

Src SUMOylation Inhibits Tumor Growth Via Decreasing FAK Y925 Phosphorylation



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Abstract

Src, a non-receptor tyrosine kinase protein, plays a critical role in cell proliferation and tumorigenesis. SUMOylation, a reversible ubiquitination-like post-translational modification, is vital for tumor progression. Here, we report that the Src protein can be SUMOylated at lysine 318 both *in vitro* and *in vivo*. Hypoxia can induce a decrease of Src SUMOylation along with an increase of Y419 phosphorylation, a phosphorylation event required for Src activation. On the other hand, treatment with hydrogen peroxide can enhance Src SUMOylation. Significantly, ectopic expression of SUMO-defective mutation, Src K318R, promotes tumor growth more potently than that of wild-type Src, as determined by migration assay, soft agar assay, and tumor xenograft experiments. Consistently, Src SUMOylation leads to a decrease of Y925 phosphorylation of focal adhesion kinase (FAK), an established regulatory event of cell migration. Our results suggest that SUMOylation of Src at lysine 318 negatively modulate its oncogenic function by, at least partially, inhibiting Src-FAK complex activity.

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Introduction

Src is a non-receptor tyrosine kinase protein encoded by the proto-oncogene SRC in normal mammalian cells [1]. Src, also referred to as pp60 c-Src, stands for “cellular Src kinase”. It is about 60KD in size [2] and belongs to Src family kinases (SFKs), where other members are Yes, Fyn, Lyn, Lck, Hck, Fgr, Blk and Yrk [3]. The Src protein is composed of 4 Src homology domains (SH4, SH3, SH2, SH1). The SH4 domain is located on the N-terminal which contains myristoylation sequence for membrane anchoring [4,5]. SH3 domain binds to proline-rich peptide ligands for protein–protein interactions [6]. SH2 domain recognizes specific phosphopeptide sequences that bind to tyrosine sites [7,8]. In the traditional model, SH1 domain (catalytic domain) has kinase activity, regulated by its tyrosine 419 autophosphorylation site [2], and the C-terminal containing Y530 which negatively regulates Src activity [9]. Mutation at Tyr530 changes Src to be active in kinase and highly oncogenesis [10]. RPTP α and CSK, as critical regulators, dephosphorylates or phosphorylates Tyr530, which cause activation or inactivation of Src [11–13]. Recently, some new evidence shows the activation of Src kinase is regulated by ATP binding site [14] and the phosphorylation of Y419 and Y530 may not be a zero-sum game [15,16]. Activated Src phosphorylates substrates in signal transduction pathways such as Ras/MAPK, PI3K/Akt, the Src/FAK complex and the β -catenin/E-cadherin complex signaling networks. Thus, Src regulates a

range of cellular processes, including apoptosis, proliferation, cell adhesion, migration, angiogenesis and metastasis.

Multiple post-translation modifications regulate Src activities and functions. The phosphorylation of Y419 and Y530 regulating mechanism has been described previously. Src is also regulated by ubiquitination, and that the active form is specifically targeted for degradation [17]. Myristoylation at Glycine2, is essential for membrane-binding and for the transforming activity [18]. Besides, certain specific point mutations convert Src activity. Mutation Src E378G in the kinase domain is dramatically more active than Src WT [19]. Src K295 is a critical lysine in

Abbreviations: SFKs, Src family kinases; SH, Src homology; SUMO, small ubiquitin-like modifier; CDS, coding sequence; IP, immunoprecipitation; GST, glutathione S-transferase; FAK, Focal adhesion kinase; SENPs, SUMO-sentrin specific proteases.

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the ATP-binding pocket, and the mutation of this site renders Src completely inactive [20]. Clustered cysteine residues Cys483/Cys487/Cys496/Cys498 in the kinase domain of Src perform critical role for protein stability and cell transformation [21]. Recently, in some of the Src involved signaling pathway, multiple protein, such as PTEN, Grb2, SHP2, FAK, AKT, has been identified to be SUMOylated. SUMOylation is a post-translational modification featured by covalent and reversible attachment of small ubiquitin-like modifier (SUMO) to protein at specific lysine [22]. SUMOylation can affect protein localization, stability, activity or protein-protein interaction [23–27]. It has not been reported whether Src can be SUMOylated. In the present study, we demonstrated that Src could be SUMOylated at K318, which could be inhibited by hypoxia in tumor *via* activating Src Y419. Besides, our results suggested that SUMOylation of Src might be a negative regulator in its oncogenic function by inhibiting Src-FAK complex activity *via* decreasing FAK Y925 phosphorylation.

Materials and Methods

Antibodies and Reagents

Anti-Src rabbit monoclonal(#2109), anti-phospho-Src (Tyr 416) rabbit polyclonal (#2101; used for detecting human Src pY419), anti-phospho-Src (Tyr 527) rabbit polyclonal (#2105; used for detecting human Src pY530), anti-FAK rabbit polyclonal(#3285), anti-phospho-FAK (Tyr 925) rabbit polyclonal(#3284), Anti-Csk rabbit monoclonal(#4980) antibody were purchased from Cell Signaling Technology. Anti-GAPDH polyclonal (#ab37168), SUMO1 (#ab32058), Senp1 (#ab108981) antibody were from Abcam. Protein A/G PLUS Agarose beads (#K0115) were obtained from Santa Cruz Biotechnology. Ni²⁺-NTA agarose beads were from Qiagen. Glutathione Sepharose 4B beads (#17-0756-01) were from GE Healthcare Life Sciences. Puromycin (#P8833) was from Sigma. Anti-RPTPα (#7-091) is kept in our lab [28].

Plasmids

The human Src CDS was cloned from pCMV-Tag2B-Src plasmid [15], digested with EcoR I and Not I and then subcloned into vector pEF5HA, CD513B, pGEX-4 T-1 and mutant Src K318R was generated using PCR-directed mutagenesis and sequenced. The shRNA sequence targeting Src 3'UTR (shSrc) was from Sigma-Aldrich "Mission shRNA" online: 5'-CATCCTCAGGAACCAACAATT-3'. The shRNA was cloned into pLKO.1 vector. The pE1E2S1 plasmid was a kind gift from Dr. Jiemin Wong in East China Normal University.

Cell Culture

HEK293T, HEK293FT, NIH/3 T3 and DU145 cell lines were obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Hyclone) at 37 °C in 5% CO₂ humidified incubator. Cell transfection was performed using Lipofectamine 2000 (Invitrogen).

SUMOylation Assays

Src SUMOylation was analyzed in HEK293T by the method of *in vivo* SUMOylation assay using Ni²⁺-NTA agarose beads as previously described [29]. Src SUMOylation analysis was also performed by the method of *in vitro* *E. coli* BL21-based SUMOylation assay with the plasmid pE1E2S1 as described [30].

Soft Agar Colony Assay

The method was performed in six-well plates with a base of 2 ml of DMEM medium containing 5% FBS with 0.6% Bacto agar

(Amresco). Stable NIH/3 T3 or DU145 cells were seeded in 2 ml of medium containing 5% FBS with 0.35% agar at 2 or 4 X 10³ cells per well and layered onto the base. The photos of the colonies developed in soft agar were taken at day 21. Three independent experiments were performed in triplicate.

Migration Assay by RTCA-DP

The method was carried out as described previously [23]. Briefly, stable NIH/3 T3 cells were starvation pre-treated with serum-free medium for 12 hours and then 4×10⁴ cells resuspended in 100 μl of serum-free medium were added into the pre-equilibrated upper chambers of the CIM-plate. The lower chamber was filled with 160 μl of normal growth medium containing 10% FBS. The kinetic cell indexes of their migration were recorded every 15 min for 2 days.

Mouse Xenograft Models

Murine xenograft models were established as described previously [31]. Briefly, 5-weeks-old nude mice were subcutaneously injected in the back with 100 μl of medium containing 2.5×10⁶ DU145 cells stably re-expressing Src WT and Src K318R. Forty-two days after injection, at the experimental endpoint, mice were sacrificed and the tumors were weighted and photographed. Statistical differences between groups were analyzed by the two-tailed Student's *t* test. *P* < .05 was considered statistically significant. Animal procedures were carried out according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University School of Medicine.

Hematoxylin and Eosin Staining (H&E)

Paraffin-embedded sample preparation, hematoxylin and eosin staining (H&E) were performed as previously described [29].

