). Mitotic SUMOylation was inhibited by the addition of a dominant-negative form of Ubc9 (dnUbc9) at a concentration of 150 ng/μl before the induction of mitosis. The localization of desired proteins was obtained by staining the chromosomes with the indicated antbodies. The DNA was stained with Hoechst 33342 dye (EMD Millipore), and the samples were mounted using Vectashield (Vector Laboratory). The specimens were analyzed using a Nikon TE2000-U microscope with Plan Apo 100X/1.40 objective, and images were taken with a Retiga SRV CCD camera (QImaging) using volocity-imaging software (Improvision).

Antibodies used

For immunofluorescence analysis,

Primary antibodies: CENPA (chicken in house antibody, 1:300), PARP1 (Rabbit in house antibody, 1:500), SETDB1 (Rabbit in house sera, 1:200), SUMO2/3 (Guinea pig in house antibody, 1:500), HP1α (Rabbit, Abcam 1:200), HP1γ (Rabbit, Cell signaling 1:200), H3K9me3 (Rabbit, Abcam 1:200), H2A.Z (Rabbit, Cell signaling 1:200).

Secondary antibodies: goat anti-rabbit Alexa 568, goat anti-mouse Alexa 488 and goat anti-guinea pig Alexa 647 (Life Technologies, Carlsbad, CA; 1:1000)

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CHAPTER 5

SUMO-regulation of Polo-like kinase 1 interacting checkpoint helicase (PICH)- current understanding and future perspectives

Understanding the regulation of the centromere structure to ensure its function in chromosome segregation is a work in progress. As mentioned in chapter one of this dissertation, epigenetics plays a critical role in the establishment of centromere structure. Epigenetics of centromeres includes chromatin features such as, unique nucleosome positioning (presence of CENPA at the centromere) and distinct histone modifications. Posttranslational modifications and nucleosome positioning play important roles in regulating histone modifications and chromatin remodeling. Understanding the molecular mechanism of the posttranslational modification, SUMOylation at the centromere is one of the major objectives of our research group. In the last decade, we have identified two SUMO substrates – Topoisomerase IIα and Poly (ADP-ribose) polymerase 1. We have further elucidated potential role(s) for Topo IIα SUMOylation in its decatenation activity and in mediating protein-protein interactions at the centromere (1,2). The work in this dissertation identified a novel SUMO substrate and a promiscuous SUMO-binding protein, Polo-like kinase 1 interacting checkpoint helicase (PICH). PICH is a mitosis-specific putative chromatin remodeler. This study has opened up new avenues in our research inter-connecting SUMO with PICH/chromatin remodeling and our previously identified SUMO substrate (Topo IIα) to ensure accurate chromosome segregation.

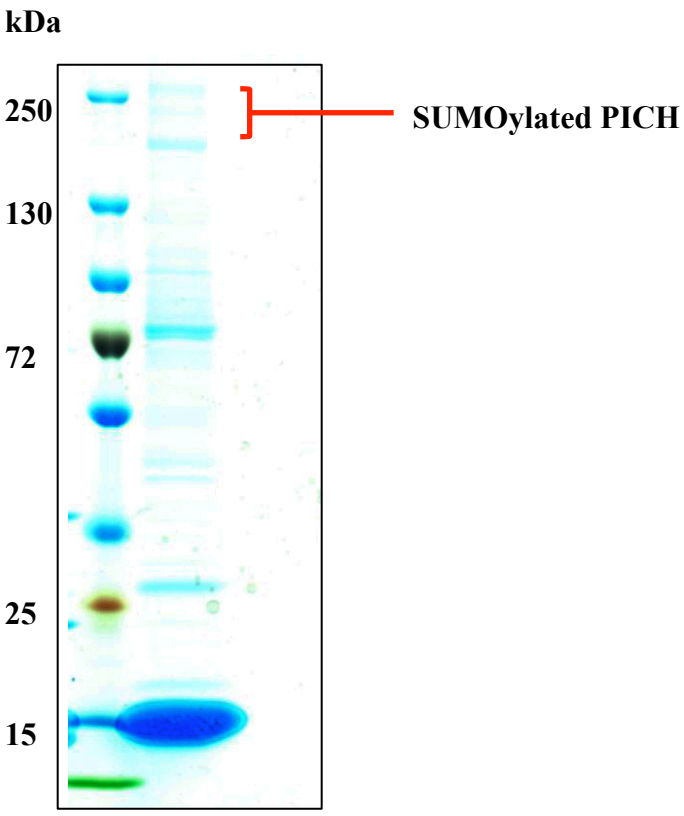
PICH: a novel SUMO substrate

Chapter 2 in this dissertation shows results that confirm PICH as a novel SUMO substrate using *Xenopus* egg extract (XEE) (Fig 2.3C&D). We showed SUMOylation of recombinant human PICH and that the SUMOylation was PIASy-dependent *in vitro* (Fig 2.4). Additionally,

