Small Ubiquitin-like Modifier (SUMO) Modification of Zinc Finger Protein 131 Potentiates Its Negative Effect on Estrogen Signaling

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Background: A poorly characterized zinc finger protein, ZNF131, is implicated as a negative regulator of estrogen receptor α target gene expression. RESULTS: ZNF131 is a target of covalent SUMOylation via hPc2, which potentiates its inhibitory effect on estrogen signaling.

Conclusion: Estrogen signaling is down-regulated through the SUMOylation of ZNF131.

Significance: Covalent SUMO1 modification of ZNF131 negatively regulates estrogen receptor-mediated signaling and estrogen-induced cell proliferation in breast cancer cells.

Like ubiquitin, small ubiquitin-like modifier (SUMO) covalently attaches to specific target proteins and modulates their functional properties, including subcellular localization, protein dimerization, DNA binding, and transactivation of transcription factors. Diverse transcriptional co-regulator complexes regulate the ability of estrogen receptors to respond to positive and negative acting hormones. Zinc finger protein 131 (ZNF131) is poorly characterized but may act as a repressor of estrogen receptor α (ERα)-mediated trans-activation. Here, we identify ZNF131 as a target for SUMO modification and as a substrate for the SUMO E3 ligase human polycomb protein 2 (hPc2). We report that the SUMO-interacting motif 1 (SIM1) and the C-box of hPc2 are critical regions required for ZNF131 SUMOylation and define the ZNF131 SUMOylation site as lysine 567. We further show that SUMO modification potentiates the negative effect of ZNF131 on estrogen signaling and consequently attenuates estrogen-induced cell growth in a breast cancer cell line. Our findings suggest that SUMOylation is a novel regulator of ZNF131 action in estrogen signaling and breast cancer cell proliferation.

Estrogen has various physiological functions throughout the body (1, 2), many of which are mediated through two structurally and functionally distinct estrogen receptors, ERα and ERβ (3). After binding to estrogen, ERα undergoes a conformational change that promotes its homodimerization, which allows the receptor to bind DNA at the estrogen response element (ERE). The estrogen-ERα-ERE complex then initiates transcription of target genes (3). The estrogen-ERα-ERE pathway is also responsible for the mitogenic effect of estrogen (4) and plays a crucial role in regulating its oncogenic expression (5). Alteration of proper growth-stimulatory signaling by estrogen and ERα has been implicated to the development of breast cancer (6, 7).

Multiple co-regulatory factors, termed co-activators and co-repressors, regulate ERα activity (3). ERα co-repressors are proposed to provide a counterbalance to estrogen-induced trans-activation and represent a potential tumor suppression mechanism for the cell (8). Zinc finger protein 131 (ZNF131) is a member of the POZ zinc finger (POZ-ZF) protein superfamily and was initially linked with developmental disorders (9). Using a high-throughput, cell-based, and functional screening platform created by linking the ERE with a reporter gene, ZNF131 has been shown to suppress ERα target gene expression (10).

Small ubiquitin-like modifier (SUMO) protein belongs to a family of ubiquitin-related proteins and is covalently conjugated to lysine residues on its substrates (11). Covalent SUMOylation of the substrate occurs through a serial enzymatic cascade, analogous to that of the ubiquitination pathway, and this process modifies the biochemical and functional properties of the substrate (11). All newly synthesized SUMO protein isoforms have a C-terminal extra-short peptide that is rapidly cleaved by SUMO-specific proteases (SENPs) to generate the mature C-terminal diglycine motif (12). There are three major SUMO protein isoforms: SUMO1, SUMO2, and SUMO3 (11). Amino acid sequence analyses of SUMO2 and SUMO3 demonstrate that these proteins share ~95% identity and ~50% identity with SUMO1, respectively. Despite the identification of three SUMO protein isoforms (11), a single heterodimeric
SUMO-activating E1 enzyme (termed SAE1/SAE2) (13–15), and a single SUMO-conjugating E2 enzyme (termed UBC9) (16–18) have been identified thus far. The absolute requirement for SUMO E3 ligase in the SUMOylation pathway is controversial (11). Nevertheless, several families of mammalian SUMO E3 ligases can promote the SUMOylation of their substrates (11).

Human polycomb protein 2 (hPc2; also known as CBX4) is a member of the polycomb repressive complex 1 (PRC1) originally identified to mediate gene silencing by altering the chromatin structure during gene transcription (19). hPc2 also functions as a SUMO E3 ligase to promote the SUMOylation of the transcriptional co-repressor CtBP (20). hPc2 contains two SUMO-interacting motifs (SIMs), both of which play a role in SUMO binding. These motifs contribute to noncovalent SUMO binding and are required for full SUMO E3 activity, as well as for SUMOylation of hPc2 itself (21).

SUMO modifies multiple targets in the estrogen-ERα-ERE pathway, including ERα (22), BRCA1 (23), KLF4 (24), SRC-1 (25), N-CoR (26), GRIP1 (27), AIB1 (28), and p300 (29). Whereas ZNF131 was previously reported to suppress ERα target gene expression (10), its molecular and regulatory mechanism has not yet been characterized. Here, we identify ZNF131 as a target for SUMOylation and report that hPc2 is its SUMO E3 ligase. Moreover, we further demonstrate that SUMO modification potentiates the co-repressor function of ZNF131 during estrogen signaling.