Immunoprecipitation (IP)

HEK293T cells transfected with the indicated plasmids were lysed in the RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM Na₃VO₄, 10 mM NaF, 40 mM N-ethylmaleimide with protease inhibitor cocktail tablet) on ice. Lysates were immunoprecipitated with appropriate antibody overnight at 4 °C and subjected to 8–12% SDS-polyacrylamide gels for Western blotting analysis.

Western Blotting

Cells were lysed in SDS-lysis buffer (25.6 mM Tris, 2% SDS, pH 6.8), and total protein concentrations were determined by Nanodrop 2000. About 100 μg of each total protein was resolved on 8–12% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was subsequently probed with the indicated primary antibodies and second antibodies, and then exposed in ImageQuant LAS 4000 (GE) after incubating with ECL substrate, then analyzed band intensities of the images with the Photoshop CS5. All primary antibodies were used at a 1:1000 dilution. All secondary antibodies were used at a 1:5000 dilution.

Results

Src can be SUMOylated Both *In Vivo* and *In Vitro*

To test whether Src protein could be SUMOylated, We transiently transfected HA-tagged Src together with His-SUMO1 and Flag-UBC9 (E2 ligase in SUMOylation) in HEK293T cells. His-SUMO-conjugated Src was pulled down by the method of

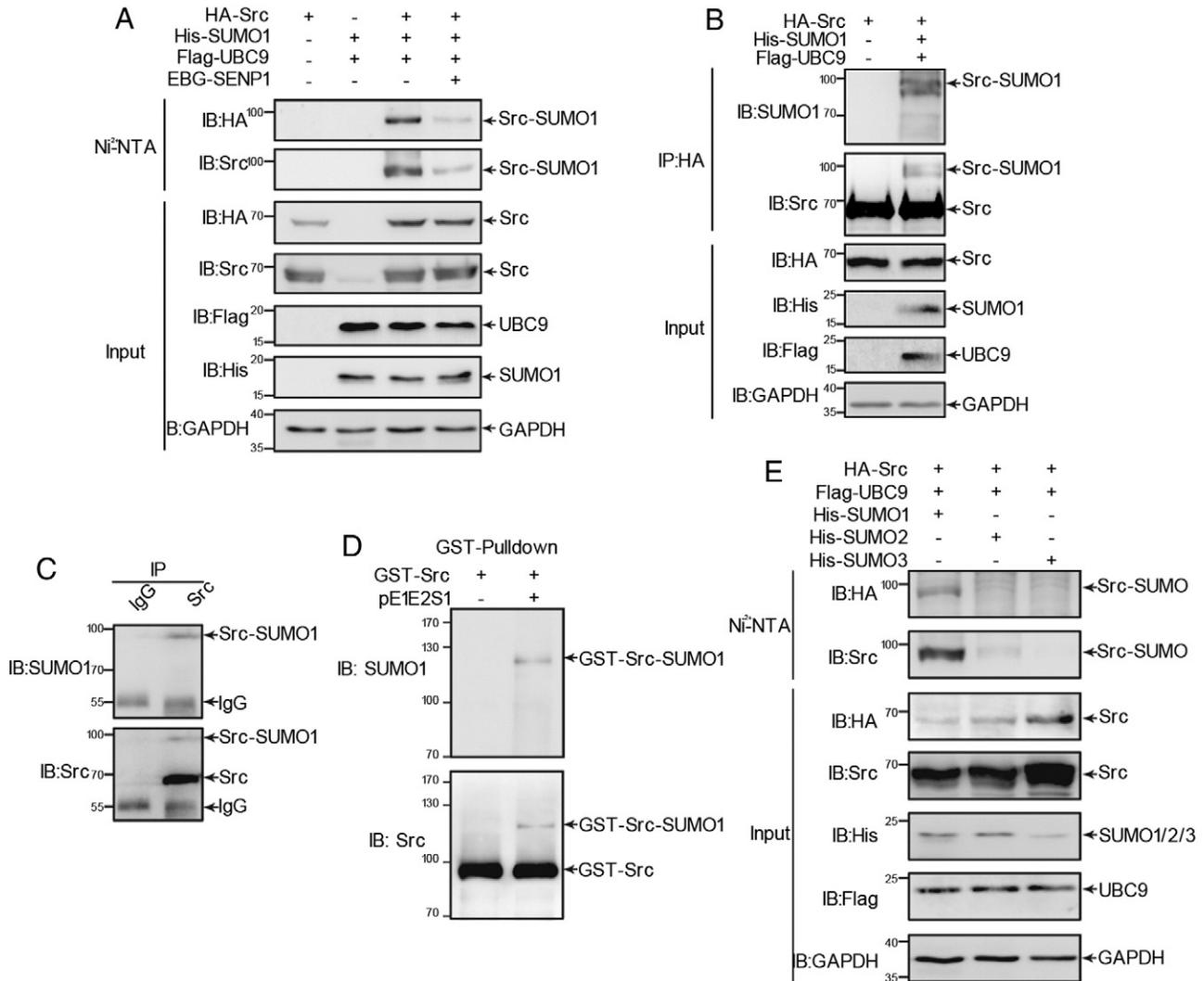


Figure 1. Src can be SUMOylated both *in vivo* and *in vitro*. (A) HEK293T cells were cotransfected with HA-Src with or without His-SUMO1/Flag-UBC9/EBG-SENP1. SUMOylated proteins were purified by Ni²⁺-NTA affinity resin following immunoblotted with anti-HA and anti-Src antibodies after 48 hours transfection. (B) HEK293T cells were transfected with indicated plasmids. Cell lysates were used for immunoprecipitation (IP) with anti-HA antibody, followed by western blot analysis with anti-SUMO1 antibody. The same membrane was re-immunoblotted with anti-Src antibody after stripping. (C) To confirm SUMOylated c-Src occurs naturally, stable SENP1-knockdown HEK293T cells were directly lysed in NEM-RIPA buffer, then immunoprecipitated with anti-Src or normal IgG, and immunoblotted with anti-SUMO1. The same membrane was re-immunoblotted with anti-Src antibody after stripping. (D) cotransformed with GST-Src with or without pE1E2S1 into *E. coli* BL21. Bacteria lysates were used for GST pull-down, and immunoblotted with anti-SUMO1 and anti-Src antibodies (same membrane, stripped). (E) HEK293T cells were cotransfected with HA-c-Src, Flag-UBC9 and His-SUMO1 or SUMO2 or SUMO3. Cell lysates were treated with Ni²⁺-NTA resin, western blotting with anti-HA and anti-Src antibodies to confirm SUMO1 is the major SUMO modification.

Ni²⁺-NTA resin precipitation as described [29]. A predicted band at about 80KD appeared with anti-HA and anti-Src antibody in HA-Src co-transfected with His-SUMO1 and Flag-UBC9 but not HA-Src alone, and this band obvious receded with SENP1 (deSUMO conjugase), which means it might be a Src-SUMO1 specific band (Figure 1A). Next, we further confirmed that with immunoprecipitation (IP) method. HA-Src with or without His-SUMO1 and Flag-UBC9 were transfected in HEK293T cells. After transfection 48 h later, the lysates were used for IP with HA antibody and immunoblotted with SUMO1 and Src antibody. As observed, same results confirmed that SUMO1 can be covalently conjugated to Src (Figure 1B). In order to examine whether endogenous Src can be SUMOylated, we immunoprecipitated Src with anti-Src antibody and immunoblotted with anti-SUMO1 antibody in shSENP1 HEK293T

cells. Results apparently revealed that Src is a SUMOylated protein *in vivo* (Figure 1C). In addition, we detected Src SUMOylation *in vitro* by a prokaryotic SUMOylation assay with pE1E2S1 [32]. pE1E2S1 is a tri-cistronic plasmid for the overexpression of SUMO-E1 enzyme (AOS1/UBA2), E2 enzyme (UBC9) and SUMO1, and modifies the substrate protein with SUMO1. We co-transformed the glutathione S-transferase (GST) tagged Src and pE1E2S1 in *E. coli* BL21, and antibiotic selection marker allows co-expression of pE1E2S1 and GST-Src of interest. As shown in Figure 1D, a clear band appeared with the molecular weight of 120kd, which is just the sum of GST-Src (about 100kd) and SUMO1. This part showed that Src could be SUMOylated *in vitro*. Vertebrates encode three main SUMO isoforms (SUMO1–SUMO3). Thus, we want to figure out which is the major SUMO modification of Src. Transfected with HA-Src and Flag-UBC9

together with His-SUMO1 or His-SUMO2 or His-SUMO3 in HEK293T cell, following by Ni²⁺-NTA resin precipitation and western blotting, the results showed that Src is modified mainly by SUMO1, not SUMO2/3 (Figure 1E). Taken together, our results suggest that Src is a SUMOylated protein covalently with SUMO1.

