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Sumoylation of p68 and p72 RNA Helicases Affects Protein Stability and Transactivation Potential†

Steven M. Mooney,‡ Joseph P. Grande,§ Jeffrey L. Salisbury,‡ and Ralf Janknecht*,‡,∥

‡*Departments of Biochemistry and Molecular Biology, and* §*Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota 55905, and* ∥*Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104*

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ABSTRACT: The p68 (DDX5) and p72 (DDX17) proteins are members of the DEAD-box (DDX) family of RNA helicases. We show that both p68 and p72 are overexpressed in breast tumors. Bioinformatical analysis revealed that the SUMO pathway is upregulated in breast tumors and that both p68 and p72 contain one consensus sumoylation site, implicating that sumoylation of p68 and p72 increases during breast tumorigenesis and potentially contributes to their overexpression. We determined that p68 and p72 are indeed sumoylated at a single, homologous site. Importantly, sumoylation significantly increased the stability of p68 and p72. In contrast to p72 and consistent with an ~3-fold lesser half-life, p68 was found to be polyubiquitylated, and mutation of the sumoylation site increased polyubiquitylation, suggesting that sumoylation increases p68 half-life by reducing proteasomal degradation. Moreover, whereas p68 robustly coactivated transcription from an estrogen response element, its sumoylation mutant showed a drastically reduced coactivation potential. In contrast, the p68 sumoylation status did not affect the ability to enhance p53-mediated MDM2 transcription. On the contrary, preventing sumoylation of p72 caused an increase in its ability to transactivate both estrogen receptor and p53. Furthermore, sumoylation promoted the interaction of p68 and p72 with histone deacetylase 1 but had no effect on binding to histone deacetylases 2 and 3, the coactivator p300, or estrogen receptor and also did not affect homo/heterodimerization of p68/p72. In conclusion, sumoylation exerts pleiotropic effects on p68/p72, which may have important implications in breast cancer by modulating estrogen receptor and p53 activity.

RNA helicases are necessary for ribosome biogenesis, pre-mRNA splicing, mRNA export from the nucleus, translation initiation, and RNA decay. In fact, RNA helicases are indispensable for all cellular functions that involve RNA (*1*). A hallmark of RNA helicases is their ability to hydrolyze ATP to dissociate RNA−RNA or RNA−DNA double strands or to modulate RNA−protein interactions. One subfamily of RNA helicases is composed of the DEAD-box (DDX)[1] proteins that are characterized by a conserved Asp-Glu-Ala-Asp sequence involved in ATP hydrolysis. Whereas the catalytic domains are highly conserved, flanking sequences are variable among the DDX RNA helicases and are thought to contain distinguishing elements that control cellular localization, catalytic activity, and binding to other proteins (*2*).

The DDX RNA helicase p68 (also called DDX5) and its paralogue, p72 (DDX17), are 92% similar within their central catalytic domains and share 71% and 44% homology in their N- and C-termini, respectively. Each of these two proteins is essential for development, since corresponding knockout mouse models display embryonic lethality (*3*). Both proteins possess RNA helicase activity and are involved in splicing, but also function in the regulation of gene transcription (*4*). Indeed, p68 and p72 RNA helicases interact with and thereby activate various transcription factors, including hormone binding proteins (estrogen receptor α (ERα) and androgen receptor), p53, MyoD, and Runx2 and the cofactors CBP/p300, PCAF, and β-catenin (*5*−*15*).

Protein expression of p68 and p72 is upregulated in colorectal, prostate, and, as shown in this study, breast cancer, but evidence suggests that this is not due to enhanced mRNA levels (*7*, *14*, *16*). This led us to hypothesize that posttranslational modifications might play a role in stabilizing p68/p72 at the protein level in tumor cells. In particular, since our analysis of the p68 and p72 amino acid sequence revealed one consensus sumoylation site, we focused in this report on the covalent attachment of SUMO (small ubiquitin-like modifier) to lysine residues within p68 and p72 and how this may affect protein stability.

Four distinct SUMO proteins exist in humans that resemble ubiquitin in their three-dimensional structure. SUMO1−SUMO3 are ubiquitously expressed and can be covalently attached to lysine residues in target proteins. This sumoylation is reversible and highly dynamic and can have profound effects, including regulating the intracellular localization, stability, and activity of target proteins (*17*, *18*). In particular, many transcription factors have been found to be sumoylated, resulting in repression of their activity (*19*). However, a few transcription factors, including the tumor suppressor p53 and heat shock transcription factors 1 and 2, are activated upon sumoylation (*20*−*23*). Interestingly, estrogen-dependent transcription is stimulated by the SUMO pathway (*24*). In part, this may be due to the reported sumoylation of ERα (*25*),

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*To whom correspondence should be addressed. Phone: 405-271-2377. Fax: 405-271-3548. E-mail: ralf-janknecht@ouhsc.edu.

[1]Abbreviations: DDX, DEAD box; ERα, estrogen receptor α; ERE, estrogen response element; HDAC, histone deacetylase; HEK, human embryonic kidney; SUMO, small ubiquitin-like modifier.

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but we speculated and therefore tested here whether sumoylation of the ERα interaction partners, p68 and p72 RNA helicases, is also involved.

EXPERIMENTAL PROCEDURES

Tumor Stainings. Breast tissue microarrays were purchased from Petagen (AccuMax Array) and then stained as described before (*26*). Antibodies directed against p68 amino acids 501−524 (*14*) and p72 amino acids 632−650 (*12*) were employed.

In Vivo Sumoylation. HEK293T cells were grown in 6 cm dishes and transiently transfected with 2 μg of 6Myc-p68/p72 or empty vector pCS3$^+$-6Myc and 4 μg of the indicated SUMO expression vector or empty vector pEV3S (*27*). Lysates were generated 36 h after transfection in 10 mM Tris-HCl, 30 mM $Na_4P_2O_7$ (pH 7.1), 200 mM NaCl, 50 mM NaF, 0.1% SDS, 1% Triton X-100, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin A, 1 mM phenylmethanesulfonyl fluoride, and 10 mM *N*-ethylmaleimide. Where indicated, immunoprecipitations were performed with anti-Myc 9E10 monoclonal antibodies as described (*28*). Alternatively, 1 μg of Flag-tagged p68/p72 was coexpressed with 2 μg of His-tagged yeast ubiquitin expression vector, and cells were treated with 20 μM MG-132 for 6 h before lysis in 8 M urea, 0.1 M Na_2HPO_4 (pH 8), and 10 mM *N*-ethylmaleimide. His-tagged proteins were then affinity purified on Ni^{2+}-NTA agarose (Qiagen) according to standard procedures.