we also showed that SUMOylation of PICH significantly reduces its ability to bind to DNA *in vitro* (Fig 2.4C&D). To understand the functional consequence of PICH SUMOylation, we wanted to identify the lysines that are SUMOylated. Identification of the lysines would enable us to generate a SUMO-deficient PICH mutant for functional analysis. We SUMOylated PICH *in vitro* as described in the materials and methods section of chapter 2 and analyzed the coommassie stained gel by LC-MS/MS (Fig 5.1).

Figure 5.1. SUMOylated PICH was ran on an 8-16% gradient gel and stained with coommassie based staining solution (instant blue).

5.1



We were able to cover ~65% of the full length PICH protein in the mass spectrometry analysis. From the covered lysines, one lysine (K853) was speculated to be SUMOylated based on LC-MS/MS data (Fig 5.2).

5.2

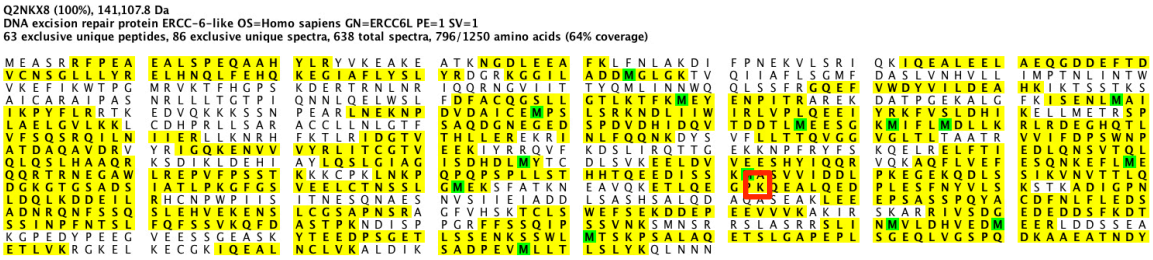


Figure 5.2. **Mass spectrometry coverage of the full length PICH protein**. Lysine 853 (highlighted in red box) identified as a potential SUMOylated site.

The identified lysine was mutated to arginine to generate a potential SUMO site-deficient PICH mutant (K853R). Potential alteration of the SUMOylation profile of the PICH K853R mutant was tested *in vitro*. No detectable reduction in the SUMOylation profile was observed *in vitro* with the K853R mutant of PICH.

5.3

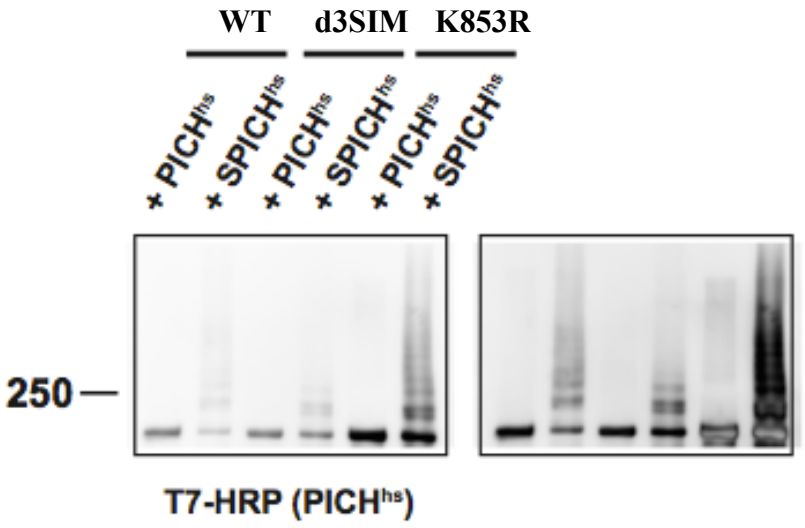


Figure 5.3. **PICH K853R SUMOylation is not detectably reduced *in vitro* in comparison to WT.** Recombinant PICH proteins (WT, d3SIM and K853R) were SUMOylated in vitro and analyzed by immunoblotting analysis using T7-HRP antibody.

Since there was no obvious reduction in the SUMOylation profile of PICH K853R, we used the SUMO site prediction program to predict potential SUMOylated lysines.

Figure 5.4. Positions of potential SUMOylated lysines predicted by the SUMO prediction program. A SUMO site prediction program was used to identify potential lysines (both consensus and non-consensus) that may be SUMOylated.