K318 is the Main SUMOylation Site of Src

Next, we focus attention on the SUMOylation sites in Src protein. Based on the SUMOylation prediction software (<http://www.abgent.com/sumoplot/>) (Fig. S1A), we constructed all the ten potential lysine→arginine (K/R) mutants and checked their SUMOylation level compared with Src wild type (WT) with the method of Ni²⁺-NTA resin precipitation. Unexpected, there was no obvious difference between all of these K/R mutants and WT, which means the main SUMO site of Src might not be in this group (Fig. S1B). After that, we wonder if the SUMO site is located in the linker between SH2 and SH1 domain with loose structure. Thus, we constructed mutations of HA-Src K252R and K260R, and tested their SUMOylation status. As shown in Fig. S1C, neither is the SUMO site. Then, we mutated all the rest lysines in Src and SUMOylation assay revealed that either K318 or K319, or both are the potential SUMO sites (Figure 2A, other negative results shown in Fig. S1 C and D). To answer this, we mutated K318 and K319 of Src individually. As shown in Figure 2B, the single mutation of K318R significantly decreased the level of SUMOylated Src equal to the double mutation K318/319R, which strongly indicated that K318R worked in Src SUMOylation in actuality. In order to validate this, we performed SUMOylation assays with IP in HEK293T and GST pulldown in *E. coli*. Both the results were in accordance with the foregoing analysis. SUMOylated band of Src K318R reduced obviously in IP experiment (Figure 2C), and nearly disappeared compared with that of Src wild type in GST pulldown test (Figure 2D, lane 3). Moreover, we checked the location of K318 site in the three-dimensional structure of Src protein, and we found that it is on the surface of the whole molecular. Thus, we deduced this position of K318 can facilitate Src SUMOylation occurrence (Figure 2E). Totally, our data demonstrated that K318 is the major SUMOylation site in Src.

Hypoxia Inhibits SUMOylation of Src via Y419 Phosphorylation

It is well known that hypoxia is one of the most important regulators in tumor microenvironment [33], and Src plays a vital role in tumor biological behaviors. We wondered if hypoxia could affect the SUMOylation of Src. In HEK293T cells, 24 hours after transfected with HA-Src along with His-SUMO1 and Flag-UBC9, we treated the co-transfected group with hypoxia (1% O₂) in different time and SUMOylation of Src was determined by western blotting. As shown in Figure 3A, the SUMOylated band of Src decreased in response to hypoxia in a time-dependent manner. It has been reported that hypoxia induced Src activation *via* phosphorylated tyrosine 419 [34,35]. Consistently, we found that hypoxia can increase Src Y419 phosphorylation with a maximal value at 3 hours in hypoxia (1% oxygen) (Figure 3B), which is similar to previous results reported by Mukhopadhyay et al. [34] and Koong et al. [35]. It is reported by Mukhopadhyay D that phosphorylation of Src Y419 is enhanced over a 60-min period of hypoxia (no oxygen) with a maximal value at 30 min in U87 cells and at 60 min in 293 cells. Another study by Koong AC showed that an increasing in Src Y419 phosphorylation and activity with 15–30 min of cellular exposure to hypoxia (0.02% oxygen) was detected. The minor time-point difference in maximal Src Y419 phosphorylation level may be due

to different cell lines used or/and different oxygen concentration. Generally, both of our result and previous results demonstrate that hypoxia can increase Src Y419 phosphorylation. Moreover, hypoxia has no effect on Src Y419F mutant and thus cannot inhibit SUMOylation of Src Y419F (Figure 3B). We supposed that hypoxia may reduce Src SUMOylation by enhancing its activity. To confirm this, we detected the SUMOylation level of constructed Flag-Src Y419F [15] comparing with that of Src WT. It is as expected that We observed up-regulated SUMOylation of Src Y419F than that of Src WT (Figure 3C). Moreover, the SUMO-defective mutant, Src K318R, hardly responded to hypoxia treatment (Figure 3D). Instead, the treatment with 100 μM H₂O₂ progressively increased the SUMOylation level of Src, and consistently, Src K318R failed to react to hyperoxia (Figure 3E). Therefore, these data demonstrated that hypoxia could inhibit SUMOylation of Src through Src activation.

SUMOylation of Src Might Weaken FAK Y925 Phosphorylation

After we identified Src is SUMOylated, we want to find out what is the function of this modification. HEK293T was transfected with constant HA-Src, co-transfected with His-SUMO1 and Flag-UBC9 in a dose gradient. Under this scenario, we built a Simulate method on detection of precise SUMOylation level of Src. Dependent on this system, we tested SUMOylation level of Src, and observed the degree of Src SUMOylation was gradually enhanced with the increase of the amount of His-SUMO1/Flag-UBC9, which means the system worked well (Figure 4A). However, RPTPα, one main Src tyrosine phosphatase [13], and CSK, Src tyrosine kinase [36], displayed no change with binding Src along with enhanced Src SUMOylation. Meanwhile, the phosphorylation status of Src Y419 or Y530, which are the indication of Src activity in conventional ways, did not change, either (Figure 4A). We put forward the idea of the Src function depending on not only its Y419/Y530 phosphorylation, but also its substrates phosphorylation. Focal adhesion kinase (FAK) is one of the prominent substrates of Src. The phosphorylation of FAK on Tyr925 is Src kinase dependent and a sign which indicates Src activity [37]. As revealed in Figure 4B, when Src was distinctly SUMOylated, the binding between FAK and Src was unchanged, while the phosphorylation of FAK on Tyr925 reduced sharply. To further confirm this, we used gradient transfection method to create a growing SUMOylation level of Src, and tested phosphorylation level of FAK on Tyr925. It is convincing that the phosphorylation level of FAK Y925 was gradually reduced with increased Src SUMOylation whereas FAK bound to Src kept still (Figure 4C). Moreover, reduction on FAK pY925 could be recovered by SENP1 induced deSUMOylation of Src (Figure 4D). It has been reported in 2003 that FAK can be SUMOylated [27]. To exclude the possibility that reduced phosphorylation of FAK on Y925 is attributed to FAK SUMOylation, We detected both the over-expressed and endogenous FAK pY925 level related to its own SUMOylation. As shown in Figure 4 E and F, there was no obvious change in phosphorylation of FAK on Y925 whether FAK was SUMOylated or not. Taking this part of data together, we consider that SUMOylation of Src might weaken FAK Y925 phosphorylation level independent of Src tyrosine phosphorylation status or binding FAK.

SUMOylation of Src May Suppress Tumor Progression

Generally, Src acts as an oncogene. To investigate whether SUMOylation of Src has biological function in tumor, we established stable cell lines by polyclonal lentiviral infections carrying vector, Src WT