**EXPERIMENTAL PROCEDURES**

**Materials**—The proteasome inhibitor MG-132 was purchased from A. G. Scientific. β-Estradiol (E2), rabbit polyclonal anti-SUMO1, and anti-actin antibodies were purchased from Sigma. Mouse monoclonal anti-HA antibody was purchased from Covance. Rabbit polyclonal anti-HA, anti-Myc, anti-pS2, anti-HSP90, mouse monoclonal anti-HA, anti-GFP, anti-Myc, anti-GAPDH, and anti-ubiquitin antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-V5 antibody was purchased from Abnova. Rabbit polyclonal anti-MBP antibody was purchased from New England Biolabs. Mouse monoclonal anti-V5 and HRP-conjugated anti-rabbit antibodies were purchased from Invitrogen. HRP-conjugated anti-mouse antibody was purchased from Thermo Scientific. Normal mouse immunoglobulin G was purchased from Upstate. DMEM, FBS, and Lipofectamine PLUS were purchased from Invitrogen. Protein A-Sepharose was purchased from Amersham Biosciences. All other chemicals were purchased from Sigma.

**DNA Constructions and RNA Interference**—The plasmid encoding 6xMyc-tagged mouse ZNF131 was a kind gift from J. M. Daniel (McMaster University, Hamilton, Canada). Human ZNF131 isoform 2 (accession number NM_003432) cDNA was PCR amplified and subcloned into modified pRK5-HA (Stratagene), pEGFP-C2 (Clontech), pDsRed2-N1 (Clontech), or pMAL-c2X (New England Biolabs) to generate HA-tagged ZNF131 (HA-ZNF131), GFP-tagged ZNF131 (GFP-ZNF131), 3’-DsRed2-tagged ZNF131 (ZNF131-DsRed2), or MBP-tagged ZNF131 (MBP-ZNF131), respectively. The plasmid encoding FLAG-tagged hPc2 (accession number NM_003655) was a kind gift from D. Wotton (University of Virginia, Charlottesville, VA). This construct served as a PCR template and was subcloned into modified pRK5-Myc, pRK5-HA, or pEGFP-C2 to generate Myc-tagged hPc2 (Myc-hPc2), HA-tagged hPc2 (HA-hPc2), or GFP-tagged hPc2 (GFP-hPc2), respectively. Human SUMO1 (accession number NM_003352), SUMO2 (accession number AK311837), and SUMO3 (accession number AK312350) cDNAs were amplified as described previously (30), and cloned into modified pRK5-V5 to generate V5-tagged wild type SUMO1 (V5-SUMO1-GG), SUMO2 (V5-SUMO2-GG), and SUMO3 (V5-SUMO3-GG). Plasmids encoding Myc-tagged PIAS1 and PIAS3 were kind gifts from K. Shuai (University of California, Los Angeles, CA) and H. Yokosawa (Hokkaido University, Sapporo, Japan), respectively. The plasmids encoding 3xMyc-tagged human UBC9, 3xMyc- and GFP-tagged human SENP1 were a kind gift from Dr. Y. K. Jang (Yonsei University, Seoul, Korea). To construct various deletion and point mutants of hPc2 and ZNF131, site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene). All cDNA sequences were verified by DNA sequencing (COMSO Genetech). The reporter plasmid pTA-4xEERE-Luc was constructed by inserting the following two annealed oligonucleotides between the MluI and XhoI sites of the luciferase reporter vector pTA-Luc (Clontech): 5’-GGTGCTCATCAGTGACCTAGGTCACAGTGCTAACATCGG-3’ and 5’-TGAGAGGTCACTGTGACCTAGGTCACTGCTACTGTCATGTCACAGTGCTAACACTGG-3’. This sequence contains four tandem repeats of the consensus ERE oligonucleotide sequence (AGTGCACAGTG-ACCT). The hPc2-specific and control siRNAs were designed and synthesized by Sigma. The hPc2 siRNA duplex sequences were 5’-UUGAAAGAUUGACAGAAAC(dTdT)-3’ and 5’-UUGUUCUUGCUUAUCUCU(dTdT)-3’. The control siRNA duplex sequences were 5’-GUCCACGCGCAAAUCU- CGU(dTdT)-3’ and 5’-ACGAAAAUUGUGGCGUAGG(dTdT)-3’. These siRNA duplexes were transfected into cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**Cell Culture and DNA Transfection**—The human embryonic kidney epithelial cell line (HEK293) and monkey kidney fibroblast cell line (COS-7) cells were maintained in DMEM supplemented with 10% FBS and 100 units/ml of penicillin/streptomycin (Invitrogen). The human breast cancer cell line (MCF-7) was maintained in DMEM supplemented with 10% FBS, 100 units/ml of penicillin/streptomycin, and 10 μg/ml of insulin (Sigma). DNA transfection was carried out using Lipofectamine PLUS reagents (Invitrogen), according to the manufacturer’s instructions.

**In Vivo SUMOylation Assay and Western Blot Analysis**—To detect protein SUMOylation, cells were lysed in lysis buffer (10 mM Tris, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 20 mM N-ethylmaleimide, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 5 mM NaF, and 0.5 mM phenylmethylsulfonyl fluoride) supplemented with 1% SDS. After sonication to reduce the viscosity, the cell lysates were mixed with Benzonase (Sigma) and incubated for 15 min.
SUMOylation Assay in E. coli

Next, we incubated with 0.2 mM isopropyl-1-thio-
31). Briefly, either the pE1:E2:SUMO1-GG or pE1:E2:SUMO2-
gene, such as pE1:E2:SUMO1 or pE1:E2:SUMO2. Analysis of
-1 min. After washing with PBS three times, the cells were ana-
3% nonfat dry milk (Bio-Rad) in TBST buffer

**Immunocytochemistry**—COS-7 cells were seeded onto cov-
inerslips in 6-well dishes at 60% confluence and incubated over-
night. Cells were washed with PBS, fixed with 4% paraformal-
dehyde in PBS for 20 min, and permeabilized with 0.2% Triton
X-100 in PBS for 30 min. Cells were then blocked with 1% BSA
in PBS for 30 min and incubated with mouse monoclonal anti-
Myc antibody overnight at 4 °C. After washing with PBS three
times, the cells were incubated with Alexa Fluor 594-conju-
gated anti-mouse antibody (Molecular Probes) for 2 h. To stain
the nuclei, cells were incubated with 1 μg/ml of DAPI in PBS for
5 min. After washing with PBS three times, the cells were ana-
lyzed using confocal microscopy (LSM510 META; Carl Zeiss)
or fluorescence microscopy (IX71; Olympus).

