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UBC9 mutant reveals the impact of protein dynamics on substrate selectivity and SUMO chain linkages

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Abstract

SUMO, a conserved ubiquitin-like protein, is conjugated to a multitude of cellular proteins to maintain genomic integrity and resist genotoxic stress. Studies of the SUMO E2 conjugating enzyme mutant, UBC9^{P123L}, suggested that altered substrate specificity enhances cell sensitivity to DNA damaging agents. Using nuclear magnetic resonance chemical shift studies we confirm that the mutation does not alter the core globular fold of UBC9, while ¹⁵N relaxation measurements demonstrate mutant-induced stabilization of distinct chemical states in residues near the active site cysteine and substrate recognition motifs. We further demonstrate that the P123L substitution induces a switch from the preferential addition of SUMO to lysine residues in unstructured sites to acceptor lysines embedded in secondary structures, thereby also inducing alterations in SUMO chain linkages. Our results provide new insights regarding the impact that structural dynamics of UBC9 have on substrate selection, and specifically SUMO chain formation. These findings highlight the potential contribution of non-consensus SUMO targets and/or alternative SUMO chain linkages on DNA damage response and chemotherapeutic sensitivity.

Graphical Abstract

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Author Contributions

C.M.W., R.H.W., and J.E.O. contributed equally. C.N.W. and J.E.O. assisted with design and executed the biochemical experiments. R.H.W. assisted with design, execution, and analysis of the NMR experiments. T.B. and C.M.W. prepared NMR protein samples. S.M., J.O and C.M.W. assisted with vector and SUMO assay development. W.J.P. collected all NMR data. M.A.B. and W.J.P. developed the initial concept and study design, oversaw data analysis, and with assistance of J.E.O., wrote the manuscript.

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PROTEIN ACCESSION IDS:

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SUPPORTING INFORMATION

A PDF-file containing 8 supporting figures is available free of charge via the internet at <http://pubs.acs.org>. This includes: S1, *In vitro* SUMOylation assay with human UBC9 and SUMO-2; S2, Representative ¹⁵N-¹H NOE crosspeaks from P123L and P123A spectra; S3, ¹⁵N backbone dynamic analysis of P123L; S4, ¹⁵N backbone dynamic analysis of P123A; S5-S6, Blots demonstrating that thioester bond formation is not impaired by the P123L mutation; S7, Blot demonstrating that P123L induces a switch in SUMO-1 chain linkage formation; and S8, Band intensity analysis of P123L SUMO conjugation at 37°C.

Keywords

SUMOylation; UBC9; NMR; DNA damage response

INTRODUCTION

Post-translational protein modifications provide cellular mechanisms to directly regulate the location, stability, and activity of diverse proteins. Moreover, the formation of reversible, covalent adducts on proteins allows cells to rapidly respond to environmental signals and threats without *de novo* protein synthesis. Protein modification by ubiquitin; small ubiquitin-related modifier (SUMO); and other ubiquitin-like proteins (ULPs) has emerged as an important regulator of cellular homeostasis, maintenance of genome integrity, and cellular responses to DNA damage ¹⁻³

In contrast to ubiquitination, SUMOylation is mediated by a single E2 enzyme, UBC9, ³⁻⁴ that contains substrate recognition motifs allowing it to recognize and modify substrates directly, in the absence of E3 proteins, ⁵ or in combination with E3 ligases. When present, SUMO E3 ligases serve to orient the UBC9~SUMO thioester and substrate to enhance SUMOylation ³⁻⁵. The ability of UBC9 to act in the absence of an E3 ligase is exploited in *in vitro* SUMO assays, where SUMOylation is driven by a higher concentration of UBC9. SUMOylated lysines in target proteins often lie within a ΨKx[D/E] consensus motif, with Ψ being a large hydrophobic amino acid and x indicating any residue. Structural evidence indicates that this motif is directly recognized by UBC9 ⁶, and that for this recognition to occur, this motif must be in an extended loop or unstructured region ⁷.

Five SUMO isoforms have thus far been identified in human cells: SUMO-1, -2, -3, -4 and, -5; with SUMO-1, -2, and -3 being ubiquitously expressed and SUMO-4 and -5 expression restricted to specific tissues ^{2,8}. Comparatively, the yeast *Saccharomyces cerevisiae* encodes a single SUMO (Smt3). An extended N-terminus, common to all SUMOs, contains lysine residues that constitute the major sites of further SUMO modification and chain formation (see Figure 1A). However, internally located, non-consensus site lysine residues, such as those embedded in conserved α-helical motifs in

UBC9, and SUMO-2, and -3, can also serve as SUMO modification sites⁹⁻¹⁰. Because SUMO-2 and SUMO-3 only differ by 3 amino acids, and are indistinguishable by antibodies, they are collectively referred to as SUMO-2/3. SUMO-1 shares ~50% homology with SUMO-2/3, and contains no consensus site lysines. Multiple SUMOylation studies in yeast and human cells have established SUMOylation as a critical regulator of cellular homeostasis, while proteomic analyses continue to expand the number of cellular proteins that are known to be SUMO substrates¹¹⁻¹⁹.

SUMOylation has been implicated as a key modulator of cellular responses to DNA damage^{11,13,21}. The role of UBC9 in protecting cells from genotoxic stress was highlighted when a *ubc9P123L* mutant (Pro123 substituted with Leu) was identified in a yeast genetic screen for temperature-sensitive mutants with enhanced vulnerability to DNA topoisomerase I (Top1)-induced lesions²²⁻²⁴. Pro123 is conserved in yeast and human UBC9, occupying the same position in X-ray crystal structures^{20,24} (Figure 1b, yellow). Yet, when Pro123 was substituted with Ala, instead of Leu, the phenotype of yeast cells expressing mutant *ubc9P123A* was indistinguishable from wild-type *UBC9* cells²³. Thus, alterations in P123L function are not simply attributable to the loss of structural rigidity imparted by proline at position 123. *ubc9P123L* mutant cells also exhibited reduced levels of both Ubc9 protein and global SUMO conjugates at the nonpermissive temperature²³. In contrast, yeast-human UBC9 chimeras, engineered to contain human residues important in substrate recognition, restored yeast cell resistance to DNA damage, but without a corresponding increase in overall protein SUMOylation²⁴. These data suggest that UBC9 substrate specificity, rather than global SUMO conjugation, is the critical determinant of cellular responses to DNA damage.