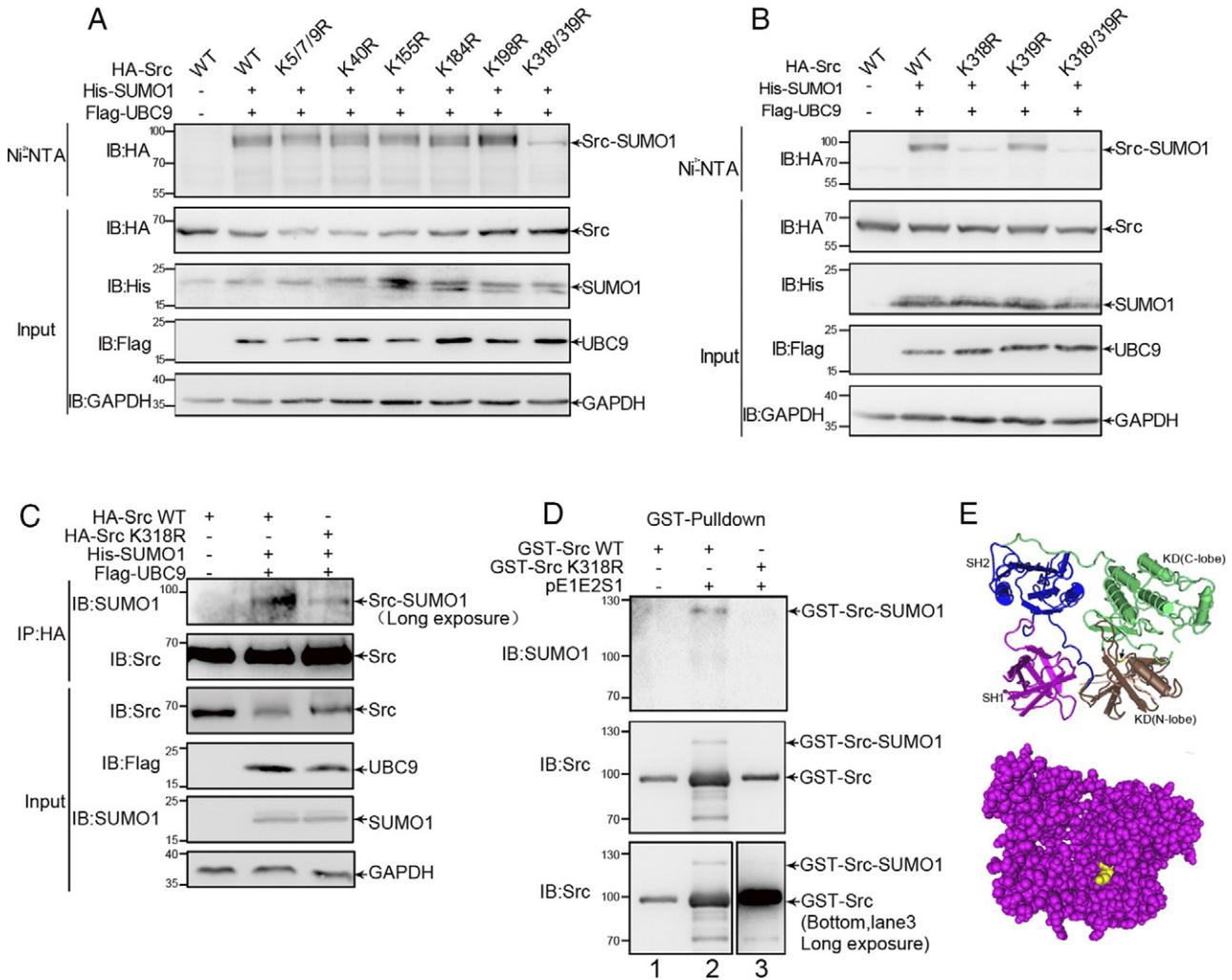


Figure 2. K318 is the main SUMO site of Src. (A) HEK293T cells were cotransfected with HA-Src or mutants and His-SUMO1/Flag-UBC9. SUMOylated proteins were purified by Ni²⁺-NTA affinity resin following immunoblotting with anti-HA antibody. Among all the Src mutants, only K318/319R double mutant abolished SUMOylation. (B) Src K318R abolished SUMOylation as same as K318/319R double mutation. The single mutation K318R or K319R or double mutation K318/319R with Flag-UBC9 and His-SUMO1 were co-transfected into HEK293T cells followed by Ni²⁺-NTA pull down for SUMOylation assay. (C) HEK293T cells were co-transfected with HA-Src WT or K318R along with His-SUMO1/Flag-UBC9; 48 hours later, cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-SUMO1 antibody. (D) GST-Src WT or K318R was co-transformed with pE1E2S1 into *E. coli* BL21. Transformed GST-Src WT only as a negative control. Lysates were immunoblotted with anti-SUMO1 and anti-Src antibodies after GST-pulldown. (E) The three-dimensional structure of Src. Ribbon diagram (top) represents crystal structures of Src. K318 located at N-terminal lobe (brown) of Kinase domain, well the magenta, blue and cyan parts represent SH3, SH2 and C-terminal lobe of Kinase domain. The figure below shows K318 (yellow) is on the surface of Src molecular.

or Src K318R, respectively, in the murine fibroblast cell line NIH/3 T3 (Figure 5A). In soft agar colony forming assay, colonies from NIH/3 T3 stable cell line with over-expressed Src K318R have more quantity and are much bigger than those with Src WT (Figure 5B), and NIH/3 T3 only with vector had no colony formed (data not shown). As Src-FAK complex plays a critical role in collective cell movement by regulating E-cadherin [38], we performed the RTCA (real-time cell analysis) migration assay [39] to evaluate the motilities of NIH/3 T3 stable cell lines that indicating ability of cell invasion. As expected, Src could enhance cell migration by comparing NIH/3 T3 stable cells carrying Src WT with those carrying only vector. However, NIH/3 T3 stable cell line carrying Src K318R displayed higher migrating ability than the cells carrying Src WT (Figure 5C). To further verify the function of Src SUMOylation in tumorigenesis *in vivo*, we generated DU145 stable cell lines by shRNA

knockdown of endogenous Src, and then re-expression of Src WT or K318R mutant (Figure 5D). The four DU145 stable cell lines were subcutaneous injected into nude mice. 6 weeks later, all the tumors were gathered for further study of characteristics. As shown in Figure 5D, DU145/Src WT cells has higher Src level than DU145/Ctrl cells, while Figure 5E and F showed that higher Src level did not lead to more tumor growth. One possibility is that Src is not a full-transforming gene, it is not high Src level enough to show more tumor growth in DU145/Src WT cells. We do not exclude other possibilities such as depending on cell type, or additional stimulation required for full oncogenic activity of Src. It is interesting to clarify the mechanism in further study. In accordance with the results of NIH/3 T3 stable cells, the tumors from Du145 stable cell line with Src K318R performed more malignant in size and weight than those with Src WT (Figure 5E

