SUMOylation in cell death: SUMO E3 ligase PIAS1 plays a critical role in determining cell survival following UV stress

By

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ABSTRACT

SUMO modification of proteins plays an important role in many cellular processes such as DNA replication, DNA repair, stress response, transcriptional regulation, signal transduction, cell cycle regulation, differentiation and cell death. SUMO modification can have varied effects on its substrates; it may alter subcellular localization, interaction partners or activity of the target proteins. SUMO modifies its substrates by forming an isopeptide bond between its own C-terminal glycine and the ε-amino group of a preferred lysine on the target protein. The modification requires three enzymes, a SUMO activating enzyme E1, a SUMO conjugating enzyme E2, and a SUMO E3 ligase, acting in a cascade. Since all eukaryotes have only one of each of the first two enzymes involved in SUMOylation, the variability in substrate selection and the choice of SUMO paralog is often attributed to a relatively small group of the third enzymes in the SUMOylation pathway, collectively called the SUMO E3 ligases. SUMO modification is also reversible and SUMO can be cleaved off of its substrates by deSUMOylating enzymes. We hypothesized that the variability in SUMOylation substrate selection is guided by the choice of SUMO E3 ligase and each of the five different PIAS isoforms regulates distinct cellular pathways, including response to DNA damage, by SUMOylation of a unique subset of substrates. To verify our hypothesis, we ectopically expressed PIAS E3 ligases in cells and provided various stress stimuli, such as UV irradiation. We then analyzed the response dictated by the expression of each of the PIAS isoforms. We observed that both the ectopic expression of PIAS1 as well as the RNAi-mediated depletion of PIAS1 in cells leads to increased sensitivity of cells to UV irradiation and cell death. This hypersensitivity to UV irradiation is unique to PIAS1 overexpression and is not observed with overexpression of other PIAS isoforms. Each PIAS

isoform shows distinct localization in cells and a unique SUMOylation profile. Our results indicate that PIAS1 induced cell death depends on the unique set of proteins it SUMOylates. Domain analysis of PIAS1 implicated the N-terminal SAP domain of PIAS1 in guiding its unique subcellular localization and the determination of substrate specificity. We identified proapoptotic protein Daxx as a mediator of PIAS1 induced apoptosis. Daxx colocalizes with PIAS1 modified SUMO foci in cells and RNAi-mediated depletion of Daxx alleviates UV-induced apoptosis in PIAS1-expresing cells. Daxx is a known transcription co-repressor and mediates its transcription repression activity by interacting with SUMOylated transcription factors, including those required for production of anti-apoptotic proteins. Daxx has two motifs, one at its N-terminus and another at the very C-terminus, called SUMO interacting motifs (SIM), that are known to be important for interaction with SUMOylated proteins. Daxx is proposed to interact with only a subset of SUMOylated proteins; however the basis for this specificity is not clear. Our data suggests that the Daxx C-terminus SIM is essential for interaction with PIAS1-mediated SUMO foci and the resulting cell death. Since cell death is regulated by a fine balance between the levels of pro- and anti-apoptotic proteins in cells, we analyzed the expression of the apoptosis mediators in cells expressing PIAS1. Results from multigene apoptotic reverse-transcription PCR arrays have revealed that the expression of a number of pro- and anti-apoptotic genes is altered in PIAS1-expressing cells. Most of these proteins appear to be targets of the NFκB family of transcription factors. We also observed a direct interaction of the NFκB transcription factor protein RelB with both PIAS1 and Daxx.

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Finally, I dedicate this dissertation to my parents. I owe my success today to them. They have always believed in me, inspired me to achieve the very best and above all, have taught me to always try to be good person. I love you Papa and Mommy.

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CHAPTER 1

An Introduction to SUMO modification

(**S**MALL **U**BIQUITIN-LIKE **MO**DIFIER)

Post-translational modifications allow rapid and transient changes in protein localization, stability, interactions or activity in response to various stimuli. Most modifications involve attachment of small chemical groups to specific residues in the protein. Some of the well characterized modifications include phosphorylation, methylation, acetylation, glycosylation, ADP-ribosylation and prenylation. A unique class of modification discovered in the past quarter century involves covalent attachment of small proteins of approximately 100 amino acids to the ε-amino group of the lysine on cellular proteins. These modifiers belong to the Ubiquitin family of proteins and include Ubiquitin, Small Ubiquitin-like Modifier (SUMO), Nedd8 and Interferon Stimulated Gene-15 (ISG-15). Ubiquitin, the founding member of this family, was first identified in 1975 as a ubiquitously expressed protein in eukaryotes (*1*). Initial work showed that modification by Ubiquitin targeted proteins to proteasomal degradation. Subsequent research showed other regulatory functions of Ubiquitin. Elucidation of the function of Ubiquitin and the modification pathway earned the researchers the Nobel Prize in Chemistry in 2004 (*2*). The focus of this chapter, however, is the SUMO protein; its discovery, the modification pathway and the functional implications of protein modification with SUMO is extensively reviewed in the following pages.

Discovery of SUMO

SUMO was identified by at least six different groups within a few months of each other in 1995-97. Meluh and Koshland (*3*) first cloned the *Saccharomyces cerevisae* SUMO gene in a screen for suppressors of temperature sensitive mutations of the MIF2 gene, a homolog of the human CENP-C centromeric protein. They named this gene SMT3 (suppressor of MIF-two protein 3). Mannen *et. al.* (*4*) discovered its homolog HSMT3 as a ubiquitously expressed cDNA in human tissues. HSMT3 and yeast SMT3 were found to share 51.1% sequence identity. Three groups independently identified SUMO-1 in yeast two-hybrid screens. Shen *et. al.*(*5*) identified a homolog of yeast SMT3 in humans as an interactor of the double-stranded break repair proteins. Since they found this protein to be a distant homolog of Ubiquitin, they named the protein Ubl-1 (Ubiquitin-like 1). Boddy *et. al.* (*6*) identified the same protein using PML as a bait and named it PIC-1 (PML interacting protein-1). They further showed that PML and PIC-1 colocalize in PODs (PML Oncogenic Domains). Okura *et. al.* (*7*) identified SUMO-1 as an interactor of the death domain of the Fas receptor and named it Sentrin based on its apparent protective function against cell-death signaling. Finally, two groups identified SUMO-1 as a protein that covalently modifies GTPase-activating protein RanGAP1. While Matunis and Blobel (*8*) called this protein GMP1 (for GAP-modifying protein 1), Mahajan *et al.* (*9*) came up with its present universally-accepted moniker SUMO-1 (Small Ubiquitin-like Modifier 1).

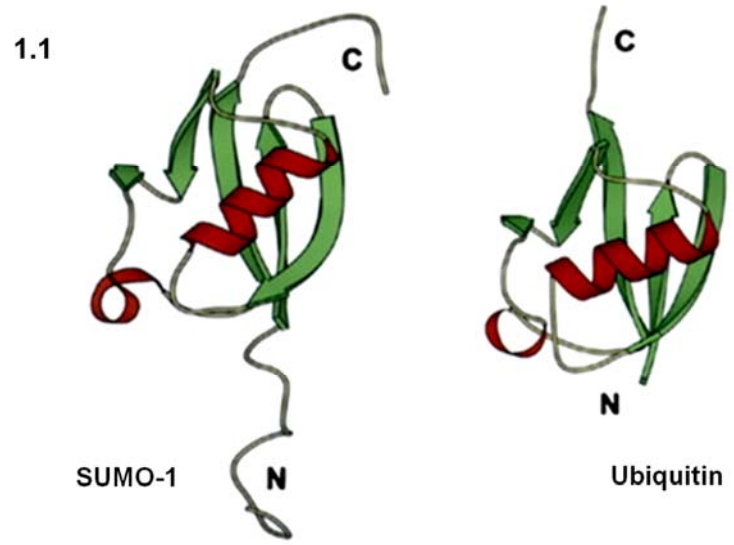

Figure 1.1. Comparison between SUMO-1 and Ubiquitin structures. Despite a sequence identity of only 18%, SUMO and Ubiquitin share the same structural fold. Figure modified and adapted from Gill G (*10*).

Ubiquitin and SUMO share only 18% sequence identity, but have the same ββαββαβ fold that has been termed the Ubiquitin fold and is shared by all Ubiquitin-like proteins. Despite a similarity in the tertiary structure, the charge distribution on Ubiquitin and SUMO is very different. SUMO also has an N-terminal extension that is not present in Ubiquitin. Also, although Ubiquitin and SUMO modification pathways are similar, they do not share the same enzymes. SUMO and Ubiquitin can modify the same sites on proteins, but the functional outcome in each case is different and sometimes antagonistic.

Homologs of SUMO have been identified in most eukaryotic life forms. Invertebrates have a single SUMO protein, whereas mammals have been shown to have four SUMO isoforms. SUMO-1, SUMO-2 and SUMO-3 share nearly 50% sequence identity with the yeast Smt3 protein. SUMO-1 is approximately 46% identical to SUMO-2 and SUMO-3. SUMO-2 and SUMO-3 are 96% identical, with a difference of only 3 amino acids in the N-terminus of the

proteins and are often thought to have the same functions in cells. Table 1 compares the sequence identity between the three SUMO isoforms and the yeast homolog Smt3p. Since there are no antibodies to differentiate between SUMO-2 and -3, they are often grouped together and referred to as SUMO-2/3. SUMO-1, -2 and -3 are ubiquitously expressed whereas SUMO-4 (*11*) shows limited expression in a few tissue types such as kidney, spleen and lymph nodes, and it is uncertain if it covalently attaches to any protein. Most of the research in the SUMO field is limited to the study of SUMO-1 and SUMO-2/3 isoforms. There are significant differences between the activity of SUMO-1 and SUMO-2/3 in cells. SUMOylated substrates often show SUMO isoform specificity *in vivo*. For example, RanGAP-1 is modified mainly by SUMO-1 (*12*), whereas Topoisomerase IIα is a target of SUMO-2/3 modification (*13*). SUMO-1 also shows different localization from SUMO-2/3. SUMO-2/3 form distinct foci in the nucleoplasm of the interphase nuclei, whereas SUMO-1 can be seen in the nucleoli, the nuclear membrane, and also the cytoplasm of interphase cells (*14*). Also, concentrations of total and unconjugated SUMO-2/3 is higher than that of SUMO-1 in cells (*12*). Additionally, cells respond to many stress stimuli, such as heat shock, oxidative stress and osmotic stress, by upregulating SUMO-2/3 modification of proteins (*12*). Another major difference between SUMO-1 and SUMO-2/3 is that SUMO-2/3 can form chains on its substrate via Lys-11 that is part of a SUMO-modification consensus motif (explained later in this chapter) (*15*).

Table 1.1. Comparison of sequence identity between the three SUMO isoforms from humans and the yeast homolog Smt3p.

	Smt3p	SUMO-1	SUMO-2	SUMO-3
SUMO-1	48%	-----	-----	-----
SUMO-2	44%	45%	-----	-----
SUMO-3	49%	50%	96%	-----
SUMO-4	40%	44%	85%	82%

The SUMO pathway enzymes

The SUMO conjugation pathway is very similar to the ubiquitination pathway and requires the sequential activity of three enzymes; a SUMO activating enzyme, which is a heterodimer of Aos1 and Uba2, a SUMO conjugating enzyme called Ubc9, and a SUMO ligase. In addition, SUMO modification requires SUMO proteases, called SENPs, which are required for both de novo generation of conjugation competent SUMO proteins from precursors and for regeneration of free SUMO by cleaving the isopeptide bond between SUMO and the substrate protein. Below is a summary of the discovery of these enzymes followed by the description of the SUMOylation pathway.

The SUMO activating enzyme

Similar to the Ubiquitin conjugation pathway, SUMO conjugation to other proteins involves three enzymes acting in a cascade. The SUMO activating enzyme E1 is a heterodimer of Aos1 (SAE2) and Uba2 (SAE1) (*16*). Uba2 was first identified as a protein similar to the C-terminal domain of the yeast Ubiquitin activating enzyme Uba1p. UBA2 was found to be an essential

gene and Uba2p localized mainly to the nucleus and could not be complemented by Uba1p. Johnson et. al. identified Uba2p as the yeast Smt3p activating enzyme and also identified the protein Aos1p as the essential Uba2p binding partner required for its activity. Aos1p is similar to the N-terminus of the Ubiquitin E1 Uba1p enzyme. Although Aos1 and Uba2 function together in the SUMOylation reaction, the expression level for the two subunits is different. Uba2 protein levels do not change substantially through the cell cycle; Aos1 protein levels, on the other hand, increase substantially during the S-phase of the cell cycle (*17*).

The SUMO conjugating enzyme

Ubc9 is the only known SUMO conjugating enzyme. Ubc9 was discovered in yeast *Saccharomyces cerevisae* before the discovery of SUMO itself and was initially thought to be one of the Ubiquitin conjugating enzymes (UBCs). Ubc9 was found to be essential for yeast viability and a temperature sensitive Ubc9-1 mutant was found to arrest at G2/M phase at 37˚C (*18*). The authors further showed the stabilization of the B-type cyclins CLB5 and CLB2 in the Ubc9-1 strain and concluded that Ubc9 was required for Ubiquitin-mediated proteasomal turnover of these cyclins (*18*). At about the same time as Ubc9 was discovered in *Saccharomyces cerevisae*, its homolog in the fission yeast was discovered in a screen for checkpoint control mutants. The homolog was named Hus5 (hydroxyurea sensitive) based on the sensitivity to hydroxyurea (a DNA synthesis inhibitor) in strains harboring mutations in this gene. Hus5 deletion mutants were almost inviable and the temperature sensitive alleles showed G2 arrest on HU or ionizing radiation treatment when coupled with other checkpoint mutations. Hus5 was also proposed to be a Ubiquitin conjugating enzyme based on sequence similarity, although no targets were identified.

By 1997, SUMO was well characterized as a distinct member of the Ubiquitin family. Johnson and Blobel (*19*) were the first to identify Ubc9p as the second enzyme involved in the SUMO-modification pathway. They showed that the yeast Ubc9p formed thioester bond with Smt3p, but not with Ubiquitin and that Ubc9p was the only Smt3p conjugating enzyme in yeast. Saitoh *et al.* (*20*) then showed that Ubc9 is required for SUMO-1 modification of RanGAP1 and RanBP2 in *Xenopus* egg extracts and that Ubc9 forms a thioester bond only with SUMO-1, and not Ubiquitin, confirming the earlier observation by Johnson and Blobel.

A number of Ubc9-interacting proteins were identified in yeast-two-hybrid screens including c-Jun and the glucocorticoid receptor, Rad51, a number of yeast centromeric proteins, and Rad52, Ubl1 and p53 (*21, 22*). Subsequently, Saitoh *et al.* (*23*) showed that RanBP2 associates with Ubc9 and a modified form of RanGAP1 (later identified as the SUMO-modified RanGAP1). Ubc9 was also shown to interact with Parp1 and Fas receptor. Tashiro *et al.* (*24*) showed that Ubc9 was essential for IκBα degradation, once again implicating it in the Ubiquitin-mediated protein degradation pathway. Hahn *et al.* were the first to report a role for Ubc9 in a regulatory pathway not leading to protein degradation (*25*). They showed that co-expression of Ubc9 with the transcriptional factor ETS-1 substantially increased its transcriptional activity but did not affect protein stability. They speculated that the modulation of ETS-1 activity by Ubc9 does not involve ubiquitination and ETS-1 stabilization.

To date Ubc9 remains the only known SUMO conjugating enzyme. In human cells, a single Ubc9 gene has been mapped to chromosome 16p13.3. Mouse cells have one Ubc9 gene and three pseudogenes, of which two have an open reading frame but show differences in key residues and fail to rescue Ubc9 temperature sensitive phenotype in yeast. Ubc9 protein shows strong sequence conservation across species. Human and mouse Ubc9 are 100% identical, and

human Ubc9 shows 65% identity to yeast Ubc9p. Structurally, Ubc9 shows the same overall fold as the ubiquitin conjugating enzymes and a similar active site cleft, but there are significant differences in the surface charge distribution, with Ubc9 having a more positively charged non-catalytic surface (*26, 27*). In fact, the positively charged surface provides the perfect binding site for the negatively charged surface of SUMO-1. As has been described earlier, the equivalent surface on Ubiquitin has an overall positive charge (*28*). This forms the basis for specific interaction of Ubc9 with SUMO-1, and not the structurally similar Ubiquitin. The binding interfaces between Ubc9 and SUMO-1 also display complementary hydrophobicity.

The SUMO E3 ligases

SUMOylation can be carried out *in vitro* using just the E1 and the E2 enzymes. It is known however, that ubiquitination requires the participation of a third class of enzymes called the Ubiquitin E3 ligases. The Ubiquitin E3 ligases are known to determine the substrate specificity in the ubiquitination pathway. The Ubiquitin ligases belong to many different families and can be either monomeric or multi-protein complexes. Many of the Ubiquitin ligases share a common Zn+ binding domain called the RING domain (RING: Really Interesting New Gene). Since SUMOylation draws many parallels from the ubiquitination pathway, and because it was shown that SUMOylation in the presence of only E1 and E2 enzymes was extremely inefficient in the absence of HeLa cell extracts or *Xenopus* egg extracts (*17*), the existence of an E3 SUMO ligase was speculated.

In 2001, two groups independently discovered SUMO E3 ligases in yeast. *Saccharomyeces cerevisae* protein Siz1 was first characterized as a SUMO E3 ligase that is required for SUMO-modification of septins and was shown to bind to both Ubc9 and yeast SUMO Smt3 (*29*). Septins are GTP binding cytoskeletal proteins required for cytokinesis in

yeast and are known to be SUMO modified (*30, 31*). Takahashi and colleagues further showed that the RING domain of Siz1 is required for Smt3p conjugation to the septin protein Cdc3 and for interaction with Ubc9 (*32*). They established Siz1 as a bonafide SUMO E3 ligase that acts as an adaptor protein between the substrate and the E2. Johnson and Gupta (*33*) identified Siz2, and the related protein Siz1, as E3 ligases in a screen for interactors of septins. They first identified Siz2 as one of the interactors of septins that contains a RING finger like domain and Siz1 was identified based on its homology to Siz2. Deletion of Siz1 and Siz2 drastically reduced SUMOylation in yeast and eliminated septin SUMOylation. However, unlike other SUMO pathway deletion mutants, deletion of Siz1 and Siz2 did not affect the viability of yeast, indicating that there were other SUMO ligases that carried out residual and essential SUMOylation in these deletion mutants (*33*).

At the same time as the Siz proteins were identified as SUMO E3 ligases in yeast, the PIAS (Protein Inhibitor of Activated Stat) family of proteins was shown to act as SUMO E3 ligases in mammalian cell lines (*34, 35*). The PIAS family of proteins was discovered in the late 1990's and consists of five members, PIAS1, PIASXα, PIASXβ, PIAS3 and PIASy. PIAS3 and PAIS1 were identified as inhibitors of Stat3 and Stat1 transcriptional activity, respectively (*36, 37*), by inhibiting DNA binding by Stat proteins. PIAS1 was isolated as a SUMO-1 binding protein in a Yeast-two-Hybrid screen and was shown to catalyze SUMOylation of p53. PIAS1 interacted with both Ubc9 and p53, whereas a RING domain mutant of PIAS1 could interact with SUMO-1 and p53, but not with Ubc9, and could not catalyze p53 SUMOylation (*34*). Sachdev and colleagues (*35*) identified PIASy as a SUMO E3 ligase in mammalian cells and a repressor of LEF1 transcription activity.

PIAS proteins share approximately 50% sequence identity with each other and less than 30% sequence identity with Siz1 and Siz2. Siz and PIAS proteins however have similar domain architecture. All of these proteins have an N-terminal SAP domain (Scaffold attachment factor, Acinus and PIAS) that is involved in interaction with DNA and other proteins such as transcription factors; a PINIT domain that is believed to be required for subcellular localization; an SP-RING domain (Siz/PIAS-RING) required for E3 ligase activity; a SIM (SUMO Interacting Motif) for non-covalent interaction with SUMO, and finally, a variable serine/threonine rich region at the C-terminus (see Figure 1.2) (*38*). PIAS domain structure is discussed in more detail in chapter 3 of this dissertation. In addition to acting as adaptors in the SUMOylation pathway, the E3 ligases are thought to promote both SUMO paralog selection and substrate selection based on their localization in the cell (*39, 40*).

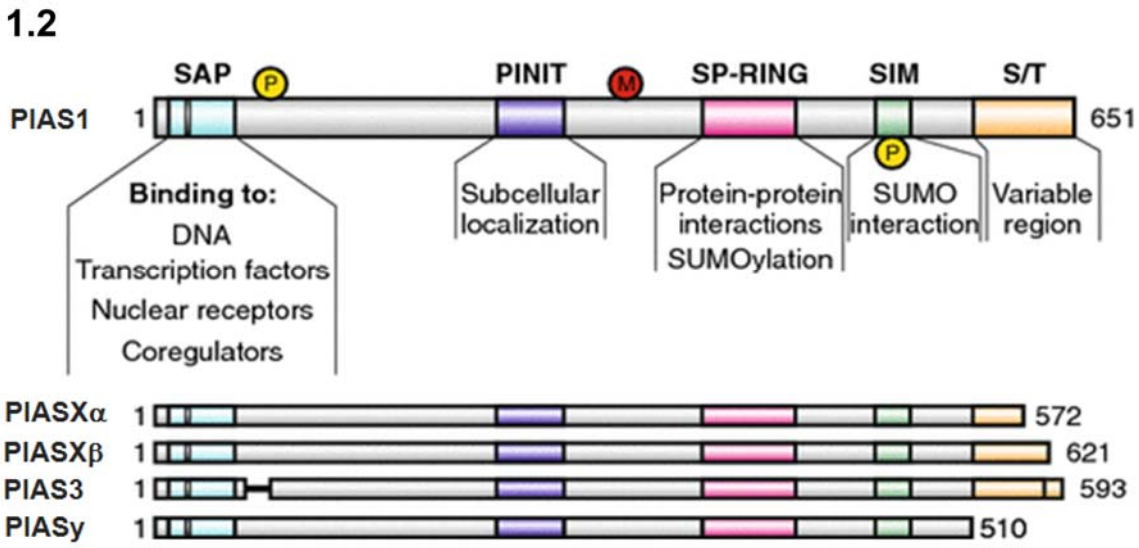

Figure 1.2: Domain architecture of PIAS family of SUMO E3 ligases. The figure has been adapted and modified from Rytinki *et al.* (*38*).

The PIAS proteins have also been shown to regulate other proteins independent of the SUMO ligase activity. For example, PIAS1 interacts with Msx-1, regulates its DNA binding activity and controls its inhibitory function in myoblast differentiation (*41*). PIASXα-dependent repression of FLI-1 derives from nuclear relocalization of this transcription factor and is independent of its SUMOylation (*42*). Another example involves transcription factor Oct4, whose transcriptional activity is repressed by its interaction with PIASy (*43*). All these interactions involve the SAP domain of the PIAS proteins. PIASXα interacts with SUMOylated transcription factor Elk-1 via its SIM domain and activates Elk-1 by displacing histone deacetylase 2 (HDAC2) from SUMOylated Elk-1 (*44*). Thus, all these transcription regulatory functions of PIASes are dependent on the PIAS SAP or SIM domain and do not require the activity of the SP-RING domain (*38*).

The SUMO proteases

Like many post-translational modifications that regulate protein function, modification by SUMO is transient and reversible. SUMO proteases cleave SUMO off of the substrates (isopeptidease activity and chain editing) and are also required for maturation of SUMO by cleaving the C-terminal residues to reveal the diglycine motif that is used for isopeptide bond formation with the substrate (endopeptidase activity/ processing). Yeast has two deSUMOylating enzymes, Ulp1 and Ulp2 (Ubiquitin-like). Ulp2 was discovered in the screen for suppressors of Mif2 along with Smt3 (*3*). Ulp1 was identified by Li and Hochstrasser (*45*) in an extensive screen for Smt3 proteases from extracts made from bacteria transformed with groups of yeast proteins. Ulp1 was found to be essential for G2/S transition and mutants displayed a phenotype identical to Ubc9 deletion mutants (*18*). The following year, SUMO specific proteases, called SENPs (sentrin proteases), were described in mammals (*46, 47*).

There are 6 SUMO-specific proteases in humans, SENP1, 2, 3, 5, 6 and 7. SENPs 1, 2, 3 and 5 are closely related to yeast Ulp1, whereas SENPs 6 and 7 are similar to yeast Ulp2. Ulp1 has both isopeptidase and endopeptidase activity, whereas Ulp2 is required for SUMO chain editing. Mammalian SENPs display SUMO paralog specificity and also differences in endopeptidase, isopeptidase, and chain editing activities (*48*). These differences have been summarized in Table 2.

Table 1.2: SUMO proteases found in humans. Adapted from Mukhopadhyay and Dasso (*48*) and Kim and Baek (*49*).

SUMO protease	SUMO Paralog preference	Processing	Deconjugation	Chain editing	Reference
SENP1	SUMO-1 and SUMO-2/3	Yes	Yes	No	(*45*, *50-52*)
SENP2	SUMO-2/3 and SUMO-1	Yes	Yes	No	(*49*, *53*, *54*)
SENP3	SUMO-2/3	Unknown	Yes	No	(*48*, *49*, *55*)
SENP5	SUMO-2/3	Yes	Yes	No	(*48*, *49*, *56*)
SENP6	SUMO-1 and SUMO-2/3	No (very low)	Yes (for SUMO-1)	Yes	(*46*, *48*, *49*)
SENP7	SUMO-2/3	No (very low)	No (very low)	Yes	(*46*, *48*, *49*)

The process of SUMOylation

SUMO-1 has a compact globular structure and the ββαββαβ fold that is characteristic of all Ubiquitin family of proteins, and is also found in a number of unrelated proteins across species

(*28*). In addition, SUMO has an N-terminal extension consisting of about 20 amino acid residues that is not present in Ubiquitin and whose function is not well characterized. SUMO proteins also have distinct surface charge topologies and present strongly polarized surfaces, with the negative and positive charges distributed to opposing faces of SUMO. This difference in surface charge distribution, despite the conserved protein fold, leads to different interaction specificities with the substrates and the modification pathway enzymes (*28, 57*). SUMO conjugation on the substrate often occurs on the ε-amino group of a lysine that is a part of the Ψ-K-x-D/E (where Ψ is a hydrophobic amino acid, K = lysine that is modified, x is any amino acid, followed by a negatively charged aspartate or glutamate residue) motif (*58, 59*). However, not all lysines in the consensus site are known to be modified *in vivo*, and there are a number of examples showing SUMOylation on lysine residues that are not a part of the consensus motif (for example; PCNA K164 and Topoisomerase IIα K660) (*60, 61*).

All SUMO isoforms, like Ubiquitin, are expressed as precursor proteins that need to be appropriately processed for conjugation to substrates. SUMO proteases cleave residues from the C-terminus of SUMO to expose the diglycine residues in a process called maturation. The first step in the process of SUMO conjugation is adenylation of SUMO followed by a thioester bond formation between the carboxyl group of the C-terminal glycine residue of SUMO and the cysteine-173 of the E1 enzyme (called the SUMO activating enzyme). The mature SUMO is recognized by the Uba2 subunit of the Aos1/Uba2 dimer. Uba2 has three domains; the catalytic cystein domain that is required for the thioester bond formation with SUMO, the adenylation domain that contains the ATP binding site, and the Ubiquitin-fold domain (UFD) that is required for interaction with the SUMO E2 Ubc9 (*62, 63*). The Aos1 subunit participates in the adenylation of SUMO along with the Uba2 subunit but does not directly interact with SUMO

(*62*). In the second step of the SUMO conjugation process, SUMO is transferred from E1 to the E2 enzyme, forming a thioester bond with the E2 (the SUMO conjugating enzyme). The E2 enzyme, Ubc9, is a central component of the SUMOylation machinery and interacts with all the proteins involved in SUMOylation. Cysteine-93 of Ubc9 is involved in the thioester bond formation with SUMO. In addition to covalent attachment to SUMO, Ubc9 can also interact non-covalently with SUMO and this interaction promotes SUMO chain formation in the case of SUMO-2/3 (*64, 65*). The final transfer of SUMO to the ε-amino group of the lysine on the substrate protein is mediated by the E3 enzyme (the SUMO ligase). The ligases bind both Ubc9 and the substrates and act as adaptor proteins, facilitating the interaction between the Ubc9-SUMO conjugate and the substrate. The SP-RING domain of the ligase is required for interaction with Ubc9 (*34*) and the N-terminal SAP and PINIT domains are required for interaction with subtrates (*63*). See Figure 1.3.

