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Detection of rapidly accumulating stress-induced SUMO in prostate cancer cells by a fluorescent SUMO biosensor

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Abstract

SUMO conjugates and SUMO chains form when SUMO, a small ubiquitin-like modifier protein, is covalently linked to other cellular proteins or itself. During unperturbed growth, cells maintain balanced levels of SUMO conjugates. In contrast, eukaryotic cells that are exposed to proteotoxic and genotoxic insults mount a cytoprotective SUMO stress response (SSR). One hallmark of the SSR is a rapid and massive increase of SUMO conjugates in response to oxidative, thermal, and osmotic stress. Here, we use a recombinant fluorescent SUMO biosensor, KmUTAG-fl, to investigate differences in the SSR in a normal human prostate epithelial cell line immortalized with SV40 (PNT2) and two human prostate cancer cell lines that differ in aggressiveness and response to androgen (LNCaP and PC3). In cells that grow unperturbed, SUMO is enriched in the nuclei of all three cell lines. However, upon 30 min of exposure to ultraviolet radiation or oxidative stress, we detected significant cytosolic enrichment of SUMO as measured by KmUTAG-fl staining. This rapid enrichment in cytosolic SUMO levels was on average fivefold higher in the LNCaP and PC3 prostate cancer cell lines compared to normal immortalized PNT2 cells. Additionally, this enhanced enrichment of cytosolic SUMO was reversible as cells recovered from stress exposure. Our study validates the use of the fluorescent KmUTAG-fl SUMO biosensor to detect differences of SUMO levels and localization between normal and cancer cells and provides new evidence that cancer cells may exhibit an enhanced SSR.

KEYWORDS

cytosolic/extra-nuclear SUMO, fluorescent SUMO-trapping biosensor protein, prostate cancer, SUMO, SUMO stress response (SSR)

1 | INTRODUCTION

Sumoylation, the protein modification with the small ubiquitin-like modifier (SUMO), is a fundamental protein modification process that is conserved from yeast to humans and controls the function, activation, localization, interaction, and half-life of specific cellular proteins (reviewed by Zhao et al., 2018). The proteins that promote sumoylation and SUMO dynamics act via a cascade of SUMO activating (E1), conjugating (E2), and ligase (E3) enzymes as well as SUMO proteases and SUMO-targeted ubiquitin ligases.[1] A recent study identified more than 4300 sumoylation sites in more than 1600 proteins from HeLa cells. This study confirmed that sumoylation is a predominantly nuclear event that regulates transcription, DNA repair, chromatin remodeling, pre-mRNA splicing, and ribosome assembly. Of note, a large number of human disease proteins were found to be targets of SUMO modification.[2,3]

An early study by Saitoh and Hinchey[4] showed that exposure to proteotoxic and genotoxic stressors leads to an extremely rapid

increase of SUMO-conjugated proteins in the cell, often within minutes. For example, SUMO-modification is rapidly enhanced when cells are subjected to reagents and conditions that damage proteins (e.g., hydrogen peroxide and increased temperature) or DNA (e.g., ultraviolet radiation [UV] irradiation). This phenomenon is now termed the SUMO stress response (SSR).[5] A long list of sumoylated proteins that accumulate in response to stress, including many chromatin remodeling factors and transcription factors, has been identified using proteomic approaches (reviewed in Golebiowski et al.[6] and references therein). It remains unclear, however, why this massive SUMO modification event unfolds as cells experience stress.

There is good evidence that organisms as diverse as yeast and humans utilize SUMO modification in their cellular stress response. This is borne out by the reduced stress tolerance of cells that lack intact sumoylation pathways. In yeast, a number of nonessential genes involved in the response to proteotoxic and genotoxic stress result in lethality when paired with genetic defects in sumoylation and desumoylation (reviewed in Seeler and Dejean[7]). Correspondingly, mammalian cells that are depleted of SUMO or the SUMO protease SENP1 show reduced ability to survive acute heat shock or exposure to ionizing radiation.[6,8] This suggests that sumoylation plays an important role in the response to proteotoxic and genotoxic stress.

SUMO's role in stress tolerance, however, remains enigmatic at best. Findings from three recent studies arrive at different, yet nonmutually exclusive conclusions.[5,6,9] One study found that, in mammalian cells, SUMO isoforms served a chaperone-like function and maintained the homeostasis of large chromatin-associated nuclear proteins during stress.[10] Another found that sumoylation temporarily stabilized denatured proteins after heat shock, preventing them from aggregating before proteasome-mediated degradation.[9] A third study found that environmental stress induced a wave of transcription-coupled sumoylation and the SSR was found to be blocked when transcription was inhibited.[5]

A rapid increase in sumoylation and the SSR occurs due to increased activity of SUMO E3 ligases, possibly coupled with a decrease in SUMO protease activity. This is borne out by the finding that initiation of the SSR in stressed cells is caused primarily by the E3 SUMO ligase Siz1 in yeast, and the combined effort of the orthologous SUMO ligases PIAS1-4 in heat-stressed osteosarcoma cells.[5,10] Additionally, several SUMO proteases (Ulp1 in yeast and SENP 1,2,3,7 in mammalian cells) are inactivated by heat and/or oxidative stress, suggesting they may act as stress sensors.[11] Inactivation of these SUMO proteases invariably results in an accumulation of SUMO-conjugated proteins as well as SUMO chains. Recent research from our lab indicates that the *S. cerevisiae* SUMO protease Ulp1 is unable to bind SUMO in the presence of extremely low levels of hydrogen peroxide (1.8 mM), underscoring a potential role of SUMO proteases as redox sensors.[12] One notable exception, the mammalian SUMO protease SENP6 is not inactivated by heat stress and becomes recruited to chromatin.[10,11] SENP6 is similar to the yeast SUMO protease Ulp2, which in turn is required for recovery from the SSR in yeast. This suggests that both SENP6 and ULP2 are involved in removing SUMO chains as a necessary step for the recovery of cells from the SSR.[5] Additionally, low levels of oxidative stress also rapidly disable the SUMO E1 (Uba2) and E2 (Ubc9) enzymes—via the formation of a disulfide bond between their catalytic cysteine residues—raising the question of how sumoylation can become amplified dramatically during the SSR when SUMO conjugation is halted.[13] The answer is unknown, but a pre-existing pool of Ubc9 with noncovalently bound SUMO, which has been shown to be involved in the formation of SUMO chains, may hint at the answer.[14]

The SSR pathway exists in single-cell eukaryotes (e.g., yeasts), in normal mammalian cells, and is generally dysregulated in cancer cells.[7] Cancer cells are subjected to a host of adverse conditions including hypoxic environments within tumors, attack by tumor-invading immune cells, and rampant aneuploidies that dysregulate cellular proteostasis. Consequently, cancer cells rely on enhanced stress response pathways to survive under these conditions. In general, sumoylation enzymes, such as activating (E1) and conjugating (E2) enzymes have been found to be elevated in tumors, potentially altering the activity of dozens of SUMO-modified tumor suppressors, oncoproteins, and stress response proteins, including heat-shock proteins (e.g., Hsp90), hypoxia-inducible factors (e.g., Hif1A), and inflammatory signaling factors (e.g., IkBalpha) (reviewed in Seeler and Dejean[7]). Specifically, the overexpression of the SUMO ligase Ubc9 in cancers has been linked to poor treatment outcomes and Ubc9 overexpression may be a useful biomarker for cervical cancer.[15,16] However, elevated levels of some SUMO proteases have also been linked to breast and prostate cancer development.[17,18] These examples suggest that accelerated SUMO dynamics, both sumoylation and desumoylation, are at play in the stress resilience of cancer cells.