Pulse−Chase Experiments. HEK293T cells grown in 6 cm dishes were transiently transfected by the calcium phosphate coprecipitation method (*29*, *30*) with 8 μg of 6Myc-p68 (wild type and K53R) or 6Myc-p72 (wild type and K50R). Thirty-six hours after transfection, cells were pulsed for 2 h with 100 μCi of [^{35}S]methionine followed by a chase with nonradioactive methionine (*31*). At various time points the cells were harvested and lysed in 10 mM Tris-HCl, 30 mM $Na_4P_2O_7$ (pH 7.1), 50 mM NaCl, 50 mM NaF, 1% sodium deoxycholate, 1% Triton X-100, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin A, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM Na_3VO_4, and 0.2 mM DTT. Then, immunoprecipitations were performed as described (*32*) with anti-p68 antibodies directed against amino acids 501−524 (*14*) or anti-Myc 9E10 monoclonal antibodies in the case of p72 (*33*). Quantitation of incorporation of radioactivity was done with a PhosphorImager.

Coimmunoprecipitation. HEK293T cells grown in 6 cm dishes were transiently transfected with plasmids encoding for 6Myc-p68 or 6Myc-p72 (8 μg) and Flag-ERα (1 μg) (*34*). Cells were lysed 36 h after transfection in 2.5 mM Tris-HCl, 7.5 mM $Na_4P_2O_7$ (pH 7.1), 12.5 mM NaCl, 12.5 mM NaF, 0.25% Triton X-100 supplemented with 10 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin A, 1 mM phenylmethanesulfonyl fluoride, 0.5 mM Na_3VO_4, and 0.2 mM DTT. Immunoprecipitations with anti-Myc 9E10 antibody were then essentially performed as described before (*35*). Coimmunoprecipitated Flag-ERα was detected by Western blotting utilizing anti-Flag M2 antibodies (*36*). Alternatively, HEK293T cells were transfected with 3 μg of p300-HA and 1 μg of 6Myc-p68 or 6Myc-p72 constructs, immunoprecipitations were performed with anti-Myc antibodies as described (*37*), and coprecipitated p300 was detected by anti-HA Western blotting (*38*). Similarly, HEK293T cells were transfected with plasmids encoding HA-p68 (8 μg) and Flag-HDAC (1 μg), and immunoprecipitations with anti-Flag M2 antibody were then performed followed by Western blotting employing anti-HA antibodies. In the case of p72, HEK293T cells grown in 10 cm dishes were transfected with 20 μg of HA-p72 and 5 μg of Flag-HDAC expression plasmids.

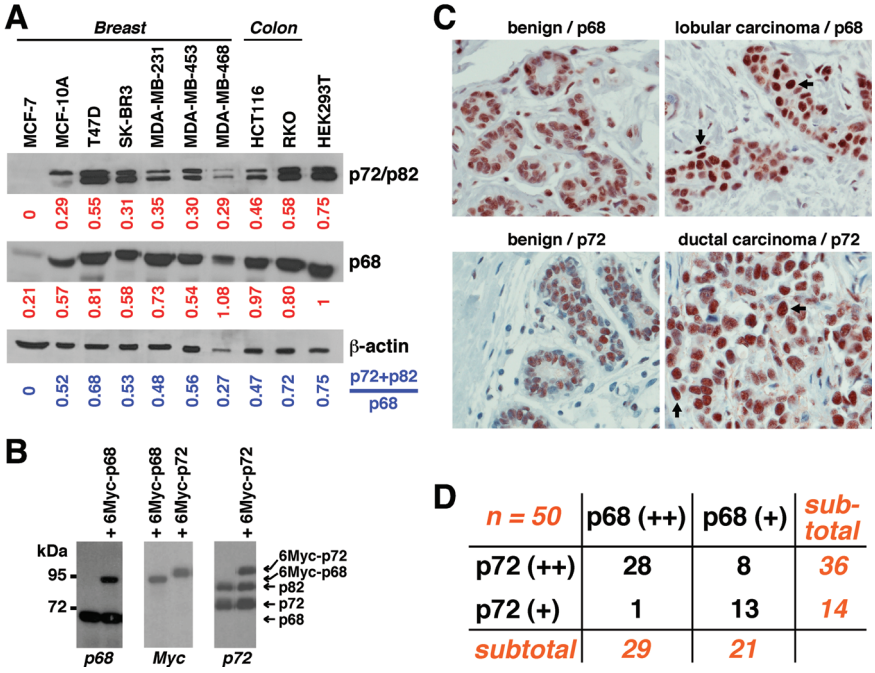
Luciferase Assays. MDA-MB-231 cells grown in 12 wells were transiently transfected using the calcium phosphate coprecipitation method (*39*, *40*). As reporter plasmid, 500 ng of ERE-luc (*41*) or 200 ng of MDM2-luc (*42*) were employed. Where indicated, 30 ng of pSG5-ERα or empty vector pSG5 and 10 ng of pcDNA3-p53 or empty vector pcDNA3 were cotransfected. HA-tagged p68 or p72 (1600 ng) or empty vector pEV3S (*43*) was employed, with the exception of wild-type p72 where 500 ng of expression vector plus 1100 ng of pEV3S was cotransfected. In the case of ERE-luc, media were replaced after transfection with one containing 5% charcoal-stripped serum with or without 1 nM estradiol. Thirty-six hours after transfection, cells were lysed, and luciferase activity was measured in a Berthold Lumat (*44*, *45*). Alternatively, protein extracts were prepared and assayed by Western blotting (*46*).

Similarly, HCT116 cells were transiently transfected utilizing 200 ng of $Gal4_2$-tk80-luc reporter plasmid (*47*), 400 ng of pAB-GAL4-linker (*48*), which encodes the DNA binding domain of the yeast protein GAL4 or the corresponding GAL4-β-catenin plasmid (*14*), 400 ng of pEV3S or HA-tagged p68 expression vector, and 25 ng of HA-SUMO1 or 50 ng of ARIP3 (*49*) expression plasmids. Further, HEK293T cells were transiently transfected by the calcium phosphate coprecipitation method (*50*) utilizing 200 ng of $Gal4_2$-tk80-luc reporter plasmid, 30 ng of pAB-GAL4-linker or GAL4-p72 (*12*), and 100 ng of SuPr-1 expression vector (*51*) or empty vector pcDNA3.