1.3

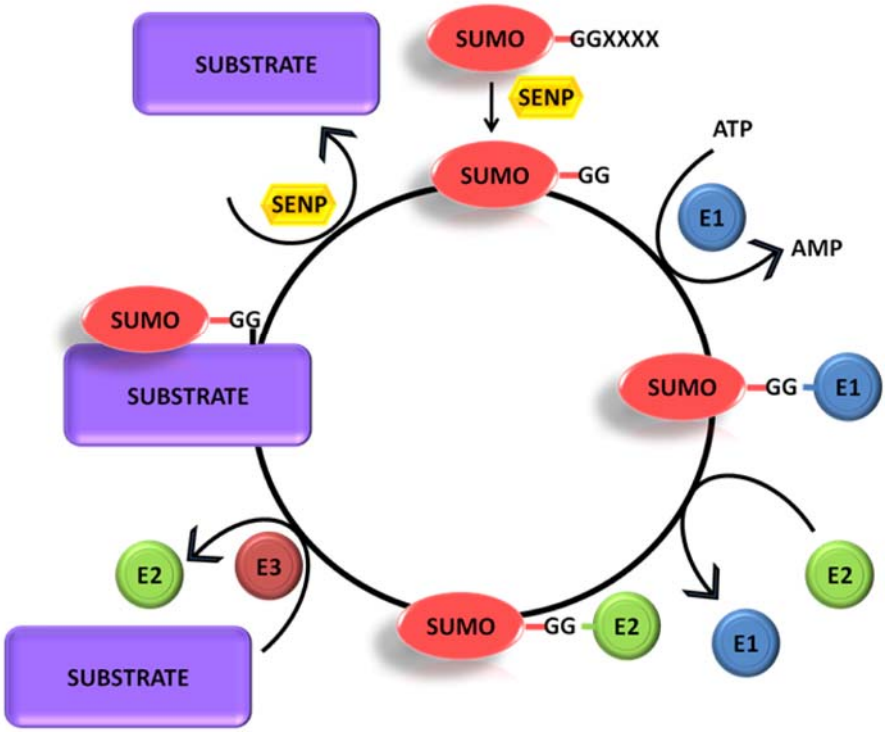

Figure 1.3: The SUMO modification pathway. SUMOylation of proteins requires three pathway enzymes, E1, E2 and E3. The substrate is modified at a lysine residue by formation of an isopeptide bond between ε amino group of lysine and the carboxyl group of the terminal glycine of SUMO. For details, see text.

Non-covalent interaction with SUMO

SUMO modification is known to enable interaction of its substrates with other proteins. While some interactions result from conformational changes in the substrate protein after SUMOylation that facilitate binding, other binding interactions often involve a direct association of the interacting protein with SUMO itself. This non-covalent interaction was shown to be mediated by a short hydrophobic motif found in many proteins called SUMO interacting motif or SIM (*66*). The SIM is made up of Val/Ile-Val/Ile-X-Val/Ile, flanked on either side by negatively charged residues or serine residues that can be phosphorylated to generate negative charges (*67*). The hydrophobic amino acids in the motif form a β-strand that can bind to the β2-strand and α-helix of SUMO in a parallel or antiparallel orientation (*68, 69*). Interaction between the β-strand of SIM and the β2-strand of SUMO results in formation of an intermolecular β-sheet. Phosphorylation of serine residues near the hydrophobic core seems to favor interaction with SUMO-1, whereas SUMO-2 binding is unaffected by the presence or absence of negative charges (*68*). The affinity of SIM to both SUMO-1 and SUMO-2/3 is in the micromolar range (*68*) and high affinity binding between a SIM-containing protein with a SUMOylated substrate can result only from simultaneous interaction with both SUMO and a low affinity site on the substrate. This ensures specificity of binding between SIM-containing and SUMOylated proteins (*70*).

SUMO is known to modify many transcription factors and regulate their activity via interactions with repressor complexes that requires SIM domains on the repressor proteins. A well studied example of this is the apoptotic pathway protein Daxx. Daxx has two SIM domains,

one at either terminus (*71, 72*). Daxx is known to interact with a number of SUMOylated transcription factors and repress their transcription activity. Well known examples include c-Jun, ETS-1, NFκB and p53 (*73*). Daxx is described in more detail in Chapter 2.

Physiological consequences of SUMO modification

SUMO modification can influence the enzymatic activity of its substrate, as shown in the case of Topoisomerase IIα where SUMO modification at lysine 660 decreases its catenation activity (*61*) and in the case of Ubiquitin ligase BRCA-1 where SUMOylation on lysine 119 increases its ubiquitination activity (*74*). SUMO modification can also change the stability of a protein, either by preventing ubiquitination and subsequent degradation (as seen for IκBα, where SUMO competes with Ubiquitin for modification on lysine 21 (*75*)) or providing a new interface for binding of SUMO-targeted Ubiquitin ligases (StUbls) that can then lead to ubiquitination and degradation of the SUMOylated proteins (*76*). However, in most cases SUMO modification has been shown to regulate molecular interactions of its substrates either by creating new binding interfaces or by masking existing ones (*70*). Physiological consequences of this are quite varied. SUMOylation is involved in chromosome organization and function, DNA repair and replication, regulation of transcription, regulation of nuclear and subnuclear localization, and modulation of signal transduction and apoptosis (*10, 77*). Table 2 lists some of the proteins known to be SUMOylated and the consequence of their SUMOylation. This list is not comprehensive but is aimed at providing a good sampling of various cellular functions regulated by SUMO modification of various proteins.

Table 1.3: SUMO modification regulates multiple cellular functions. The table presents examples of SUMOylated proteins in various cellular pathways and a brief summary of the effect of SUMOylation.

Cellular Function	Substrate (s)	Consequence of SUMOylation	References
Transcription	Ets-1, c-Myb, AR, C-EBP, c-Jun, p53	Repression of transcritptional activation by recruitment of co-repressors such as Daxx, histone deacetylases, etc.	(*78-82*)
	p53	Activation of transcriptional activity by preventing Mdm2 mediated degradation.	(*83*)
	Ikaros	Activation of transcription by disruption of interaction with repressor complexes NuRD and Sin3.	(*84*)
DNA Replication	PCNA	Prevents recombination at stalled forks during replication	(*85*)
DNA damage response and repair	BRCA-1	Activation of BRCA-1 ubiquitination activity and DNA double strand break foci.	(*74, 86*)
	PARP-1	Alters PARP-1 stability and affects other modifications on PARP-1 and PARP-1 activity	(*87-89*)
	TIP-60	Required for its UV-mediated localization to PML bodies; increases its acetyltransferase activity and p53-mediated DNA damage response.	(*90*)
DNA damage response and repair	BLM helicase	Regulates BLM's localization and prevents its anti-recombinogenic function during homologous recombination repair.	(*91*)
	Thymine-DNA glycosylase (TDG)	TDG excises a mismatched base during repair but remains tightly bound to DNA after excision. SUMOylation reduces TDG's affinity to the abasic site on DNA, allowing its release from the DNA and continuation of repair.	(*92, 93*)

Cell division and checkpoint control	Topoisomerase IIα	Required for chromosomal segregation, possibly by regulating its DNA catenation activity at the centromere during anaphase	(*61*)
	Aurora-B	Regulates its autophosphorylation and activation	(*94*)
Signal Transduction	IκBα	Stabilization of IκBα and inhibition of NFκB transcription activity	(*75*)
	MAPK kinase MEK1	In Dictostylium discoidium, MEK1 is SUMOylated following cAMP stimulation and translocates to plasma membrane. This in turn translocates ERK1 to plasma membrane and activates it. Required for chemotaxis and aggregation.	(*95*)
	Mdm2	Stabilizes Mdm2 by preventing self-ubiquitination and increases p53 ubiquitination and degradation.	(*96*)
Nuclear Transport	RanGAP	Promotes its interaction with RanBP2 and localization to nuclear pore complex from the cytoplasm	(*8, 9*)
	NEMO (IkB kinase)	Promotes nuclear localization and subsequent modulation of NFkB activity in response to genotoxic stress.	(*97, 98*)

SUMOylation in development and diseases

The importance of SUMO modification in whole organism development has been studied using various model organisms. Complete abrogation of SUMO modification using Ubc9 deletion mutants, the sole SUMO E2 conjugating enzyme, leads to cell cycle and chromosome segregation defects in yeast. SUMOylation regulates both the degradation of cyclinB (*18*) and

the proteolytic activity of the anaphase promoting complex/cyclosome (APC/C) (*99*). In the *Xenopus* egg extract system, inhibition of SUMOylation on Topoisomerase IIα causes segregation defects during anaphase (*13*). In most multicellular organisms, loss of Ubc9 leads to embryonic lethality (*100, 101*). Figure 4 below presents a general summary of the developmental pathways regulated by SUMO modification.

In addition, defects in SUMOylation have been linked to many disease states. Increased production of Ubc9 and SUMO E3 enzymes is seen in a number of cancers and is associated with poor prognosis. SUMO has been shown to regulate the activity of a number of tumor suppressors. A number of proteins known to be involved in neurological disorders such as Huntington's, Parkinson's and Alzheimer's have been shown to be regulated by SUMO and in each of the cases, either an increase in SUMO modification or defects in SUMOylation has been associated with the disease state. Lamin A, which is associated with several cardiomyopathies and progeria, has also been shown to be SUMOylated. SUMOylation of Lamin A is important for its correct localization and two different diseases have been linked to reduced SUMOylation of Lamin A due to mutations in the SUMOylation consensus motif (*102*).

A number of human pathogens also target the host SUMO pathways to establish infection. Viruses of the adenoviridae and herpeviridae families are known to regulate the host cell SUMOylation, especially in the promyelocytic leukemia nuclear bodies (PML-NBs). Viral proteins are also known to be targets of SUMO modification. For example, SUMOylation of HIV-1 integrase is important for both infectivity and replication efficiency of this virus. Pathogenic bacteria also target the SUMO modification pathways in the host cells. One of the effector proteins secreted by *Yersinia* species in host cells is YopJ, which is a homolog of SUMO proteases. *Listeria monocytogenes* targets Ubc9 and SUMOylated proteins for degradation (*103*).

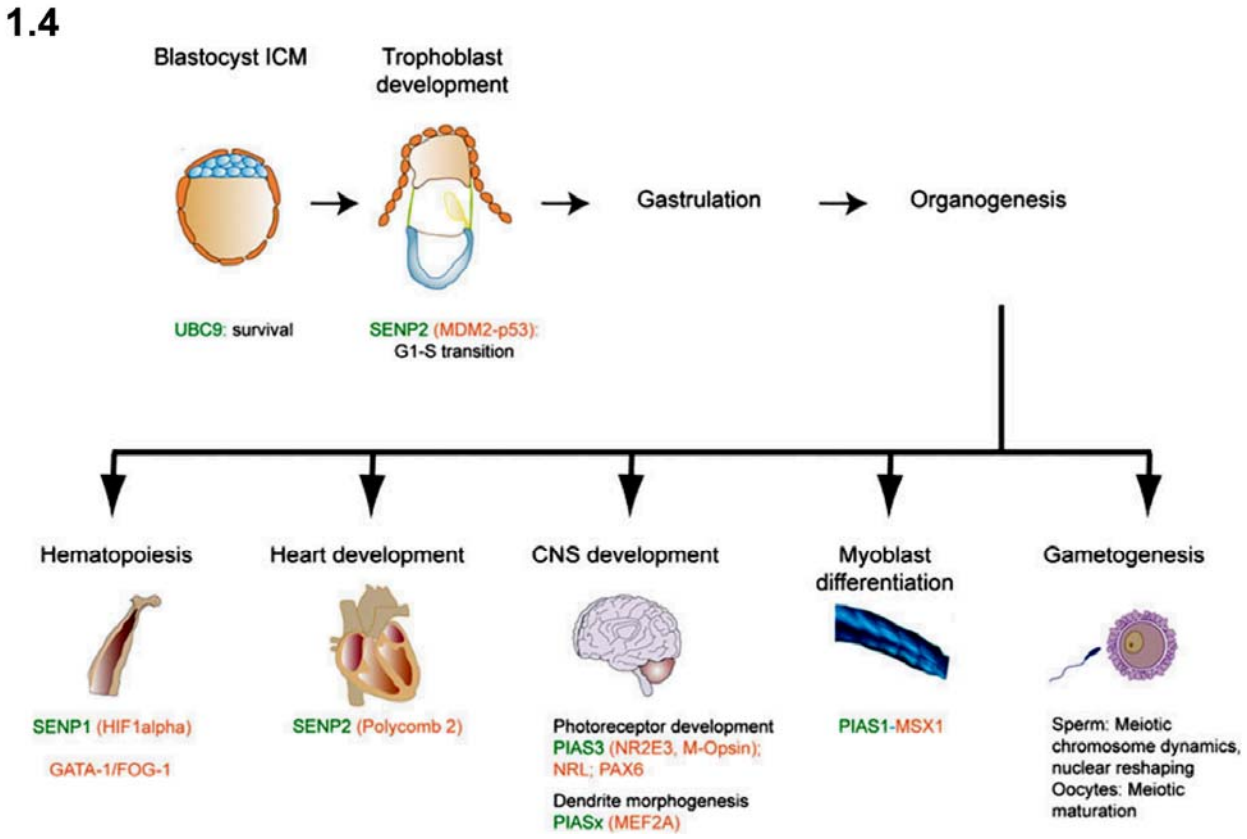

Figure 1.4: Role of SUMO modification in mouse development. Target genes are in parentheses in orange, specific SUMO pathway member involved in regulation is in green. Figure adapted from Lomeli and Vasquez (*104*).

Thus, SUMO pathway proteins have very important roles in development and maintenance of homeostasis. Disruptions in SUMO modifications lead to developmental abnormalities and promote various diseases.

Understanding the functions of SUMO modification

Undoubtedly, SUMO plays very important roles in all of the life processes and defects in SUMO modification lead to disease states. A lot of advances have been made in understanding the importance of this post-translational modification in the past fifteen years and many of the

SUMO substrates have been identified. Despite these advances, SUMO remains a difficult protein to study and characterizing its function in regulation of various proteins and cellular pathways presents many challenges. First, a very small percentage of SUMO substrates are actually found conjugated to SUMO at any given time in cells (*77*). Estimates of any given substrate being SUMOylated range from less than 1 percent to up to 5 percent. This variability makes it difficult to identify new SUMO substrates and to understand the functional significance of SUMO modification of a protein. Second, SUMO proteases are very active enzymes and very difficult to inhibit chemically. These proteases cause rapid turnover of SUMOylated proteins in cells and on cell lysis (*77*). Third, SUMO modification is highly dependent on the local concentration of the SUMO-pathway enzymes. For example, SUMO modification can be carried out efficiently *in vitro* even in the absence of E3 ligases if the E2 enzyme Ubc9 is used at very high concentrations. Not all lysines that are modified *in vitro* are targets of SUMO modification *in vivo*, where a more complex milieu regulates both the accessibility of the lysines available for modification and the localization of SUMO E3 enzyme. Fourth, mutation of individual SUMOylation sites on a protein can often present very subtle effects on protein function that might be difficult to characterize. In some cases, SUMOylation of a nearby lysine can sufficiently substitute for the loss of the original target lysine. Also, in the case of transcription regulation at least, SUMO modification does not regulate basal transcription but often contributes to regulation of combinatorial transcription factor binding; this effect may not be very apparent under normal conditions (*105*). Finally, whole organism knock-out studies of SUMO isoforms and SUMO E3 enzymes show remarkable redundancy. SUMO-1 deficient mouse develop normally and most of the SUMO-1 functions are efficiently substituted by SUMO-2/3 (*106*), whereas SUMO-2/3 gene silencing has more significant effects on the

expression of many cellular genes and on cell division rates (*107*). Similarly, knockout studies of the individual PIAS family members present very modest effects in animal models. PIASXα knockout mice present mild defects in testicular development and PIASy deficiency causes minor defects in interferon and Wnt signaling in various cell types (*105*). PIAS1 knockout mice are viable but show defects in the innate immune system development and noticeable defects in the regulation of NFκB and Stat1 signaling (*108, 109*) . PIAS1/PIASy double knockout mice show early embryonic lethality (*110*). As a result of the difficulties presented here, SUMO modification is often studied using conditions wherein either SUMO itself or the modification enzymes are overexpressed.

In this thesis, I have tried to characterize the importance of SUMO E3 ligase PIAS1 in regulating cellular responses to genotoxic stress following UV irradiation. This project was initiated with the aim of understanding the importance of the PIAS family of SUMO E3 ligases in various cellular pathways and specifically in genome maintenance following stress. Overexpression of each of the PIASes leads to a distinct SUMOylation pattern in the cell. The PIASes also show unique localization in the nucleus. In chapter 2, we show that the overexpression of PIAS1 in HeLa cells, but not of any other PIAS isoforms, leads to increased cell death following UV irradiation. PIAS1 localization is not altered in the nucleus on UV irradiation and there appears to be no global changes in SUMOylation following UVR. PIAS1 expression itself is not toxic to cells, but cells undergo rapid apoptosis after UV irradiation. Based on these observations, we hypothesized that overexpressed PIAS1 modifies one or more distinct proteins in the absence of UV irradiation. This leads to alterations in the levels of pro- and anti-apoptotic proteins, presumably by changes in transcription. Thus, the cells remain in a "sensitized" state and immediately initiate apoptosis following genotoxic stress. We identified

Daxx as a downstream effector protein of PIAS1-mediated apoptosis following UV irradiation. In chapter 3, we show that the PIAS1 N-terminal SAP domain is essential for its correct nuclear localization and hence for the PIAS1-mediated sensitivity to UV. We also show that Daxx itself is not a target of PIAS1 SUMOylation but interacts with proteins SUMOylated by PIAS1 via its C-terminal SIM domain. In chapter 4, we have identified NFκB transcription factor RelB as an interactor and potential regulatory target of PIAS1. Chapter 5 summarizes the dissertation and also presents future perspectives and avenues for further research in this subject.

Our research provides new insights into the distinct roles of the PIAS family of SUMO ligases in regulating cellular response to genomic insult. This research will lead to a better understanding of how cells respond to DNA damaging stimuli and switch between a pro-survival and apoptotic response, of the factors involved in this decision making, and the role of SUMOylation in these responses. Our study has important implications in the field of DNA repair and apoptosis research.

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CHAPTER 2

The SUMO ligase PIAS1 regulates UV-induced apoptosis by recruiting Daxx to SUMOylated foci

INTRODUCTION

The Small Ubiquitin-like Modifier (SUMO) proteins regulate the activity, localization and/or stability of various cellular proteins through a reversible covalent modification or non-covalent interactions with their substrates (*1*). Three well-characterized SUMO isoforms, numbered 1 to 3, exist in vertebrates; SUMO-1, SUMO-2 and SUMO-3 covalently attach to a substrate by forming an isopeptide linkage with one or more specific lysine residues on the protein. In addition, SUMO-2 and SUMO-3, which share 95% sequence identity, are known to form poly-SUMO chains (*2*). SUMO modification involves the sequential transfer of a processed active SUMO protein from an E1 SUMO-activating enzyme to an E2 SUMO-conjugating enzyme and the subsequent covalent conjugation to the substrate in a reaction that involves a SUMO-E3 ligase. In their substrate-conjugated form, SUMO proteins can interact with other proteins containing a SUMO-interacting motif (SIM), which is characterized by ΨΨXΨ or ΨXΨΨ residues (where Ψ is a hydrophobic amino acid) and is often flanked by negatively charged residues (*3, 4*). Therefore, protein modification by SUMO can lead to new protein-protein interaction modules.

There is only one SUMO E1 enzyme and only one SUMO E2. Mutations that completely abrogate the function of either of these two enzymes lead to a complete absence of SUMOylation

and are lethal. The diversity of SUMO substrates is presumably governed by a small number of SUMO E3 ligases that exist in cells. The PIAS family of SUMO E3 ligases contains homologs in both vertebrates and invertebrates, and is characterized by the presence of a zinc-binding SP-RING domain (*5, 6*). In addition, PIAS family members all contain an N-terminal SAP (scaffold attachment factor, acinus and PIAS) domain that is involved in DNA binding and protein-protein interactions, a PINIT domain that has been proposed to be required for subcellular localization and substrate interactions (*7*), a SUMO-interacting motif (SIM), and a variable C-terminal serine-threonine-rich region. Mammals contain five major PIAS isoforms, PIAS1, PIAS2α/Xα and 2β/Xβ, PIAS3 and PIAS4/y. PIAS proteins are functionally similar to RING finger ubiquitin ligases and act as adaptor proteins that bridge the SUMO-conjugated E2 enzyme and the substrate in the SUMOylation reaction. The SP-RING finger domain is essential for the SUMO-ligase activity of PIAS proteins. It has also been suggested that PIAS proteins promote SUMO paralog selection (*6, 8*).

Various cellular stress stimuli have been demonstrated to induce global protein modification by SUMO-2/3, which suggests that cells may use SUMO-modification as a protective response (*9*). A number of DNA damage repair (DDR) pathway proteins have recently been shown to be modified by SUMO. For example, the SUMO-conjugation of the base excision repair (BER) enzyme thymine DNA-glycosylase (TDG) has been shown to regulate its DNA-binding activity (*10*). Similarly, NER, NHEJ and HR pathway proteins have been shown to be SUMOylated. PIAS1 and PIAS4 have been implicated in DNA double-strand break repair that depends on the SUMO-2/3 conjugation of BRCA1 and the SUMO-1 conjugation of 53BP1, respectively (*11*). PIAS proteins are also known to promote apoptosis. UV irradiation-induced TIP60 SUMOylation has been shown to be essential for its localization to PML bodies and the

activation of the p53-dependent transcriptional response (*12*). PIAS1 has been shown to initiate apoptosis in response to the presence of reactive oxygen species by JNK activation (*13*).

Daxx is another PML (promyelocytic leukemia protein) -associated protein that has been shown to participate in UV-induced cell death via the activation of the JNK pathway (*14*). Daxx is a well-characterized SUMO-interacting protein and interacts with SUMO-modified proteins via its N- and C-terminal SIMs (*15*). Daxx acts as a transcriptional co-repressor for a number of transcriptional factors, including ETS-1 and NF-κB, and has been shown to both activate and repress p53 transcriptional activity (*16*). Daxx may also regulate chromatin dynamics by binding to histone deacetylases and chromatin remodeling proteins (*17*). Daxx interacts with the SUMOylated form of the PML protein (*18*), and the sequestration of Daxx in PML bodies suppresses its transcription inhibition function (*19*). Various lines of evidence suggest that Daxx can act as both a pro- and an anti-apoptotic protein. Daxx is known to be involved in the axin-mediated activation of p53 transcription, which leads to apoptosis (*20*). Daxx has been shown to suppress anti-apoptotic gene transcription by repressing NFκB transcriptional activity (*21*). Conversely, RNAi-induced Daxx silencing has been shown to increase apoptosis (*22*) and sensitize cells to cellular stressors, such as UV and TNF-α (*23*).

In this chapter, we present our finding that the exogenous expression of PIAS1, but not other members of the PIAS family of SUMO E3 ligases, increases UV-induced apoptosis in HeLa cells. PIAS1's SUMO-ligase activity is essential for increased UV sensitivity and that increased apoptosis is mediated by the recruitment of Daxx to SUMOylated PIAS1 foci. Based on these results, I propose a novel, specific role for PIAS1-mediated SUMOylation in sensitizing cells to UV damage through Daxx.

RESULTS

PIAS1-transfected cells display increased sensitivity to UV irradiation

To understand the specific roles of the PIAS family of SUMO ligases in the DNA damage response, we expressed C-terminal mCherry-tagged PIAS1, PIAS2α/Xα, PIAS3 or PIAS4/y in HeLa cells and subjected the cells to 30 J/m^2 of UV irradiation, twenty-four hours after transfection. Four hours post-UV irradiation, apoptotic cells were counted based on their aberrant nuclear morphology and fragmentation. Fixed cells were also stained with an anti-Parp1 antibody (Fig. 1A). Parp1 is a known substrate of caspase-3 during apoptosis (*24*). In non-apoptotic cells, Parp1 is present only in the nucleus, whereas in apoptosis-initiating cells, cleaved Parp1 can be detected in the cytoplasm. Based on nuclear morphology and Parp1 staining, we determined that less than 2% of the untransfected cells underwent apoptosis within four hours after irradiation. In cells expressing PIAS family proteins, 5-20% of each group of PIAS-expressing cells were apoptotic in the absence of UV irradiation. Four hours after irradiation, the PIAS1-expressing cells contained significantly more apoptotic cells compared with the other PIAS-expressing cells; hereafter, we refer to this phenotype as "UV-hypersensitive apoptosis" (Fig. 1B). In the mock-irradiated condition, the PIAS1-expressing cells displayed better survival when compared with the other PIAS-expressing cells, which indicates that PIAS1 expression is less toxic to HeLa cells than that of other PIAS proteins (Fig. 1A). UV-hypersensitive apoptosis was observed in several different cell lines, suggesting that the role of PIAS1 is not specific to the genetic background of the HeLa cells. We calculated the apoptotic ratio by normalizing UV irradiation induced apoptosis to the apoptosis in mock-irradiated cells. As shown in Fig. 1C, PIAS1-expressing cells displayed more than twice the amount of apoptosis compared to other PIAS-expressing cells 4 hours after irradiation. These results suggest a specific role for PIAS1 in

UV-induced apoptosis. This increase in apoptosis in cells expressing PIAS1 could be suppressed by treating cells with caspase-3 inhibitors before and during recovery from the UV-treatment (Fig. 1D).

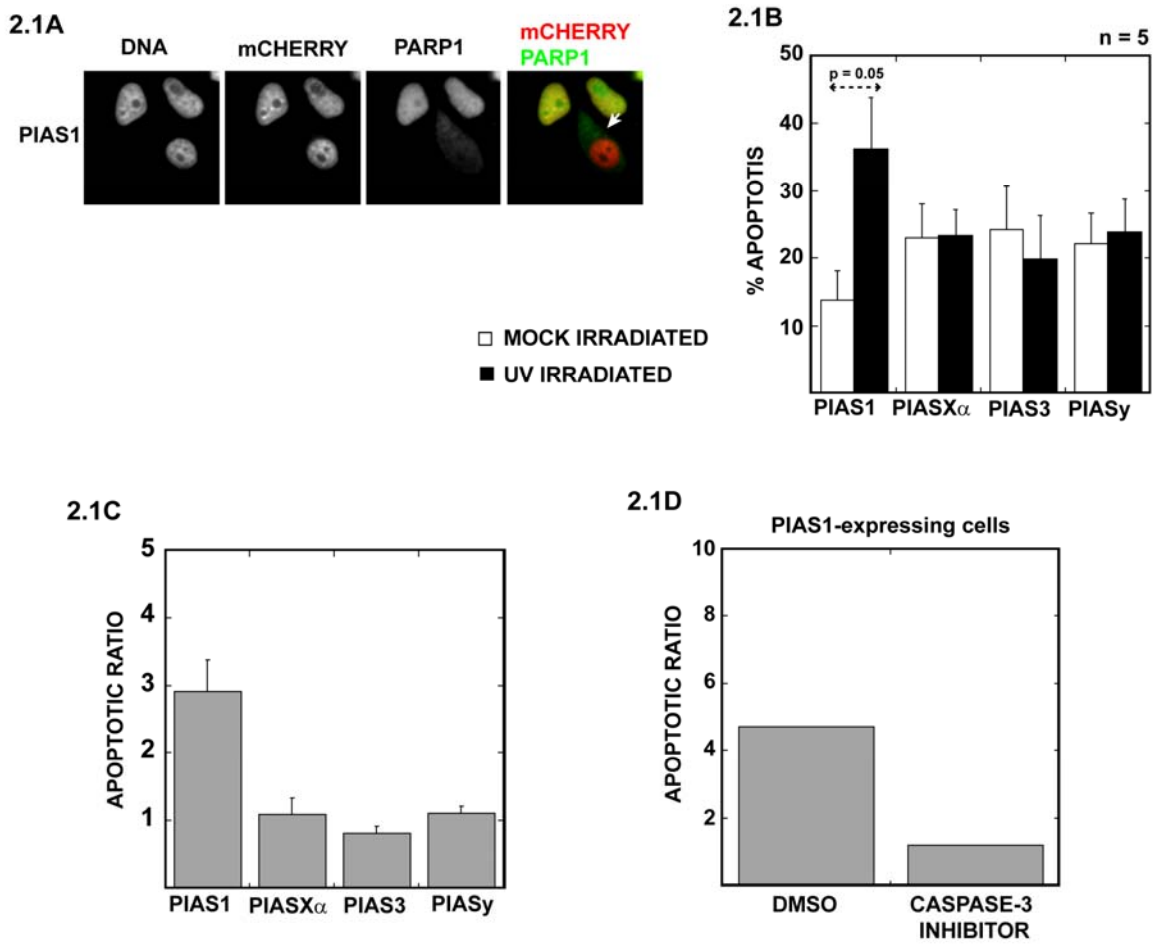

Figure 2.1: **PIAS1 overexpression sensitizes HeLa cells to UV irradiation. (A)** Parp1 localization changes in apoptotic cells. Parp1 is a known substrate of activated caspase3. Uncleaved Parp1 mainly resides in the nucleus, but after cleavage by caspase3, the cleaved fragment can be detected in the cytoplasm as well. This change in Parp1 localization often precedes a change in nuclear morphology. **(B)** PIAS1-expressing cells undergo increased UV irradiation-induced apoptosis. HeLa cells were UV-irradiated (30 J/m^2) or mock-irradiated 24 hours after transfection with the PIASes. After a 4-hour recovery period, the numbers of dead and surviving cells were counted based on their nuclear morphologies and Parp1 staining patterns. The plotted values are the average of five independent experiments. The error bars represent 1 standard error of the mean. **(C)** The differences between UV-

irradiation induced apoptosis in various PIAS isoform-expressing cells is emphasized by plotting the ratio of the percentage of apoptotic cells after UV-irradiation over the percentage of apoptotic cells in mock-irradiated samples, as calculated for Fig. 1B. **(D)** Caspase inhibitor reduces PIAS1-mediated UV-induced apoptosis. PIAS1-transfected cells were treated with either DMSO or 200uM caspase-3 inhibitor III (DEVD-CMK, EMD Biosciences, Massachusetts, USA) for 2 hours before UV irradiation and also during the four hour recovery period post-irradiation. Apoptotic cells were counted based on nuclear morphology and Parp1 staining of fixed cells.

