Sumoylation-dependent Control of Homotypic and Heterotypic Synergy by the Krüppel-type Zinc Finger Protein ZBP-89

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The Krüppel-like transcription factor ZBP-89 is a sequence-specific regulator that plays key roles in cellular growth and differentiation especially in endodermal and germ cell lineages. ZBP-89 shares with other members of the Sp-like family an overlapping sequence specificity for GC-rich sequences in the regulatory regions of multiple genes. Defining the mechanisms that govern the intrinsic function of ZBP-89 as well as its competitive and non-competitive functional interactions with other regulators is central to understand how ZBP-89 exerts its biological functions. We now describe that post-translational modification of ZBP-89 by multiple small ubiquitin-like modifier (SUMO) isoforms occurs at two conserved synergy control (SC) motifs flanking the DNA binding domain. Functionally sumoylation did not directly alter the ability of ZBP-89 to compete with other Sp-like factors from individual sites. At promoters bearing multiple response elements, however, this modification inhibited the functional cooperation between ZBP-89 and Sp1. Analysis of the properties of ZBP-89 in cellular contexts devoid of competing factors indicated that although on its own it behaves as a modest activator it potently synergizes with heterologous activators such as the glucocorticoid receptor. Notably we found that when conjugated to ZBP-89, SUMO exerts a strong inhibitory effect on such synergistic interactions through a critical conserved functional surface. By regulating higher order functional interactions, sumoylation provides a reversible post-translational mechanism to control the activity of ZBP-89.

Eukaryotic ZBP-89² (also known as BERF-1, BFCOL1, or ZNF148) is a widely expressed Krüppel-type transcription factor that harbors an N-terminal cluster of four (three canonical C2H2 and a variant C2H2) zinc finger motifs. In vivo experiments indicate that ZBP-89 plays important roles in the generation and maintenance of specific cell lineages because haploinsufficiency causes infertility in mice due to apoptosis of germ cells (1), whereas knockdown of ZBP-89 in zebrafish disrupts early steps in hematopoiesis (2). ZBP-89 also appears to play important roles in the gastrointestinal tract because mice exclusively expressing a splice variant lacking the first 127 residues display increased sensitivity to colitis (3), whereas intestinal overexpression of ZBP-89 activates apoptosis and mitigates neoplasia burden in ApcMin/+ mice (4). Functionally, ZBP-89 has been most often linked to transcriptional inhibition of genes such as those for gastrin, vimentin, or ornithine decarboxylase (ODC) (5–10). ZBP-89 shares with Sp1 and other Sp-like factors the ability to recognize GC-rich sequences in target genes. This overlapping DNA recognition has led to a competitive model of inhibition in which ZBP-89 represses gene transcription by displacing proteins such as Sp1 or Sp3 (8, 11). Thus, an analysis of the proximal ODC gene promoter revealed that Sp1 and ZBP-89 bind to GC elements in a mutually exclusive manner (6). In other cases, ZBP-89 appears to inhibit by binding to DNA independently of Sp1 (12). In addition to its role in transcriptional inhibition, ZBP-89 has been implicated as a modest positive regulator of a set of genes including p21, lymphocyte-specific protein-tyrosine kinase (lck), and stromelysin (13–15). In the case of the p21 promoter, recruitment of the coactivator p300 appears to contribute to activation of this gene (16). The overlap in DNA binding specificity with widely expressed Sp-like factors, however, makes the mechanistic analysis of ZBP-89 function particularly challenging. Broad dissection of ZBP-89 suggests that distinct regions are involved in negative and positive regulation with repressive and transactivating functions in the N-terminal and C-terminal regions, respectively (17, 18). How these still poorly defined functions are selectively utilized in different contexts and how ZBP-89 engages in competitive and non-competitive functional interactions with other factors remain poorly understood.

An emerging mechanism to regulate higher order interactions among transcription factors involves the function of synergy control (SC) motifs (19). These short regulatory sequences, which we first identified in the glucocorticoid receptor (19), are present in multiple factors across a wide range of families. SC motifs are distinct from transcriptional activation functions and often map to negative regulatory regions. Functionally SC motifs limit the synergistic transcriptional output from complexes assembled at multiple response elements with-
out altering the activity of a regulator from a single site (19–21). Mechanistically, we and others have demonstrated that SC motifs operate in an autonomous manner by serving as sites for reversible post-translational modification by members of the small ubiquitin-like modifier (SUMO) family of proteins (20–23).

