

SUMOylation of GPS2 protein regulates its transcription-suppressing function

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ABSTRACT G-protein pathway suppressor 2 (GPS2) is a human suppressor of G protein-activated mitogen-activated protein kinase signaling. It is involved in many physiological processes, including DNA repair, cell proliferation, apoptosis, and brain development. In this study, we show that GPS2 can be modified by the small ubiquitin-like modifier (SUMO) SUMO-1 but not SUMO-2 or -3. Two SUMOylation sites (K45 and K71) are identified in the N-terminal coiled-coil domain of GPS2. Substitution of K45 with arginine reduces SUMOylation, whereas substitution of K71 or both K45 and K71 with arginine abolishes SUMOylation, with more of the double mutant GPS2 appearing in the cytosol than in the nucleus compared with wild type and the two-single-mutant GPS2. SUMOylation stabilizes GPS2 protein by promoting its interaction with TBL1 and reducing its ubiquitination. SUMOylation also enhances the ability of GPS2 to suppress transcription and promotes its ability to inhibit estrogen receptor α -mediated transcription by increasing its association with SMRT, as demonstrated in MCF-7 and T47D cells. Moreover, SUMOylation of GPS2 also represses the proliferation of MCF-7 and T47D cells. These findings suggest that posttranslational modification of GPS2 by SUMOylation may serve as a key factor that regulates the function of GPS2 in vivo.

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INTRODUCTION

G-protein pathway suppressor 2 (GPS2) was first identified as a human suppressor of G protein-activated mitogen-activated protein kinase signaling in both yeast and mammalian cells (Spain *et al.*,

1996). It was later shown to be involved in many cellular processes, including proliferation (Cheng and Kao, 2009), apoptosis (Peng *et al.*, 2001), DNA repair (Lee *et al.*, 2006), brain development (Wu *et al.*, 2006), and metabolism (Venteclef *et al.*, 2010). GPS2 is a 37-kDa protein that is expressed in most human tissues. GPS2 contains an N-terminal coiled-coil domain (amino acids 12–101), which is essential for interaction with other proteins. GPS2 interacts with SMRT/N-CoR through its coiled-coil domain in the N-terminus, acting as a subunit of SMRT/N-CoR complex, which is important for the inhibition function of the complex (Zhang *et al.*, 2002). Another GPS2-interacting protein in the SMRT/N-CoR complex is transducin β -like 1 (TBL1), and SMRT and GPS2 are both protected from proteolysis when in complex with TBL1 (Oberoi *et al.*, 2011). Besides suppressing the activity of certain transcription factors, GPS2 also interacts with some transcription factors through its C-terminal domain to promote the transcriptional activities of these proteins. GPS2 interacts with papillomavirus E2 protein and p300, increasing the transactivation ability of papillomavirus E2 (Peng *et al.*, 2000). In all cases, the C-terminal domain of GPS2 is necessary for interaction between GPS2 and other proteins. Moreover, GPS2 can undergo oligomerization through its N-terminal region, and the process of oligomerization increases the transcriptional activity of p53 (Peng *et al.*, 2001).

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H.W. supervised the research, including the conception and design of experiments and writing of the manuscript. H.B. and S.L. performed the experiments and analyzed the data. H.B., S.L., M.W., Z.J., and P.P. assisted with preparation of reagents and other materials. A.K.C. revised the manuscript. All authors read and approved the final manuscript.

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Abbreviations used: AIB1, amplified in breast cancer 1; CHX, cycloheximide; CRABP-II, cellular retinoic acid-binding protein II; E2, estrogen; ER α , estrogen receptor α ; ERE, estrogen-responsive element; GPS2, G-protein pathway suppressor 2; HDAC3, histone deacetylase 3; NEMO, NF- κ B essential modulator; NLS, nuclear localization signal; RA, retinoic acid; SENPs, SUMO-specific proteases; SUMO, small ubiquitin-like modifier.

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GPS2 is predominantly localized in the nucleus of multiple cell types, where it can carry out the function of transcription regulation. However, a small fraction of GPS2 is also localized in the cytosol. The cytoplasmic function of GPS2 is to protect RIP1 from ubiquitination by inhibiting the TRAF2/Ubc13 pathway, and this is required for precise control of immunity and homeostasis. The mechanism that mediates passage of GPS2 between nucleus and cytosol is unknown (Cardamone *et al.*, 2012).

GPS2 is a multifunction protein that participates in a number of cellular pathways. GPS2 can interact with several nuclear receptors and orphan receptors, including LXR, FXR, LRH-1, HNF4 α , and SHP, and thus plays an active and important role in the metabolism of bile acid, lipid, and cholesterol and also in anti-inflammation response (Sanyal *et al.*, 2007; Venticlef *et al.*, 2010). GPS2 interacts with the cyclinA1-CDK2 complex, and the complex plays a role in cell cycle control, DNA repair, and meiosis (Diederichs *et al.*, 2004). Because GPS2 is a stable component of the SMRT corepressor complex, overexpression of GPS2 increases and knockdown of GPS2 eliminates SMRT-mediated repressive activity. Furthermore, GPS2 knockdown also enhances estradiol (E2)-induced gene expression and proliferation of MCF-7 (Cheng and Kao, 2009).

Modification of proteins by small ubiquitin like-modifier (SUMO) is an important mechanism for dynamical posttranslational regulation of protein functions. In the process of SUMOylation, SUMO is covalently attached to a lysine residue of the substrate via its C-terminal diglycine (Seeler and Dejean, 2003). SUMO conjugation often requires the ψ KXE sequence, where ψ is a large hydrophobic amino acid and X represents any amino acid (Rodriguez *et al.*, 2001). Four isoforms of mammalian SUMO have been identified: SUMO-1, 2, 3, and 4. SUMO-1 shares 48% identity with SUMO-2/3. SUMO-2 and -3 are closely related (Enserink, 2002). SUMO-4 has so far been detected only at the RNA level (Fox *et al.*, 2006). SUMO is cleaved from the SUMO-protein conjugate by SUMO-specific proteases (SENPs). In the recent years, more and more proteins have been found to be modified by SUMO-1, and these include transcription factors, nuclear receptors, and transcriptional cofactors that are involved in many cell processes, such as p53 (Stindt *et al.*, 2011), androgen receptor (Mukherjee *et al.*, 2009), and amplified in breast cancer 1 (AIB1; Wu *et al.*, 2006). Modification of these proteins by SUMO has a range of effects on the modified proteins, including changes in subcellular localization/transport, protein stability, interaction with other factors, and changes in ability to activate or repress transcription. The subunits of SMRT/N-CoR complex, N-CoR and TBL1, are subject to SUMOylation. SUMOylation of TBL1 plays a role in β -catenin-mediated Wnt signaling (Choi *et al.*, 2011). Moreover, SUMOylation-deficient mutants of N-CoR have reduced ability to repress transcription (Tiefenbach *et al.*, 2006). Because GPS2 has two consensus SUMO acceptor sites, K45 and K71, both of which are contained

within the ψ KXE sequence, we speculated that GPS2 might be a target of SUMOylation.

In this article, we show that GPS2 can be modified by SUMO1 and demonstrate that SUMOylation occurs at K45 and K71. SUMOylation of GPS2 appears to promote nuclear localization and increases the protein stability of GPS2 by enhancing the interaction between GPS2 and TBL1 and reducing the ubiquitination of GPS2, consequently leading to enhanced transcriptional suppression activity of GPS2. SUMOylation of GPS2 enhances the ability of GPS2 to suppress estrogen receptor α (ER α)-mediated transcription and cell proliferation.