RESULTS

Overexpression of p68 and p72 in Breast Tumors. Instigated by the facts that ERα plays an oncogenic role in many human breast tumors and that p68 and p72 are coactivators of ERα (*5*, *6*), we assessed the level of their expression in breast tumors. To this end, we first determined the expression of p68 and p72 in established breast cancer cell lines. With the exception of MCF-7 cells, all of the other six breast cancer cell lines displayed robust expression of p68 and p72 that was comparable to two colon cancer cell lines (RKO and HCT116) and transformed human embryonic kidney (HEK) 293T cells (Figure 1A); note that due to an alternative start codon in the p72 mRNA (*52*), a slightly longer p82 isoform was also detectable with the anti-p72 antibody. Since RKO and HCT116 colon cancer cells have previously been shown to overexpress p68 and p72 (*14*), these data suggested that p68 and p72 might also be overexpressed in breast tumors.

To estimate the molar ratio of p68 to p72/p82 in the various cell lines shown in Figure 1A, we employed 6Myc-tagged versions of p68 and p72 that have an ~20 kDa larger apparent molecular mass than respective endogenous proteins. We mixed comparable amounts of 6Myc-p68 or 6Myc-p72, as determined by anti-Myc Western blotting (Figure 1B, middle panel), into HEK293T cell extract and then probed with anti-p68 (Figure 1B, left panel) or anti-p72 antibodies (Figure 1B, right panel). This allowed us to estimate that the molar ratio of p72 + p82 to p68 is 0.75 in HEK293T cells. Then, we normalized p68 protein levels to β-actin levels by densitometric scanning of the respective Western blots in Figure 1A. The resulting unitless numbers were then calibrated to the one from HEK293T cells, where the β-actin-normalized p68 protein level was arbitrarily set to 1. The resulting



D

n = 50	p68 (++)	p68 (+)	subtotal
p72 (++)	28	8	36
p72 (+)	1	13	14
subtotal	29	21	

FIGURE 1: Overexpression of p68 and p72 in breast cancer. (A) Expression of p68 and p72 in cell lines derived from breast or colon tumors and in HEK293T cells. Shown are Western blots for p72/p82 and p68 as well as β-actin as a loading control. Red numbers are levels of p68 or p72/p82 normalized to β-actin and calibrated to p68 levels in HEK293T cells (set to 1) or p72/p82 levels in HEK293T cells (set to 0.75 based on the results shown in panel B). In blue, the molar ratios of p72 plus p82 to p68 in all cell lines are presented. (B) Comparable amounts of 6Myc-tagged p68 or p72 were mixed into HEK293T cell extract as indicated, and anti-p68, anti-Myc, or anti-p72 Western blotting was performed to estimate the molar ratio of endogenous p68 to endogenous p72 plus p82 in HEK293T cells. (C) Representative anti-p68 and anti-p72 staining in normal/benign breast tissue and breast carcinomas. Arrows point out examples of cells strongly overexpressing p68 or p72. (D) Summary of the immunohistochemical analysis of p68 and p72 expression in 50 breast tumor samples. Overexpression is indicated by (++), whereas (+) indicates a staining level comparable to normal breast tissue.

numbers indicate how p68 protein levels (normalized to β-actin) vary between the various cell lines. Based on these numbers, MCF-7 cells have ~5 times less p68 than HEK293T cells, whereas all of the other cell lines have half to equal levels of p68 compared to HEK293T cells (Figure 1A, red numbers underneath the p68 blot). Similarly, p72 + p82 levels were normalized to β-actin and then calibrated to a value of 0.75 in HEK293T cells (this calibration follows our determination that the molar ratio of p72 + p82 to p68 is 0.75 in HEK293T cells). With the exception of MCF-7 cells, all of the other cell lines had a β-actin-normalized p72 + p82 level that was between 39% and 77% of the respective level in HEK293T cells. Dividing the calibrated levels of p72 + p82 by the calibrated levels of p68 gave the molar ratio of p72 + p82 to p68 (Figure 1A, blue numbers), which was 0 in MCF-7 cells but ranged from 0.27 to 0.68 in the other breast cell lines. Thus, with the exception of MCF-7 cells, p72 + p82 protein levels are less, but not drastically reduced, compared to p68 protein levels in the breast cancer cell lines studied, implicating that p68 and p72/p82 are expressed at similar levels in breast tumors.

Next, we stained human breast tissue microarrays with anti-p68 and anti-p72 antibodies. Normal/benign breast tissue showed nominal expression of both p68 and p72 (Figure 1C). In contrast, carcinomas displayed robust expression of these two proteins; in particular, a higher frequency of cells with dark staining was observed. Overall, we found that 29 and 36 out of 50 breast tumor cases displayed overexpression of p68 and p72, respectively (Figure 1D). Thus, p68 and p72 are overexpressed in breast cancer. Furthermore, since breast tissue microarrays from the same batch were employed to stain with anti-p68 or anti-p72 antibodies, we could correlate p68 with p72 expression and found a significant co-overexpression (Figure 1D; exact McNemar significance probability = 0.0391).

Sumoylation of p68 and p72. One possible way of how p68/p72 might become overexpressed in breast tumors is through the inhibition of ubiquitylation and subsequent protein degradation. Since analysis of the p68 and p72 amino acid sequences revealed a consensus sumoylation site in their N-termini (see below), we hypothesized that sumoylation of p68/p72 may interfere with their ubiquitylation. However, apart from a study indicating that the SUMO ligase PIAS3 is overexpressed in breast tumors (*53*), there have been no reports that the SUMO pathway is dysregulated in breast tumors. Thus, we utilized ONCOMINE and analyzed published microarray data (*54*) for any changes of SUMO pathway components in breast tumors versus normal breast tissue. Interestingly, we found that several transcripts encoding proteins in the SUMO pathway show modified expression in breast tumors based on the following results: (i) mRNA of the SUMO conjugating enzyme Ubc9 is upregulated, (ii) SUMO1 mRNA is also upregulated, and (iii) mRNA levels of the SUMO protease SENP6 that reverses sumoylation are downregulated (Figure 2A). All of this suggests that sumoylation of proteins, including possibly p68 and p72, is generally enhanced in breast tumors.

