SUMOylation of the transcription factor ZFHX3 at Lys-2806 requires SAE1, UBC9, and PIAS2 and enhances its stability and function in cell proliferation

SUMOylation is a posttranslational modification (PTM) at a lysine residue and is crucial for the proper functions of many proteins, particularly of transcription factors, in various biological processes. Zinc finger homeobox 3 (ZFHX3), also known as AT motif-binding factor 1 (ATBF1), is a large transcription factor that is active in multiple pathological processes, including atrial fibrillation and carcinogenesis, and in circadian regulation and development. We have previously demonstrated that ZFHX3 is SUMOylated at three or more lysine residues. Here, we investigated which enzymes regulate ZFHX3 SUMOylation and whether SUMOylation modulates ZFHX3 stability and function. We found that SUMO1, SUMO2, and SUMO3 each are conjugated to ZFHX3. Multiple lysine residues in ZFHX3 were SUMOylated, but Lys-2806 was the major SUMOylation site, and we also found that it is highly conserved among ZFHX3 orthologs from different animal species. Using molecular analyses, we identified the enzymes that mediate ZFHX3 SUMOylation; these included SUMO1-activating enzyme subunit 1 (SAE1), an E1-activating enzyme; SUMO-conjugating enzyme UBC9 (UBC9), an E2-conjugating enzyme; and protein inhibitor of activated STAT2 (PIAS2), an E3 ligase. Multiple analyses established that both SUMO-specific peptidase 1 (SENP1) and SENP2 deSUMOylate ZFHX3. SUMOylation at Lys-2806 enhanced ZFHX3 stability by interfering with its ubiquitination and proteasomal degradation. Functionally, Lys-2806 SUMOylation enabled ZFHX3-mediated cell proliferation and xenograft tumor growth of the MDA-MB-231 breast cancer cell line. These findings reveal the enzymes involved in, and the functional consequences of, ZFHX3 SUMOylation, insights that may help shed light on ZFHX3’s roles in various cellular and pathophysiological processes.

Posttranslational modifications (PTMs), including ubiquitination, acetylation, methylation, and SUMOylation of the lysine residues, are essential for a variety of cellular processes and human diseases, such as Parkinson’s and Huntington’s diseases (16–18) and cancer (19). Abnormalities in the SUMO pathway thus impact various biological processes, such as nuclear transport, transcription, chromosome segregation, and DNA repair (15). As a result, SUMOylation plays important roles in various human diseases, such as Parkinson’s and Huntington’s diseases (16–18) and cancer (19).

Zinc finger homeobox 3 (ZFHX3), originally named ATBF1 for AT motif–binding factor 1, is a 404-kDa transcription factor that comprises four homeodomains, 23 zinc finger motifs,
and several other domains. ZFHX3 was originally discovered as a negative transcriptional regulator of the AFP (α fetoprotein) gene in a hepatocellular carcinoma cell line (20), but it also plays roles in multiple pathophysiological processes, such as atrial fibrillation (21), myogenic differentiation (22), embryonic development (23), circadian regulation (24), and carcinogenesis (25, 26). For example, ZFHX3 is frequently mutated in advanced prostate cancer (27), deletion of Zfhx3 in mouse prostates induces and promotes neoplastic lesions, and ZFHX3 is essential for ERβ to inhibit cell proliferation via the down-regulation of MYC and cyclin D1 in prostate cancer cells (28). ZFHX3 can also be oncogenic in other contexts, as ZFHX3 is integral to the angiogenic activity of HIF1α/VEGFA signaling in liver cancer cells.3 ZFHX3 is rarely mutated in breast cancer (29), and ZFHX3 interacts with estrogen receptor α to modulate gene expression and cell proliferation in breast cancer cells (30, 31). During postnatal development of mouse mammary glands, Zfhx3 is essential for the progesterone signaling to induce cell proliferation, side branching, and alveologenesis (32–34). ZFHX3 has also been implicated in other types of cancers, including gastric, cervical, and head and neck (35).

Biochemically, ZFHX3 can be degraded by the ubiquitin proteasome pathway, and EFP, an estrogen-responsive RING finger ubiquitin E3 ligase, mediates the ubiquitination and degradation of ZFHX3 in breast cancer cells (29). In addition, ZFHX3 can be SUMOylated endogenously (36), and expression of ZFHX3 makes diffusely distributed nuclear SUMO1 proteins form nuclear body-like structures that are associated with PML nuclear bodies (26). Whereas SUMOylation of ZFHX3 occurs at multiple lysine residues and is nucleus-specific, the Pias3 SUMO E3 ligase, which interacts with ZFHX3 directly, diminishes rather than enhances ZFHX3 SUMOylation (26). At present, the activating, conjugating, and ligating enzymes for ZFHX3 are unknown, and so is whether SUMOylation impacts ZFHX3 stability and function.