RESULTS

GPS2 is modified by SUMO1

To examine whether GPS2 can be modified by SUMO, we cotransfected COS-7 cells with hemagglutinin (HA)-tagged GPS2 and Myc-tagged SUMO-1, 2, or 3. Western blot analysis of GPS2 in the cell extracts using anti-HA antibody detected a band with molecular mass much higher than GPS2 when GPS2 was coexpressed with SUMO-1 (Figure 1A), suggesting that GPS2 was conjugated to SUMO-1. To further confirm the SUMOylation of GPS2, we cotransfected COS-7 cells with HA-tagged GPS2 and green fluorescent protein (GFP)-tagged SUMO-1 or GFP-SUMO-1/GA. SUMO-1/GA is a SUMO mutant that lacks the ability to join with the substrate due to a C-terminal diglycine substitution (GG to GA). Extracts prepared from these cells were then subjected to Western blot analysis with anti-HA antibody. As shown in Figure 1B, a band of higher molecular

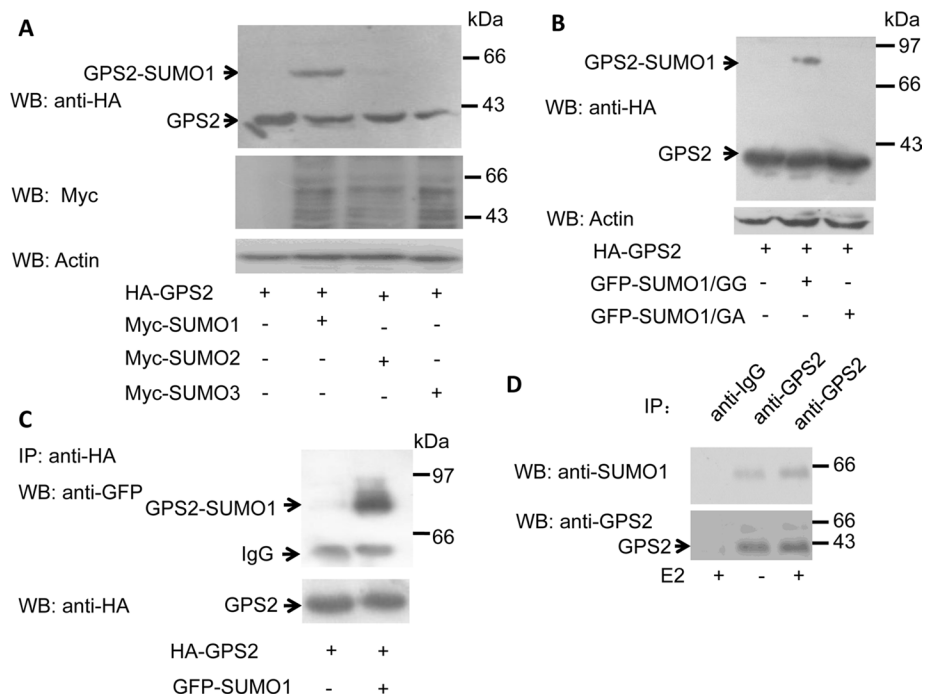


FIGURE 1: Modification of GPS2 by SUMO1. (A) COS-7 cells were cotransfected with HA-tagged GPS2 and Myc-tagged SUMO1, 2, or 3 and then subjected to Western blot with anti-HA antibody. (B) COS-7 cells were cotransfected with HA-tagged GPS2 and GFP-tagged SUMO1/GG or SUMO1/GA and then subjected to Western blot with anti-HA antibody. (C) COS-7 cells were cotransfected with HA-tagged GPS2 together with or without GFP-tagged SUMO1 and then subjected to immunoprecipitation with anti-HA antibody, followed by Western blot with anti-GFP antibody. (D) MCF-7 cells were subjected to serum starvation for 48 h, followed by treatment with 10 nM E2 for 1 h, and the cells were collected and lysed in the presence of *N*-ethylmaleimide (NEM) to prevent deSUMOylation of SUMO-modified proteins. The cell extract was then subjected to immunoprecipitation with anti-GPS2 antibody or anti-immunoglobulin G, followed by Western blot analysis with anti-SUMO1 or anti-GPS2 antibody.

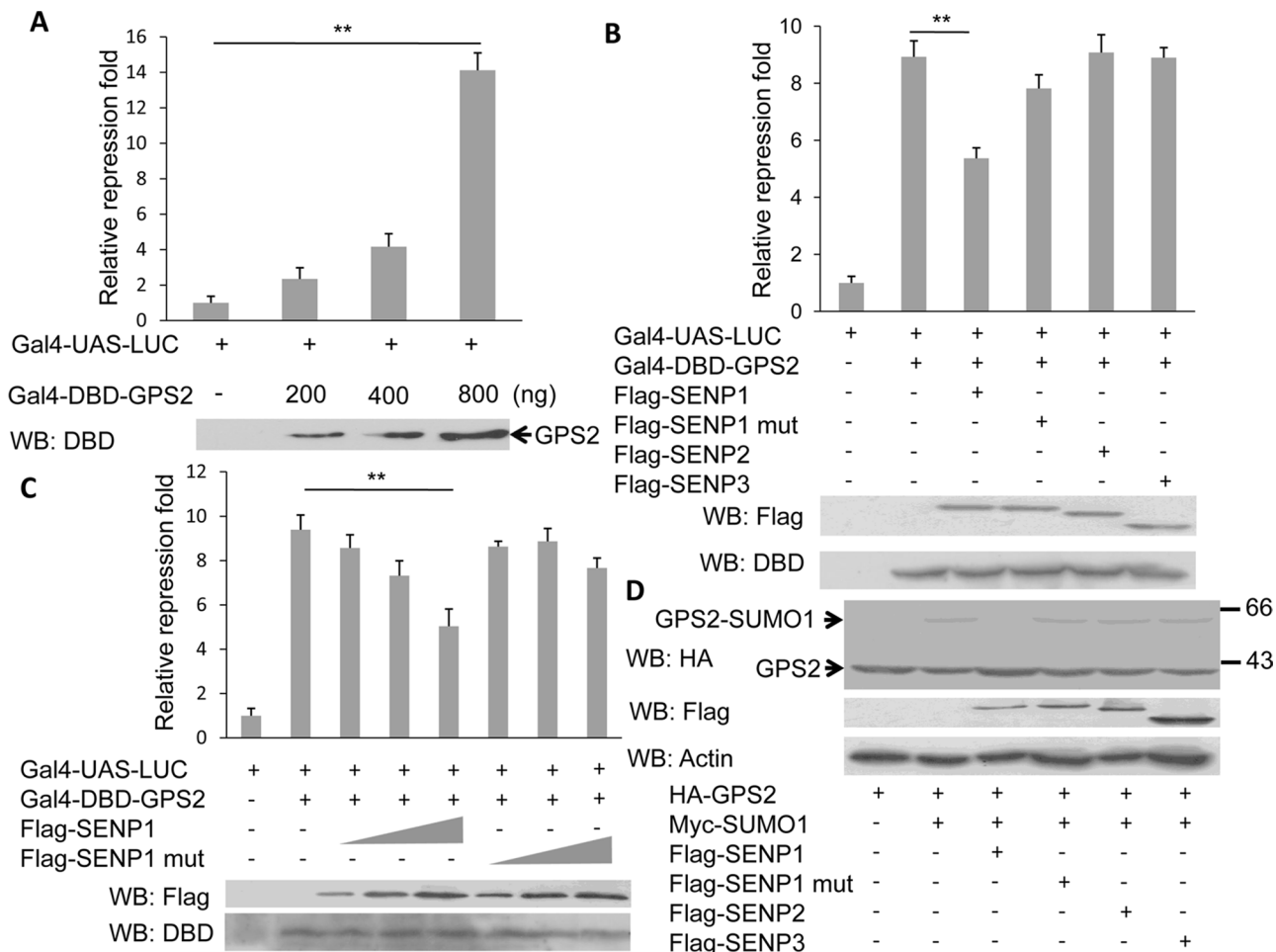


FIGURE 2: Sensitivity of GPS2 SUMOylation to SENP1. (A) COS-7 cells were transfected with GAL4-UAS-Luc and increasing amounts of GAL4-DBD-GPS2. After 24 h, the cells were collected and luciferase activity was measured. $**p < 0.01$ compared with cells transfected with GAL4-DBD-Luc only. (B) COS-7 cells were transfected with GAL4-UAS-luc without and with GAL4-DBD-GPS2 plus Flag-SENP1, SENP1 mut, SENP2, or SENP3. Cells were harvested, and luciferase activity was measured 24 h after transfection. (C) Similar experiments as described in B were performed but with increasing amounts of Flag-SENP1 or SENP1 mut (10, 100, 300 ng). $**p < 0.01$ compared with cells transfected with GAL4-DBD-GPS2 only. (D) COS-7 cells were transfected with different combinations of constructs as indicated below the blot, followed by Western blot with anti-HA antibody. Each bar in the graphs represents the mean \pm SD from three independent experiments.

mass indicative of GPS2-SUMO was detected only when GPS2 was coexpressed with wild-type SUMO1 in the cells (Figure 1B).