We recently developed a novel SUMO-trapping protein from a thermotolerant yeast, *Kluyveromyces marxianus* (Km).[12] Specifically, we generated a catalytically inactive recombinant SUMO-trapping fragment of the UD domain of Ulp1 (the yeast SUMO protease), termed KmUTAG. KmUTAG can efficiently bind to a variety of purified SUMO isoforms and immobilized SUMO1 with nanomolar affinity (Kd ~12.8 nM). Moreover, KmUTAG has high affinity for SUMO and SUMO-modified proteins even in the presence of oxidative stressors (170 mM H_2O_2), reducing agents (5 mM TCEP Tris(2-carboxyethyl)phosphine hydrochloride), denaturants (up to 2 M UREA), and temperature stress (42°C) that induce protein misfolding.[12]

Next, to visualize SUMO within cells with the KmUTAG, we developed a Recombinant Fluorescent SUMO biosensor KmUTAG-fl.[19] KmUTAG-fl is a recombinant mCherry-tagged SUMO-trapping fusion protein. This stress-tolerant pan-SUMO specific biosensor is produced recombinantly in bacteria. Once purified it recognizes and traps native SUMO- conjugated proteins and SUMO chains in fixed permeabilized cells. This biosensor protein compares favorably to staining protocols with SUMO-specific antibodies as it has reduced affinity for free, unconjugated SUMO. Meanwhile, it can be used to analyze SUMO variants from additional model and non-model systems. In the present study, we used KmUTAG-fl to detect SUMO in a

variety of human prostate cells lines; a non-tumorigenic SV40-immortalized prostate epithelial cell line (PNT2), an aggressive androgen-insensitive prostate cancer cell line derived from bone metastasis with high metastatic potential (PC3), and a hormone-sensitive metastatic prostate cancer cell line derived from lymph node metastasis with low metastatic potential (LNCaP). Using the fluorescent KmUTAG-fl biosensor, we compared SUMO levels in the nuclei and extra-nuclear compartment (cytosol) in untreated, UV-treated, and H_20_2-treated cells. We detected significant differences in the SUMO profiles between normal (from here on defined as nonmalignant, immortalized PNT2 prostate epithelial cells) and cancer prostate cancer cells. After stress exposure, both prostate cancer cell lines showed a cytosolic SUMO enrichment that was approximately fivefold higher than normal PNT2 cells. The cytosolic SUMO enrichment was detected within 30 min of stress exposure and was completely reversible after recovery in fresh media. While there was a clear difference between cancer and normal prostate cells, we did not detect a difference between cells exhibiting low (LNCaP) and high (PC3) metastatic potential. Our study suggests that differences in the SSR may be linked to the enhanced robustness of cancer cells and therefore, SUMO profile visualization using the KmUTAG-fl biosensor could be a useful tool to differentiate normal and tumorigenic cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture and maintenance

PC3, PNT2, and LNCaP cells were grown in RPMI media with 10% heat-inactivated FBS (Thermo Fisher Scientific; #10438018) and 1% antifungal/antibiotic (anti/anti) (Thermo Fisher Scientific; #15240062). All cells were grown at 37°C in a humidified incubator which is kept constant at 5% CO_2.

2.2 | Expression of KmUTAG-fl and in vitro assays

A codon-optimized bacterial overexpression clone of mCherry-KmUTAG was generated as previously described.[19] KmUTAG-fl biosensor were overexpressed in BL21-STAR(DE3) cells. Purification of KmUTAG-fl in these cells was as previously described[19] except that KmUTAG-fl was bound to magnetic SPOT-trap beads (Chromotek). To assess the SUMO-trapping activity of KmUTAG-fl, SUMO-binding reactions were performed on SUMO1 beads and with the recombinant SUMO-CAT fusion protein.[12]

For SUMO staining, PC3, PNT2, or LNCaP cells were grown to 80% confluency and counted using a hemocytometer. Each well in a 6-well plate (Thermo Fisher Scientific; 07-200-83) received 300,000 cells in 2 ml media. Cells were incubated for 24 h until 80% confluent. After fixing the cells with fresh 4% paraformaldehyde (PF) for 20 min at room temperature, cells were permeabilized for 15 min with 0.1% Triton X-100 in dPBS. Next, the cells were incubated with 0.1 M glycine-HCL (pH 2.0) for 10 s. The pH was immediately neutralized with 500 µL of 10X SPB (500 mM Tris-HCL, pH 8.0 2% NP-40, 1.5 M NaCl)[12] and coverslips were removed from the well and placed into humidity chambers. For KmUTAG-fl staining, 2 µg of recombinant KmUTAG-fl was added to 100 µl 1X SPB containing 5 mM TCEP. The mix was transferred onto the coverslip and incubated at room temperature for 1 h in the humidity chamber. For KmUTAG-fl and anti-SUMO2/3 antibody co-staining, 2 µg of KmUTAG-fl and 0.5 µl SUMO2/3 8A2 (obtained for Developmental Studies Hybridoma Bank - DSHB Hybridoma Product SUMO-2 8A2)[20] were mixed with 100 µl blocking buffer, pipetted onto the coverslip, and incubated in room temperature for 1 h. After washing the coverslips with dPBS, 0.5 µl anti-mouse Alexa Fluor 488–conjugated antibody (Jackson ImmunoResearch 115-545-003) in 100 µl blocking buffer (Prometheus 20-313), was pipetted onto the coverslip of KmUTAG-fl and SUMO2/3 co-staining slides, and incubated at room temperature for 1 h. After washes with dPBS, the coverslips were inverted onto pre-cleaned microscopy slides with FLUORO-GEL II with DAPI (Electron Microscopy Sciences 50-246-93). The slides were stored at −20°C overnight before viewing under the microscope.

2.3 | Stress treatment

To test the SSR to UV damage, cells were grown until ~80% confluent on coverslips and subjected to UV irradiation before proceeding with fixation and staining. The coverslips were first transferred from the six-well plate to a humidity chamber. Excess media on the coverslip was removed and the humidity chamber containing the coverslip was put into a UV chamber (GS Gene Linker). The UV intensity was adjusted per the manufacturer's protocol. After irradiation, coverslips were immediately placed back into the culture medium and placed back into the tissue culture incubator for an additional 30 min. Fixation and staining were completed as detailed above.

For peroxide stress treatment, H_2O_2 was added from a 3 M stock solution to 2 ml cultures in a six-well plate to achieve the desired final concentration. Cells were then incubated for an additional 30 min in the tissue culture incubator. After incubation, the tissue culture supernatant was removed and the cells were washed in dPBS before fixation and staining with KmUTAG-fl. All experiments were completed at least in duplicate and include 2–10 slides with cells per condition tested. Representative images are shown after normalization.