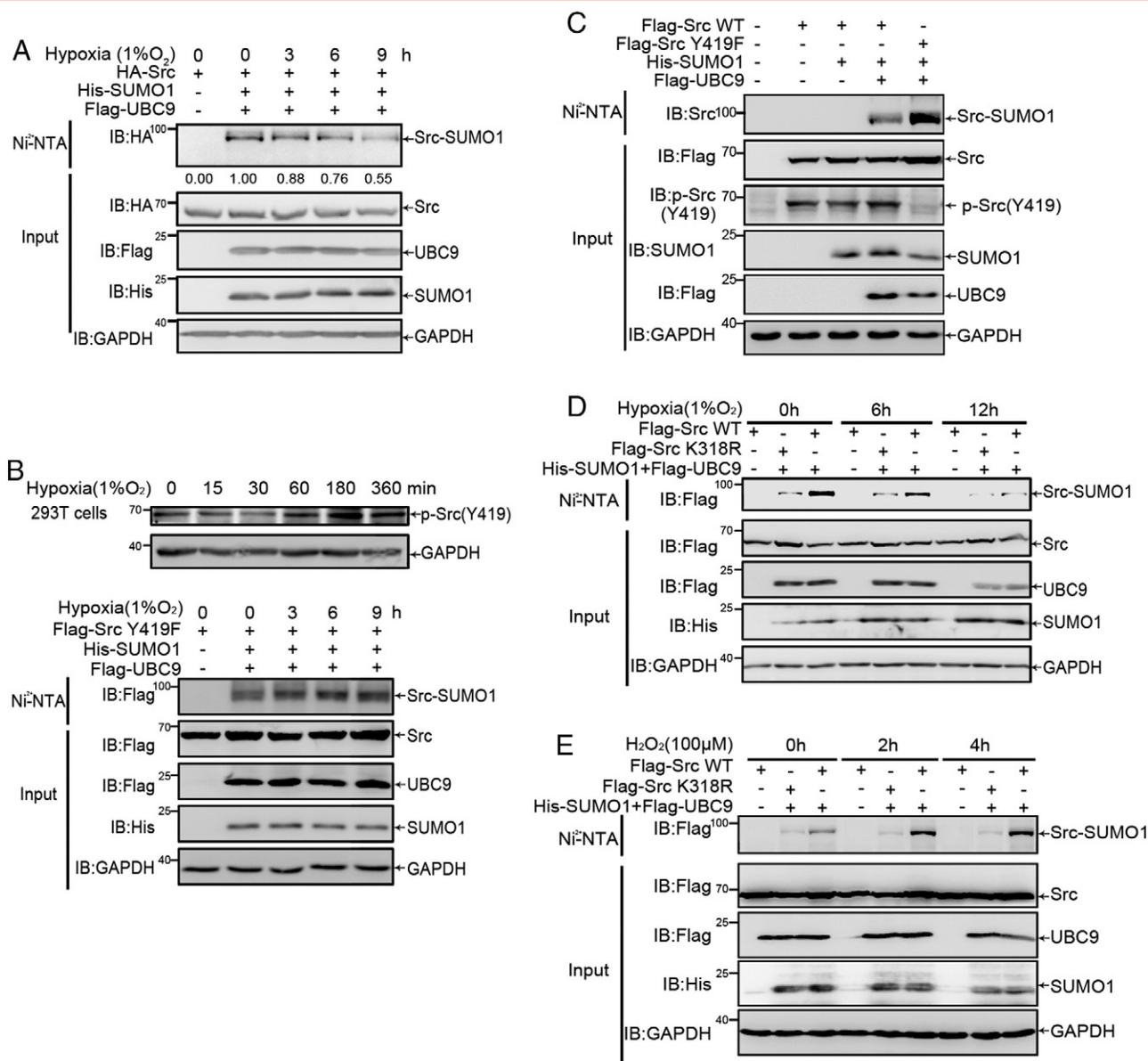


Figure 3. Hypoxia inhibits SUMOylation of Src *via* Y419 phosphorylation. (A) HEK293T cells were transfected with HA-Src, Flag-UBC9 and His-SUMO1. After 36 hours transfection, cells were transferred to hypoxic incubator with 1% oxygen concentration. Cells were harvested at 0, 3, 6, 9 hours, respectively. (B) (Upper) HEK293T cells were transferred to hypoxic incubator with 1% oxygen concentration. Cells were harvested at 0, 15, 30, 60, 180, 360 min, respectively. (lower) HEK293T cells were transfected with Flag-Src Y419F, Flag-UBC9 and His-SUMO1. After 36 hours transfection, cells were transferred to hypoxic incubator with 1% oxygen concentration. Cells were harvested at 0, 3, 6, 9 hours, respectively. (C) Flag-Src WT or Y419F with Flag-UBC9/His-SUMO1 were transfected into HEK293T cells. (D) Flag-Src WT or SUMO-defective K318R with Flag-UBC9/His-SUMO1 were transfected into HEK293T cells. After 36 h transfection, cells were transferred to hypoxic incubator with 1% oxygen concentration. Cells were harvested at 0, 6, 12 hours, respectively. (E) HEK293T cells were cotransfected with Flag-Src or K318R with Flag-UBC9/His-SUMO1. After 44 h transfection, cells were treated with 100 μ M H₂O₂ for 0, 2, 4 hours before being harvested. SUMOylated proteins were purified by Ni²⁺-NTA affinity resin for SUMOylation assay (A-E).

and *F*). Moreover, HE stain was performed to evaluate the pathological feature. As shown in Figure 5G, there were many infiltration of inflammation cells, hemorrhagic spots and large necrosis area in tumors from Src K318R, and these changes happened slightly in tumors from Src WT. All the results in tumor cell lines support the view that SUMOylation of Src may suppress tumor progression.

Discussion

Src, the normal cellular homologue of v-Src, is the first cloned proto-oncogene and has been the subject of intensive investigation for

the past nearly four decades [1]. Src, as a non-receptor protein tyrosine kinase, extensively affects multiple signaling pathways, such as Ras/Raf/MAPK, PI3K/Akt [40] and FAK-Src-p130^{cas} [41], which are involved in a multitude of tumor progression properties including proliferation, differentiation, angiogenesis, migration, and adhesion [42]. In this study, we were first to identify that Src can be SUMOylated at lysine318 both *in vitro* and *in vivo*. In general, the consensus SUMOylation motif is sequence Ψ KxE/D, where K is modified, Ψ stands for a large hydrophobic amino acid, generally isoleucine, leucine, or valine; x is any residue [43]. However, we tried to construct ten potential