In addition, immunoprecipitation analysis of the cell extracts prepared from COS-7 cells cotransfected with HA-tagged GPS2 and GFP-tagged SUMO1 also showed that GPS2 was SUMOylated by SUMO-1 (Figure 1C). The effect of E2 on endogenous SUMOylated GPS2 was also investigated. As shown in Figure 1D, immunoprecipitated GPS2 from MCF-7 cells was detected by anti-SUMO1 antibody, and its level increased when the cells were treated with E2. These data suggested that GPS2 could be modified by SUMO-1 but not SUMO-2 or 3.

SUMOylation of GPS2 is reversed by SENP1

SUMOylation can be reversed by SENP. Three SENPs are present in the cell nucleus: SENP1, 2, and 3. GPS2 is known to function as a transcriptional corepressor (Cheng and Kao, 2009), so we constructed GAL-DBD-GPS2 to identify the effects of SENPs on the activity of GPS2.

The level of reporter activity in COS-7 cells cotransfected with increasing amounts of GAL4-DBD-GPS2 and GAL4-UAS-luc was repressed by GPS2 in a dose-dependent manner (Figure 2A). To determine which SENPs could affect the activity of GPS2, we cotransfected COS-7 cells with GAL4-UAS-luc, Flag-SENP1, SENP2, or SENP3 with GAL4-DBD-GPS2, and measured the level of reporter activity. Only COS-7 cells cotransfected with GAL4-DBD-GPS2 and SENP1 showed reduced level of reporter activity, ~50% the level of cells transfected with GAL4-DBD-GPS2 alone (Figure 2B). In contrast, COS-7 cells cotransfected with GAL4-DBD-GPS2 and SENP2, SENP3, or SENP1 mut (R630L/K631M) had similar levels of reporter activity as cells transfected with GAL4-DBD-GPS2 alone (Figure 2B). Because SENP1 mut is deficient in catalytic activity, the lack of inhibition of reporter activity observed for cells transfected with GAL4-DBD-GPS2 and SENP1 mut was consistent with expectation. The suppression of GPS2-driven reporter activity by overexpression of wild-type SENP1 was dose dependent (Figure 2C), and overexpression of SENP1 without GAL4-DBD-GPS2 exerted almost no effect

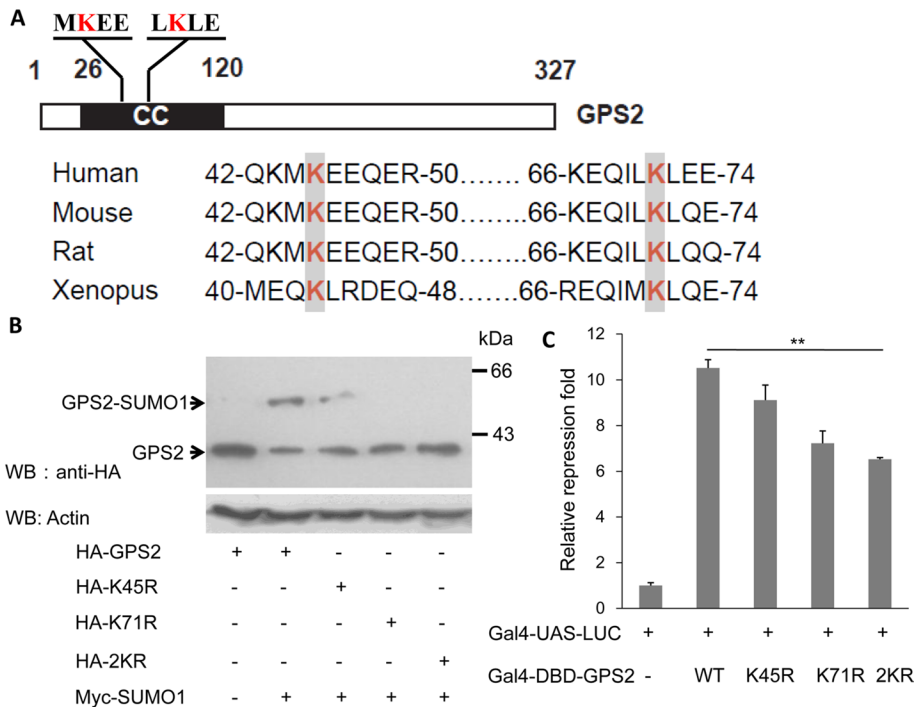


FIGURE 3: Identification of SUMOylation sites in GPS2. (A) Schematic representation of mouse GPS2 (accession number NP_062700.2), showing the conserved lysine residues within the coiled-coil region. The amino acids surrounding the two conserved lysine residues were compared with those of GPS2 from three other species: human (accession number AAB60432.1), rat (NP_001017477.1), and *Xenopus* (AAI58526.1). Conserved lysine residues are boxed. (B) COS-7 cells were transfected with Myc-tagged SUMO1 and HA-tagged wild-type GPS2 or its mutant (K45R, K71R, or K45R/K71R [2KR]) and then subjected to Western blot with anti-HA antibody. For negative control, the cells were transfected with HA-tagged wild-type GPS2 only. (C) COS-7 cells were transfected with Gal4-DBD-tagged wild-type GPS2 or its mutant K45R, K71R, or 2KR together with Gal4-UAS-luc reporter, and luciferase activity was determined 24 h after transfection. ** $p < 0.01$ compared with cells transfected with GAL4-DBD containing wild-type GPS2. Each bar in the graph represents the mean \pm SD from three independent experiments.

on the reporter activity (Supplemental Figure S1). The amount of SUMOylated GPS2 in cells transfected with wild-type SENP1 was reduced compared with the level in the transfected with SENP1 mut, SENP2, or SENP3 (Figure 2D). The data showed that only SENP1 could affect the activity of GPS2 through deSUMOylation, which resulted in lower level of SUMOylated GPS2 and less GPS2-mediated suppression of gene transcription.

SUMOylation of K45 and K71 in GPS2 enhances GPS2-mediated transcriptional repression

Analysis of the GPS2 amino acid sequence revealed two SUMOylation consensus sites (ψ KXE) in the coiled-coil domain, one corresponding to K45 and the other to K71 (Figure 3A). Changing K45 to arginine (K45R) resulted in reduced SUMOylation of GPS2, whereas changing K71 (K71R) or both K45 and K71 to arginine (2KR) appeared to abolish SUMOylation of GPS2 (Figure 3B). This indicated that both K45 and K71 of GPS2 could be SUMOylated and K71 seemed to be the key site. Alternatively, the two SUMOylation sites might interact with each other such that SUMOylation of K71 would facilitate the SUMOylation of K45, and therefore when K71 was changed to arginine, the loss of SUMOylation virtually resulted in little or no detectable SUMOylation at K45.