Pro123 (Figure 1B, yellow) lies in the long loop region between the second and third alpha helices ($\alpha 2\alpha 3$ loop, residues 121–131) of UBC9's ubiquitin-conjugating (UBC) fold. The UBC fold is the common fold for ULP catalytic E2 proteins²⁵. Residues in the $\alpha 2\alpha 3$ loop serve to promote interaction with consensus site motifs in substrate proteins (Figure 1B, green), orient the thioester-linked SUMO di-glycine tail (Figure 1B, orange), and regulate access to the active site (Figure 1B, blue). Mutant cells expressing UBC9P123L (hereafter referred to as P123L) were viable in the presence of different environmental stresses, but exhibited enhanced sensitivity to a variety of DNA-damaging agent at the non-permissive temperature of 36°C, relative to that observed with isogenic wild-type *UBC9* cells^{11,13,21}. These studies demonstrate that the P123L substitution successfully uncouples the catalytic function of UBC9 necessary to protect cells against DNA damage, from the essential activity of this enzyme required to maintain cell viability in the absence of genotoxic stress²³⁻²⁴. However, the structural and/or mechanistic basis for this separation of functions remains unclear.

Here we report on the specific effects that the P123L mutation has on UBC9 structure and function. To define the structural basis for this alteration in substrate specificity, we assessed NMR chemical shift perturbation (CSP) and backbone dynamic analyses of wild-type UBC9, P123A, and P123L proteins. Our findings indicate that substitution of Leu for Pro123 perturbs a network of hydrophobic interactions that modulate UBC9 protein dynamics around substrate recognition motifs in helix $\alpha 3$ and the active site cysteine. These

mutant-induced changes promote the stabilization of distinct conformational states. We then employed recombinant wild-type and mutant versions of human UBC9 in *in vitro* SUMOylation assays (Supplementary Figure S1) to assess how these mutations alter the selection of SUMO chain linkages. We observed that a distinct pattern of SUMO chain linkages are formed by P123L. In contrast to the preferential SUMOylation of lysine residues in the unstructured N-terminal domain of SUMO-2 by wild-type UBC9 [or P123A], P123L catalyzed the modification of lysine residues that reside in structured, non-consensus sites in SUMO-2. These findings provide critical new insights into the structural basis for SUMO chain linkage and site selection.

METHODS

DNA manipulation and expression vectors.

IPTG-inducible expression vectors, pGEXhUBC9, pGEXSAE1/SAE2, pGEXSUMO-1, and pGEXSUMO-2, were the kind gift of Dr. Brenda Schulman (St Jude Children's Research Hospital, Memphis, TN), which allowed for expression of human UBC9, E1 (SAE1/SAE2 heterodimer), SUMO-1 and SUMO-2, respectively, as thrombin-cleavable N-terminal GST-fusions in bacteria. For efficient ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ -labeling of human UBC9 for NMR studies, a codon optimized human UBC9 cDNA was synthesized (Genewiz) and cloned into the pET28a bacterial expression vector (Thermo Fisher) to include a cleavable N-terminal hexa-histidine purification tag. UBC9_{P123L} and UBC9_{P123A} constructs were subsequently generated in both pGEXhUBC9 and pETHUBC9 vectors by site directed mutagenesis (Agilent), as were the SUMO-2(K11R) and SUMO-2(K5,7,11R) mutants in pGEX. All constructs were confirmed by DNA sequencing.

Protein production.

For purification of GST-fusions, all pGEXhUBC9, pGEXSUMO1 and pGEXSUMO-2 constructs were transformed into BL21(DE3) competent *Escherichia coli* cells (Thermo Fisher). pGEXSAE1/SAE2 was transformed into either BL21(DE3)RIL or BL21(DE3)Rosetta2 cells. For the pGEXSAE1/SAE2 construct, only the larger SAE2 subunit was GST-labeled; however, heterodimers were stable throughout the purification process. In all cases, transformed *E. coli* cells were grown in 1L of LB with antibiotic selection at 37°C to an OD_{600 nm} of ~0.8, then induced with 1 mM IPTG overnight at 25°C, except for P_{123L} expression, which was achieved at 16°C. Cells were pelleted at 4°C, resuspended in 10 ml resuspension buffer (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 5 mM DTT, 2.5 mM PMSF) and lysed on ice by sonication. Clarified lysates were mixed with 4 ml of a 50% slurry of glutathione sepharose (GE Healthcare), pre-equilibrated in resuspension buffer. After gentle rocking at 4°C, the mixtures were packed into disposable columns (BioRad) and washed with resuspension buffer. The beads were incubated with 80 units of Thrombin (GE Healthcare) in 1 ml PBS (pH 7.4) and 2.5 mM CaCl₂ at 16°C overnight with gentle rocking in a 50 ml conical tube. The cleaved proteins were recovered from the beads in disposable columns with elution buffer [50 mM Tris•HCl (pH 7.6), 100 mM NaCl, 1 mM DTT] and then subjected to ion-exchange chromatography with the same buffer, either on a 2 ml Q-Sepharose column (Sigma) for UBC9 and SAE1/SAE2 proteins, or a 2 ml SP Sepharose column (Sigma) for SUMO-1 and SUMO-2 proteins. Purified proteins were

concentrated on Amicon Ultra centrifugal filtration devices [YM-3 for SUMO-1 and SUMO-2, YM-10 for UBC9, and YM-50 for E1 (SAE1/SAE2)] in elution buffer.

For ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ -labeled UBC9 proteins, BL21(DE3) cells transformed with pETHUBC9 constructs were induced at an $\text{OD}_{600\text{ nm}}$ of ~ 0.4 with 1 mM IPTG in 2 X 250 ml M9 minimal media + kanamycin, using 1 g $^{15}\text{NH}_4\text{Cl}$, or 1 g $^{15}\text{NH}_4\text{Cl}$ and 4 g $^{13}\text{C}_6$ D-glucose as the sole nitrogen, or nitrogen and carbon sources, respectively. Induced cells were incubated overnight at 25°C (for UBC9 and P₁₂₃A) or 16°C (P₁₂₃L). Cells were pelleted by centrifugation and resuspended in 5 ml/ 250 ml culture in Buffer A [PBS (pH 7.4), 20 mM imidazole, 2 mM β ME) with 2.5 mM PMSF and disrupted by sonication. Cleared lysates were combined with 4 ml of a 50% slurry of Ni-NTA beads (Qiagen) that had been pre-equilibrated in buffer A, and rocked at 4°C. The bead-bound His-tagged proteins were washed with buffer A in disposable columns and eluted with buffer A containing a final 250 mM imidazole. Purified proteins were subjected to Amicon Ultra 10,000 Da centrifugal filtration with storage buffer [PBS (pH 7.0), 2 mM β ME, 0.1 % NaAzide] to achieve a final concentration of ~ 1 mM. Storage buffer for P123L also included 10 mM glutamate. All protein preparations were assessed for purity and integrity by SDS-PAGE / SYPRO ruby staining, and aliquots were flash frozen and stored at -80°C .

***In vitro* SUMOylation assays.**—Purified human heterodimeric E1, SUMO, and UBC9 proteins were thawed on ice, protein concentrations were determined using a Bradford assay (BioRad), and the proteins were added in the following order to assay buffer (50mM MgCl_2 , 25mM Tris pH 7.6, 100mM NaCl with or without 20mM ATP): E1, SUMO, and UBC9. For 30 μL reactions, 0.05 pmol of E1 was used, with UBC9 and SUMO proteins added at the indicated molar ratios. Reactions were incubated at the indicated temperatures for 2 hrs, and the products resolved by NuPAGE 4–12% Bis-Tris polyacrylamide gel electrophoresis in MES buffer (Thermo Fisher), and transferred to PVDF by Transblot Turbo (Biorad). Antibody staining was performed using anti-SUMO1 antibody (Abcam), anti-SUMO-2 antibody (Abcam) or anti-UBC9 antibody (BD Bioscience), as per the manufacturer's instructions, and visualized with SuperSignal West Dura (Thermo Fisher).

For SUMOylation time course experiments, reactions were first prepared in the absence of ATP. 20 μL aliquots were removed for the zero time point, ATP was added, and the reactions were split and placed at the indicated temperatures. At each time point, 20 μL aliquots were removed and boiled with SDS-PAGE sample buffer. SDS-PAGE and immunoblots were performed as indicated above. The use of infrared fluorescence, dual antibody staining with IRDye secondary staining and Odyssey Imaging (LI-COR) is described in the supplemental data.

NMR spectroscopy.

NMR spectra were collected at 298 K, unless specified in text, with Bruker Avance 600 MHz or Avance 850 MHz spectrometers equipped with gradient triple resonance probes. The spectra were processed using TOPSPIN 3.2 (Bruker Biospin) and analyzed with CARA (cara.nmr.ch)²⁶. The sequence-specific ^{15}N , H_N , C_α , and C_β as signments for UBC9,

UBC9P123L, and UBC9P123A were obtained from 3D HNCA, 3D HNCACB, and 3D CBCAcoNH spectra. Complete assignment was obtained for both UBC9 and UBC9P123A (including the new A123 crosspeak in the UBC9P123A 2D [^{15}N , ^1H]-HSQC). The assignment of UBC9P123L was complete excluding backbone assignments for E118, L120, N121, E122, L123, I125, D127, Q130, I136, R149, A150, K153, K154, and F155. For chemical shift perturbation studies, 2D [^{15}N , ^1H]-HSQC spectra (16 ppm x 32 ppm) were collected with 2048 direct and 256 indirect points and a ^{15}N frequency off-set of 118 ppm. Chemical shift values were calculated using the equation $\delta = \text{SQRT}[(\delta^1\text{H})^2 + (0.2 * \delta^{15}\text{N})^2]$. Perturbation of UBC9P123L was compared to UBC9 with both in modified Buffer A containing 10 mM glutamic acid. Steady-state ^{15}N - ^{1}H -NOEs were measured on a Bruker Avance 600 MHz spectrometer using TROSY-based experiments²⁸ with an interscan delay of 2.5 s and a saturation period of 3 s. For each UBC9 variant, 3 separate protein preparations were tested, peak values were integrated in CARA, and the resulting NOE/NONOE values were plotted against the protein sequence in GraphPad Prism. Secondary structure probability plots were calculated using PECAN (pine.nmr.fam.wisc.edu/PECAN)²⁹ based on assigned C α and C β chemical shift assignments. Longitudinal (R_1) and transverse (R_2) relaxation rates were measured at 600 MHz. For R_1 delays of 10, 40, 80, 120, 160, 200, 240, 280, 350, and 800 msec were collected with an interscan delay of 1.5sec. Replicate spectra at 80 and 200 ms were collected to determine spectral noise. For R_2 delays of 20, 34, 41.8, 50, 58.6, 75.3, 108.8, 142.3, and 204 ms were collected with interscan delays of 1.5sec. Replicate spectra were collected for 20, 58.6, and 108.8 ms points to determine spectral noise. Peak volumes were calculated and fit to a single exponential decay function using GraphPad Prism. Resulting R_2 rates were determined for all residues with signal >2.5 x noise and fitted to a gradient color scheme as outlined in Figure 6 using NESSY (home.gna.org/nessy)³⁰ and plotted using PyMOL (www.pymol.org). S^2 order parameters were calculated using Rotdif 3.1³¹.

RESULTS

Chemical shift analysis of the effect of the P123L mutation.