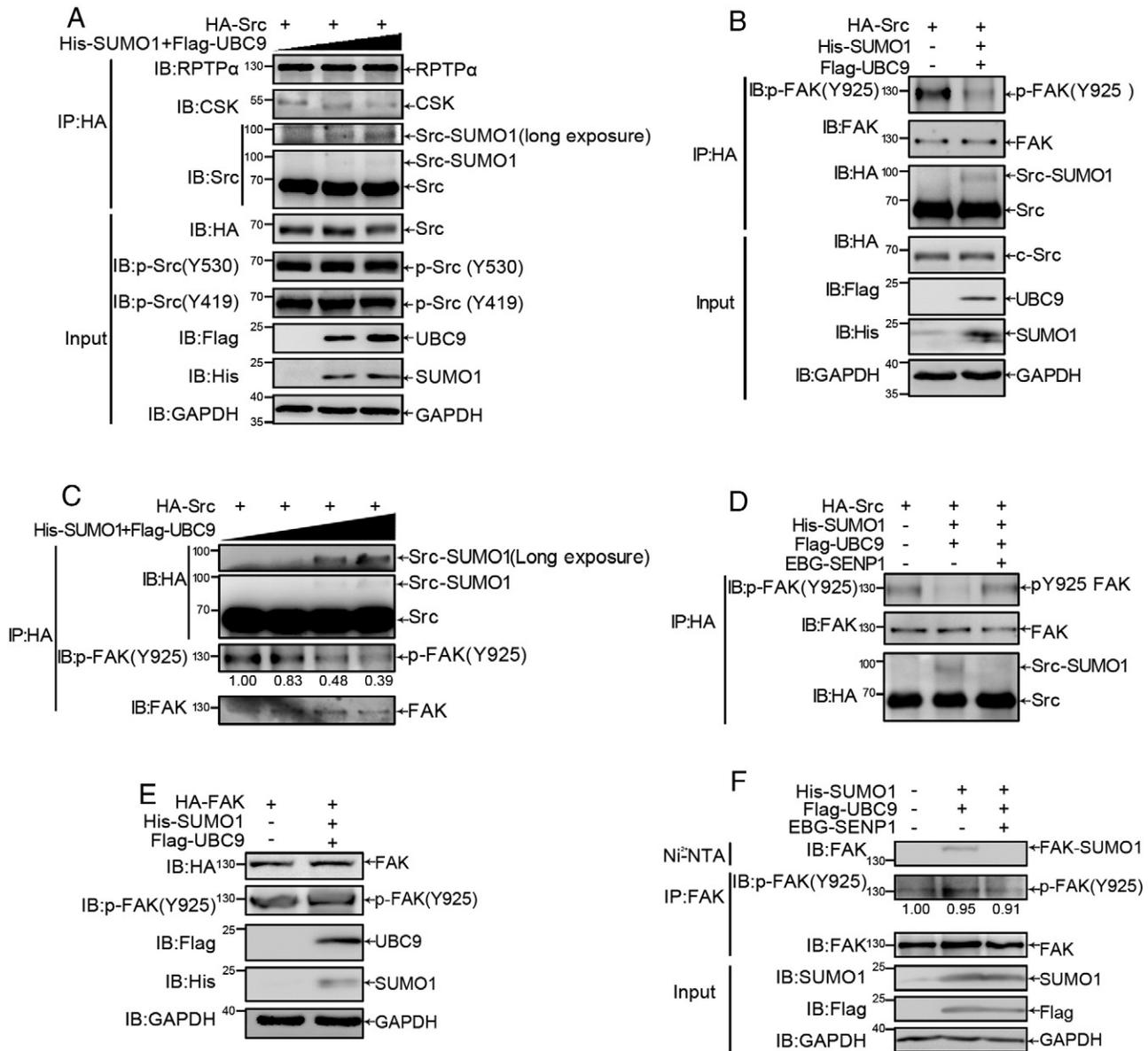


Figure 4. SUMOylation of Src might weaken FAK Y925 phosphorylation. (A) HEK293T cells were transfected with HA-Src along with the increased amount of Flag-UBC9/His-SUMO1. Cells were lysed after 48 hours transfection and used for immunoprecipitation with anti-HA antibody and subsequently immunoblotted with anti-RPTPα and anti-CSK antibodies to analyze protein-protein interaction. Whole cell lysates were immunoblotted with indicated Src phosphorylation antibody. (B) HA-Src with or without Flag-UBC9/His-SUMO1 were transfected into HEK293T cells. 48 hours later, cells lysates were used for immunoprecipitation with anti-HA antibody and followed by Western blot analysis with anti-FAK pY925, anti-FAK and anti-HA antibodies. (C) HA-Src with the increased amount Flag-UBC9/His-SUMO1 was transfected into HEK293T cells. 48 hours later, cells lysates were used for immunoprecipitation with anti-HA antibody and followed by Western blot analysis with anti-FAK pY925, anti-FAK and anti-HA antibodies. (D) HA-c-Src with or without Flag-UBC9/His-SUMO1 and EBG-SEN1 plasmids were co-transfected into HEK293T cells. After 48 hours cells culture, cells were lysed by NEM-RIPA buffer, immunoprecipitated with anti-HA antibody, following immunoblotted with indicated antibodies. (E) HEK293T cells were transfected with HA-FAK with or without Flag-UBC9/His-SUMO1. Cells were lysed in SDS-lysis buffer and immunoblotted with antibodies indicated. (F) HEK293T cells were transfected with Flag-UBC9/His-SUMO1 with or without EBG-SEN1. Ni²⁺-NTA resin affinity pulldown was used to purify SUMOylated protein of endogenous FAK. Cells lysates were immunoprecipitated with anti-FAK antibody following immunoblotted with anti-FAK pY925 antibody.

lysine-arginine (K/R) mutations belonging to the classical SUMO motif and found that none of lysine was SUMO site. Then, we detected all the other lysine sites and confirmed that K318 is the major SUMO site where MK(318)KL is not the full consensus motif. It has been reported that substitution of leu with phe or met (M) did not alter the efficiency

of SUMO-1 conjugation in RanGAP1 containing sequence LKSE [43]. In addition, some new SUMOylation motifs emerged in a recent study [44]. Lamoliatte et al. determined that only 12% of all the identified sites resided in the full consensus motif ΨKxE/D and 13% in the partial consensus motif xKxE/D, even 25% of all the sites were not attributed

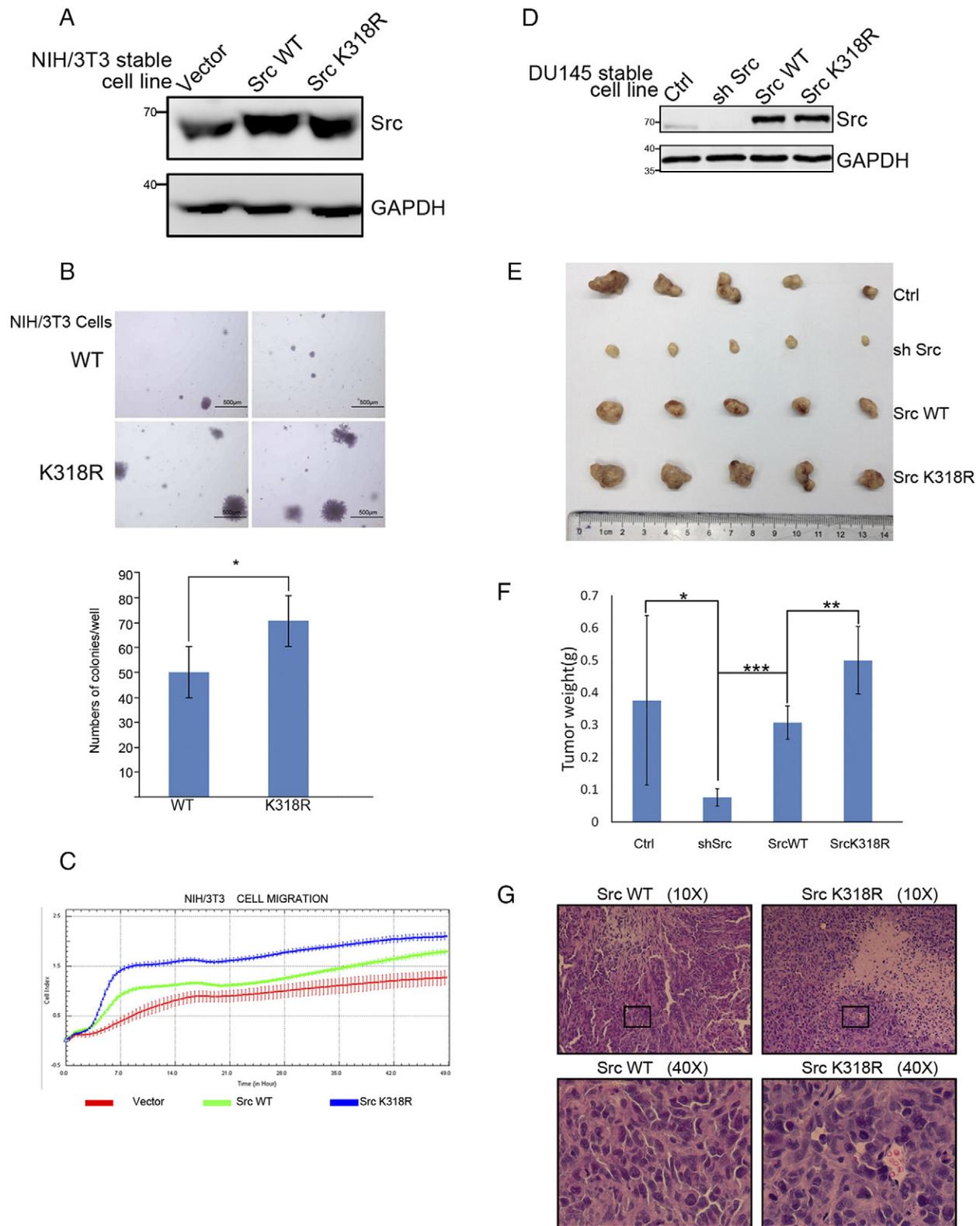


Figure 5. SUMOylation of Src may suppress tumor progression. (A) Exogenous Src WT or Src K318R were introduced into the murine fibroblast cell line NIH/3T3 cells by lentiviral system. Western Blotting showed the expression of Src in these cells. (B) Soft agar assay performed the colony formation of NIH/3T3 stable cell lines. Each of them pictured two fields. Cells were seeded in 2 ml medium composing 5% FBS with 0.35% soft agarose at 4×10^3 cells per well. (C) In migration assay of NIH/3T3 stable cell lines, the kinetic curves were graphed by the xCELLigence RTCA-DP system. (D) Endogenous Src of prostate cancer cell line DU145 was knockdown by shRNA targeting 3'UTR of Src mRNA in the lentiviral plko.1 system. Exogenous Src WT or Src K318R was re-expressed into these cells by lentiviral system. Western Blotting showed the expression of Src in DU145 stable cell lines. (E) The male BALB/c nude mice ($n = 5$) were given a suspension DU145 stable cells subcutaneously (2.5×10^6 cells/each). Xenografted tumors were taken out after 6 weeks, and tumors were dissected and weighed (F). (G) Hematoxylin and eosin staining of Xenografted tumor sections at 42 days after subcutaneous injections.