5.4

Position	Peptide	Score	Cutoff	Type
287	KTFKMEY	1.308	0.17	TypeI: ?-K-X-E
685	LSVKEEL	2.133	0.17	TypeI: ?-K-X-E
853	EGPKQEA	1.128	0.17	TypeI: ?-K-X-E

Position	Peptide	Score	Cutoff	Type
287	KTFKMEY	1.308	0.13	TypeI: ?-K-X-E
330	RRTKEDV	2.956	2.64	TypeII: Non-consensus
621	YFSKQEL	2.647	2.64	TypeII: Non-consensus
651	AQRKSDI	3.25	2.64	TypeII: Non-consensus
655	SDIKLDE	3.147	2.64	TypeII: Non-consensus
685	LSVKEEL	2.133	0.13	TypeI: ?-K-X-E
786	EGEKQDL	2.985	2.64	TypeII: Non-consensus
853	EGPKQEA	1.128	0.13	TypeI: ?-K-X-E
937	SEAKLEE	0.621	0.13	TypeI: ?-K-X-E

A few of the predicted lysines were selected (based on where they were located-HELICc domain and PFD domain) and mutated. None of the mutant proteins showed obvious reduction in the SUMOylation profile *in vitro*. Lysines that were mutated and tested were: double mutant K287R/K685R and triple mutant K330R/K651R/K853R (K3R).

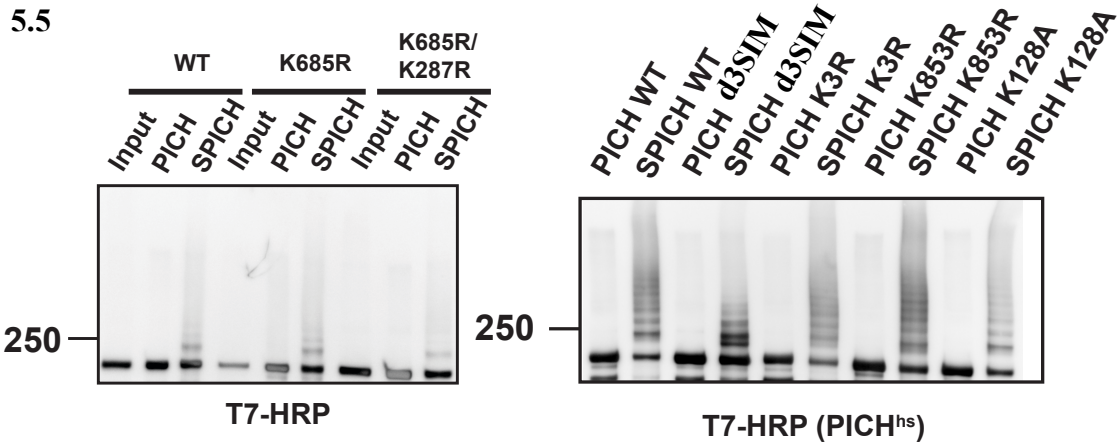


Figure 5.5. Mutations of some of the program-predicted lysines did not affect SUMOylation of PICH *in vitro*. Five of the predicted SUMOylated lysines in PICH were mutated to arginines and then SUMOylation profiles were tested by immunoblotting analysis. PICH 4SIM is PICH d3SIM as described in chapter 3. PICH K128A is the ATPase deficient mutant.

Though these mutants did not show a promising reduction in the SUMOylation of PICH *in vitro*, it is still possible that one or more of these lysines are SUMOylated *in vivo*. Chromatin isolation from XEE as described in chapter 2 (Fig 2.3C&D) with the addition of the lysine mutants, followed by immunoblotting analysis will clarify if these lysines are important for SUMOylation of PICH. Furthermore, we can aim to enrich for SUMOylated peptides of PICH using our SUMO peptide antibody for future mass spectrometric analysis. The SUMO peptide antibody only recognizes the peptide (QQTGG) of SUMO that is left on the SUMOylated

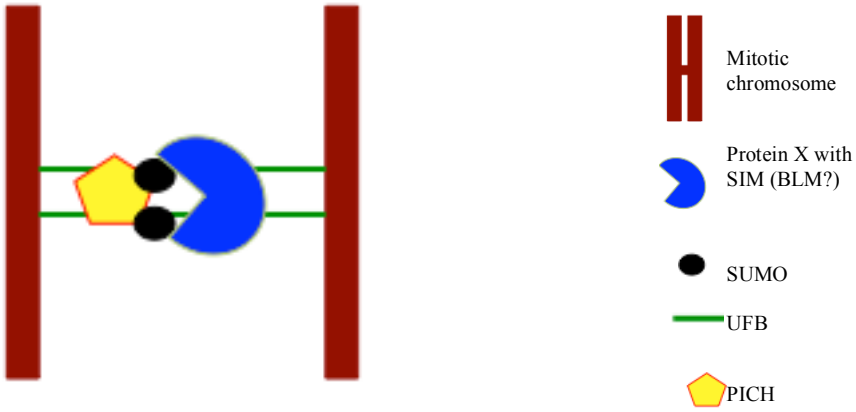
substrate after trypsin digestion when a C-terminal Q in SUMO2 is mutated to R. SUMOylated PICH can be trypsin digested and the SUMO peptide antibody can enrich the trypsin peptide fragments for LC-MS/MS analysis.

