SUMOylation of Csk Negatively Modulates its Tumor Suppressor Function

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Abstract

Csk, a non-receptor tyrosine kinase, serves as an indispensable negative regulator of the Src family kinases (SFKs). However, little is known about regulation of Csk expression so far. SUMOylation, a reversible post-translational modification, has been shown to regulate many biological processes especially in tumor progression. Here we report that Csk is covalently modified by SUMO1 at lysine 53 (K53) both in vitro and in vivo. Treatment with hydrogen peroxide inhibited this modification to a certain extent, but PIAS3, identified as the main specific SUMO E3 ligase for Csk, could significantly enhance SUMO1-Csk level. In addition, phosphorylation at Ser364, the active site in Csk, had no effect on this modification. Ectopic expression of SUMO-defective mutant, CskK53R, inhibited tumor cell growth more potentially than Csk wild-type. Consistent with the biological phenotype, the SUMO modification of Csk impaired its activity to interact with Cbp (Csk binding protein) leading to decreased c-Src phosphorylation at Y527. Our results suggest that SUMOylation of Csk mainly at lysine 53 negatively modulates its tumor suppressor function by reducing its binding with Cbp and consequently, inducing c-Src activation.

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Introduction

In the late 1980’s, one tyrosine kinase, selectively catalyze the C-tail phosphorylation at tyrosine 527 site in c-Src kinase, was identified and named Csk (C-terminal Src kinase) [1,2]. Soon it was shown that Csk also phosphorylates the conserved negative regulatory sites in other Src family kinases (SFKs) [3,4]. Phosphorylation by Csk stabilizes an inactive form of the Src kinases, in which the phosphorylated tail binds to the Src homology 2 (SH2) domain [5–7]. Its in vivo function has been demonstrated by the phenotypes of mice deficient in Csk. Homozygous mutant embryos exhibit a complex phenotype that includes defects in the neural tube and die at day 9–10 of gestation [8]. The kinase activity of SFKs, such as c-Src, Fyn and Lyn in these embryos, is greatly enhanced as an apparent consequence of enhanced specific activity [9]. Recently, it has been identified that deficiency of Csk results in the elevated activation of the Src family kinases c-Src, c-Yes and Lyn in intestinal epithelial cells, which is implicated in the pathogenesis of DSS-induced colitis [10–12]. These findings indicate that Csk is crucial to act as a negative regulator of SFKs. On the other hand, it is also mentioned that Csk could regulate the tricellular junction protein Gliotactin endocytosis independent of c-Src activity [13]. And Csk-mediated phosphorylation of eEF2 (eukaryotic elongation factor 2) enhances its proteolytic cleavage and the nuclear translocation [14].

Csk is ubiquitously expressed in mammalian cells and evolutionarily conserved from early-diverging metazoan Hydra to humans [15]. The Csk protein is about 50KD and composed of three Src homology domains (SH3, SH2, kinase domain). SH3 domain binds to proline-rich peptide ligands for protein–protein interactions [16].
SH2 domain recognizes specific phosphopeptide sequences that bind to tyrosine sites [17]. Csk is predominantly present in cytosol because it lacks a transmembrane domain and an N-terminal fatty acylation signal, whereas its substrates SFKs are anchored to the membrane via their N-terminal myristate and palmitate moieties. Therefore, the relocation of Csk to the membrane, where SFKs are activated, is thought
to be a critical step for Csk activity. One transmembrane phosphoprotein, Cbp/PAG1 (Csk binding protein/phosphoprotein associated with glycosphingolipid-enriched membrane) has been identified as a membrane anchor of Csk [18,19]. Cbp is localized in lipid rafts where SFKs is located, so it is a readily available substrate of SFKs. Activation of SFKs results in the phosphorylation of Cbp followed by recruitment of Csk to the membrane and consequently efficient inactivation of the SFKs by Csk [20–22]. This negative-feedback signaling loop likely plays a critical role in preventing tumorigenesis and controlling the cell mitotic signals from activation of growth factor receptors.