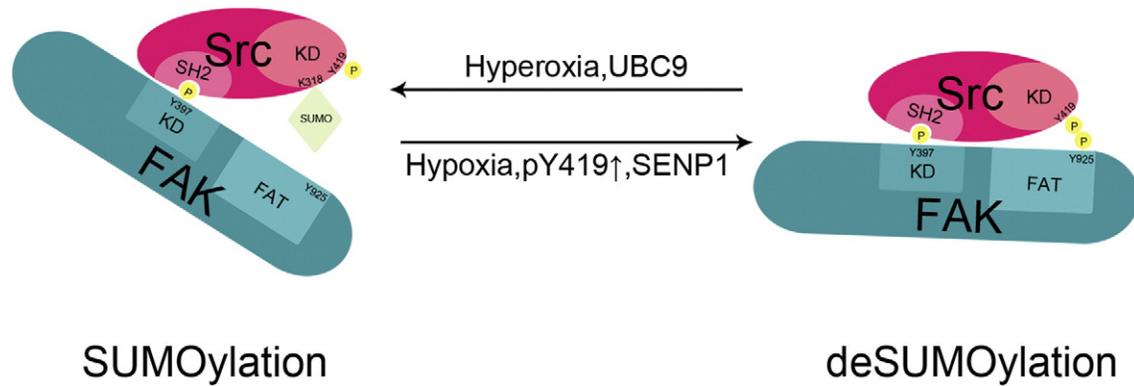


Figure 6. Src SUMOylation was a novel regulation mechanism in Src-FAK complex. SUMOylation of Src occurs mainly at lysine 318 located at kinase domain. This post-translational modification makes FAK tyrosine 925 phosphorylation refrained which is dependent on Src. When Src is SUMOylated, SUMO1 molecule conjugated with Src at K318 could spatially interfere phosphorylation process of FAK Y925 by Src pY419. On certain conditions, such as hypoxia, SUMO1 was removed by activated Src pY419, consequently FAK Y925 was phosphorylated. Meanwhile, the binding between Src and FAK was irrelevant to Src SUMOylation that mediated by SH2 domain of Src.

to a consensus sequence. Thus, the SUMO site of Src that we first identified was located at ΨK motif, and we believe there should be some other rules of SUMO modification motif worth further exploration.

The factors influencing the SUMOylation of Src are one point we focus on. In early reports, hypoxia could increase the kinase activity of Src and its phosphorylation on tyrosine 419 within 15–30 min in NIH/3 T3 or U87 cells, leading to Ras/NF-κB activation [35] or VEGF expression [34]. Here, we provided evidence that hypoxia could inhibit Src SUMOylation in a time course (0–9 hours) in HEK293T cells (Figure 3 A and C), but Src Y419F, the kinase inactive form, could rescue the SUMOylation level (Figure 3D), which implied that SUMOylation of Src is negatively regulated by hypoxia induced Src activation, and this modification might have some suppression effect in Src related tumorigenesis.

In neoplastic diseases, the SUMOylation pathway is regulated extensively [45]. SUMO-conjugating enzyme E2 UBC9 (also known as UBE2I) was associated with higher cancer risk or poor prognosis in breast cancer [46,47]. Besides, SUMO-sentrin specific proteases (SENP) were highly expressed in some cases [48,49]. Here, we figured out how the SUMOylated Src affected tumor progression. We did not detect any obvious change at Y530 phosphorylation level along with SUMOylation, which was corresponding to unchanged binding between Src and RPTPα/CSK, the main Src Y530 phosphorylation regulators (Figure 4A). Notably, the SUMO site K318 and autophosphorylation site Y419 are both located in the kinase domain. In fact, the kinase domain contains two parts, N-terminal (or small) lobe and C-terminal (or big) lobe. The N-lobe is composed of five β-strands and a single α-helix, named C helix, which is an important component of regulation in Src kinase. C-lobe contains the regulatory activation loop, which is the site of activating tyrosine 419 phosphorylation. Nucleotide binding and phosphotransfer occur in the cleft between the two lobes [50]. The K318 site is exactly located in the N-, but not in the C-lobe, which could explain why SUMOylation at K318 has no effect on phosphorylation at Y419. However, how phosphorylation at Y419 down-regulating SUMOylation at K318 was still unclear. It was likely that there is not a direct regulation since Src pY419 has many downstream signaling. Interestingly, FAK pY925, which was a Src dependent phosphorylation event [37], was demonstrated to be reduced gradually with Src SUMOylation increased (Figure 4B-D). One

reasonable explanation model was shown as Figure 6, in which, SUMO1 molecule conjugated with Src at K318 could spatially interfere phosphorylation process of FAK Y925 by Src pY419. On certain conditions, such as hypoxia, SUMO1 was removed by activated Src pY419, consequently FAK Y925 was phosphorylated. In addition, we confirmed that SUMOylation at K318 did not alter protein stability or localization (Fig. S2 A and B).

Src-FAK complex was reported to be critical to cell migration and invasion. Phosphorylation at Y925 defined a role for FAK activity in promoting a MAPK-associated angiogenic switch during tumor progression. FAK inhibition reduced vascular endothelial growth factor (VEGF) expression, which resulted in small avascular tumors in mice [51]. As shown in Figure 5, the stable cell lines with SUMO-defective Src K318R showed more malignant in migration assay, soft agar assay, and xenograft tumor model, all of which might be caused by FAK Y925 phosphorylation.

Conclusions

In summary, our findings are summarized in Figure 6. The SUMOylation status of Src is influenced by negative regulators, such as hypoxia, pY419, and SENP1, while hydrogen peroxide can enhance this modification. DeSUMOylation of Src leading to FAK Y925 phosphorylation might be a tumor-promoting cause. Given that Src-FAK complex plays an important role in carcinogenesis that could be regulated by SUMO modification, our study offers a novel strategy for clinical treatment of cancer.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2017.09.001>.

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Author Contributions

J.H. and J.Y. supervised the project. J.H. and J.W. designed the experiments. J.W. performed most experiments. R.D., N.C., H.Z.,

T.L., J.D., X.Z., R.C. and Y.W. helped with all the experiments. J.H., J.Y. and J.W. discussed the results. J.H. and J.W. wrote the manuscript. All authors read and approved the final manuscript.

