Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;45:2283-2292
DOI: 10.1159/000488174
Published online: March 16, 2018
Accepted: February 13, 2018







**Original Paper**

# Sumoylation of EphB1 Suppresses Neuroblastoma Tumorigenesis via Inhibiting PKCγ Activation

Qin Chen[a, c] Rong Deng[a] Xian Zhao[a] Haihua Yuan[a] Hailong Zhang[a]
Jinzhuo Dou[a] Ran Chen[a] Hui Jin[a] Yanli Wang[a] Jian Huang[a] Jianxiu Yu[a, b]

[a]Department of Biochemistry and Molecular Cell Biology, Shanghai Key Laboratory of Tumor Microenvironment and Inflammation, Shanghai Jiao Tong University School of Medicine, Shanghai, [b]State Key Laboratory of Oncogenes and Related Genes, Shanghai Jiao Tong University School of Medicine, Shanghai, [c]Department of Chemistry, Fudan University, 220 Handan Lu, Shanghai, China

**Key Words**
Neuroblastoma • EphB1 • SUMOylation • PKCγ • Tumorigenesis

**Abstract**
***Background/Aims:*** An increasing number of studies have linked erythropoietin-producing hepatocellular carcinoma (Eph) family receptor tyrosine kinases to cancer progression. However, little knowledge is available about the regulation of their functions in cancer. ***Methods:*** SUMOylation was analyzed by performing $Ni^{2+}$-NTA pull-down assay and immunoprecipitation. Cell proliferation, anchorage-independent growth, and tumorigenesis *in vivo* were examined by cell counting kit-8, soft agar colony formation assay, and a xenograft tumor mouse model, respectively. ***Results:*** We found that EphB1 was post-translationally modified by the small ubiquitin-like modifier (SUMO) protein at lysine residue 785. Analysis of wild-type EphB1 and SUMOylation-deficient EphB1 K785R mutant revealed that SUMOylation of EphB1 suppressed cell proliferation, anchorage-independent cell growth, and xenograft tumor growth. Mechanistic study showed that SUMOylation of EphB1 repressed activation of its downstream signaling molecule PKCγ, and consequently inhibited tumorigenesis. A reciprocal regulatory loop between PKCγ and SUMOylation of EphB1 was also characterized. ***Conclusion:*** Our findings identify SUMO1 as a novel key regulator of EphB1-mediated tumorigenesis.



## Introduction

The erythropoietin-producing hepatocellular carcinoma (Eph) receptors represent the largest family of receptor tyrosine kinases and are comprised of two subclasses, EphA and EphB. These receptors have been implicated in regulating cancer progression. However,

Q. Chen and R. Deng contributed equally to this work.

Qin Chen
and Jianxiu Yu

Department of Biochemistry and Molecular Cell Biology,
Shanghai Jiao Tong University School of Medicine (SJTU-SM), Shanghai (China)
Tel. 86-21-54660870, 86-21-54660870, E-Mail qinchen77@hotmail.com, Jianxiu.Yu@gmail.com

they act as both tumor promoters and suppressors depending on cancer type and cellular context [1-3]. EphB1 is overexpressed in migrating medulloblastoma cells and contributes to medulloblastoma cell migration, growth, and radiosensitization [4, 5]. Conversely, in renal cell carcinomas, glioma, and ovarian, gastric, and colorectal cancers, EphB1 expression is downregulated [6-9]. EphB1 plays a tumor suppressor role in acute myelogenous leukemia (AML) by regulating DNA damage response [10]. However, the role of EphB1 in neuroblastoma has not been clarified. Neuroblastoma is a common biologically and clinically heterogeneous pediatric tumor of neural crest origin. These reported paradoxical functions of EphB1 make it of interest to investigate the role of EphB1 in neuroblastoma.

Despite accumulating evidence linking EphB1 to cancer, little is known about the regulation of EphB1 by post-translational modification. So far, phosphorylation and ubiquitination have been reported to be involved in post-translational modulation of EphB1 activity. Ligand binding induces EphB1 oligomerization and subsequent phosphorylation at specific tyrosine residues in the cytoplasmic domain, which interacts with a number of cytoplasmic signaling molecules such as Grb2, Nck, and Src [11-13]. Following ligand stimulation, EphB1 is ubiquitinated by the E3 ubiquitin ligase Cbl and is subsequently degraded through the lysosomal pathway [14].

SUMOylation is an enzymatic cascade reaction that involves covalent and reversible conjugation of small ubiquitin-like modifiers (SUMO) to specific lysine residues in protein substrates. This form of post-translational modification regulates protein activity, sub-cellular localization, and stability [15]. Several studies show that SUMOylation can target various proteins including nuclear and cytoplasmic proteins, but little is known about SUMOylation of transmembrane proteins [16-19].

Here, we show that receptor tyrosine kinase EphB1 is modified by SUMO1 at lysine residue K785. EphB1 SUMOylation then suppresses activation of PKCγ, and thus inhibits tumorigenesis. This study therefore uncovers a novel regulatory mechanism by which the function of EphB1 in tumorigenesis is specifically modulated.

## Materials and Methods

*Antibodies and reagents*

Antibodies against Flag M2 (#F1804), HA (#H3663), and His-tag (#SAB2702218) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-SUMO1 (#ab32058) and anti-SENP1 (#ab108981) antibodies were from Abcam Inc. (Cambridge, MA). Antibodies against EphB1 (#sc-926), PKCγ (#sc-211) and normal mouse IgG (#sc-2025) were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphor-PKCγ antibody (#9379) was purchased from Cell Signaling Technologies Inc., (Danvers, MA). $Ni^{2+}$-NTA agarose beads were obtained from Qiagen (Valencia, CA). Protein G plus / Protein A agarose suspension (#IP05) was from Calbiochem (San Diego, CA). Puromycin (#P8833) and polybrene (#H9268) were from Sigma-Aldrich (St. Louis, MO).