Several different mechanisms are involved in the activity regulation of Csk. Cbp protein positively regulates Csk function not only by recruiting Csk to the membrane but also by induction of 2–4 fold Csk activity [23,24]. Another regulatory mechanism is that Csk activity can be regulated by the oxidation state of the disulfide bond in the SH2 domain, implying that Csk could be regulated by the redox state within the cells [25]. Furthermore, phosphorylation of Csk at Ser364 by PKA increases its kinase activity up to 2–4 fold [26].

One protein post-translational modification (PTM) by small ubiquitin-like modifier (SUMO), termed SUMOylation, has become widely recognized that targets a myriad of proteins in many physiological processes. The SUMO conjugation to the lysine(s) of substrates is carried out by SUMO E1, E2, and E3 enzymes [27]. Organisms examined so far contain only a single SUMO E1 and E2 enzyme. In striking contrast with the ubiquitination system, where hundreds of E3 ligases are known, there is only the PIAS (protein inhibitor of activated STAT) family and few other SUMO E3 ligases have been described [28]. The correlation between SUMOylation and cancer has been clearly established that SUMO regulation exists in all cancer hallmark functions [29]. However, the exact role of SUMOylation, considered either tumor promoting or tumor suppressive, is not completely defined yet. For example, although much is known about tumor suppressor p53, the function of p53 SUMOylation is still controversial [30]. Recently, we have demonstrated that c-Src is a SUMOylated protein [31]. In the present study, we report that Csk could be SUMOylated at lysine53 both in vitro and in vivo, and this modification is modulated by hydrogen peroxide or SUMO E3 ligase PIAS. Moreover, Csk SUMOylation negatively regulates its tumor suppressor function by reducing its binding with Cbp, therefore decreasing its phosphorylation on c-Src.

Materials and Methods

Antibodies and Reagents

Antibodies against Csk (#2109 and #4980), phospho-Src (Tyr 416) (#2101; used for detecting human Src pY419), phospho-Src (Tyr 527) (#2105; used for detecting human Src pY530), His-Tag Csk (2366) were purchased from Cell Signaling Technology. Anti-Csk antibody (#17720–1–AP) for immunoprecipitation was purchased from proteinTech. Antibodies against GAPDH (#ab37768), SUMO1 (#ab32058) were from Abcam. Anti-Flag (M2), anti-HA and anti-Myc antibodies were from Sigma-Aldrich. Protein A/G PLUS Agarose beads (#K0115) were obtained from Santa Cruz Biotechnology. Ni2+-NTA agarose beads (#K0115) were obtained from Santa Cruz Biotechnology. Puromycin (#P8833) and EDTA-free Protease Inhibitor Cocktail were from Sigma-Aldrich.

Plasmids

The human Csk CDS was cloned by RT-PCR and sequenced, and then digested with BamH I and Not I, and sub-cloned into pEF-5HA. The forward primer is CGGGATCCCATGTCAGCAATACAGGGCCCGCGGGGAGCCTGCTGG and the reverse primer is ATAAAAATGCAGCTCTGTGG. For other constructs based on pEF-5HA-Csk, PCR and digested with Hind III and Xhol I for pCMV-Tag2B-Csk, digested with Xbal and Not I for CD513B-HA-Csk and pGEX-4 T-1-Csk. Flag-SUMO1-Csk was constructed by inserting SUMO1(2-96aa) into the Flag-Csk plasmid with restriction enzyme sites BamH I and Hind III, then CD513B-Flag-Csk and CD513B-Flag-SUMO-Csk were constructed by digesting with Xbal and Not I from Flag-SUMO1-Csk plasmid. The Cbp CDS was cloned by RT-PCR and sequenced, and then digested with EcoR I and Xba I, and sub-cloned into pCMV-c-Myc to generate the Myc-Cbp. The forward primer is CGGAGTTCCGACCACCTGGGCACCAGCCGGGGAGCCTGCTGG and the reverse primer is ATCTGCTGAGCTGCCTGTATCTCTGCGTGTG.