2.4 | Microscopy and data analysis

Images were acquired using a fully automated Nikon A1R inverted confocal microscope or a Zeiss Axioscope using the appropriate filter sets. Fluorescence staining intensity was quantified using CellProfiler (www.cellprofiler.org).[21] Nuclei and cytosol of imaged cells were automatically detected as DAPI-stained nuclei and kmUTAG-fl

stained nuclei and cytosol, respectively. For normalization between images, the background fluorescence intensity between cell features was subtracted from the mean intensity of each compartment of individual cells to obtain the cytoplasmic and nuclear staining intensity. The relative cytosolic enrichment was calculated as the ratio between cytoplasmic and nuclear staining intensities per cell. Number of cells used for evaluation is listed in the individual figure legends. Unpaired parametric t-test was used to assess significant differences in staining intensity of cytosolic and nuclear kmUTAG-fl before and after stress exposure. Significance level signs were displayed to indicate the result of the *t* test (NS: $p > 0.05$, $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$). The percentage difference between mean fluorescence intensity levels per treatment group was calculated to further describe the change in kmUTAG-fl signal intensity before and after stress. Data were graphed using R software.

3 | RESULTS

3.1 | Cytosolic SUMO levels increase in PC3 prostate cancer cells upon UV irradiation

We previously showed that KmUTAG-fl is a single-chain recombinant pan-SUMO binding protein that reliably detected SUMO conjugates in fixed nematode gonads and in mammalian cells.[19] In unperturbed mammalian cells, the KmUTAG-fl signal colocalized with SUMO2 in the nucleoplasm and in distinct nuclear foci.[12,19] To investigate the differences of the SSR in normal and cancer cells, we used KmUTAG-fl to investigate the impact of UV-induced stress on SUMO levels in two human prostate cell lines, immortalized normal PNT2 cells derived from prostate epithelium and PC3 adenocarcinoma cells with high metastatic potential.[22,23] PC3 and PNT2 cells were grown on coverslips and subjected to various doses of UV irradiation (50 mJ/m^2 and 150 mJ/m^2). After irradiation, the cells were allowed to recover for 30 min in culture medium before fixing and staining with KmUTAG-fl. A rapid increase in KmUTAG-fl staining after UV exposure (150 mJ/m^2) was visually apparent in PC3 cells (Figure 1A, left panel), but was not discernible in PNT2 cells (Figure 1A, right panel). Levels of KmUTAG-fl in PC3 cells were measured and quantified using CellProfiler, revealing a significant increase in SUMO levels between the unirradiated (zero "0" mJ/m^2) and irradiated (150 mJ/m^2) samples (Figure 1B, top panels). Specifically, we found that after UV treatment (150 mJ/m^2) KmUTAG-fl staining in the cytosol (denoting the extra-nuclear region of the cell) increased by ~54% in PC3 cells (Figure 1C). In comparison, no significant change in SUMO accumulation was detected in the cytosol of PNT2 (Figure 1B, bottom left panel). Therefore, the difference in cytosolic SUMO levels between UV-treated PC3 and PNT2 cells (150 mJ/m2) was approximately 14-fold. Concomitantly, UV-treatment also increased SUMO levels in the nucleus of PC3 cells, albeit only by 27% (Figure 1B, top right panel). No significant change in SUMO accumulation was detected in nuclei of PNT2 (Figure 1B, bottom right panel). Importantly, taking into account nuclear and cytosolic KmUTAG-fl signals, we recorded an increasing relative cytosolic enrichment (RCE) of SUMO after UV irradiation (Figure 1D). These data indicated that the SSR in PC3 cancer cells involved a significant increase of SUMO or SUMO conjugates in the cytosol.

Next, we wanted to know whether this phenomenon of increased extra-nuclear SUMO accumulation could be recapitulated using the monoclonal anti-SUMO2 8A2 antibody to visualize SUMO.[20] Therefore, we compared the ability of KmUTAG-fl and the anti-SUMO2 8A2 antibody to detect a change in the localization and levels of SUMO following UV-irradiation of PC3 cells. As expected, nonirradiated PC3 cells stained with the 8A2 antibody revealed SUMO2 localization in the nucleus. However, upon UV irradiation (250 mJ/m^2), SUMO2 was also detected in the cytosol of these cells (Figure 2A). Quantitation of KmUTAG-fl and 8A2 signal revealed that cytosolic SUMO/SUMO2 levels increased by 67% and 52%, respectively (Figure 2C). However, unlike the KmUTAG-fl stained sample, 8A2 antibody staining did not reveal a significant change of nuclear SUMO levels in irradiated PC3 cells (Figure 2B, left panel). In summary, these data confirmed that KmUTAG-fl was an effective reagent to detect increased SUMO levels, especially in the cytosol of PC3 cancer cells after acute UV exposure.

3.2 | Cytosolic SUMO levels increase in PC3 prostate cancer cells due to oxidative stress

The rapid accumulation of extra-nuclear SUMO levels due to UV irradiation prompted us to investigate the SSR in PC3 and PNT2 cells after exposure to oxidative stress. First, we treated PC3 cells with varying concentrations of hydrogen peroxide (0.5 µM to 30 mM H_2O_2) for 30 min, and immediately fixed and stained with KmUTAG-fl. Our analysis of PC3 cell staining revealed a significant increase of cytosolic and nuclear KmUTAG-fl signal after treatment with 25 µM to 30 mM H_2O_2 (Figure 3A,B). Overall SUMO accumulation and relative cytosolic enrichment peaked at 1 mM hydrogen peroxide exposure (Figure 3A,B, left panel, and C). The most significant median increase of SUMO staining intensity in PC3 cells was observed after 1 mM H_2O_2 treatment both in the cytosol (216%) and nucleus (87%) (Figure 3B,G).

By comparison, the cytosolic and nuclear KmUTAG-fl signal in PNT2 cells did not reveal a steady trend of increasing SUMO signal following treatment (Figure 3D–F). Yet a statistically significant increase in cytosolic PNT2 SUMO staining intensity was observed after treatment with 20 µM H_2O_2 (~40%). However, cytosolic and nuclear SUMO accumulation in PNT2 cells fell below the intensity of the untreated control (28% reduction) at 5 mM H_2O_2. The reason behind this observation is not entirely clear but may be linked to the reduced ability of PNT2 cells to withstand even brief treatment with 5 mM hydrogen peroxide (Figure 3E,F). Importantly, when comparing hydrogen peroxide-treated PC3 and PNT2 cells, a clear trend of nuclear and cytosolic SUMO accumulation emerges only in the carcinogenic PC3 cells (Figure 3G).

Simultaneously, we also quantitated nuclear SUMO foci in H_2O_2-treated PNT2 and PC3 cells. Nuclear foci of SUMO2/3 are a hallmark of SUMO-specific localization in mammalian cells and colocalize with a variety of nuclear proteins including the PML protein. PML nuclear bodies are implicated in nuclear stress response and reported to increase over