**SUMOylation Assay in Escherichia coli**—The pE1:E2:SUMO1-
GG and pE1:E2:SUMO2-GG plasmids were constructed as
described previously (30). Using these two constructs, E. coli
SUMOylation assays were performed, as previously described (30,
31). Briefly, either the pE1:E2:SUMO1-GG or pE1:E2:SUMO2-
GG plasmid was co-transformed into the BL21(DE3) E. coli strain
with pMAL-c2X or pMAL-c2X-ZNF131. After the bacteria were
incubated with 0.2 mm isopropyl 1-thio-β-D-galactopyranoside for
12 h at 25 °C, MBP-tagged recombinant proteins were purified
using amylose beads (New England Biolabs), according to the
manufacturer’s protocol.

**Luciferase Reporter Assay**—After maintaining MCF-7 cells in
pheno red-free DMEM (HyClone) containing 5% charcoal-
stripped FBS (Sigma), cells were co-transfected with pTA-
4xERE-Luc and pRL plasmids (Promega). The pRL plasmid
constitutively expresses Renilla luciferase and is used to nor-
malize transfection efficiency. Cells were lysed and analyzed
using the Dual Luciferase Reporter Assay system (Promega).
Significant differences in luciferase activity were analyzed using
the Student’s t test in the Sigma Plot 11 program (Systat Soft-
ware Inc.).

**Cell Growth Analysis**—MCF-7 cells were maintained in pheno-
ol red-free DMEM containing 5% charcoal-stripped FBS for
24 h. After DNA transfection for 24 h, cells were treated with
vehicle (ethanol) or E2 (10 nM) for the indicated times. The
number of viable cells was determined using Cell Counting
Kit-8 (Dojindo Molecular Technology). Significant differences
in cell viability were analyzed using the Student’s t test in Sigma
Plot 11.

**RESULTS**

ZNF131 Physically Interacts with hPc2—To isolate novel
binding partners of hPc2, we performed a yeast two-hybrid
assay using full-length hPc2 as bait. Using a human fetal brain
cDNA library, we identified many previously reported binding
partners of hPc2, including RING1 (32) and UBC9 (33). In addi-
tion, we found that ZNF131 interacts with hPc2 in yeast cells
(data not shown). To examine whether ZNF131 also interacts
with hPc2 in mammalian cells, HEK293 cells were transfected
with plasmids encoding epitope-tagged ZNF131 and epitope-
tagged hPc2. The lysates were immunoprecipitated followed by
Western blot as shown in Fig. 1, A and B. The results reveal that
ZNF131 binds to hPc2 in HEK293 cells (Fig. 1, A and B). Immu-
nostaining of COS-7 cells transfected with GFP-ZNF131 or
Myc-hPc2 demonstrates that ZNF131 alone exhibits diffuse
nuclear localization (Fig. 1C, top panels) and hPc2 is exclusively
localized to nuclear speckles called polycomb bodies (Fig. 1C,
middle panels). This confirms previous findings that ZNF131 is
a nuclear protein (34), and that hPc2 is a component of poly-
comb nuclear bodies (19). Interestingly, when cells co-express
ZNF131 and hPc2, the proteins co-localize within the poly-
comb bodies (Fig. 1C, bottom panels). GFP alone did not co-
localize with hPc2 (Fig. 1C, middle panels), further suggesting
that ZNF131 physically interacts with hPc2 in mammalian cells.

ZNF131 Is Covalently Modified by SUMO1—Next, we
assessed whether ZNF131 could be a substrate for SUMOyla-
ation. HEK293 cells were transfected with HA-ZNF131 alone or
together with V5-tagged wild type SUMO1 (SUMO1-GG) or its
conjugation-defective mutant (SUMO1-AA). The formation of
SUMOylated ZNF131 was determined by immunoprecipitat-
ing the cell extracts with HA antibody followed by Western
blotting with the V5 or SUMO1 antibodies. These bands
were not observed in cells transfected with ZNF131 alone or with
SUMO1-AA mutant, and likely correspond to the size of the
mono- or di-SUMOylated forms of ZNF131, respectively (Fig.
2A). The same pattern of higher molecular weight ZNF131
bands was also observed in COS-7 and MCF-7 cells (data not
shown). To verify ZNF131 SUMOylation, we performed a
SUMOylation assay in E. coli. This system efficiently generates
multiple SUMO1- and SUMO2-conjugated products using
recombinant mammalian substrates (30, 31). E. coli was trans-
formed with bacterial expression vectors encoding recombi-
nant ZNF131 plus components of the SUMOylation machin-
ery, such as pE1:E2:SUMO1 or pE1:E2:SUMO2. Analysis of
the reaction products indicate that mono-SUMOylation of
ZNF131 occurs in E. coli in the presence of SUMO1 or SUMO2
(supplemental Fig. S1). Finally, immunoprecipitating HEK293
cells with the ZNF131 antibody followed by immunoblot with the SUMO1 antibody generated a SUMO1-modified endogenous ZNF131 protein band (Fig. 2A). Overall, these results demonstrate that endogenous ZNF131 is SUMOylated in mammalian cells and that the covalent attachment of SUMO1 to ZNF131 is not an artifact of ectopic DNA transfection.