As a transcription repressor, GPS2 could suppress the basal transcription of a target gene (as illustrated by the activity of luc reporter gene) in a dose-dependent manner (Figure 2A). The N-terminus of

GPS2 comprising the first 120 amino acids (including the two SUMOylation sites) has been demonstrated to be the minimal domain of GPS2 required for its repression of ER activity (Cheng and Kao, 2009). To determine how much influence SUMOylation may exert on the transcriptional suppression activity of GPS2, we cotransfected COS-7 cells with GAL4-DBD-tagged wild-type or mutant GPS2 together with GAL4-UAS-luc and measured the level of reporter activity. Wild-type GPS2 strongly suppressed the reporter activity, but this effect of GPS2 was compromised when either of the two SUMOylation sites was mutated, with further loss (up to 40% of wild type) when both SUMOylation sites were abolished (2KR; Figure 3C). It was obvious that mutation of both K45 and K71 did not abrogate the ability of GPS2 to suppress the activity of the reporter gene, suggesting that besides SUMOylation, there may be other mechanisms regulating the GPS2-mediated suppression of the transcription of the reporter gene.

SUMOylation of GPS2 alters its nuclear distribution

GPS2 is predominantly localized in the nucleus in multiple cell types, such as MCF-7, CV-1, and HeLa cells. Nonetheless, a small fraction of GPS2 is localized in the cytosol. The mechanism that is involved in nuclear-cytosolic trafficking of GPS2 is not clear. Because SUMOylation is known to affect the subcellular localization of a protein (Hong *et al.*, 2011), we speculated that SUMOylation might affect the subcellular localization of GPS2. To test our speculation, we transfected MCF-7 cells with HA-tagged wild-type GPS2 or its mutants K45R, K71R, and 2KR (lacking both SUMOylation sites) and then subjected them to immunofluorescent assay. Much more of the GPS2 protein was localized in the nucleus than in the cytosol in the case of the wild type and the two single mutants, K45R and K71R, with K45R having slightly lower and K71R having higher nuclear level compared with wild type (Figure 4, A and B). This suggested that SUMOylation does have an effect on the subcellular localization of GPS2, since abolishing the K45 site seemed to decrease, whereas abolishing K71 seemed to increase, the level of the mutant protein in the nucleus. On the other hand, the double GPS2 mutant 2KR was found almost equally distributed between the nucleus and cytosol, and this was rather unexpected since we would expect the effect of K45R and K71R to likely cancel out in the double mutant, resulting in a somewhat closer subcellular distribution to that of wild-type GPS2. Nevertheless, the result did suggest that SUMOylation could affect GPS2 shuttling between the nucleus and cytosol.

SUMOylation stabilizes GPS2 by increasing its interaction with GPS2 and TBL1 and reduces its ubiquitination

Because SUMOylation is usually involved in the regulation of protein stability, we also examined the effect of SUMOylation on GPS2 stability. MCF-7 cells were transfected with HA-tagged wild-type or

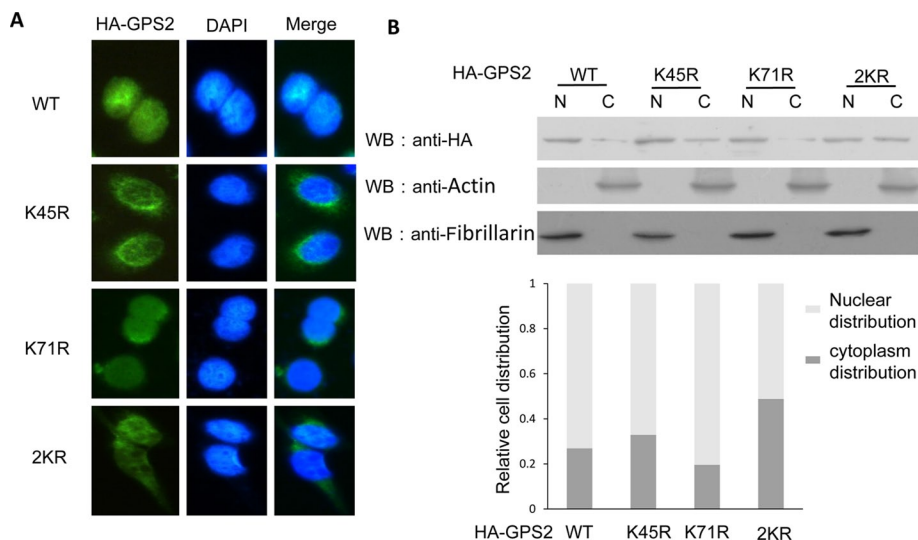


FIGURE 4: Effect of SUMOylation on nucleolar distribution of GPS2. (A) MCF-7 cells transfected with HA-tagged wild-type GPS2 or its mutant K45R, K71R, or 2KR were stained with mouse anti-HA antibody (green) and then counterstained with DAPI (blue) for nucleus detection. (B) MCF-7 cells were transfected with HA-tagged wild-type GPS2 or its mutant K45R, K71R, or 2KR. Cytosolic and nuclear fractions of the cell were subjected to Western blot using anti-HA antibody. Fibrillarlin and β -actin were measured to monitor the efficiency of nuclear and cytosolic preparations, respectively.

mutant GPS2, followed by treatment with the protein translation inhibitor cycloheximide (CHX) for five different time periods. The half-life of wild-type GPS2 was between 6 and 12 h, whereas the half-life of the mutant 2KR was <6 h (Figure 5A).

It has been reported that the stability of GPS2 protein is related to TBL1, since upon interacting with TBL1, GPS2 can become more resistant to proteolysis (Oberoi *et al.*, 2011). The stability of GPS2 mediated by TBL1 is important for it to suppress the activities of other transcription factors. The region of GPS2 that interacts with TBL1 appears to be located in the coiled-coil domain containing the SUMOylation site (K71), suggesting that SUMOylation may effectively influence the interaction between GPS2 and TBL1. MCF-7 cells were cotransfected with Flag-tagged TBL1 and HA-tagged wild-type GPS2 or its mutant 2KR, and the corresponding cellular extracts were then subjected to immunoprecipitation with anti-HA antibody and Western blot analysis with anti-Flag antibody. The band representing TBL1 was not detected in the immunoprecipitation result when the cells were transfected with the GPS2 mutant 2KR (Figure 5B), indicating that SUMOylation of GPS2 was necessary for its association with TBL1. Furthermore, overexpression of TBL1 also increased the protein level of wild-type GPS2 but not of its mutant 2KR (Figure 5C).

Degradation of GPS2 via the 26S proteasome pathway requires the covalent attachment of multiple ubiquitin molecules to GPS2. To investigate whether the increased stability of GPS2 that resulted from its SUMOylation was a result of suppression of its ubiquitination, we transfected MCF-7 cells with HA-tagged GPS2 or mutant 2KR and Myc-tagged ubiquitin and compared the level of GPS2 ubiquitination with that of MCF-7 cells transfected with HA-tagged wild-type GPS2 and Myc-tagged ubiquitin (Figure 5D). Ubiquitination of wild-type GPS2 was reduced compared with the mutant, indicating that SUMOylation of GPS2 may inhibit its ubiquitination, resulting in increased stability for the protein. As for the relationship between TBL1 and the ubiquitination of GPS2, its clarification requires further study.

SUMOylation of GPS2 suppresses ER α -mediated transcriptional activity and cell proliferation

Previous studies showed that GPS2 acts as a transcription repressor that suppresses ER α -mediated transcription by being recruited along with SMRT and other corepressors (e.g., HDAC3) onto the promoter of a target gene and then forming a transcription-repressive complex (Cheng and Kao, 2009). Thus we first examined whether SUMOylation of GPS2 could influence its interaction with SMRT and HDAC3.