In this study, we identified the modifying enzymes for ZFHX3 SUMOylation and determined whether SUMOylation impacts ZFHX3 stability and function. We found that SUMO1, SUMO2, and SUMO3 can be conjugated to ZFHX3, and among the lysines that can be SUMOylated, Lys-2806 was the major SUMOylation site of ZFHX3. In addition, Lys-2806 is evolutionarily conserved among ZFHX3 orthologous of different animal species (26). In this study, we were able to detect a shifted band of ZFHX3 when SUMO1, SUMO2, or SUMO3 was ectopically expressed with HA-ZFHX3 in HEK293T cells (Fig. 1A). In HA-ZFHX3 proteins precipitated with the anti-HA antibody, the shifted band was detected with both anti-SUMO1 and anti-ZFHX3 antibodies, whereas the lower unshifted band was detected only with anti-ZFHX3 antibody (Fig. 1B), confirming that the shifted band represented SUMOylated ZFHX3. In HeLa cells, RNAi-mediated silencing of SENP1 clearly increased the intensity of the upper ZFHX3 band, suggesting that SENP1 deSUMOylates endogenous ZFHX3 (Fig. 1C). Consistently, in the SUMO1-associated proteins pulled down by immunoprecipitation (IP) with anti-SUMO1 antibody in HeLa cells, ZFHX3 was also detected, and the ZFHX3 band(s) were shifted up when compared with the ZFHX3 band from cell lysate before IP (Fig. 1D). Therefore, endogenous ZFHX3 is SUMOylated.

Lysine residues undergoing SUMOylation are typically found within a SUMO modification consensus motif, ψKXE, where ψ is a large hydrophobic residue and X is any residue (37). Analysis of human ZFHX3 with the SUMOsp software (RRID: SCR_018261) identified three potential SUMOylation sites: Lys-1218, Lys-2806, and Lys-3258 (Fig. 1E), the latter two of which have been experimentally confirmed to undergo SUMOylation along with the Lys-2349 nonconsensus site (26). Each of these four lysines was mutated to arginine in the full-length ZFHX3 (FLAG-tagged, this vector is shorter than the one with an HA tag) and analyzed for SUMOylation in HEK293T cells (Fig. 1F) and HeLa cells (data not shown). Compared with WT ZFHX3, whereas mutants K1218R, K2349R, and K3258R did not cause obvious changes in ZFHX3 SUMOylation levels, mutant K2806R dramatically decreased ZFHX3 SUMOylation (Fig. 1F), indicating that Lys-2806 is the major SUMOylation site of ZFHX3. The result was similar when SUMO2 or SUMO3 was co-expressed (Fig. 1G), indicating that Lys-2806 can be SUMOylated with all three SUMO isoforms. We also constructed FLAG- and HA-tagged SUMO1 expression constructs and co-expressed each of them with Myc-tagged ZFHX3 in HEK293T cells with GFP-tagged SUMO1 as a control. Different tags of SUMO1 showed similar effects on ZFHX3 SUMOylation (Fig. 1H). The shifted band was detectable only when the WT SUMO1 or its active form SUMO1-GG was expressed and not when the SUMOylation-dead mutant SUMO1-GA was expressed. Mutation of SUMO2 or SUMO3 to the ginkgolic acid (GA) form also prevented ZFHX3 SUMOylation when co-expressed with HA-ZFH3 (Fig. 1I), suggesting that Lys-2806 is important for ZFHX3 function.

Results

Lys-2806 is the major SUMOylation site of ZFHX3

Our previous study demonstrated that ZFHX3 can be SUMOylated with SUMO1 at lysines 2349, 2806, and 3258 (26), but the enzymes for ZFHX3 SUMOylation are unknown. In this study, we were able to detect a shifted band of ZFHX3 when SUMO1, SUMO2, or SUMO3 was ectopically expressed with HA-ZFHX3 in HEK293T cells (Fig. 1A). In HA-ZFHX3 proteins precipitated with the anti-HA antibody, the shifted band was detected with both anti-SUMO1 and anti-ZFHX3 antibodies, whereas the lower unshifted band was detected only with anti-ZFHX3 antibody (Fig. 1B), confirming that the shifted band represented SUMOylated ZFHX3. In HeLa cells, RNAi-mediated silencing of SENP1 clearly increased the intensity of the upper ZFHX3 band, suggesting that SENP1 deSUMOylates endogenous ZFHX3 (Fig. 1C). Consistently, in the SUMO1-associated proteins pulled down by immunoprecipitation (IP) with anti-SUMO1 antibody in HeLa cells, ZFHX3 was also detected, and the ZFHX3 band(s) were shifted up when compared with the ZFHX3 band from cell lysate before IP (Fig. 1D). Therefore, endogenous ZFHX3 is SUMOylated.

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Identification of activating, conjugating, and ligating enzymes for ZFHX3 SUMOylation

The E1 activating enzyme in the SUMO cycle of human cells is a heterodimer containing SAE1 and SAE2 subunits (37). We found that ectopic expression of SAE1 increased and its knockdown decreased SUMOylated ZFHX3, respectively, in HEK293T cells (Fig. 2, A and B), confirming that SAE1 is also the E1 for ZFHX3 SUMOylation. UBC9 is the only known E2 conjugating enzyme in the SUMO cycle (38, 39), which was also confirmed to be the case for ZFHX3, as ectopic expression and knockdown of UBC9 enhanced and reduced, respectively, ZFHX3 SUMOylation (Fig. 2, C and D). In addition, UBC9 was present in the ZFHX3 protein complex, as detected by IP and Western blotting (Fig. 2 E). Therefore, SUMOylation of ZFHX3 also depends on the SAE1 E1 activating and the UBC9 E2 conjugating enzymes.