Previously, we have shown that a fusion between β-catenin and the DNA binding domain of the yeast protein GAL4 can be activated by p68 (*14*). Accordingly, we observed that GAL4-β-catenin-mediated transcription from a GAL4 binding site-driven reporter construct was stimulated by p68, whereas p68 did not activate the GAL4 DNA binding domain itself (Figure 2B). Importantly, coexpression of SUMO1 with GAL4-β-catenin and p68 raised luciferase levels by 7.1 units, whereas SUMO1 elicited

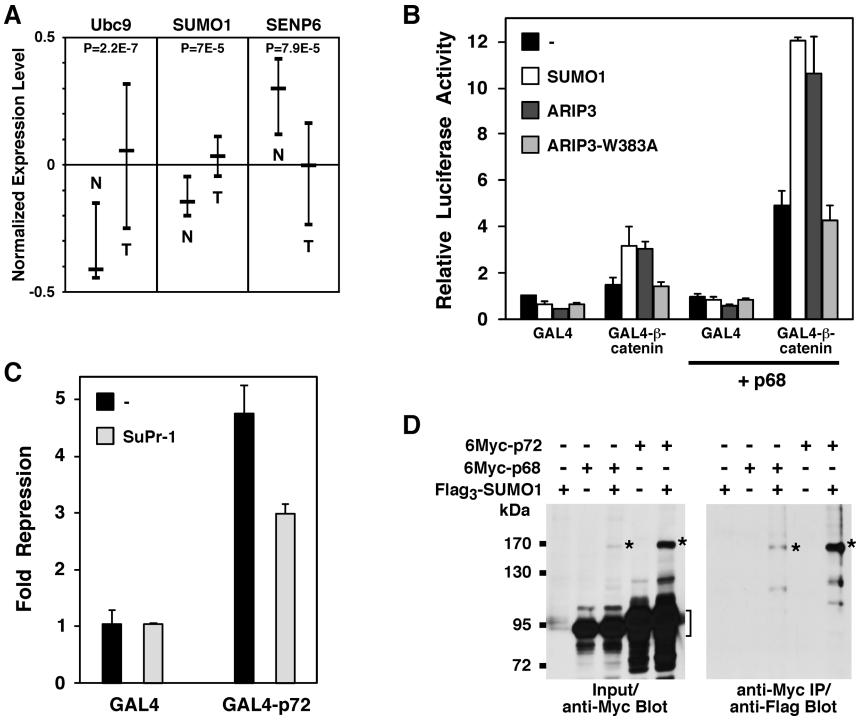


FIGURE 2: Changes in transcripts encoding for sumoylation pathway components in breast cancer and evidence for sumoylated p68 and p72. (A) Analysis of microarray data from 40 tumor (T) and 7 normal (N) breast tissues. Normalization was done by log 2 transforming mRNA data sets, with the median set to 0 and the standard deviation set to 1. Shown are medians and 25th/75th percentile ranges. Significance was determined with Student's *t* test. (B) SUMO1 or the SUMO ligase ARIP3 stimulate p68. As indicated, GAL4 or GAL4-β-catenin were cotransfected with p68 into HCT116 cells and luciferase activities from a GAL4 binding site-driven reporter construct measured. (C) The SUMO protease SuPr-1 alleviates p72-dependent transcriptional repression. GAL4 or GAL4-p72 were expressed with or without SuPr-1 in 293T cells, and repression of the cotransfected GAL4 binding site-driven luciferase reporter was measured. (D) 6Myc-tagged p68 or p72 were coexpressed with $Flag_3$-SUMO1 in HEK293T cells. The left panel shows an anti-Myc Western blot of cell lysates and the right panel an anti-Flag Western blot after anti-Myc immunoprecipitation. Asterisks mark higher molecular mass species representing sumoylated p68/p72. The bracket indicates unmodified p68/p72.

only a small increase (1.6 units) of GAL4-β-catenin luciferase activity in the absence of overexpressed p68, implicating that sumoylation may activate p68. Accordingly, overexpression of ARIP3, a SUMO ligase also called PIASxα (*49*), synergized with p68 and GAL4-β-catenin, whereas the ARIP3-W383A mutant that is impaired in its SUMO ligase activity had no effect (Figure 2B).

Further, we previously reported that GAL4-p72 exerts a repressive effect on GAL4 binding site-driven transcription (*12*). Surprisingly, overexpression of a SUMO protease, SuPr-1 (*51*), alleviated this repression mediated by GAL4-p72, whereas SuPr-1 had no effect on the GAL4 moiety itself (Figure 2C). These data suggested that sumoylation of p72 might repress its transcriptional function.

Prompted by the aforementioned results raising the possibility that p68 and p72 are sumoylated and thereby regulated in their activities, we wished to determine whether p68 and p72 are indeed posttranslationally modified by SUMO. Thus, we expressed Myc-tagged p68 and p72 with and without Flag-tagged SUMO1 in HEK293T cells. Anti-Myc Western blotting revealed an additional band at higher molecular mass (see asterisks in Figure 2D, left panel) only in the presence of coexpressed $Flag_3$-SUMO1, suggesting that both p68 and p72 become sumoylated. Indeed, anti-Myc immunoprecipitation followed by anti-Flag Western blotting proved that these higher molecular mass bands are sumoylated forms of p68 and p72 (Figure 2D, right panel).

To identify the sumoylation sites in p68/p72, we searched for the sumoylation consensus motif ψ**K**xE, where ψ is an aliphatic branched amino acid and x any amino acid (*18*). p68 and p72 were found to have only one such site, which was located within their N-termini at K53 in p68 and K50 in p72 (Figure 3A). Thus, we mutated K53 to arginine in p68 and assessed how this would affect sumoylation. As shown in Figure 3B (left panel), the respective K53R mutant did not display a higher molecular mass band in the presence of HA-SUMO1 or $Flag_3$-SUMO1, indicating that indeed K53 is the attachment site for SUMO in p68. Similarly, we assessed whether the homologous K50 is the sumoylation site in p72. Indeed, p72-K50R lost the higher molecular mass band in the presence of HA-SUMO1 or $Flag_3$-SUMO1 (Figure 3B, right panel), demonstrating that K50 is the site for SUMO attachment in p72. While this work was in progress, another laboratory also reported that p68 becomes sumoylated on K53 but did not assess sumoylation of p72 (*55*).