Our data suggests that SUMOylation of PICH is most likely important for its activity since DNA binding of SUMOylated PICH is significantly lower than non-SUMOylated PICH *in vitro* (Fig 2.4C&D). We know that inhibition of PICH by siRNA results in chromosome segregation defects, but the precise functional activity of PICH at the centromere during mitosis is not well understood. Based on our finding of PICH as a SUMO substrate, one hypothesis is that SUMOylated PICH can undergo a conformational change that can allow further cooperation with Topoisomerase IIα at the centromere to allow chromosome segregation. Multiple groups have shown links between Topo IIα and PICH during mitosis (3-6). Spence et al. showed an increase in PICH-coated ultrafine DNA threads (UFBs) when TopoIIα was inhibited, suggesting a functional connection between PICH and Topo IIα (3). UFBs as mentioned in chapter 1 of this dissertation are DNA threads that cannot be stained with DAPI. Wang et al. showed a similar result where they inhibited the activity of Topo IIα by addition of an inhibitor and observed a defect in the resolution of PICH-positive threads (4). Rouzeau et al. showed that PICH functions to make centromeric catenates more accessible to Topo IIα for resolution during anaphase (5). Recently, Nielsen et al. showed that cells with PICH deleted are hypersensitive to a Topo IIα inhibitor (6). Additionally, they showed that PICH stimulates the decatenation activity of Topo IIα *in vitro*. Similar to the cells treated with Topo IIα inhibitor, PICH-depleted cells also showed chromosome instability, chromosome condensation and decatenation defects in the study by Nielsen et al. (6). Because of numerous evidence of potential cooperative function between PICH and Topo IIα, it would be interesting to test if SUMOylation of PICH results in a

conformational change that is critical to regulate the catalytic activity of Topo IIα (Fig. 5.6B). PICH could also be cooperatively functioning with SUMOylated Topo IIα and this possibility is discussed in the next section of this chapter (PICH: a promiscuous SUMO- interacting protein).

Other than Topo IIα, multiple groups have shown the concerted role of PICH partnering with BLM (Bloom's syndrome helicase) (7). Ian Hickson's group identified BLM decorating the UFBs around the same time as Erich Nigg's group discovered PICH on those unique DNA threads (8). BLM was shown to interact with PICH *in vitro* and its localization on these UFBs is dependent on PICH (7). Though BLM could interact with PICH *in vitro*, it is possible that SUMOylation of PICH is assisting in the recruitment of BLM *in vivo* (Fig. 5.6A). To bolster this hypothesis, the SIM prediction program shows a few sites on BLM that could potentially act as SUMO-interacting motifs. To test the above-mentioned hypotheses, identification of SUMOylation sites on PICH is critical.