Cell Cultures

Human embryonic kidney cell line 293 T and 293FT, prostate cancer cell line DU145 and cervical cancer cell line HeLa were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) (Hyclone) at 37°C in a 5% CO2 humidified incubator. Cell transfection was performed using Lipofectamine 2000 (Invitrogen).
Figure 2. K53 is the main SUMOylation site of Csk. (A and C) HEK293T cells were transfected with either HA-Csk or KR mutants, alone or with His-SUMO1. SUMOylated proteins were purified by Ni²⁺-NTA affinity resin following immunoblotted with anti-HA antibody. GAPDH is a loading control. (B) The single-mutant HA-Csk K53R or HA-Csk K196R or double-mutants HA-Csk K53/196RR with His-SUMO1 were co-transfected into HEK293T cells followed by Ni²⁺-NTA pull down for SUMOylation assay. GAPDH is a loading control.
[33] was modified and performed. Generally, SENP−/− HeLa cells or HeLa cells or SENP−/− HEK293T (1.5 × 10^5) were lysed in 0.3 ml of RIPA buffer (20 mM sodium phosphate (NaP), pH 7.4, 120 mM NaCl, 1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 20 mM NEM, 1 mM Na_3VO_4, 10 mM NaF, 5% glycerol, protease inhibitor cocktail). The viscous lysate was sonicated until it became fluid. The cell lysate was then supplemented with 50 mM dithiothreitol (DTT), boiled for 10 min and finally diluted 1:10 with RIPA buffer without SDS. The lysate was centrifugal and the supernatant were immunoprecipitated with appropriate antibody overnight at 4°C and subjected to 8% SDS-polyacrylamide gels for Western blotting analysis.

**Cell Growth, Colony Formation Assay and Soft-Agar Colony Assay**

For cell growth assay, 1 x 10^4 /well DU145 cells were inoculated in 96-well plates. Each sample equally spread 6–8 wells. 3–4 h before measuring, each well was added with 10 μl CCK 8 solution. The absorbance was finally determined at 450 nm using a microplate reader. The absorbance values of the cells were recorded at 0, 24 h, 48 h, 72 h and 96 h, respectively. Data are expressed as mean + SD. Statistical differences between groups were analyzed by the two-way ANOVA test. P < .05 was considered statistically significant.

For colony formation assay, 2 x 10^2 /well DU145 cells or HeLa cells were seeded in 6-well plates. Each sample equally spread 3–4 wells. Cells were cultured in DMEM medium containing 10% FBS for 2–3 weeks. To visualize cell colonies, cells were fixed with 10% formaldehyde and stained with Giemsa stain.

For soft-agar colony assay, the method was described before [52]. This assay was performed in 6-well plates in triple with a base of 2 ml of DMEM medium containing 5% FBS with 0.6% Bacto agar (Amresco). DU145 cells were seeded at a density of 2 x10^3 cells /well of DMEM medium containing 5% FBS with 0.6% Bacto agar and stained with Giemsa. For soft-agar colony assay, the method was described before [52]. This assay was performed in 6-well plates in triple with a base of 2 ml of DMEM medium containing 5% FBS with 0.6% Bacto agar (Amresco). DU145 cells were seeded at a density of 2 x10^3 cells /well of DMEM medium containing 5% FBS with 0.6% Bacto agar and stained with Giemsa.

**Comunmunoprecipitation (co-IP)**

Cells transfected with the indicated plasmids were lysed in the RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 20 mM NEM, 5% glycerol, protease inhibitor cocktail with 250 mM NaCl for Cbp, and 350 mM NaCl for PIASs, respectively) on ice. The lysate was centrifugal and the supernatant were immunoprecipitated with appropriate antibody overnight at 4°C and subjected to 8% SDS-polyacrylamide gel for Western blotting analysis.

**Western Blotting**

Western blot analysis was performed as previously described [52]. The proteins either from whole cell lysates in the SDS-lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, and 1 mM Na_3VO_4), or from immunoprecipitations, were resolved on 8% SDS-PAGE, then transferred to a polyvinylidene difluoride (PVDF) membrane. Blocking was performed with 5% nonfat milk in Tris-buffered saline and Tween 20 (TBST) buffer. The membrane was subsequently probed overnight at 4°C with the indicated primary and second antibodies (at 1:5000 dilutions in TBST with 5% non-fat milk), and then exposed in ImageQuant LAS 4000 (GE) after incubating with ECL substrate.