We also tested whether SUMO2 is capable of sumoylating p68 and p72. SUMO2 was expressed at a similar level as SUMO1 in HEK293T cells (Figure 3C). However, we were unable to detect significant sumoylation with SUMO2 on p68, and also SUMO2 attachment to p72 was less efficient than attachment of SUMO1 (Figure 3C). This indicates that SUMO1, rather than SUMO2, is the main SUMO variant modifying p68 and p72.

Sumoylation Increases the Half-Life of p68 and p72. Previously, it was reported that p68 is polyubiquitylated (*16*) and its stability may therefore be regulated through the ubiquitin pathway. Indeed, we found that p68 was significantly polyubiquitylated in HEK293T cells (Figure 4A). In contrast, very little monoubiquitylation and no polyubiquitylation were observable in the case of p72. Thus, we focused on p68 and found that sumoylation of p68 appears to suppress ubiquitylation, since

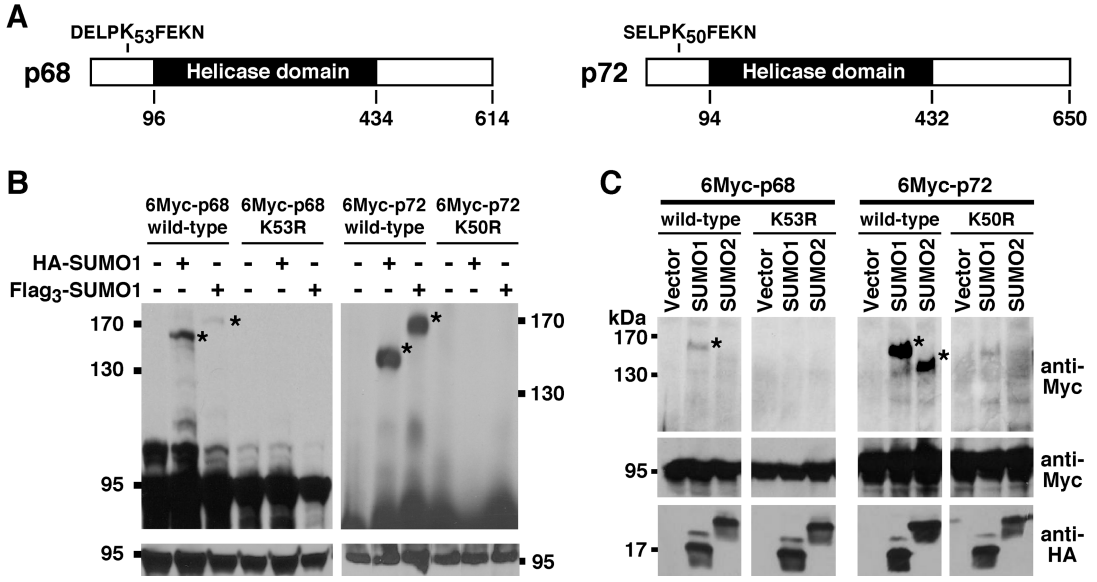


FIGURE 3: Mapping of sumoylation sites. (A) Diagrams of p68 and p72. The sequences surrounding the predicted sumoylation sites K53 in p68 and K50 in p72 are presented. (B) HEK293T cells were transfected with indicated expression constructs. The top shows a longer exposure of an anti-Myc Western blot, with asterisks marking the sumoylated proteins. The bottom panels are shorter exposures to show that comparable amounts of respective Myc-tagged proteins were expressed. (C) HA-tagged SUMO1 or SUMO2 were coexpressed with 6Myc-tagged p68 or p72 as indicated. The top panel shows a longer exposure of an anti-Myc Western blot and the middle panel a shorter exposure to reveal comparable loading of p68 and its K53R mutant or of p72 and its K50R mutant. The bottom panel is an anti-HA blot showing input levels of SUMO1 and SUMO2.

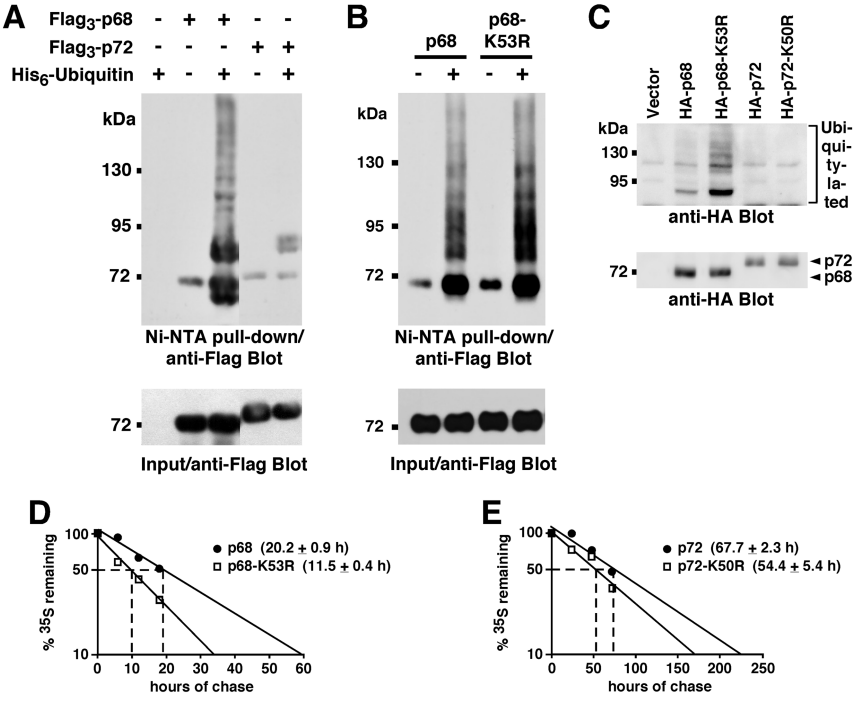


FIGURE 4: Ubiquitylation and half-lives of p68 and p72. (A) Flag-tagged p68 or p72 were coexpressed with His-tagged ubiquitin in HEK293T cells. After pull-down of His-tagged proteins with Ni^{2+}-NTA, ubiquitylated p68 and p72 were found by anti-Flag Western blotting. The bottom panel shows the input levels of p68 and p72 at a shorter exposure. (B) Analogous comparison of the ubiquitylation of wild-type p68 and its K53R mutant. (C) HA-tagged indicated p68 and p72 proteins were expressed in HEK293T cells and their ubiquitylated forms detected by anti-HA Western blotting of cell extracts. The bottom blot is a shorter exposure showing the mutated proteins to be expressed at the same level as respective wild-type ones. (D) Representative pulse–chase experiment with [^{35}S]methionine. The decay of radioactive p68 (wild-type and K53R mutant) over time is displayed. Half-lives correspond to the hours of chase where 50% of radioactivity (plotted on a logarithmic scale) remains. Mean half-lives with standard deviations are given in brackets. (E) Similar for p72.