hPc2 Acts as a SUMO E3 Ligase of ZNF131—We next assessed how hPc2 affects the extent of ZNF131 SUMOylation. Although SUMOylated ZNF131 was relatively undetectable in cells co-expressing ZNF131 and SUMO1-GG, the presence of hPc2 greatly increased the amount of SUMOylated ZNF131 (Fig. 3A, top panel). In contrast, the amount of auto-SUMOylated hPc2 was not considerably altered by the presence of ZNF131 (Fig. 3A, second panel). To determine the identity of the higher molecular weight ZNF131 bands, we examined the effect of the SUMO-specific SENP1 protease on ZNF131 SUMOylation. As shown in Fig. 3B, addition of SENP1 completely abolished the hPc2-induced higher molecular weight ZNF131 bands (Fig. 3B). Moreover, there was an additive and dose-dependent effect of hPc2 on ZNF131 SUMOylation (Fig. 3C). As expected, hPc2 did not mediate ZNF131 SUMOylation in the presence of the conjugation-defective SUMO1-AA mutant (Fig. 3C). Interestingly, we observed multiple bands of hPc2-mediated ZNF131 SUMOylation even in the input Western blots (Fig. 3, A–C). The same pattern of hPc2-enhanced ZNF131 SUMOylation was also observed in COS-7 and MCF-7 cells (data not shown). Compared with hPc2, two members of the PIAS family, such as PIAS1 and PIAS3, did not directly bind to ZNF131 (data not shown). In addition, these enzymes had no significant effect on ZNF131 SUMOylation (supplemental Fig. S2). As expected, overexpression of the SUMO E2-conjugation enzyme UBC9 remarkably promoted SUMOylation of ZNF131 (supplemental Fig. S3). Furthermore, we found that the three major SUMO isoforms (SUMO1, SUMO2, and SUMO3) all conjugate to ZNF131 in the presence of hPc2 (supplemental Fig. S4).

To prove that hPc2 acts as an endogenous SUMO E3 ligase for ZNF131, we first examined the effect of knocking down hPc2 on ZNF131 SUMOylation. Unfortunately, none of commercially available hPc2 antibodies we tested were able to detect endogenous hPc2 protein. Therefore, we could only assess the knockdown effect of hPc2-siRNA on hPc2 mRNA expression through RT-PCR. Treatment with hPc2-siRNA reduces the expression of hPc2 mRNA by ~30% and the level of mono-SUMOylated ZNF131 protein was decreased by ~40% (Fig. 3D). Moreover, this was not seen with the nonspecific siRNA control (Fig. 3D). Second, we examined the effect of hPc2 on the endogenous ZNF131 SUMOylation. As expected, overexpression of hPc2 led to the increase of endogenous

FIGURE 1. ZNF131 and hPc2 physically interacts in fibroblast cells. A, HEK293 cells were transfected for 24 h with 6xMyc-ZNF131, in the presence or absence of HA-hPc2, and immunoprecipitated (IP) with the HA antibody. Immunoprecipitates were then immunoblotted with the antibodies as indicated. HSP90 was used as a protein loading control. B, HEK293 cells were transfected for 24 h with Myc-hPc2, in the presence or absence of HA-ZNF131, and immunoprecipitated with the HA antibody. Immunoprecipitates were then immunoblotted with the antibodies as indicated. Actin served as a protein loading control. C, COS-7 cells were transfected for 24 h with GFP-ZNF131, in the presence or absence of Myc-hPc2, and analyzed by immunocytochemistry with the Myc antibody. Expression of GFP-ZNF131 (green), Myc-hPc2 (red), and the DAPI-stained nuclei (blue) were analyzed using confocal microscopy. The merged image (yellow) indicates the co-localization of Myc-hPc2 and GFP-ZNF131. WB, Western blot; M, relative molecular mass.

FIGURE 2. ZNF131 is posttranslationally modified by SUMO1. A, HEK293 cells were transfected for 24 h with HA-ZNF131 and either V5-SUMO1-GG or V5-SUMO1-AA. Lysates were then immunoprecipitated (IP) with the HA antibody and immunoblotted with the indicated antibodies. Actin was used as a protein loading control. B, HEK293 cell extracts were immunoprecipitated with control or ZNF131 antibodies followed by immunoblot with the indicated antibodies. GAPDH served as a protein loading control. ●, nonspecific band. WB, Western blot; M, relative molecular mass.
**Functional Modulation of ZNF131 by SUMOylation**

ZNF131 SUMOylation (supplemental Fig. S5). These data suggest that hPc2 is an endogenous SUMO E3 ligase of ZNF131.

**SUMO E3 Ligase Activity of hPc2 on ZNF131 Is Dependent on Central SIM1 Domain**—To determine which region(s) of hPc2 are important for ZNF131 SUMOylation, we constructed several deletion mutants of hPc2 as well as point mutants at several previously reported critical residues (33, 35) (Fig. 4A). Compared with full-length hPc2, the amount of ZNF131 SUMOylation was greatly reduced in the presence of the mutants without SIM1 (ΔSIM1) or HCR (ΔHCR) (Fig. 4B). However, the hPc2 SIM2 domain deletion mutant (ΔSIM2) and a point mutant at the self-SUMOylation site (K494R) had no significant effect on ZNF131 SUMOylation (Fig. 4B). The other point mutant we constructed, T497A, had a modest effect on ZNF131 SUMOylation (Fig. 4B and supplemental Fig. S6). These results suggest that the SIM1 domain and threonine 497 of hPc2 are important regions for its SUMO E3 ligase activity on ZNF131.