*Cell culture*

Human neuroblastoma SH-SY5Y cells were purchased from ATCC (Manassas, VA) and cultured in RPMI 1640 containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. NG108, Neuro-2a, HEK293T, and 293FT cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. All the cells were grown at 37 °C in a humidified incubator under 5% $CO_2$.

*Plasmids construction and transfection*

The pcDNA3.1-EphB1 plasmid was kindly provided by Dr Nanjie Xu. HA-EphB1 cDNA was subcloned into the lentiviral vector (System Biosciences LLC, Palo Alto, CA) carrying *puromycin* and *EGFP* genes. The shRNAs were subcloned into pLKO.1 vector. PKCγ shRNA CCGGGCCTCCACTCTAGTTCTAGATCTCGAGATCTAGAACTAGAGTGGAGGCTTTTTTG; SENP1 shRNA#1 CCGGTGGGAACATTCAGTACATGACTCGAGTCATGTACTGAATGTTCCCTTTTTG; SENP1 shRNA #2 CCGGTAGAATACTCTTGCAATACCCTCGAGGGTATTGCAAGAGTATTCTT TTTTG.

The above plasmids were co-transfected with the packaging plasmids into 293FT cells using

Lipofectamine® 2000, respectively. Supernatant was collected after 48 hr and incubated with either SH-SY5Y or HEK293T cells in the presence of 5 mg/mL polybrene for 24 hr. After infection, stably transfected cells were selected by adding puromycin.

*$Ni^{2+}$-NTA pull-down assays*

HEK293T cells were transfected with HA-EphB1, His-SUMO1, and Flag-Ubc9 with or without GFP-PKCγ for 48 hr. His-SUMO1-modified EphB1 was pulled down with $Ni^{2+}$-NTA resin as previously described [20], followed by immunoblotting with antibody against HA. As loading control, the whole cell lysate before $Ni^{2+}$-NTA pull-down was stained for HA, β-actin, Ubc9, and GFP.

*Immunoprecipitation*

HEK293T cells were co-transfected with HA-EphB1, His-SUMO1, and Flag-Ubc9 for 48 hr. After washing with NEM-PBS buffer (20 mM N-ethylmaleimide in PBS), cells were lysed in NEM-RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 20 mM N-ethylmaleimide, and complete protease inhibitor cocktail). Then, 1 mg of the cell lysates was immunoprecipitated with anti-HA antibody and then immunoblotted with anti-His antibody.

*Quantitative realtime PCR (qRT-PCR)*

qRT-PCR was performed as described before [21]. Briefly, total RNA was extracted from three neuroblastoma cell lines and reverse transcribed. Real-time PCR was performed and EphB1 expression was normalized to that of GAPDH. Primers for qRT-PCR are listed as follows. EphB1 forward primer: GATGAAGATCTACATTGACCC; reverse primer: TAGATTTCCCTCTTGCCTGG. GAPDH forward primer: GATGACATCAAGAAGGTGG; reverse primer: ATGAGGTCCACCACCCTGTT.

*Western blot*

Cells were lysed in SDS lysis buffer (62.5 mM Tris pH 6.8, 1% SDS) and the protein concentration of the cell lysates determined by NanoDrop Spectrophotometer. Equal amounts of protein were loaded onto SDS-PAGE gel and transferred to nitrocellulose membranes. Western blot was performed using primary antibodies and secondary antibodies conjugated with HRP. Protein bands were visualized by enhanced chemiluminescence (ECL kit, Pierce Biotechnology, Rockford, IL).

*Soft agar colony formation assay*

Anchorage-independent growth of tumor cell lines was assessed by soft agar colony assays. Briefly, $1 \times 10^3$ cells were suspended in 2 mL of 0.35% agar gel with 2% FBS and then seeded in 6-well plates with a base of 2 mL of 0.6% agar gel containing 2% FBS. After incubation at 37 °C for two weeks, photographs of the colonies were taken after crystal violet staining and the number of colonies was counted.

*Mouse xenograft model*

SH-SY5Y cells expressing empty vector, wild-type EphB1, or EphB1 K785R mutant ($2 \times 10^6$) were suspended in 100 μL of serum-free medium and subcutaneously injected into 6-week-old male nude mice. Tumor growth was measured every 4 days and tumor volume was calculated as $1/2 \times \text{length} \times \text{width}^2$. Tumors were harvested 3 weeks after injection, and then photographed and weighed. All animal studies were conducted with the approval and guidance of Shanghai JiaoTong University Medical Animal Ethics Committee.

*Statistical Analysis*

Statistical significance was calculated using Student's t-test. $p<0.05$ was considered statistically significant.