**Results**

**Csk Protein Occurs SUMOylation Both in vitro and in vivo**

SUMOylation is an important mechanism for modulation of cellular functions by regulating tyrosine kinase activity. Most recently, our study demonstrated that SUMOylation of c-Src at lysine 318 negatively modulates its oncopgenic function by inhibiting Src-FAK complex activity. Since Csk is the specific negative regulator of c-Src, we tend to ask whether Csk can also be SUMOylated. To verify this hypothesis, we transiently transfected HEK293T cells with HA-tagged Csk and His-tagged SUMO1. Then the His-tagged SUMO1 conjugates enriched with Ni^{2+}-NTA resin pull-down assay as described before [32] were immunoblotted with appropriate antibody. A predicted band at about 70 kD (the expected size of Csk is about 50 kD, and SUMO1 is 15–20 kD) appeared with anti-HA or anti-Csk antibody in cells transfected with HA-Csk / His-SUMO1, but not in HA-Csk or His-SUMO1 alone (Figure 1A). Moreover, the specific 70 kD band was greatly enhanced by the SUMO E2 ligase Ubc9 but attenuated by SENP1, a main de-SUMO enzyme (Figure 1B, lane 4 and 5). Both the results suggest that Csk can be SUMOylated in cells. Additionally, one immunoprecipitation (IP) method described as sensitive to detecting endogenous SUMO targets was performed [33]. HA-Csk with or without Flag-SUMO1 were transfected in HEK293T cells. 48 h later after transfection, the lysates, boiled for 10 min with 1% SDS and 50 mM DTT, then diluted 1:10 with RIPA buffer, were used for IP with Flag beads overnight, and immunoblotted with HA antibody. As shown in Figure 1C, as expected, SUMO1 can be covalently conjugated with Csk. Furthermore, we investigated SUMOylation of Csk by using an in vitro E.coli-based SUMOylation assay by pE1E2S1 [34,35]. 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. Co-expression of GST-Csk with pE1E2S1 in bacteria BL21 (DE3) showed that GST-Csk were SUMOylated by SUMO1 with two bands but not in the E.coli transformed with GST-Csk alone (Figure 1D). This result suggested that there might be more than one SUMO sites for Csk. Significantly, we also detected endogenous SUMO1-Csk form both in SENP1−/− HeLa cells (Figure 1E) and SENP1−/− HEK293T cells (Figure 1F) by the method of immunoprecipitation described above. Meanwhile, we also detected weaker endogenous SUMO1-Csk in HeLa cells compared with SENP1−/− HeLa cells (Figure 1G). Collectively, all the results indicate that Csk is a SUMOylated protein covalently with SUMO1.

**K53 is the Main SUMOylation Site of Csk**

To determine the SUMOylation sites in Csk protein, based on the SUMOylation prediction softwares (Figure S1), we constructed seven single lysine-arginine (K/R) mutants and checked their SUMOylation level compared with Csk wild type (WT) with the method of Ni^{2+}-NTA resin pulldown assay. Unexpected, there was no obvious different between each of these K/R mutants and WT, which means the main SUMOylation Site of Csk do not be in this group (Figure S2). After that, we mutated all the rest lysines in Csk and SUMOylation assay revealed that only K53 is a potential SUMOylation site (Figures 2A and C and S3). What is more, in our
In the project process, we searched one paper concerning large-scale SUMOylation and ubiquitylation crosslink, in which K196 was identified as the SUMOylation site of Csk [36], so we verified Csk<sup>53R</sup> and Csk<sup>196R</sup> SUMOylation in one same test. As shown in Figure 2B, the SUMOylation level of Csk<sup>53R</sup> was much lower than that of Csk WT, but SUMOylation of Csk<sup>196R</sup> looked like as that...
of Csk WT. Moreover, the low SUMOylation level of CskKK53/196RR was same as that of CskK53R, strongly suggesting that K53, but not K196, was the major SUMOylation site of Csk protein.