5.6A



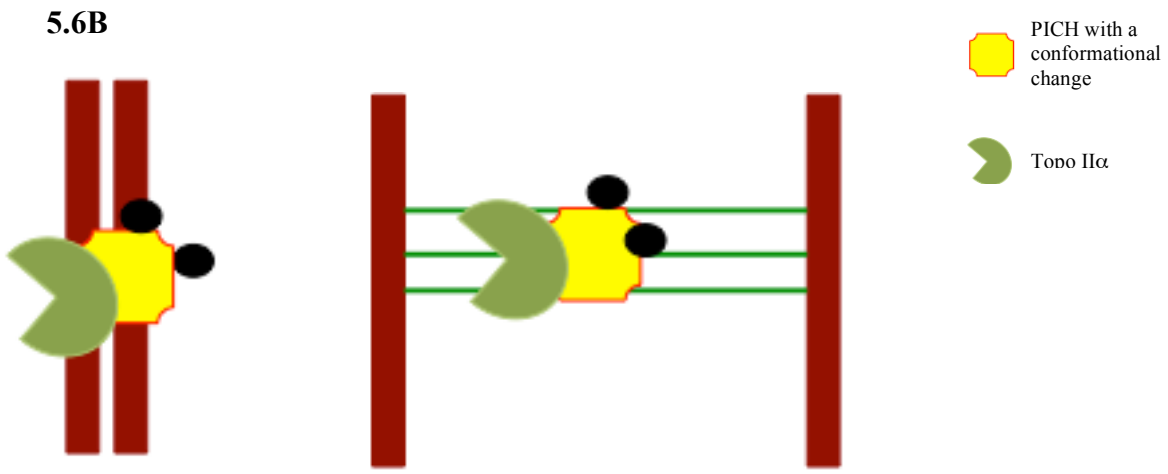


Figure 5.6. Model of potential role(s) of SUMOylated PICH on mitotic chromosomes. A&B) Based on the literature and our results, we hypothesize that SUMOylated PICH interacts with either Topo IIα to assist in decatenation at the centromere or Topo IIα or BLM to ensure UFB resolution for proper chromosome segregation during mitosis.

PICH: a promiscuous SUMO-interacting protein

The non-covalent SUMO interaction of PICH was the first observation that connected potential SUMO regulation with PICH as described in chapter 2 of this dissertation. We studied this property of PICH in more detail by the identification of SUMO-interacting motifs (SIMs) as described in detail in chapter 3. To summarize some of the key results from our study on the PICH-SIM interaction, we showed that PICH has three SIMs that cooperatively bind to SUMOylated substrates *in vitro* (Fig 3.1). We showed that these SIMs were not critical for PICH's localization at UFBs but were essential for PICH's robust localization at the centromere (Fig 3.2). Surprisingly, we saw that PICH SIM3 contributed to the robust localization of PICH at the centromere more significantly than SIM1&2 combined (Fig 3.3). Interestingly, despite of having defective centromeric localization, PICH dSIM3 was able to rescue the chromatin bridge

phenotype in cells treated with PICH siRNA (Fig 3.4). In contrast, PICH dSIM1&2, with no defective centromere localization was not able to rescue the chromatin bridge phenotype in cells (Fig 3.4). These results leave us with many unanswered questions. First, we know that SIM3 is more critical for PICH's robust centromeric localization but how SUMOylation regulates PICH's localization is not understood. Identification of the SUMOylated protein that binds to PICH SIM3 for its robust centromere localization is a key to start understanding the significance of PICH centromere localization. Chromatin immunoprecipitation from mitotic chromosomes using a PICH/EGFP antibody (for EGFP-PICH exogenously expressed protein) and identifying potential SUMOylated binding protein is one approach. A few possible candidates based on the literature are SUMOylated Topo IIα (9,10) and/or SUMOylated histones at the centromere (11,12).

Inhibition of PICH by siRNA or genome editing in mammalian cells causes chromosome segregation defects (6,13). But the precise molecular mechanism of how PICH plays a role in the prevention of chromosome segregation defects is not well understood. We made a very interesting observation with a PICH SIM1&2 mutant. PICH deficient in SIM1&2 (PICH dSIM1&2) was able to localize to the centromere but resulted in chromatin bridge phenotype in the absence of endogenous PICH in cells. This suggests that PICH SIM1&2 is important for its activity during mitosis, most likely via SUMO-mediated interaction. Since PICH dSIM3 has a localization defect and not a chromatin bridge phenotype (as discussed above), a SUMO-binding protein of PICH SIM1&2 is most likely different from that of PICH SIM3. Identification of SUMO-interacting proteins of PICH in its native condition will be challenging. Chromatin immunoprecipitation as discussed in the above paragraph is one approach. Potential candidates that may be acting as the SUMOylated interacting protein of PICH are Topo IIα and BLM. Both

these proteins are known to localize at the UFBs along with PICH (6,8) and are SUMO substrates (9,10,14). Previous results from other groups suggest that both Topo IIα and BLM function cooperatively with PICH to prevent chromosome segregation defects (5-7). Additionally, localization of BLM on UFBs is dependent on PICH. In the above section of this chapter (PICH: a novel SUMO substrate), it was discussed how SUMOylated PICH could be recruiting BLM to UFBs. Since BLM is a known SUMO substrate and PICH has multiple SIMs, we cannot rule out the possibility of SUMOylated BLM getting recruited to UFBs via SIMs on PICH. Independent studies have shown the importance of all three molecules (PICH, BLM and Topo IIα) in the resolution of UFBs (6,7). It would be interesting to test if SUMOylation is the key regulator in the cooperative function of these proteins during mitosis. We currently don't have evidence that defects in the resolution of UFBs lead to the formation of chromatin bridges. Recently, Nielsen et al. showed that >90% of cells that have UFB during late anaphase resolve with binucleation, suggesting the importance of resolving UFBs during anaphase. In our current study we didn't investigate the resolution of UFBs in our SIM mutants. It would be insightful to test if PICH dSIM3 and/or dSIM1&2 result in defective UFB resolution during anaphase. This observation would connect the importance of SIM1&2- and SIM3-mediated centromere localization in UFB resolution.