## Results

*EphB1 is modified by SUMO1*

To determine if EphB1 undergoes SUMOylation, HEK293T cells were co-transfected with HA-tagged EphB1 (HA-EphB1), the SUMO conjugating enzyme Ubc9, and His6-tagged SUMO1 (His-SUMO1). SUMOylated proteins were enriched by $Ni^{2+}$-NTA pull-down assays, followed by immunoblotting with anti-HA antibody. HA-EphB1 was detected as a single band of the expected 120 kDa molecular weight. When cells were transfected with HA-EphB1, His-SUMO1, and Ubc9, we observed two strong higher molecular weight bands of around 135 and 150 kDa (Fig. 1A), which corresponded to mono- and di-SUMOylated EphB1. The intensity of these two bands was reduced after co-transfection of SUMO1-specific protease SENP1 (Fig. 1B). To further confirm that the shift resulted from SUMOylation, we stably knocked down SENP1 in HEK293T cells by using SENP1 shRNA #1 or #2. Then EphB1, Ubc9, and SUMO1 were co-transfected and $Ni^{2+}$-NTA pull-down assays were performed. As shown in Fig. 1C, SUMOylated EphB1 protein increased after SENP1 was down-regulated, which was further validated by immunoprecipitation assay (Fig. 1D).

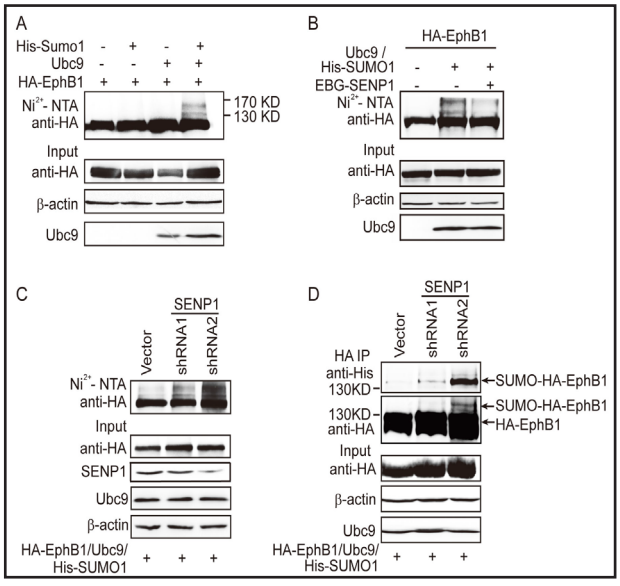


**Fig. 1.** EphB1 is modified by SUMO1. (A) HEK293T cells were co-transfected with HA-EphB1, Ubc9, and His-SUMO1. Cells were lysed 48 h after transfection and pulled down by $Ni^{2+}$-NTA resin. SUMOylated EphB1 was detected by immunoblotting with anti-HA antibody. (B) HEK293T cells were co-transfected with HA-EphB1, Ubc9, His-SUMO1, with or without EBG-SENP1; $Ni^{2+}$-NTA pull-down assay was performed. (C) SENP1 expression was stably knocked down in HEK293T cells by infecting with virus-expressing SENP1 shRNA1 or shRNA2; plasmids were transfected and then $Ni^{2+}$-NTA pull-down assays were performed. (D) HEK293T with stably silenced SENP1 were transfected with HA-EphB1, Ubc9, and His-SUMO1. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-His antibody. A representative blot of three experiments is shown.

*K785 is a major site for SUMOylation*

SUMOs are conjugated to the lysine residues of target proteins often found within the consensus sequence ψKX(D/E), where ψ is a hydrophobic amino acid and X is any amino acid. Seven potential SUMOylation sites of EphB1 were predicted by SUMOplot (www.abgent.com/sumoplot) (Fig. 2A). We then mutated these lysine residues to arginine residues individually in HA-EphB1 and examined whether these mutants could be SUMOylated. The results from $Ni^{2+}$-NTA pull-down assays showed that SUMOylation of EphB1 was strongly reduced by mutation of K785 to arginine (Fig. 2B). Although mutation of K643 to arginine also had a slight effect on EphB1 SUMOylation, the level of SUMOylation was comparable between single mutation K785R and double mutation K643/785R (Fig. 2C), indicating that K785 is the major site for SUMOylation.

To confirm the results of $Ni^{2+}$-NTA pull-down assay, we also examined the SUMOylation of EphB1 with K785R mutation by immunoprecipitation assay. HEK293T cells were transfected with HA-EphB1 or the K785R mutant, His-SUMO1, and Ubc9. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-His antibody. Consistently, mutation of K785 to arginine reduced the SUMOylation of EphB1 (Fig. 2D).