PIAS isoforms show distinct nuclear distribution and SUMOylation profiles

The SUMO substrate selectivity of PIASes can stem from their distinct localizations within the cell (*8, 25*). To obtain additional insight into the substrate specificity of PIAS proteins, we compared the localization of each of the PIAS isoforms. All of the mCherry-tagged PIAS isoforms localized to the nucleus, and each of the PIAS isoforms was uniquely distributed in the nucleus. PIAS1 showed numerous (>30) small foci in the nucleus at low expression levels and comparatively fewer (<10) but larger foci at high expression levels. The foci that were formed by PIAS2α/Xα were more numerous (>100) and had low PIAS2α/Xα expression levels. PIAS3, when expressed at low levels, showed a similar localization pattern to that of PIAS1 high expression foci, whereas PIAS3 was homogenously distributed in the nucleus at a high expression level. Finally, PIAS4/y-mCherry was homogenously distributed within the nucleus, and a small number of faint foci were visible (Figure 2A). All of the isoforms showed varying levels and patterns of colocalization with the SUMO isoforms and strongly recruited SUMO to foci, which yielded SUMO localization patterns that are distinct from the SUMO distribution that was observed in untransfected cells (Figure 2A).

Because the SUMO ligase activity of PIAS1 is required for UV-hypersensitive apoptosis, we examined whether all PIAS proteins have similar SUMO ligase activity in transfected cells. To compare the SUMOylation profiles of the PIAS proteins, we enriched PIAS-expressing cells

based on the mCherry signal using a cell sorter (MoFlo XDP, Beckman-Coulter) from both mock-irradiated and UV-irradiated samples. The SUMOylation profile of the sorted cells was analyzed by immunoblotting with either anti-SUMO-1 or anti-SUMO-2/3 antibodies (Fig. 2B), and anti-β-actin was used as a loading control. All of the PIAS isoforms increase the levels of SUMO-1 modification on several proteins that range in molecular weight from 95 kD to 300 kD. However, no significant difference was observed in the pattern of SUMO-1-modified proteins between the cells that expressed PIAS1 and those that expressed other PIASes, suggesting that SUMO-1 modification may not be relevant to UV-hypersensitive apoptosis. Conversely, the SUMO-2/3 immunoblot shows clear differences in the SUMOylation profile upon the expression of various PIAS proteins. Each of the PIAS proteins produce a number of unique SUMO-2/3 modified bands, indicating that each PIAS protein has a unique subset of substrates for SUMO-2/3 modification. The molecular weights of most of the unique bands are greater than 130 kD. PIAS2α/Xα appears to give the most intense SUMOylation signals, although it does not show UV-hypersensitive apoptosis. This suggests that increasing SUMOylation in cells alone does not contribute to UV-hypersensitivity. We did not observe any significant differences in the SUMOylation pattern between the mock- and UV-irradiated cells in any of the samples. In general, HeLa cells seem to slightly repress SUMOylation in response to UV irradiation. These results indicate that all PIAS proteins promote SUMOylation of their SUMO-2/3-specific substrates in cells, and PIAS1-specific substrates may contribute to the sensitization of cells to UV irradiation and apoptosis induction.

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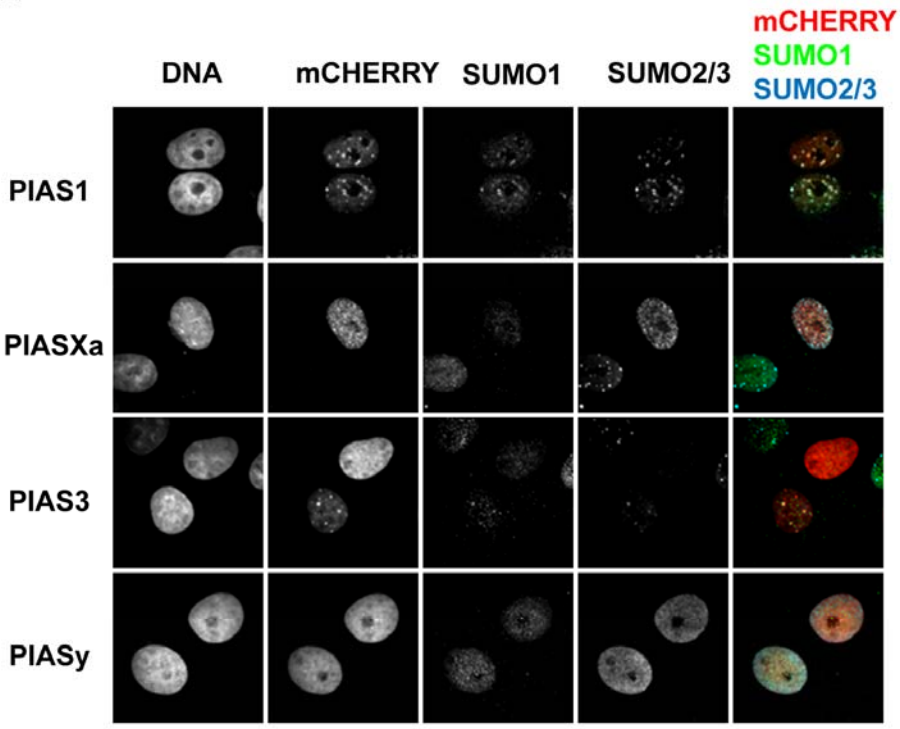
2.2A


DNA
mCHERRY
SUMO1
SUMO2/3
mCHERRY
SUMO1
SUMO2/3
PIAS1
PIASXa
PIAS3
PIASy

2.2B

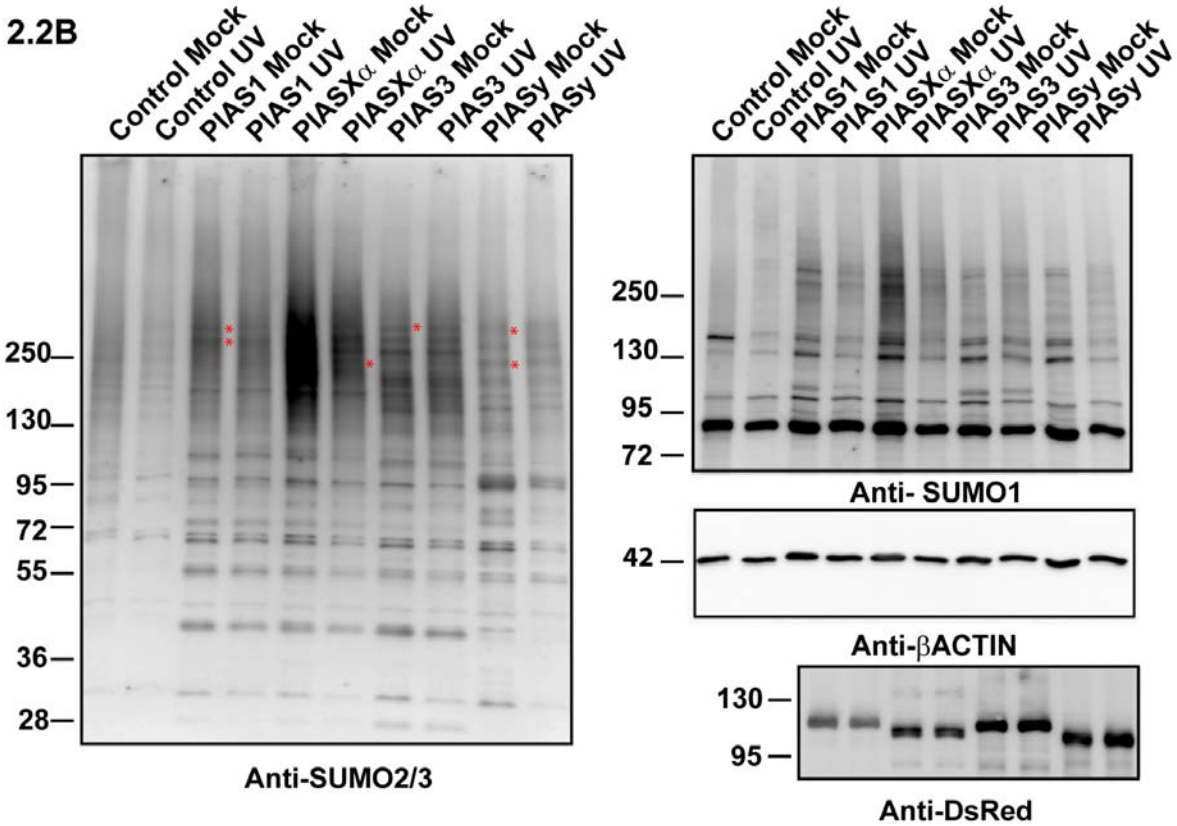

Control Mock
Control UV
PIAS1 Mock
PIAS1 UV
PIASXα Mock
PIASXα UV
PIAS3 Mock
PIAS3 UV
PIASy Mock
PIASy UV
250
130
95
72
55
36
28
Anti-SUMO2/3
Anti- SUMO1
42
Anti-βACTIN
130
95
Anti-DsRed

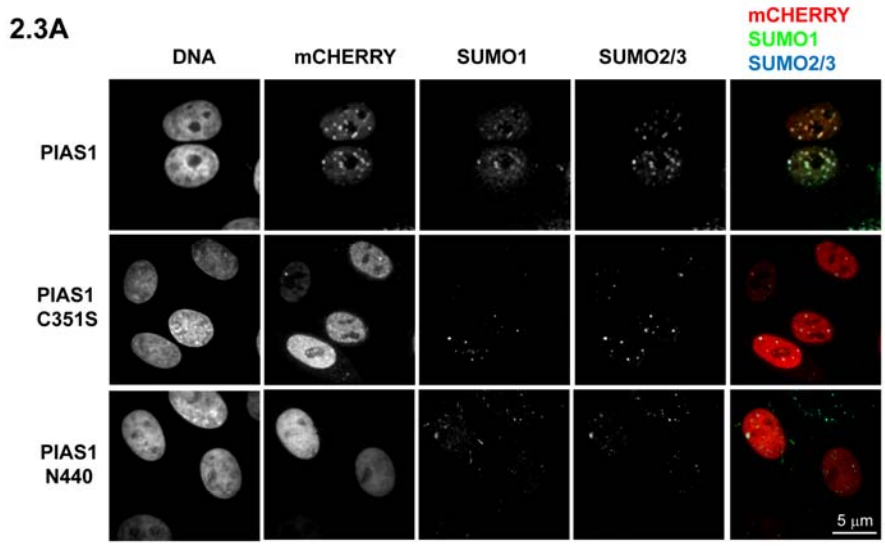
Figure 2.2: PIAS family SUMO ligases show distinct localization and SUMOylation substrates in cells. (A) Differential localization of PIAS family of SUMO ligases in HeLa cells. PIAS1, PIASXα, PIAS3 and PIASy C-terminal mCherry fusion constructs were transfected into HeLa cells. Cells were immunostained with anti-SUMO1 and anti-SUMO2/3 antibodies 24 hours after transfection. **(B)** PIAS family of SUMO ligases have distinct substrates in cells. HeLa cells overexpressing mCherry fusion PIASes were sorted from untransfected cells and SUMOylation in transfected cells analyzed using either anti-SUMO1 or anti-SUMO2/3 antibody after western blot. Some of the unique SUMOylated bands have been marked with an asterisk

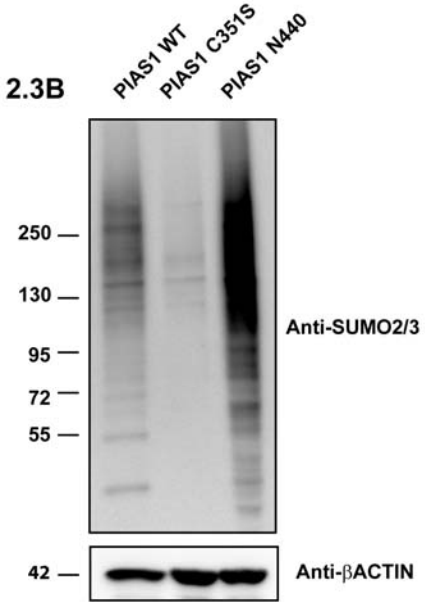
PIAS1's SUMO ligase activity is required for UV-sensitivity

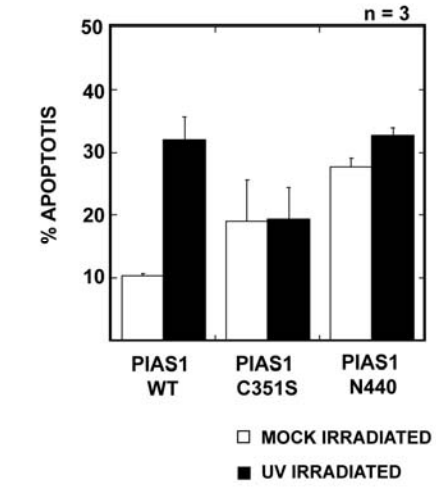
PIAS family SUMO E3 ligases have been shown to regulate a number of cellular pathways independent of their SUMO ligase activity (*6*). PIASes are known to regulate the activity of other proteins by altering their localization via direct interactions that do not depend on the presence of a functional SP-RING domain. For example, PIAS1 has been shown to regulate apoptosis-related proteins, such as p53 and Msx1, independent of its SUMO ligase activity (*26-28*). To determine whether PIAS1's SUMO ligase activity is required for UV-hypersensitive apoptosis, we expressed the PIAS1 C351S mutant and a PIAS1 N440 deletion mutant in HeLa cells and compared the rate of UV-hypersensitive apoptosis to that in cells expressing wild-type PIAS1 (PIAS1wt). The C351S mutant contains a mutation in the SP-RING domain that disrupts RING finger formation; therefore, it lacks SUMO E3 ligase activity (*29*). The PIAS1 N440 deletion mutant lacks the C-terminal SIM domain that has been shown to increase the affinity of PIAS for SUMO, although it is not required for SUMOylation, *in vitro* (*7*). PIAS1wt and the two mutants did not show similar localization in the nucleus. PIAS1 forms numerous (>30) small foci in the nucleus at low expression levels and comparatively fewer (<10) but larger foci at high expression levels. In contrast, the PIAS1 C351S mutant has a more homogenous nuclear distribution and forms very few foci (Fig. 3A). PIAS1 N440 also shows a homogenous nuclear distribution and does not form clear foci.

As we predicted, PIAS1 C351S did not increase the SUMO-2/3 modification of cellular proteins. Rather, it suppressed most SUMO-2/3 modifications and acted in a dominant-negative manner (Fig. 3B). PIAS1 N440, however, showed higher SUMOylation activity than PIAS1wt. Our results are in agreement with earlier studies showing that the SIM domain of yeast PIAS homolog Siz1 is dispensable for specific substrate recognition and site selective SUMOylation of PCNA (*7*). HeLa cells expressing PIAS1 C351S and PIAS1 N440 showed more cell death compared with PIAS1wt-expressing cells. Four hours after UV irradiation, the PIAS1 C351S-expressing cells did not show elevated apoptosis levels, unlike the PIAS1wt-expressing cells (Fig. 3C). This result indicates that the SUMO ligase activity of PIAS1 is required for UV-hypersensitive apoptosis, and that neither the inhibition of SUMOylation by the PIAS1 C351S mutant nor the aberrant SUMOylation by PIAS1 N440 contribute to UV-hypersensitive phenotype.

Figure 2.3. PIAS1's SUMOylation activity is required for UV sensitivity. (A) The ligase-dead mutant (PIAS1 C351S) and the SIM domain truncation mutant (PIAS N440) do not exhibit nuclear punctate localization that is shown by wild-type PIAS1. C-terminal mCherry-tagged PIAS1wt, PIAS1 C351S or PIAS1 N440 were expressed in HeLa cells for 24 hours. Fixed cells were stained with anti-SUMO-1 and anti-SUMO-2/3 antibodies. The images were acquired using a confocal microscope using a 100X objective. Scale bar: 5 μm. **(B)** Altered SUMOylation profile in PIAS1-mutant-expressing cells. The cells expressing mCherry-tagged PIAS1 C351S and PIAS1 N440 were sorted, and their SUMO-2/3 profile was compared with those of PIAS1-expressing cells by immunoblotting using the anti-SUMO-2/3 antibody. **(C)** Wild-type PIAS1, but neither PIAS1 C351S nor PIAS1 N440, promotes increased apoptosis upon UV irradiation. The plotted values are an average of three independent experiments. The error bars represent 1 SEM. The percentage of apoptotic cells was determined, as explained in **Figure 2.1A**.


2.3A
DNA
mCHERRY
SUMO1
SUMO2/3
mCHERRY
SUMO1
SUMO2/3
PIAS1
PIAS1
C351S
PIAS1
N440
5 μm


2.3B
PIAS1 WT
PIAS1 C351S
PIAS1 N440
250
130
95
72
55
Anti-SUMO2/3
42
Anti-βACTIN


2.3C
n = 3
% APOPTOTIS
50
40
30
20
10
PIAS1
WT
PIAS1
C351S
PIAS1
N440
MOCK IRRADIATED
UV IRRADIATED

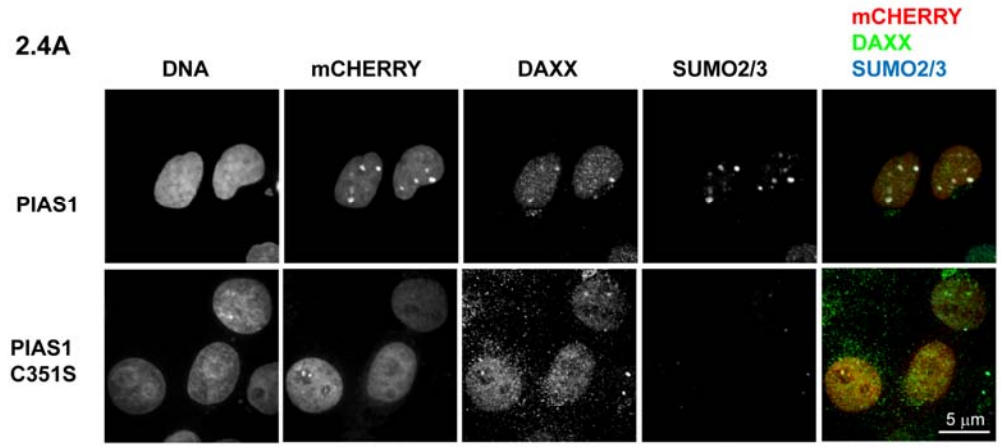
PIAS1 colocalizes with Daxx in cells

Because PIAS1 promotes SUMOylation of specific cellular proteins, we aimed to identify the protein(s) that interact with SUMOylated substrates in PIAS1-expressing cells. Daxx was one of the logical candidates for this approach because it is a known pro-apoptotic protein that contains two SUMO-interaction motifs (one at each terminus) (*15*). We immunostained PIAS1-transfected, mock- or UV-treated, cells with an anti-Daxx antibody. In untransfected cells, Daxx showed small punctate localization in the nucleus which largely coincided with promyelocytic leukemia nuclear bodies (PML-NBs). Daxx localization was altered in the PIAS1-expressing cells. In approximately 20 to 30 percent of the PIAS1-expressing cells, Daxx colocalized with some of the PIAS1 foci (Figure 4A). This colocalization was more easily observed in the high-expression PIAS1 foci in both the mock-irradiated and UV-irradiated cells.

Daxx is known to localize to PML-NBs in cells and this localization requires PML SUMOylation. The colocalization of PML and Daxx has been proposed to regulate Daxx's co-repressor function (*19*). We therefore examined whether the PML/Daxx interaction was altered by PIAS1 expression. By immunofluorescence staining, we observed that PIAS1 also colocalizes with PML, but Daxx and PML show very little colocalization in PIAS1-expressing cells. The colocalization of PIAS1 with PML and Daxx appears to be mutually exclusive. However, in PIAS1 C351S-expressing cells, PML and Daxx largely remain colocalized (Figure 4B). We calculated the colocalization between PML and Daxx in PIAS1wt- and PIAS1 C351S-expressing cells using the cell image analysis software, CellProfiler v2.0 (*30*). The correlation between each focus of PML and Daxx was calculated using Pearson's correlation coefficient. As shown in Figure 4C, in PIAS1 C351S-expressing cells, over 35% of Daxx foci strongly correlate with the PML foci. In contrast, PIAS1wt-expressing cells display a strong correlation in less than 5% of

Daxx and PML foci. To further evaluate PML/Daxx interaction in each cell, we counted the number of cells showing a strong correlation (greater than 0.5) coefficient between PML and Daxx. As shown in Figure 4D, in nearly 80% of the PIAS1-expressing cells, less than 5% PML foci showed a strong correlation with Daxx. In contrast, nearly 40% of the C351S-expressing cells showed greater than 50% of the PML foci strongly colocalizing with Daxx, and almost an equal number of cells showed between 26-50% of the PML foci colocalizing with Daxx. This suggests that the inhibition of SUMOylation by PIAS1 C351S expression does not disrupt the PML/Daxx interaction, but increased SUMOylation by PIAS1wt promotes the dissociation of Daxx from PML. These observations strongly suggest that Daxx preferentially localizes to PIAS1-induced SUMO foci, which may then lead to UV-hypersensitive apoptosis.

Figure 2.4. PIAS1 colocalizes with Daxx in cells and regulates its localization to PML bodies. (A) Daxx colocalizes with the SUMOyalted foci in PIAS1-expressing cells, but not in PIAS1 C351S-expressing cells. PIAS1-mCherry and PIAS1 C351S-mCherry fusions were expressed in HeLa cells. The cells were stained with an anti-Daxx antibody 24 hours after transfection. The stained samples were imaged using confocal microscopy using a 100X objective. Scale bar: 5 μm. **(B)** PIAS1 expression prevents Daxx from colocalizing with PML bodies. PIAS1- or PIAS1 C351S-transfected cells were stained with anti-Daxx and anti-PML antibodies. Daxx colocalizes with PML bodies in PIAS1 C351S-expressing HeLa cells but not in PIAS1-expressing cells. Scale bar: 5 μm. PIAS1-Daxx and PIAS1-PML colocalization is enlarged (inset). **(C)** Quantitative measurement of colocalization correlation between Daxx and PML in PIAS1 vs PIAS1 C351S expressing cells. PIAS1 or PIAS1 C351S expressing cells were stained with anti-Daxx and anti-PML antibodies. Over sixty images were acquired using a confocal microscope from three independent experiments. Pearson's correlation between Daxx and PML was calculated in the transfected cells from the images using CellProfiler v2.0 software.


2.4A
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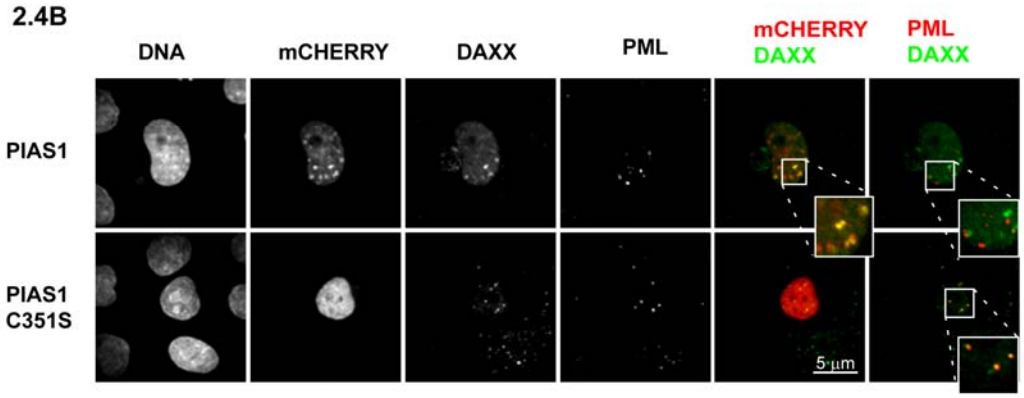
mCHERRY
DAXX
SUMO2/3
mCHERRY
DAXX
SUMO2/3
PIAS1
PIAS1
C351S
5 µm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mCHERRY
DAXX
PML
mCHERRY
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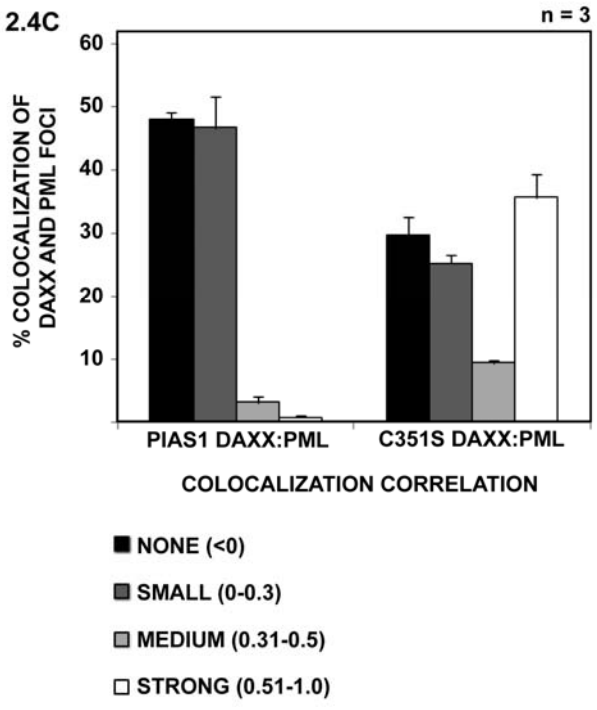
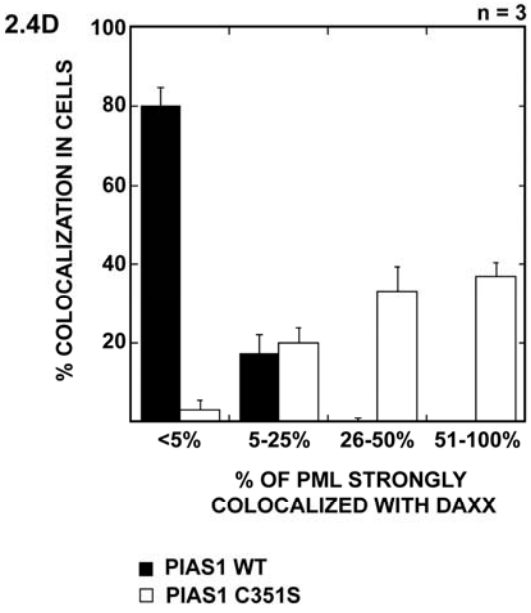
PML
DAXX
PIAS1
PIAS1
C351S
5 µm


2.4C
n = 3
% COLOCALIZATION OF DAXX AND PML FOCI
60
50
40
30
20
10
PIAS1 DAXX:PML
C351S DAXX:PML
COLOCALIZATION CORRELATION
NONE (<0)
SMALL (0-0.3)
MEDIUM (0.31-0.5)
STRONG (0.51-1.0)


2.4D
n = 3
% COLOCALIZATION IN CELLS
100
80
60
40
20
<5%
5-25%
26-50%
51-100%
% OF PML STRONGLY COLOCALIZED WITH DAXX
PIAS1 WT
PIAS1 C351S

The depletion of Daxx alleviates PIAS1-induced apoptosis

Because we observed that PIAS1-mediated SUMOylation alters the localization of Daxx, which is known to regulate apoptosis, we examined whether Daxx acts as a pro-apoptotic protein downstream of PIAS1. We utilized Daxx siRNA knockdown to elucidate the role of Daxx in PIAS1-mediated UV-hypersensitive apoptosis. We used two different siRNAs against Daxx and depleted approximately 70% of Daxx within 48 hours of treatment using either siRNA (Figure 5A). Under these conditions, we expressed PIAS1-mCherry fusion proteins to measure UV-hypersensitive apoptosis. In the Daxx depleted cells, PIAS1-mediated UV-hypersensitive apoptosis was significantly reduced. Compared with the control siRNA-treated cells, the cells that were treated with Daxx siRNAs showed 50% less apoptosis following UV irradiation. There was a slight increase in non-UV-induced apoptosis in the PIAS1-expressing cells that were treated with Daxx siRNAs (Figure 5B). This result indicates that Daxx plays a critical role in PIAS1-mediated UV-hypersensitive apoptosis and acts downstream of PIAS1-induced SUMOylation.