PIASs are the major class of SUMO E3 ligases, and five mammalian PIAS proteins, including PIAS1, PIAS2 (commonly known as PIASX, which has two isoforms, /H9251 and /H9252), PIAS3, and PIAS4 (also known as PIASY), have been identified, and they function as SUMO E3 ligases with substrate specificity (37, 40, 41). To determine the E3 ligase for ZFHX3 SUMOylation, we co-expressed each of the five PIAS E3 ligases with ZFHX3 and SUMO1 in HEK293T cells and examined ZFHX3 SUMOylation by Western blotting. PIAS2/ /H9251 and -2/ /H9252 clearly increased, PIAS1 and PIAS4 did not change, and PIAS3 decreased ZFHX3 SUMOylation (Fig. 2 F), indicating that both isoforms of PIAS2 are E3 ligases for ZFHX3 SUMOylation. Furthermore, when FLAG-tagged UBC9 and HA-tagged PIAS2/ /H9251 were simultaneously expressed in HeLa cells, an upper band of endogenous ZFHX3 was detected (Fig. 2 G), supporting both UBC9 and PIAS2/ /H9251 as SUMOylation enzymes for ZFHX3. Both PIAS2 isoforms were associated with ZFHX3, as demonstrated by IP and Western blotting (Fig. 2 H), and knockdown of PIAS2 (α isoform) by siRNA clearly decreased, and PIAS3 increased ZFHX3 SUMOylation (Fig. 2 F), indicating that both isoforms of PIAS2 are E3 ligases for ZFHX3 SUMOylation. Furthermore, when FLAG-tagged UBC9 and HA-tagged PIAS2α were simultaneously expressed in HeLa cells, an upper band of endogenous ZFHX3 was detected (Fig. 2 G), supporting both UBC9 and PIAS2α as SUMOylation enzymes for ZFHX3.

Both PIAS2 isoforms were associated with ZFHX3, as demonstrated by IP and Western blotting (Fig. 2 H), and knockdown of PIAS2 (α isoform) by siRNA clearly decreased the SUMOylation of ZFHX3 (Fig. 2 F). Ectopic expression of PIAS3 diminished rather than enhanced ZFHX3 SUMOylation, which is consistent with a previous study (26). These findings demonstrate that PIAS2 functions as an E3 ligase for ZFHX3 SUMOylation.

To test whether UBC9 and PIAS2 act on lysine 2806 of ZFHX3, we ectopically expressed FLAG-tagged UBC9 or HA-tagged PIAS2α with GFP-tagged SUMO1 in HEK293T cells, with the conserved lysine shown in boldface type.

Identification of activating, conjugating, and ligating enzymes for ZFHX3 SUMOylation

Figure 1. Lysine 2806 is the major SUMOylation site of ZFHX3. A, ZFHX3 can be SUMOylated by SUMO1, SUMO2, and SUMO3. HEK293T cells were transfected with expression plasmids of HA-tagged ZFHX3 (HA-ZFHX3) and GFP-tagged SUMO1, SUMO2, or SUMO3, and ZFHX3 was detected by Western blotting with anti-HA-beads, and eluted proteins were immunoblotted (IB) with anti-SUMO1 (top) or anti-ZFHX3 (middle) antibody. The middle membrane was stripped and reprobed with anti-SUMO1 antibody (bottom). C and D, detection of endogenous ZFHX3 SUMOylation in HeLa cells with RNAi-mediated SENP1 knockdown by IP and Western blotting with anti-HA and anti-GFP antibodies. F, detection of ZFHX3 SUMOylation in HEK293T cells ectopically expressing FLAG-, HA-, and GFP-tagged SUMO1 and Myc-tagged ZFHX3 by Western blotting with the indicated antibodies. J, alignment of ZFHX3 sequences from different species surrounding the human Lys-2806 SUMOylation site, with the conserved lysine shown in boldface type.
tagged WT ZFHX3 and ZFHX3-K2806 mutant in HEK293T cells. Western blotting demonstrated that, whereas UBC9 and PIAS2/H9251 obviously promoted SUMOylation of WT ZFHX3, neither UBC9 nor PIAS2/H9251 had a detectable effect on the ZFHX3-K2806R mutant (Fig. 2, J and K). Therefore, UBC9 and PIAS2 indeed can act on Lys-2806 in ZFHX3 SUMOylation.