Despite success by multiple groups in resolving the crystal structures of UBC9 alone and in multiple complexes^{4-7, 24}, the P123L mutant has defied crystallization, which necessitated employing NMR to interrogate its structure and dynamics in solution. To determine the effect of the P123L mutation on protein structure, we used NMR CSP to map local changes in the chemical environment of the backbone amide bonds. Uniformly ^{15}N -labeled and double ^{13}C , ^{15}N -labeled human protein was expressed and purified from *E. coli*. The resulting sample enabled the collection of 3D-HNCA, 3D-HNCACB, and 3D-CBCA(CO)NH spectra, which were used to assign the backbone ^{15}N , $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and $^1\text{H}_\text{N}$ resonances for 91% of the P123L protein. Due to low peak intensity/separation, we were unable to complete assignment for residues E118, L120, N121, E122, L123, I125, D127, Q130, I136, R149, A150, K153, K154, and F155 in P123L. Parallel and complete backbone assignment of wild-type UBC9 matched the previously published back-bone assignment³². Comparison of 2D [^{15}N , ^1H]-HSQC spectra of UBC9 and P123L (Figure 2A) identified significant CSP induced by the mutation, where $\delta > 0.05$ ppm corresponds to a cutoff of three standard deviations above the average perturbation observed for residues 10–40, which

exhibited minimal perturbation. Structural mapping of P123L CSP identified a localized concentration of moderately perturbed ($\delta > 0.05$ ppm), highly perturbed ($\delta > 0.1$ ppm), very highly perturbed ($\delta > 0.15$ ppm), and missing assignments surrounding the site of the mutation (Figure 2B and C). The regions that underwent the largest perturbation were the $\alpha 3$ and $\alpha 4$ helices, the $\alpha 2\alpha 3$ and $\beta 4\alpha 2$ loops, and the active site cysteine C93. Minimal perturbation was observed in the C-terminal region of helix $\alpha 4$ and the $\beta 2\beta 3$ loop where they make contact in the native UBC9 fold.

P123A demonstrates the specific nature of the leucine side-chain in perturbing UBC9.

To determine if the CSP effects were due to loss of proline-induced backbone rigidity at the P123 site following mutation to Leu, or a specific effect of the Leu sidechain, we prepared both double ^{13}C , ^{15}N -labeled and ^{15}N -labeled P123A protein; completed the P123A backbone assignment, as for P123L; and quantified the CSP response of the P123A mutant compared to wild-type UBC9. As with wild-type UBC9 protein, P123A exhibited uniform [^{15}N , ^1H]-HSQC peak intensity enabling complete backbone resonance assignment (including the A123 crosspeak). We assessed the magnitude of P123A CSP using equivalent cutoffs as for P123L analysis. Comparison of CSP induced by the P123A mutation to wild-type UBC9 identified moderate perturbation that are mostly localized around the site of the mutation (Figure 3), but a significant decrease in CSP when compared to P123L. These findings suggest that the bulky hydrophobic leucine side-chain induces specific changes in the UBC9 protein other than simply removing proline-imposed rigidity in the $\alpha 2\alpha 3$ loop

P123L mutation retains secondary structure.

The significant perturbation of both helices $\alpha 3$ and $\alpha 4$ in the P123L protein led us to determine if the substitution of Leu for Pro123 disrupted folding of these two secondary structured elements. Prior studies have demonstrated that $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ chemical shifts can predict secondary structured regions when compared to random coil values³³.

We analyzed the $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ assignments using PECAN²⁹ to determine regular secondary structure probability for each amino acid. The resulting probability plots for UBC9 and P123L are shown in Figure 4. The only location that exhibits significant changes in predicted backbone dihedral angle derived secondary structure as well as CSP perturbation are the residues 93 – 96 (Figure 4D), which include the catalytic C93 and the 3_{10} helix that immediately follows it. Of note, there is a decrease in predicted β -strand propensity for $\beta 1$ in P123L, though a lack of CSP in this region makes it unlikely that this secondary structural element is altered by the mutation. Similar analysis of the P123A mutant found no predicted change in secondary structure from wild-type (Figure 4B). The CSP data combined with these probability plots strongly suggest that the core β -strands ($\beta 1$ - $\beta 4$) and α -helices ($\alpha 1$ - $\alpha 4$) are retained in the P123L protein.

P123L mutant suppresses fast-timescale motions in UBC9.

The presence of a collection of unassignable residues, indicative of residues undergoing intermediate timescale motions, near the mutation site led us to further interrogate the backbone dynamics of the two mutants compared to wild-type UBC9. Multiple studies have highlighted that backbone dynamics can impact both protein function and stability with fast-

timescale motions indicative of high-flexibility or intrinsically disordered regions, while slow-timescale dynamics report on proteins existing in multiple conformations in solution³⁴. Prior backbone dynamic studies of wild-type UBC9 had identified the protein as a relatively rigid molecule with conformational flexibility restricted to the $\alpha 2\alpha 3$ and $\beta 4\alpha 2$ loops³⁵. Structural and dynamic analysis of other ubiquitin E2 enzymes has highlighted these loops and their motions as key regulators of substrate recognition and E2 function³⁶⁻³⁹.

As a first step to assessing the impact of mutation on backbone dynamics, we recorded in interleaved fashion, $^{15}\text{N}\{-^1\text{H}\}$ heteronuclear NOE experiments using a 2.5 second recycle delay. Resulting NOE/NONOE signal intensities for UBC9, P123A, and P123L were calculated for 139, 138, and 122 out of the 143 non-prolyl residues, respectively, and plotted against the primary amino acid sequence of UBC9 (Figure 5). The average wild-type UBC9 heteronuclear NOE values of 0.73 (± 0.15) for all residues and 0.77 (± 0.09) for all structured residues agree with the previously published results³⁵. In comparison, the P123A and P123L mutants each exhibited a slight increase in rigidity with average heteronuclear NOE values of 0.81 (± 0.13) for all residues and 0.82 (± 0.09) for structured residues in P123A and 0.79 (± 0.18) for all residues and 0.81 (± 0.14) for all structured residues in P123L. These data confirm the overall structural integrity of the mutant and wild-type enzymes. We did observe that several residues (E132, Y134, and R141) in the P123L spectra exhibited multiple cross-peaks with low signal intensity in the $^{15}\text{N}\{-^1\text{H}\}$ spectra, indicative of their occupation of multiple distinct chemical states (highlighted in Figure 5C and D using magenta markers and side chains, respectively). The detection of distinct states is illustrated by the presence of multiple peaks in the saturated (NOE) spectrum and single peaks in the unsaturated (NONOE) for residues E132 and R141 (Figure 5E). For Y134, distinct chemical conformations are evident even in the NONOE spectrum, and amplified in the NOE spectrum. Summation of the intensities of these individual states resulted in a combined intensity that is comparable in intensity to the majority of the P123L protein [$^{15}\text{N}\{-^1\text{H}\} \sim 0.7$], and that observed for both wild-type and P123A. Representative analyses of surrounding residues showing single NOE peaks in P123A and P123L are presented in Supplementary Figure S2. Overall, the P123L spectrum shows no indication of increased backbone flexibility compared to either UBC9 or P123A. Rather, the NOE experiments suggest a model wherein the P123A and P123L mutations slightly increase rigidity of the proteins. Further, the P123L mutant appears to selectively induce the stabilization of specific chemical conformations of residues surrounding the $\alpha 3$ helix. To assess if this stabilization was instead a second state that arose from an initial unfolding or aggregation, which ultimately drives the temperature sensitive effect observed in the enzymatic studies, we collected [^{15}N , ^1H]-HSQC spectra of P123L at 310K (36°C). At this temperature we observed no increase in crosspeaks in the unstructured region of the spectrum, thus providing no evidence of structural unfolding. Further, while the spectra underwent considerable perturbation as an effect of temperature increase, we observed no evidence of line broadening.