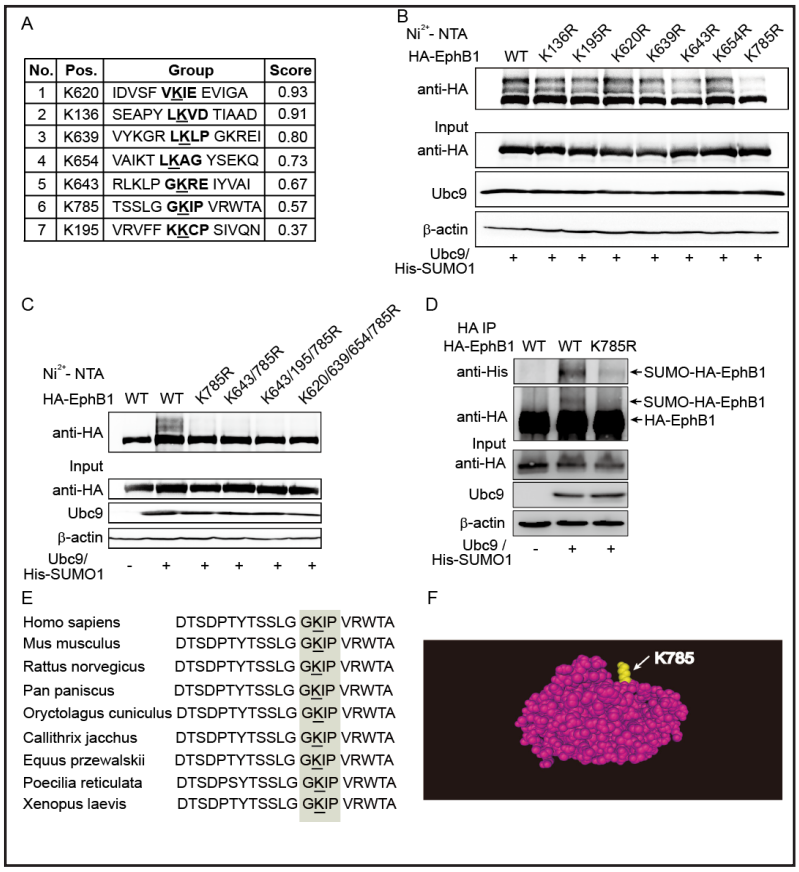


**Fig. 2.** K785 is the main lysine residue in EphB1 that conjugates SUMO1. (A) Seven potential SUMOylation sites of EphB1 are predicted by SUMOplot (www.abgent.com/sumoplot). (B) EphB1 mutants were generated by site-directed mutagenesis and SUMOylation was detected by $Ni^{2+}$-NTA pull-down assay. (C) $Ni^{2+}$-NTA pull-down assay was performed on EphB1 with single-, double-, triple-, or quadruple-mutation. (D) HEK293T cells were transfected with HA-EphB1 wild-type (WT) or K785R mutant, along with Ubc9 and His-SUMO1. Cells were lysed and immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-His antibody. (E) Amino acid sequence alignment from different species showed that the highlighted motif containing the underlined K785 was conserved. (F) The major site for SUMO1 protein conjugation (K785) is mostly exposed. The crystal structure depicts the tyrosine kinase domain of EphB1 (amino acid 602 - 896, taken from Protein Data Bank [PDB]: 3ZFX_A).

The motif containing K785 is located in the kinase domain and remains highly conserved in vertebrates (Fig. 2E).

Using the crystal structure depicting the tyrosine kinase domain of EphB1 [22] (amino acids 602 - 896, taken from Protein Data Bank [PDB]: 3ZFX_A), we found that the major site for SUMO1 protein conjugation (K785) was mostly exposed and accessible to SUMO1 conjugation (Fig. 2F).

*SUMOylation of EphB1 inhibits tumorigenesis*

To examine the function of SUMOylated EphB1 in neuroblastoma, we first evaluated the expression of EphB1 in three neuroblastoma cell lines. As shown in Fig. 3A and B, EphB1 was widely expressed in neuroblastoma and SH-SY5Y cells had the highest EphB1 mRNA and protein expression levels among these three cell lines. Next, we overexpressed EphB1 and K785R mutant in neuroblastoma cells SH-SY5Y (Fig. 3C). Overexpression of EphB1 significantly reduced cell proliferation, while abolishment of EphB1 SUMOylation reversed the inhibition (Fig. 3D). Soft agar colony formation assays were performed to measure malignant transformation in cells and similar results were obtained (Fig. 3E).

To verify the role of EphB1 SUMOylation in tumorigenesis *in vivo*, cells stably transfected with vector, EphB1, or K785R mutant were injected subcutaneously into nude mice. As shown in Fig. 3F, overexpression of EphB1 slightly suppressed tumor growth. In contrast, expression of K785R mutation remarkably promoted tumor growth compared with wild-type EphB1 (Fig. 3G and H). Thus, our data revealed that SUMOylation of EphB1 was required for EphB1-mediated inhibition of tumorigenesis.