The mammalian SUMO family consists of four genes designated SUMO1–4. SUMO2 and -3 are closely related, whereas SUMO1 shares ~48% identity to either SUMO2 or -3 (24, 25). A more recently identified gene encodes a fourth isoform very similar to SUMO2/3 (26). SUMO proteins are structurally related to ubiquitin, and the analogous SUMO modification pathway is carried out by a distinct, SUMO-specific set of enzymes. Following translation, processing by SUMO proteases removes C-terminal residues in SUMO to expose a conserved diglycine motif (27). Notably SUMO4 harbors a Pro residue at position 90 that prevents initial processing by known SUMO protease enzymes and subsequent conjugation (28). Whether this member functions through non-covalent interactions only remains to be determined. After this initial cleavage, SUMO is then activated in an ATP-dependent manner by the heterodimeric E1-activating enzyme SAE1/SAE2. The thioester-linked SUMO is then transferred to the SUMO-specific E2-conjugating enzyme Ubc9, which in turn recognizes specific substrates and catalyzes the formation of an isopeptide bond between SUMO and the target lysine. This step is facilitated by SUMO E3 ligases such as RanBP2 and members of the protein inhibitor of activated STAT (signal transducers and activators of transcription) family (29–31). Finally, SUMO, conjugation is reversed through the isopeptide activity of SUMO-specific proteases (32). Recent functional and structural analysis by our group (33) indicates that once conjugated, individual SUMO isoforms mediate their distinct effects through a conserved surface that interacts with Val/Leu-rich motifs in target proteins (34–37). The nature of the SUMO-interacting proteins responsible for SUMO-dependent transcriptional inhibition remains poorly defined but may include histone deacetylases and DAXX (34–37). The nature of the SUMO-interacting proteins responsible for this regulatory mechanism on factors that are generically referred to as Sp-like factors remains largely unexplored.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids—**An N-terminal, hexahistidine-tagged, cytomegalovirus-driven human ZBP-89 expression plasmid was generated in two steps. Oligonucleotides 5′-CTAGC-GGCACCATGAGCATCACCCATTACATCG-3′ and 5′-GATTCATTGATGTGGTATGCTTCCATGTTGG-CCG-3′, which contain a starting ATG codon within a consensus Kozak sequence followed by a hexahistidine tag, were annealed and ligated into the BamHI and Nhel sites of pcDNA3.1(+) to yield pcDNA3.1(+) His. Human ZBP-89 cDNA was then PCR-amplified using forward primer 5′-GATGGATCATGACATTGAGCACAATCAGGAAGG-3′ and reverse primer 5′-CATCTCGAGTTAGCCAAAACGTCTGGCAGTGGTG-3′. The resulting fragment was digested with BamHI and Xhol and ligated into the same sites of pcDNA3.1(+) His to yield pcDNA3.1(+) His-ZBP-89. Single and double point mutations that replaced Lys-115 and -356 with Arg were prepared using the QuikChange™ site-directed mutagenesis approach using pcDNA3.1(+) His-ZBP-89 as a template. N-terminal HA-tagged ZBP-89 expression plasmids were generated by ligating the 3092-bp BamHI/Xbal fragment from pcDNA3.1(+) His-ZBP-89 into the same sites of pcDNA3.1(+) HA (23). The expression vector for the non-cleavable fusion of HA-SUMO1 at the N terminus of ZBP-89 K115R/K356R was generated through excision of the HA-SUMO portion from pcDNA3 HA-SUMO1(−Gly) Gal4 (23) as an Nhel/BamHI fragment and ligation into the same sites of pcDNA3.1(+) His-ZBP-89 K115R/K356R. Fusions to HA-SUMO2, HA-SUMO2 K33E/K42E, and HA-SUMO3 were generated by ligating the 3092-bp BamHI/Xbal fragment from pcDNA3.1(+) His-ZBP-89 into the same sites of pcDNA3.1(+) HA-SUMO(−Gly) Gal4 vectors (23, 33). The Rous sarcoma virus long terminal repeat-drivers as transcription factors (p6RGR) and WT rat GR (p6RGR) expression vectors as well as the cytomegalovirus-driven (pcDNA3) expression vectors for HA-tagged SUMO1, SUMO2, and SUMO3 have been described previously (23, 39).

**PCR**-β-gal is a cytomegalovirus-driven β-galactosidase expression vector and was used to correct for transfection efficiency.

To generate ZBP-89 *Drosophila* expression plasmids driven by the actin 5C promoter, two oligonucleotides, 5′-GATCAGCTAGGCTATTCTCCTAGAA-3′ and 5′-AGCTTCTGAGAATTCTGACT-3′, containing Nhel and Xbal restriction sites were annealed and ligated into the HindIII and BamHI sites of pA5C (kindly supplied by Dr. Thomas Kobr, University of California, San Francisco) to yield pA5C®. Nhel/Xbal fragments containing its tagged WT or mutant ZBP-89 wereexcised from the corresponding pcDNA3.1(+) His-ZBP-89 plasmids and ligated into the same sites of pA5C® to yield pA5C® His-ZBP-89 and pA5C® His-ZBP-89 K115R/K356R, respectively. Non-cleavable fusions of HA-SUMO isoforms at the N terminus of ZBP-89 K115R/K356R were generated by insertion of Nhel/BamHI fragments exactly as described above for the mammalian vectors. The *Drosophila* vector for expression of rat GR (pA5C® GR) was generated by transferring the GR coding region (as a BamHI fragment) from p6RGR into the BamHI site of pA5C®. pPac Sp1 (40) and pActin β-gal (a gift from Dr. Ken Cadigan, University of Michigan) are
For in vivo sumoylation in AGS cells, 2 × 10⁶ cells were seeded in 10-cm plates and transiently transfected using the calcium phosphate method with the constructs indicated in the figure legends. 40 h post-transfection, cells were lysed for 10 min on ice with 0.75 ml of high salt lysis buffer (20 mM Hepes, pH 7.5, 5 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 400 mM NaCl, 20 mM N-ethylmaleimide, and EDTA-free protease inhibitors (one tablet/10 ml of buffer; Roche Applied Science). After 5 min, N-ethylmaleimide was quenched by addition of dithiothreitol to 40 μM final concentration and a further 2-min incubation. 5% of the sample was reserved for further analysis, and the remaining cell extracts were incubated with monoclonal HA-11 antibody (Covance, Berkeley, CA) and protein A-agarose (Invitrogen) at 4 °C overnight. The immunoprecipitates were washed four times in low salt lysis buffer (high salt lysis buffer containing 200 mM NaCl) and eluted in 2 × Laemmli sample buffer. Samples were resolved by 7.5% SDS-PAGE and processed for immunoblotting as described below.

For functional assays, AGS cells (2 × 10⁴) were seeded (0.5 ml) onto 24-well plates. Cells were transfected 24 h later with the indicated amounts of ZBP-89 expression plasmids. To control for promoter dosage effects, cells treated with submaximal amounts of ZBP-89 expression vector also received the required amount of empty expression vector to maintain equimolar doses of expression plasmid. Cells also received 25 ng of the indicated luciferase reporter plasmid and 25 ng of pCMV β-gal. For Fig. 5D, cells also received 20 ng of p6RGR expression plasmid. In all cases, the total amount of DNA was supplemented to 0.3 μg/well with pBSKS (+). For experiments including GR, medium was supplemented 16 h post-transfection with 10 nM dexamethasone (Dex) or vehicle (0.1% ethanol). Cells were lysed 36 h after transfection, and luciferase and β-galactosidase activities were determined as described previously (39). Functional assays in S2 cells were carried out by seeding in 24-well plates (8 × 10⁴ cells/well, 0.5 ml) followed 24 h later by transfection with the amounts of expression plasmids indicated in the figure legends. All conditions contained equimolar amounts of each type of expression vector to control for promoter dosage effects. Cells also received 50 ng of the indicated luciferase reporter plasmid and 50 ng of pActin β-gal. For the GR experiments, transfections included 100 ng of pA5C GR, and cells were stimulated with 100 nM Dex for the last 24 h. The total amount of DNA was supplemented to 0.3 μg/well with pBSKS (+). Cells were harvested 48 h post-transfection and processed as described above.