To complete backbone dynamic analysis of the mutant, we collected complementary ^{15}N longitudinal relaxation (R_1) and transverse relaxation (R_2) rates for the observable residues in P123L using 2D [^{15}N , ^1H]-HSQC-based experiments. Collected R_1 spectra of P123L,

similar to the NOE measurements, contained a series of peaks near the mutation site that exhibited multiple crosspeaks (75, 83, 92, 109, 124, 141, and 156). This observation further supports the presence of multiple conformational states in the P123L mutant. For these residues, we were unable to calculate R_1 values. The remaining residues in P123L had an average R_1 of $2.39(\pm 0.79)$ (Supplementary Figure S3). For P123A, the average R_1 of $1.89(\pm 0.54)$ matched the value for WT UBC9. Transverse relaxation (R_2) rates report on fast-timescale (ps-ns) motions of the backbone ^{15}N such that decreases in R_2 signal denote regions with increased backbone flexibility, while increases in R_2 rates would support the presence of different chemical states. The average R_2 values for wild-type UBC9 of $20.96(\pm 2.18)$ indicated free exchange for most residues, except for G55 and residues around A131, which lie above the dashed threshold values in Figure 6A, and are indicated by red and orange shading in Figure 6E. A similar collection of R_2 values was observed for P123A with an average of $19.28(\pm 4.27)$ with increased R_2 rates for G55 and E132 (Supplementary Figure S4). In contrast, we found that while the average R_2 rates in the P123L mutant were similar to wild-type [$21.1(\pm 2.42)$], the P123L mutant induces low or missing R_2 spectral intensity, or more extensive positive shifts in R_2 rates around residues W53, F82, A129, and C138 (Figure 6B and F), which is consistent with the adoption of multiple distinct chemical conformations. Representative transverse relaxation curve fitting for select residues (shown in Figure 6C and D) highlight the reproducibility of the curve fitting for F24 and D100, the elevated R_2 rates for G55 in wild-type UBC9 and P123L, and the increase in R_2 rates for A129 and C138 in P123L alone. The amino acids in P123L that exhibit these changes are clustered along a planar interface between the $\alpha 3$ and $\alpha 4$ helices and the UBC9 core (Figure 6F), and include residues in $\beta 2\beta 3$ loop, $\beta 4\alpha 3$ loop, and $\alpha 3\alpha 4$ loop. R_2/R_1 ratio calculations further localize the changes in backbone dynamics to two locations: near the G55 and at either end of the helix $\alpha 3$. These P123L mutant-induced changes are consistent with the leucine substitution impacting the interactions of residues within the hydrophobic core, and stabilizing $\alpha 3$ in multiple conformations. Complete dynamic analyses (R_1 , R_2 , NOE, and S^2) for P123L and P123A are presented in Supplementary Figures S3 and S4, respectively.

Reduced SUMOylation by P123L is not due to impaired UBC9~SUMO thioester bond formation.

In addition to determining the structural and dynamic impact of the P123L mutation, we also set out to determine how this mutation affects UBC9 enzyme function. To assess alterations in P123L catalysis, purified human SUMO pathway components [E1 (SAE1/2 heterodimer), wild-type or mutant UBC9, and SUMO-1 or SUMO-2] were incubated in *in vitro* SUMOylation assays at molar ratios of 1:2.5:3 (E1:UBC9:SUMO) plus ATP. DTT was added to resolve thioester linkages between the active site cysteines of UBC9 and the E1 with SUMO, and the products were resolved by SDS-PAGE. Infrared fluorescent imaging of dual SUMO and UBC9 antibody staining (Supplemental Figure S1) reveal isopeptide linkages of SUMO to lysine residues in SUMO itself or in UBC9.

In agreement with prior studies of yeast SUMO conjugation, we observed a temperature-dependent decrease in P123L mediated SUMO chain formation with increasing temperature (Figure 7A) ²³. Interestingly, wild-type (UBC9) and the P123A mutant exhibit a similar pattern of SUMO-2 conjugation at 37°C but P123A exhibits a slight preference for the

lower di-SUMO band at 30°C and the higher di-SUMO band at 42°C. In contrast, a more severe reduction in SUMO conjugates was formed by P123L at 42°C, and this deficit in extended chain formation was only partially restored with increased P123L protein. These reactions further resolved two distinct di-SUMO bands produced by UBC9, P123A, and P123L (Figure 7A, red box area and darker exposure below). However, P123L failed to generate the faster migrating di-SUMO form at 42°C.