Another interesting possibility for regulation of PICH via SUMO/SIM is intra-molecular interaction. SIMs on PICH could be interacting with SUMOylated PICH resulting in either intra- or inter-molecular PICH-SUMO interactions resulting in conformational changes that assists in PICH's activity. Alternatively, PICH SIM-mediated binding with SUMO substrate can also promote SUMOylation of PICH. There are known examples where both SUMO sites and SIM sites play a role in the regulation of the protein's activity (15). To test if SUMOylation of PICH

was dependent on its SIM site, we tried to SUMOylate PICH d3SIM *in vitro*. Interestingly, PICH d3SIM showed defective SUMOylation in comparison to PICH WT (Fig 5.6A). We also tested whether the DNA binding was inhibited in SUMOylated PICH d3SIM like PICH WT (Chapter 2). Since the SUMOylation profile was defective, SUMOylated PICH d3SIM could not inhibit DNA binding *in vitro*. Surprisingly, PICH d3SIM by itself could bind DNA more effectively than PICH WT (Fig 5.6B). Our hypothesis for this difference is that it results from PICH d3SIM's inability to translocate on DNA in the presence of ATP, resulting in more stable binding to DNA *in vitro*.

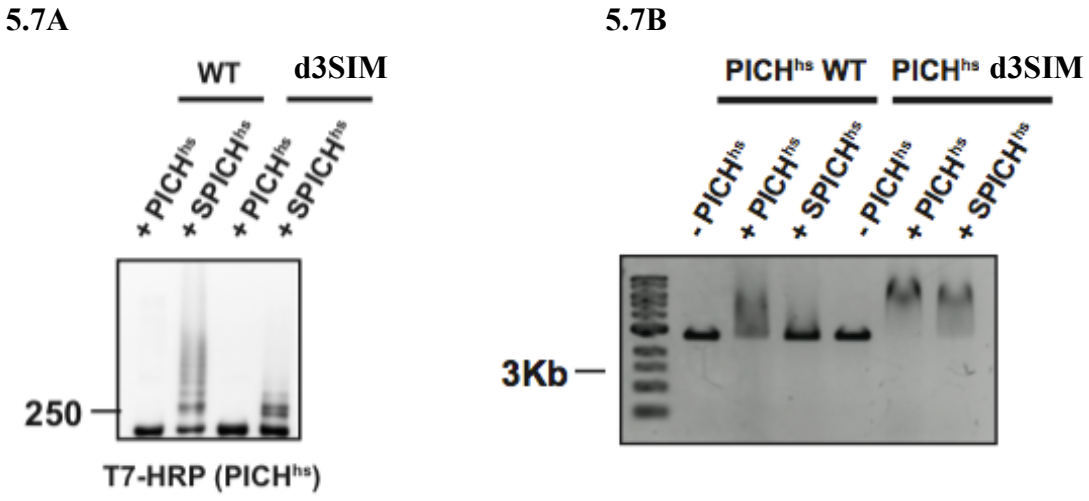
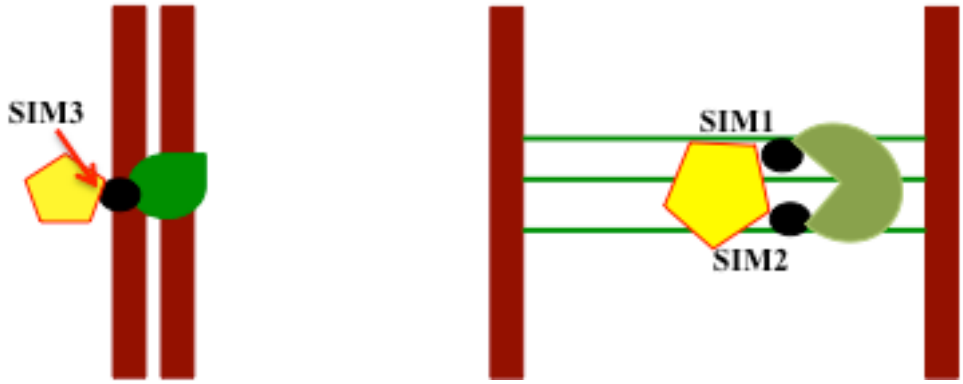


Figure 5.7. PICH d3SIM has defective SUMOylation profile *in vitro*. A) PICH WT and d3SIM (4SIM) were SUMOylated *in vitro* and analyzed by immunoblotting analysis. **B)** Non-SUMOylated and SUMOylated PICH WT and d3SIM (4SIM) were subjected to EMSA *in vitro* (similar to Fig 2.4 in the chapter 2).