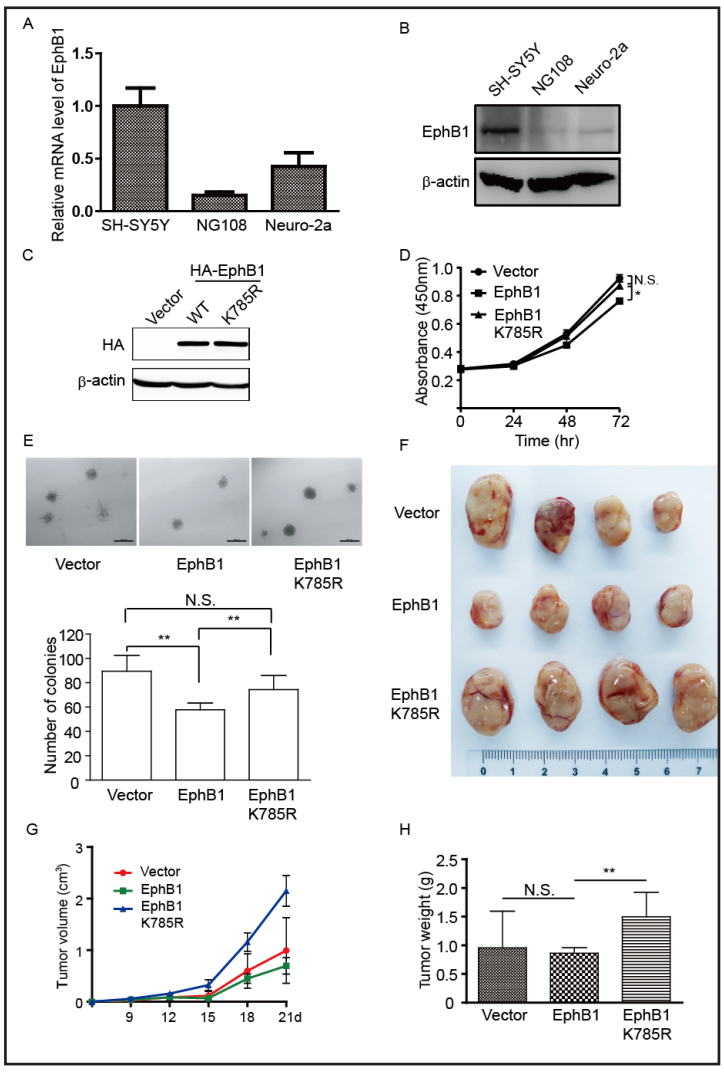


**Fig. 3.** SUMOylation of EphB1 enhances its tumor suppressive activity. (A) EphB1 mRNA levels in three neuroblastoma cell lines SH-SY5Y, NG108, and Neuro-2a was examined by qRT-PCR. Expression values of EphB1 were normalized to that of GAPDH. (B) Protein level of EphB1 in three neuroblastoma cell lines was detected by Western blotting. (C) HA-EphB1 WT or K785R mutant was stably introduced into SH-SY5Y cells by lentivirus infection and HA-EphB1 expression was detected by immunoblotting with HA. (D) Stably transfected cells were plated in 96-well plates (3000 cells/well) and cell proliferation was determined by cell counting kit-8 at indicated time points. (E) $1 \times 10^3$ cells stably expressing vector, EphB1-WT, or K785R were mixed with medium containing 0.35% agar and 2% FBS, and seeded in 6-well plates with a bottom layer of 0.6% agar. Colonies were stained with crystal violet after culture for 14 days and the number of colonies was counted. (F) $2 \times 10^6$ SH-SY5Y cells overexpressing vector, EphB1-WT, or K785R were injected subcutaneously into the dorsal flank of 6-week-old male nude mice (n=4). Three weeks after injection, resulting tumors were dissected and photographed. (G) Tumor growth was measured every 4 days from 9 days after injection and tumor growth curves were plotted. (H) Tumor weight was measured and data are presented as mean ± SD.

*SUMOylation of EphB1 suppresses tumorigenesis via down-regulation of PKCγ signaling*

To further investigate the signaling pathway involved, we first examined the classic cancer-related pathways that have been reported to be affected by EphB1, such as Erk and Akt. However, we did not observe any difference in the level of Erk and Akt phosphorylation after overexpression of wild-type and mutant EphB1 (Fig. 4A). Then phosphorylation of PKCγ, which was reported to regulate EphB-mediated spinal pain [23], was detected. Interestingly, overexpression of EphB1 reduced the phosphorylation of PKCγ, whereas K785R mutation had the opposite effect (Fig. 4A).

Given that the role of PKCγ in tumorigenesis is unclear, we stably knocked down PKCγ in cells that have been stably transfected with vector, EphB1, and K785R mutant (Fig. 4B), respectively, and tested its effect on colony formation. As shown in Fig. 4C, knock-down of PKCγ in SH-SY5Y-Vector cells significantly inhibited colony formation, suggesting that increased tumorigenesis observed in K785R mutant transfected cells may be due to up-regulated phosphorylation of PKCγ. Indeed, knock-down of PKCγ in SH-SY5Y-EphB1 K785R cells

dramatically inhibited colony formation, while down-regulation of PKCγ had no significant effect on colony formation of SH-SY5Y-EphB1 cells. These results suggested that SUMOylation of EphB1 suppressed tumorigenesis through down-regulation of PKCγ signaling.

*Phosphorylation of EphB1 inhibits its SUMOylation*

Protein phosphorylation can regulate its SUMOylation both positively and negatively [24, 25]. Given that two potential PKC phosphorylation sites (S780 and S781) in EphB1 localize near the K785 SUMOylated site, we speculated if phosphorylation of the two sites could affect the SUMOylation of EphB1. We mutated S780 and S781 to phosphomimetic aspartic acid residues and performed $Ni^{2+}$-NTA pull-down assay. Both S780D and S781D phosphomimetic mutants exhibited reduced SUMOylation (Fig. 5A), which was confirmed by immunoprecipitation assays (Fig. 5B). Consistently, SUMOylation of EphB1 was enhanced when S780 and S781 were mutated to non-phosphorylatable alanine residues (Fig. 5C). To verify the specificity of Ser phosphorylation of EphB1 by PKCγ, HEK293T cells were co-transfected with HA-EphB1, Ubc9, and His-SUMO1, with or without GFP-PKCγ, and then $Ni^{2+}$-NTA pull-down

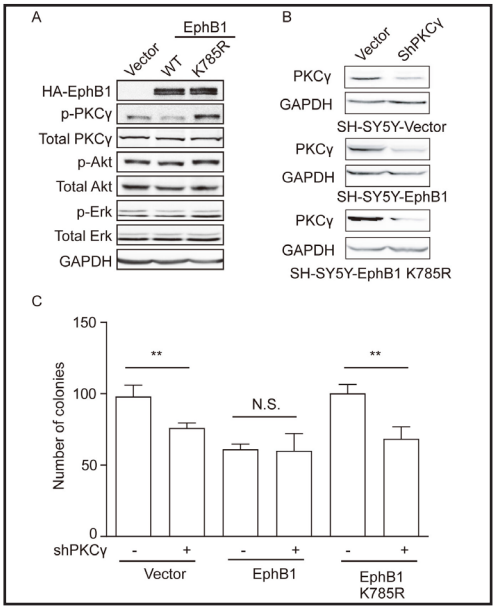


**Fig. 4.** PKCγ functions downstream of EphB1. (A) Total cell lysates from SH-SY5Y cells overexpressing vector, EphB1-WT, or K785R were immunoblotted with antibodies as indicated. (B) PKCγ was stably knocked down in cells that had been stably transfected with vector, EphB1, or K785R mutant, by transfecting virus expressing PKCγ shRNA. (C) Effect of PKCγ knock-down on anchorage-independent growth was examined by soft agar colony formation assay.