SENP1 is a major deSUMOylating enzyme for ZFHX3

SUMOylation is a dynamic process that can be reversed by deSUMOylating enzymes known as SENPs. Six SENPs have been identified in humans, and they have different cellular localization and substrate specificity (42). To identify the SENP(s) for ZFHX3, SUMO1 and each of the six SENPs were co-transfected with HA-ZFHX3 into HEK293T cells, and ZFHX3 SUMOylation was assessed. SENP1 and SENP2 decreased ZFHX3 SUMOylation, whereas other SENPs did not (Fig. 3A). Co-IP and Western blotting demonstrated that SENP1 had a stronger interaction with ZFHX3 than SENP2 (Fig. 3B), so we focused on SENP1 for additional analyses. Consistent with its deSUMOylating effect on ZFHX3, silencing the endogenous SENP1 by RNAi increased (Fig. 3C) and ectopic expression of SENP1 abrogated ZFHX3 SUMOylation. The C603A mutant of SENP1, which no longer has a catalytic activity, failed to decrease ZFHX3 SUMOylation. Similar results were observed when SUMO2 or SUMO3 was co-expressed with SENP1 and its mutant (Fig. 3D). Ectopic expression of increasing amounts of SENP1 plasmids resulted in a dose-dependent decrease in ZFHX3 SUMOylation (Fig. 3E), further confirming the deSUMOylating activity of SENP1 for ZFHX3.

In addition, whereas ectopic expression of SENP1 significantly decreased the SUMOylation of WT ZFHX3, it had little effect on the SUMOylation-deficient ZFHX3-K2806R mutant (Fig. 3F). Similarly, whereas knockdown of SENP1 significantly increased the SUMOylation of WT ZFHX3, it did not cause a similar change when the ZFHX3-K2806R mutant was expressed (Fig. 3G), even though other lysines of ZFHX3 can also be SUMOylated. These results firmly establish SENP1 as a deSUMOylating enzyme for ZFHX3.

SENP1 interacts with ZFHX3

We then characterized the interaction between ZFHX3 and SENP1. Both ectopically expressed SENP1 and ZFHX3 (Fig. 4A) and the endogenous SENP1 and ZFHX3 showed a protein–protein interaction, as demonstrated by IP and Western blotting (Fig. 4B). The interaction was independent of SENP1’s catalytic activity, as ZFHX3 also interacted with the catalytically inactive C603A mutant of SENP1 (Fig. 4C).
and treatment with the N-ethylmaleimide (NEM) inhibitor of SENP1 did not decrease the interaction (Fig. 4D). To map the interacting domains of SENP1 and ZFHX3, different fragments of both SENP1 and ZFHX3 were co-expressed in HEK293T cells, and co-IP and Western blotting were performed. A fragment of ZFHX3 (residues 1334–2667) showed an interaction with SENP1 (Fig. 4, E and G), whereas a fragment of SENP1 (residues 181–419) interacted with ZFHX3 (Fig. 4, F and H).

SUMOylation of ZFHX3 enhances its protein stability

Although SUMOylation itself does not directly mediate the degradation of a protein as ubiquitination does through the proteasome pathway, it can affect the ubiquitination of proteins and thus indirectly modulate protein stability. For example, SUMOylation enhances the stability of Smad4 (43) and PCNA (44). EFP is a ubiquitin E3 ligase that mediates the ubiquitination and subsequent degradation of ZFHX3 via the ubiquitin proteasome pathway (29). In testing whether SUMOylation alters the ubiquitination and degradation of ZFHX3, HEK293T cells were transfected with ZFHX3 and EFP in the presence of SUMO1, and protein levels were measured. Overexpression of SUMO1, which enhanced ZFHX3 SUMOylation, attenuated EFP-mediated degradation of ZFHX3 (Fig. 5A). Consistently, ectopic expression of ubiquitin increased ZFHX3 ubiquitination, which was also decreased by SUMO1 overexpression (Fig. 5B). As expected, knockdown of EFP significantly increased both SUMOylated and total ZFHX3 (Fig. 5C), further suggesting that SUMOylation protects ZFHX3 from proteasome-mediated degradation. Ectopic expression of SUMO1 also weakened the interaction of ZFHX3 with EFP (Fig. 5D), and consistently, knockdown of SENP1, which enhanced ZFHX3 SUMOylation, significantly attenuated the ZFHX3-EFP interaction (Fig. 5E). These results further indicate that SUMOylation of ZFHX3 protects ZFHX3 from EFP-mediated ubiquitination and degradation.

To further test the role of SUMOylation in ZFHX3 stability, we analyzed whether SUMOylation of ZFHX3 affects its ubiquitination. Mutation of the major SUMOylation site (i.e. Lys-2806) significantly enhanced the ubiquitination of ZFHX3 (Fig. 5F).
and the global ubiquitination level also appeared to be increased (Fig. 5F, bottom). As expected, the half-life of ZFHX3 was significantly reduced by the K2806R mutation (Fig. 5G).

We also tested whether SUMOylation affects the stability of endogenous ZFHX3. UBC9 and PIAS2/H9251 was knocked down by RNAi in HeLa cells, and the cycloheximide (CHX) assay was performed. Knockdown of either UBC9 or PIAS2/H9251 significantly reduced the half-life of endogenous ZFHX3 (Fig. 5, H and I). These findings support a role for SUMOylation in protein stability of ZFHX3.