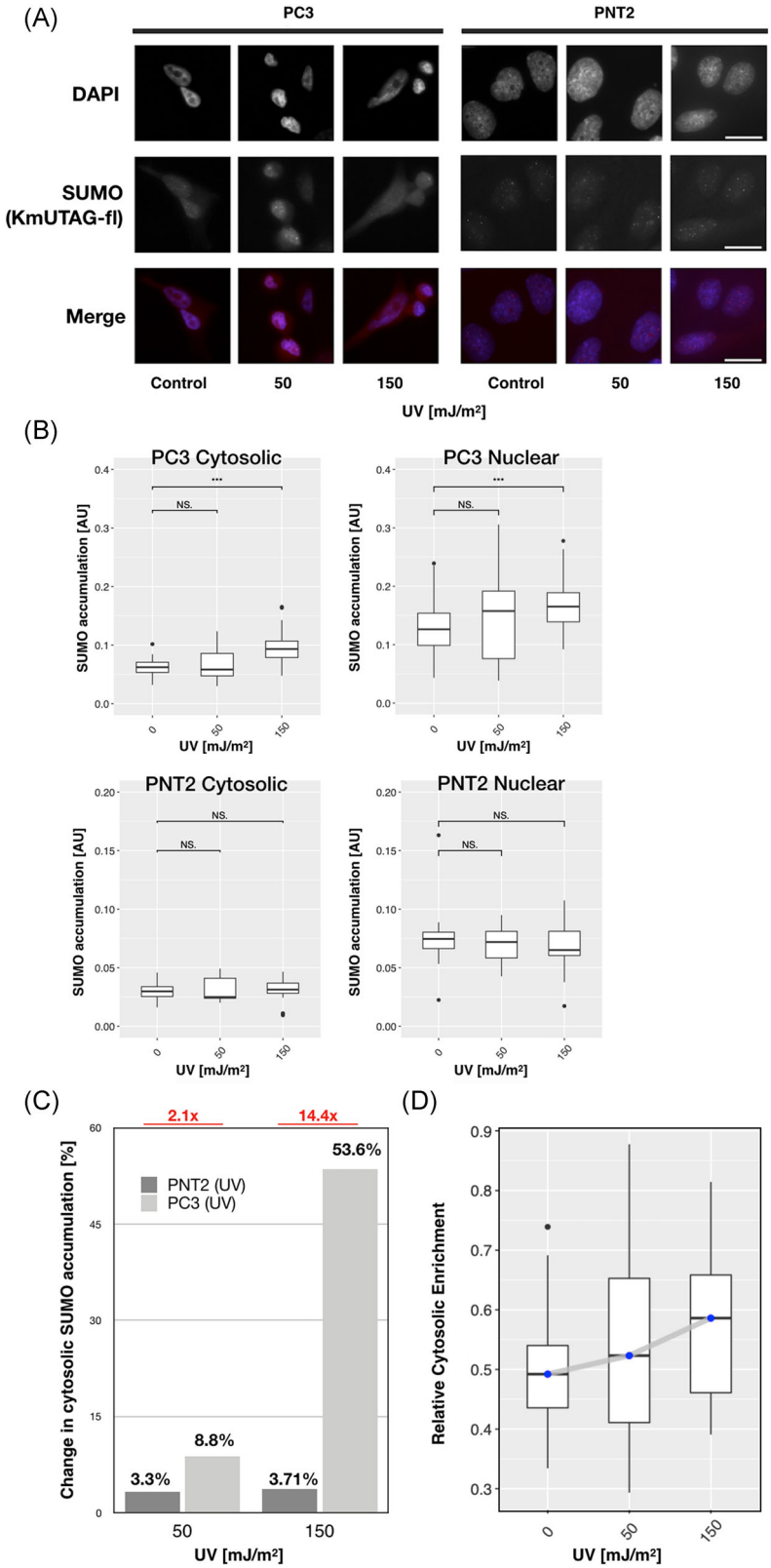

FIGURE 1 SUMO enrichment of UV irradiated PC3 and PNT2 cells.(A) UV dosage changed the SUMO profile of PC3 cells but not of PNT2 cells. UV irradiated PC3 and PNT2 cells (0 (Control), 50 mJ/m^2, and 150 mJ/m^2) were stained with KmUTAG-fl (SUMO), and visualized using confocal microscopy for mCherry (KmUTAG-fl, red) and DAPI (blue). (B) Nuclear and cytosolic KmUTAG-fl signal intensities in PC3 and PNT2 cells were quantified with CellProfiler (Carpenter et al., 2006 PMID: 17076895). PC3: 0 mJ/m^2, $n = 54$; 50 mJ/m^2, $n = 27$; 150 mJ/m^2, $n = 68$. PNT2: 0 mJ/m^2, $n = 20$; 50 mJ/m^2, $n = 20$; P 150 mJ/m^2, $n = 29$. (C) Increased cytosolic SUMO accumulation (KmUTAG-fl signal intensity) of PC3 cells compared to PNT2 cells. Intensity change was calculated as the difference between the UV irradiated and untreated Control cells. (D) Relative cytosolic enrichment (RCE) ratio of SUMO (KmUTAG-fl signal) in PC3 cells increased with UV dosage. The RCE was calculated as the ratio between cytosolic and nuclear fluorescence intensity. Statistical analysis by unpaired t test (NS: $p > 0.05$, $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$). Scale bar = 20 µm [Color figure can be viewed at wileyonlinelibrary.com]

time when cells are exposed to genotoxic stressors.[24] However, our short (30 min) incubation with H_2O_2 did not statistically affect the frequency, size, or intensity of SUMO foci in either PC3 or PNT2 cells (data not shown).

In summary, our data suggest that both UV irradiation and oxidative stress resulted in a rapid and concentration-dependent accumulation of SUMO in PC3 cancer cells, especially in the cytosol. These differences were not observed under similar conditions in