Taken together, these data indicate that PIAS1 has a distinct role in regulating Daxx activity among the PIAS family of SUMO ligases. PIAS1-specific SUMOylation alters Daxx's function by recruiting Daxx to SUMOylated proteins, which allows Daxx to mediate UV-hypersensitive apoptosis.

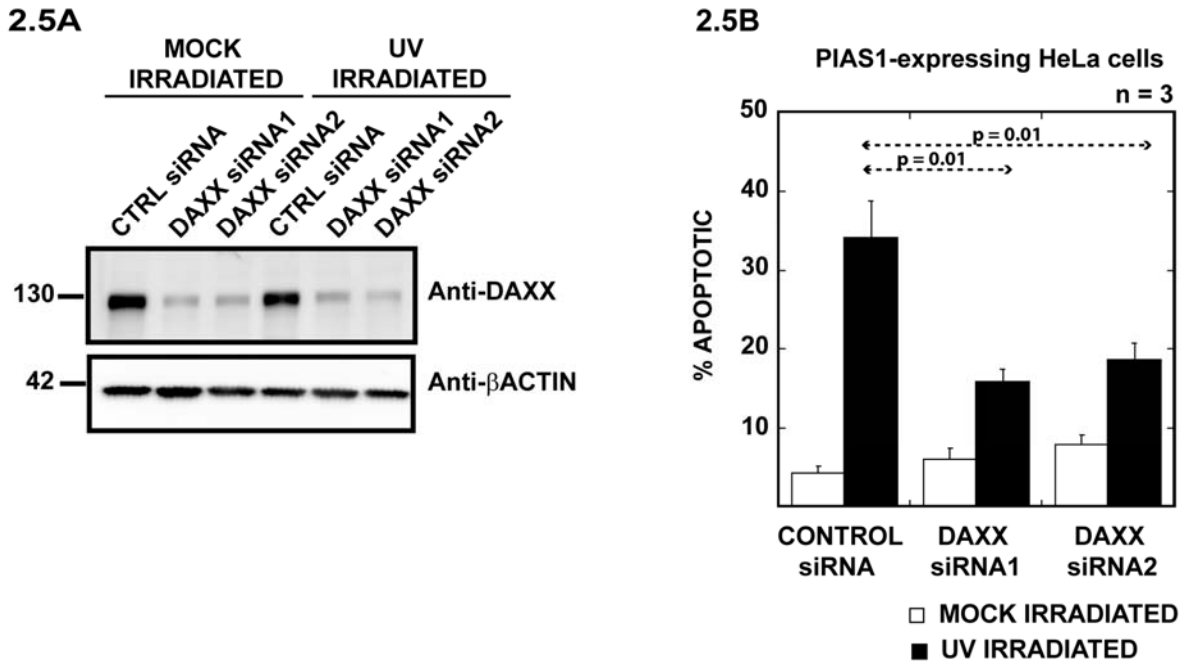


Figure 2.5. Daxx depletion alleviates UV sensitivity in PIAS1-expressing cells. (A) Treatment with either siRNA reduced cellular Daxx by nearly 70%. Daxx was depleted from HeLa cells using two different siRNAs against Daxx. mCherry-tagged PIAS1 was transfected into HeLa cells 24 hours after the Daxx siRNA treatment. siRNA-treated HeLa cell extracts were immunostained with anti-Daxx antibody after Western blot. Anti-β-actin antibody was used as a loading control. **(B)** Expression of Daxx siRNA but not the control siRNA significantly reduced apoptosis in cells expressing PIAS1 after UV treatment. Daxx siRNA treated, PIAS1 expressing cells were UV irradiated, and the apoptotic cells were counted, as described above 48 hours after Daxx depletion. The plotted values are an average of three independent experiments. The error bars represent 1 s.e.m.

DISCUSSION

Many different stress stimuli lead to the SUMOylation of several cellular proteins that act in the damage repair pathway or lead to apoptosis (*6, 9*). Members of the PIAS family of SUMO E3 ligases have been shown to have distinct substrates and contribute to SUMO paralog selectivity in response to various stress pathways (*6*). Various pieces of evidence indicate that PIAS1 is an important regulator of the cellular stress response. Galanty *et al.* (*11*) have shown that PIAS1 stimulates double strand break repair by modifying BRCA1 with SUMO-2/3. PIAS1 has also

been shown to participate in cellular responses to oxidative stress by regulating the JNK pathway and PIAS1 knockdown prevents reactive oxygen species-induced apoptosis (*31, 32*). PIAS1 also SUMOylates p53 and leads to an increase in p53 transcriptional activity (*26, 33*). In this chapter, we described a specific role of PIAS1 in regulating apoptosis in response to UV irradiation. The overexpression of PIAS1 in HeLa cells leads to increased sensitivity to UV irradiation and results in an increase in cell death by four hours after UV irradiation. The expression of other members of the PIAS family does not lead to increased apoptosis after UV irradiation. PIAS1's SUMO ligase activity is required for inducing hypersensitivity to UV irradiation; a ligase inactive mutant of PIAS1 fails to elicit UV-hypersensitivity in cells. Each of the PIASes displays a distinct SUMO-substrate profile and localization in the nucleus, but only PIAS1 expression leads to increased apoptosis following UV irradiation. This indicates that substrates unique to PIAS1 participate in UV-induced cell death response. The identification of PIAS isoform-specific substrates and the mediators of specific localization of each PIAS protein will be essential to comprehensively understand PIAS-mediated SUMOylation in stress-related apoptosis.

PIAS1-SUMOylated foci recruit the apoptotic protein Daxx. Daxx is a well-known apoptosis regulator that acts primarily as a transcriptional regulator, although its status as a pro- or anti-apoptotic factor is debated (*16, 17, 34*). Daxx contains two well-characterized SUMO-interacting motifs (SIM) at its termini that allow it to interact with SUMOylated proteins in cells (*15, 35, 36*). In response to UV irradiation, Daxx appears to act as a pro-apoptotic protein downstream of PIAS1. We confirmed the involvement of Daxx using a knockdown approach. PIAS1-induced UV sensitivity is alleviated when Daxx levels are reduced using siRNAs against

Daxx. Overall, our data show that the SUMOylation-dependent regulation of Daxx is critical for sensitizing cells to UV irradiation, which is consistent with the previously proposed model (*37*).

Daxx is known to localize to PML bodies in cells (*18*). The PML-NB recruitment of Daxx has been shown to attenuate its repressive activity toward several promoters (*19*). In PIAS1-expressing cells, Daxx does not localize to PML bodies; instead, it relocates to PIAS1 SUMOylation foci. Because a large number of known SUMOylated proteins are transcription factors, and many SUMOylated transcription factors are known targets of Daxx-mediated transcriptional repression, PIAS1 may mediate the SUMO-2/3 modification of transcription factors, and Daxx may act as a co-repressor in a SUMOylation-dependent manner. Therefore, Daxx may suppress the production of anti-apoptotic factors, which may therefore sensitize cells to cellular stressors, such as UV irradiation. Daxx has been shown to repress the transcription of anti-apoptotic proteins downstream of NFκB (*21*), and it would be interesting to examine whether this pathway is also targeted downstream of PIAS1 during UV-hypersensitive apoptosis.

In conclusion, we show that PIAS1 sensitizes cells to UV stress-induced apoptosis through the recruitment of Daxx to PAIS1-specific SUMOylated substrates. Daxx itself does not appear to be a substrate of PIAS1, but interacts with SUMOylated substrates. The identification of the specific PIAS1 substrates, their mechanism of interaction with Daxx and Daxx's downstream targets will be important to promote our understanding of PIAS1-mediated cell death in response to UV irradiation.

EXPERIMENTAL PROCEDURES

DNA constructs and cell transfection: mCherry DNA was amplified and cloned into the pCDNA His maxC vector (Invitrogen, Carlsbad, CA) using NotI and XhoI restriction enzymes to create a pCDNA4 His maxC mCherry vector. DNA sequences encoding PIAS1, PIAS2/Xα, PIAS3 and PIAS4/y were amplified and subcloned into the pCDNA4 His maxC mCherry vector using *Eco*RI and *Not*I restriction enzymes; this produced a C-terminal mCherry fusion for the PIAS proteins. The PIAS1 C351S mutant was generated using a QuikChange II XL Site-Directed Mutagenesis kit from Agilent Technologies (Santa Clara, CA). HeLa cells were grown to 50% confluence in McCoy's medium (Mediatech Inc., Manassas, VA) that was supplemented with 10% FBS and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions.

UV irradiation and apoptotic assays: The transfected HeLa cells were either mock-irradiated or irradiated with 30 J/m^2 of UV using an Entela UV crosslinker. In brief, the cells were washed twice with PBS and then irradiated in PBS. After irradiation, the PBS was removed and replaced with growth medium. The cells were analyzed 4 hours or 24 hours after UV irradiation. Cells that were grown on coverslips were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X-100. The cells were then stained with an anti-Parp1 antibody and Hoechst 33342 dye (which stains DNA) for immunofluorescence analysis. The apoptotic cells were counted based on DNA morphology and the re-distribution of Parp1 into the cytoplasm.

Cell sorting and Western blot analysis: HeLa cells that were transfected with mCherry-tagged PIAS family members were sorted from the untransfected cells using a Beckman Coulter MoFlo XDP sorter (Fort Collins, CO) with a 488 nm excitation filter and a 620/29 nm emission filter. The sorting was performed 4 hours after the mock or UV irradiation. The sorted cells were

subjected to a Western blot analysis, and the protein levels were normalized by staining for β-actin (Cell Signaling Technology, Danvers, MA).

Immunofluorescence analysis: Transfected and treated cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X-100. The cells were then blocked for 15 min with 5% BSA and 2.5% fish gelatin prepared in PBS-T. After washing, the cells were stained for one hour with the appropriate antibody following the manufacturer's dilution instructions, which was followed by staining with an Alexa dye-tagged secondary antibody (Invitrogen, Carlsbad, CA) and Hoechst 33342 dye. The cells were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and imaged using a DSU-10 spinning disk (Yokugawa-type) confocal microscope that was equipped with an Olympus 100X objective (with a numerical aperture of 1.45), Hamamatsu EMCCD and the Slidebook imaging package (Intelligent Imaging Innovations, Denver, CO). An anti-human Daxx antibody was purchased from Sigma and an anti-PML antibody from SantaCruz Biotechnology Inc. (Santa Cruz, CA). The other antibodies that were used in this study were generated in the lab.

Daxx silencing using siRNAs: siRNAs against human Daxx were obtained from Invitrogen (Carlsbad, CA) (siRNA1:AUCCAAUUCUGAGAGAUCCAACUCC and siRNA2: AUCAGAGUCUGAGAGCACGAUGAUC). HeLa cells were transfected with each of the siRNAs using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) using the reverse transfection method. The cells were plated to obtain a next-day confluency of 50%. Twenty-four hours after the siRNA transfection, the cells were transfected with pCDNA4 His maxC Cherry. The cells were UV-irradiated 48 hours after the siRNA treatment as described above, and the apoptotic cells were counted 4 hours later.

Colocalization correlation calculations

The colocalization correlation between PML and Daxx was calculated using 90 confocal images acquired from three independent experiments as described above. CellProfiler v2.0 (*30*) cell image analysis software was used to calculate colocalization using Pearson's correlation. The Otsu Adaptive threshold was used to identify and select all nuclei expressing mCherry. The correlation was calculated between Daxx and PML spots within the selected nuclei.

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CHAPTER 3

Domain analysis reveals essential domains of PIAS1 and Daxx required for UV-sensitivity

INTRODUCTION

SUMO E3 ligases can be grouped into four distinct families; nucleoporin RanBP2, polycomb protein Pc2, MMS21, and the PIAS family of SUMO E3 ligases. Of these, the PIAS (Protein Inhibitors of Activated Stats) are the most extensively studied and show a high degree of conservation across eukaryotes. Yeast have two PIAS isoforms (Siz1 and Siz2), whereas humans have five: PIAS1, PIASXα, PIASXβ, PIAS3 and PIASy (*1*). All members of the PIAS family have a conserved domain structure comprised of an N-terminal SAP domain, followed by a PINIT domain, SP-RING finger domain, SIM domain and a variable S/T rich region at the C-terminus (*1*) (See Fig. 1.2, chapter 1 or Fig. 3.1, this chapter for a schematic representation of PIAS domain organization)

The SAP (SAF-A/B, Acinus and PIAS) domain is a putative DNA binding domain that is found in many nuclear proteins. The SAP domain is comprised of a four helix bundle with strong affinity for DNA, but has a topology distinct from other DNA-binding domains (*2*). The SAP domain is also known to mediate interaction of PIASes with specific transcription factors (*2, 3*). In addition, our lab has recently shown that the PIASy SAP domain is essential for its recruitment to the centromeric region of mitotic chromosomes and this recruitment is mediated by an interaction between the PIASy SAP domain and the Rod/Zw10 proteins (*4*). The SAP domain is followed by a PINIT domain which is believed to be important for subcellular

localization of PIAS proteins as well as interaction with substrates (*5*). It is named after the Pro-Ile-Asn-Ile-Thr (P-I-N-I-T) motif present in all PIAS proteins. After the PINIT domain is the SP-RING domain (Siz/PIAS-RING). This domain is similar to the Ubiquitin E3 ligase RING (Really Interesting New Gene) domains and is essential for SUMO ligase activity of PIASes. The SP-RING has been shown to interact with the SUMO E2 enzyme Ubc9 (*6, 7*) and is proposed to promote SUMO modification by forming a complex containing the substrate and the E2. The SP-RING domain coordinates one Zn^{+2} ion via a tetrahedral configuration of three cysteines and one histidine residue. Mutation of any of the cysteines leads to a loss of SUMO ligase activity (*8*). The SIM (SUMO interacting motif) domain mediates non-covalent interactions with SUMO and is comprised of Val/Ile-Val/Ile-X-Val/Ile flanked either by negatively charged residues, or serines that can gain negative charge by phosphorylation (*9*). The SIM domain on PIAS is not essential for SUMOylation *in vitro*, but increases the affinity of PIAS for SUMO (*8*).

The SIM domain is also found in many effector proteins that interact with SUMOylated proteins. Daxx has two SIM domains, one at either terminus that has been shown to mediate its interaction with a number of SUMOylated transcription factors (*10-12*). Daxx was first described as a pro-apoptotic protein that acts downstream of the Fas death receptor (*13*). Subsequent research has shown that Daxx localizes in the nucleus where it acts mainly as a co-repressor for SUMOylated transcription factors (*14*), but also as a transcription co-activator in some cases (*10, 15, 16*). Daxx also associates with SUMOylated PML (Promyelocytic leukemia) protein. The two SIM domains present in Daxx can interact independently with SUMOylated proteins (*10*), albeit with different binding affinities. The Daxx N-terminal SIM has been shown to have a lower binding affinity towards both SUMO-1 and SUMO-2/3 compared to the Daxx C-terminal SIM (*12*). In addition to its ability to interact with SUMOylated proteins, Daxx itself has been

shown to be modified by SUMO (*17*), although a distinct SUMO E3 ligase for Daxx has not been identified.

In Chapter 2, we showed that Daxx localizes to PIAS1 SUMOylated foci and depletion of Daxx alleviates UV-hypersensitivity induced by PIAS1 overexpression in cells. Based on these results, we hypothesized that the UV-hypersensitivity is dependent on Daxx recruitment to protein(s) specifically SUMOylated by PIAS1. This localization to SUMOylated foci and the resulting UV-hypersensitivity is dependent on either of the Daxx SIM domains. We also hypothesized that similar to PIASy, the specific localization of PIAS1 in the nucleus requires the PIAS1 N-terminal SAP domain and is also important for UV-hypersensitivity. In this chapter, we present results that validate these hypotheses.

RESULTS

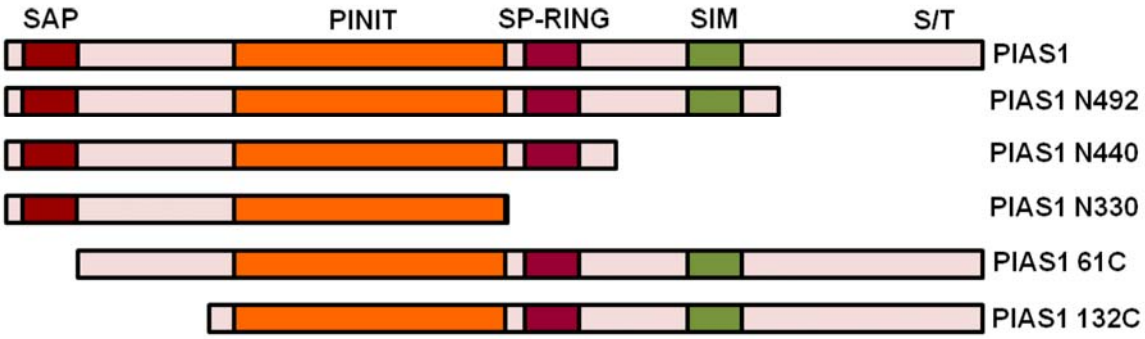
PIAS1 domain deletion mutants show differences in localization

In order to understand the importance of various PIAS1 domains for its correct localization, we constructed a series of deletion mutants lacking one or more PIAS1 domains. A schematic representation of these mutants is depicted in Figure 3.1A. The PIAS1 C-terminus truncation mutant N492 lacks the final 159 amino acids including the S/T-rich region, N440 lacks the C-terminal 211 amino acids including the SIM domain and N330 is truncated at amino acid 330 and has all domains including and downstream of the SP-RING domain deleted. The PIAS1 61C mutant has the first 61 amino acids deleted and thus lacks the SAP domain; the PIAS1 132C mutant has the first 132 amino acids deleted and starts just before the PINIT domain. All the mutants were expressed in HeLa cells with a C-terminal mCherry tag. The localization of these truncation mutants and their colocalization with SUMO2/3 in cells is compared to that of full

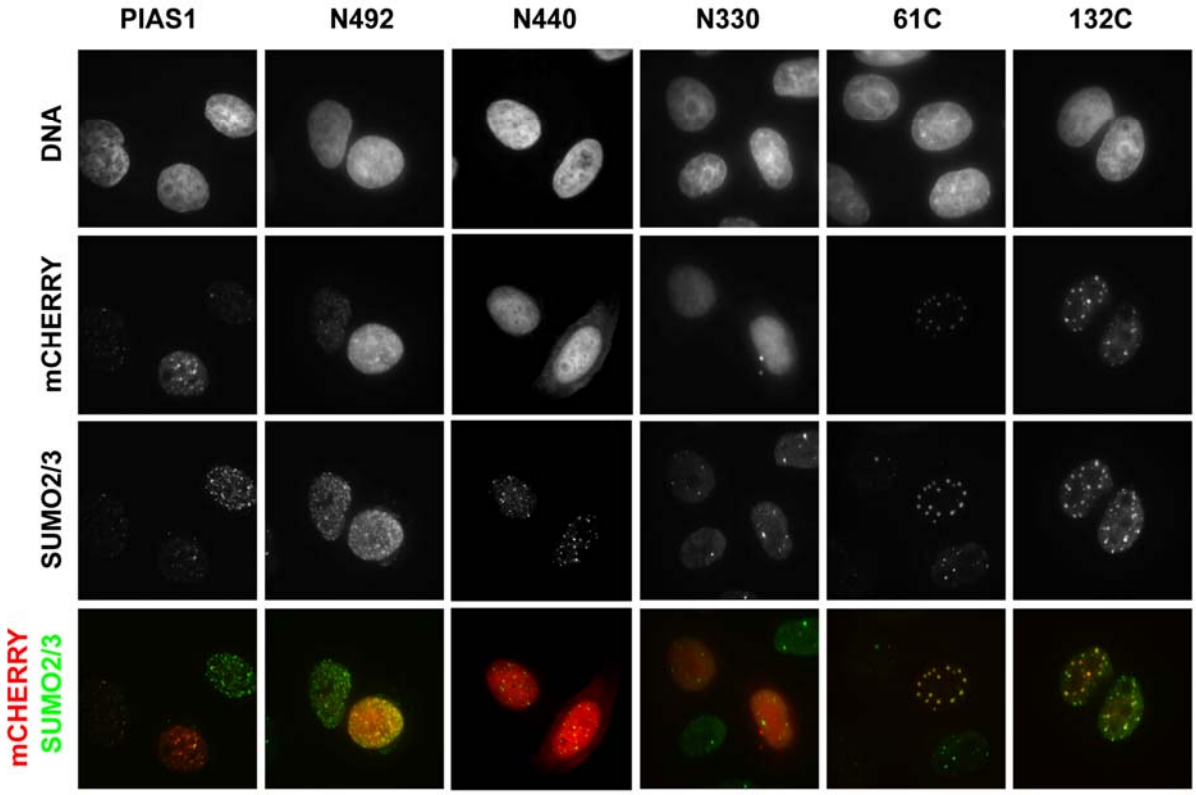
length PIAS1 in Figure 3.1B. As described earlier, PIAS1 forms distinct nuclear foci in cells. PIAS1 N492 mutant displays similar punctate nuclear distribution and colocalization with SUMO-2/3. Both PIAS1 N440 and N330 mutants fail to form foci in HeLa cell nuclei and they also localize in the cytoplasm. We therefore conclude that PIAS1 SIM domain is essential for complete nuclear retention and foci formation. The N-terminal mutants display more interesting nuclear localization. Both the PIAS1 61C and 132C mutants are retained completely in the nucleus where they form more numerous and larger foci than PIAS1 and colocalize with SUMO2/3. Since the PIAS1 N492 mutant looks virtually identical to PIAS1wt in localization and nuclear distribution, and the remaining C-terminus truncation mutants show only homogenous distribution in cells, we further explored only the N-terminal PIAS1 truncations that form nuclear foci distinct from PIAS1wt protein.

Figure 3.1: PIAS1 domain deletion mutants show nuclear localization distinct from PIAS1 wt. (A) Diagrammatic representation of PIAS1 domain deletion mutants. N- and C-terminal truncations were designed to eliminate one or more PIAS1 domains. Each deletion mutant was constructed with a C-terminal mCherry tag as described in the methods section. **(B)** C-terminal mCherry-tagged PIAS1 wt and domain deletion mutants were expressed in HeLa cells for 24 hours. Fixed cells were stained with anti-SUMO-2/3 antibody and colocalization of the deletion mutants with SUMO-2/3 was analyzed. Images were acquired using a confocal microscope and a 100X objective.

3.1A



3.1B



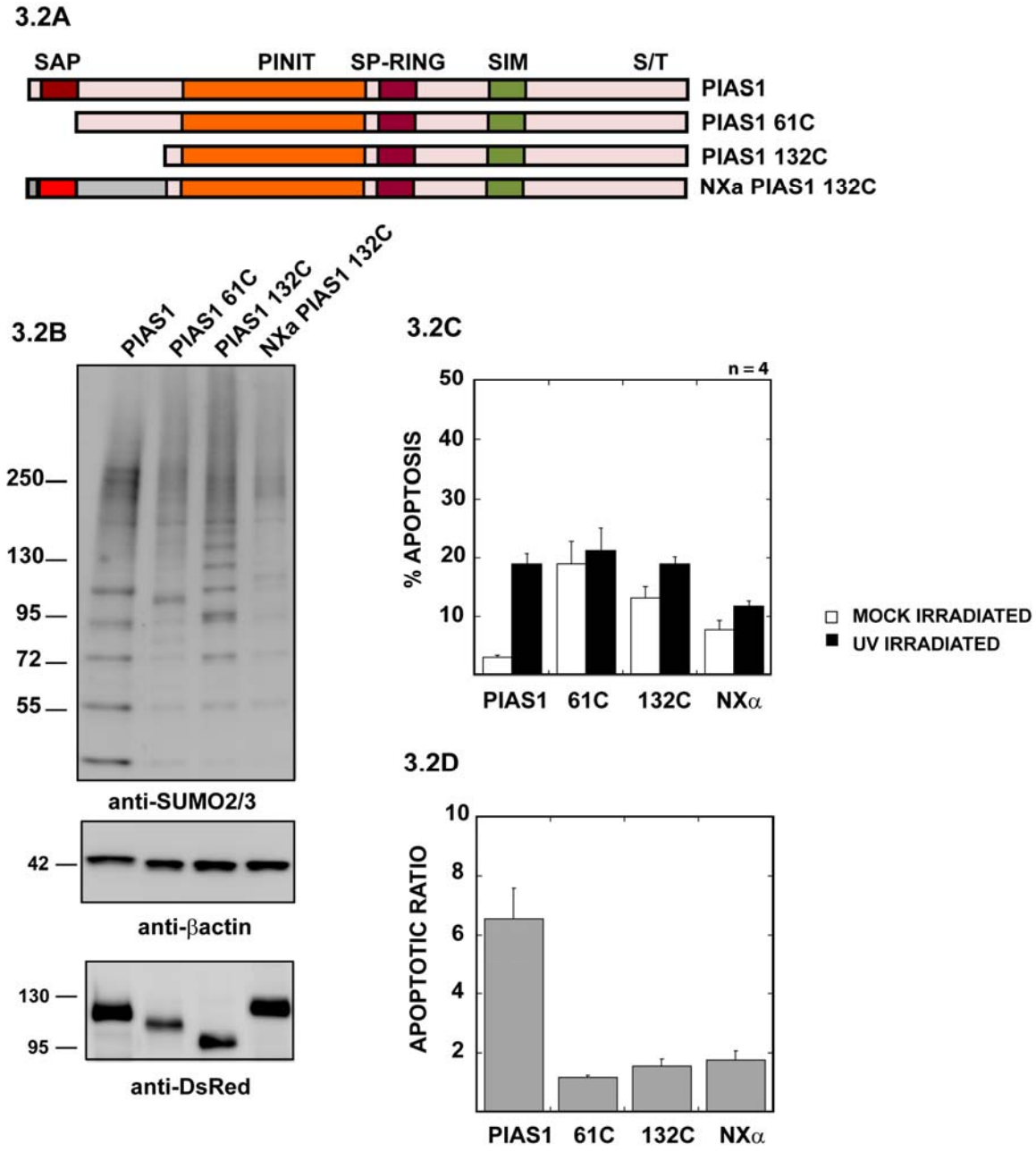
PIAS1 N-terminus is required for UV-induced hypersensitivity

The SUMO substrate selectivity of PIASes can stem from their distinct localizations within the cell (*4, 18*). We have shown earlier that PIASy localization is determined by its N-terminal SAP

domain. We hypothesized that the PIAS1 N-terminus may similarly be important for correct localization and substrate selectivity of PIAS1 and hence for the UV-hypersensitivity displayed by PIAS1-expressing cells. To test our hypothesis, we used the two N-terminal truncation mutants (61C and 132C) and an N-terminal domain-swap mutant of PIAS1 (NXα PIAS1 132C) that has the N-terminal 155 amino acids of PIASXα fused before the PIAS1 132C truncation mutant (Fig. 3.2A). We expressed these PIAS1 mutants in HeLa cells and sorted the cells as earlier to compare the SUMOylation profile for the mutants and PIAS1wt by immunoblotting for SUMO-2/3. SUMO-2/3 modifications appear less efficient in the mutants, with many of the higher molecular weight bands lacking in the deletion mutants (Figure 3.2B). Moreover, the PIAS1 N-terminal deletion mutants do not induce apoptosis in UV-treated cells (Fig. 3.2C). The basal cell death in cells expressing PIAS1 mutants is higher in the mock-irradiated samples, but there is no significant increase in apoptosis four hours after UV irradiation. More significantly, fusing N-terminus of PIASXα to PIAS1 132C truncation mutant does not restore the UV-hypersensitivity of cells. Once again, we calculated the apoptotic ratio by normalizing UV irradiation-induced apoptosis to apoptosis in mock-irradiated cells and we see three-fold more apoptosis in cells expressing PIAS1wt when compared to cells expressing PIAS1 N-terminal deletion mutants (Fig. 3.2D). These results indicate the importance of the PIAS1 N-terminal domain in substrate selection and in UV-hypersensitivity.

Figure 3.2: PIAS1 N-terminal SAP domain is essential for correct localization and UV-hypersensitivity. (A) Diagrammatic representation of PIAS1 N-terminal truncation and domain swap mutant design. The truncation and the PIASXα domain swap mutants were designed with a C-terminal mCherry fusion, as described in the methods section. **(B)** PIAS1 N-terminal is required for correct substrate modification. Cell expressing PIAS1 wt or truncation/swap mutants were sorted based on mCherry signal and the SUMOylation profile was analyzed by immunostaining with anti-SUMO-2/3 antibody. Anti-DsRed antibody marks the mCherry-tagged PIAS1 or PIAS1 truncation mutants and anti-β-actin antibody has been used as a loading control. **(C)** PIAS1 mutants lacking the

PIAS1 N-terminus do not increase apoptosis following UV irradiation. The plotted values are an average of four independent experiments. The error bars represent 1 standard error of the mean. **(D)** Apoptotic ratio was determined by plotting the ratio of the percentage of apoptotic cells after UV-irradiation over the percentage of apoptotic cells in mock-irradiated samples, using values calculated for (C).