In addition to stability, SUMOylation is also known to regulate substrate subcellular distribution (4, 45), so we also tested whether ZFHX3’s cellular localization is affected by SUMOylation. In HeLa cells transfected with WT ZFHX3 or its K2806R mutant, immunofluorescence staining demonstrated that both ZFHX3 and ZFHX3-K2806R massed to form nuclear body-like dots in the nucleus, and no apparent differences were noticeable (Fig. 6A). However, elevated SUMOylation, as induced by the SENP1 inhibitor NEM, increased the amount of nuclear ZFHX3, as detected by Western blotting in the cytoplasmic and nuclear fractions of cells (Fig. 6B). Consistently, the intervention of ZFHX3 SUMOylation by different concentrations of GA (Fig. 6C), an inhibitor of protein SUMOylation (46, 47), reduced the amount of nuclear ZFHX3 (Fig. 6D).

SUMOylation at Lys-2806 is necessary for ZFHX3 to promote cell proliferation and tumor growth

Given that Lys-2806 is the major SUMO modification site of ZFHX3 and SUMOylation often modulates molecular functions, we determined whether SUMOylation modulates ZFHX3 function. In prostate cancer cells, ZFHX3 coordinates with ERα to inhibit cell proliferation via the down-regulation of MYC and cyclin D1 (48). Therefore, we stably expressed ZFHX3 and its K2806R mutant in several prostate cancer cell lines (PC-3, 22Rv1, and C4-2B) and breast cancer ones (MCF-7, T-47D, Hs 578T, and MDA-MB-231) to prepare cells that stably express ZFHX3 and its K2806R mutant. Unfortunately, we were only able to obtain such cells for MDA-MB-231 and Hs 578T triple-negative breast cancer cell lines. Compared with parental cells, whereas ectopic expression of WT ZFHX3 in MDA-MB-231 cells significantly increased cell proliferation, as indicated by the SRB and colony formation (data not shown) assays in two-dimensional culture and the sphere formation assay in Matrigel (Fig. 7, A and B), expression of the K2806R mutant showed opposing effects. In addition, the larger spheres in Matrigel from the WT ZFHX3 formed branches, an indicator of higher motility (Fig. 7B, bottom panels), whereas the K2806R mutant did not. Similar results were obtained in the Hs 578T breast cancer cell line (Fig. 7C). Consistent with these in vitro
assays, subcutaneous injection of MDA-MB-231 cells expressing different forms of ZFHX3 into nude mice demonstrated that whereas the WT ZFHX3 significantly promoted tumor growth, as indicated by tumor images, growth curves, and tumor weights at 35 days after injection (Fig. 7D), the K2806R mutant significantly suppressed tumor growth. These results indicate that SUMOylation is necessary for ZFHX3 to promote the proliferation of breast cancer cells.

MYC and cyclin D1 are involved in the suppressive function of ZFHX3 in the proliferation of prostate cancer cells (48), so we
determined whether they are also involved in ZFHX3 function in breast cancer cells. Ectopic expression of WT ZFHX3 in MDA-MB-231 cells up-regulated MYC and cyclin D1, but the K2806R mutant did not (Fig. 7E). In xenograft tumors, immunohistochemical (IHC) staining further revealed that WT ZFHX3 increased the expression of the cell proliferation marker Ki-67 and proliferation-promoting cyclin D1 and MYC, but the K2806R mutant did not (Fig. 7F). Collectively, these data suggest that SUMOylation of ZFHX3 at Lys-2806 is necessary for ZFHX3 to promote cell proliferation and tumor growth.

Discussion

The ZFHX3 transcription factor modulates multiple pathophysiological processes, such as embryonic development, carcinogenesis, and atrial fibrillation (23–25), and can be posttranslationally modified by SUMOylation (26) and ubiquitination (29). In this study, we determined how ZFHX3 is SUMOylated and deSUMOylated, and whether SUMOylation impacts the stability and function of ZFHX3.

SUMOylation is an enzymatic process that covalently conjugates SUMOs to the lysines of proteins by enzymes analogous to those of ubiquitination, including the E1 activating enzyme, the E2 conjugating enzyme, and the specific SUMO E3 ligase. For the SUMOylation of ZFHX3, we demonstrated that SUMO1, SUMO2, and SUMO3 can each be conjugated to the protein (Fig. 1). In addition, whereas multiple lysines in ZFHX3, including Lys-2349, Lys-2806, and Lys-3258, can be SUMOylated, which is consistent with a previous study (26), we demonstrated that Lys-2806 is the major acceptor site because the K2806R mutation significantly reduced SUMO1/2/3-modified ZFHX3 (Fig. 1). As expected, SUMOylation of ZFHX3 required the established E1 and E2 enzymes, including the SAE1 E1 activating enzyme and the UBC9 E2 conjugating enzyme, because their ectopic expression and knockdown clearly increased and decreased, respectively, the level of SUMOylated ZFHX3 (Fig. 1). As expected, SUMOylation of ZFHX3 required the established E1 and E2 enzymes, including the SAE1 E1 activating enzyme and the UBC9 E2 conjugating enzyme, because their ectopic expression and knockdown clearly increased and decreased, respectively, the level of SUMOylated ZFHX3 (Fig. 2).