Immunoblotting—For the in vivo sumoylation experiments, immunoblots were probed with primary mouse anti-tetra-His (Qiagen), rabbit anti-ZBP-89 antiseraum, or HA-11 (Covance) monoclonal antibodies followed by goat anti-mouse or donkey anti-rabbit IgG peroxidase-conjugated (Bio-Rad) secondary antibodies. ZBP-89 and GR expression levels were confirmed by Western blotting. Cells were transfected as described for the functional assays and lysed in 1 × SDS-PAGE sample buffer containing 150 mM NaCl 36 h after transfection, resolved by 7.5% SDS-PAGE, and processed for immunoblotting. Mammalian transfection samples were probed with HA-11 (Covance) monoclonal antibody followed by goat anti-mouse IgG-peroxidase conjugate (Bio-Rad) as secondary antibody. For transfec-

expression plasmids for Sp1 and β-galactosidase, respectively, and are driven by the actin 5C promoter.

Reporter Plasmids—The pΔODLO 02 reporter plasmid in which a minimal Drosophila distal alcohol dehydrogenase promoter (−33 to +55) drives the luciferase gene has been described (20). Luciferase reporter plasmids bearing one or more ZBP binding sites were generated as follows. The oligonucleotides 5′-GAGCCCCGCCCCCTCCCCCGCA-3′ and 5′-GATCCTGAGAATGGGAGCGG-3′ containing a single ZBP-89 binding site from the ODC promoter (−114 to −100) (6) were annealed and ligated into the BamHI and BglII sites of pΔODLO 02 to yield pΔ(ODC)₃-Luc. Similar ligation of oligonucleotides 5′-GATCCTGTAACAGGATGTTCA-3′ and 5′-GATCTAGAACATCCTGTACAG-3′ bearing a glucocorticoid response element from the tyrosine aminotransferase gene (41) yielded pΔ(TAT)₃-Luc. Reporter plasmids containing multiple ZBP-89 or GR binding sites were generated as follows. Ligation of independent BseRI/BglII and BamHI/BseRI fragments of pΔ(ODC)₃-Luc yielded pΔ(ODC)₅-Luc. The same operation applied to pΔ(ODC)₅-Luc and pΔ(TAT)₃-Luc yielded pΔ(ODC)₈-Luc and pΔ(TAT)₅-Luc. Ligation of BseRI/ BglII and BamHI/BseRI fragments of pΔ(ODC)₈-Luc and pΔ(ODC)₅-Luc, respectively, yielded pΔ(ODC)₁₀-Luc. The reporter plasmid bearing one ZBP-89 site upstream of a single glucocorticoid response element (pΔ(ODC)₅(TAT)₃-Luc) was generated by ligation of BseRI/BglII and BamHI/BseRI fragments from pΔ(ODC)₅-Luc and pΔ(TAT)₅-Luc, respectively. The same strategy using pΔ(ODC)₈-Luc and pΔ(TAT)₅-Luc yielded pΔ(ODC)₁₀(TAT)₅-Luc. The pODC-Luc reporter plasmid harbors the region from −400 to +79 of the human ornithine decarboxylase gene in the pGL2 plasmid (Promega, Madison, WI).

Cell Culture and Transfections—Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% fetal bovine serum. Human AGS gastric carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Drosophila melanogaster S2 cells were grown in Schneider’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum at 25 °C. Except for data in Fig. 2B, all cell lines were transfected by using Lipofectamine™ and plus™ reagent (Invitrogen). For in vivo sumoylation experiments, 293T cells (2 × 10⁶) were seeded onto 10-cm plates. After 24 h, cells were co-transfected with 3 μg of His-tagged ZBP-89 expression plasmids and 7 μg of HA-SUMO expression plasmids or empty vector as indicated in Fig. 3. Cells were harvested 24 h after transfection in 0.75 ml of urea lysis buffer (8 M urea, 0.5 M NaCl, 45 mM Na₂HPO₄, 5 mM NaH₂PO₄, 10 mM imidazole, pH 8.0) and sonicated. Lysates were incubated with 0.1 ml of 50% nickel-nitritolriacetic acid-agarose (Qiagen) equilibrated in lysis buffer for 1 h at room temperature with rocking. The resin was then washed three times with 10 bed volumes of wash buffer 1 (8 M urea, 0.4 M NaCl, 17.6 mM Na₂HPO₄, 32.4 mM NaH₂PO₄, 10 mM imidazole, pH 6.75) and two times with wash buffer 2 (buffer 1 with 150 mM NaCl and no urea). Proteins were eluted by boiling in 50 μl of 2 × Laemmli sample buffer, resolved by 7.5% SDS-PAGE, and processed for immunoblotting as described below.
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tions in Drosophila cells, ZBP-89 and derivatives were detected with an anti-ZBP-89 rabbit serum followed by secondary goat anti-rabbit IgG-peroxidase conjugate (Bio-Rad). GR was detected using BuGR2 (Covance) primary and goat anti-mouse IgG peroxidase-conjugated secondary antibodies. Images were captured in a Kodak Image Station 440 CF using Super Signal West Femto substrates (Pierce). All the experiments were performed at least thrice with similar results.

Protein-Protein Interaction and in Vitro SUMO Conjugation Assays—ZBP-89 forms or luciferase was synthesized in vitro using the T7-Tnt Quick®TM Coupled Transcription-Translation system (Promega) in the presence of [35S]methionine using pcDNA3.1(+)-His-ZBP-89 or luciferase plasmids as templates. Binding reactions (50 µl) were performed at 4 °C and contained 1.2 nmol of GST or GST-Ubc9 fusion protein (20) bound to 20 µl of glutathione-Sepharose 4B (Amersham Biosciences) in binding buffer (50 mM NaCl and 1 mg/ml bovine serum albumin) and 10 µl of radiolabeled proteins. After a 1-h incubation with rocking, the resin was washed four times with 0.1% Nonidet P-40 in phosphate-buffered saline. Samples were eluted by boiling in 40 µl of SDS-PAGE sample buffer and resolved by 7.5% SDS-PAGE, and radioactive proteins were visualized using a PhosphorImager screen (Amersham Biosciences).