In chapter 2, we showed that some of the PIAS1 foci colocalize with PML and some with Daxx, but the two colocalizations are mostly independent of one another. In contrast to PIAS1wt, the

two PIAS1 truncation mutants showed strong colocalization with PML-NBs and disrupted Daxx distribution in cells (Fig. 3.3). PIAS1 61C showed weak colocalization with Daxx in the PML bodies, but PIAS1 132C failed to colocalize with Daxx completely. The NXα PIAS1 132C mutant showed much reduced localization with PML bodies, but also failed to localize significantly with Daxx (Fig. 3.3). These results indicate that the N-terminus of PIAS1 is required for correct localization in the nucleus and for recruitment of Daxx to SUMOylated foci.

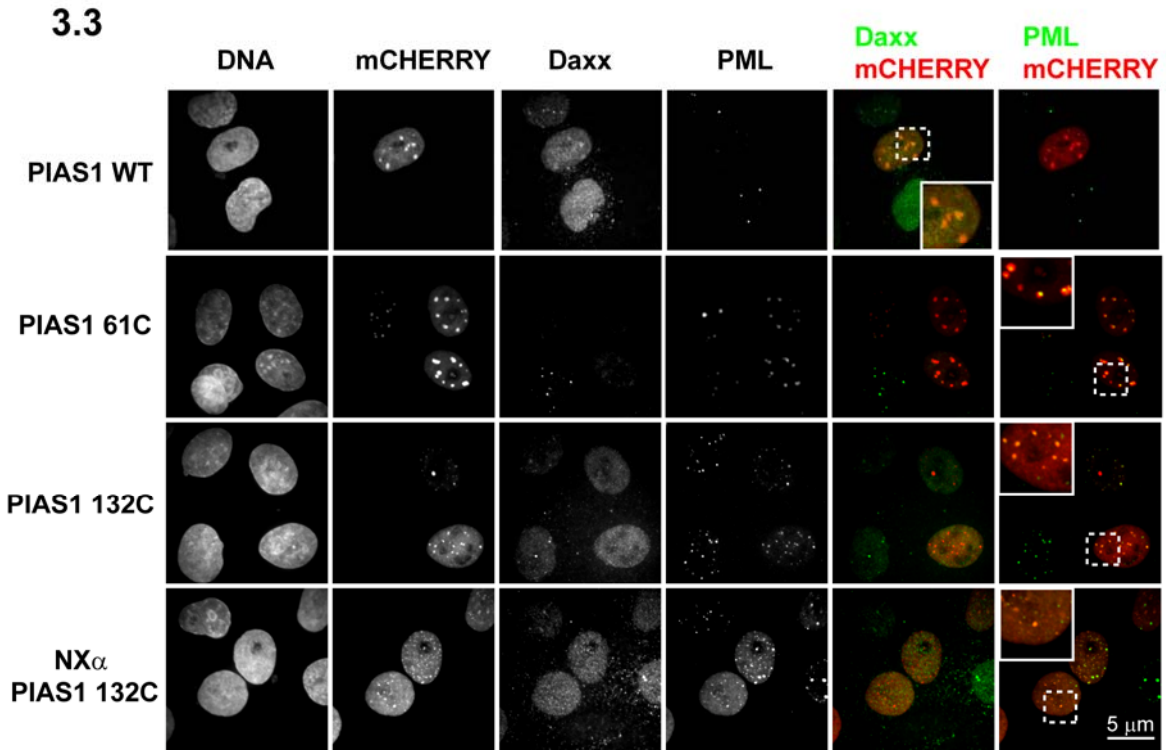
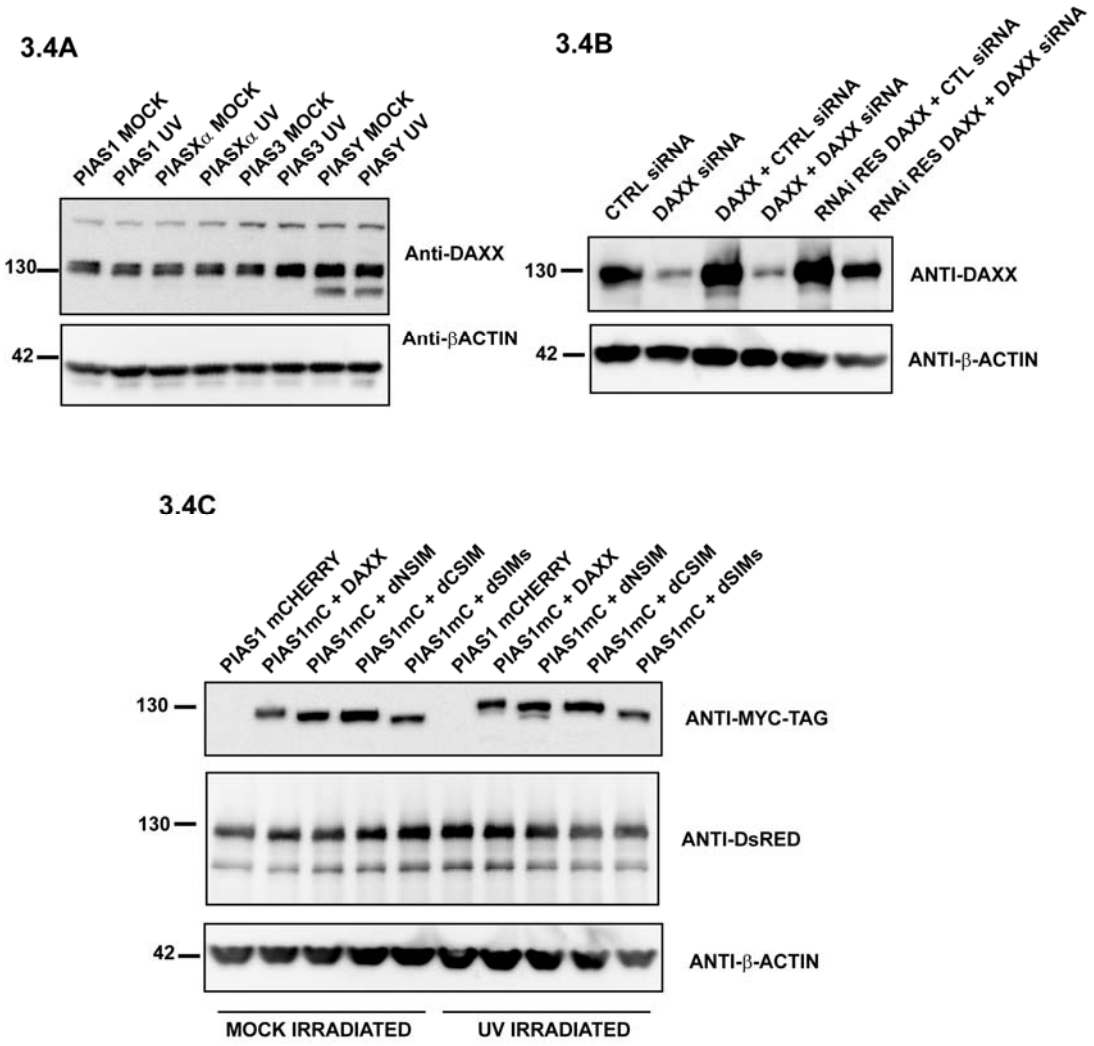


Figure 3.3: PIAS1 N-terminal mutants localize preferentially to PML-bodies but do not colocalize with Daxx. C-terminal mCherry-tagged PIAS1wt, PIAS1 61C, 132C and NXα 132C mutants were expressed in HeLa cells for 24 hours. Fixed cells were stained with anti-Daxx and anti-PML antibodies. Images were acquired using a confocal microscope with a 100X objective.

Daxx C-terminal SIM domain is important for PIAS1-mediated UV-hypersensitivity

Daxx is also known to be SUMOylated in cells. Over 15 different lysines in Daxx can be modified, primarily by SUMO-1 (*11*). Using immunoblotting, we investigated whether any of the PIASes can act as SUMO E3 ligases for Daxx. We could not detect higher molecular weight bands in any of the PIAS-transfected cells, which indicates that Daxx is not a major SUMOylated protein in PIAS-expressing cells (Fig. 3.4A).Daxx has two SUMO Interacting Motifs (SIM); one at each terminus. The SIMs allow Daxx to interact with SUMOylated proteins including PML and a number of transcription factors. The two SIM domains have been shown to display different affinities for SUMO (*12*). In order to understand the requirement of either Daxx SIM domain for PIAS1-mediated UV-hypersensitivity, we first constructed RNAi-resistant Daxx by introducing five silent mutations in the Daxx DNA in a stretch of base pairs targeted by Daxx siRNA. Figure 3.4B shows that this protein is resistant to Daxx siRNA induced degradation. Daxx SIM deletion mutants were constructed from the RNAi-resistant Daxx (Fig. 3.4C) and expressed along with PIAS1 in HeLa cells treated with Daxx siRNA.

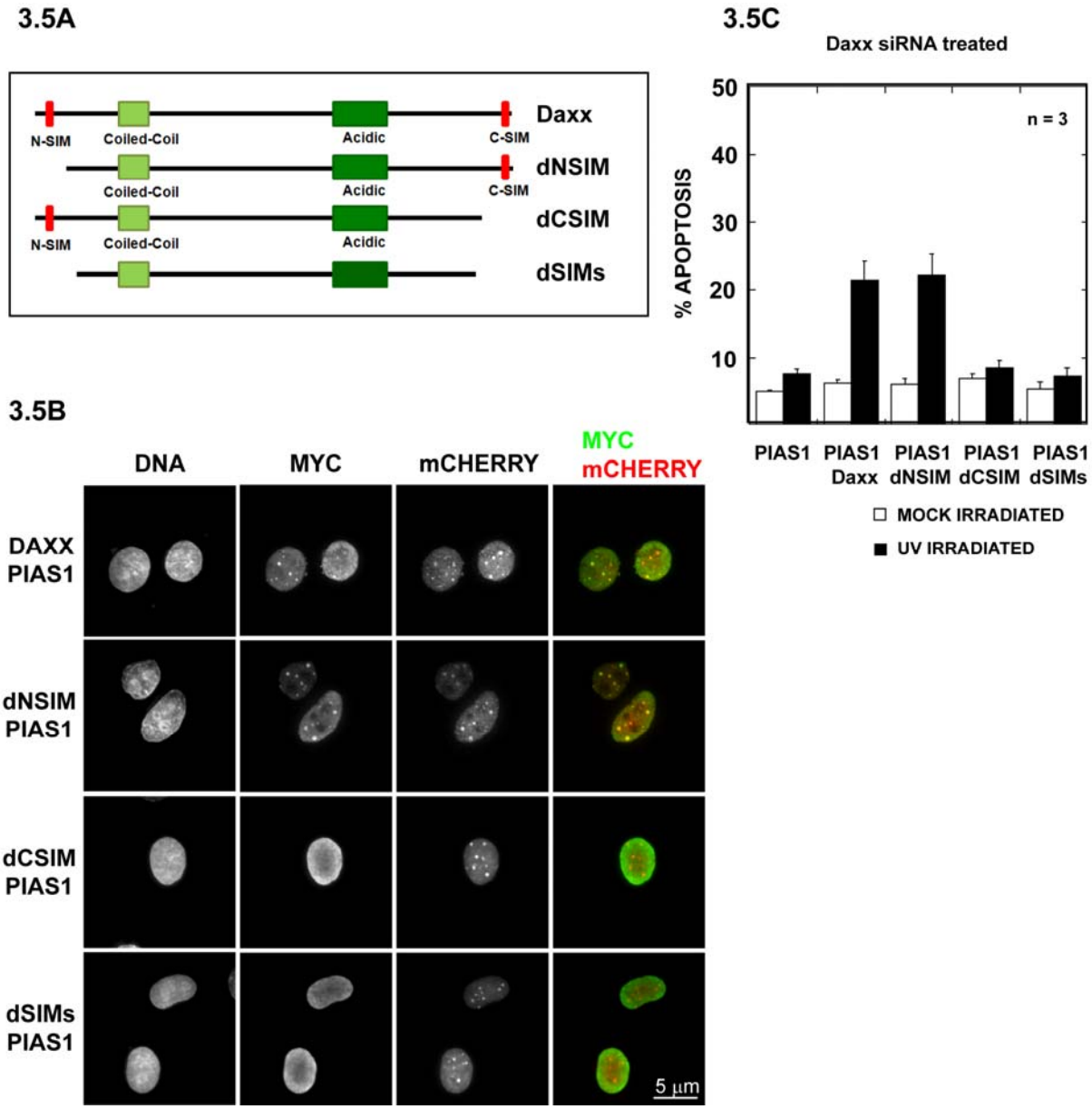
Figure 3.4: Analysis of Daxx SUMOylation and expression of Daxx SIM truncation mutants. (A) Daxx does not appear to be a major SUMOylation substrate. HeLa cells were transfected with each of the PIAS isoforms. Mock and UV-treated cells were sorted based on mCherry signal and lysates were immunostained with anti-Daxx antibody after western blotting. Anti-β-actin was used as a loading control. **(B)** RNAi resistant Daxx was made by introducing five silent mutations in Daxx cDNA in the region affected by siRNA1. HeLa cells expressing empty vector, Daxx, or RNAi resistant Daxx were treated with either control siRNA or Daxx siRNA for 48 hours. Whole cell extracts were probed with anti-Daxx antibody after western blotting. β-actin was used as a loading control. **(C)** Daxx siRNA-treated, mock irradiated or UV-irradiated samples of HeLa cells expressing either PIAS1mCherry alone or in combination with Myc-tagged RNAi-resistant Daxx fl or Daxx SIM deletion mutants were tested for the presence of Daxx and PIAS1 by probing with anti-Myc-tag antibody or anti-DsRed antibody, respectively. β-actin was used as a loading control.



The exogenously expressed Daxx, and Daxx N-terminal SIM deletion mutant (dNSIM), form distinct foci in cells that colocalize with PIAS1 (Fig. 3.5A and B). The Daxx C-terminal SIM deletion mutant (dCSIM) and the Daxx deletion mutant lacking both the SIMs (dSIMs) fail to form distinct foci and thus do not show colocalization with the PIAS1 foci (Fig. 3.5A and B). As expected, PIAS1 expression alone in Daxx siRNA-treated cells did not increase apoptosis following UV irradiation (Fig. 3.5C). We saw a more than two-fold increase in the percentage of apoptotic cells when RNAi resistant Daxx was expressed in addition to PIAS1 in Daxx siRNA-treated cells. Expression of Daxx lacking the dNSIM mutant also increased apoptosis to a similar

extent. However, expression of Daxx lacking the C-terminal SIM (dCSIM) or both the SIMs (dSIMs) failed to increase apoptosis in PIAS1-expressing cells. These results indicate that Daxx C-terminus SIM is important for PIAS1-mediated hypersensitivity to UV irradiation. Together, these results indicate that Daxx is recruited to PIAS1 foci by interaction with PIAS1-SUMOylated substrates via its C-terminal SIM domain and that this recruitment of Daxx, and probably not Daxx SUMOylation itself, is important for PIAS1-mediated UV-induced apoptosis in cells.

Figure 3.5 Daxx C-terminal SIM domain is required for PIAS1-mediated UV-hypersensitivity. (A) Diagrammatic representation of Daxx SIM domain truncation mutants. The truncation mutants were designed as described in the methods section. Daxx dNSIM mutant lacks the first 17 amino acids, dCSIM lacks the final 20 amino acids, and dSIMs mutant lacks both the first 17 amino acids and the last 20 amino acids, thus eliminating both SIM domains from Daxx. All Daxx constructs have an N-terminal Myc-tag. **(B)** Daxx C-terminal SIM domain is essential for proper Daxx localization. HeLa cells transfected with the bicistronic vector carrying PIAS1-mCherry and Myc-Daxx or Myc-Daxx truncations. Fixed cells were stained with anti-Myc-tag antibody. Images were acquired using a confocal microscope using a 100X objective. Scale bar: 5 μm. **(C)** Expression of Daxx C-terminal SIM deletion mutants rescues UV-hypersensitivity phenotype in PIAS1 expressing cells. Apoptotic cells were counted as described in the methods section. The plotted values are an average of three independent experiments. The error bars represent 1 s.e.m.



DISCUSSION

PIASes have highly conserved domain organization, yet show selectivity towards their substrates. Reindle *et al.* (*19*) have shown that in the case of the yeast SUMO E3 ligases Siz1 and Siz2, the localization of the ligases dictates the substrate selectivity. In chapter 2, we showed that the UV-hypersensitivity phenotype is specific to PIAS1. We also showed that each of the PIASes

shows a unique nuclear distribution and appears to have a unique subset of substrates. Based on these results, we hypothesized that PIAS1's distinctive localization and the resulting substrate selectivity are responsible for the PIAS1-specific cellular response to UV irradiation. This hypothesis is supported by the results that the PIAS1 N-terminus truncation mutants that are retained in the nucleus but do not display the same localization pattern as wild-type PIAS1, do not activate apoptosis in response to UV irradiation. This localization-dependent regulation of SUMO ligase activity is consistent with our published results showing PIAS4/y mediates mitotic chromosomal SUMOylation in *Xenopus* egg extract assays (*4, 18*). We have earlier shown that PIASy's centromeric localization to the mitotic chromosome requires it's N-terminal SAP domain (*4*). We show a similar requirement of the PIAS1 N-terminus for its correct localization and SUMOylation activity. Mutants lacking PIAS1 SAP domain localize preferentially to PML bodies and do not display the same SUMOylation profile as PIAS1wt. PIAS1 SAP domain deletion mutants also fail to elicit sensitivity to UV irradiation. The PIAS1 N-terminus domain is specifically required for UV-hypersensitivity; a substitution of the domain with the equivalent domain from PIASXα does show localization similar to PIAS1wt and also does not cause increased cell death following UV irradiation. The PIASy SAP domain specifically binds to the Rod/Zw10 proteins and this interaction is required for localizing PIASy to the centromere in mitotic chromosomes. It will be interesting to see if the PIAS1 SAP domain also has specific binding partner(s) that recruit it to distinct nuclear foci.

In chapter 2 we showed that Daxx is a mediator of UV-hypersensitivity caused by PIAS1 overexpression in cells. Daxx can both interact with SUMOylated proteins and can be SUMOylated in cells. Fifteen different lysine residues can be potentially SUMOylated *in vitro*, however, mutation of these residues has not been shown to have any effect on Daxx's

localization or function as a transcriptional regulator (*11*). On the other hand, the Daxx C-terminal SIM domain has been shown to be essential for recruitment of Daxx to PML bodies and SUMOylated transcription factors (*11*). Our data also suggests that Daxx C-terminal SIM is essential for its recruitment to PIAS1 SUMOylated foci and also for PIAS1-induced UV hypersensitivity. Overexpression of Daxx C-terminal SIM truncation mutant in cells treated with Daxx RNAi leads to a loss of PIAS1-induced UV hypersensitivity. Cells expressing of Daxx N-terminal SIM truncation mutant along with PIAS1 undergo cell death after UV irradiation.

Many different transcription factors are known to be SUMOylated and Daxx has been shown to regulate gene expression by both acting as a repressor and an activator of transcription from SUMOyalted transcription factors. Our results suggest a similar mechanism for PIAS1-induced UV hypersensitivity, where PIAS1-mediated SUMOylation of specific substrates may lead to recruitment of Daxx and changes in gene regulation that sensitize cells to UV irradiation. This premise is further explored in the next chapter.

EXPERIMENTAL PROCEDURES

DNA constructs and cell transfection: PIAS1 truncations were amplified from full length PIAS1 cloned into pCDNA4 his maxC mCherry vector described in chapter 2. The amplified PIAS1 truncation fragments were cloned into the same vector, so that expressed proteins will have a C-terminal mCherry fusion. NXα-PIAS1 132C was constructed by amplifying the DNA encoding the first 155 amino acids of PIASXα and inserting it into the EcoRI site preceding the PIAS1 132C DNA. Daxx cDNA was amplified from HeLa cells and cloned between NheI and EcoRI sites in pEGFPC1 vector (Clontech Laboratories Inc., Mountain View, CA). The Daxx RNAi-resistant mutant was generated by introducing 5 silent mutations in Daxx DNA [(**620**-

aaaaagaattggatctttcagagttagatga-**650**, the mutations are underlined] that disrupt Daxx siRNA (DAXX-HSS102654, Invitrogen, Carlsbad, CA) binding using the Quikchange II XL Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Daxx SIM deletion mutants were created by amplifying Daxx DNA coding for amino acids 18-740 (dNSIM), 1-720 (dCSIM) and 18-720 (dSIMs). A Myc-tagged RNAi-resistant Daxx and Daxx deletion mutants were cloned into the multiple cloning site-A of the bicistronic pIRES vector (Clontech Laboratories Inc., Mountain View, CA). HeLa cells were grown to 50% confluence in McCoy's medium (Mediatech Inc., Manassas, VA) that was supplemented with 10% FBS and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions.

UV irradiation and apoptotic assays: The transfected HeLa cells were either mock-irradiated or irradiated with 30 J/m^2 of UV using an Entela UV crosslinker. In brief, the cells were washed twice with PBS and then irradiated in PBS. After irradiation, the PBS was removed and replaced with growth medium. The cells were analyzed 4 hours or 24 hours after UV irradiation. Cells that were grown on coverslips were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X-100. The cells were then stained with an anti-Parp1 antibody and Hoechst 33342 dye (which stains DNA) for immunofluorescence analysis. Apoptotic cells were counted based on DNA morphology and the re-distribution of Parp1 into the cytoplasm.

Cell sorting and Western blot analysis: HeLa cells that were transfected with mCherry-tagged PIAS family members were sorted from the untransfected cells using a Beckman Coulter MoFlo XDP sorter (Fort Collins, CO) with a 488 nm excitation filter and a 620/29 nm emission filter. The sorting was performed 4 hours after the mock or UV irradiation. The sorted cells were subjected to a Western blot analysis, and the protein levels were normalized by staining for β-actin (Cell Signaling Technology, Danvers, MA).

Immunofluorescence analysis: Transfected and treated cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton-X-100. The cells were then blocked for 15 min with 5% BSA and 2.5% fish gelatin that was prepared in PBST. After washing, the cells were stained with the appropriate antibody following the manufacturer's dilution instructions for one hour, which was followed by staining with an Alexa dye-tagged secondary antibody (Invitrogen, Carlsbad, CA) and Hoechst 33342 dye. The cells were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA), imaged using a DSU-10 spinning disk (Yokugawa-type) confocal microscope that was equipped with an Olympus 100X objective (with a numerical aperture of 1.45), Hamamatsu EMCCD and the Slidebook imaging package (Intelligent Imaging Innovations, Denver, CO). An anti-human Daxx antibody was purchased from Sigma and an anti-PML antibody and the anti-Myc-tag antibody from SantaCruz Biotechnology Inc. (Santa Cruz, CA). The other antibodies that were used in this study were generated in the lab.

Daxx silencing using siRNAs: siRNAs against human Daxx were obtained from Invitrogen (Carlsbad, CA) (siRNA1: DAXX-HSS102654 and siRNA2: DAXX-HSS175936). HeLa cells were transfected with 200pmols of either siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) using the reverse transfection method, as described by the manufacturer. The cells were plated to obtain a next-day confluency of 50%. Twenty-four hours after the siRNA transfection, the cells were transfected with pCDNA4 His maxC PIAS1 mCherry or the bicistronic pIRES contruct expressing PIAS1 and Myc-tagged RNAi resistant Daxx or Daxx truncation mutants. The cells were UV-irradiated 48 hours after the siRNA treatment as described above, and the apoptotic cells were counted 4 hours later.

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CHAPTER 4

Exploring downstream targets of PIAS1: Potential regulation of NFκB pathway

INTRODUCTION

SUMOylation can regulate gene expression via modification of transcription factors, their co-regulators and chromatin modifiers. Other than a few exceptions, SUMO modification of transcription factors has an inhibitory effect on their activity (*1*). PIASes (Protein Inhibitors of Activated Stat) were initially identified as proteins that can inhibit function of members of the Stat family of transcription factors (*2, 3*). PIAS proteins interact with many different proteins, a large number of which are transcription factors. In addition to acting as a SUMO E3 ligase for many of these transcription factors, PIASes have also been shown to regulate the activity of some transcription factors in a SUMO-ligase independent manner (described in Chapter 1) (*4*). Thus, PIASes are also transcriptional regulators.

The NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factors are dimers of Rel Homology Domain (RHD) containing proteins and play important roles in regulation of cell survival, immune responses, differentiation and development. The NFκB family consists of five members that can be segregated into two groups. The first group consists of RelA (p65), RelB and c-Rel proteins; the second group includes repressive precursor proteins p105 and p100 that are processed to the transcriptionally active forms p50 and p52, respectively. The p105 protein is constitutively cleaved to its active form p50, whereas the p100 protein is

cleaved only when activating stimuli are received by specific cell surface receptors. These proteins can form fifteen different homo- or heterodimers, only nine of which (RelA:p50, RelA:p52, RelB:p50, RelB:p52, c-Rel:p50, c-Rel:p52, c-Rel:RelA, c-Rel:c-Rel, and RelA:RelA) have DNA-binding and transcription regulation activities (see Figure 4 below). In the canonical pathway, NFκB dimers are held inactive in the cytoplasm by the IκB (Inhibitors of NFκB) family of inhibitory proteins. Activating stimuli leads to phosphorylation of IκB proteins by IκB kinases (IKK) and Ubiquitin mediated degradation that then allows for the translocation of NFκB dimers (mainly RelA:p50) to the nucleus.

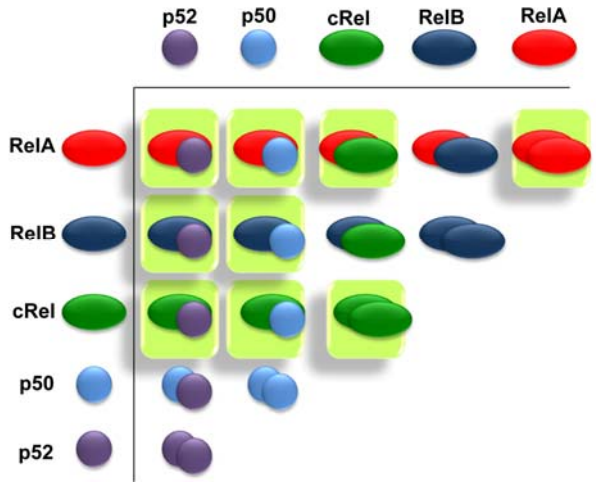

Figure 4.0: **NFκB family of transcription factors.** The members of the NFκB family of transcription factors can form 15 different dimers of which 9 are transcriptionally active (highlighted in yellow). Image modified from Ellen O'Dea and Alexander Hoffman, Regulatory logic of the NF-κB signaling system (2009) Cold Spring Harbor Perspectives in Biology.

The non-canonical or alternate pathway involves the p52 precursor p100 and RelB. Both p105 and p100 proteins contain IκB-like domains in their C-terminus. p100 binds to RelB in the cytoplasm. An activating stimulus leads to stabilization of the NFκB-Inducing-Kinase (NIK) that in turn phosphorylates p100. Phosphorylation causes proteolytic cleavage of p100 to the active p52 form. The RelB:p52 dimer then translocates to the nucleus where it acts as a transcription regulator. The kinetics of the canonical and the non-canonical pathways is very different. The canonical NFκB pathway is a rapid but transient response to activating stimuli. The non-canonical NFκB response on the other hand develops slowly but persists for a longer period (*5*).

Daxx has been shown to repress the transcriptional activity of both RelA (*6*) and RelB (*7*). In the case of RelA, Daxx binding has been shown to inhibit CBP-mediated acetylation and activation of RelA (*6*). Interaction of Daxx with RelB has been shown to repress transcription of a subset of anti-apoptotic genes and involves recruitment of DNA methyl transferase enzyme 1 (DNMT1) to RelB promoters (*7, 8*). Daxx has two SUMO interaction motifs (SIMs) and interacts with and represses many SUMOylated transcription factors, but the role of SUMOylation in the interaction of Daxx with RelA or RelB has not been tested.

Based on the data presented in chapter 2 and 3, we hypothesized that the expression of PIAS1 in cells changes the balance of pro- and anti-apoptotic factors. We tested our hypothesis by analyzing the transcription levels of several pro- and anti-apoptotic proteins. Expression data reveals the NFκB pathway to be an important target of PIAS1 in cells. These results are presented and discussed in this chapter.

RESULTS

Exogenous expression of PIAS1 in HeLa cells affects the transcription of both pro- and anti-apoptotic genes

In order to test our hypothesis that PIAS1 expression imbalances the levels of pro- and anti-apoptotic factors, we first evaluated the levels of transcripts of the Bcl2 family of apoptosis-related proteins. The Bcl2 family consists of both pro- and anti-apoptotic proteins that regulate the intrinsic or mitochondrial pathway of apoptosis. Total RNA was purified from sorted mCherry or PIAS1-mCherry expressing cells. DNA fragments corresponding to the anti-apoptotic (Bcl2, Bcl-xl and Mcl-1) and pro-apoptotic (Bax-α, Bak and Bik) Bcl2 family members was amplified from the cDNA using exon-specific primers restricted to a single exon. Levels of transcripts from mCherry- and PIAS1-mCherry-expressing cells were compared from

three replicates based on their intensity on agarose gels. We did not see differences between the levels of transcripts of any of the Bcl2 family members between mCherry- and PIAS1 mCherry-expressing cells (Figure 4.1A). This result led us to conclude that the Bcl2 family may not be regulated by PIAS1 and Daxx.