Among the common E3 ligases (i.e. the PIAS family members PIAS1, PIAS2, PIAS3, and PIAS4), only PIAS2 has the ligase activity for ZFHX3, because its expression modulates ZFHX3 SUMOylation and impacts on stability and function.
SUMOylation level, and it interacts with ZFHX3 (Fig. 2). Interestingly, the PIAS3 E3 ligase in fact decreased ZFHX3 SUMOylation, which is consistent with previous studies where PIAS3 physically interacts with ZFHX3 to reduce its SUMOylation (26, 49).

Among the common deSUMOylating enzymes, SENP1 and SENP2 have deSUMOylating activities for ZFHX3, because their ectopic expression reduced ZFHX3 SUMOylation, and both SENP1 and SENP2 interacted with ZFHX3, even though SENP2 was weaker than SENP1 in the interactions (Fig. 3). Further supporting this conclusion, knockdown of SENP1 or mutation of its catalytic domain increased ZFHX3 SUMOylation (Fig. 3), endogenous ZFHX3-SENP1 interaction also occurred (Fig. 4), and the ZFHX3-SENP1 interaction regions were mapped to residues 1334–2667 of ZFHX3 and 181–419 of SENP1 (Fig. 4). Notably, the catalytic domain of SENP1 is dispensable for its interaction with ZFHX3, as neither the C603A inactivating mutation nor the NEM inhibitor of SENP1 affected the ZFHX3-SENP1 interaction (Fig. 4).

Similar to ubiquitin in both structure and biochemistry, SUMO commonly conjugates to lysine residues of its substrate proteins (2). In addition, SUMOylation of lysines can prevent the same residue’s ubiquitination and subsequent protein degradation, as seen for Mdm2, PCNA, 1xBo, and other proteins (8, 37, 44, 50). On the other hand, SUMO can also act as a signal for the recruitment of a ubiquitin E3 ligase to a substrate to promote the substrate’s ubiquitination and degradation, as seen for PML (15). For ZFHX3, SUMOylation prevents its ubiquitination and subsequent degradation via the ubiquitin proteasome pathway, which is based on multiple lines of evidence. For example, SUMOylation reduced the ubiquitination and degradation of ZFHX3 by EFP (Fig. 5, A–E), a known ubiquitin E3 ligase for ZFHX3, and interruption of SUMOylation at Lys-2806 by the K2806R mutation increased the ubiquitination of ZFHX3 and led to more degradation of ZFHX3 (Fig. 5, F and G).

At present, it remains unknown which lysines are responsible for the ubiquitination and degradation of ZFHX3, even though the EFP E3 ubiquitin ligase is clearly involved. In addition, it is unknown whether Lys-2806 can be ubiquitinated, although its lack of SUMOylation enhanced the overall ubiquitination and degradation of ZFHX3 (Fig. 5).

SUMOylation often occurs in the nucleus, and its interruption can lead to cytoplasmic translocation of a target protein by changing its inter- or intramolecular interactions (15, 51). For ZFHX3, its nuclear localization is required for its SUMOylation, as failure to enter into the nucleus prevents its SUMOylation (26), and ZFHX3 is normally localized in the nucleus to regulate gene transcription via promoter binding (52). Interestingly, deSUMOylation does not appear to cause detectable cytoplasmic translocation of ZFHX3, because the K2806R mutation, which attenuated ZFHX3 SUMOylation to a large extent, did not change ZFHX3’s nuclear localization (Fig. 6A), and neither did chemical activation or inhibition of ZFHX3 SUMOylation (Fig. 6, B–D). On the other hand, cytoplasmic translocation likely alters the function of ZFHX3, as its cytoplasmic localization often occurs in different types of cancers, including gastric cancer and those of the skin, head and neck, and bladder, and cytoplasmic translocation correlates with worse survival in head and neck cancer patients (53–55). In addition, multiple nuclear localization signals (NLSs) have been identified for ZFHX3, including NLS1387, NLS2947, and NLS2987 defined in one study (56) and NLS KRK2615-2617 defined in another study (26). Currently, it is unknown how ZFHX3 shuttles between the cytoplasm and the nucleus.

SUMOylation clearly plays important roles in a variety of biological processes, such as development and carcinogenesis (19). SUMOylation prevents the ubiquitination and subsequent degradation of ZFHX3 (Fig. 5), and quantitative reduction in ZFHX3 has functional consequences because ZFHX3 is haploinsufficient (23, 35). More specifically, SUMOylation is essential for ZFHX3 to promote cell proliferation and tumor growth at least in some breast cancer cell lines (i.e. MDA-MB-231 and Hs 578T), as the K2806R mutation, which clearly interrupts ZFHX3 SUMOylation, significantly attenuated the promoting effects of ZFHX3 on colony and sphere formation and xenograft tumor growth (Fig. 7).

Whereas SUMOylation at Lys-2806 is crucial for the function of ZFHX3 in the proliferation of some breast cancer cell lines and this SUMOylation site is evolutionarily conserved among different animal species, it is unknown whether the promoting effect of SUMOylated ZFHX3 on cell proliferation is also there for normal mammary epithelial cells, where Zfhx3 is essential for the progesterone signaling to induce ductal cell proliferation during side branching (34). In addition, it is also unknown whether SUMOylation modulates the function of ZFHX3 in prostate cancer cells, where ZFHX3 clearly possesses a tumor suppressor activity based on its frequent inactivating mutations in advanced human prostate cancer and the induction of neoplastic lesions by its deletion (35). We are in the process of generating a transgenic mouse line in which the K2806R mutation can be induced by Cre expression, which will enable us to examine whether Zfhx3 SUMOylation affects its functions in mammary gland development, prostate tumorigenesis, and likely other pathophysiological processes.