MCF-7 cells were cotransfected with Myc-tagged SMRT and Flag-tagged HDAC3 together with HA-tagged wild-type GPS2 or its mutant 2KR, followed by immunoprecipitation with anti-HA antibody and Western blot analysis with anti-Myc, Flag, or HA antibody. The result showed that only the wild-type GPS2 coprecipitated with SMRT and HDAC3 (Figure 6A), suggesting that SUMOylation of GPS2 was necessary for the formation of the SMRT repressive complex.

We also used the estrogen-responsive element-reporter gene construct (ERE-luc) to examine whether SUMOylation would affect the suppressive function of GPS2 with respect to ER α -mediated transcriptional activity.

MCF-7 and T47D cells transfected with ERE-luc and wild-type GPS2 showed reduced level of ER α -mediated transcriptional activity compared with the same cells transfected with ERE-luc and K45R, K71R, or 2KR (Figure 6B). The effect of GPS2 SUMOylation on the regulation of *cyclin D1* expression, a well-established ER α -target gene, was also examined. Wild-type GPS2 suppressed the expression of *cyclin D1* in the presence or absence of E2, but this effect of GPS2 was compromised when either of the two SUMOylation sites was mutated, whereas the double mutant 2KR did not suppress the expression of *cyclin D1* in the absence and presence of E2 (Figure 6C).

GPS2 is important for maintaining normal proliferation of MCF-7, and knockdown of GPS2 promotes cell proliferation (Cheng and Kao, 2009). The role of GPS2 SUMOylation in cell proliferation was investigated by determining the effect of GPS2 SUMOylation on the growth of MCF-7 and T47D cells. As shown in Figure 6D, MCF-7 cells overexpressing wild-type GPS2 showed weaker growth than those overexpressing 2KR. Similar results were observed for T47D cells (Figure 6D). These data indirectly demonstrated that SUMOylation of GPS2 could repress the proliferation of breast cancer cells.

DISCUSSION

Posttranslational modification of proteins endows proteins with multiple functions. SUMOylation, an important posttranslational modification, plays a major role in regulating protein stability, localization, protein-protein interaction, and transcriptional activity. GPS2 is a suppressor of the G protein pathway, and it plays important roles in many physiological processes. However, very few studies have focused on the posttranslational modification of GPS2. We first analyzed the primary sequence of GPS2 and found two potential SUMOylation sites. Subsequent experiments demonstrated that GPS2 could be modified by SUMO-1 but not SUMO-2/3, and SUMOylation of GPS2 promoted its stability, nuclear localization, and ability to suppress transcription.

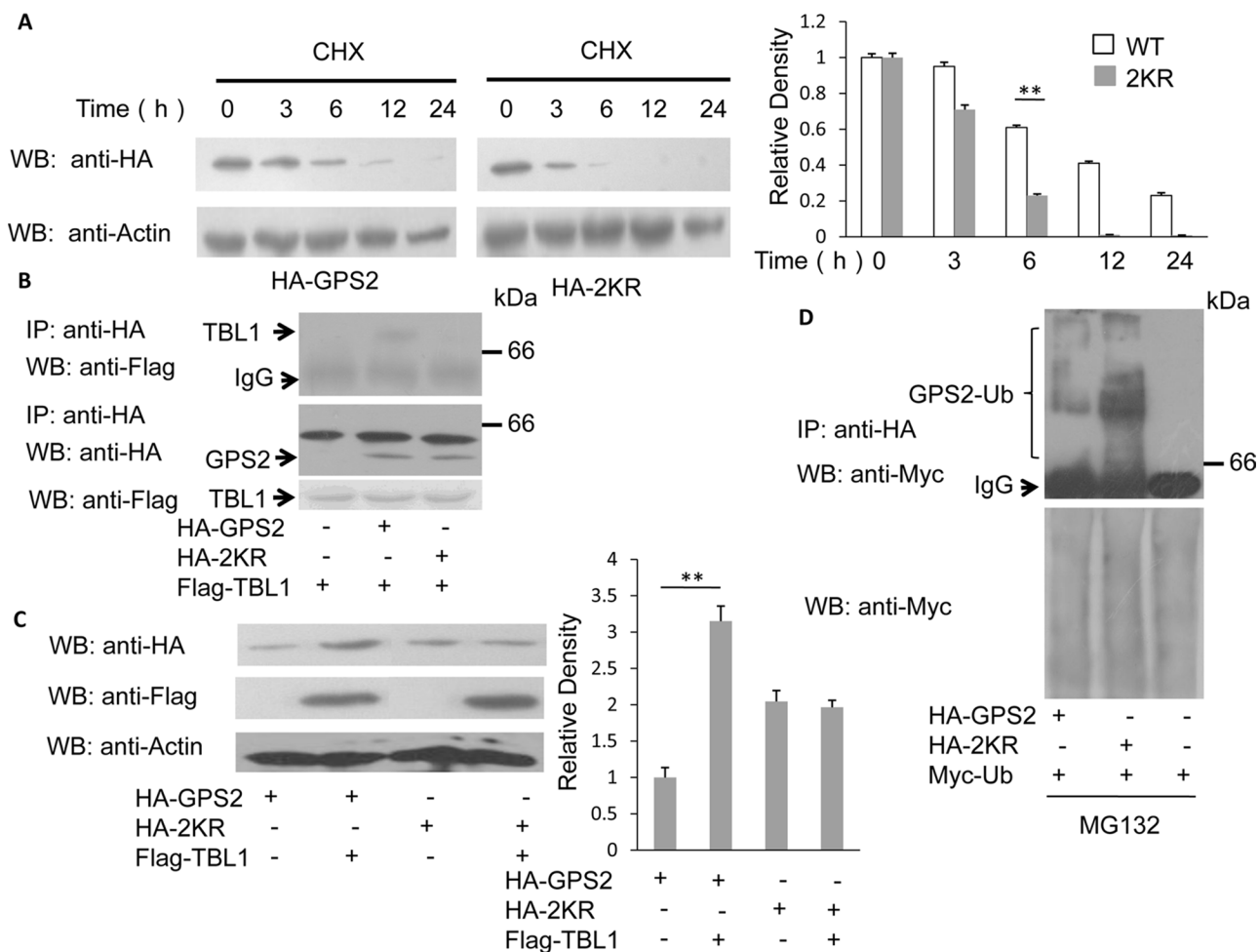


FIGURE 5: Effect of SUMOylation on the protein stability of GPS2. (A) MCF-7 cells were transfected with HA-tagged wild-type GPS2 or its mutant 2KR and then treated with CHX at the indicated time periods. Cell lysate was subjected to Western blot with anti-HA antibody. The graph shows the relative intensity of the GPS2 bands at those time points. $**p < 0.01$ compared with cells transfected with HA-GPS2. Each bar in the graphs represents the mean \pm SD from three independent experiments. (B) MCF-7 cells were cotransfected with Flag-tagged TBL1 together with HA-tagged GPS2 or its mutant 2KR and then subjected to immunoprecipitation with anti-HA antibody, followed by Western blot with anti-Flag or anti-HA antibody. (C) MCF-7 cells were cotransfected with HA-tagged GPS2 or its mutant 2KR without or with Flag-tagged TBL1 and then subjected to Western blot with the antibodies indicated on the left side of the blot. $**p < 0.01$ compared with cells transfected with HA-GPS2 only. Each bar in the graphs represents the mean \pm SD from three independent experiments. (D) MCF-7 cells were transfected with HA-tagged wild-type GPS2 or its mutant 2KR together with Myc-tagged ubiquitin and then treated with MG132, a proteasome inhibitor, for 6 h before harvest. Cell lysate was subjected to immunoprecipitation with anti-HA antibody, followed by Western blot with anti-Myc antibody.