Sumoylation reactions were carried out in 20 µl of 50 mM Tris (pH 7.5), 5 mM MgCl2 and in the presence (as indicated) of 1 µg of GST-SAE2/SAE1, 5 µg of GST-Ubc9, 5 µg of His-SUMO-1GG (20), and 5 µl of in vitro translated 35S-labeled His-ZBP-89 WT, its sumoylation-deficient mutants, or luciferase as indicated. Reactions were initiated by the addition of an ATP regeneration system (final concentrations: 10 units/ml creatine kinase, 25 mM phosphocreatine, 5 mM ATP) and pyrophosphatase (0.6 units/ml final concentration). Samples were incubated at 30 °C for 90 min, and reactions were terminated by addition of 20 µl of disruption buffer (50 mM Tris-HCl, pH 6.8, 1.67% SDS, 10% glycerol, 0.24 mM β-mercaptoethanol, 0.015% bromphenol blue) and boiling for 5 min. Samples were resolved by 7.5% SDS-PAGE, and radioactive proteins were visualized as described.

RESULTS

ZBP-89 Interacts with Ubc9 and Is a Target of Sumoylation at Two Conserved SC Motifs—Bioinformatics analysis of ZBP-89 revealed the presence of two sequences resembling synergy control motifs located in the N-terminal half of the protein surrounding the zinc finger DNA binding domain (Fig. 1). Analysis of the sequence conservation in the SC motif regions showed that the cardinal features of SC motifs, including flanking proline residues, are conserved across vertebrate species, including distant teleost fish. In contrast, surrounding sequences and the third and non-essential position of the SC motif are more variable, suggesting the presence of a selective pressure for the conservation of the SC motifs and their associated functions. SC motifs serve as sites for sumoylation through their direct interaction with Ubc9. Notably we recently isolated Ubc9 in a yeast two-hybrid screen using ZBP-89 as bait (data not shown). To confirm this interaction, we carried out in vitro interaction assays using immobilized GST-Ubc9 fusion protein as an affinity resin. As can be seen in Fig. 2A, in vitro transcribed and translated ZBP-89 was efficiently retained on this matrix, but no binding was observed to an equimolar amount of GST. In contrast, firefly luciferase, which lacks sequences resembling SC motifs, did not bind to either matrix. Consistent with the known binding properties of Ubc9, replacement of the lysine residues within the SC motifs by arginines (ZBP-89 mut) did not interfere with this interaction (Fig. 2B).

The identification of Ubc9 as an interaction partner of ZBP-89 coupled with the presence of well conserved synergy control motifs within its sequence strongly suggested that this factor is likely to be subject to SUMO modification. To examine this possibility directly, we transiently transfected human AGS gastric carcinoma cells with expression vectors for HA-tagged SUMO3 and ZBP-89 (Fig. 2). Immunoblotting of cell extracts with the anti-ZBP-89 antibody demonstrated the expression of both endogenous as well as exogenous ZBP-89 (middle panel). Similarly probing with an anti-HA antibody demonstrated the incorporation of SUMO3 into cellular proteins (lower panel). Analysis of HA immunoprecipitates from cells expressing both ZBP-89 and HA-SUMO3 revealed the presence of an ~160-kDa slower mobility form in addition to smaller amounts of unmodified ZBP-89 (Fig. 2B, top panel). The upper band is consistent with SUMO modification, whereas the unmodified material probably is derived from dimerization of modified and non-modified forms of ZBP-89. Notably small amounts of a similar slow migrating species were also detected from cells expressing exclusively HA-SUMO3, suggesting that endogenous ZBP-89 is also modified. To confirm and characterize the
sumoylation of ZBP-89, we expressed N-terminally His-tagged WT ZBP-89 and HA-tagged SUMO1, SUMO2, or SUMO3 in HEK 293T cells. Cells were lysed under denaturing conditions (8 M urea) in an effort to preserve the sumoylation status of cellular proteins and to disrupt non-covalent interactions. The cleared lysates were subjected to Ni\(^{2+}\) chelate chromatography to isolate His-tagged ZBP-89, and samples were resolved by SDS-PAGE and analyzed by Western blotting. As can be seen in Fig. 3A, co-expression of ZBP-89 with any of the three SUMO isoforms resulted in the appearance of an ~160-kDa HA-immunoreactive band. This species was not observed when either SUMO or ZBP-89 were expressed alone and is indicative of a SUMO-modified form of ZBP-89. As in the case of other transcription factors (27, 42), the stoichiometry of modification appears to be relatively low because the SUMO-modified form was difficult to detect with the ZBP-89-directed antibodies we examined. Although we have not analyzed the linkage topology, the large apparent size of this form may be consistent with a single SUMO modification because branched SUMO-modified proteins often run anomalously in SDS-PAGE. Moreover ZBP-89 may be particularly prone to such anomalous behavior because the full-length protein migrates substantially slower than the predicted 89 kDa (Fig. 3B). Analysis of crude cell lysates revealed that SUMO conjugates as well as free SUMO accumulate to substantially higher levels in the case of SUMO2 and -3 when compared with SUMO1 (see supplemental Fig. 1 and Ref. 20). Nevertheless, comparable levels of ZBP-89 sumoylation were observed with all three SUMO isoforms. Whether this reflects a relative preference for SUMO1 modification of ZBP-89 remains to be explored in more detail. To examine the role of the putative SC motifs in ZBP-89 sumoylation, samples were isolated from cells expressing ZBP-89 mutants bearing a Lys to Arg substitution in either the first (K115R), second (K356R), or both SC motifs (K115R/K356R). As can be seen in Fig. 3A, each substitution did not substantially reduce the ~160-kDa form, whereas the double mutant essentially eliminated it. Probing of the same samples with an anti-His-tag antibody (Fig. 3B) indicated that neither the expression of SUMO...
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isoforms nor the presence of the mutations alter the expression or recovery of ZBP-89. Taken together, the results strongly support the idea that in vivo ZBP-89 is a target of SUMO modification, that all three SUMO isoforms can be conjugated to ZBP-89, and that the main sites of modification correspond to the proposed SC motifs.

To further investigate the mechanism of ZBP-89 SUMO modification, we used a reconstituted in vitro sumoylation assay consisting of purified recombinant SUMO1 as well as E1 (SAE1/SAE2) and E2 (Ubc9) enzymes. Sumoylation targets consisted of in vitro translated 35S-labeled proteins. As in the in vivo experiments, in vitro synthesized His-tagged ZBP-89 migrated with an apparent molecular mass of \( \sim 110 \) kDa (Fig. 4A). Incubation with all sumoylation machinery components led to the generation of slower migrating bands of \( \sim 160 \) and \( 220 \) kDa consistent with mono- and diSUMOylated forms of ZBP-89. Omission of the E1 or E2 enzymes or SUMO itself during the reaction prevented the appearance of the slowly migrating species (Fig. 4A), indicating that they represent specific covalent modification of ZBP-89 by SUMO1. In contrast, in vitro transcribed firefly luciferase, which lacks sequences related to SC motifs, did not serve as substrate (Fig. 4A, right). Similar results were obtained with SUMO2 (not shown).