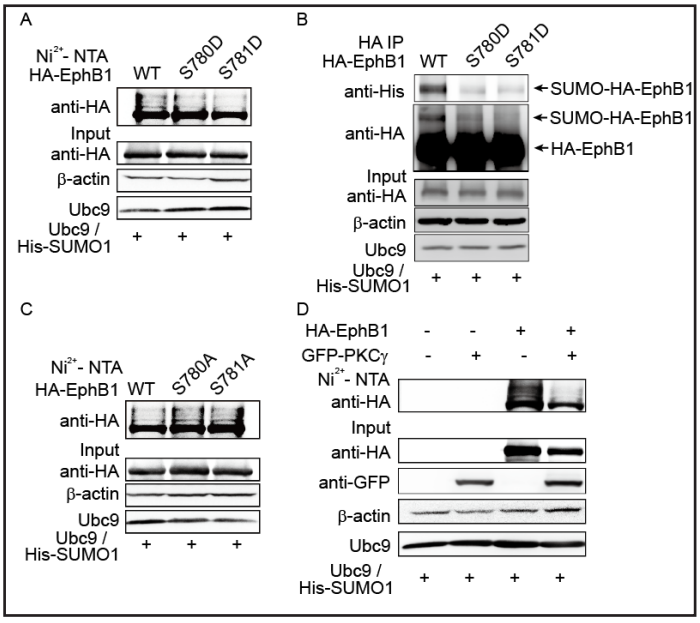


**Fig. 5.** Phosphorylation of PKCγ inhibits SUMOylation of EphB1. (A) HEK293T cells were co-transfected with HA-EphB1 WT, S780D, or S781D mutants, along with Ubc9 and His-SUMO1; $Ni^{2+}$-NTA pull-down assay was performed. (B) HEK293T cells were co-transfected with HA-EphB1 WT, S780D, or S781D mutants, along with Ubc9 and His-SUMO1, and cell lysates were immunoprecipitated with anti-HA antibody followed by immunoblotting with anti-His antibody. (C) HEK293T cells were co-transfected with HA-EphB1 WT, S780A, or S781A mutants, along with Ubc9 and His-SUMO1; $Ni^{2+}$-NTA pull-down assay was performed. (D) HEK293T cells were co-transfected with HA-EphB1, Ubc9, and His-SUMO1 with or without GFP-PKCγ. SUMOylation of EphB1 was detected by $Ni^{2+}$-NTA pull-down assay. Blots are representative of three independent experiments.

assays were performed. As shown in Fig. 5D, overexpression of GFP-PKCγ significantly reduced the SUMOylation of EphB1.

## Discussion

Here, we provide evidence that receptor tyrosine kinase EphB1 is modified by SUMOylation at lysine 785. Conjugation of SUMO1 to EphB1 suppresses the phosphorylation of its downstream signaling molecule PKCγ, and consequently inhibits tumorigenesis. In contrast, activation of PKCγ inhibits EphB1 SUMOylation (Fig. 6).

To the best of our knowledge, this is the first study demonstrating the posttranslational modification of EphB1 by SUMO1 protein. However, K785 mutation did not completely abolish the SUMOylation, suggesting that other lysine residues may also be SUMOylated.

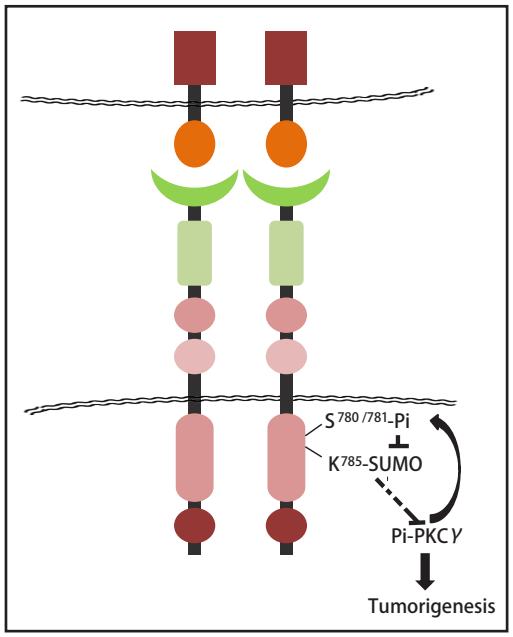


**Fig. 6.** A model for the role of SUMOylation of EphB1 in tumorigenesis. Pi - Phosphorylation; SUMO - SUMOylation

The biological function of EphB1 in tumor progression is associated with cancer type. So far, no study has yet reported EphB1 in association with neuroblastoma, in which SUMOylation has been shown to be critical [26-28]. We found that overexpression of EphB1 significantly reduced malignant transformation in neuroblastoma cells, while K785R mutation reversed EphB1-mediated inhibition of tumorigenesis, suggesting that SUMOylation enhanced the role of EphB1 in tumorigenesis. Intriguingly, overexpression of EphB1-K785R promoted tumor growth *in vivo* compared with control, whereas *in vitro* experiments showed that cells transfected with EphB1-K785R grew as fast as vector-transfected cells. This may be due to the difference in detection sensitivity of the two assays. Moreover, given that EphB1 plays a role in corneal neovascularization [29], SUMOylation of EphB1 may also play a role in tumor angiogenesis, which could only manifest in tumor growth *in vivo* instead of the *in vitro* assays we used. This deserves further study.