SUMO is conjugated to a lysine residue on a protein (Gill, 2004). The classic strategy for studying the SUMOylation of proteins is to mutate a specific lysine residue believed to be the SUMOylation site. The K-to-R mutation might slightly influence the structure of substrate proteins. However, this strategy would effectively eliminate its ability to conjugate with SUMO while introducing minimal change to the structure of the protein, since the lysine-to-arginine change is considered to be a conservative substitution because of the greater similarity between these two amino acids compared with other amino acids. As expected, changing K45 and K71 to arginine (2KR) abolished SUMOylation of GPS2 (Figure 3B) because arginine could not form a covalent bond with SUMO. It also resulted in a somewhat reduced level of noncovalent interaction between GPS2 and SUMO (Supplemental Figure S2), probably because of the change that the substitution had on the structure of GPS2. However, the effect was still small, and therefore the inability of the

mutant GPS2 to become modified by SUMO was a result of its inability to form a covalent bond with SUMO. In addition to being subject to SUMOylation, GPS2 also interacts with other SUMOylated proteins. For example, in the hepatic acute-phase response, GPS2 interacts with SUMOylated LXR β or LRH-1 via its SUMO-interacting motif (SIM), and such interaction prevents the dissociation of the N-CoR complex, leading to the repression of acute-phase genes (Venteclef *et al.*, 2010). This suggests that changing the amino acid of a residue within the SIM (residues 61–94) of GPS2 to a different amino acid will likely affect its interaction with SUMO or SUMO-bearing proteins. Because K71 is within the SIM, some reduction in noncovalent interaction between GPS2 and SUMO from the replacement of K71 with arginine is consistent with the role of SIM. However, the loss of noncovalent interaction between SUMO and GPS2 was small in the case of GPS2 mutant 2KR. Thus it is relevant to conclude that SUMOylation does play an important role in

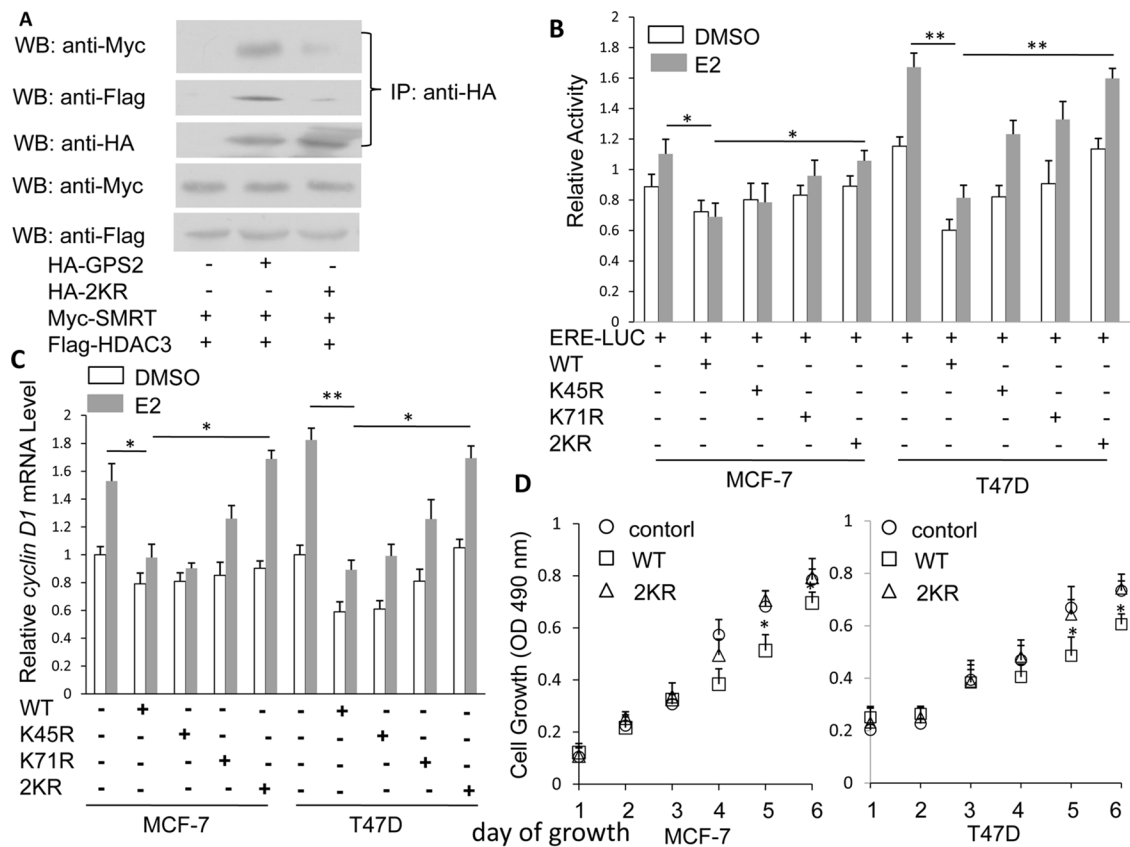


FIGURE 6: Effect of GPS2 SUMOylation on ER α -mediated transcriptional activity and cell proliferation. (A) MCF-7 cells were cotransfected with Myc-tagged SMRT and Flag-tagged HDAC3 together with HA-tagged GPS2 or its mutant 2KR and then subjected to immunoprecipitation with anti-HA antibody, followed by Western blot with anti-Myc, anti-Flag, or anti-HA antibodies. (B) MCF-7 and T47D cells were transfected with HA-tagged wild-type GPS2 or its mutants K45R, K71R, and 2KR together with ERE-luc. The cells were subjected to serum starvation for 48 h and then treated with or without E2 (10 nM) for 16 h before luciferase activity was determined. * $p < 0.05$ and ** $p < 0.01$ compared with cells transfected with ERE-Luc only or ERE-Luc and GPS2 2KR in the presence of E2. Each bar represents the mean \pm SD from three independent experiments. (C) MCF-7 and T47D cells were transfected with HA-tagged GPS2 or its mutant K45R, K71R, or 2KR and then subjected to serum starvation for 48 h, followed by E2 or no E2 treatment for 4 h before analysis of *cyclin D1* expression by reverse transcription PCR. The mRNA level of *cyclin D1* was expressed relative to *GAPDH* transcription level. * $p < 0.05$ and ** $p < 0.01$ compared with cells transfected with control vector or GPS2 2KR in the presence of E2. Each bar represents the mean \pm SD from three independent experiments. (D) MCF-7 and T47D cells were transfected with HA-tagged GPS2 or its mutant 2KR, and subjected to serum starvation for 48 h, and then treated with E2 (10 nM) for the indicated times. The cells were then subjected to MTT assay performed according to the manufacturer's instructions (KeyGEN, Nanking, China). * $p < 0.05$ compared with cells transfected with control vector. Each bar represents the mean \pm SD from five independent experiments.

regulating the activity and stability of GPS2 and its subsequent interaction with other transcription factors.

Besides SUMOylation, several other protein modifications, such as methylation, acetylation, and ubiquitination, also occur on lysine residues. Histone H3K9, which can be modified by acetylation and trimethylation, is associated with activation and repression of transcription, respectively (Babbio *et al.*, 2012; Kimura, 2013). To our knowledge, there has been no other reported study investigating the posttranslational modification of GPS2. Although we identified two lysine residues of GPS2 that could be modified by SUMO, we do not know whether they are also subject to other forms of modification, and therefore further study is needed to clarify this issue. Nevertheless, our result did show that modification of K45 and K71 by SUMOylation would affect the function of GPS2.

Protein modification by SUMOylation is a dynamical process, driven by SUMOylation and deSUMOylation. Overexpression of

SUMO in the cells would help to push the process toward more SUMOylation, effectively making it easier to detect any changes associated with the phenomenon, such as a change in the level of SUMOylated target protein, which in our case is GPS2. For this reason, we therefore resorted to overexpression of GPS2 and SUMO in order to amplify the signal, making it easier to detect the changes associated with GPS2 SUMOylation that would otherwise be difficult to discern if one relied entirely on the endogenous SUMO and GPS2.