5.8A


SIM3
SIM1
SIM2

5.8B

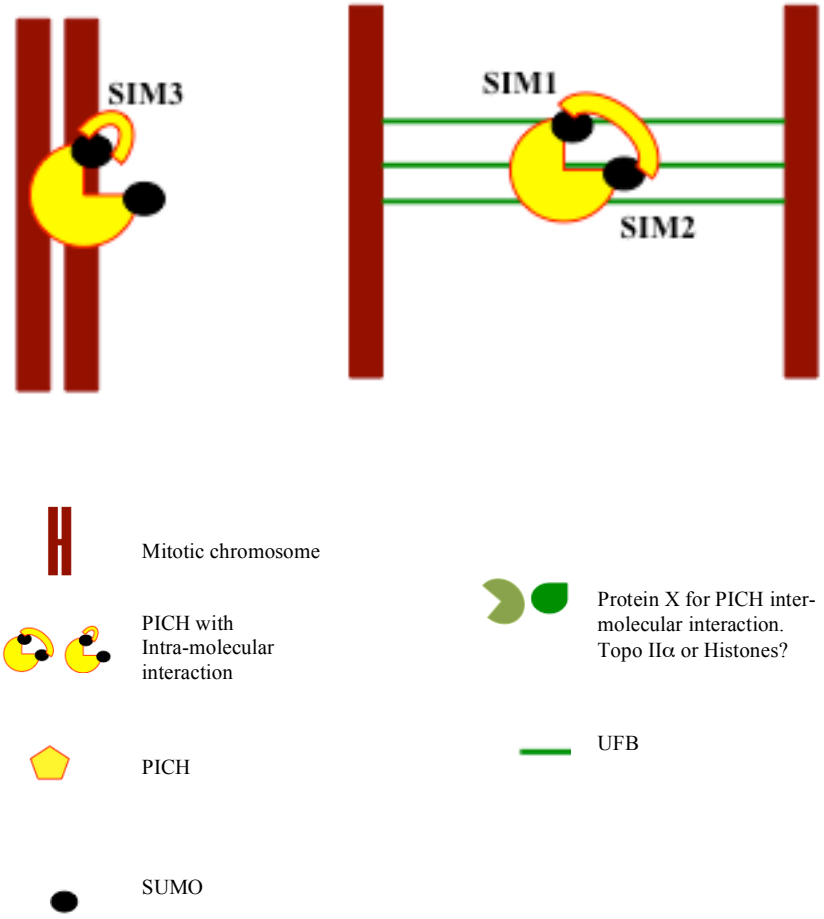

SIM3
SIM1
SIM2
Mitotic chromosome
PICH with
Intra-molecular
interaction
PICH
SUMO
Protein X for PICH inter-
molecular interaction.
Topo IIα or Histones?
UFB

Figure 5.8. Model for potential PICH SIM mediated regulation. A) Inter-molecular interaction with protein X regulates SIM3 mediated PICH localization and SIM1&2 mediated PICH's activity at the centromere or UFBs **B)** Intra-molecular interaction of PICH when SUMOylated with SIM3 for localization and SIM1&2 for its activity at the centromere or UFBs.

Lastly, PICH dSIM3 was able to rescue the chromatin bridge phenotype in endogenous PICH-inhibited cells despite having significant reduction in its centromere localization compared to PICH WT (Fig 3.3&3.4). Formation of chromatin bridges is one of the consequences of chromosomal abnormalities. Nielsen et al. showed extensively the possible chromosomal abnormalities in PICH-deficient cells including, chromatin bridges, micronucleation, binucleation and polyploidy. Though PICH dSIM3 does not result in chromatin bridge phenotype, it is plausible that absence of centromeric PICH results in other chromosomal abnormalities. Chromosomal analysis post anaphase on cells expressing PICH dSIM3 would give some insight on the importance of centromeric localization of PICH to ensure accurate chromosome segregation.

PICH: regulation of nucleosome remodeling?