In contrast to WT ZBP-89 and in agreement with the in vivo experiments, the higher molecular weight species were absent in the case of the K115R/K356R mutant in which both potential sumoylation sites are disrupted (Fig. 4B). Interestingly the Lys to Arg substitution at position 115 produced a more significant reduction in sumoylation than that observed with the substitution at position 356, suggesting a preferential targeting of Lys-115 under our in vitro conditions. This mild bias, however, was not detected in the in vivo experiments. Taken together the above results argue strongly that the lysine residues within the first and second SC motifs of ZBP-89 (Lys-115 and Lys-356) serve as the main sites for conjugation of all three SUMO isoforms both in vivo and in vitro.

The SC Motifs in ZBP-89 Exert a SUMO-dependent Inhibitory Effect Only at Compound Response Elements—Given our previous characterization of SC motifs in other factors, we anticipated that the functional effects of the SC motifs in ZBP-89 would be restricted to contexts where multiple instances of one or more SUMOylated factors are recruited to independent sites on DNA (19, 23). To determine the functional role of the SC motifs in ZBP-89, we generated HA-tagged expression vectors for the K115R/K356R double mutant (ZBP-89 mut) as well as for a non-cleavable fusion between SUMO1 and the ZBP-89 mutant lacking its own sumoylation sites (SUMO1-ZBP-89 mut). The former plasmid generates a ZBP-89 form with disabled SC motifs (due to the loss of SUMOylation), whereas the latter mimics a persistently SUMOylated form of ZBP-89. The fusion strategy is particularly useful because it allows for selective recruitment of SUMO to a specific factor without interfering with the endogenous complement of SUMO-modified proteins. Moreover in the cases where it has been examined in detail, the fusions accurately recapitulate the principal effects of sumoylation (23). We first compared the activity of the sumoylation mutant and SUMO-fused forms to that of WT ZBP-89 at reporter genes bearing either one or three copies of a GC site from the ODC gene (6). Sp1 and ZBP-89 bind this element in a mutually exclusive manner, and ZBP-89 antagonizes the effects of Sp1 through a displacement mechanism (6). In human AGS gastric carcinoma cells, these reporters displayed substantial basal activity compared with a control reporter lacking ODC sequences (Fig. 5, A and B). This activity is mainly attributable to endogenous Sp1 (6). Transfection of increasing doses of expression plasmid encoding ZBP-89 led to a dose-dependent inhibition of both promoters. At the reporter containing a single ZBP-89 binding site (Fig. 5A) where presumably only one protein can be present at a time, the sumoylation mutant and the SUMO fusion displayed activities comparable to WT ZBP-89, indicating that sumoylation does not appear to influence the ability to bind the response element and displace endogenous Sp1-like factors. The comparable activities are also consistent with the equivalent expression levels of the three forms (Fig. 5, inset). At the reporter bearing three copies of the element (Fig. 5B), however, we consistently observed that at intermediate doses the sumoylation-deficient ZBP-89 was a weaker inhibitor, whereas the
SUMO1-fused ZBP-89 was a stronger inhibitor compared with WT ZBP-89. Given that the intact proximal promoter of the ODC gene harbors multiple GC-rich elements, we anticipated that ZBP-89 sumoylation would also influence transcriptional inhibition at the native promoter. As seen in Fig. 5C, this appears to be the case because the inhibition pattern is similar to that observed at the simpler (ODC)1 reporter. In addition, we obtained similar results in the case of the proximal gastrin promoter (not shown). Given that at both the natural and (ODC)3 reporters Sp1-like factors and ZBP-89 can be recruited simultaneously through occupancy of independent sites, the data indicate that sumoylation of ZBP-89 exerts a negative influence on the transcription complexes that assemble at compound response elements.

If ZBP-89 sumoylation acts to inhibit mixed or heterotypic complexes, this modification should also influence the activity of unrelated activators bound nearby. To examine this point, we took advantage of the well described synergistic cooperation between CACCC box binding factors such as Sp1 and the glucocorticoid receptor (43, 44). As can be seen in Fig. 5D, the activity of a target gene bearing a single ODC element upstream of a single glucocorticoid response element from the tyrosine aminotransferase gene ((ODC)1(TAT)1) was substantially higher upon activation of the glucocorticoid receptor with dexamethasone than in its absence (endogenous Sp-like factors alone). The activity was also higher when compared with a reporter bearing only a single tyrosine aminotransferase element (GR activity alone, data not shown). Expression of ZBP-89 in this context resulted in a dose-dependent inhibition of activity. Notably, the sumoylation-deficient ZBP-89 was a weaker inhibitor as evidenced by a rightward shift in the dose response. This difference is due to loss of sumoylation because fusion of SUMO1 to the ZBP-89 mutant restored its inhibitory potential. A similar pattern, with slightly more pronounced differences, was observed at a reporter bearing two copies of each response element (not shown). Taken together, the above results indicate that sumoylation of ZBP-89 modulates its ability to regulate transcription in mammalian cells mainly by altering cooperation with other factors. Thus, at compound response elements, complexes including SUMOylated ZBP-89 are less conducive to transcriptional activation.

ZBP-89 Antagonizes Sp1 through Displacement and Sumoylation-dependent Inhibition of Synergy at Compound Response Elements in Drosophila S2 Cells—As mentioned above, the effects of ZBP-89 in mammalian cells likely depend on both its intrinsic transcriptional properties as well as its ability to compete for response element occupancy with other factors. To evaluate the role of sumoylation in each of these contexts and to define more clearly the intrinsic properties of ZBP-89, we turned to Drosophila Schneider S2 cells. This model has proven extremely valuable to study Sp-like factors because, unlike most mammalian cells, they are functionally devoid of this class of regulators (40). To determine whether it is possible to recapitulate the effects observed in mammalian cells, we examined the ability of ZBP-89 to inhibit Sp1-driven activity at promoters bearing one or three copies of the ODC response element (Fig. 6). As expected, in the absence of Sp1, the activity of these reporters was negligible (indistinguishable from the background signal from untransfected cells). Expression of Sp1 led to a robust activation of these reporters with the activity at the (ODC)3 reporter being nearly 4-fold higher than that of the reporter bearing a single site. As in mammalian cells, expression of ZBP-89 led to a dose-dependent inhibition of activity. At the reporter with a single ODC response element, we did not detect significant differences between WT ZBP-89 and the SC motif mutant or the SC motif mutant SUMO1 fusion (Fig. 6A). These data parallel the observations in mammalian cells (Fig. 5A) and further support that sumoylation of ZBP-89 does not alter its ability to compete with Sp1 for occupancy of the response element. In the case of the reporter bearing three response elements (Fig. 6B), disruption of the SC motifs in ZBP-89 reduced whereas the SUMO1 fusion enhanced the ability of ZBP-89 to inhibit transcription. The data reiterate the observations in mammalian cells (Fig. 5B) and indicate that SUMO modification of ZBP-89 reduces the transcriptional output of complexes assembled at compound response elements. Clearly the ability to recapitulate the ZBP-89-mediated transcriptional effects obtained in mammalian cells validates...
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A