The intracellular localization of several proteins, such as cellular retinoic acid-binding protein II (CRABP-II), Daxx, and NEMO, is regulated by SUMOylation. GPS2 is synthesized in the cytosol but is transported into the nucleus, where it functions as a transcription factor. The transport of a protein from the cytosol into the nucleus is mediated by the protein's nuclear localization signal (NLS), which binds to a heterodimeric receptor on the membrane. This receptor consists of importin- α and β subunits (Kohler *et al.*, 1999). Mutation

of K102 (a SUMOylation site) of CRABP-II abolishes the ability of CRABP-II to localize to the nucleus in response to retinoic acid (RA), which shows that SUMOylation is critical for the RA-induced nuclear mobilization of CRABP-II (Majumdar *et al.*, 2011). Daxx is another NLS-containing protein that is also subject to modification by SUMOylation, and its SUMOylation site is located just within the NLS sequence. SUMOylation of Daxx enhances its transport into the nucleus, whereas loss of SUMOylation site in Daxx leads to a reduction in its nuclear level (Jang *et al.*, 2002). Passage of proteins into the nucleus facilitated by SUMOylation has also been demonstrated with SUMO-1–fused NF- κ B essential modulator (NEMO), in which wild-type NEMO tends to localize in the cytosol, whereas SUMO-1–fused NEMO is found in both cytosol and nucleus (Huang *et al.*, 2003). These findings suggest that SUMOylation regulates the transport of certain proteins into and out of the nucleus. The N-terminal 50 residues of GPS2 also contained a putative NLS sequence, as predicted by computer program. GPS2 is mainly localized in the nucleus but is also found in the cytosol in some cells. Similarly, SUMOylation of GPS2 also promoted its localization to the nucleus (Figure 4). The attachment of SUMO to a protein often causes a change in the conformation of the protein, especially the region close to the site of attachment. Unlike the SUMOylation sites of Daxx, the SUMOylation site of GPS2, K45, was located near the putative NLS, meaning that SUMOylation at K45 may have an effect on the function of the NLS of GPS2, probably by reducing its transportation into the nucleus, since abolishing SUMOylation at this K45 slightly reduced the nuclear distribution of the mutant GPS2. From the perspective of single mutation, the lightly lower distribution of K45R in the nucleus might have resulted in loss of repression of reporter activity shown in Figure 3C. However, in the case of K71R, one would expect its slightly higher nucleus distribution to have compensated for the loss of transcriptional repression activity of the individual protein. Instead of seeing a similar level of repression as for K45R, K71R showed a greater loss of repression, and this clearly indicated that loss of transcriptionally repressive activity of GPS2 mutants was due mainly to loss of SUMOylation and not mislocalization. The data for the subcellular localization of the double mutant 2KR (Figure 4) appeared to suggest that change in the transcriptional repression activity of GPS2 was probably due to its mislocalization, which was in turn caused by deficiency in SUMOylation. However, the extent of reduction in nucleus distribution for 2KR did not parallel the extent of loss of transcriptionally repressive activity compared with K71R. This could be taken as supporting evidence that loss of SUMOylation and not mislocalization was the major factor leading to loss of transcriptional repression activity of GPS2.

SUMOylation is also known to regulate the stability of a protein. Consistent with this feature is the shorter half-life (<6 h) of the mutant GPS2 compared with wild type (6–12 h; Figure 5A). SUMOylation is also known to influence protein–protein interaction, and we also demonstrated that interaction between GPS2 and TBL1 inhibited the proteasome-mediated degradation of GPS2, suggesting that SUMOylation might regulate the interaction between GPS2 and TBL1. SUMOylated GPS2 contributed to its association with TBL1 and therefore increased its stability, a property that would be favorable to its regulation of cell functions. Because SUMOylation and ubiquitination are both lysine-targeted modifications, the antagonistic relationship between SUMOylation and ubiquitination may play an important role in regulating GPS2 activity.

SUMOylation regulates the transcriptional activity of a range of transcription factors, such as AIB1 (Wu *et al.*, 2006), CLOCK (Li *et al.*, 2013), and DEC1 (Hong *et al.*, 2011). For example, phosphorylation of AIB1 by ERK promotes the transcriptional activity of AIB1, whereas

SUMOylation of AIB1 has the opposite effect, and these two modifications seem to antagonize each other in regulating the activity of AIB1 (Wu *et al.*, 2006). Another example is DEC1, in which SUMOylation has the effect of promoting its interaction with HDAC1, an important component that contributes to the repressive activity of DEC1 (Hong *et al.*, 2011). The GC box–binding transcription factor SP3 is a substrate of SUMO-1, and it has both transcriptional activation and repression activities. Modification of SP3 by SUMO1 is an important switch that determines whether SP3 functions as an activator or a repressor. SUMOylated SP3, which corresponds to the transcriptionally repressed form, is localized to the nuclear periphery and nuclear dots, whereas the deSUMOylated form SP3 or SP3 mutant lacking the SUMOylation site corresponds to the transcriptionally active form and exhibits a more diffuse nuclear distribution (Ross *et al.*, 2002; Sapetschnig *et al.*, 2002). Thus it is obvious that SUMOylation regulates the transcriptional activity of different proteins in multiple ways.

GPS2 was previously shown to interact with N-CoR/SMRT complex through its N-terminal domain, and the resulting GPS2-N-CoR/SMRT complex has a repressive effect on JNK signaling pathway (Zhang *et al.*, 2002). The two SUMOylation sites of GPS2 are localized in the coiled-coil domain, which is in the N-terminus, and SUMOylation of GPS2 is expected to affect its conformation, possibly promoting its interaction with SMRT.

As a component of SMRT corepressor complexes, GPS2 is important for optimal TAM-mediated repression of ERE-reporter activity or ER α target genes, which also provides mechanistic insights into how the SMRT corepressor complex mediates transcriptional repression of ER target genes (Cheng and Kao, 2009). Because GPS2 does not have a nuclear receptor-interacting motif (LXXLL) and probably does not bind ER directly, it may in fact be recruited to the SMRT complex via SMRT itself. Thus the interaction between GPS2 and SMRT could be crucial for negatively regulating the transcriptional activity of ER that is mediated by GPS2. SUMOylation of GPS2 may regulate the activity of ER in two ways: by promoting its ability to suppress other transcription factors, and by enhancing the interaction between GPS2 and SMRT. The latter would be necessary for GPS2-mediated suppression of ER activity, which has the effect of modulating the transcriptional regulation of ER-target genes.

Cell cycle proteins play important roles in cell proliferation and differentiation. Those cell cycle proteins that control the G1- to S-phase transition, particularly cyclin D1, are commonly upregulated in breast cancer cells, leading to defects in mammary development and contributing to breast tumorigenesis, presumably by increasing cell proliferation (Caldon *et al.*, 2010). Previous study shows that knockdown of GPS2 in MCF-7 cell increases E2-induced expression of *cyclin D1* and *pS2* and promotes the proliferation of the cell (Cheng and Kao, 2009). This is consistent with our data, which showed that overexpression of GPS2 reduced the expression of *cyclin D1* in MCF-7 and T47D cells and the proliferation of these two cell lines (Figure 6, C and D). The suppression of *cyclin D1* by GPS2 is subject to regulation by SUMOylation, since mutant GPS2 that could not be SUMOylated clearly had no effect on the expression of *cyclin D1* and cell proliferation (Figure 6, C and D). One possible explanation could be that the effect of SUMOylated GPS2 on the proliferation of breast cancer cells was regulated by the recruitment of SMRT, which could effectively enhance the transcriptional-repressive activity of GPS2.

In summary, we demonstrated in this study that the function and stability of GPS2 may depend on its SUMOylation status and identified K45 and K71 as the SUMOylation sites, with K71 appearing to be the principal site. SUMOylation of GPS2 also promoted its

nuclear localization and enhanced its interaction with SMRT. The ultimate effect of GPS2 SUMOylation at the cell level was the suppression of cell proliferation, as demonstrated for two different breast cancer cell lines. This extends our understanding of the mechanism behind GPS2-mediated regulation of transcription factors and the consequent inhibition of breast cancer cell proliferation. However, much still needs to be explained, such as how SUMOylation can change the activity of GPS2 and what changes occur in the structure of SUMOylated GPS2.