To exclude the possibility that this is an artifact of overexpressing SUMO protein, we examined the effect of the hPc2-SIM1 domain on ZNF131 SUMOylation in the absence of exogenously added SUMO1. As shown in Fig. 4C, ZNF131 is mono-SUMOylated by wild type hPc2 in the absence of SUMO1 overexpression (Fig. 4C). As expected, co-expression of ZNF131 and hPc2-ΔSIM1 failed to generate the ZNF131 SUMOylation (Fig. 4C). This result indicates that hPc2 mediates the mono-SUMOylation of ZNF131 using endogenous SUMO1 protein.

We next examined whether deletion of the SIM1 region diminishes the enzymatic function of hPc2 and/or causes a decrease in its substrate binding. Co-immunoprecipitation experiments reveal that the binding affinity of hPc2 to ZNF131 was greatly decreased when the SIM1 domain was deleted (Fig. 4D). To test whether co-localization of hPc2 with ZNF131 is also altered by the SIM1 deletion, ZNF131-DsRed2 and GFP-hPc2 were co-transfected into COS-7 cells and visualized by fluorescence microscopy. As previously reported (21), GFP-hPc2-ΔSIM1 was selectively localized to polycomb bodies (Fig. 4E). However, ZNF131-DsRed2 displayed a diffuse nuclear localization and did not localize to polycomb bodies (Fig. 4E). These results suggest that hPc2 E3 ligase activity on ZNF131 is dependent on the SIM1 domain of hPc2, which is critical for the binding to ZNF131.

**SUMO E3 Ligase Activity of hPc2 on ZNF131 Is Dependent on Localization to Polycomb Bodies**—The C-terminal C-box domain of hPc2 is required for interaction with the polycomb complex protein RING1, which subsequently elicits hPc2 localization in polycomb bodies (36). To examine whether the localization of hPc2 to polycomb bodies is necessary for ZNF131 SUMOylation, we created an hPc2 deletion mutant lacking the C-box (1–531) (Fig. 4A). Expression of the hPc2-(1–531) mutant greatly reduces the amount of SUMOylated ZNF131 as compared with wild type hPc2 (Fig. 5A). Next, we examined how deletion of the C-box affects the binding affinity of hPc2 to ZNF131. Co-immunoprecipitation experiments demonstrate that the lack of hPc2 C-box does not diminish hPc2 binding to ZNF131; on the contrary, the interaction between the two proteins was slightly increased (Fig. 5B). To further test whether co-localization of hPc2 and ZNF131 is affected by
deleting the C-box, DsRed2-ZNF131 and GFP-hPc2-(1–531) were co-transfected into COS-7 cells and visualized by fluorescence microscopy. As shown in Fig. 5C, GFP-hPc2-(1–531) did not localize to polycomb bodies, but showed a diffuse nuclear localization (Fig. 5C). Furthermore, the two proteins completely co-localized within the nucleoplasm (Fig. 5C). These results suggest that the SUMO E3 ligase activity of hPc2 on ZNF131 is dependent on the proper localization of hPc2 to the polycomb bodies.

Lysine 567 of ZNF131 Is a Major SUMO Acceptor Site—To identify the SUMO modification site(s) in ZNF131, we analyzed several ZNF131 deletion and point mutants using in vivo SUMOylation assays. Although ZNF131 contains a single consensus SUMOylation motif ψKXX(D/E) at amino acids 254–257 that is well conserved across species (supplemental Fig. S7A), the mutants deleting 242–270 or ZF1 domain or having arginine substitutions at Lys-255 (supplemental Fig. S7B) showed no significant alteration in the extent of ZNF131 SUMOylation, eliminating the possibility of ZNF131 conjugation at Lys-255 (supplemental Fig. S7B). Through the analysis of serial ZNF131 deletion mutants, we found that a ZNF131 mutant lacking C-terminal 177 amino acids (amino acids 413–589) was not SUMOylated by hPc2 (supplemental Fig. S7C). Sequence analysis revealed that this C-terminal region of ZNF131 contains three lysine sites (Fig. 6A and supplemental Fig. S7A), and the mutant having arginine mutations at Lys-443, Lys-567, and Lys-576 was not SUMOylated by hPc2 (supplemental Fig. S7B). Interestingly, the amino acid sequence surrounding lysine 567 is conserved in mammals and fits the inverted SUMOylation motif sequence reported by Matic et al. (37) (Fig. 6B). Of these
three residues, the ZNF131-K567R mutant had significantly reduced SUMOylation in cells transfected with hPc2 alone (Fig. 6C) or together with SUMO1 (Fig. 6D). Next, we examined whether lysine 567 is a major targeting site for ZNF131 SUMOylation in the resting state. When cells were transfected with ZNF131-K567R, we observed reduced mono-SUMOylation of ZNF131 using endogenous SUMOylation machinery (Fig. 6E). These data suggest that lysine 567 is the major SUMOylation site in ZNF131.

To assess the functional consequences of ZNF131 SUMOylation, we investigated whether SUMOylation affects the intranuclear localization of ZNF131. Immunostaining COS-7 cells transfected with wild type ZNF131 or SUMOylation-defective ZNF131-K567R mutant demonstrates that both constructs localize in the nucleoplasm (Fig. 6F, left panels). In addition, analysis of cells co-transfected with hPc2 and either wild type ZNF131 or the mutant ZNF131-K567R demonstrates that both ZNF131 proteins equally co-localize with hPc2 in polycomb bodies (Fig. 6F, right panels, and supplemental Fig. S8). These findings suggest that SUMOylation does not affect the intranuclear localization of ZNF131 or its co-localization with hPc2.