In order to analyze a larger set of apoptosis-related transcripts, we used the RT^2 Profiler PCR Array (SA Biosciences/ Qiagen) that can screen for expression levels of 84 apoptosis-related genes. Three independent PCR arrays were performed and only the genes whose expression levels were observed to be changed consistently in each array in response to PIAS1 overexpression were considered for further analysis. The result of this analysis is summarized in Figure 4.1B and Table 4.1 and discussed in further detail in the next subsection. Briefly, we saw higher than three-fold change in the expression levels of a number of apoptosis-related genes from cells expressing PIAS1. More significantly, we saw an increase in the levels of pro-apoptotic transcripts CASP14 and CD40, and a decrease in the levels of anti-apoptotic transcripts BIRC3, XIAP, and a protein important for apoptosis regulation following UV irradiation, Gadd45α. A detailed analysis of these results is presented in the next subsection.

We would like to clarify at this point that the results from the apoptotic PCR array is only being used a guide for designing further experiments. These results will be verified independently by quantitative reverse-transcriptase PCR for each of the genes that show a reproducible change in gene expression and also by analyzing the protein-levels in PIAS1-expressing cells using specific antibodies.

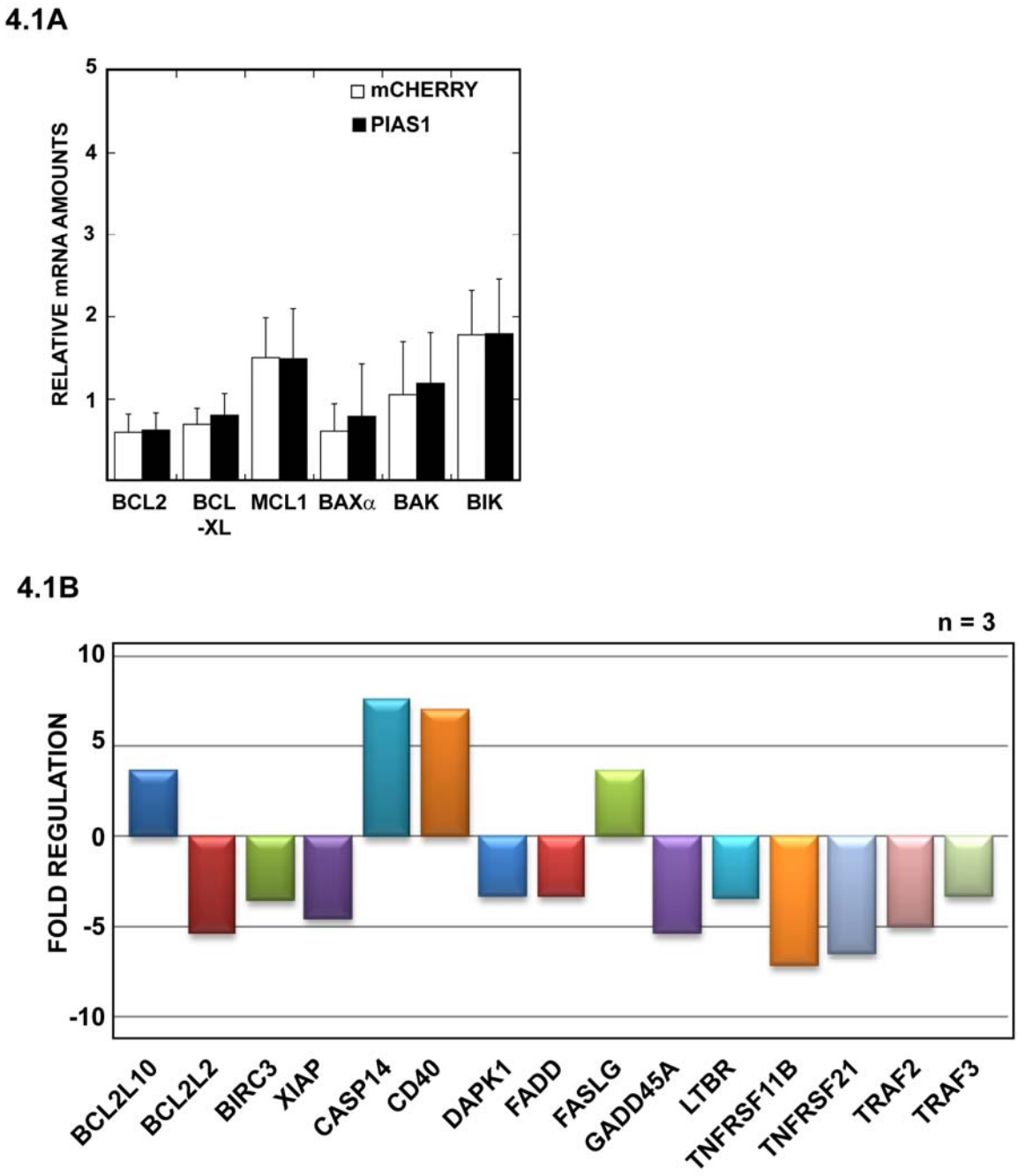

Figure 4.1: PIAS1 expression leads to changes in transcript level of several apoptosis related genes. (A) Relative mRNA amounts of pro- and anti-apoptotic Bcl-2 family genes from cells expressing mCherry or PIAS1 does not vary significantly. HeLa cells transfected with mCherry or PIAS1 were sorted. Total cellular RNA was extracted from cells and used to amplify anti- (Bcl2, Bcl-xl, Mcl1) and pro- (Bax-α, Bak, Bik) apoptotic Bcl-2 family genes. RNA amounts were quantified by measuring band intensities on agarose gel and normalized with β-actin. The plotted values are an average of three independent experiments. The error bars represent 1 s.e.m. **(B)** Apoptosis array data reveals several apoptosis related genes whose transcript level is modulated after PIAS1

expression. mCherry or PIAS1 transfected HeLa cells were sorted, total cellular RNA was purified, and expression of 84 apoptosis related genes was analyzed using real-time PCR analysis. Gene expression was analyzed using web-based analysis software from SA Biosciences. The software performs $\Delta\Delta C_T$ based fold-change calculations from uploaded raw threshold cycle (C_T) data. C_T values obtained from mCherry-expressing cells were used as a control group. Expression data was normalized using house-keeping genes β-2-microglobulin (B2M) and hypoxanthine phosphoribosyl transferase1 (HPRT1). Fold change was calculated as $2^{(-\Delta\Delta CT)}$; all fold change values greater than 1 were reported as fold upregulation and for fold-change values less than 1, the negative inverse of the result was reported as fold downregulation. Three independent experiments were performed, and only genes showing at least a 2 fold change in gene expression are shown in the figure.

Table 4.1: Apoptosis-related transcripts regulated by expression of PIAS1 in HeLa cells.

Gene	Pro/Anti Apoptotic	Fold Regulation	Transcription regulated by NFκB
BCL2L10	**Anti**	**3.66**	No
BCL2L2	**Anti**	**-5.31**	No
BIRC3	**Anti**	**-3.52**	**Yes** (RELB)
XIAP	**Anti**	**-4.56**	**Yes** (RELB)
CASP14	**Anti**	**7.56**	**Yes** (RELB)
CD40	**Pro**	**7.02**	**Yes**
DAPK1	**Pro**	**-3.26**	**Yes** (RELB)
FADD	**Pro**	**-3.3**	No
FASLG	**Pro**	**3.56**	No
GADD45A	**Anti**	**-5.36**	Yes* (see text below) (RELB)
LTBR	**Pro**	**-3.47**	**Yes**
TNFRSF11B	**Anti**	**-7.2**	**Yes**
TNFRSF21	**Pro**	**-6.46**	No
TRAF2	**Anti**	**-5.03**	**Yes**
TRAF3	**Anti**	**-3.34**	**Yes**

PIAS1 expression potentially regulates the NFκB pathway

In chapters 2 and 3, we showed that PIAS1 recruits Daxx to SUMOylated foci and recruitment of Daxx is required for PIAS1-induced UV hypersensitivity. Daxx binds to SUMOylated transcription factors and acts as a co-repressor (*9, 10*). To identify any transcription factors that might be common to the gene transcripts down- or up-regulated by PIAS1 expression in cells, we used a bioinformatics search engine from SA Biosciences that catalogs known regulatory elements upstream of genes included in their microarrays. We found that 9 out of 15 genes whose expression levels changed consistently in response to PIAS1 overexpression are known to be directly regulated by the NFκB family of transcription factors (see Table 4.1). Expression of four of the genes, Birc3, XIAP, DAPK (*7, 8*), and Caspase14 (*11*), has been shown to be regulated by RelB. Of these, Birc3 and XIAP are anti-apoptotic whereas DAPK is pro-apoptotic. Caspase14 is expressed in differentiated keratinocyte and plays a protective role against UV radiation (*12, 13*).

Two other down-regulated genes that are regulated by NFκB at the transcriptional level, TRAF2 and TRAF3 (Tumor necrosis factor receptor associated factor), have been shown to be important regulators of the non-canonical NFκB (RelB) pathway. TRAF2 and TRAF3 form a multi-component Ubiquitin ligase (TRAF2-TRAF3-cIAP E3 ligase) that negatively regulates the levels of NIK (NFκB inducing kinase) in the cells. Receptor stimulation leads to degradation of the Ubiquitin ligase components. This reduces NIK turnover in cells and increases the levels of NIK. NIK-induced p100 phosphorylation and subsequent proteolytic processing generates the active p52 that translocates with RelB to the nucleus. Genetic deficiencies in TRAF2 or TRAF3 cause accumulation of NIK and aberrant p100 processing (*5*). Thus, RelB translocation might be upregulated in PIAS1 expressing cells due reduced levels of TRAF2 and TRAF3.

TNFRSF11b (tumor necrosis factor receptor superfamily, member 11b) acts as an anti-apoptotic protein by inhibiting TRAIL binding to its receptors (TRAIL-R1/R2) (*14*). TRAIL has been shown to activate apoptosis in response to UV irradiation (*15*). TNFRSF11b also acts as an inhibitor of RANKL (Receptor activator of NFκB ligand)-RANK interaction by sequestering RANKL. Both RANK and CD40 receptors activate non-canonical NFκB pathway strongly by binding to TRAF3 and inducing NIK stabilization (*16*). Thus, a downregulation of TNFRSF11b, which itself is regulated by NFκB at the transcriptional level, can both sensitize cells to UV and upregulate RelB translocation to the nucleus.

Gadd45α is a member of the GADD family (Growth arrest and DNA damage inducible) of genes that have been shown to be important modulators of DNA damage response and act by initiating growth arrest, DNA repair and regulating cell death or survival (*17*). Gadd45α expression is stimulated by UV irradiation. Both the canonical and the non-canonical NFκB pathways can downregulate Gadd45α transcription indirectly by increasing c-Myc transcription. c-Myc acts as a transcriptional co-repressor of C/EBP-α mediated Gadd45α transcription (*17*). In different studies, Gadd45α has been shown to both activate and suppress apoptosis by activation of p38 MAPK pathway in a cell-type specific manner (*18, 19*). Interestingly, Gadd45α mediated suppression of apoptosis by p38 activation also involves the activation of the canonical NFκB pathway (*20*).

Taken together, our data suggest that the NFκB pathway is a potential regulatory target of exogenous PIAS1 expression in cells. Changes in the NFκB pathway may contribute directly to PIAS1 induced UV hypersensitivity.

RelB interacts with PIAS1 and Daxx in HeLa cells overexpressing PIAS1

Since our PCR array data point to an involvement of the NFκB pathway in PIAS1 mediated UV hypersensitivity, we decided to test for a direct interaction between PIAS1 and NFκB members. We expressed PIAS1-mCherry or PIAS1 132C-mCherry (truncation mutant lacking the PIAS1 SAP domain) in HeLa cells and then used the cell-permeable reversible protein cross-linker DTBP (dimethyl 3, 3'-dithiobispropionimidate) to crosslink interacting proteins. We immunoprecipitated crosslinked proteins using anti-PIAS1 or anti-Daxx antibody (Figure 4.2). As expected, we were able to co-immunoprecipitate (Co-IP) Daxx using an anti-PIAS1 antibody that recognizes the C-terminus of PIAS1 from PIAS1 cell lysate. We could also co-immunoprecipitate (IP) significantly lesser amounts of Daxx from PIAS1 132C lysate, though we suspect that this could be due to immunoprecipitation of endogenous PIAS1 along with overexpressed PIAS1 132C truncation. Conversely, we were able to coimmunoprecipitate PIAS1-mCherry with Daxx using anti-Daxx antibody, but not PIAS1 132C mCherry. This agrees with our immunofluorescence data that PIAS1, but not PIAS1 132C, shows significant colocalization with Daxx in cells. We detected minimal amounts of RelA in both anti-PIAS1 and anti-Daxx IP and there are no differences between the PIAS1 and PIAS1 132C lysates. We could detect significant enrichment of RelB in both anti-PIAS1 and anti-Daxx IP from PIAS1 lysate compared to PIAS1 132C lysate (Figure 4.2). (Some RelB was immunoprecipitated non-specifically with rabbit IgG. Although we could immunoprecipitate PIAS1 132C less efficiently compared to PIAS1wt using PIAS1 antibody, we do see an enrichment of RelB in IP from PIAS1-lysate over the background level. RelB co-immunoprecipitated with PIAS1 132C is not significantly higher than the non-specific immunoprecipitation we see with IgG). This result shows that there is significant association of RelB with Daxx and PIAS1 in cells exogenously

expressing PIAS1. Overall, our data suggests that exogenously expressed PIAS1 is able to recruit RelB and Daxx to a common focus where these proteins potentially interact.

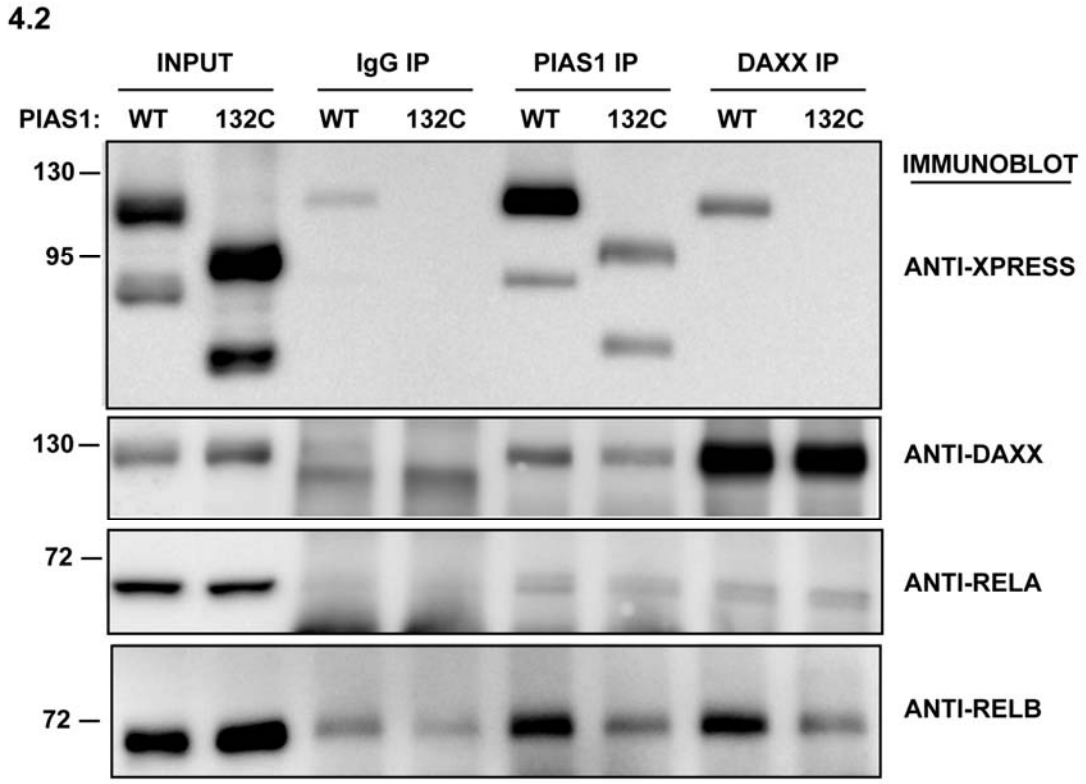

Figure 4.2: The NFκB family protein RelB interacts with PIAS1 and Daxx in HeLa cells expressing PIAS1. Cells expressing PIAS1 or PIAS1 132C deletion mutant were treated with the cell permeable reversible crosslinker DTBP before lysis. Immunoprecipitation was performed using anti-PIAS1 antibody or anti-Daxx antibody and rabbit IgG was used as control. Anti-PIAS1 antibody could co-immunoprecopitate RelB in more amounts from PIAS1-expressing cell lysate compared to PIAS1 132C lysate. Similarly, anti-Daxx antibody could co-immunoprecipitate more RelB from PIAS1 lysate than from PIAS1 132C lysate. RelA was immunoprecipitated at similar levels by anti-PIAS1 and anti-Daxx antibodies from both PIAS1 and PIAS1 132C lysates.

DISCUSSION

Our data suggests that PIAS1 interacts with NFκB pathway protein RelB in cells. We see significant differences in PIAS1:RelB and Daxx:RelB interaction from cells expressing the

PIAS1wt or PIAS1 132C mutant. RelB shows a stronger interaction with PIAS1wt and also with Daxx in PIAS1wt expressing cells. Thus RelB appears to be a relevant target of exogenously expressed PIAS1 in cells.

The canonical NFκB pathway is known to be regulated by PIAS1 (*21*). RelA DNA binding activity is inhibited by PIAS1. This inhibition requires PIAS1 SUMO E3 ligase activity, although PIAS1 itself has not been shown to SUMOylate RelA (*21*). Instead, RelA was shown to be SUMOylated by PIAS3 and this modification results in repression of NFκB transcriptional activity (*22*). On the other hand, PIAS1-null cells show increased expression of certain RelA regulated genes (*21*). Daxx also regulates RelA transcriptional activity, though the mechanism described does not involve RelA SUMOylation (*6*). Our data shows that both PIAS1 and the PIAS1 132C mutant is able to interact with endogenous RelA and that interaction of endogenous Daxx with RelA is not affected by the expression of either the wildtype or the mutant PIAS1 (Figure 4.2).

While our data does not explicitly prove the involvement of NFκB in PIAS1-induced UV hypersensitivity, the gene expression data does suggest that PIAS1 expression leads to an altered transcriptional state that sensitizes cells to UV irradiation. We showed that many of the genes whose expression is altered in PIAS1-expressing cells are transcriptionally controlled by NFκB, or specifically, by RelB. The current model of PIAS1-mediated regulation presents only a repressive interaction between PIAS1 and NFκB. Interestingly, only a subset of κB promoters are subjected to regulation by PIAS1 (*23*). It is not currently understood what drives this gene-selective regulation. Non-canonical κB promoters have been described (*24*) and a subset of these might be specific for PIAS1-dependent regulation. Further analysis of this will require chromatin

immunoprecipitation-sequencing (ChIP-Seq) assays using both anti-PIAS1 and anti-RelB antibodies.

RelA is known to be induced by UV radiation. RelA translocates into the nucleus and binds to DNA in response to UV irradiation (*25*). A role for RelB in UV response has not been described, but RelB regulates transcription of anti-apoptotic factors and thus, has a role in cell survival (*7*). Substantial cross-talk occurs between RelA and RelB and each one can regulate the activity of the other (*26, 27*). It is possible that both RelA and RelB are induced in PIAS1-expressing cells and that they regulate each other. Suppression of RelA-regulated genes (such as TRAF2, TRAF3) might increase nuclear translocation of RelB; RelB can then be regulated in the nucleus by Daxx and also directly by PIAS1. RelB has not been shown to SUMOylated, but there are potential SUMOylatable lysines on RelB that can be analyzed for PIAS1-directed SUMOylation. In this regard, generation of RelB mutants that cannot interact with PIAS1 will be valuable for further analysis role of PIAS1 in regulation of RelB.

Finally, since we see changes in the expression of both RelB regulated genes and genes that regulate RelB activation, PIAS1-mediated regulation of NFkB pathway will be complex with active negative and positive feedback loops. Discerning the sequence of events leading to the regulation of RelB activity will be essential for determining the mechanism of PIAS1 induced UV-hypersensitivity.

EXPERIMENTAL PROCEDURES

RNA Extraction and Bcl2 family gene expression analysis: Total cellular RNA was extracted from sorted mCherry-expressing or PIAS1mCherry-expressing cells using the cell lysis buffer from Signosis Single Cell RT-PCR Assay kit (Signosis Inc., Sunnyvale CA) as described in the

manufacturer's protocol. Expression of Bcl2 family genes was analyzed using the TaKaRa Inc. ApoPrimer Set (Bcl2 family) (Clontech Labs, Mountain View, CA) following manufacturer's protocol. Expression of β-actin was used as a control for normalizing gene expression. Transcript amounts was analyzed by measuring the intensity of bands on agarose gel following electrophoresis using Image Station 4000R (Kodak).

Apoptotic genes PCR Array: RT^2 PCR Array that can screen for transcript levels of 84-apoptosis related genes was obtained from SABiosciences/Qiagen. PIAS1mCherry- or mCherry-expressing HeLa cells were sorted and total RNA extracted using Qiagen RNeasy kit. 1μg of RNA was used in a reverse transcription reaction to generate cDNA using the Qiagen RT^2 First Strand kit. PCR Array was used as per manufacturer's protocol using reagents specified by the manufacturer. Applied Biosystem's Step One Plus Real-Time PCR machine was used for amplification. Expression data was analyzed using web-based analysis software from SABiosciences (sabiosciences.com/pcrarraydataanalysis.php). $\Delta\Delta C_T$ based fold change calculation was used to identify genes with changes in gene expression. Genes that showed fold-regulation ($2^{\Delta\Delta C_T}$) value of 2 or higher in three independent arrays were considered for further analysis.

Cross-linked Immunoprecipitation: HeLa cells were transfected with PIAS1wt or PIAS1 132C expression vectors at approximately 60-70% confluency (10cm culture dishes). 24 hours after transcfection, cells were washed twice with PBS, pH8.5 and incubated in 5mM DTBP (Dimethyl 3,3´-dithiobispropionimidate•2 HCl; Thermo Fisher Scientific, Rockford, IL) in PBS, pH 8.5 for 15 min at room temperature. Cells were washed twice in PBS and incubated in DTBP quenching buffer (100mM Tris HCl, pH 8.0, 150mM NaCl) for 10 min. Cells were washed with PBS, scraped off the dishes and pelleted. Cells were then lysed in RIPA buffer (50mM Tris, pH8.0,

150mM NaCl, 1% NP40, 0.5% Na.deoxycholate, 0.1% SDS) for 30 min at 4C, sonicated to disrupt DNA and then incubated with rabbit IgG, anti-PIAS1 antibody or anti-Daxx antibody for 1 hour at 4C. Dynabeads coupled to Protein-A (Invitrogen, Carlsbad, CA) was added to the lysate and antibody solution and incubated overnight at 4C. Beads were washed four-times with a series of wash buffers (RIPA, RIPA+250mM NaCl, PBS-T, PBS-T+250mM NaCl, PBS) for 5 min each at room temperature and the bound proteins were eluted by adding 1X SDS-PAGE buffer and heating at 95C for 10 min with vigorous vortexing every 5 min.

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CHAPTER 5

PIAS1-induced UV-hypersensitivity: current understanding and future perspectives

UV radiation induces apoptosis in cells

Apoptosis is an essential process for both the regular turnover of old and damaged cells as well as for regulated differentiation and development of multicellular organisms. Loss of regulated apoptosis leads to various degenerative diseases as well as tumorigenesis. Cells undergoing apoptosis round up and shrink; the chromatin condenses and degrades and nuclear envelope-enclosed nuclear bodies are generated; cells fragment into apoptotic bodies, the phosphatidylserine, which is normally present in the inner leaflet of the plasma membrane, is exposed on the surface of apoptotic bodies and triggers engulfment by macrophages (*1*). Apoptosis progresses through both extrinsic and intrinsic pathway. Although the two pathways are biochemically distinct, there is a lot of crosstalk between them (*2*). The extrinsic pathway is initiated by binding of cell surface death receptors with their cognate ligands (*3*). Clustering of receptors and ligand binding leads to recruitment of cytoplasmic adaptor proteins, such as FADD (Fas activated death domain), that in turn recruits procaspase-8. Together, this large complex called the Death Inducing Signaling Complex (DISC) (*4*) leads to autocatalytic activation of pro-caspase8. The intrinsic pathway is also called the mitochondrial pathway. Triggering of the intrinsic pathway leads to a loss of mitochondrial transmembrane potential, opening of the mitochondrial transition pore and release of certain pro-apoptotic proteins from the intermembrane space, including cytochrome-c. Cytochrome-c release is regulated by the Bcl-2

family of proteins that include both pro- and anti-apoptotic proteins. Cytochrome-c binds Apaf-1 (Apoptotic protease activating factor-1) which in turn recruits procaspase9 and leads to its proteolytic cleavage and autoactivation. Caspase8, caspase9 and caspase10 lead to the activation of the effector caspases such as caspase3, caspase6 and caspase7. The effector caspases in turn activate endonucleases that cause DNA fragmentation as well as proteases that degrade cytosolic and nuclear proteins (*5*).

UV radiation (UVR) can activate either the DNA repair pathway or apoptosis in cells. UVR is a short wavelength electromagnetic radiation that is somewhat arbitrarily divided into three components; UV-A (320-400 nm), UV-B (290-320 nm) and UV-C (200-290 nm). UV-B and UV-C cause lesions in DNA by forming cyclobutane pyrimidine dimers and (6-4) pyrimidine pyrimidone photoproducts. These lesions can be repaired by the Nucleotide Exchange Repair (NER) pathway. UVR can trigger both the extrinsic and intrinsic pathways of apoptosis. The intrinsic pathway is triggered by DNA damage, may be p53-dependent or -independent depending on the cell type, and involve DNA-mismatch repair system (MMR) (*1, 6*). UVR can also cause activation of Fas, TNF-R1 and TRAIL (TNF Receptor Apoptosis Inducing Ligand) receptors, and thus trigger the extrinsic pathway. UV is also known to induce the expression of the Fas ligand (FasL) (*7*). Although UVR triggers both the extrinsic and the intrinsic pathways of apoptosis, the two pathways act in an independent and additive manner. Inhibition of either the DNA damage induced pathway by expression of photolyase in cells, or the extrinsic pathway by blocking receptor clustering at low temperatures results in only a partial inhibition of apoptosis. However, DNA damage appears to be a more potent inducer of apoptosis (*6, 8*).

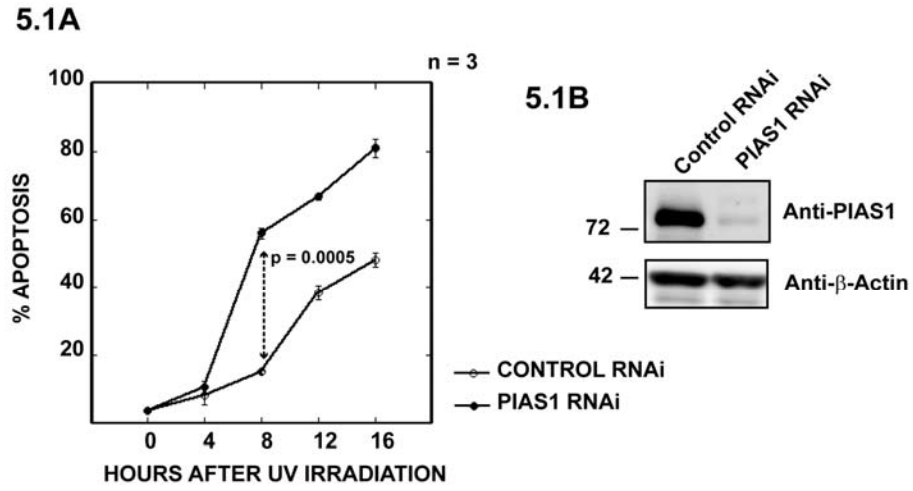
UV-B and UV-C also modulate transcription activity in cells by activating a number of transcription factors including p53, AP-1 (c-fos and c-jun) and NFκB (*9*). Activation of p53 leads to induction of p21 and cell cycle arrest (*10, 11*). Transcription factors c-Jun and c-fos are activated by phosphorylation mediated by upstream kinases c-Jun N-terminal Kinase (JNK) and p38 mitogen activated protein kinases (MAPKs). JNK activation is associated with increased sensitivity of cells to UVR (*12*) and does not require damage to DNA (*13*). The canonical NFκB is also induced by UVR (*13*) in a DNA damage independent manner. UVR causes degradation of the inhibitory cytosolic protein IκBα and nuclear translocation and DNA binding by RelA (*9*). UV-C has been shown to induce RelA to act as a repressor of certain anti-apoptotic genes such as XIAP and Bcl-xl (*14*).