Prior structural analyses of UBC9 indicated that Pro123 contributes to the coordination of hydrogen bonds that stabilize the carboxy-terminal di-Gly of SUMO bound to the active site cysteine of UBC9⁶. This UBC9~SUMO thioester reaction intermediate then acts to catalyze the conjugation of SUMO to acceptor site lysine residues in target proteins. Given the importance of thioester bond formation in UBL transfer we next asked if the defect in P123L SUMO conjugation was a consequence of reduced P123L~SUMO thioester bond formation. To test this, *in vitro* SUMOylation reactions were sampled at varying time points and treated with SDS buffer (minus or plus DTT, which reduces UBC9~SUMO thioester bonds). As seen in Supplementary Figures S5 and S6, thioester linkages of UBC9~SUMO intermediates are efficiently resolved by DTT. In the (+) DTT reactions, the same pattern of reduced P123L•SUMO conjugates (as in Figure 7A) were observed. However, in the absence (–) of DTT, comparable levels of UBC9~SUMO or UBC9–di-SUMO thioester intermediates were detected with UBC9, P123A or P123L, at 37°C or 42°C, even at early time points. These data indicate that P123L selectively impairs the formation of isopeptide linkages, but not the generation of the UBC9~SUMO thioester reaction intermediate.

P123L substitution induces a switch from N-terminal to internal lysine modification.

We next considered if the di-SUMO bands observed in Figure 7A result from the formation of different branch points (chain linkages), which could induced distinct effects on electrophoretic mobility. To address this question, we performed SUMOylation reactions with specific SUMO–2 Lys to Arg mutants (Figure 7B).

Previous studies have defined K11 as the major SUMOylation site in SUMO–2. K11 is located in an unstructured N-terminal consensus site motif, with K5 and K7 serving as minor acceptor sites (Figure 1A and 7B). SUMOylation of structured and α -helical residues such as K42 in SUMO–2 (K41 in SUMO–3) have also been reported⁹⁻¹⁰. In reactions with SUMO–2 K11R, both di-SUMO bands were detected, albeit with reduced efficiency. In contrast, mutation of all three N-terminal Lys residues in SUMO–2 K5, 7, 11R abolished the formation of the lower di-SUMO isoform. Based on these data, and prior proteomics analyses¹⁰, we assign K5,7,11 linkages to the lower di-SUMO band and internal lysine linkages, e.g. K42,45, to the upper band. Similar defects in SUMO–1 N-terminal chain linkages were also observed with P123L (Supplementary Figure S7).

P123L mutant suppresses extended SUMO chain formation.

In time course experiments with wild-type UBC9 and P123A (Figure 8A), the accumulation of the upper di-SUMO band precedes formation of higher order SUMO chains, UBC9-SUMO conjugates, and di-SUMO(K5,7,11) at 37 and 42°C. In contrast, the accumulation of high molecular weight SUMO conjugates (extended SUMO chains) and di-SUMO(K5,7,11)

were selectively impaired in reactions containing P123L. Quantitation of relative band intensities obtained over a 60 minute time frame at 37°C (Supplementary Figure S8) and 42°C (Figure 8B) reveals a switch in P123L substrate selection, from Lys residues in the extended N-terminal domain of SUMO-2 [lower di-SUMO(K5,7,11) band] to nonconsensus site Lys acceptors embedded in α -helical secondary structures in

SUMO (K42,45) (upper di-SUMO band), and UBC9 (K14) (UBC9•SUMO band). Moreover, the corresponding decrease in di-SUMO(K5,7,11) and global SUMOylation suggest that di-SUMO comprised of N-terminal K linkages, and especially K11, is a preferred substrate for higher order SUMO chain formation. This interpretation is also consistent with the appearance of di-SUMO(K5,7,11) in UBC9 and P123A reactions at later times, when free SUMO is largely depleted, effectively limiting additional chain formation.

DISCUSSION

The SUMO pathway plays a crucial regulatory role in maintaining genomic stability and cellular responses to DNA damage^{11,13,21,40}. Our previous studies determined that substitution of Leu for Pro123 in yeast UBC9 uncouples UBC9's function in suppressing cell sensitivity to genotoxic stress (at high temperature) from the essential catalytic function necessary to maintain cell viability in the absence of DNA damage²³. Studies with chimeric yeast/human UBC9 constructs highlighted similar effects of P123L on human UBC9 function, and implicated alterations in UBC9 target substrate discrimination²⁴. However, the mechanistic basis for discriminating between UBC9 target proteins required for resistance to DNA damage versus essential SUMO substrates was unclear.

Using NMR, we identified that the P123L mutant displays significant CSPs in those residues in the $\alpha 2\alpha 3$ loop, which are critical for the interaction between UBC9 and the hydrophobic residue in the $\Psi Kx[D/E]$ consensus motif, and in $\alpha 3$ and $\alpha 4$ residues implicated in substrate binding. Yet, using ^{13}C chemical shifts, we were able to demonstrate that the P123L mutant retains its secondary structure. The P123A mutant, which exhibits wild-type enzyme activity in our *in vitro* assays, does not exhibit the magnitude of CSPs observed in P123L, and its secondary structure are no different from wild-type UBC9. Our observation that multiple residues in the $^{15}N\{-^1H\}$ NOE spectra displayed decreased peak intensity and indicative of the presence of intermediate exchange in the $\alpha 2\alpha 3$ loop in P123L. Similar peak multiplets were observed in the R^1 spectra and led us to interrogate the backbone motions of the mutants in relation to wild-type UBC9. These studies revealed that P123L does not induce significant increases in backbone flexibility, but R_1 , R_2 , and $^{15}N\{-^1H\}$ NOE backbone dynamic analyses further support that the P123L mutation stabilizes specific states in the $\alpha 3$ helix. This could impair the physical interaction of P123L with select substrates, such as the lysine residues in the unstructured N-termini of SUMO-1 or SUMO-2/3. We confirmed that there are significant changes in the internal motions of P123L compared to wild-type UBC9 through collection of $^{15}N\{-R_2\}$ rates and calculation of generalized S^2 order parameters, which exhibited increased R_2 values and decreased S^2 values for select residues in the $\beta 2\beta 3$, $\beta 4\alpha 2$, and $\alpha 2\alpha 3$ loops. These residues lie along an interface that connects the core α/β fold of UBC9 to the $\alpha 3$ and $\alpha 4$ helices (Figure 6F), residues of which are implicated in substrate selection. Collectively, these data suggest that the spatial network of hydrophobic residues

underlying Pro123 plays a critical role in maintaining the dynamic interactions between the UBC9 core and the $\alpha 3/\alpha 4$ helices that are necessary for wild-type UBC9 substrate selection and SUMOylation.