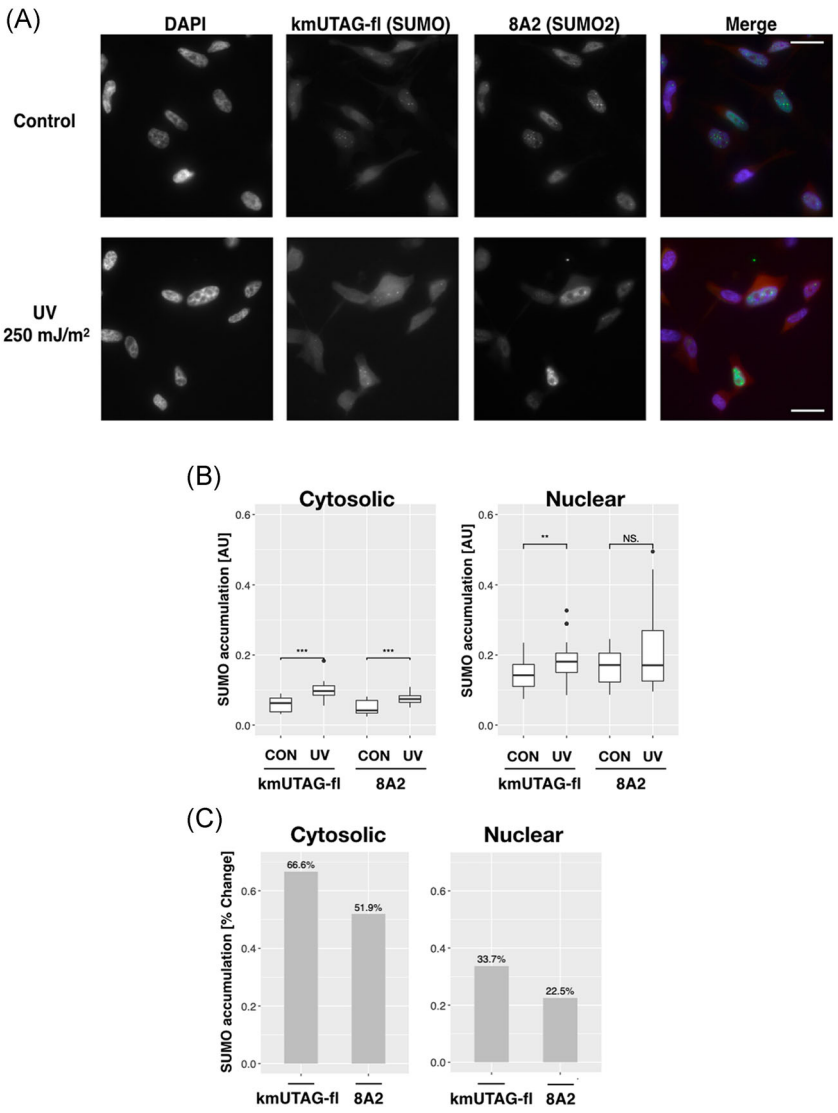

FIGURE 2 KmUTAG-fl and anti-SUMO2 8A2 antibody co-staining of UV irradiated cancer cells.(A) Similar SUMO profiles were observed using the KmUTAG-fl biosensor (red) and the anti-SUMO antibody (green). PC3 cells were irradiated with 250 mJ/m^2 UV. Fixed cells were co-stained with KmUTAG-fl (SUMO), anti-SUMO2 8A2 antibody (SUMO2), and DAPI (nucleus, blue). Control cells were not UV-irradiated. (B) Average cytosolic and nuclear KmUTAG-fl and 8A2 signal intensities (SUMO accumulation) were quantified with CellProfiler (km_Control $n = 23$; km_UV $n = 22$; 8A2_Control $n = 23$; 8A2_UV $n = 22$). (C) Percent change in cytosolic and nuclear KmUTAG-fl and antibody signal intensity in PC3 cells. Statistical analysis by unpaired t test (NS: $p > 0.05$, $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$). Scale bar = 20 µm. AU, arbitrary units [Color figure can be viewed at wileyonlinelibrary.com]

PNT2 cells. Therefore, we focused on this novel stress-induced accumulation of SUMO conjugates specifically in the cytosol.

3.3 | Recovery from peroxide stress is accompanied by the reduction of cytosolic SUMO levels

We evaluated the possibility that the rapid increase of cytosolic SUMO levels in PC3 cancer cells was a reversible process. PC3 and PNT2 cells were treated for 30 min with H_2O_2 before recovery in fresh media for 1–5 h. As before, treatment with 1 mM peroxide resulted in a significant and rapid increase of cytosolic (246%) and nuclear (104%) SUMO signals in PC3 cells (Figure 4A,C,E). Remarkably, the nuclear SUMO signal of PC3 cells remained elevated (108%) 1 h into the recovery period (Figure 4C, right panel, and E, bottom panel). During recovery, a significant reduction of cytosolic and nuclear SUMO levels was apparent after 2 h and SUMO levels returned to pretreatment levels after 5 h (Figure 4C,E). Taking into account nuclear and cytosolic KmUTAG-fl signals, we found a robust RCE of SUMO that decreased steadily during recovery (Figure 4F, left panel). These data indicated a rapid and dynamic fluctuation of SUMO levels in the nucleus and cytosol of PC3 cells as they responded to and recovered from oxidative stress.

In contrast, the increase of cytosolic (28%) and nuclear (44%) SUMO levels in PNT2 cells was significantly less pronounced (compare Figure 4C and D). Notably, the cytosolic and the nuclear SUMO signal of PNT2 cells peaked between 1 and 3 h into the recovery period, suggesting slower SSR response kinetics in PNT2 cells (Figure 4D,E). This was also apparent when comparing the RCE of PC3 and PNT2 cells. The RCE of SUMO in PC3 cells peaked immediately after H_2O_2 treatment (0.85) while the RCE of SUMO in PNT2 cells peaked after 1 h (0.58, Figure 4F). In summary, these data suggest that SUMO levels of the PC3 prostate cancer cell line significantly increased after H_2O_2-induced stress and subsequently decreased as part of a recovery process.