In summary, we have identified the enzymes for ZFHX3 SUMOylation, which include the SAE1 E1 activating enzyme, the UBC9 E2 conjugating enzyme, the PIAS2 E3 ligase, and the SENP1 and SENP2 deSUMOylating enzymes. We have also demonstrated that among the lysines of ZFHX3 that can be SUMOylated, Lys-2806 is the major SUMOylation site and SUMOylation at Lys-2806 enhances ZFHX3 stability by preventing its ubiquitination and proteasome-mediated degradation. While not affecting ZFHX3’s nuclear localization, SUMOylation is essential for ZFHX3 to promote cell proliferation and xenograft tumor growth in the MDA-MB-231 breast cancer cell line. These findings will be helpful for understanding how ZFHX3 functions in different pathophysiological processes.

**Experimental procedures**

**Cell lines**

All cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA). HEK293T, HeLa, and Hs 578T cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum...
**ZFHX3 SUMOylation and impacts on stability and function**

(Hyclone, Logan, UT). MDA-MB-231 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. All cells were maintained at 37 °C with 5% CO2.

**Plasmid preparation and transfection**

Expression plasmids for ZFHX3, ZFHX3-NLSm, SUMO1, EFP, and ubiquitin were described previously (26, 29). PCR and cloning of PCR products were used to generate expression plasmids for SUMO1 in pcdNA3.0-FLAG and pCMV-HA, for SUMO2 and SUMO3 in pEGFP-C1, and for SENP1-C603A and UBC9 in p3xFLAG-CMV-10 following standard procedures. Mutants of ZFHX3 and SENP1 plasmids were generated by PCR-mediated site-directed mutagenesis. PCR primers for gene cloning and site-directed mutagenesis are listed in Table 1. Expression plasmids for all PIASs and SENPs were kindly provided by Dr. Jinke Cheng of Shanghai Jiao Tong University School of Medicine (57). Identities of all expression plasmids were confirmed by DNA sequencing. Plasmid was transfected into cells using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**Antibodies and reagents**

Monoclonal anti-FLAG M2 and anti-HA affinity gels, NEM, MG132, CHX, and ginkgolic acid C15:1 were purchased from Sigma. Normal rabbit IgG was from Santa Cruz Biotechnology, Inc. (Dallas, TX). Protein A- and G-agarose were from GE Healthcare. Complete protease inhibitor mixture tablet was from Roche Applied Science. Details of primary antibodies are listed in Table 2. Secondary antibodies, including horseradish peroxidase–conjugated goat anti-rabbit IgG (catalogue no. 7074) and goat anti-mouse IgG (catalogue no. 7076), were from Cell Signaling (Danvers, MA).

**Establishment of cell lines ectopically expressing ZFHX3**

MDA-MB-231 and Hs 578T breast cancer cells were transfected with pcdNA3.0-FLAG and FLAG-tagged WT ZFHX3 (ZFHX3-WT) or its K2806R mutant (ZFHX3-K2806R) and selected with 1 mg/ml G418 for 14 days. Clones were isolated and subjected to Western blotting with anti-FLAG antibody to identify those expressing FLAG-tagged ZFHX3.

**Immunofluorescence staining**

Cells were transfected with the indicated plasmids for 24 h and then seeded onto coverslips in 6-well plates overnight, washed with PBS, fixed in 4% paraformaldehyde under room temperature for 30 min, permeabilized with 0.2% Triton X-100 for 10 min, and then blocked with 5% bovine serum in PBS. Cells on coverslips were then incubated with primary antibody at 4 °C overnight, washed three times with PBS, incubated with secondary antibody conjugated with TRITC (Life Technologies; A11008) at room temperature for 2 h in a lightproof box, counterstained with 4’,6-diamino-2-phenylindole (Sigma) for 5 min, rinsed with PBS, mounted to a glass slide with a drop of mounting medium, and sealed with nail polish. The immunofluorescence images were taken by a laser-scanning confocal microscope (Zeiss, LSM710, Jena, Thuringen, Germany).

**Immunoprecipitation and Western blotting**

For IP, cells were washed twice with cold PBS after various treatments, lysed at 4 °C for 30 min by gentle shaking in IP buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.5, and 1% Nonidet P-40, supplemented with 1% protease inhibitor mixture). For the detection of SUMOylated proteins, NEM was added to the lysis buffer (20 μM) to preserve SUMOylation of ZFHX3 during the experiment. After centrifugation at 12,000 × g for 15 min, cell lysates were incubated with anti-FLAG or anti-HA-agarose
beads at 4 °C for 2 h. For endogenous co-IP, cell lysates were incubated with anti-ZFHX3 antibody or normal IgG for 4 h at 4 °C. Protein A/G-agarose beads were then added and incubated for 2 h at 4 °C. After washing with cold IP buffer five times, immune complexes were collected by centrifugation at 2000 rpm for 2 min, boiled in 2 × SDS sample buffer for 10 min, and subjected to Western blotting.