the K53R mutant was approximately twice as much ubiquitylated as wild-type p68 (Figure 4B); this difference was also present when no ectopic ubiquitin was expressed (Figure 4C). These data suggest that sumoylation enhances the stability of p68 by preventing proteasomal degradation.

To test this hypothesis, we performed pulse–chase experiments with [^{35}S]methionine and then determined the decay of radioactively labeled p68 over time; please note that levels of nonmodified ^{35}S-labeled p68 were measured, since the amounts of both sumoylated and ubiquitylated p68 were below the

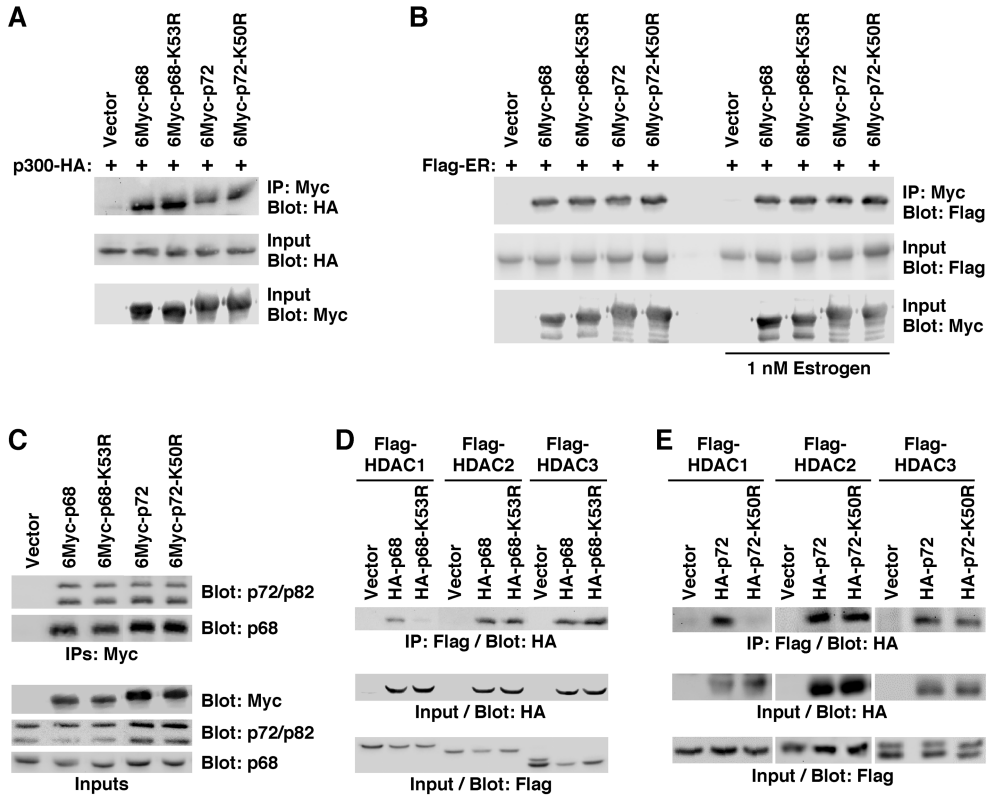


FIGURE 5: Impact of sumoylation on protein interactions. (A) Interaction with p300. Myc-tagged p68/p72 were coexpressed with HA-tagged p300. After anti-Myc immunoprecipitation, coprecipitated p300 was revealed by anti-HA Western blotting. The bottom two panels show input levels of p300-HA and Myc-tagged p68/p72. (B) Similar interaction of Myc-tagged p68/p72 with Flag-tagged ERα in the presence and absence of estrogen. (C) Homo- and heterodimerization of Myc-tagged p68/p72 with endogenous p68 or p72/p82. (D) Coimmunoprecipitation of HA-tagged p68 with Flag-tagged HDACs. (E) Similar interaction of p72 with HDACs.

detection limit in this experiment. Whereas wild-type p68 had a half-life of 20.2 h, its K53R mutant displayed a markedly shorter half-life of 11.5 h (Figure 4D). Consistent with being much less ubiquitylated than p68, p72 displayed a >3-fold longer half-life compared to p68 (Figure 4E). Again, the sumoylation site mutant was less stable than wild-type p72 (54.4 versus 67.7 h half-life), although the difference was less pronounced than that of p68. Altogether, these results indicate that sumoylation increases the half-lives of p72 and especially p68.

Impact of Sumoylation on Binding to Partner Proteins. Posttranslational modifications can affect protein−protein interactions. Several interactants of p68 and p72 have been uncovered, including the coactivator p300 and ERα (*5*, *6*, *11*, *12*). Thus, we tested whether p68/p72 and their respective sumoylation site mutants differentially bind to these two proteins in coimmunoprecipitation experiments. Compared to wild-type p68/p72, there was no difference in the ability of the K53R/K50R mutants to bind to p300 or ERα (Figure 5A,B). Since p68 and p72 form both homo- and heterodimers (*56*), we also assessed the interaction of 6Myc-tagged p68/p72 with endogenous p68/p72. Note that 6Myc-tagged p68 and p72 electrophorese at ∼20 kDa above and are therefore easily separated from endogenous p68 and p72. Again, we did not find a difference in the ability of the 6Myc-p68-K53R/6Myc-p72-K50R mutants to form homo- or heterodimers with endogenous p68 or p72 compared to wild-type 6Myc-p68/p72 (Figure 5C).