(ODC)$_1$-Luc

![Graph A](image)

B

(ODC)$_3$-Luc

![Graph B](image)

FIGURE 6. SUMO modification enhances the ability of ZBP-89 to antagonize Sp1 in Drosophila cells only at promoters harboring multiple binding sites. Drosophila S2 cells were co-transfected with the indicated amounts of expression vectors for WT ZBP-89 (open circles), the K115R/K356R double mutant that disrupts both SC motifs (open diamonds), or an N-terminal fusion of SUMO1 to the double mutant ZBP-89 (shaded diamonds). Cells received a constant amount (10 ng) of Sp1 expression plasmid and luciferase reporter plasmids containing one (pΔ(ODC)$_1$-Luc, A) or three (pΔ(ODC)$_3$-Luc, B) ZBP-89 binding sites from the ODC gene. Data represent the average ± S.E. of at least three independent experiments performed in triplicate and are expressed as a percentage of the activity in the presence of Sp1 alone, which amounted to 2.44 ± 0.18 and 9.39 ± 0.71 for pΔ(ODC)$_1$-Luc and pΔ(ODC)$_3$-Luc, respectively.

FIGURE 7. In the absence of competing factors, homotypic synergistic activation by ZBP-89 is strongly inhibited by SC motifs. Drosophila S2 cells were co-transfected with 100 ng of expression vectors for ZBP-89 WT (solid bars), ZBP-89 SC motif double mutant (K115R/K356R, hatched bars), or equimolar amounts of control vector (pA5C, open bars) together with luciferase reporters harboring the indicated numbers of ZBP-89 response elements (RE). Data represent the average ± S.E. of at least three independent experiments performed in triplicate and are expressed as a fold induction over the activity obtained for the vector alone at the pΔ(ODC)$_0$-Luc reporter (0.29 ± 0.02). The dashed reference lines indicate the predicted activity assuming simple additive effects. The inset shows the expression levels of the ZBP-89 variants.

Schneider S2 cells as an adequate model to probe the effects of SUMO modification on ZBP-89-mediated transcriptional effects.

In the Absence of Sp1, ZBP-89 Functions as an Activator Whose Synergy Potential Is Restricted by SC Motifs—The functional antagonism evident in Figs. 5 and 6 indicates that ZBP-89 can compete with Sp1 for common binding sites. The substantial residual activity at the highest doses of ZBP-89 expression plasmid, however, suggested that ZBP-89 may possess intrinsic transcriptional activation properties that could be modulated by SUMO modification. To probe this question directly, we examined the activity of ZBP-89 in the absence of competing factors (S2 cells) at reporter genes harboring from zero to four copies of the ODC response element. As can be seen in Fig. 7, expression of WT ZBP-89 caused a ~3-fold activation of the reporter bearing a single ODC response element. Notably the presence of additional response elements did not lead to significant enhancements in activity (Fig. 7, closed bars). This indicates that ZBP-89 can function as a modest activator, but its ability to engage in homotypic synergistic interactions is severely limited. Consistent with our model of synergy control, disruption of the SC motifs in ZBP-89 did not affect its activity at a single site but led to a substantial enhancement at reporters bearing multiple sites (Fig. 7, hatched bars). Notably the expansion of the number of response elements results in synergistic effects because the activity is higher than predicted from purely additive effects (dashed lines). Taken together, these results indicate that ZBP-89 is capable of activating transcription and that the SC motifs in ZBP-89 limit its homotypic synergy potential at compound response elements. Given the much greater relative activity of Sp1 (>10-fold compared with ZBP-89), it is therefore clear how in cellular contexts where Sp1 is present replacement by ZBP-89 leads to a lower overall activity.
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in S2 cells. At a promoter harboring a single ZBP-89 binding site (Fig. 8A), both the WT (solid bar) and SC motif mutant (hatched bar) forms of ZBP-89 displayed identical activities (~3-fold over basal). In contrast, at the promoter bearing three response elements, disruption of the SC motifs in ZBP-89 led to substantially greater (~2.6-fold) activation compared with WT ZBP-89 (Fig. 8B). Fusion of either SUMO1, -2, or -3 to the SC motif mutant ZBP-89, which mimics the SUMOylated form of ZBP-89, led to a dramatic inhibition of activity at the promoter bearing three response elements (Fig. 8B, shaded hatched bars). Such severe inhibitory effects of SUMO isoforms suggest that even at the low stoichiometry of modification observed in vivo sumoylation could have profound consequences on activity. Interestingly, the SUMO fusions also led to a striking inhibition of activity at the promoter bearing a single response element (Fig. 8A, shaded hatched bars). The fact that the WT and SC motif mutant have comparable activities at this promoter may indicate that the stoichiometry of modification may not be sufficiently high to cause an effect in this context. It is also possible that the N-terminal fusions do not fully reproduce all of the properties of ZBP-89 when SUMOylated at its endogenous SC motifs. The results from both promoters, however, clearly indicate that ZBP-89 activity is exceedingly sensitive to the inhibitory effects of all three SUMO isoforms. Through a detailed analysis we have recently identified a highly conserved surface in SUMO that is responsible for its inhibitory properties on multiple transcription factors (33). Disruption of this SUMO effector surface by substitution of two critical basic residues (Lys-33 and Lys-42 in SUMO2) by glutamic acid dramatically affects the ability of SUMO to inhibit transcription (33). Importantly and in contrast to WT SUMO2, fusion of the K33E/K42E SUMO2 mutant to ZBP-89 failed to inhibit activity at the (ODC)_1 promoter and was substantially less inhibitory than WT SUMO2 at the (ODC)_3 promoter (Fig. 8A and B). Because all SUMO fusions were expressed at comparable levels, these results indicate that the inhibitory properties of SUMO on ZBP-89 depend on its critical effector surface and are not simply due to steric hindrance effects.