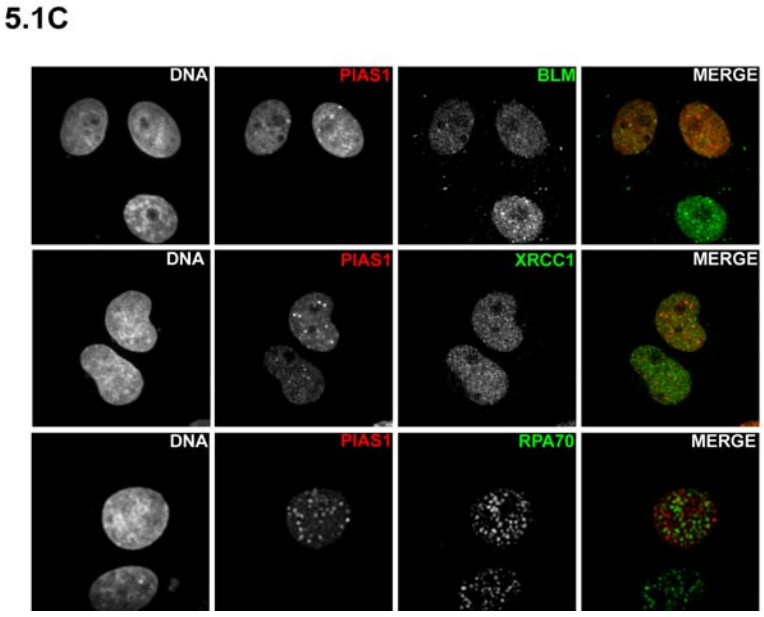
PIAS1 plays an important role in UV-induced apoptosis

In this dissertation, we showed that exogenous expression (Figure 2.1) of SUMO E3 ligase PIAS1 causes increased sensitivity of cells to UVR. PIAS1 SUMO E3 ligase activity is essential for increased sensitivity to UV as a ligase-dead mutant of PIAS1 fails to elicit this response in HeLa cells (Figure 2.3). Using immunoblot, we compared the SUMOylation profile of the PIAS family by overexpressing them in cells. We observed several distinct SUMO-modified bands on expression of each of the PIASes in cells (Figure 2.2B). Also, each of the PIASes shows distinct nuclear localization and foci formation and colocalization with both SUMO-1 and SUMO-2/3 (Figure 2.2A). We showed that the PIAS1 N-terminus SAP domain as well as the SIM domain is essential for its correct localization and substrate selection in cells (Figure 3.2). Thus, PIAS1-induced hypersensitivity to UV may be mediated by distinct substrates SUMOylated by PIAS1 expression in cells. To further understand the role of PIAS1 in UV-induced apoptosis, we

depleted PIAS1 from HeLa cells using siRNA (Figure 5.1A). Interestingly, depletion of PIAS1 in HeLa cells using RNAi also caused increased apoptosis following UVR, although the kinetics of apoptosis induction is different. Increased apoptosis was seen 4 hours after UVR in cells exogenously expressing PIAS1 whereas in PIAS1-depleted cells, apoptosis occurred 8 hours after UVR. This difference in kinetics could reflect different pathways being targeted by PIAS1. As discussed earlier in this chapter, UVR initiates a DNA damage response, modulates transcriptional activity and activates apoptotic pathways in cells. We first tested some of the known DNA damage response pathway proteins that are known to be SUMOylated in cells. However, we did not observe distinct colocalization of XRCC1, BLM or RPA70 (*15-17*) with PIAS1 (Figure 5.1B).

Figure 5.1(A) and (B): PIAS1 depletion sensitizes HeLa cells to UV irradiation. PIAS1 RNAi-treated HeLa cells show increased apoptosis after UV irradiation. HeLa cells were treated with PIAS1 siRNA or control siRNA for 48 hours before treatment with 30J/m^2 UV irradiation. Apoptotic cells were counted by Annexin V staining every four hours. PIAS1 siRNA-treated HeLa cells show an almost 40% increase in apoptotic cells 8 hours after UV irradiation compared with control siRNA-treated cells. The plotted values are an average of three independent experiments, and the error bars represent 1 SEM. PIAS1 RNAi reduces the amount of PIAS1 in HeLa cells to less than 10% of the control RNAi treatment. siRNA against PIAS1 was also obtained from Invitrogen (PIAS1 siRNA: PIAS1VHS41400). Cells were transfected with 200 pmol PIAS1 siRNA and UV irradiated 48 hours after transfection. The depletion efficiencies of the target proteins were estimated by immunoblotting followed by measuring the intensity of the bands with Image Station 4000R (Kodak). **(C) PIAS1 does not colocalize with BLM, XRCC1 or RPA70.** PIAS1-mCherry expressing cells were irradiated with 30J/m^2 of UV. The cells were fixed 4 hours after irradiation and immunostained with anti-BLM (Calbiochem), anti-XRCC1 (Santa Cruz Biotechnology) or anti-RPA70 (Abcam) antibodies, followed by Alexa dye-tagged secondary antibodies (Invitrogen) and Hoechst 33342 dye. None of the proteins tested showed colocalization with PIAS1.





Daxx acts as an effector of PIAS1-mediated UV-hypersensitivity

We next aimed to identify the proteins that colocalize with PIAS1 at the PIAS1-SUMO foci as these could potentially interact with PIAS1-SUMOylated substrates and act as downstream effectors of PIAS1-induced UV hypersensitivity. We identified Daxx as an apoptotic protein that localizes significantly at PIAS1 SUMOylated foci but not with other PIASes (Figure 5.2). Daxx is an apoptotic pathway protein that functions as a transcriptional co-regulator for a number of

SUMOylated transcriptional factors (*11*). Daxx has been mainly shown to act as a co-repressor but can also act as a co-activator in some cases (*18-20*). Daxx itself is a known SUMO-modified protein (*21*), but we did not observe a stimulation of Daxx SUMOylation in PIAS1-expressing cells (Figure 3.4A). Thus, although Daxx is recruited to PIAS1-SUMOylated foci, it is not a substrate of SUMO in PIAS1-expressing cells.). Furthermore, PIAS1 interaction with Daxx occurs even in the absence of UV (Figure 5.3).

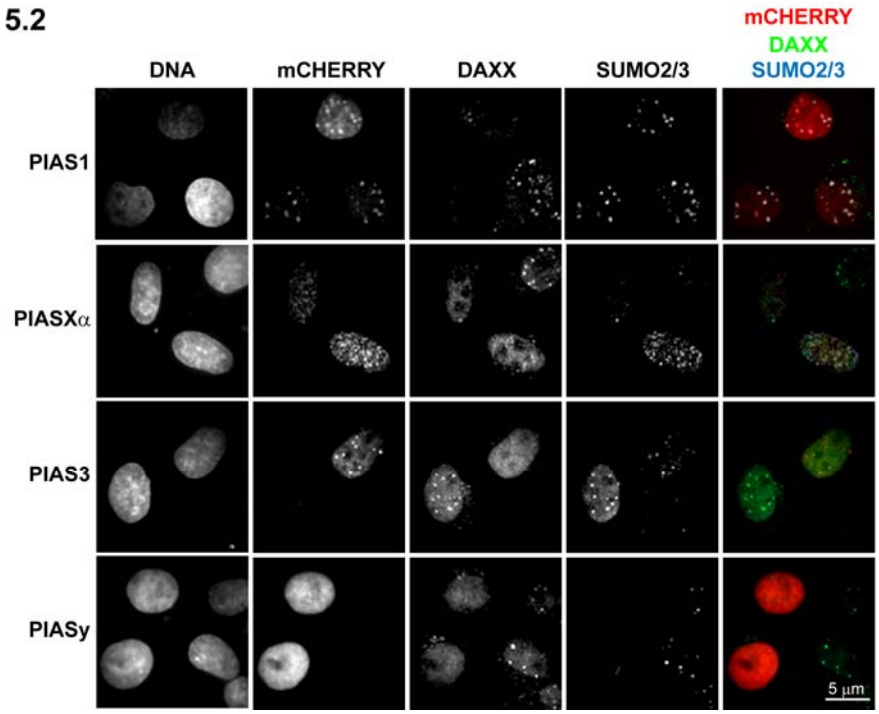
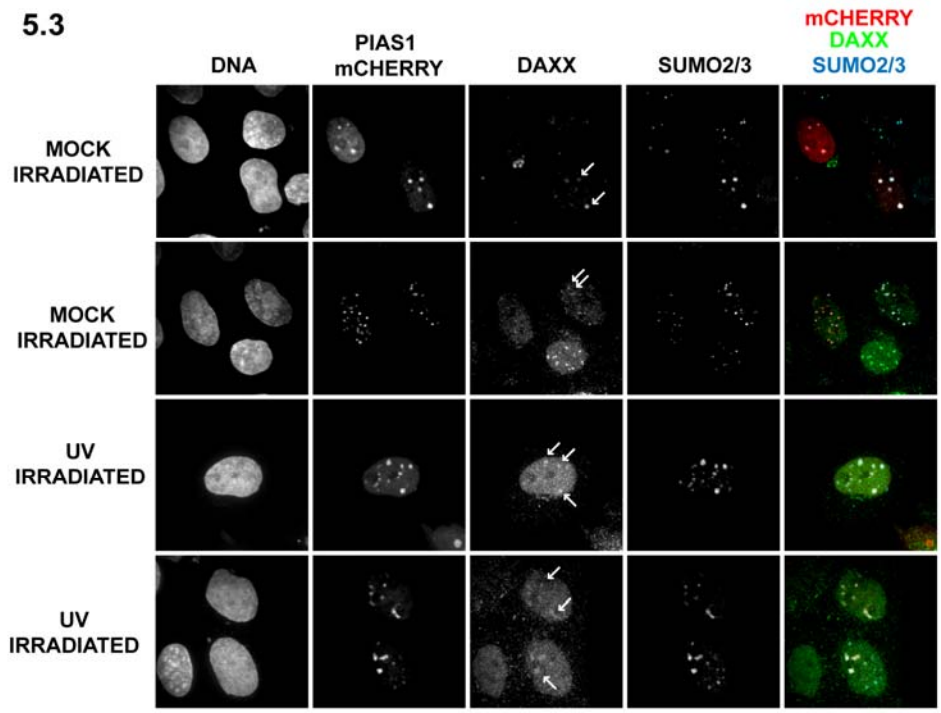


Figure 5.2: PIAS1, but not other PIASes, colocalize with Daxx. The PIAS1, PIAS2/Xα, PIAS3 and PIAS4/y C-terminal mCherry fusion constructs were transfected into HeLa cells. The cells were immunostained with anti-Daxx and anti-SUMO-2/3 antibodies 24 hours after transfection. All PIASes disrupt normal Daxx foci in cells, but only PIAS1 shows significant colocalization with Daxx.

Figure 5.3: PIAS1 and Daxx colocalize in both mock- and UV-irradiated cells. PIAS1 recruits Daxx (indicated by arrows) to SUMOylated foci in both mock- and UV-irradiated cells. PIAS1 localization in the nuclei is not discernibly affected in mock- or UV-irradiated cells.



Daxx has two SUMO interaction motifs, one at either terminus, that allow it to interact with SUMOylated transcription factors and also with SUMOylated PML (promyelocytic leukemia) bodies. Daxx interaction with PML is thought to preclude its transcriptional co-regulator activity. We observed a decrease in PML-Daxx interaction in cells expressing PIAS1, suggesting that Daxx was preferentially recruited to PIAS1-SUMOylated substrates. Of the two SIMs present in Daxx, we identified the C-terminal SIM to be essential for Daxx localization with PIAS1 and distinct nuclear foci formation (Figure 3.5B and 3.5C). Further, we showed that Daxx is required for PIAS1-induced UV-hypersensitivity as cells expressing PIAS1, but depleted in Daxx, showed significant reduction in apoptosis after UVR (Figure 2.5). In light of the data presented so far, we proposed a model wherein PIAS1 expression leads a cell to alter its transcription

profile to a stress-like state. Exogenously expressed PIAS1 mediates SUMOylation of certain substrates which may include transcription factors, co-regulators or chromatin modifiers that lead to recruitment of Daxx and changes in expression of genes that regulate apoptosis (Figure 5.4).

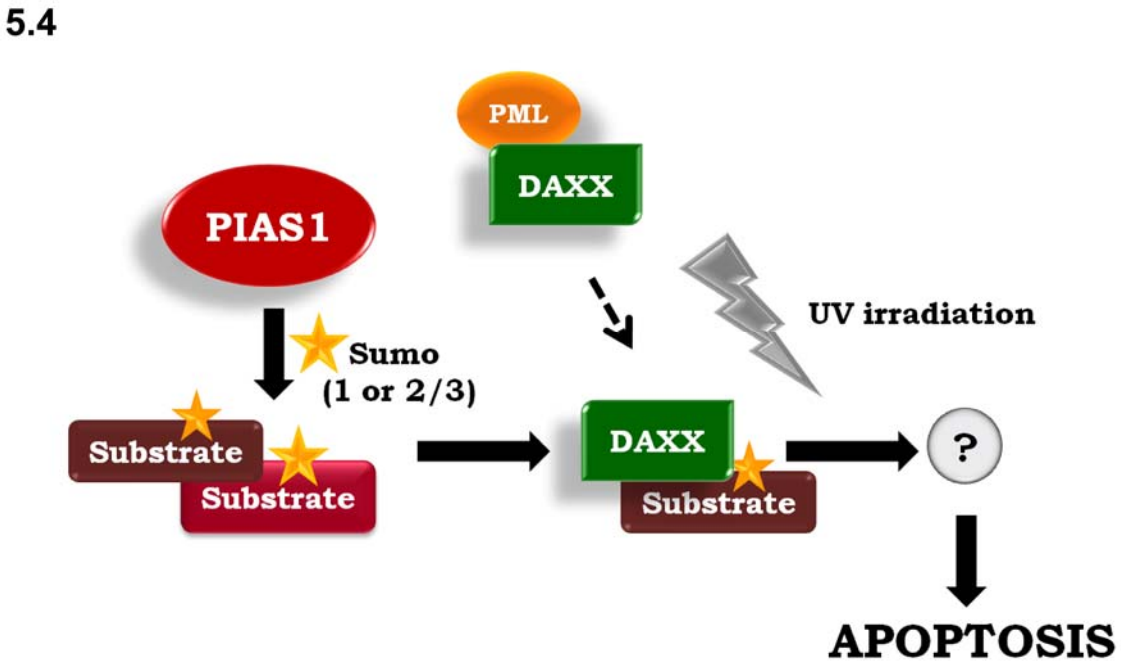


Figure 5.4 PIAS1-directed SUMO-modified substrates recruit Daxx to mediate UV-hypersensitive apoptosis. Based on our data, we propose a model wherein the ectopic expression of PIAS1 promotes SUMOylation of specific substrates. One or more of these SUMOylated proteins recruit Daxx. The Daxx C-terminal SIM domain is necessary for this interaction. The Daxx-mediated corepression of certain transcription factors may then contribute to apoptosis after UV irradiation.

PIAS1 potentially provides an alternative, p53-independent pathway for stress-induced apoptosis

PIASes are known to regulate many transcription factors through SUMOylation and also by SUMO-independent mechanisms (*22*). SUMOylation often represses transcriptional activity by modifying the localization or DNA-binding ability of transcription factors. SUMOylation of

transcription factors, transcriptional co-regulators or chromatin modifiers also leads to formation of repressor complexes (*22, 23*). Tumor suppressor p53, which is an important modulator of cellular response to UV, is also SUMOylated by PIAS1 (*24, 25*). Expression of PIAS1 in both HeLa cells and in Hct116 cells causes increased cell death following UVR (Figure 5.4A and 5.4B). Parental HeLa and Hct116 cells have similar response to UVR and show significant increase in apoptosis between 12 and 16 hours after UVR (Figure 5.4C). Hct116 cells have wild-type levels of p53, whereas HeLa cells do not contain detectable levels of p53 [HeLa cells are cervical cancer cell lines that are positive for Human Papilloma Virus (HPV)-18; the E6 oncoprotein from HPV-18 promotes Ubiquitin-mediated degradation of p53 (*26, 27*)]. Thus, PIAS1-mediated UV-sensitivity appears to be independent of the p53 pathway. As discussed earlier, p53 is an important effector of UV-induced apoptosis. Loss of p53 tumor suppressor activity is seen in most cancers. Thus, a p53-independent pathway to apoptosis in response to DNA damage appears to be an important alternative to ensure elimination of damaged, and potentially carcinogenic, cells.

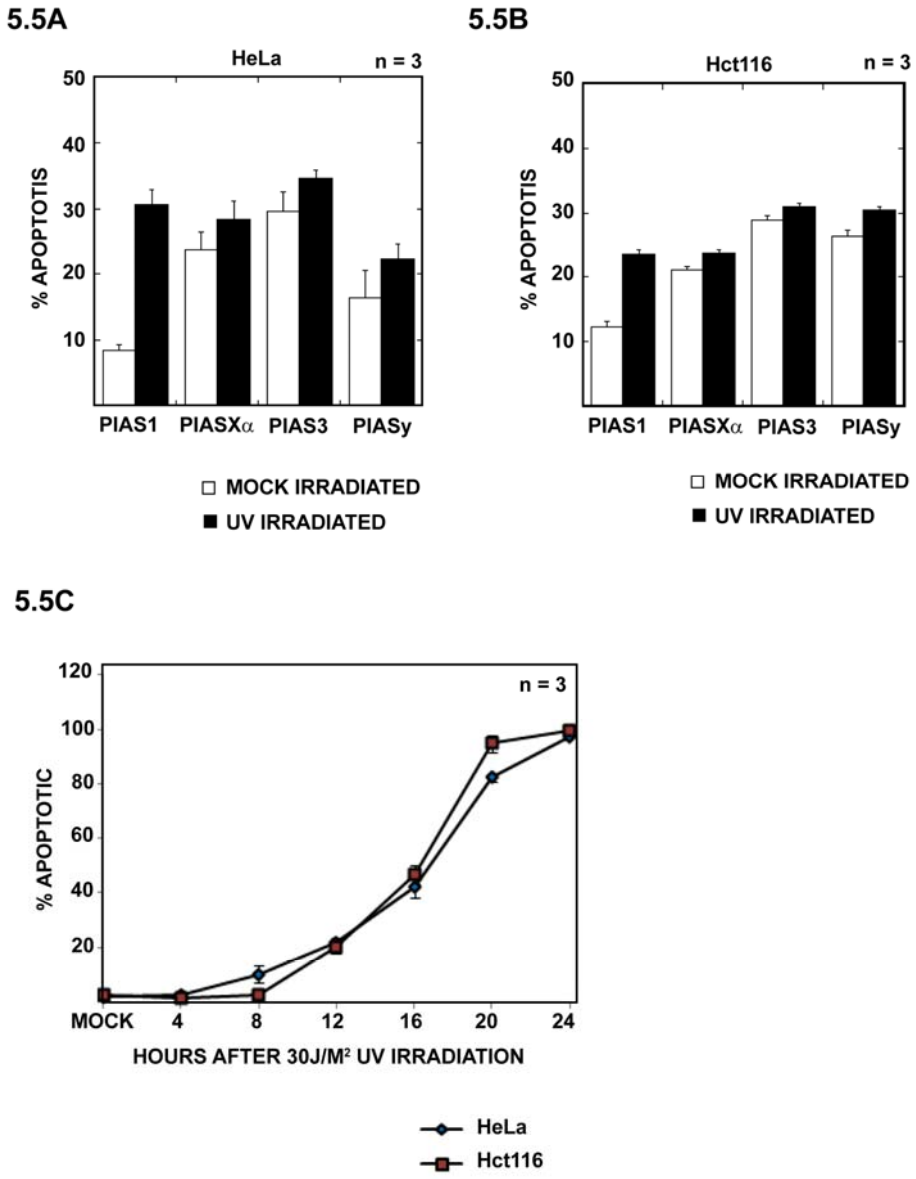


Figure 5.5 Both HeLa and Hct116 cells show PIAS1 induced UV hypersensitivity (A) and **(B)** HeLa or Hct116 cells were transfected with the PIASes, and apoptosis was calculated based on Annexin V staining four hours after mock or UV irradiation. **(C)** Apoptosis induced by UV irradiation in untransfected HeLa and Hct116 cells was determined by Annexin V staining every four hours over a 24-hour period after UV irradiation.

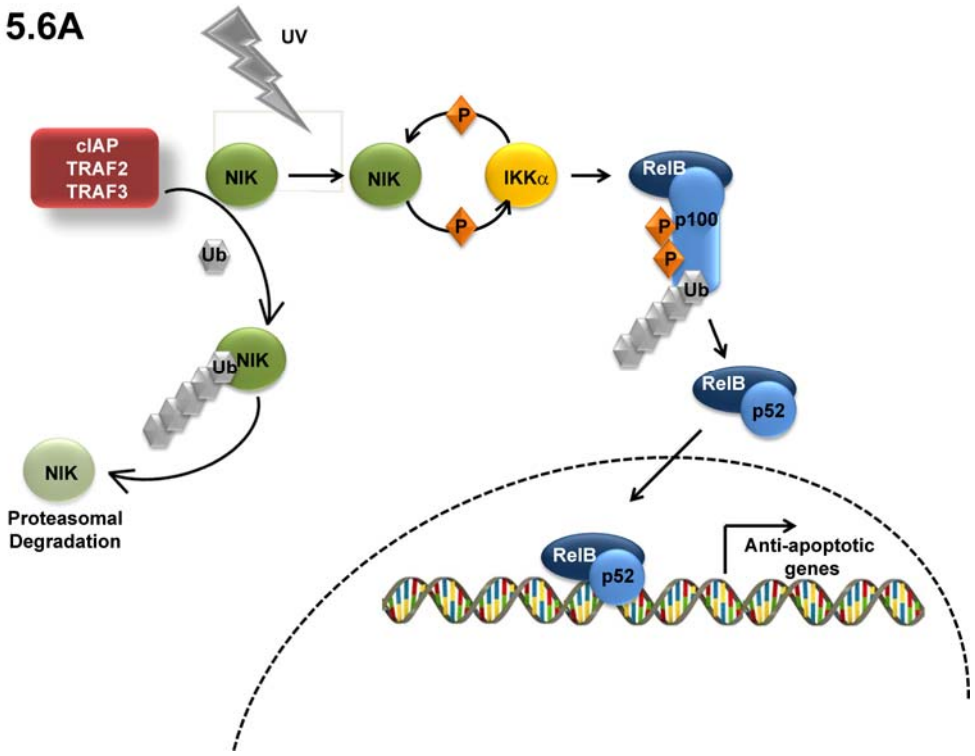
PIAS1 potentially regulates NFκB transcriptional activity

PIAS1 expression in HeLa cells changes gene expression of many apoptotic proteins. We showed that the expression of many of these proteins is controlled by the NFκB family of

transcription factors and some of these proteins themselves act as regulators of NFκB activity, specifically the non-canonical NFκB pathway that involves transcription factor RelB (Figure 4.1B and Table 4.1). We also showed that both PIAS1 and Daxx are able to specifically interact with NFκB member RelB in PIAS1-expressing cells (Figure 4.2). Thus, NFκB appears to be a credible target of PIAS1 and Daxx in cells.

The canonical NFκB pathway (RelA:p52 heterodimer) is known to be induced by UVR (*9, 13*). The non-canonical pathway (RelB:p50) has been studied extensively only in immune system development (*28*) and its role in stress response is not well analyzed. However, transcription of a number of anti-apoptotic genes has been shown to be controlled by RelB (*29*) (Table 4.1). The NFκB pathway is regulated by SUMOylation of upstream NFκB regulatory proteins such as IκBα (Inhibitor of NFκB) (*30*) and NEMO (NFκB Essential Modulator) (*31*). Both PIAS1 and PIAS3 have been shown to negatively regulate RelA transcriptional activity. PIAS1 inhibits DNA binding of RelA (*32*), whereas PIAS3 SUMOylates RelA only when it is bound to DNA and inhibits transcription presumably by preventing its interaction with co-activators (*33, 34*). Transcription activity of both RelA and RelB are also repressed by Daxx. Daxx prevents CBP mediated acetylation and activation of RelA (*35*). And represses RelB by recruiting DNA methyl transferase 1 enzyme (DNMT1) to RelB promoters (*29*). It is not known if SUMOylation of the NFκB factors is required for recruitment of Daxx to either protein. Since we showed RelB to interact with PIAS1, it would be interesting to examine RelB SUMOylation in cells. We have identified four potential SUMOylation sites on RelB (K232, K256, K330 and K362). Of these, lysines 232 and 330 lie in the groove that binds DNA and thus SUMOylation on either of these two residues can potentially interfere with DNA binding; whereas

SUMOylation on lysines 256 and 362 can alter interactions with co-activators or co-repressors. We will test each of these sites for SUMOylation *in vitro* and *in vivo*.



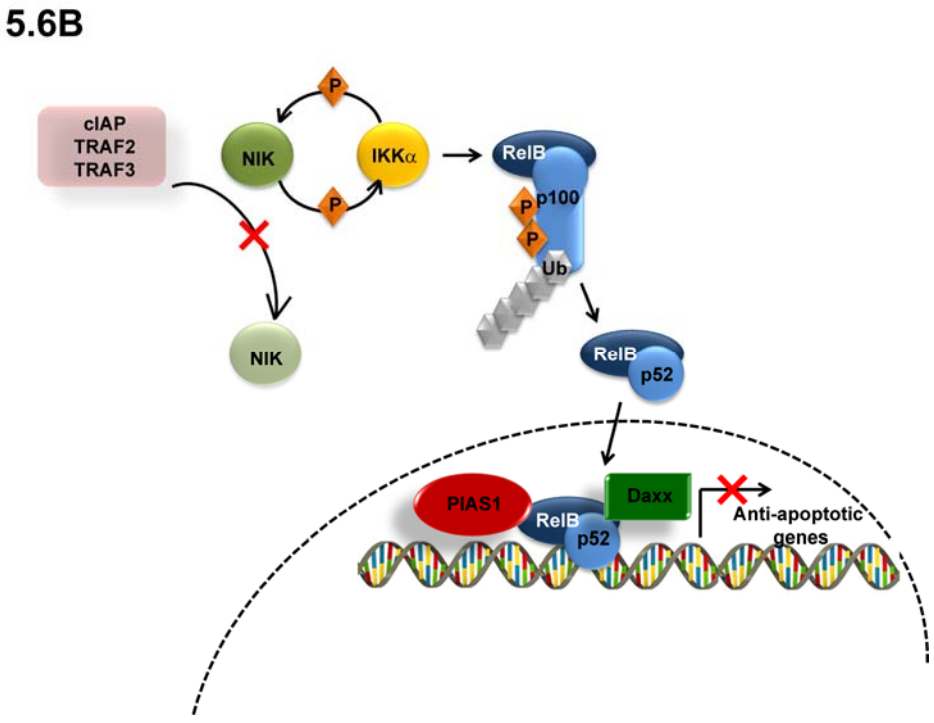


Figure 5.6 The non-canonical NFκB pathway is potentially regulated by PIAS1 expression. (A) Stress stimulus induces translocation of RelB to the nucleus where it induces transcription of anti-apoptotic genes. **(B)** RelB translocates to the nucleus in the absence of stress stimulus but its transcription activity is repressed by PIAS1 and Daxx. (See text for detailed explanation).

As described in chapter 4, RelB is held inactive in the cytoplasm by the p100 protein. TRAF2 and TRAF3 (Tumor Necrosis Factor Receptor Associated Factor) proteins form a Ubiquitin-ligase complex along with cIAP that continuously degrades NFκB-Inducing Kinase (NIK). Activating stress stimuli stabilizes NIK and leads to phosphorylation and degradation of the IκB-like domain of the p100 protein to generate the active p52 fragment. RelB and p52 heterodimer translocates to the nucleus where it activates transcription of certain anti-apoptotic genes (Figure 5.6A) (*36*). Based on our data, we propose a model wherein PIAS1-expressing cells express lower amounts of TRAF2 and TRAF3 proteins (presumably by also modulating the canonical NFκB pathway) that leads to increased stabilization of NIK and hence increased translocation of RelB to the nucleus. This resembles a stressed-state, similar to that induced by a stress stimulus. However, PIAS1 also suppresses activation of genes downstream of RelB by recruitment of Daxx to RelB promoters (Figure 5.6B).

Thus, our data suggests that exogenous PIAS1 expression induces a stress-like state in cells by changing the expression of pro- and anti-apoptotic genes and regulating the NFκB pathway. Data from pias1$^{-/-}$ mice shows an increased activation of certain NFκB target genes (*32*). It would be interesting to identify apoptosis-related genes whose expressions are changed significantly in PIAS1-depleted cells and see if the same genes are targeted in both PIAS1-overexpression and PIAS1-depletion models. PIAS1 has been shown to induce cell death in response to reactive oxygen species (ROS) via induction of JNK (*37*). PIAS1 also localizes to

double strand break foci generated by ionizing radiation (*38*). Thus, PIAS1 appears to be an important player in various stress response pathways.