Previously, it was shown that p68 and p72 also interact with the histone deacetylase (HDAC) 1 (*57*). Thus, we additionally tested the ability of p68 and p72 to interact with various HDACs. p68 bound expectedly to HDAC1 and also to HDAC2 and HDAC3 as revealed by coimmunoprecipitation assays (Figure 5D). Importantly, the sumoylation site mutant of p68 bound less well to HDAC1 than wild-type p68, whereas there was no difference in the case of HDAC2 or HDAC3. Similarly, mutation of K50 in p72 resulted in drastically diminshed binding to HDAC1 but did not affect binding to HDAC2 or HDAC3 (Figure 5E). These data indicate that sumoylation selectively promotes the interaction of p68 and p72 with HDAC1. While this work was in progress, another report also demonstrated that p68 sumoylation enhances binding to HDAC1 (*55*).

Sumoylation Modulates Transcriptional Coactivation. Both p68 and p72 have been reported to coactivate ERα (*5*, *6*). Thus, we studied how sumoylation might affect this property of p68/p72 by utilizing MDA-MB-231 breast cancer cells that are ERα-negative and a luciferase reporter that is driven by an estrogen response element (ERE). Expectedly, in the absence of cotransfected ERα or in the absence of estrogen stimulation, no luciferase activity was measurable. However, when ERα was stimulated with estrogen, robust luciferase activity was observed (Figure 6A). When wild-type p68 was coexpressed, it stimulated ERα activity >6-fold. In contrast, the K53R mutant showed a much reduced ability to coactivate (∼2-fold). These data indicate that sumoylation enhances the ability of p68 to coactivate ERα.

Another promoter we investigated was MDM2, a target of the tumor suppressor p53 and whose transcription has been shown to be enhanced by p68 and p72 (*8*, *12*). We employed an MDM2 luciferase construct in the p53-negative MDA-MB-231 cells to determine the impact of p68 sumoylation on this promoter. p68 synergized with p53 in the activation of the MDM2 promoter (Figure 6B). However, there was no difference in this ability when

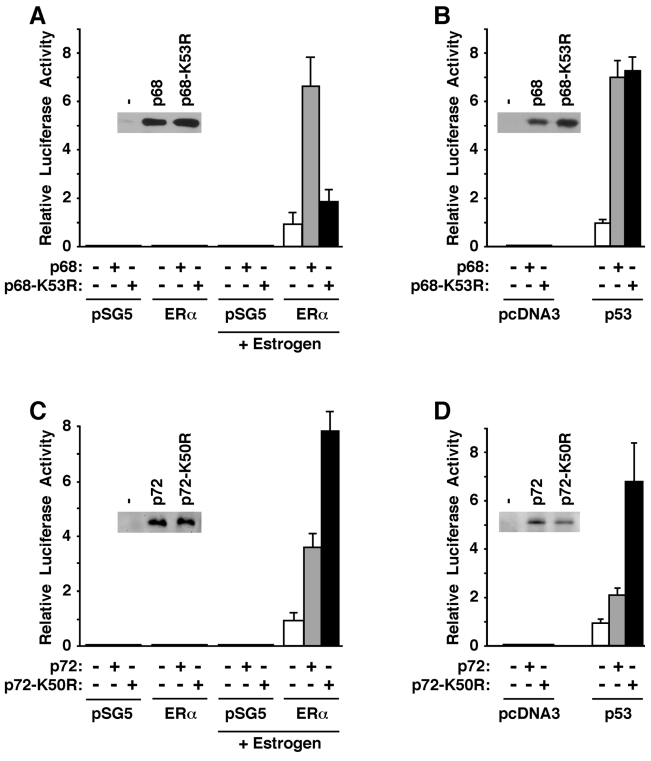


FIGURE 6: Sumoylation of p68/p72 modulates transcription. (A) HA-tagged p68 (wild type or K53R mutant) was expressed in MDA-MB-231 cells. ERα or empty expression vector pSG5 were cotransfected and cells stimulated with estrogen as indicated. Resultant luciferase activities derived from the cotransfected ERE-luc reporter are depicted. The inset shows an anti-HA blot indicating that comparable amounts of wild-type and K53R p68 were expressed upon ERα cotransfection and estrogen stimulation. (B) Similar stimulation of the MDM2 promoter by p68. Where indicated, p53 or the empty pcDNA3 expression vector was cotransfected. The inset is an anti-HA blot showing the comparable expression of wild-type and K53R p68 upon p53 coexpression. (C, D) Analogous activation of the ERE-luc reporter and MDM2-luc promoter by p72 (wild type or K50R mutant).

utilizing the K53R mutant. Thus, sumoylation affects the transactivation potential of p68 in a promoter-specific context.

Similarly, we tested the impact of sumoylation on the p72 coactivation potential. Surprisingly, the p72-K50R mutant was more active than wild-type p72 at both the ERE and MDM2 promoters (Figure 6C,D). This suggests that sumoylation of p72 represses its coactivation function and marks an unexpected difference between p68 and p72. These findings are consistent with our results shown in Figure 2B,C, where sumoylation appeared to stimulate the coactivation potential of p68 and to enhance p72-mediated transcriptional repression.

Sumoylation has been shown to influence the intracellular localization of several proteins (*17*, *18*). Thus, it is conceivable that the altered coactivation potential of the p68 and p72 sumoylation mutants is simply a reflection of a changed intracellular localization. However, we did not find any evidence that the p68-K53R or p72-K50R mutants localize differently within cells compared to the respective wild-type proteins (Supporting Information Figure 1).

DISCUSSION

About 70% of breast tumors are ERα-positive, and therapies targeting estrogen or ERα have been developed and proven effective to fight early stage breast cancer (*58*). However, acquired or inherent endocrine resistance is a major problem in the clinic. One mechanism by which ERα may become independent of estrogen or insensitive to antiestrogens is through enhanced stimulation by cofactors (*59*). This report is the first demonstration that the ERα cofactors p68 and p72 are overexpressed in the majority of human breast tumors and may thereby cause aberrant activation of ERα and contribute to carcinogenesis.

In addition, we demonstrated that p68 and p72 can be sumoylated on one particular lysine residue, K53 or K50, respectively, which are at homologous sites in the N-termini of these RNA helicases. While this work was in progress, another report established similarly that K53 is the sumoylation site of p68 but did not address sumoylation of p72 (*55*). Our results showed that SUMO1 was more efficiently added onto p68 and p72 than SUMO2 in HEK293T cells, but this may be cell-type dependent since the opposite preference was reported for p68 in COS-7 cells (*55*).