SUMOylation Does Not Influence ZNF131 Ubiquitination but Treatment with Proteasomal Inhibitor Stimulates ZNF131 SUMOylation—Based on the mechanistic and functional similarities between ubiquitination and SUMOylation, we examined whether SUMOylation affects the ubiquitination of ZNF131. When HEK293 cells were transfected for 24 h with HA-tagged ZNF131, immunoprecipitation analysis of ubiquitination with endogenous ubiquitin protein was not remarkable (Fig. 7A). However, treatment of the cells with the proteasomal inhibitor MG-132 significantly promoted ZNF131 ubiquitination using endogenous ubiquitin (Fig. 7A). Next, we tested whether ZNF131 ubiquitination induced by MG-132 treatment could affect ZNF131 SUMOylation, or vice versa. As shown in the top panel of Fig. 7A, ZNF131 ubiquitination induced by MG-132 was not significantly affected by co-expression with hPc2 and SUMO1, indicating that ZNF131 SUMOylation has no apparent effect on ZNF131 ubiquitination. However, treatment with the proteasomal inhibitor largely increased the mono-SUMOylation of ZNF131 using endogenous SUMO1 protein (Fig. 7A, second panel). This stimulatory effect is masked in cells transfected with SUMO1 and hPc2 due to the high level of ZNF131 SUMOylation (Fig. 7A, second and third panels). Next, we assessed whether mono-SUMOylation of ZNF131 still occurs at lysine 567 in the presence of MG-132. As shown in Fig. 7B, mono-SUMOylation of the ZNF131 protein was increased by treating cells with MG-132 (Fig. 7B). This stimulatory effect of MG-132 was virtually undetectable in cells overexpressing the ZNF131-K567R mutant. Overall, these results suggest that treatment with a proteasomal inhibitor stimulates the mono-SUMOylation of ZNF131 at lysine 567.
SUMO Modification Potentiates Negative Effect of ZNF131 on Estrogen Signaling—Next, we examined whether ZNF131 negatively regulates estrogen signaling, based on a previous report that ZNF131 inhibits ligand-dependent trans-activation by ERα (10). ERα-positive MCF-7 breast cancer cells were transfected with a luciferase reporter plasmid containing four tandem EREs in the presence or absence of HA-ZNF131. The luciferase reporter assay confirms that the ZNF131 protein significantly inhibits 17β-estradiol (E2)-induced expression of estrogen-reporter gene products (p < 0.001, Fig. 8A). However, E2 treatment did not alter the SUMOylation status of ZNF131 (data not shown). This result suggests that ZNF131 inhibits the transcriptional activity of ERα. We next investigated whether SUMOylation influences the inhibitory action of ZNF131 on estrogen signaling. As shown in Fig. 8B, co-expression with wild type SUMO1-GG potentiated the negative effect of ZNF131 on estrogen signaling (p < 0.01, Fig. 8B). This stimulatory effect was not detected in cells transfected with conjugation-defective SUMO1-AA mutant. However, ERα itself could be a target for SUMOylation, thus modulating ERα-mediated trans-activation (22). Therefore, this stimulatory effect is not conclusive evidence that ZNF131 SUMOylation modulates its inhibitory effect on estrogen signaling. But, in luciferase reporter assays in MCF cells transfected with either wild type or SUMOylation-defective ZNF131 mutant (K567R) in the presence or absence of exogenous hPc2, ZNF131 had increased negative regulation of estrogen signaling in the presence of hPc2 (p < 0.01, Fig. 8C). This stimulatory effect was not observed in cells transfected with the ZNF131-K567R mutant (Fig. 8C). In addition, the SUMOylation-defective ZNF131-K567R mutant blocked the inhibition of estrogen signaling mediated by ZNF131 (Fig. 8, D–F). Furthermore, this effect on estrogen signaling was more pronounced upon treatment with E2 for longer periods of time (Fig. 8, D and E) and with the addition of MG-132 (Fig. 8F). Overall, these data suggest that SUMO modification potentiates the inhibitory effect of ZNF131 on estrogen signaling.

We next examined the effect of ZNF131 SUMOylation on the expression of an endogenous estrogen-responsive gene, pS2, in MCF-7 cells to reflect better estrogen signaling in a physiologically relevant cellular environment. In the same way as the luciferase reporter assay, wild type ZNF131 significantly inhibits estrogen-induced expression of the pS2 gene (p < 0.01, supplemental Fig. S9), whereas this inhibitory effect was significantly reduced in cells
transfected with the ZNF131-K567R mutant (p < 0.05, supplemental Fig. S9). These data indicate that SUMO modification potentiates the inhibitory effect of ZNF131 on estrogen signaling in a physiologically relevant cellular environment.

ZNF131 SUMOylation Attenuates Estrogen-induced Cell Growth in MCF-7 Cells—Based on the previous finding that estrogen stimulates cell proliferation in MCF-7 cells (38), we examined the effect of ZNF131 and ZNF131 SUMOylation on MCF-7 cell growth rate. Although estrogen stimulates the growth rate of MCF-7 cells, this effect was greatly attenuated in the presence of exogenous ZNF131 (p < 0.01, Fig. 9). However, the SUMOylation-defective ZNF131-K567R mutant reversed the ZNF131-mediated attenuation of MCF-7 cell growth (p < 0.01, Fig. 9). This result indicates that ZNF131 SUMOylation attenuates estrogen-induced cell growth in MCF-7 cells.