MATERIALS AND METHODS

Cell culture and plasmids

COS-7, MCF-7, and T47D cells were used in our previous work (Venteclef *et al.*, 2010; Li *et al.*, 2013). COS-7 and MCF-7 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Beijing, China) and penicillin-streptomycin (100 U/ml penicillin and 0.1 mg/ml streptomycin). T47D cells were maintained in RPMI-1640 medium (Invitrogen, Auckland, New Zealand) supplemented with 0.2 U/ml insulin and 10% FBS. Cells were incubated at 37°C in a humidified incubator with 5% CO₂. HA-GPS2 was a gift kindly provided by Darryl C. Zeldin (Duke University Medical Center, Durham, NC). GFP-SUMO1 and its mutant GFP-SUMO1/GA were provided by Jorma J. Palvimo (University of Eastern Finland, Kuopio, Finland). The construct Myc-SMRT was obtained from Ho Geun Yoon (Yonsei University College of Medicine, Seoul, South Korea), and Flag-TBL1 was provided by Stephan Herzig (University Hospital Heidelberg, Heidelberg, Germany). The mutants HA-K45R, K71R, and 2KR were prepared using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA) with HA-GPS2 as template. pcDNA3.1/pGal4-DBD-GPS2 was prepared by cloning the full-length GPS2 gene into pcDNA3.1-Gal4-DBD. pcDNA3.1/ERE luciferase reporter was provided by Carolyn L. Smith (Baylor College of Medicine, Houston, TX).

GAL4-DBD-GPS2 is a fusion construct containing the DNA-binding domain of the yeast transcription activator protein (GAL4) and the DNA sequence of GPS2. GAL4-UAS-Luc reporter construct used in our previous study was purchased from Promega (E1370; Madison, WI; Li *et al.*, 2013; Yang *et al.*, 2013). It contains 9×GAL4 UAS and the DNA sequence of luciferase. The UAS effectively acts as the promoter of the luciferase. The DBD portion of DBD-GPS2 fusion protein binds to the UAS in the GAL-UAS-Luc construct, enabling GPS2 to regulate the activity of the luciferase. Thus change in luciferase activity reflects the effect of GPS2 on the expression of luciferase, and this indirectly shows the transcriptional activity of GPS2.

Antibodies and reagents

Rabbit polyclonal anti-Flag, anti-GFP, anti-HA, and anti-GPS2, and mouse monoclonal anti-actin and anti-SUMO1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Myc and mouse monoclonal anti-Flag (M2) antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Cycloheximide was obtained from Sigma-Aldrich, and MG132 was obtained from Calbiochem (San Diego, CA). Clean Blot IP Detection Reagent was purchased from Thermo Scientific (Rockford, IL).

Reporter assays

COS-7 cells were grown in 24-well plates and transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's specification. At 24 h after transfection, the cells were rinsed with phosphate-buffered saline (PBS)

and subjected to luciferase and *Renilla* activity assays using a dual luciferase kit (Promega, Madison, WI). MCF-7 and T47D cells were plated in 24-well plates and transfected with the appropriate plasmids. Eight hours after transfection, the cells were switched to phenol red-free medium containing 10% charcoal-dextran-treated FBS for 48 h, followed by treatment with or without 10 nM 17-estradiol (E2; Abcam, Hong Kong, China) for another 16 h. The cells then were harvested and subjected to luciferase and *Renilla* activity assays.

Western blot and immunoprecipitation

MCF-7 and COS-7 cell were lysed in cold buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, and protease inhibitor mixture (Roche Applied Science, Indianapolis, IN) and then subjected to SDS-PAGE. Proteins in the gel were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA) and probed with the specified primary antibody, followed by the appropriate secondary antibody, and then visualized using the enhanced chemiluminescence detection reagents (Thermo) according to the manufacturer's instructions.

Immunoprecipitation experiments were carried out using COS-7 and MCF-7 cell extracts. Cells were lysed in cold buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, and protease inhibitor mixture. The cell lysate was centrifuged at 12,000 × *g*/4°C for 10 min, and the supernatant was incubated with protein A-Sepharose (GE Healthcare Bio-Sciences, Uppsala, Sweden) at 4°C for 1 h. It was then centrifuged at 5000 × *g*/4°C for 10 min, and the supernatant was incubated with fresh protein A-Sepharose and the desired antibody at 4°C for overnight. After that, the mixture was centrifuged at 5000 × *g*/4°C for 5 min, and the pellet was retained and washed twice with wash buffer I (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1% NP-40, and 0.05% sodium deoxycholate) and once with wash buffer II (50 mM Tris-HCl, pH 7.5, 500 mM sodium chloride, 0.1% NP-40, and 0.05% sodium deoxycholate). After washing, it was resuspended in SDS-PAGE loading buffer, heated at 100°C for 5 min, and then resolved in 8 or 10% gel.

RNA extract and reverse transcription-PCR

MCF-7 cells were starved for 48 h and then treated with 10 nM E2 for 4 h before they were harvested. Total RNA was extracted from the cells using Takara RNAiso Reagent (Takara, Dalian, China). Total RNA (1 µg) was reverse transcribed using oligo (dT) primer and a Reverse Transcription System (Takara). The single-stranded cDNA was amplified by PCR using specific primers, and the PCR products were analyzed by 1% agarose gel electrophoresis. The following primers were used: *cyclin D1*, 5'-GCTGCTCCTGGTGAACAAGC-3' and 5'-AAGTGTTCATGAAATCGTGCG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GGGTGTGAACCATGAGAAGT-3' and 5'-GACTGTGGTCATGAGTCCT-3'. *cyclin D1* mRNA level was normalized with a GAPDH mRNA level.

Immunofluorescence

MCF-7 cells cultured on coverslips were cotransfected with HA-tagged GPS2 or its mutant K45R, K71R, or 2KR. Cells were then washed three times with cold PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, permeabilized with cold methanol for 15 min at -20°C, and then blocked with 0.8% bovine serum albumin for 30 min at room temperature. Finally, the cells were stained with rabbit anti-HA antibody (1:100), followed by anti-rabbit secondary antibody (1:100). The cell nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI; Roche Applied Science, Mannheim, Germany).

Preparation of cytoplasmic and nuclear fractions

Cells were washed with PBS three times and collected in a microcentrifuge tube. The cell pellet was resuspended in cytoplasmic extraction buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 5 mM dithiothreitol [DTT]) containing protease inhibitors, followed by incubation on ice for 10 min. The cell suspension was vortexed for 10 s to break the cytoplasmic membrane. The extract was centrifuged at 2000 × g/4°C for 10 min. The supernatant was collected and designated as the cytoplasmic fraction. The pellet was dissolved in nuclear extraction buffer (20 mM Tris-HCl, pH 7.4, 400 mM KCl, 2 mM EDTA, 2 mM MgCl₂, 1 mM DTT, 10% glycerol) containing protease inhibitors, followed by freezing at -80°C and thawing at 42°C. The freeze/thaw cycle was repeated five times. After vigorous vortexing, the suspension was centrifuged at 12,000 × g/4°C for 10 min, and the supernatant was collected and designated as the nuclear fraction.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell growth assay was performed using the MTT assay. MCF-7 and T47D cells were stably transfected with the control vector or HA-GPS2 (wild-type or mutant). MTT assay was performed as previously described (Venteclef *et al.*, 2010).

Statistical analysis

All statistical analyses of data were performed with analysis of variance with the least significant difference method. Data are given as mean ± SD, and significance was considered at either the $p < 0.05$ or 0.01 level. All experiments were repeated at least three times.

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