This dissertation emphasizes the essential role that PIAS1 plays in UV-stress response and has made significant contributions towards identification of some of the major interactors of PIAS1 involved in cellular apoptotic response to UV. While both Daxx and NFκB are important for regulation of cell death following UVR, we do not yet have a coherent explanation for the complete pathway. While it is clear that Daxx acts on PIAS1-SUMOylated substrates, the substrates themselves remain to be identified and substantial efforts are being directed towards identification of global substrates of PIAS1 and also of other PIASes. NFκB itself seems to be regulated at multiple levels and the initial events that change gene regulation on PIAS1 expression have not been determined. There is substantial cross-talk between the canonical and the non-canonical NFκB pathway and we envisage a similar interaction induced by PIAS1 overexpression in cells. Future studies to further delineate the mechanism of PIAS1-induced UV-hypersensitvity will benefit significantly from generation of inducible PIAS1 expressing cell lines. Further experiments to comprehend the involvement of the NFκB pathway will be required for a complete understanding of PIAS1-induced UV-hypersensitivity.

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APPENDIX: Analysis of vertebrate PCNA SUMOylation

INTRODUCTION

Proliferating Cell Nuclear Antigen (PCNA) is the processivity factor for replicative DNA polymerases in eukaryotes. PCNA aids replicative polymerases delta and epsilon to remain on DNA for longer period during replication and thus allow replication of thousands of bases with a single replisome loading (*1*, *2*). PCNA protein forms a homotrimer (*3*) and functions as a double homotrimer complex (*4*) that assembles around the DNA with the help of a clamp loader chaperone-like complex called Replication Factor C (RFC) (*5*). PCNA itself is devoid of any enzymatic activity but it functions as a platform for assembly of multi-protein complexes.

PCNA has since been identified as a master player in a number of cellular pathways. PCNA interacts with Fen-1 endonuclease, which is required for processing of Okazaki fragments, during replication and enhances its activity (*6*). PCNA also binds to DNA ligase 1 at the ends of Okazaki fragments where DNA ligase 1 catalyzes phosphodiester bond formation between adjacent Okazaki fragments (*7*). In *Xenopus* egg extract, PCNA prevents reinitiation of replication by recruiting Cdt1-DDB Ubiquitin ligase complex to the DNA and thus ensuring degradation of replication licensing factor Cdt1 (*8*). In addition, PCNA participates in DNA repair by interacting with a host of proteins involved in mismatch repair, base excision repair and nucleotide excision repair; interacts with sister chromatid cohesion factors; chromatin remodelers; cell cycle regulators and apoptotic factors (*9*). In yeast, PCNA also prevents recombination between sister chromatids by recruiting helicase Srs2 to the chromosomes (*10, 11*).

PCNA has been shown to be both ubiquitinated and SUMOylated. PCNA modifications are very well studied in yeast. PCNA is ubiquitinated in response to DNA damage. Ubiquitination at lysine-164 (K164) recruits polymerases involved in translesion synthesis (TLS) to the site of DNA damage (*12*). TLS is a damage bypass pathway used during replication. During replication, when a replicative polymerase encounters damaged bases, the replication fork stalls. This triggers a damage response pathway culminating in mono-ubiquitination of PCNA on K164. This form of PCNA recruits low fidelity polymerases that are able to incorporate mismatched bases opposite to the damaged base site and thus continue with replication, albeit in an error-prone manner. PCNA can also be polyubiquitinated at the same lysine. The polyubiquitin chain is formed by Ubiquitin linking through K63 (not K48 on Ubiquitin, which is a signal for protein degradation). Polyubiquitin chain on PCNA recruits homologous recombination machinery that repairs DNA in an error-free manner (*12*). Lysine 164 can also be SUMOylated and this modification leads to recruitment of a helicase Srs2 that prevents unnecessary recombination between sister chromatids (*11*). K164 SUMOylation also also controls recruitment of Eco1, a protein involved in sister chromatid cohesion during S-phase (*13*). Establishment of proper sister chromatid cohesion is important for faithful chromatid segregation in mitosis. In addition, PCNA is also SUMOylated on lysine 127, although the significance of this SUMOylation is not well-studied (*12*).

PCNA is highly conserved from yeast to mammals; however, PCNA SUMOylation is not well-studied in vertebrates. *Xenopus laevis* PCNA is 62% similar to *Saccharomyces cerevisae* PCNA and has been shown to be modified by both SUMO and Ubiquitin on K164 during S-phase. *Xenopus* PCNA is also diubiquitinated on DNA damage (*14*). However, K127 is not conserved between *Xenopus* and the yeast PCNA. PCNA SUMOylation and ubiquitination has

also been described in chicken DT40 cells (*15*), but only mono-ubiquitination on K164 has been described in response to DNA damage to mammalian cells. Human PCNA and *Xenopus* PCNA share 89% identity at the protein sequence level. Monoubiquitination of PCNA in mammalian cells is required for recruitment of DNA polymerase η (eta) to damaged DNA and translesion synthesis (*16, 17*). In addition, no homolog of yeast PCNA-SUMO interacting protein helicase Srs2 has been identified in higher eukaryotes. We sought to further elucidate the role of PCNA SUMOylation in *Xenopus* egg extract and identify SUMOylation of PCNA in mammalian cells.

RESULTS

PCNA is SUMOylated and ubiquitinated only during the S-phase in *Xenopus* egg extracts

We first repeated the earlier observations that PCNA is SUMOylated and ubiquitinated in *Xenopus* egg extracts during normal S-phase. *Xenopus* egg extract is very well-suited to study proteins regulated in a cell cycle dependent manner. The egg extract is prepared by spinning eggs at high centrifugal forces that separates lipid and nuclear components from the crude soluble fraction (CSF) that represents the mitotic stage of cell cycle. When sperm DNA is added at this stage, mitotic chromatin is assembled. Interphase (S-phase) can be activated by addition of Ca^{+2} and the extract can be forced to re-enter mitosis by addition of more crude soluble fraction (CSF) to the interphase extract (Figure A1a).

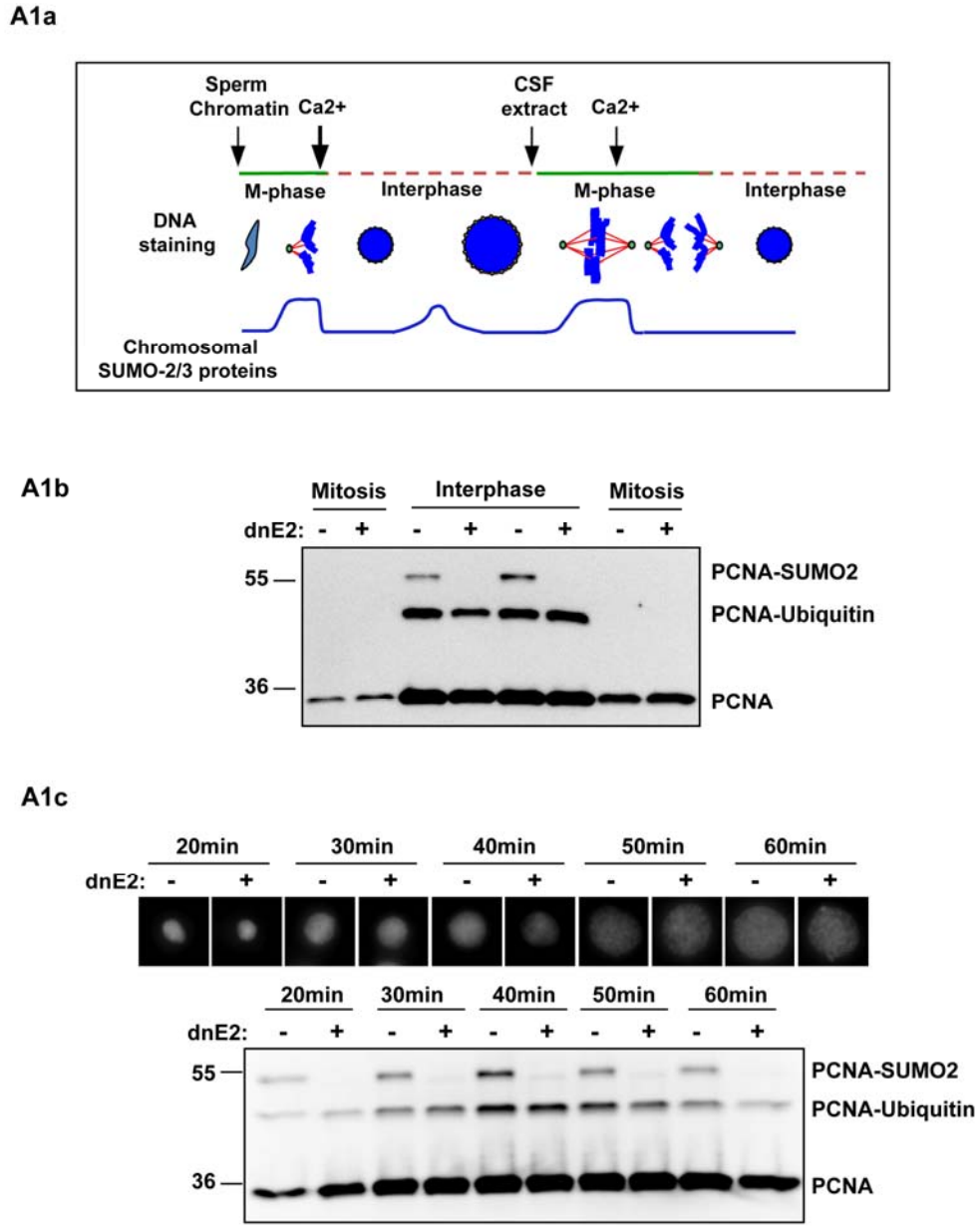

Figure A1: PCNA is modified during S-phase in *Xenopus* egg extract. (a) Schematic representation showing *Xenopus* egg extract cycling through mitosis and interphase. **(b)** PCNA is SUMOylated and ubiquitinated during S-phase, but not during mitosis on un-replicated or replicated chromosomes. Chromosomes were isolated from the respective stage of egg extract and chromosomal proteins immunoblotted with anti-PCNA antibody. **(c)** PCNA SUMOylation and ubiquitination first increases and then decreases as the chromosome replicates during S-phase.

Majority of chromosomal SUMOylation takes place during mitotic stage, but a small number of SUMOylated proteins can be identified during S-phase as well (Figure A1a). We observed slower mobility PCNA bands associated with the chromosome only during the interphase or the S-phase of egg extract (Figure A1b). Of the two slower mobility PCNA bands, the SUMOylated PCNA can be identified by disappearance of one of the bands when the extract is incubated with a dominant negative E2 enzyme (C93S) (*18, 19*). PCNA ubiquitination and SUMOylation cannot be observed during mitosis or when replicated chromosomes are induced to re-enter mitosis (Figure A1b). Amount of PCNA associated with chromosomes is also greatest during the S-phase. Figure A1c (courtesy, Dr. Lin Cong) shows PCNA ubiquitination and SUMOylation increase steadily through the S-phase and then decreases as the chromosomes are fully replicated. Addition of dominant negative Ubc9 does not interfere with progression through S-phase, thus, PCNA SUMOylation does not appear to be critical for replication of chromosomes.

PCNA is modified by SUMO-2/3 in *Xenopus* egg extracts

Leach and Michael (*14*) showed that PCNA is modified by SUMO-1 at a higher efficiency than SUMO-2 in *Xenopus* egg extract. However, they added excess recombinant GST-tagged SUMO to the extract to arrive at this conclusion. Endogenous situation may not necessarily reflect a preference for SUMO-1 over SUMO-2. In order to identify the SUMO isoforms that modifies PCNA in the egg extract, we performed PCNA IP from interphase chromosomes under denaturing conditions that prevents deSUMOylation by SUMO proteases. We identified only SUMO-2 modified form of PCNA associated with chromosome in the egg extracts (Figure A2, courtesy of Dr Lin Cong). Thus, under native conditions, PCNA is modified by SUMO-2.

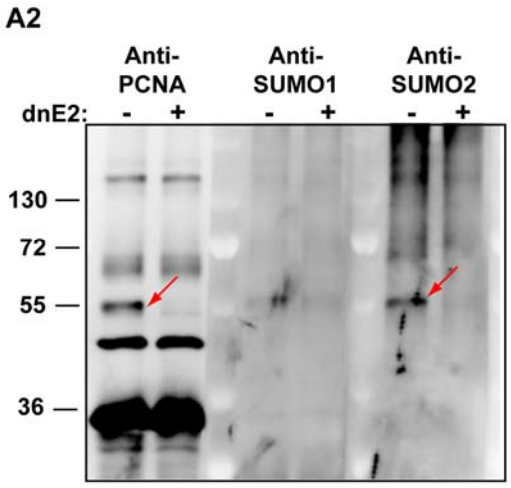

Figure A2: PCNA is modified by SUMO-2 in *Xenopus* egg extract. PCNA bound to interphase chromosome was immunoprecipated under denaturing conditions and immunoblotted with anti-PCNA, anti-SUMO-1 and anti-SUMO-2/3 antibodies. A 55 kD band corresponding to the SUMO-modified form of PCNA was identified only in the SUMO-2 immunoblot (indicated by red arrows). Figure courtesy, Dr Lin Cong.

PCNA SUMOylation *in vitro* requires SUMO E3 ligase

In order to further analyze PCNA SUMOylation *in vitro*, we cloned and purified recombinant *Xenopus* PCNA protein. We used this protein in an *in vitro* SUMOylation assay with varying concentrations of E2 SUMO conjugating enzyme (Ubc9) and E3 SUMO ligase (PIASy). We compared *in vitro* PCNA SUMOylation to Parp1 SUMOylation, a SUMO substrate that has been well characterized in the lab earlier (*20*). *In vitro* PCNA SUMOylation is very inefficient compared to Parp1 SUMOylation (compare Figures A3a and A3b). Also, while Parp1 can be SUMOylated by E2 alone, PCNA SUMOylation requires the presence of an E3 ligase. In addition, we saw two PCNA-SUMO bands (Figure A3a), suggesting *in vitro*, *Xenopus* PCNA can be modified at two sites.

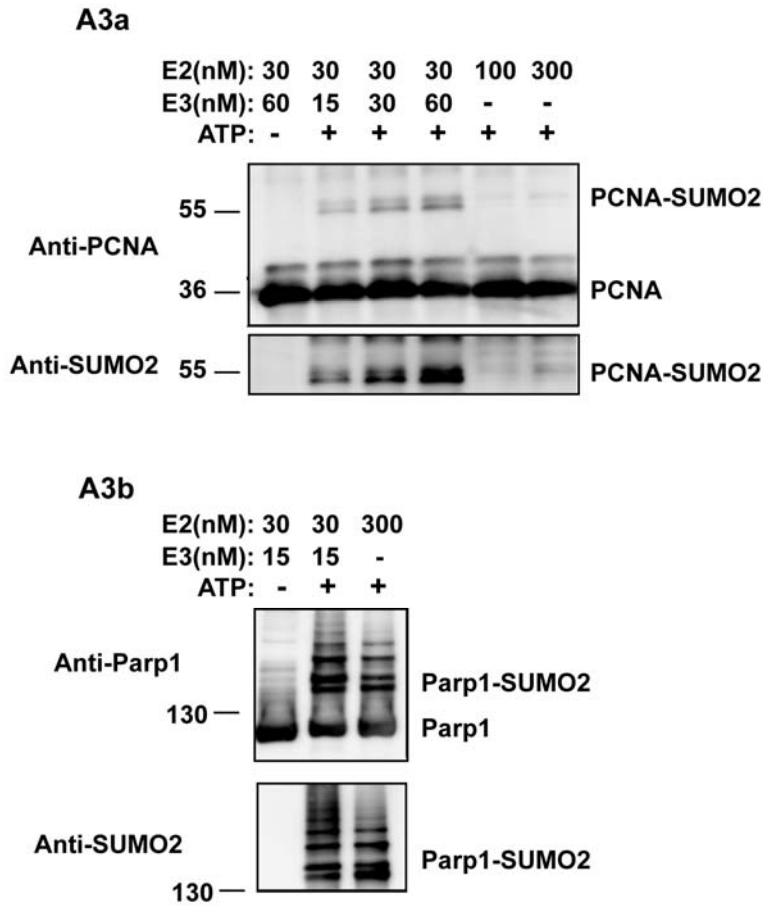

Figure A3: *In vitro* PCNA SUMOylation is E3-dependent. (a) *In vitro* SUMOylation of *Xenopus* is dependent on the presence of E3 SUMO ligase enzyme. *Xenopus* PCNA is modified at two sites *in vitro*. **(b)** *In vitro* SUMOylation of Parp1 as a control. Parp1 can be SUMOylated even in the absence of an E3 enzyme.

PCNA can be modified at lysines 164 and 254 *in vitro*

PCNA SUMOylation at K164 is already described. K164 is a non-consensus SUMOylation site. We studied the PCNA structure (*3*) to identify surface-exposed lysine residues and used the SUMOsp 2.0 online software (*21*) to identify potential SUMOylation sites. We identified lysine 254 as a second potential SUMOylation site on *Xenopus* PCNA. Lysine 254 is part of a consensus SUMO motif ΨKXE (Ψ=hydrophobic amino acid, K=lysine, X=any amino acid, E=glutamate). We created lysine to arginine mutants of each of these lysines and also a double

mutant (DM = K164, 254R) on PCNA and verified K164 and K254 to be the sites of SUMO modification *in vitro*. Also, our data suggests that K164 SUMOylation efficiency can be slightly improved by increasing the concentration of E2, whereas, K254 SUMOylation is independent of the E2 concentration. We also, at times, saw weak SUMOylation on additional sites on PCNA in the double mutant (Figures A4a and A4b). This is not unusual, as *in vitro*, additional lysines can be modified when the primary SUMOylation sites have been mutated, although such modifications may be restricted *in vivo*.

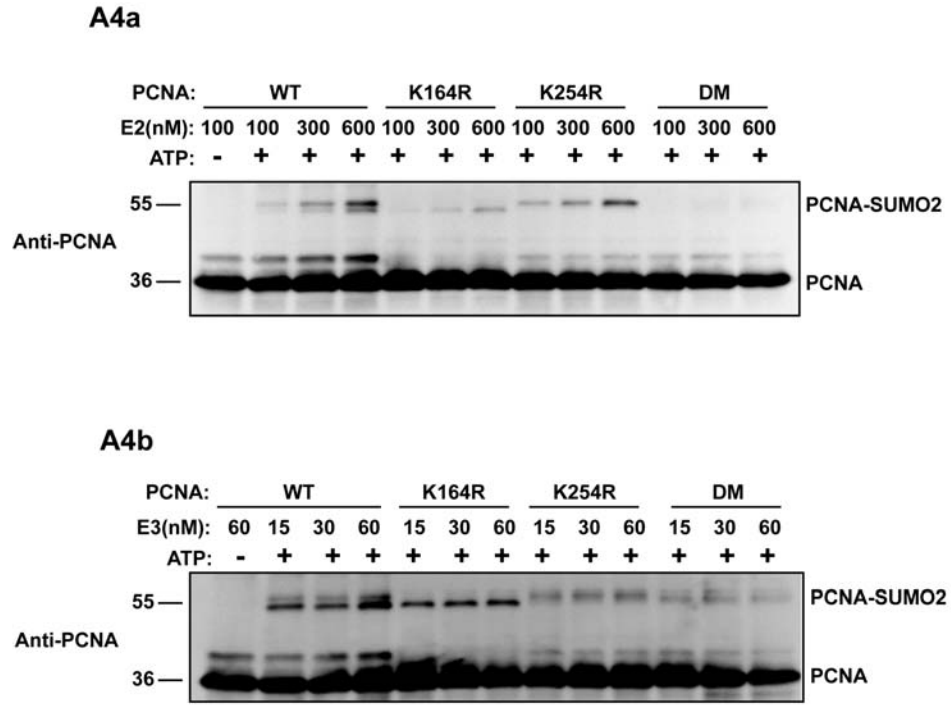

Figure A4(a) and (b): *Xenopus* PCNA can be modified on lysine-164 and lysine-254 *in vitro*. Both lysine 164 and lysine 254 in *Xenopus* PCNA were identified as SUMO-modification sites *in vitro*. *In vitro* reaction contained 60nM E3 ligase in **(a)** and 30nM E2 in **(b)**.

Both the SUMOylation sites in PCNA show a preference for SUMO-2 over SUMO-1 *in vivo*

In order to identify any SUMO isoforms preference for both lysines 164 and 254, we used equal concentrations of SUMO-1 and SUMO-2 in *in vitro* SUMOylation reactions (Figure A5). Both the lysines were observed to be SUMOylated at a higher efficiency by SUMO-2.

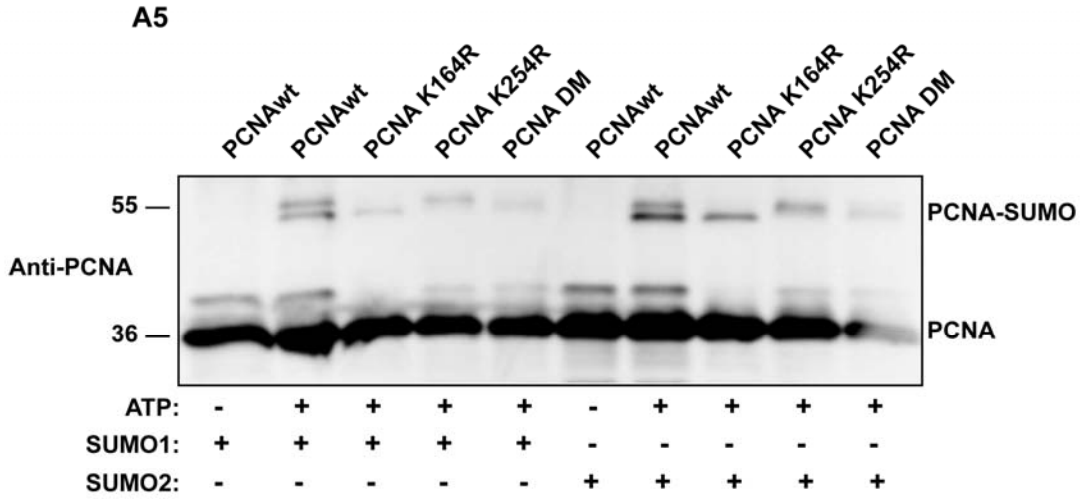

Figure A5: PCNA is modified preferentially by SUMO-2 *in vitro* PCNA SUMOylation was carried out in the presence of equal concentration of either SUMO-1 or SUMO-2. SUMO-2 modification of PCNA was found to be more efficient.

PCNA is not modified at K254 in *Xenopus* egg extract

Since PCNA ubiquitination and SUMOylation has been described only at K164 in *Xenopus* egg extract, we sought to identify secondary SUMOylation sites on PCNA in the egg extract. We added recombinant his-tagged PCNA wild-type and PCNA mutants to the egg extract and incubated the extracts either in the presence or absence of aphidicolin. Aphidicolin is a reversible inhibitor of DNA polymerase and thus inhibits cells progression through the S-phase. Addition of aphidocolin prolongs retention of replication machinery on the chromosome. We then pulled down his-tagged PCNA assembled on purified interphase chromosomes. Wild-type as well as the mutant PCNAs were able to bind to the chromosome. Addition of excess wild-type or mutant

PCNA did not interfere with entry or progression of the extract through interphase. Aphidicolin addition prevented replication of chromosomal DNA but not loading of PCNA on the chromosomes. We could observe both the ubiquitinated and the SUMOylated forms of PCNAwt as well as PCNA K254R in either aphidicolin-treated or untreated samples. In addition, we could identify a di-ubiquitinated form of PCNA (indicated by asterisk in the figure) in the aphidicolin-treated samples in PCNAwt and PCNA K254R mutant, as reported earlier (*14*). PCNA K164R and PCNA DM did not show either ubiquitination or SUMOylation under conditions of normal DNA replication or when replication was inhibited using aphidicolin. Thus, our data suggests that PCNA K254 may not be a SUMOylation site in egg extracts.

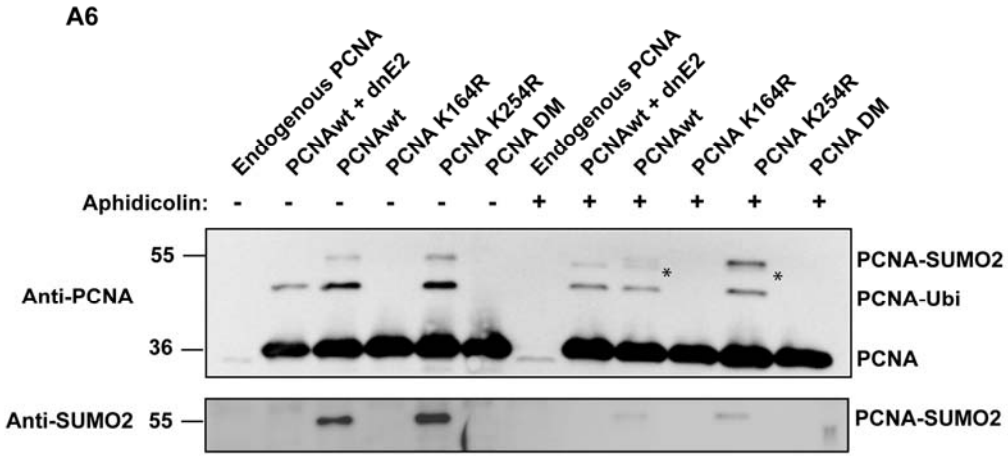

Figure A6: PCNA lysine 254 is not a site of modification in *Xenopus* egg extracts. Interphase was induced in *Xenopus* egg extract by addition of Ca^{+2}. PCNAwt or PCNA mutants were added to the extract in the presence or absence of replication inhibitor Aphidicolin. Lysine 254 was not shown to be SUMOylated or ubiquitinated under the conditions tested.

DISCUSSION

We showed that *Xenopus* PCNA can be SUMOylated at two sites (lysines 164 and 254) *in vitro* but could not identify any conditions wherein lysine 254 is modified in the egg extracts. In 2010, Xu *et al.* (*22*) showed that in mammalian PCNA from HEK293T cells is modified by Ubiquitin.

They also showed that ubiquitination on K254, but not K164, increased significantly when HEK293T cells were treated with mitotic spindle inhibitor drug colchicine. Although ubiquitination of mammalian PCNA at K164 has been described before, SUMOylation of PCNA in mammalian cells was not shown until recently. We tried to observe PCNA SUMOylation in HeLa, Hct116 as well as U2OS cell lines but were not successful. We also used a U2OS line overexpressing SUMO-2, but could not detect any slow-migrating PCNA bands from cell extracts. More recently, Gali *et al.* (*23*) were able to detect SUMO modified forms of PCNA when they transiently overexpressed PCNA, Ubc9 and one of the SUMO isoforms in HEK293T cells. They also identified K254 as a secondary SUMO modification site *in vitro*. Further, they showed that PCNA SUMOylation protects against double-strand break formation at stalled replication forks.

Any future work in the field of PCNA SUMOylation in mammalian cells will be aimed at identifying specific interactors of SUMOylated PCNA. Since PCNA SUMOylation can be detected in mammalian cells only when the substrate as well as Ubc9 and SUMO are overexpressed, identifying specific conditions under which PCNA SUMOylation occurs in cells will be important for further analysis. Also, since PCNA SUMOylation is easily detected in B-cell derived DT40 chicken cell lines, PCNA SUMOylation in mammals may similarly occur more efficiently in B-cells that undergo DNA recombination at high rates. Thus, it maybe advantageous to study PCNA SUMOylation in mammalian B-cell lines.

EXPERIMENTAL PROCEDURES

Cloning and recombinant protein purification: *Xenopus* PCNA was cloned into pET28b vector. PCNA SUMOylation site mutants were generated by site-directed mutagenesis using

Stratagene Quikchange mutagenesis kit. Proteins were expressed in E.coli BL21DE3 strain and purified using Ni-NTA beads followed by further purification on a Q-sepharose column.

***In vitro* SUMOylation assay:** *In vitro* SUMOylation was performed as described in (*20*). Briefly, 15nM E1, 5uM SUMO-1 or SUMO-2, 2.5mM ATP and 500nM PCNA was incubated in the reaction buffer (20mM Tris, 100mM NaCl, 10mM $MgCl_2$, 0.05% Tween-20 and 0.5mM DTT) together with the indicated concentrations of E2 (Ubc9) and E3 (PIASy) enzymes. Reactions were incubated at 25˚C for 1 hour.

***Xenopus* egg extract assay:** *Xenopus* egg extract was prepared as described in (*24*). CSF extracts were induced to enter interphase by addition of 0.6mM $CaCl_2$. Demembranated sperm chromatin were added to the interphase extract and incubated for 1 hour. Chromosome was isolated as described (*24*).

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