When beginning these experiments, our initial hypothesis was that the P123L mutation induces a dynamically unstable protein centered on the mutation site as a result of the loss of backbone rigidity imparted by the proline residue. However, only upon critical review of our data did we arrive at the current model - that the leucine substitution makes critical hydrophobic contacts, which stabilize the mutant protein. Three key points led us to this model. First, we only observed changes in dynamics at the ends of each of helices $\alpha 3$ and $\alpha 4$, and not across the entire helix. Nor did we observe CSP of residues across both the $\alpha 3$ and $\alpha 4$ helices. These observations do not support a model that assumes the mutation is generally unfolding the alpha helices, as such unfolding should significantly impact dynamics and CSP across the entire helix and not only in localized regions. Secondly, the $\alpha 3$ and $\alpha 4$ helices make contact to the core UBC9 fold through three sites. The first two involve the two hydrophobic residues Y137 and Y144 from each helix, respectively. The third contact occurs between the C-terminus of helix $\alpha 4$ and the $\beta 2\beta 3$ loop. Notably, we observed neither CSP nor dynamic changes in the region of this third contact in the P123L mutant. This result strongly suggests that this contact is not perturbed. Thirdly, the kinetics of P123L catalyzed formation of UBC9-SUMO conjugates and di-SUMO conjugates formed at lysine residues embedded in structured elements of SUMO were unaffected at 42°C versus lower temperature. If, in fact, there was progressive unfolding of P123L at higher temperatures, we would anticipate a progressive reduction in efficiency of all conjugates. Instead, we observe a selective alteration in substrate specificity with increasing temperature with the P123L mutant. These observations obligated us to follow the data to the currently presented model.

Pro123 is in the $\alpha 2\alpha 3$ loop, and lies on a hydrophobic surface formed by residues in helices $\alpha 3$ and $\alpha 4$ and the loop that connects $\beta 4$ with the 310 helix. Our observed CSPs and alterations in R_1 and NOE spectra suggest that the introduction of the bulky hydrophobic side chain of leucine at this position impacts the ns- μ s timescale backbone motions of UBC9, stabilizing distinct chemical exchange states. To our knowledge, this is a unique report of a mutant-induced stabilization of distinct protein conformations resolvable in the timespan of the R_1 and $^{15}\text{N}-\{^1\text{H}\}$ NOE spectral acquisition but not R_2 or $[^{15}\text{N}, ^1\text{H}]\text{RHSQC}$ acquisition. These data raise the intriguing possibility that a distinct conformation in P123L may facilitate α -helical substrate binding to P123L to promote efficient conjugation of SUMO to acceptor lysines, while the less restricted movement of the $\alpha 3$ helix in wild-type UBC9 (and P123A), facilitates an induced fit model of binding to lysine acceptors in unstructured SUMO sites. Since the $\Psi\text{Kx[D/E]}$ consensus motif must lie within an extended region in order to be recognized by UBC9⁶, the deficiency exhibited by P123L might translate to an impairment in consensus lysine modification *in vivo*. In future studies, it will be interesting to address this model using different peptide substrates and additional backbone dynamic studies of other UBC9 mutants.

Here, we establish the temperature-dependent defect in P123L mutant enzyme catalysis in *in vitro* SUMO conjugation assays. In contrast to the preferential modification of lysine acceptor sites in the unstructured N-terminus of SUMO-2 (K7,11,15) catalyzed by wild-

type UBC9, P123L preferentially links SUMO to nonconsensus site lysine residues embedded in α -helical secondary structures (K42/45 in SUMO-2, K14 in UBC9). This switch in substrate specificity was also observed with SUMO-1, and coincided with a decrease in global SUMO conjugates. Our findings argue against P123L-induced defects in (1) the stabilization of the SUMO carboxy-terminal di-glycine motif, as efficient UBC9~SUMO2 thioester linkage formation was observed *in vitro*, or (2) in the catalytic priming of the acceptor Lys, as the kinetics of UBC9-SUMOylation and di-SUMO(K42/45) are not as impaired as SUMOylation of acceptor sites in the unstructured N-terminal residues. However, our data cannot exclude the possibility of selective defects in P123L catalysis induced by interaction with Lys residues in unstructured substrates. Additionally, our studies indicate that di-SUMO(K7,11,15), and in particular di-SUMO(K11), is more efficiently converted to longer chains than di-SUMO isoforms with an atypical internal Lys linkage, such as K42/45.

Our findings also represent the first evidence that distinct poly-SUMO linkages might have distinct biological consequences – in this case – the response to DNA damage. While we have not linked the switch in P123L chain formation directly to DNA damage hypersensitivity, neither wild-type UBC9 nor P123A exhibit altered chain linkages or the CSPs seen with P123L. The concept of different chain linkages has been robustly explored for ubiquitin¹, and in the formation of mixed SUMO-ubiquitin chains^{11,41-42}. However, there has been little evidence of atypical SUMO chain linkages (either as homotypic or heterotypic chains) mediating distinct cellular processes. In part, this may be due to the technical challenges in detecting branched SUMO-peptides by standard proteomic approaches, and the relatively low abundance of SUMO conjugates^{11-19,40,43-45}. The sensitivity of proteomic determinations of global SUMOylation sites has been improved using cell lines engineered to express His10-tagged K0-SUMO-2^{11,13-14,17}. However, the mutation of all lysine residues in the ectopically expressed K0-SUMO precludes chain formation, making the identification of rare chain linkages with endogenous SUMO-2 even more challenging. Nevertheless, should more direct evidence emerge linking catalysis of particular SUMO modifications to protection from DNA damage, such findings would support selectively targeting the DNA damage response function of UBC9 by disrupting its catalysis of particular poly-SUMO linkages. As UBC9 overexpression is correlated with increased malignancy and poor prognosis in Myc-dependent cancer and some cancers with activated Ras⁴⁶⁻⁴⁸, this approach could provide a better way to target cell SUMOylation without indiscriminately inhibiting all SUMOylation catalyzed by UBC9.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

SUMO	Small ubiquitin-related modifier
ULP	ubiquitin-like protein
UBC	ubiquitin conjugating fold
NMR	nuclear magnetic resonance
HSQC	heteronuclear single-quantum correlation
CSP	chemical shift perturbation

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A

SMT3	MSDSEVNQEA	KPEVK	--PEVK	PETHINL	26
SUMO-1	MSD----	QEAKPSTEDLGD	KKEGEYIKL		24
SUMO-5	MSD----	QEAKLSTEH LGD	KIKDEDIKL		24
SUMO-2	MAD----	EKPKEGV	--TENN--	DHINL	20
SUMO-3	MSE----	EKPKEGV	--TEN---	DHINL	19
SUMO-4	MAN----	EKPTEEV	--TENNN--	HINL	20

Figure 1: SUMO consensus interactions with UBC9.