Sumoylation of the SC Motifs in ZBP-89 Limits Its Ability to Engage in Heterotypic Synergy with Other Activators—The above analysis indicates that ZBP-89 can function as an activator and that modification by SUMO is an effective mechanism to inhibit its ability to self-synergize (homotypic synergy). To examine both the ability of ZBP-89 to cooperate with other activators and the role of sumoylation in such heterotypic synergy, we examined in S2 cells the functional interaction between WT GR and ZBP-89 at a promoter bearing two ZBP-89 binding sites upstream from two glucocorticoid response elements (Fig. 9). In the absence of GR, the WT and mutant forms of ZBP-89 displayed a modest pattern of activity similar to that observed at the (ODC)_1-Luc reporter. Exclusive expression of GR, however, resulted in substantial ligand-dependent activation (~7-fold higher than WT ZBP-89). Notably, co-expression with WT ZBP-89 led to a 3-fold enhancement of activity compared with GR alone. Thus, although ZBP-89 is a modest activator, it is able to synergize very effectively with GR. Furthermore disruption of the SC motifs in ZBP-89 led to an even more substantial (~6.5-fold) enhancement of GR activity. This indicates that, as in the case of homotypic interactions, the SC motifs strongly inhibit the heterotypic synergy potential of ZBP-89. Remarkably, although fusion of any of the three SUMO isoforms to the SC motif-deficient ZBP-89 did not affect expression, this maneuver essentially eliminated the amplifying
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| Activity (fold over GR alone) |
|-----------------------------|
| 0                           |
| 1                           |
| 2                           |
| 3                           |
| 4                           |
| 5                           |
| 6                           |
| 7                           |

**FIGURE 9.** Sumoylation of ZBP-89 potently inhibits its ability to synergize with GR. Drosophila S2 cells were co-transfected with 100 ng of control vector (pASC, open bars) or expression vectors for ZBP-89 WT (solid bars), ZBP-89 SC motif double mutant (K115R/K356R, hatched bars), or ZBP-89 SC motif mutant fused to the indicated SUMO isoforms (shaded hatched bars). Cells also received 200 ng of expression vector for WT GR as indicated and the pΔ(ODC)2(TAT)2-Luc reporter. The SUMO2 mutant harbors the K33E and K42E mutations. Cells were treated with 100 μM dexamethasone 24 h after transfection and were processed as described under “Experimental Procedures.” Data represent the average ± S.E. of at least three independent experiments performed in triplicate and are expressed relative to the activity obtained for GR alone (10.03 ± 2.01). The expression of the individual ZBP-89 variants and GR as determined by immunoblotting is shown below the corresponding bars.

Effect on GR activity because the resulting activity is comparable to that of GR alone. Furthermore, the inhibitory effect of SUMO requires an intact effector surface because fusion of the K33E/K42E SUMO2 mutant was much less effective (Fig. 9, S2 mut). Taken together, these results indicate that, by serving as sites of post-translational modification for SUMO, the SC motifs in ZBP-89 play an important role in controlling the transcriptional activity of this ubiquitous regulator.

**DISCUSSION**

**The Synergy Control Motifs in ZBP-89 Are Sites for SLUMO Modification**—Our finding that ZBP-89 interacts with Ubc9 and that the two SC motifs in ZBP-89 correspond to the main sites of SUMO modification extends the impact of the synergy control mechanism to this family of transcription factors. The very recent demonstration of SUMO modification of other distantly related Krüppel-type transcription factors (45–47) as well as Sp1 (48) indicates that it is a widespread mechanism to regulate this broad class of regulators. Although all three SUMO isoforms can be conjugated to ZBP-89, the fact that SUMO2 and -3 accumulated to substantially higher levels than SUMO1 argues for a preference for SUMO1 modification in vivo. Although the intranuclear localization of ZBP-89 has not been defined, the distinct subnuclear targeting of isoform-specific SUMO proteases suggests that ZBP-89 may accumulate in regions enriched in SUMO2/3-specific proteases such as the nucleoli (49, 50). Initial analysis of green fluorescent protein fusions indicates that both WT and SC mutant ZBP-89 localize exclusively to the nucleus (not shown). Likewise the fact that the apparent preference for sumoylation of Lys-115 over Lys-356 in vitro was not observed in vivo may be due to the actions of cellular activities such as E3 ligases that are absent in the in vitro assay. Although lysine residues can be targets for multiple modifications, we did not observe alterations in the steady-state levels of ZBP-89 upon disruption of the SC motifs, suggesting that they are unlikely to contribute to ubiquitination-dependent degradation. Furthermore the ability of SUMO fusions to reverse the effects of SC motif disruption argues strongly that sumoylation is responsible for the function of the SC motifs.

ZBP-89 as an Activator and Synergy Partner Regulated by Sumoylation—Although in most cases ZBP-89 appears to inhibit transcription of target genes, functional data suggest that it can exert a positive effect on transcription in certain contexts (14, 16, 51). Whether this is due to a displacement mechanism or an intrinsic activation function remains unresolved. We obtained evidence that ZBP-89 can function as a direct albeit weak activator by taking advantage of S2 cells, which provide a cellular context devoid of competing factors. This analysis is consistent with data derived from Gal4 DNA binding domain fusions to recruit ZBP-89 domains because Gal4 fusions to C-terminal fragments of ZBP-89 activate transcription from reiterated Gal4 sites (17). In contrast to most activators, increasing the number of response elements did not lead to enhanced transcription. This lack of synergy was relieved by disruption of the SC motifs indicating that sumoylation exerts a strong inhibitory effect on the ability of ZBP-89 to engage in homotypic synergy. Such behavior can be viewed as an extreme example of the synergy control mechanism observed in other activators such as GR, ETS-1 (19), and CCAAT/enhancer-binding protein α (20) and is akin to the properties of Sp3 (52, 53). Notably although on its own ZBP-89 appears to be a weak activator, its remarkable ability to cooperate with other factors such as GR indicates that it has the capacity to exert quite substantial effects on activation when embedded in a complex promoter. Sumoylation may therefore modulate the proposed cooperation between ZBP-89 and JKTBP1 in the activation of the cytochrome c oxidase Vb gene (10). In this view, ZBP-89 may serve a function similar to that of the “accessory factors” that are required for full glucocorticoid induction of metabolic genes such as PEPCK (54, 55). Consistent with a role in the regulation of synergistic transcription, the ability of ZBP-89 to cooperate with GR is inhibited by SUMO modification. These data extend the role of SUMO modification to include heterotypic cooperation between different factors and suggest that sumoylation is likely to be an important mechanism to regulate the concerted output of the transcription factor complexes that assemble at natural enhancers. In contexts where ZBP-89 competes with other factors for a com-
mon site, the final functional effect of ZBP-89 will therefore depend not only on the relative intrinsic activities of the competing factors but also on their sumoylation-regulated ability to cooperate with additional factors bound nearby.