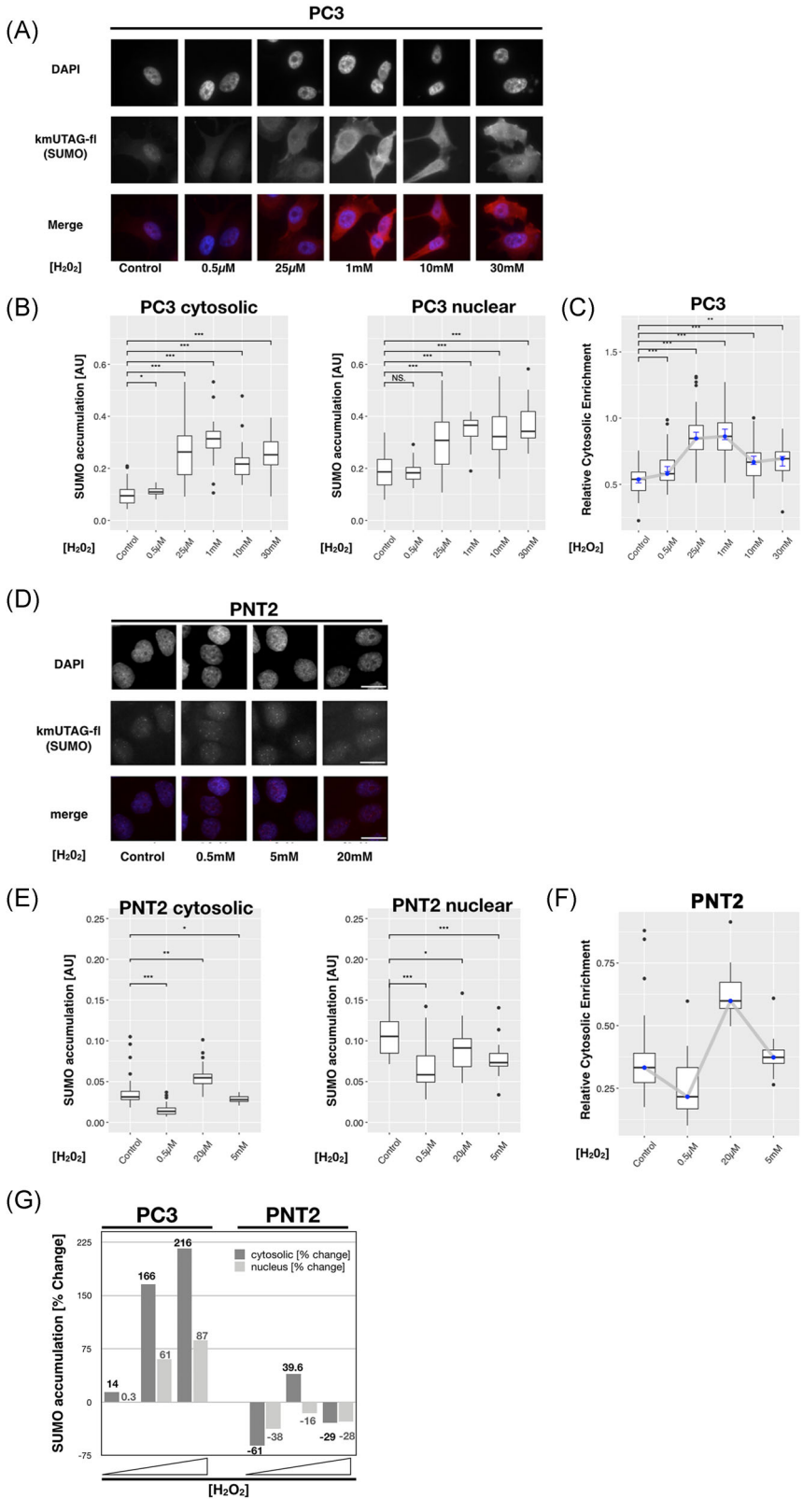

FIGURE 3 SUMO enrichment of H_2O_2treated PC3 and PNT2 cells. (A) H_2O_2 treated (0.5 μM, 25 μM, 1 mM, 10 mM, 30 mM) or control PC3 cells were fixed and stained for SUMO with KmUTAG-fl, visualized and quantified as previously described. (B) Average cytosolic and nuclear KmUTAG-fl signal intensity (SUMO accumulation) were quantified with CellProfiler (PC3 Control $n = 47$; PC3 0.5 μM $n = 44$, PC3 25 μM $n = 54$; PC3 1 mM $n = 23$; PC3 10 mM $n = 25$; PC3 30 mM $n = 17$). (C) Relative cytosolic enrichment (RCE) ratio of SUMO (KmUTAG-fl signal) in PC3 cells increased with H_2O_2 concentration. The RCE was calculated as the ratio between cytosolic and nuclear fluorescence intensity. (D) H_2O_2 treated (0.5 μM, 20 μM, 5 mM) or control PNT2 cells were fixed and stained for SUMO with KmUTAG-fl, visualized and quantified as above. (E) Average cytosolic and nuclear KmUTAG-fl (SUMO accumulation) were quantified with CellProfiler PNT2 Control $n = 24$; PNT2 0.5 μM $n = 27$, PNT2 20 μM $n = 33$; PNT2 5 mM $n = 20$. (F) Variability in the relative cytosolic enrichment (RCE) ratio of SUMO (KmUTAG-fl signal) in PNT2 cells at various H_2O_2 concentrations. The RCE was calculated as the ratio between cytosolic and nuclear fluorescence intensity. (G) Trend of SUMO accumulation in PC3 and PNT2 cells at increasing hydrogen peroxide concentrations as measured in (B) and (E). Variability statistical analysis by unpaired t-test (NS: $p > 0.05$, $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$). Scale bar = 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

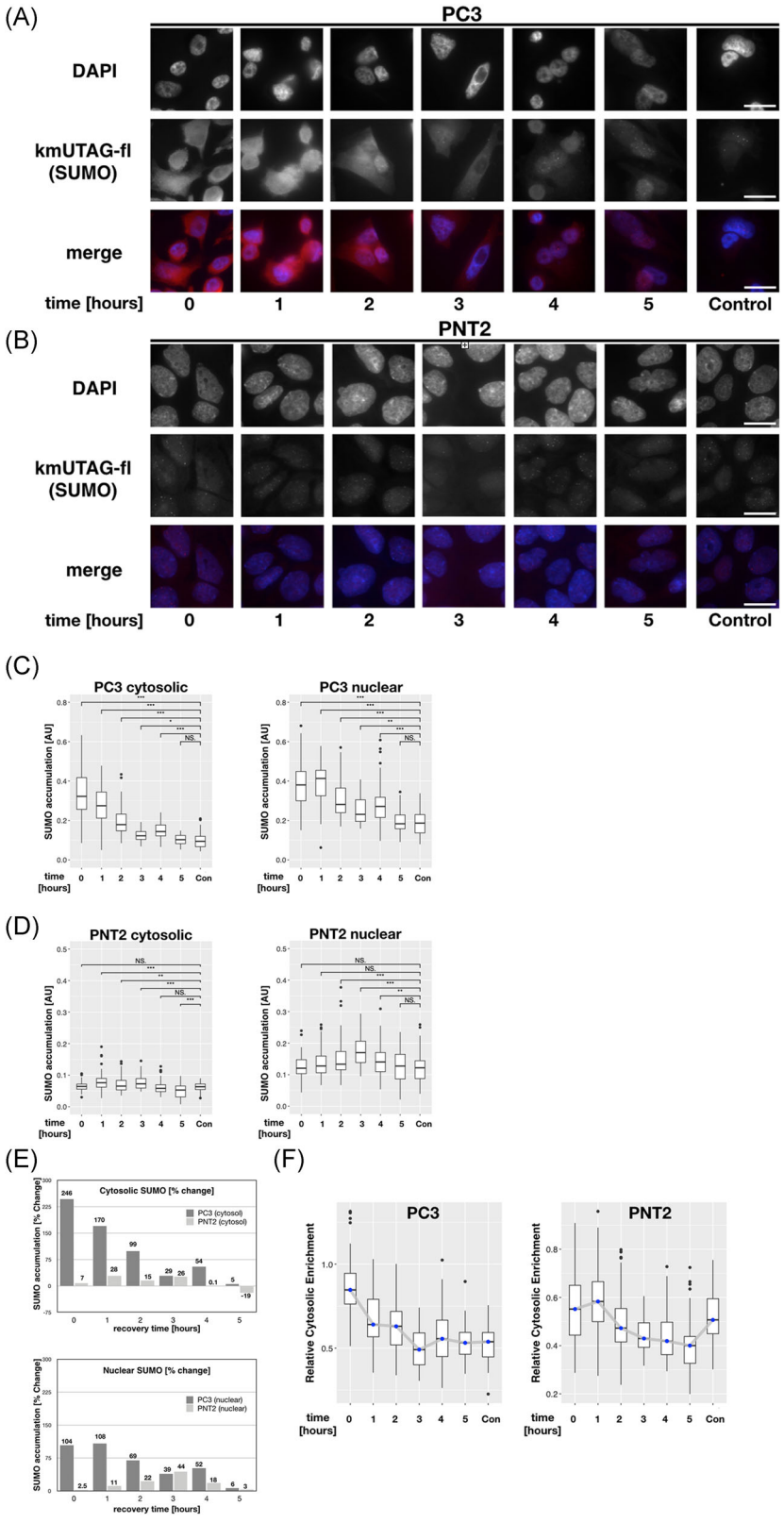

FIGURE 4 Recovery from peroxide stress is accompanied by gradually decreasing SUMO level. (A) H_2O_2 treated (1 mM) or control PC3 cells were fixed and stained for SUMO with KmUTAG-fl after the indicated recovery times (0–5 h). Representative cells samples are shown. Merged cells: DAPI (blue), KmUTAG-fl (red) (B) Same as (A) except PNT2 cells. (C) Decreasing KmUTAG-fl signal intensity (cytosolic and nuclear) in recovering PC3 cells (PC3 0 h $n = 54$; PC3 1 h $n = 60$; PC3 2 h $n = 93$; PC3 3 h $n = 15$; PC3 4 h $n = 51$; PC3 5 h n = 54; PC3 Control $n = 47$). (D) Little or no significant change in cytosolic and nuclear KmUTAG-fl signal intensity in recovering PNT2: (0 h $n = 72$; PNT2 1 h $n = 95$; PNT2 2 h $n = 103$; PNT2 3 h $n = 60$; PNT2 4 h $n = 102$; PNT2 5 h $n = 103$; PNT2 Control $n = 70$). (E) Comparison of the change (%) of cytosolic and nuclear (SUMO) KmUTAG-fl signal intensity in PC3 and PNT2 cells. Top panel: change (%) of cytosolic (SUMO) KmUTAG-fl signal. Bottom panel: change (%) of nuclear (SUMO) KmUTAG-fl signal. The intensity change was calculated as the intensity difference between the treated cells and control cells. (F) The relative cytosolic enrichment (RCE) ratio of the KmUTAG-fl signal in PC3 cells (left panel) and PNT2 cells (right panel) after the indicated recovery times. Note the delayed and reduced RCE of PNT2 cells compared to PC3 cells. Statistical analysis by unpaired t-test (NS: $p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$). Scale bar = 20 µm [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Stress-induced increase of cytosolic SUMO levels is observed in LNCaP prostate cancer cells with low metastatic potential