EphB1 suppressed phosphorylation of PKCγ, while SUMOylation deficiency abrogated this effect. Our study also demonstrated that PKCγ was oncogenic in neuroblastoma cells. Down-regulation of PKCγ inhibited tumorigenesis in EphB1-K785R stably transfected cells, but barely had any effect on EphB1-mediated tumorigenesis. Taken together, we propose that PKCγ acts as a downstream effector and participates in EphB1-regulated tumorigenesis. However, further verification will be required to further address this question. It has been reported that PKCγ promoted anchorage-independent growth of mammary epithelial cells through ERK activation [30]. Because we did not observe any change in the level of phosphorylated Erk1/2 in EphB1-overexpressing cells, other downstream signaling such as Hsp90α plasma membrane translocation [31] may be involved, rather than decreased Erk1/2 signaling.

Phosphorylation in the extension of SUMOylation motifs has been reported to either promote or inhibit SUMOylation. Our study demonstrated that phosphorylation of Ser780/781 which may be mediated by PKCγ inhibited the SUMOylation of EphB1. Previous studies showed that phosphorylation of IκBα, c-Jun, c-Fos, and p53 also inhibited their modification by SUMO [32-35]. We speculated that phosphorylation of Ser780 and Ser781 might inhibit the interaction between EphB1 and the E3 SUMO-protein ligase and this requires future investigation.

## Conclusion

Summarily, we identify SUMOylation as a novel regulatory mechanism of EphB1-mediated tumorigenesis, which provides a better understanding of EphB1 function and may shed light on the development of new therapeutic strategies.

## Acknowledgements

We thank Dr. Nanjie Xu for the pcDNA3.1-EphB1 plasmid and Dr. Yong Li for PKCγ antibody. This work was supported by the National Natural Science Foundation (81572695, 31301066, 81472571) and the Innovation Program of Shanghai Municipal Education Commission (14YZ048).

## Disclosure Statement

No conflict of interests exists.

## References

1 Miao H, Li DQ, Mukherjee A, Guo H, Petty A, Cutter J, Basilion JP, Sedor J, Wu J, Danielpour D, Sloan AE, Cohen ML, Wang B: Epha2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with akt. Cancer Cell 2009;16:9-20.

2 Genander M, Halford MM, Xu NJ, Eriksson M, Yu Z, Qiu Z, Martling A, Greicius G, Thakar S, Catchpole T, Chumley MJ, Zdunek S, Wang C, Holm T, Goff SP, Pettersson S, Pestell RG, Henkemeyer M, Frisen J: Dissociation of ephb2 signaling pathways mediating progenitor cell proliferation and tumor suppression. Cell 2009;139:679-692.

3 Chen J, Zhuang G, Frieden L, Debinski W: Eph receptors and ephrins in cancer: Common themes and controversies. Cancer Res 2008;68:10031-10033.

4 Morrison LC, McClelland R, Aiken C, Bridges M, Liang L, Wang X, Di Curzio D, Del Bigio MR, Taylor MD, Werbowetski-Ogilvie TE: Deconstruction of medulloblastoma cellular heterogeneity reveals differences between the most highly invasive and self-renewing phenotypes. Neoplasia 2013;15:384-398.

5 Bhatia S, Baig NA, Timofeeva O, Pasquale EB, Hirsch K, MacDonald TJ, Dritschilo A, Lee YC, Henkemeyer M, Rood B, Jung M, Wang XJ, Kool M, Rodriguez O, Albanese C, Karam SD: Knockdown of ephb1 receptor decreases medulloblastoma cell growth and migration and increases cellular radiosensitization. Oncotarget 2015;6:8929-8946.

6 Zhou S, Wang L, Li G, Zhang Z, Wang J: Decreased expression of receptor tyrosine kinase of ephb1 protein in renal cell carcinomas. Int J Clin Exp Pathol 2014;7:4254-4260.

7 Wang H, Wen J, Guo Q, Shi S, Shi Q, Zhou X, Liu Q, Lu G, Wang J: Loss of expression of ephb1 protein in serous carcinoma of ovary associated with metastasis and poor survival. Int J Clin Exp Pathol 2014;7:313-321.

8 Teng L, Nakada M, Furuyama N, Sabit H, Furuta T, Hayashi Y, Takino T, Dong Y, Sato H, Sai Y, Miyamoto K, Berens ME, Zhao SG, Hamada J: Ligand-dependent ephb1 signaling suppresses glioma invasion and correlates with patient survival. Neuro Oncol 2013;15:1710-1720.

9 Wang JD, Dong YC, Sheng Z, Ma HH, Li GL, Wang XL, Lu GM, Sugimura H, Jin J, Zhou XJ: Loss of expression of ephb1 protein in gastric carcinoma associated with invasion and metastasis. Oncology 2007;73:238-245.

10 Kampen KR, Scherpen FJ, Garcia-Manero G, Yang H, Kaspers GJ, Cloos J, Zwaan CM, van den Heuvel-Eibrink MM, Kornblau SM, De Bont ES: Ephb1 suppression in acute myelogenous leukemia: Regulating the DNA damage control system. Mol Cancer Res 2015;13:982-992.

11 Stein E, Cerretti DP, Daniel TO: Ligand activation of elk receptor tyrosine kinase promotes its association with grb10 and grb2 in vascular endothelial cells. J Biol Chem 1996;271:23588-23593.