**Inhibitory Effect of ZBP-89 Sumoylation Depends on Recruitment to Multiple Binding Sites and a Critical Surface in SUMO**—In most cases, ZBP-89 functions as an inhibitor of transcription, and displacement of stronger activators from GC-rich sites is likely to be an important and widespread mechanism. Notably, sumoylation did not alter the ability to compete with Sp1 at a single site. In contrast, at promoters bearing multiple sites where ZBP-89 can engage in heterotypic interactions with Sp1, ZBP-89 SUMO modification limited transcriptional activation. This is consistent with the role of sumoylation as a mechanism to regulate higher order interactions among transcription factors. It is important to note that many sequence-specific factors are targets of sumoylation, and therefore at complex promoters there is substantial redundancy in SUMO-mediated inhibition. Consistent with this notion, our analysis of the effects of SC motifs in the glucocorticoid receptor indicates that the most dramatic effects of loss of sumoylation are observed when all potential sumoylation sites in a complex are disrupted (19, 23). The recent demonstration that Sp1 is also subject to SUMO modification (48) suggests that the relatively modest effect of disrupting the sumoylation sites in ZBP-89 that we observed in mammalian cells may be due to a reduction but not elimination of all potential sumoylation sites at the promoter. In addition, the analysis of Gal4 DBD fusions suggests that ZBP-89 harbors an intrinsic repressor function in the N-terminal region (17, 18). The implicated domain spans the two SC motifs, and in the case of the vimentin gene, a direct repression mechanism involving histone deacetylase recruitment has been invoked (12, 56). Whether the SC motifs are sufficient to account for this activity or additional direct repressive functions are present in this N-terminal domain remains to be determined. In the case of KLF-8 however, Wei et al. (46) argue that sumoylation attenuates the ability of KLF-8 to repress transcription at the KLF-4 promoter. Given the overlap in sequence specificity with other GC box-binding proteins, however, it is difficult to determine whether this is due to a direct influence on an intrinsic repressive function or to factor displacement and altered cooperation effects. Our data, however, clearly indicate that the sumoylation-dependent effects of SC motifs are restricted to promoter contexts where functional interactions involving multiple response elements take place. Based on our mechanistic analysis, we have proposed that SUMO-dependent recruitment of inhibitory cofactors may require a multivalent SUMO-target interaction. Conversely, the cofactors or machinery that mediates the inhibitory effects of SUMO may be able to antagonize only the activity emanating from stable complexes such as those that assemble at reiterated sites. In this view, SUMO may inhibit from a single site as long as the stoichiometry is high and the DNA-activator binding is stable. The reduced activity of the ZBP-89 SUMO fusion at a single site may be an example of this scenario. As in essentially all other examples of sumoylation effects on transcription factors, this modification can exert a substantial effect even when the steady-state stoichiometry of modification that can be detected is low. Although the exact mechanism is unclear, it is possible that sumoylation facilitates the establishment of an altered functional state (by facilitating or disrupting protein interactions for example). Once established, such a state may not require persistent sumoylation for its maintenance. Through an extensive, structurally based mutagenesis analysis, we (33) and others (36) have identified a key conserved surface in SUMO responsible for its inhibitory effects on transcription. X-ray (35) and NMR structural analyses (34) indicate that this effector region in SUMO interacts with Val/Leu-rich SUMO binding motifs in context-specific target proteins. In this regard, certain histone deacetylases (38) and DAXX (37), which harbor such motifs, have been implicated in SUMO-dependent inhibition of transcription. It is likely, however, that additional processes are at play because we have been unable to demonstrate a role for these proteins in SC motif function. It is clear, however, that the effects of SUMO on ZBP-89 do rely on this conserved functional surface of SUMO.

The substantial impact of sumoylation on the activity of ZBP-89 argues that it is likely to contribute to its regulation in vivo. Given the important role of ZBP-89 for germ line and hematopoietic lineages, it is possible that developmental changes in ZBP-89 sumoylation could contribute to the temporally regulated and cell type-restricted activity of this otherwise mostly ubiquitous factor. The recent demonstration that variations in the levels and activity of sumoylation machinery components can accompany cellular differentiation programs (57) lends support to this view. Identifying the proteins and signals that regulate ZBP-89 sumoylation and interaction with other modifications will thus be informative. Overexpression of ZBP-89 leads to cell cycle arrest and apoptosis both in cell culture (58) and in intestinal tissues upon targeted transgenic expression (4). A direct interaction between the zinc finger region of ZBP-89 and p53 likely contributes to this effect (58). Interestingly disruption of the SC motifs did not affect the ability of ZBP-89 to induce cell cycle arrest (not shown). This suggests that sumoylation may not interfere with p53 interactions or that arrest may depend on ZBP-89 transcriptional effects that involve displacement mechanisms insensitive to sumoylation. The influence of sumoylation on transcription factor-driven cell cycle effects appears to depend on the nature of the factor because sumoylation of KLF-8 appears to inhibit the ability of this factor to promote entry into G1 (46). We have recently shown that a splice variant of ZBP-89 generated by alternative promoter usage yields an N-terminally truncated isoform. Because the first SC motif is absent in this form, alterations in sumoylation may contribute to the shortened lifespan and increased sensitivity to dextran-sulfate-sodium-induced colitis displayed by mice exclusively expressing this N-terminally deleted form (3). Taken together, our analysis indicates that by modulating the ability of ZBP-89 to engage in higher order functional interactions sumoylation offers a context-depend-ent post-translational mechanism to regulate the activity of this multifunctional transcription factor.

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