Our finding that stress rapidly increased cytosolic SUMO levels in the highly aggressive PC3 cell line prompted us to question whether this accumulation of SUMO is linked to metastatic potential. To assess this possibility, we compared stress-induced SUMO levels in the LNCaP cell line, possessing low metastatic potential, to the highly aggressive PC3 and the nonmalignant PNT2 cells.[25] For this experiment, we simultaneously treated LNCaP, PC3, and PNT2 cells with H_2O_2 (1 mM, 30 min) as detailed above (Figures 3 and 4) and compared cytosolic and nuclear SUMO levels before and after treatment. SUMO levels in nuclei and cytosol of untreated LNCaP, PC3, and PNT2 cells showed different levels of SUMO before treatment (Figure 5A). After H_2O_2 treatment, cytosolic SUMO levels of all cell lines increased significantly (Figure 5B, left). The largest increase in cytosolic SUMO signal after peroxide exposure was detected in LNCaP cells (71%), followed by PC3 (44%), and lastly PNT2 cells (13%) (Figure 5B,C). Therefore, cytosolic SUMO levels in LNCaP cells and in PC3 cells were 5.5- and 3.5-fold higher respectively than PNT2 cells after exposure. As before, a small but significant change in nuclear SUMO levels was detected in PC3 and PNT2 cell lines: SUMO levels were decreased by 10.3% and 7.8% below the untreated control samples, respectively (Figure 5C). Concomitantly, H_2O_2-treated LNCaP cells did not show a significant change in nuclear SUMO levels (Figure 5B, right). Reduced nuclear SUMO levels after peroxide exposure were likely an authentic effect, as a large number of cells ($n > 300$ for each) were scored for this comparison. Importantly, taking into account nuclear and cytosolic KmUTAG signals, a robust increase of the RCE of SUMO was detected for both LNCaP and PC3 cells (64% increase for LNCaP and 55% increase for PC3 cells) but this change was much less pronounced in normal PNT2 cells (27% increase) (Figure 5D). Importantly, the RCE of PC3 and LNCaP cells showed similar slopes and magnitude, suggesting the observed cytosolic enrichment of SUMO may not be related to the differences in cancerous potential. In summary, our data obtained with the KmUTAG-fl SUMO biosensor indicate that the detected increase in the RCE of SUMO levels is a quantifiable hallmark of the SSR specific to cancerous cells.

4 | DISCUSSION

The KmUTAG-fl biosensor reports on the presence and distribution of untagged, native SUMO conjugates in a variety of eukaryotic cells.[19] Here, we used KmUTAG-fl to detect, quantitate, and analyze the cellular distribution of SUMO conjugates before and after

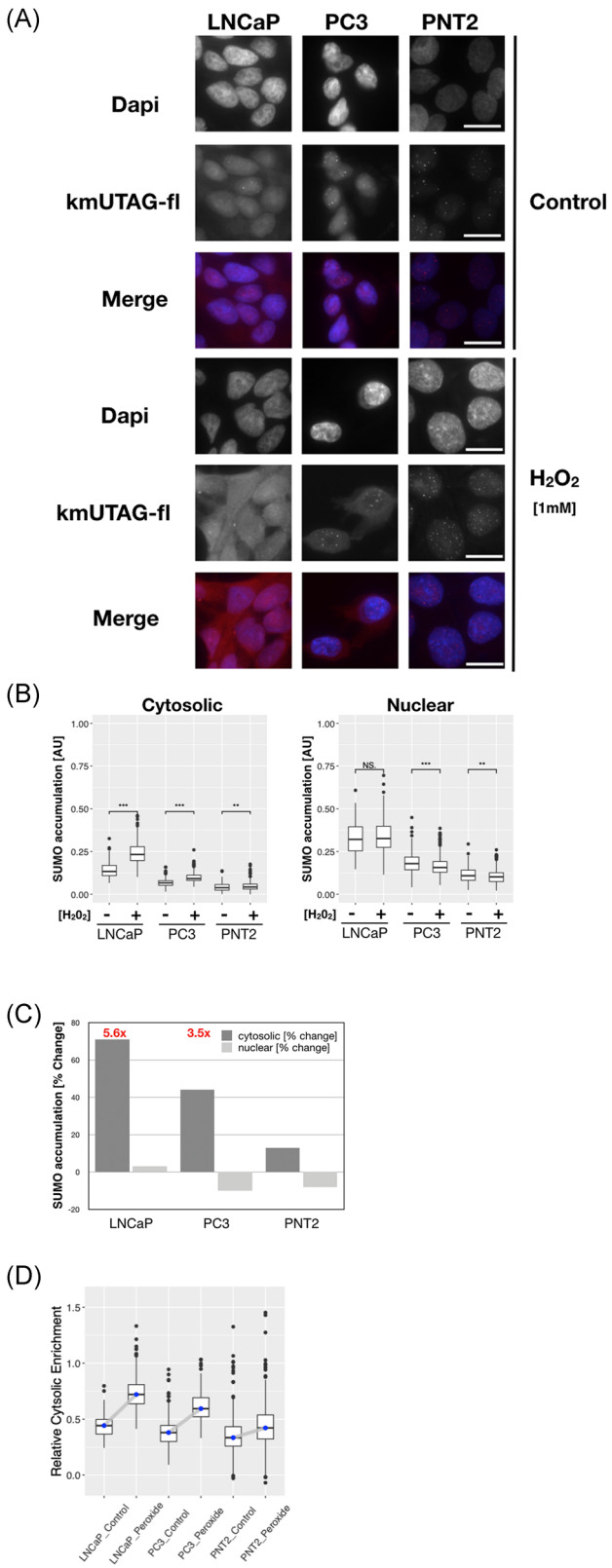

FIGURE 5 (See caption on next page)

exposure to acute proteotoxic and genotoxic stress. Using this approach, we detected significant, stress-induced differences in the distribution of cytosolic (extra-nuclear) SUMO between a normal (PNT2) and two cancer cell lines (PC3 and LNCaP). While the increase in cellular SUMO conjugate levels in response to stress has previously been observed, it is unknown how the SSR propagates throughout the cell or the extent of extra-nuclear SUMO distribution changes when cells undergo acute stress. In this report, we observed a significant increase in cytosolic SUMO in response to oxidative and UV-irradiation stress within 30 min of exposure, which was dependent on the concentration of the stressor and the time after stress exposure. Additionally, after recovery cytosolic SUMO levels returned to normal levels within a 5-h interval. Nuclear SUMO levels were also altered during the SSR but overall the amplitude of this stress-induced modulation was lower or not significantly changed in the cell lines tested. The increase of cytosolic SUMO in response to various peroxide treatments ranged from 12.6% to 70.6% (average increase ~5-fold) and was statistically significant for both PC3 and LNCaP cell lines when compared to PNT2 cells (unpaired *t* test), indicating this stress-induced effect was consistent and reproducible. Overall, our findings demonstrate that the SUMO biosensor KmUTAG-fl can differentiate between the SSR of cancerous and normal cells. Additionally, our work offers new insights into the dynamics of the SSR and how cancer cells modulate SUMO levels and subcellular distribution in response to stress. Ultimately, we hope to determine whether this enhanced SSR response facilitates the robustness of cancer cells.