FIGURE 7. SUMOylation does not influence the ubiquitination of ZNF131 but treating cells with a proteasomal inhibitor stimulates SUMOylation. A, HEK293 cells were transfected for 24 h with V5-SUMO1-GG, HA-ZNF131, or Myc-hPc2 alone or in combination. Cells were then left untreated or treated with MG-132 (20 μM) for 9 h. The cell lysates were immunoprecipitated (IP) with the HA antibody followed by Western blot with the indicated antibodies. Actin was used as a protein loading control. B, HEK293 cells were transfected for 24 h with HA-ZNF131-WT or HA-ZNF131-K567R. Cells were then left untreated or treated with MG-132 (20 μM) for 9 h, as indicated. Cell lysates were immunoprecipitated with the HA antibody followed by immunoblot with the indicated antibodies. *, unidentified non-ZNF131 band; IgHC, immunoglobulin heavy chain; Mr(K), relative molecular mass; WB, Western blot.
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trans-activation (22). Nuclear receptor co-repressor (N-CoR), another member of this pathway, is also modified by SUMO, which contributes to its transcriptional repression activity (26). Interestingly, the majority of the estrogen-ERα-ERE pathway SUMOylation targets utilize PIAS1 or PIAS3 as their SUMO E3 ligase (39). The present study reveals that the presumed ERα co-repressor ZNF131 is an additional target of covalent SUMO modification, consequently regulating its modulatory activity toward ERα. Furthermore, we demonstrate that instead of the PIAS family, the SUMO E3 ligase hPc2 significantly enhances the SUMOylation of ZNF131, suggesting that hPc2 is a unique SUMO E3 ligase for ZNF131.

In mammals, there are three SUMO isoforms: SUMO1, SUMO2, and SUMO3. Although SUMO2 and SUMO3 form polymer chains and are occasionally conjugated to their targets through poly-SUMOylation according to the existence of a SUMO consensus motif (supplemental Fig. S4), SUMO1 is predominantly conjugated to the substrate via mono-SUMOylation (40). Here we demonstrate that hPc2 efficiently promotes covalent conjugation of all three SUMO isoforms to ZNF131 (supplemental Fig. S4). Contrary to a previous report (40), our findings indicate that SUMO2 and SUMO3 conjugation to ZNF131 do not form polymer chains through poly-SUMOylation according to the existence of a SUMO consensus motif (supplemental Fig. S4). SUMO1 is predominantly conjugated to the substrate via mono-SUMOylation (40). Here we demonstrate that hPc2 efficiently promotes covalent conjugation of all three SUMO isoforms to ZNF131 (supplemental Fig. S4). Contrary to a previous report (40), our findings indicate that SUMO2 and SUMO3 conjugation to ZNF131 do not form polymer chains through poly-SUMOylation according to the existence of a SUMO consensus motif (supplemental Fig. S4). SUMO1 is predominantly conjugated to the substrate via mono-SUMOylation (40). Here we demonstrate that hPc2 efficiently promotes covalent conjugation of all three SUMO isoforms to ZNF131 (supplemental Fig. S4). Contrary to a previous report (40), our findings indicate that SUMO2 and SUMO3 conjugation to ZNF131 do not form polymer chains through poly-SUMOylation according to the existence of a SUMO consensus motif (supplemental Fig. S4). Moreover, endogenous SUMO2 and SUMO3 are conjugated to the ZNF131 at lysine 567 via mono-SUMOylation (supplemental Fig. S4). These results suggest that ZNF131 may be mono-SUMOylated at lysine 567 using either endogenous-SUMO1 or -SUMO2/3 in physiological conditions. However, overexpression of SUMO protein generated di- and mono-conjugates as well.

FIGURE 8. SUMO modification potentiates the negative effect of ZNF131 on estrogen signaling. A, MCF-7 cells were transfected for 24 h with either pTA-Luc or pTA-4xERE-Luc alone or together with pTA-4xERE-Luc plus HA-ZNF131, as indicated. After cells were treated for 6 h with vehicle or E2 (10 nM), cell extracts were subjected to luciferase assays. B, MCF-7 cells were transfected for 24 h with pTA-4xERE-Luc plasmid, HA-ZNF131-1WT, HA-ZNF131-K567R, or Myc-hPc2 alone or in combination. Cells were then treated with E2 (10 nM) for 6 h and luciferase assays were performed with cell extracts. Inset, relative differences of luciferase activities were calculated from cells with (a) ZNF131-1WT alone versus ZNF131-1WT plus hPc2, and (b) ZNF131-K567R alone versus ZNF131-K567R plus hPc2. D-F, MCF-7 cells were transfected for 24 h with pTA-4xERE-Luc plasmid alone or together with HA-ZNF131-1WT or HA-ZNF131-1K567R. After cells were treated with E2 (10 nM) for the indicated times (D and E) or MG-132 (20 μM) for 9 h followed by E2 (10 nM) for 6 h (F), cell extracts were subjected to luciferase assays. All error bars represent mean ± S.D.; n.s., not significant; **, p < 0.01; ***, p < 0.001.

FIGURE 9. ZNF131 SUMOylation attenuates estrogen-induced cell growth in MCF-7 cells. MCF-7 cells were transfected for 24 h with either ZNF131-1WT or ZNF131-K567R, and treated with vehicle or E2 (10 nM) for the indicated times. Cell growth was determined by counting cell number using Cell Counting Kit-8. Error bars represent the mean ± S.D. **, p < 0.01.