The primary sequence of PICH has a predicted HELICc domain and walker A/walker B motifs (mediates ATP binding and hydrolysis), identifying PICH as a SNF2 class ATPase of the superfamily 2 helicases (16-18). Though PICH has helicase in its name, it does not have a helicase strand separation catalytic activity as other members of SNF2 family proteins (7). The SNF2 family of proteins typically, by their translocation activity, can perform chromatin remodeling. PICH has been previously shown to possess ATPase and DNA translocase activity *in vitro* (13,19). Kaulich et al. demonstrated the ATPase activity of PICH *in vitro* was defective

when walker A/walker B motifs were mutated (13). Biebricher et al. showed that PICH could translocate on double stranded DNA *in vitro* (19) and the translocase activity was lost when walker A/walker B motifs were mutated. Ke et al. showed that PICH possesses nucleosome-remodeling activity *in vitro* (7). The remodeling activity of PICH in the Ke et al. study required higher concentration of PICH recombinant protein compared to the positive controls used in the study to give minimal remodeling activity. Therefore, the *in vivo* functional capabilities of PICH are not yet well understood. Some of the key questions that need to be answered are: 1) Can PICH remodel nucleosomes at the centromere? 2) Is this nucleosome activity of PICH essential at the centromere or UFBs or both? 3) Can SUMOylation of PICH or other proteins regulate PICH's remodeling activity? 4) Does PICH act as a chromatin remodeler by recognizing a particular centromeric histone? 5) Does PICH alter centromere structure and organization?

Based on our results of SIM-mediated localization of PICH as well as identification of PICH as a novel SUMO substrate, SUMOylation is most likely an important posttranslational modification in the regulation of PICH's activity *in vivo*. Biebricher et al. did an extensive *in vitro* study on PICH binding to DNA under tension. The research group also tested if PICH had *in vitro* nucleosome remodeling activity in nucleosomes assembled with canonical histones as well as octamers with centromeric histone H3 variant, CENPA but PICH did not show any significant ability to remodel nucleosomes *in vitro* (19). Their data suggested that there might be extra cofactors playing a role in the remodeling activity of PICH *in vivo*. The centromere has unique histone markers that might be required for PICH to recognize and function. Additionally, our data described in the chapter of this dissertation on SUMO-dependent localization of H2A.Z and HP1γ leads to a hypothesis that SUMO can regulate the centromere organization during mitosis. PICH is a promising candidate for a chromatin remodeler regulating centromere

organization due to the following reasons: 1) PICH associates with chromosomes only in mitosis, making it a unique putative mitotic remodeler. 2) SUMOylation regulates PICH's localization and functional activity (both *in vitro* and *in vivo*). 3) Depletion of PICH results in chromosome segregation defects and chromosome instability, suggesting that it is important for genome maintenance. Based on SUMO-regulation of PICH and SUMO-mediated localization of H2A.Z, it is promising to hypothesize that PICH regulates H2A.Z nucleosomes at the centromere during mitosis. Depletion of PICH and looking into H2A.Z at the centromere will give insight into this hypothesis. Alternatively, it is plausible that SUMOylated histones (including CENPA) are required for the remodeling activity of PICH. It would be interesting to reconstitute octamers with SUMOylated histones and perform *in vitro* PICH remodeling activity as previously described (7,19).

On a broader thought, if PICH is important for centromeric nucleosome remodeling and its activity is dependent on SUMOylation then it would be interesting to compare the overall centromere epigenetics with PICH WT and PICH d3SIM. Research from our group has also shown that Topo IIα SUMOylation is required for Haspin localization, which is an important mitotic kinase that regulates Histone H3 threonine3 phosphorylation (H3T3P) (Yoshida et al. manuscript in press, Journal of Cell Biology). It is plausible that the remodeling activity of PICH assists in these chromatin changes at the centromere. Chromatin immunoprecipitation with CENPA antibody from samples expressing either PICH WT or PICH d3SIM and analysis of the centromeric histones/general centromere composition might be insightful. Further this ChIP assay can also be used to dissect the precise roles of SIM3 versus SIM1&2 on PICH with respect to the regulation of centromeric epigenetics. One of the strengths of working with *Xenopus* egg extracts is the ability to do biochemical analysis with it. We currently have a PICH antibody that

can deplete PICH from XEE and analysis of the centromere region by ChIP using CENPA antibody might be informative towards the molecular mechanism of the role of PICH.

In summary, this dissertation delineates a novel regulation of PICH via SUMOylation. While we learned that PICH can be SUMOylated and SUMOylated PICH significantly reduces DNA binding *in vitro*, we currently do not understand the significance of SUMOylated PICH *in vivo*. This study is the first report that shows a mechanism for positive recruitment/localization of PICH at the centromere via its SUMO-interacting motif. Additionally, the identified SIMs most likely have functional roles other than localization at the centromere. We currently do not know the SUMOylated proteins that play a role in PICH's localization and activity at the centromere to prevent chromatin bridges. We currently don't clearly understand if PICH has a nucleosome remodeling activity at the centromere. Further studies to identify the SUMOylated proteins involved, understanding the significance of PICH SUMOylation *in vivo* and delineating alternation in centromeric organization leading to segregation defects will be essential for comprehensive understanding the molecular function of PICH at the centromere during mitosis. This would further bridge the gap in understanding the role of SUMO in centromere epigenetics.

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