(A) Sequence alignment of N-terminal residues from yeast SMT3, and human SUMO-1, SUMO-2, SUMO-3, SUMO-4, and SUMO-5. Consensus lysine residues are highlighted in red while non-consensus lysines are highlighted in cyan. (B) Ribbon presentation of UBC9 depicting location of P123 (yellow) and residues that are critical for SUMO transfer. Pro123 is located in the $\alpha 2$ - $\alpha 3$ loop, and in coordination with Leu94, Ser95, Glu122, and Asn124 (orange), forms a series of stabilizing hydrogen bonds with the SUMO carboxy-terminal di-glycine motif [7]. Further along the same loop, residue Asp127, in concert with Asn85 and Tyr87 (blue), has been shown to catalyze SUMO conjugation by manipulating the pKa of the acceptor lysine residue [4, 7]. The carboxy-terminus of the $\alpha 2$ - $\alpha 3$ loop

contains residues Pro128, Gln130, and Ala131 (green), which make Van der Waals contacts with the conserved hydrophobic residue in the consensus SUMO acceptor site [Ψ Kx(D/E)] [7, 25]. Hydrogen bonding interactions with the consensus acidic D/E residues (in the acceptor site) is mediated by interactions with residues Lys74, Ser89, and Thr91 (red) [7], which reside in the loop that connects β 4 with the 310 helix and also contains the active site cysteine, C93 (yellow). PDB: 1A3S²⁰

Figure 2: Spectral response to P123L mutation.

(A) Overlay of 2D [^{15}N , ^1H]-HSQC spectra of wild-type (black) and P123L mutant (red) UBC9. (B) Se-quene mapping of weighted [^{15}N , ^1H] chemical shift perturbation resulting from mutation. Thresholds corresponding to 3 (0.05 ppm), 6 (0.1 ppm), and 9 (0.15 ppm) standard deviations are shown marked by yellow, orange or red dotted lines, respectively. Unassigned residues in the mutant spectra are denoted by negative cyan bars and the mutation site is identified by a negative gold bar. (C) Ribbon representation of the human UBC9 structure with locations of unassigned, moderately shifted and significantly shifted residues colored as described in (B). PDB: 1A3S²⁰

Figure 3: Spectral response to P123A mutation.

(A) Ribbon representation of the human UBC9 structure with locations of moderately shifted and significantly shifted residues colored as outlined below. (B) Sequence mapping of weighted $[\text{^{15}N}, \text{^1H}]$ chemical shift perturbation resulting from P123A mutation. Threshold corresponding to 0.05 ppm and 0.1 ppm are marked by yellow or orange dotted lines, respectively. PDB: 1A3S²⁰

Figure 4: P123L retains secondary structured elements.

Plots of predicted alpha helical (positive, green) and beta strand (negative, blue) secondary structure derived from $C\alpha$ and $C\beta$ chemical shift values for (A) wild-type UBC9, (B) P123A, and (C) P123L. Unassigned residues in P123L are identified by red dashes. (D) Overlay of (A) and (C) showing a strong retention of secondary structure in the P123L mutant.

Figure 5: P123L mutation does not increase backbone flexibility.

Sequence plot of $^{15}\text{N}\{-^1\text{H}\}$ NOE intensities for (A) WT UBC9, (B) P123A, and (C) P123L. Unassigned residues and weak peaks (cyan dashes), split peaks (magenta circles), and a dashed line indicating significance thresholds (0.6; 0.0) are identified. (D) Mapping of the data illustrated in (C) on the structure of UBC9. Side chains of the hydrophobic residues F82, Y137, and Y144 are shown in grey. (E) Comparison of peak intensities obtained with (NONOE) and without (NOE) proton saturation, are presented in black and red, respectively. PDB: 1A3S²⁰

Figure 6: Transverse relaxation rates (R_2) indicate P123L stabilizes ns- μ s chemical states. Sequence plots of R_2 values for (A) UBC9 and (B) P123L. Open circles mapped for residues with large and uncertain R_2 relaxation rates. Representative ^{15}N - R_2 relaxation data for (C) UBC9 and (D) P123L. The solid lines are single-exponential best fits. Cartoon plots of (E) UBC9 and (F) P123L, respectively mapping R_2 rates on the structures. Color is based on R_2 rates. Blue represents residues below the dashed threshold, indicative of free exchange, orange residues indicate a moderate increase in R_2 rates, and red indicates residues with open circles in (A), (B) with large R_2 relaxation rates. Missing assignments and residues K59, H83, and S89 with low R_2 spectral intensity are colored in light blue. PDB: 1A3S²⁰

Figure 7: P123L induces a switch in SUMO-2 chain linkage formation.

(A) Purified human E1, UBC9 (wild-type, P123A, or P123L) and SUMO-2 (molar ratio of 1:2.5:10, respectively) were incubated at 30°C, 37°C, or 42°C for 2 hours. 3X reactions contained 3-fold higher levels of P123L. Samples were immunoblotted with anti-SUMO-2 antibodies. A darker exposure of the portion of the gel boxed in red is shown below. (B) As in A, except as indicated wild-type or mutant SUMO-2 were included, and reactions sampled at varying times. Representative images of n=3 experiments.

Figure 8: Decreased global SUMO conjugation and N-terminal SUMO chain linkage formation by P123L.

(A) As in Figure 7, SUMOylation reactions were sampled at the indicated times, and immunoblotted with SUMO-2 antibodies. * position of free SUMO-2, ** indicates di-SUMO bands. (B) Band pixel densities (n=3 independent experiments) from 0–60 minutes, at 42°C, were quantified, with values plotted relative to those obtained with wild-type UBC9 at 60 minutes.