12 Stein E, Huynh-Do U, Lane AA, Cerretti DP, Daniel TO: Nck recruitment to eph receptor, ephb1/elk, couples ligand activation to c-jun kinase. J Biol Chem 1998;273:1303-1308.
13 Vindis C, Cerretti DP, Daniel TO, Huynh-Do U: Ephb1 recruits c-src and p52shc to activate mapk/erk and promote chemotaxis. J Cell Biol 2003;162:661-671.
14 Fasen K, Cerretti DP, Huynh-Do U: Ligand binding induces cbl-dependent ephb1 receptor degradation through the lysosomal pathway. Traffic 2008;9:251-266.
15 Gareau JR, Lima CD: The sumo pathway: Emerging mechanisms that shape specificity, conjugation and recognition. Nat Rev Mol Cell Biol 2010;11:861-871.
16 Kang JS, Saunier EF, Akhurst RJ, Derynck R: The type i tgf-beta receptor is covalently modified and regulated by sumoylation. Nat Cell Biol 2008;10:654-664.
17 Rajan S, Plant LD, Rabin ML, Butler MH, Goldstein SA: Sumoylation silences the plasma membrane leak k+ channel k2p1 Cell 2005;121:37-47.
18 Benson MD, Li QJ, Kieckhafer K, Dudek D, Whorton MR, Sunahara RK, Iniguez-Lluhi JA, Martens JR: Sumo modification regulates inactivation of the voltage-gated potassium channel kv1.5 Proc Natl Acad Sci U S A 2007;104:1805-1810.
19 Qi Y, Wang J, Bomben VC, Li DP, Chen SR, Sun H, Xi Y, Reed JG, Cheng J, Pan HL, Noebels JL, Yeh ET: Hyper-sumoylation of the kv7 potassium channel diminishes the m-current leading to seizures and sudden death. Neuron 2014;83:1159-1171.
20 Yu J, Zhang SS, Saito K, Williams S, Arimura Y, Ma Y, Ke Y, Baron V, Mercola D, Feng GS, Adamson E, Mustelin T: Pten regulation by akt-egr1-arf-pten axis. EMBO J 2009;28:21-33.
21 Sun Q, Zhao X, Liu X, Wang Y, Huang J, Jiang B, Chen Q, Yu J: Mir-146a functions as a tumor suppressor in prostate cancer by targeting rac1 The Prostate 2014;74:1613-1621.
22 Overman RC, Debreczeni JE, Truman CM, McAlister MS, Attwood TK: Completing the structural family portrait of the human ephb tyrosine kinase domains. Protein Sci 2014;23:627-638.
23 Zhou XL, Zhang CJ, Wang Y, Wang M, Sun LH, Yu LN, Cao JL, Yan M: Ephrinb-ephb signaling regulates spinal pain processing via pkcgamma. Neuroscience 2015;307:64-72.
24 Wilkinson KA, Henley JM: Mechanisms, regulation and consequences of protein sumoylation. Biochem J 2010;428:133-145.
25 Hietakangas V, Anckar J, Blomster HA, Fujimoto M, Palvimo JJ, Nakai A, Sistonen L: Pdsm, a motif for phosphorylation-dependent sumo modification. Proc Natl Acad Sci U S A 2006;103:45-50.
26 Kobayashi T, Masoumi KC, Massoumi R: Deubiquitinating activity of cyld is impaired by sumoylation in neuroblastoma cells. Oncogene 2015;34:2251-2260.
27 Masoumi KC, Massoumi R: Cyld and sumo in neuroblastoma therapy. Oncoscience 2016;3:3-4.
28 Xiang-Ming Y, Zhi-Qiang X, Ting Z, Jian W, Jian P, Li-Qun Y, Ming-Cui F, Hong-Liang X, Xu C, Yun Z: Senp1 regulates cell migration and invasion in neuroblastoma. Biotechnology and applied biochemistry 2016;63:435-440.
29 Kojima T, Chang JH, Azar DT: Proangiogenic role of ephrinb1/ephb1 in basic fibroblast growth factor-induced corneal angiogenesis. The American journal of pathology 2007;170:764-773.
30 Mazzoni E, Adam A, Bal de Kier Joffe E, Aguirre-Ghiso JA: Immortalized mammary epithelial cells overexpressing protein kinase c gamma acquire a malignant phenotype and become tumorigenic *in vivo*. Mol Cancer Res 2003;1:776-787.
31 Yang J, Song X, Chen Y, Lu XA, Fu Y, Luo Y: Plcgamma1-pkcgamma signaling-mediated hsp90alpha plasma membrane translocation facilitates tumor metastasis. Traffic 2014;15:861-878.
32 Desterro JM, Rodriguez MS, Hay RT: Sumo-1 modification of ikappabalpha inhibits nf-kappab activation. Mol Cell 1998;2:233-239.
33 Muller S, Berger M, Lehembre F, Seeler JS, Haupt Y, Dejean A: C-jun and p53 activity is modulated by sumo-1 modification. J Biol Chem 2000;275:13321-13329.
34 Lin JY, Ohshima T, Shimotohno K: Association of ubc9, an e2 ligase for sumo conjugation, with p53 is regulated by phosphorylation of p53. FEBS Lett 2004;573:15-18.
35 Bossis G, Malnou CE, Farras R, Andermarcher E, Hipskind R, Rodriguez M, Schmidt D, Muller S, Jariel-Encontre I, Piechaczyk M: Down-regulation of c-fos/c-jun ap-1 dimer activity by sumoylation. Mol Cell Biol 2005;25:6964-6979.