Cancer cells are known to modulate their SUMO dynamics as part of a strategy to become more stress tolerant.[7] Cancer cells are under constant threat of adverse conditions, including hypoxia within tumors, immune invasions, and aneuploidies that threaten protein homeostasis.[26,27] Thus, they require enhanced stress response pathways to mitigate these effects and to maintain proteostasis and genome integrity. For our study, we used three different cell lines to observe the SSR and to identify unique cell-type specific features. PNT2 cells were established by SV40-mediated immortalization of normal adult prostatic epithelial cells, PC3 cells represent an adenocarcinoma cell line with high metastatic potential, and the LNCaP cell line has low metastatic potential. These human-derived prostate cell lines differ not only in their tumorigenicity but also their chromosomal make-up. PNT2 cells are nonmalignant immortalized normal prostate epithelium, PC3 have 62 chromosomes,[23] and LNCaP harbor 79-91 chromosomes.[28] Therefore, both the expression levels and copy number of SUMO genes may be increased in these tumorigenic cell lines (Kerscher, unpublished results).

Our observation that SUMO levels are significantly increased after stress exposure of PC3 and LNCaP cells may be linked to the increased expression levels of SUMO in comparison to PNT2 cells. However, it is important to note that de novo protein synthesis (translation) is not required for the SSR to proceed. Rather, it has been reported that pre-existing SUMO is dynamically transferred to specific proteins in response to stress exposure and as part of transcriptional reprogramming in the nucleus. Therefore, it is possible that the change in SUMO accumulation observed by us represents a stress-dependent re-distribution rather than an increase in SUMO per se.

Regarding the observed stress-dependent cytosolic enrichment of SUMO, one possibility is that after stress sumoylated nuclear proteins may "spill" into the cytosol. This is unlikely because in our study SUMO levels do not simply equilibrate across the cell (e.g., see Figure 3G). We posit that sequestered SUMO is released, for example, from a pool of Ubc9 with noncovalently bound SUMO, and becomes redistributed and detectable. One possible sumoylation target in the cytosol could be proteins involved in ribosomal activity and translation. Work in yeast has established that Siz/PIAS SUMO E3 ligases drive the SSR and SUMO protease Ulp2 is required for inactivation of the SSR.[5] In mammalian cells certain SUMO E3 ligases (e.g., PIAS4) and SUMO proteases (e.g., SENP2) localize to the nucleus as well as the cytosol, suggesting that the observed increase and decrease of SUMO conjugates may take place outside of the nucleus without transport across the nuclear envelope.

Next to SUMO, SUMO E1, E2, and E3 enzyme levels are also altered in cancer cells and this likely further modulates the dynamics of stress-induced sumoylation. For example, the SUMO E2 ligase Ubc9 is upregulated in breast, lung, neck, and head cancer specimens, and is elevated 5.7-fold in breast tumors compared to normal breast tissues.[16] Ubc9 may represent a useful biomarker for cervical cancer and is linked to the progression of HPV's oncogenicity.[15] Additionally, hundreds of proteins in the cytosol may be targets of stress-induced sumoylation. As outlined below, sumoylation may serve to stabilize proteins during acute proteotoxic stress. Additionally, chains of SUMO monomers likely make up a considerable part of stress-induced sumoylated products.[29] The identification of these proteins, while of interest, is beyond the scope of the present analysis.

Sumoylation is considered a predominantly nuclear event, so the rapid stress-induced increase and decrease of SUMO conjugates in the cytosol of cancer cells is an interesting finding. A recent review on subcellular sumoylation in the heart posits that sumoylation in the

FIGURE 5 H_2O_2stress induces increase in cytosolic SUMO levels in LNCaP cells with low-metastatic potential. (A) H_2O_2 treated (1 mM) or Control LNCaP, PC3, and PNT2 cells were fixed and stained for SUMO with KmUTAG-fl. Representative cells of each cell line are shown. Merged cells: DAPI (blue) and KmUTAG-fl (red). (B) KmUTAG-fl signal intensity in nuclei and cytosol of LNCaP, PC3, and PNT2 cells across treatment groups. (LNCaP_Control $n = 402$; LNCaP_Peroxide $n = 345$; PC3_Control $n = 368$; PC3_Peroxide $n = 422$; PNT2_Control $n = 342$; PNT2_Peroxide $n = 331$). (C) The largest change (%) in KmUTAG-fl signal intensity is observed in nuclei and cytosol of LNCaP and PC3 cells when compared to PNT2 cells. The intensity change was calculated as the intensity difference between the peroxide-treated cells and control cells. (D) The relative cytosolic enrichment (RCE) ratio of the KmUTAG-fl signal is most pronounced in LNCaP, PC3 when compared to PNT2 cells. Statistical analysis by unpaired *t* test (NS: $p > 0.05$, $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$). Scale bar = 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

extra-nuclear compartment of cardiomyocytes is generally cardioprotective.[30] More specifically, results from a study on SUMO2/3 suggest that stress-induced sumoylation serves to temporarily stabilize (keep soluble) misfolded proteins and targets those that can't be refolded for proteasomal degradation.[9] Nevertheless, most information on stress-induced sumoylation concerns its nuclear effects and ignores the cytoplasmic roles of SUMO. For example, SUMO isoforms may serve a chaperone-like function to maintain the homeostasis of large chromatin-associated nuclear proteins during stress[10] and stress-induced sumoylation is required for transcriptional reprogramming.[5] In this respect, our observation of a transient increase of SUMO due to acute UV and oxidative stress underscores the dynamic nature of the SSR in the entire cell. Additionally, it is an important reminder that the effects of SUMO (and especially the SSR in normal and transformed cell lines) are likely to produce very different, cell line-specific outcomes. Many proteomics studies on the SSR in mammalian cell lines are conducted in cancer-derived cell lines and these studies do not always include normal, immortalized comparators.

In summary, using the pan-SUMO specific KmUTAG-fl biosensor, we have measured a transient, reversible, stress-induced increase of SUMO conjugates in the cytosol of PC3 and LNCaP cells. This stress-induced cytosolic SUMO enrichment clearly distinguishes PC3 and LNCaP cells from normal immortalized PNT2 cells, suggesting that it may be part of a stress-tolerance pathway that is specific for cancer cells. One of our future goals to identify these stress-induced SUMO targets and conjugates after cell fractionation. The latter is technically difficult as SUMO is rapidly deconjugated or degraded during cell fractionation and will be part of a future study.

While the cytosolic increase of SUMO in response to acute stress may be a feature of these prostate cancer cells, we have not yet shown that this enhanced SSR is correlated with enhanced oncogenic potential (defined as an increase in cellular migration, invasiveness, and aggressive cell proliferation). Looking forward, we will investigate a potential link between transiently increased SUMO levels and oncogenic potential that would underscore the importance that the SSR plays during cellular transformation. Additionally, to further understand how the SSR unfolds in cancer cells, we plan to use a cell-penetrating CPP-adaptor system to deliver and release the KmUTAG-fl biosensor into the cytoplasm of mammalian cells.[31] The successful delivery of KmUTAG-fl may allow us to visualize the propagation of the SSR inside living cells and study the SUMO-specific features as cells go from non-cancer to malignant. These experiments chart an important new route in our studies of the SSR and ultimately will help us understand the role that SUMO plays in the enhanced stress tolerance of eukaryotic pathogens and abnormal cells.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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