References

- [1] Stehelin D, Varmus HE, Bishop JM, and Vogt PK (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260**, 170–173.
- [2] Kim LC, Song L, and Haura EB (2009). Src kinases as therapeutic targets for cancer. *Nat Rev Clin Oncol* **6**, 587–595.
- [3] Yeatman TJ (2004). A renaissance for Src. *Nat Rev Cancer* **4**, 470–480.
- [4] Xu W, Harrison SC, and Eck MJ (1997). Three-dimensional structure of the tyrosine kinase c-Src. *Nature* **385**, 595–602.
- [5] Martin GS (2001). The hunting of the Src. *Nat Rev Mol Cell Biol* **2**, 467–475.
- [6] Yu H, Rosen MK, Shin TB, Seidel-Dugan C, Brugge JS, and Schreiber SL (1992). Solution structure of the SH3 domain of Src and identification of its ligand-binding site. *Science* **258**, 1665–1668.
- [7] Songyang Z, Shoelson SE, Chaudhuri M, Gish G, Pawson T, Haser WG, King F, Roberts T, Ratnofsky S, and Lechleider RJ, et al (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767–778.
- [8] Sadowski I, Stone JC, and Pawson T (1986). A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps. *Mol Cell Biol* **6**, 4396–4408.
- [9] Cartwright CA, Eckhart W, Simon S, and Kaplan PL (1987). Cell transformation by pp60c-src mutated in the carboxy-terminal regulatory domain. *Cell* **49**, 83–91.
- [10] Shalloway D, Johnson PJ, Freed EO, Coulter D, and Flood Jr WA (1987). Transformation of NIH 3T3 cells by cotransfection with c-src and nuclear oncogenes. *Mol Cell Biol* **7**, 3582–3590.
- [11] Imamoto A and Soriano P (1993). Disruption of the csk gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. *Cell* **73**, 1117–1124.
- [12] Levinson NM, Seeliger MA, Cole PA, and Kuriyan J (2008). Structural basis for the recognition of c-Src by its inactivator Csk. *Cell* **134**, 124–134.
- [13] Zheng XM, Wang Y, and Pallen CJ (1992). Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase. *Nature* **359**, 336–339.
- [14] Pucheta-Martinez E, Saladino G, Morando MA, Martinez-Torrecuadrada J, Lelli M, Sutto L, D'Amelio N, and Gervasio FL (2016). An allosteric cross-talk between the activation loop and the ATP binding site regulates the activation of Src kinase. *Sci Rep* **6**, 24235.
- [15] Lai X, Chen Q, Zhu C, Deng R, Zhao X, Chen C, Wang Y, Yu J, and Huang J (2015). Regulation of RPTPalphac-Src signalling pathway by miR-218. *FEBS J* **282**, 2722–2734.
- [16] Min J, Reznichenko M, Poythress RH, Gallant CM, Vetterkind S, Li Y, and Morgan KG (2012). Src modulates contractile vascular smooth muscle function via regulation of focal adhesions. *J Cell Physiol* **227**, 3585–3592.
- [17] Harris KF, Shoji I, Cooper EM, Kumar S, Oda H, and Howley PM (1999). Ubiquitin-mediated degradation of active Src tyrosine kinase. *Proc Natl Acad Sci U S A* **96**, 13738–13743.
- [18] Kamps MP, Buss JE, and Sefton BM (1985). Mutation of NH2-terminal glycine of p60src prevents both myristoylation and morphological transformation. *Proc Natl Acad Sci U S A* **82**, 4625–4628.
- [19] Bjorge JD, Bellagamba C, Cheng HC, Tanaka A, Wang JH, and Fujita DJ (1995). Characterization of two activated mutants of human pp60c-src that escape c-Src kinase regulation by distinct mechanisms. *J Biol Chem* **270**, 24222–24228.
- [20] Piwnica-Worms H, Saunders KB, Roberts TM, Smith AE, and Cheng SH (1987). Tyrosine phosphorylation regulates the biochemical and biological properties of pp60c-src. *Cell* **49**, 75–82.
- [21] Senga T, Miyazaki K, Machida K, Iwata H, Matsuda S, Nakashima I, and Hamaguchi M (2000). Clustered cysteine residues in the kinase domain of v-Src: critical role for protein stability, cell transformation and sensitivity to herbimycin A. *Oncogene* **19**, 273–279.
- [22] Mahajan R, Delphin C, Guan T, Gerace L, and Melchior F (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* **88**, 97–107.
- [23] Qu Y, Chen Q, Lai X, Zhu C, Chen C, Zhao X, Deng R, Xu M, Yuan H, and Wang Y, et al (2014). SUMOylation of Grb2 enhances the ERK activity by increasing its binding with Sos1. *Mol Cancer* **13**, 95.
- [24] Deng R, Zhao X, Qu Y, Chen C, Zhu C, Zhang H, Yuan H, Jin H, Liu X, and Wang Y, et al (2015). Shp2 SUMOylation promotes ERK activation and hepatocellular carcinoma development. *Oncotarget* **6**, 9355–9369.
- [25] Huang J, Yan J, Zhang J, Zhu S, Wang Y, Shi T, Zhu C, Chen C, Liu X, and Cheng J, et al (2012). SUMO1 modification of PTEN regulates tumorigenesis by controlling its association with the plasma membrane. *Nat Commun* **3**, 911.
- [26] Li R, Wei J, Jiang C, Liu D, Deng L, Zhang K, and Wang P (2013). Akt SUMOylation regulates cell proliferation and tumorigenesis. *Cancer Res* **73**, 5742–5753.
- [27] Kadare G, Toutant M, Formstecher E, Corvol JC, Carnaud M, Bouterin MC, and Girault JA (2003). PIAS1-mediated sumoylation of focal adhesion kinase activates its autophosphorylation. *J Biol Chem* **278**, 47434–47440.
- [28] Huang J, Yao L, Xu R, Wu H, Wang M, White BS, Shalloway D, and Zheng X (2011). Activation of Src and transformation by an RPTPalphac splice mutant found in human tumours. *EMBO J* **30**, 3200–3211.
- [29] Yu J, Zhang SS, Saito K, Williams S, Arimura Y, Ma Y, Ke Y, Baron V, Mercola D, and Feng GS, et al (2009). PTEN regulation by Akt-EGR1-ARF-PTEN axis. *EMBO J* **28**, 21–33.
- [30] Uchimura Y, Nakamura M, Sugasawa K, Nakao M, and Saitoh H (2004). Overproduction of eukaryotic SUMO-1- and SUMO-2-conjugated proteins in *Escherichia coli*. *Anal Biochem* **331**, 204–206.
- [31] Chen C, Zhu C, Huang J, Zhao X, Deng R, Zhang H, Dou J, Chen Q, Xu M, and Yuan H, et al (2015). SUMOylation of TARBP2 regulates miRNA/siRNA efficiency. *Nat Commun* **6**, 8899.
- [32] Saitoh H, Uwada J, and Azusa K (2009). Strategies for the expression of SUMO-modified target proteins in *Escherichia coli*. *Methods Mol Biol* **497**, 211–221.
- [33] Liu C, Gao S, Qu Z, and Zhang L (2007). Tumor microenvironment: hypoxia and buffer capacity for immunotherapy. *Med Hypotheses* **69**, 590–595.
- [34] Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS, and Sukhatme VP (1995). Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* **375**, 577–581.
- [35] Koong AC, Chen EY, Mivechi NF, Denko NC, Stambrook P, and Giaccia AJ (1994). Hypoxic activation of nuclear factor-kappa B is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). *Cancer Res* **54**, 5273–5279.
- [36] Okada M (2012). Regulation of the SRC family kinases by Csk. *Int J Biol Sci* **8**, 1385–1397.
- [37] Brunton VG, Avizienyte E, Fincham VJ, Serrels B, Metcalf 3rd CA, Sawyer TK, and Frame MC (2005). Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. *Cancer Res* **65**, 1335–1342.
- [38] Serrels A, Canel M, Brunton VG, and Frame MC (2011). Src/FAK-mediated regulation of E-cadherin as a mechanism for controlling collective cell movement: insights from in vivo imaging. *Cell Adh Migr* **5**, 360–365.
- [39] Liu X, Chen Q, Yan J, Wang Y, Zhu C, Chen C, Zhao X, Xu M, Sun Q, and Deng R, et al (2013). MiRNA-296-3p-ICAM-1 axis promotes metastasis of prostate cancer by possible enhancing survival of natural killer cell-resistant circulating tumour cells. *Cell Death Dis* **4**, e928.
- [40] Summy JM and Gallick GE (2006). Treatment for advanced tumors: SRC reclaims center stage. *Clin Cancer Res* **12**, 1398–1401.
- [41] Hsia DA, Mitra SK, Hauck CR, Streblov DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, and Leng J, et al (2003). Differential regulation of cell motility and invasion by FAK. *J Cell Biol* **160**, 753–767.
- [42] Batuello CN, Hauck PM, Gendron JM, Lehman JA, and Mayo LD (2015). Src phosphorylation converts Mdm2 from a ubiquitinating to a neddylation E3 ligase. *Proc Natl Acad Sci U S A* **112**, 1749–1754.
- [43] Rodriguez MS, Dargemont C, and Hay RT (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* **276**, 12654–12659.
- [44] Lamoliatte F, McManus FP, Maarifi G, Chelbi-Alix MK, and Thibault P (2017). Uncovering the SUMOylation and ubiquitylation crosstalk in human cells using sequential peptide immunopurification. *Nat Commun* **8**, 14109.
- [45] Seeler JS and Dejean A (2017). SUMO and the robustness of cancer. *Nat Rev Cancer* **17**, 184–197.
- [46] Dunnebie T, Bermejo JL, Haas S, Fischer HP, Pierl CB, Justenhoven C, Brauch H, Baisch C, Gilbert M, and Harth V, et al (2009). Common variants in the UBC9 gene encoding the SUMO-conjugating enzyme are associated with breast tumor grade. *Int J Cancer* **125**, 596–602.
- [47] Synowiec E, Krupa R, Morawiec Z, Wasylecka M, Dzik L, Morawiec J, Blasiak J, and Wozniak K (2010). Efficacy of DNA double-strand breaks repair in breast cancer is decreased in carriers of the variant allele of the UBC9 gene c.73G>A polymorphism. *Mutat Res* **694**, 31–38.

- [48] Xiang-Ming Y, Zhi-Qiang X, Ting Z, Jian W, Jian P, Li-Qun Y, Ming-Cui F, Hong-Liang X, Xu C, and Yun Z (2016). SENP1 regulates cell migration and invasion in neuroblastoma. *Biotechnol Appl Biochem* **63**, 435–440.
- [49] Sun Z, Hu S, Luo Q, Ye D, Hu D, and Chen F (2013). Overexpression of SENP3 in oral squamous cell carcinoma and its association with differentiation. *Oncol Rep* **29**, 1701–1706.
- [50] Boggon TJ and Eck MJ (2004). Structure and regulation of Src family kinases. *Oncogene* **23**, 7918–7927.
- [51] Mitra SK, Mikolon D, Molina JE, Hsia DA, Hanson DA, Chi A, Lim ST, Bernard-Trifilo JA, Ilic D, and Stupack DG, et al (2006). Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. *Oncogene* **25**, 5969–5984.