For Western blotting, cell lysates or immunoprecipitates were separated by 4% (for full-length ZFHX3) or 10% (for all other proteins) SDS-PAGE, and proteins were then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were then blocked with 5% nonfat milk, incubated with horseradish peroxidase–linked secondary antibody overnight at 4 °C, and then incubated with the primary antibody overnight at 4 °C, and then incubated with horseradish peroxidase–linked secondary antibody for 2 h at room temperature. After adding Western Bright ECL reagents (Advansta, Menlo Park, CA), protein signals were detected with a luminescent image analyzer (Jun Yi Dong Fang, Beijing, China).

**RNAi**

For gene silencing by RNAi, siRNAs (details are shown in Table 3) were synthesized by Sangon Biotech (Shanghai, China) and transiently transfected into cells using the Lipofectamine RNAiMAX reagent according to the manufacturer’s instructions. Cells were harvested after transfection for 48–72 h and analyzed by Western blotting.

**CHX assay**

To examine protein stability, cells were seeded into 12-well plates at a density of 2 × 10⁵ cells/well, cultured for 24 h, and transfected with the indicated plasmids. After treatment with CHX (100 μg/ml) for the indicated times, cells were then harvested and subjected to Western blotting.

**Subcellular fractionation**

Cells were collected, resuspended in lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, and 1% protease inhibitor mixture), incubated for 5 min on ice, and centrifuged at 450 × g for 5 min. Cell pellets were then resuspended and incubated in lysis buffer with 0.6% IGEPAL CA-630 for 15 min on ice. After centrifugation at 12,000 × g for 30 s, the supernatant was collected as the cytoplasmic fraction. The pellets were washed at least three times in lysis buffer and lysed in extraction buffer (20 mM HEPS, pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, and 1% protease inhibitor mixture) for 30 min on ice. After centrifugation at 20,000 × g for 5 min, the supernatant was collected as the nuclear fraction. Western blotting was applied to detect cytoplasmic and nuclear proteins.

**SRB and colony formation assays**

The SRB assay was performed to monitor cell proliferation and death, as described previously (27). Briefly, cells were plated into 24-well plates with 4 replicates/group, incubated for different times, fixed, stained with SRB, and measured for optical intensities, which indicated cell numbers.

For the colony formation assay, 1000 cells were plated into 6-well plates in triplicate. Cells were cultured for 10 or more days until colonies were clearly visible, fixed in 4% paraformaldehyde, and stained with 0.25% crystal violet. The numbers of colonies were scored by using the ImageJ program. Each experiment was repeated at least twice.

**Matrigel assay**

Eight-well chamber slides (FALCON, Corning, Inc., catalogue no. 353097) were precoated with 40 μl of growth factor–reduced Matrigel (BD Biosciences, catalogue no. 354230) and set in the cell culture incubator for 30 min to allow Matrigel solidification. A total of 1600 cells were overlaid onto the gel in medium supplemented with 2% Matrigel in a well. The medium was replenished every 3 days. 12 or 15 days later, images of spheres were taken, and the number of spheres at each well was counted by using the ImageJ program. Each experiment was repeated twice.

**Mouse xenograft tumor growth assay**

The animal studies were approved by the Nankai University School of Life Science Animal Care and Use Committee. For MDA-MB-231 cells expressing vector, FLAG-ZFHX3-WT, or FLAG-ZFHX3-K2806R, 1.5 × 10⁶ cells in 100 μl of PBS/Matrigel (2:1) (catalogue no. 354234) were injected into the flanks of a 4-week-old nude mouse. Tumor volumes were recorded every 7 days using a Vernier caliper, and the tumor volume was estimated as follows: V = (length × width × height × 0.5) mm³. Five weeks after injection, mice were sacrificed, and the tumors were isolated, photographed, weighed, and sectioned for IHC staining.

**IHC staining**

Tissue sections of xenograft tumors (4 μm thick) were immunostained with anti-MYC, anti-cyclin D1, or anti-Ki-67

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**Table 2**

Antibodies used in the study

<table>
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<th>Antibody</th>
<th>Company</th>
<th>Catalog no.</th>
<th>Dilution</th>
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**Table 3**

Sequences of siRNAs used in the study

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<td>PIAS3</td>
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*WB, Western blotting; IF, immunofluorescence; IHC, immunohistochemistry.*
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antibodies overnight at 4 °C. The peroxidase-conjugated streptavidin method was performed, followed by the 3,3’-diaminobenzidine procedure according to the manufacturer’s protocols (Dako, Agilent Pathology Solutions). Hematoxylin was used for counterstaining.

Statistical analysis

All in vitro experiments were repeated at least twice unless stated otherwise, and results from one experiment are shown. Prism 6 software (GraphPad Software, San Diego, CA) was used for all statistical analyses. All quantitative data are expressed as mean ± S.E. Differences were analyzed using Student’s t test or one-way analysis of variance for multiple group comparisons. \( p < 0.05 \) was considered as statistically significant difference.

Data availability

All of the data are contained within the article.


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References

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