The SUMO pathway is hyperactivated in breast cancer based on published data indicating that the SUMO ligase PIAS3 is overexpressed (*53*) and our bioinformatical analysis revealing that SUMO1 and the SUMO conjugating enzyme Ubc9 are upregulated whereas the SUMO protease SENP6 is downregulated in breast tumors. Importantly, overexpression of Ubc9 in MCF-7 breast cancer cells led to larger tumors in a xenograft mouse model, while a dominant-negative Ubc9 molecule suppressed tumor growth (*60*). These data suggest that a hyperactivated SUMO pathway contributes to breast tumor growth, possibly by stimulating estrogen-dependent transcription that is activated by the SUMO pathway (*24*). Therefore, sumoylation of the ERα cofactors, p68 and p72, is likely to be relevant in breast carcinogenesis.

Mutation of the sumoylation site in p68 markedly decreased its half-life, and the mutation of K50 in p72 also resulted in a small but significant decrease of protein stability. This indicates that sumoylation stabilizes p68/p72 and provides one explanation of how a hyperactivated SUMO pathway causes, at least in part, the observed overexpession of p68/p72 in breast tumors. Sumoylation may increase the stability of a protein by simply preventing ubiquitylation at the same lysine residue as shown, for instance, for IκBα or MDM2 (*61*, *62*). This is not the case for p68, since the K53R mutant, which can neither be sumoylated nor be ubiquitylated at position 53, is even more ubiquitylated than wild-type p68. Rather, sumoylation appears to prevent ubiquitylation at a lysine residue(s) other than K53 in p68, possibly by sterically inhibiting the docking of ubiquitin ligases to p68.

Sumoylation also affected the transcriptional activity of p68 and p72. Our data revealed a significant enhancement of ERα-mediated transcription by sumoylated p68, which demonstrates how sumoylation of p68 may promote breast tumorigenesis. However, the opposite held true for p72: sumoylation appeared to repress the ERE luciferase reporter. Thus, enhanced sumoylation may dampen the activating effect of p72 overexpression on ERα in breast tumors.

In the case of the MDM2 promoter, a prominent target of the tumor suppressor p53 (*63*), sumoylation of p68 had no impact on its ability to coactivate p53, which indicates that the importance of sumoylation on p68 is promoter specific. In contrast to our data, a previous report showed that the K53R mutant of p68 activated a luciferase gene driven by multiple synthetic p53 sites 1.5 times more efficiently than wild-type p68 (*55*), further suggesting that sumoylation of p68 has promoter-dependent

effects on p53 and may be neutral or repressing. Of note, advanced, chemotherapy-resistant breast cancer is characterized by loss of p53 transcriptional activity (*64*), indicating that p68 and p72 overexpression may play an oncogenic role by upregulating MDM2 transcription and thereby facilitating MDM2-mediated destruction of the tumor suppressor p53. Unlike p68, mutation of the p72 sumoylation site enhanced the ability of p72 to coactivate the MDM2 promoter, suggesting that sumoylation of p72 could suppress tumor formation by reducing MDM2 transcription and thereby stabilizing p53.

How can one explain the impact of sumoylation on the coactivation potential? One property that could be influenced by sumoylation is the recruitment of other cofactors. However, we found no impact of p68/p72 sumoylation on the recruitment of the coactivator p300, the histone deacetylases HDAC2 and HDAC3, or ERα and also no change in the ability to homo/heterodimerize. But we observed that HDAC1 recruitment is suppressed when the sumoylation site in p68 or p72 was mutated. This clearly represents a proof of principle that sumoylation can modulate p68/p72 by selectively regulating the binding to another protein (HDAC1) and should instigate further studies on how association with other p68/p72 interaction partners is modulated by sumoylation.

Consistent with many reports describing the sumoylation of various proteins (*17*−*19*), only a very small fraction of p68 or p72 is sumoylated (see Figure 2D), raising the issue of how this may affect p68 and p72 function. In particular, when we determined the ability of p68 or p72 to coimmunoprecipitate with HDAC1, we did not measure how much sumoylated p68/p72 coimmunoprecipitated (this was below our detection limit) but how much nonsumoylated p68/p72 did, and this was different between wild type and sumoylation mutants. Thus, it appears that sumoylation of p68/p72 positively affects binding of non-modified p68/p72 to HDAC1.

Several models have been put forward to explain such a fact, all taking into account the high dynamics of intracellular sumoylation guaranteeing any target protein to be modified by SUMO for at least a very short time span (*18*, *65*). One model suggests that sumoylation induces a conformational change in a target protein that persists after desumoylation. Accordingly, such an irreversible conformational change may be required for p68/p72 to interact with HDAC1. A second model proposes that sumoylation increases the rate constant for complex association but does not affect the dissociation constant. If so, sumoylation of p68/p72 is predicted to greatly increase the association with HDAC1, whereas subsequent desumoylation of p68/p72 will not affect the release of HDAC1 from p68/p72 complexes. Again, p68/p72 sumoylation, albeit transient and at a low steady-state level, will thereby greatly promote the complex formation with HDAC1. These models also explain how sumoylation increases the half-lives of nonsumoylated p68 and p72: an irreversible conformational change or sequestration into a protein complex induced by transient sumoylation conceivably precludes ubiquitylation and thus proteasomal destruction.

Histone deacetylation is commonly thought to suppress gene transcription. In line with this, reduced HDAC1 binding of the K50R mutant of p72 correlated with an enhanced coactivation potential. However, a few studies indicate that HDAC1 can also activate transcription, for instance, at certain interferon target genes (*66*, *67*). Thus, it remains to be studied whether enhanced interaction with HDAC1 is one of the underlying causes of why sumoylation of p68 leads to increased coactivation of ERα.

In conclusion, our study has revealed that p68 and p72 are overexpressed in breast tumors and may therefore contribute to breast carcinogenesis by augmenting ERα. In addition, hyperactivation of the SUMO pathway and the resultant enhanced sumoylation of p68 and p72 may be an underlying cause of their overexpression in breast cancer. Moreover, p68 sumoylation increases its coactivation potential and will thereby further stimulate ERα function. By contrast, sumoylation of p72 reduces estrogen-dependent transcription and MDM2 promoter activity. It is suspected that the balance of all these effects determines whether sumoylation of p68 and p72 promotes breast tumorigenesis.

SUPPORTING INFORMATION AVAILABLE

One figure as described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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