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FIGURE 9. ZNF131 SUMOylation attenuates estrogen-induced cell growth in MCF-7 cells. MCF-7 cells were transfected for 24 h with either ZNF131-1WT or ZNF131-K567R, and treated with vehicle or E2 (10 nM) for the indicated times. Cell growth was determined by counting cell number using Cell Counting Kit-8. Error bars represent the mean ± S.D. **, p < 0.01.
Growing evidence indicates that SUMOylation of proteins increases in response to diverse cellular stresses. For example, there is a large pool of free SUMO2/3 in resting COS-7 cells (41) but a marked increase in SUMO2/3 conjugation occurs following heat stress. Similarly, oxidative, ethanol, and osmotic stresses cause a rapid increase in global SUMO conjugation (41). Stress-dependent increases in SUMO conjugation have been reported in various cellular systems including oxygen/glucose deprivation and hypothermia in neurons (42, 43). However, despite extensive effort, we failed to find the exact physiological condition or diverse cellular stress that induces the SUMOylation of ZNF131. It could be that, notwithstanding enhanced global protein SUMOylation during cellular stress, a specific target or a set of substrates are differentially modified. For example, although there is a net increase in SUMOylation following heat shock, the SUMOylation of some substrates is unaffected and even decreases for others (44). Moreover, differential sensitivity to deSUMOylating proteases contributes to the target-specific response for cellular stress (45).

Many proteins are substrates for both SUMO and ubiquitin, often at the same lysine residue, which suggests that SUMO and ubiquitin may compete with each other. Previous studies reveal that in many cases SUMO and ubiquitin are conjugated either sequentially or simultaneously to functionally regulate the substrate protein (11, 46, 47). For example, the RING domain E3 ligase MDM2 induces p53 ubiquitination, which recruits the SUMO E3 ligase PIASy, and consequently promotes SUMOylation of p53 (48). Similar to many other targets, ZNF131 is a target of ubiquitination as well as SUMOylation.

In addition to co-regulating a single substrate protein, ubiquitination and SUMOylation can directly regulate each other by modifying components of their respective enzymatic machinery (11). For example, SUMOylation of the ubiquitin E2 enzyme E2–25k inhibits its ability to conjugate ubiquitin (49). Interestingly, SUMOylation of ZNF131 does not significantly affect the ubiquitination pattern of ZNF131, but treatment with the proteasomal inhibitor MG-132 markedly induces SUMOylation of ZNF131 in the absence of exogenously added SUMO1 or hPc2 (Fig. 7). Based on the finding the mono-SUMOylation of endogenous ZNF131 still occurs in the absence of exogenous hPc2 (Fig. 2B), these data suggest that hPc2 promotes the SUMOylation of ZNF131 at resting state. Accumulation of ubiquitinated ZNF131 (as when the proteasome is blocked by MG–132) might recruit a SUMO E3 ligase, specifically hPc2, which then promotes ZNF131 SUMOylation. We also hypothesize that unspecified ZNF131-ubiquitination pathway(s) are closely linked to subsequent SUMOylation, which occurs mainly through the action of hPc2 and consequently regulates the extent of ZNF131 SUMOylation.

hPc2 is a member of the polycomb repressive complex 1 (PRC1) and localizes to polycomb bodies within the nucleus (19). ZNF131 co-localizes with hPc2 in polycomb bodies in a SIM1- and C-box-dependent manner. The SUMO E3 ligase activity of hPc2 on ZNF131 is also dependent on the proper localization of hPc2 to polycomb bodies. These results suggest that ZNF131 may be a potential component of PRC1, which controls global gene silencing (50). Further characterization of ZNF131 as a member of PRC1 would provide crucial information regarding the physiological role of ZNF131. Interestingly, we found that two other POZ-ZF proteins, ZBTB16 (PLZF) and ZBTB17 (MIZ1), also interact with hPc2 in yeast cells (data not shown). Due to this finding, we speculate that hPc2 may act as a scaffold protein at polycomb bodies to recruit various target POZ-ZF transcriptional regulator proteins.

Although ZNF131 was previously reported to inhibit ligand-dependent trans-activation by ERα (10), its molecular and regulatory mechanisms have not yet been characterized. Our results confirm that ZNF131 inhibits the transcriptional activity of ERα. Furthermore, we demonstrate that SUMO modification of ZNF131 modulates its inhibitory effect on estrogen signaling. Similarly, another ERα co-repressor, KLF4, also suppresses estrogen signaling by inhibiting the transcriptional activity of ERα via direct interaction (51). Moreover, KLF4 is found to be SUMOylated at a single lysine residue (24). Interestingly, we found that ZNF131 directly binds to ERα and consequently inhibits its homodimerization,3 an effect that may underlie the activity of ZNF131 to act as an ERα co-repressor. We also speculate that SUMOylation of ZNF131 may potentiate the binding affinity of ZNF131 to ERα, which needs to be verified by further experiments. Recently, Donaldson et al. (52) reported that ZNF131 directly binds the ZNF131-binding element via its zinc finger domain in vitro and acts as a transcriptional activator of an artificial ZNF131-binding element promoter reporter. Because an additional and putative function of ZNF131 is a transcriptional activator, it would also be interesting to test whether SUMOylation may also affect this activity of ZNF131.

We have shown that SUMOylation of ZNF131 attenuates estrogen-induced cell growth in MCF-7 cells. The MCF-7 breast cancer cell line is widely used as a model of hormone-dependent and ERα-positive breast cancer. Moreover, the growth stimulatory effects of estrogen and ERα have been closely linked to many types of breast cancers (6, 7). Therefore, the data suggest the importance of ZNF131 SUMOylation during estrogen-mediated carcinogenesis in breast cancer. Consistent with our findings, analysis of a microarray data base using the Genevestigator program identified that ZNF131 and hPc2 are repressed in metastatic breast cancer cells as compared with primary breast cancer cells (ZNF131, −1.13; hPc2, −1.07; data are represented as log2 signal ratio). This digital Northern analysis suggests that ZNF131 and hPc2 are implicated in breast cancer progression. Taken together, these data demonstrate that estrogen signaling could be down-regulated through SUMOylation of the ERα-co-repressor protein ZNF131.

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3 Y. Oh and K. C. Chung, unpublished data.
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