**Csk SUMOylation Independent of its Phosphorylation is Inhibited by Hydrogen Peroxide**

Oxidative stress can affect apoptosis and proliferation of cells and change the development of tumor. We have found that hypoxia inhibits SUMOylation of Src via Y419 phosphorylation in tumor biological behaviors previously [31]. It has been reported that the disulfide bonds in the structure domain of Csk SH2 can be affected by oxidative stress. Therefore, we want to explore whether oxidative stress affects the SUMO modification of Csk. We designed experiments as following. HA-Csk and His-SUMO1 were over-expressed in 293 T cells, with only one mixed system and evenly divided into each cell culture dish to ensure that each group was the same. 48 h later after transfection, hydrogen peroxide (H2O2) with a final concentration of 100 uM was added to simulate the hyperoxic state. The samples were treated for 0, 3 and 6 hours respectively. According to the experimental result, the SUMOylation of Csk decreased with hydrogen peroxide treatment in a time-dependent manner (Figure 3A). Similarly, endogenous Csk SUMOylation in SENP−/− HeLa cells was also inhibited obviously 6 hours later after treated with hydrogen peroxide (H2O2) with a final concentration of 100 μM (Figure 3B). On the other hand, previous studies have shown that Csk phosphorylation at Ser 364 is induced by PKA. So we ask whether this phosphorylation affect Csk SUMOylation. With the method of Ni2+-NTA resin pulldown assay, we found that the level of SUMOylation of CskS364A was equal to that of Csk WT (Figure 3C). Taken together, our results suggest that SUMOylation of Csk is down-regulated by hyperoxia and unaffected with its phosphorylation status.

**PIAS3 is the Main E3 Ligase of Csk SUMOylation**

Recent evidence indicates that SUMOylation is directly involved in the regulation of oncogenic networks. Although it is not completely understood how substrate recognition is achieved in the SUMOylation system, one SUMO E3 ligase family, PIAS, are often over-expressed in cancer [37]. In mammals, there are four PIAS proteins exist: PIAS1, PIAS2 (also known PIASx, two splice variants α and β), PIAS3 (also known PIASβ) and PIAS4 (also known PIASγ). PIAS proteins are emerging as key positive regulators of many cancer-related proteins as PML [38], p53 and PTEN [39]. We have set up coimmunoprecipitation experiment (co-IP) to explore the specific PIASs interacting with Csk. As shown in Figure 3D, PIAS1, PIAS3 and PIAS4 could bind to Csk. Moreover, comparing the binding intensity in IP test and expression level in input group, we supposed that PIAS3 was the main E3 ligase for Csk SUMOylation. To enforce this viewpoint, we constructed a RING domain deletion form of PIAS3, and found that PIAS3 ΔRING could still bind to Csk (Figure 3E), which is consistent with the reports that both PIAS1 and PIAS4 regulate LEF-1 and p53, regardless of the integrity of the RING domain, which is necessary for their E3 ligase activity [40,41]. Furthermore, as expected, PIAS3 could significantly enhance the SUMOylation of Csk in SUMOylation assay using Ni2+-NTA resin affinity pulldown (Figure 3F). However, none of PIAS1, PIAS4 or ΔRING PIAS3 could enhance the SUMOylation of Csk (Figure 3G). All the data support that PIAS3 is the main SUMO E3 ligase for Csk.

**Csk SUMOylation Negatively Regulates its Anti-Oncogenic Function**

We next examined further the role of the Csk SUMOylation in proliferation and tumorigenesis. To investigate this, we generated stable DU145 cell lines and HeLa cell lines over-expressing the lentivirus, HA-Csk or HA-CskK53R with the polyclonal lentiviral infections (Figures 4A and S4). Then Cell Counting Kit 8 (CCK8) assay was used to examine the cell proliferation. As shown in Figure 4B, the rate of cell proliferation was moderately decreased in the HA-Csk group compared with lenti-vector group, which is consistent with Csk tumor suppressor function. However, the SUMO-defective form, HA-CskK53R cells displayed a slower, even less significant, proliferation rate than HA-Csk cells, which indicated that SUMO modification might have a negative effect for Csk function. To support this hypothesis, plate-colony forming assay and soft-agar anchorage-independent growth test were carried out. As expected, the numbers of plate clones from stable HA-CskK53R cells were much less than those from HA-Csk cells (Figures 4C and S5). Consistently, CskK53R effectively suppressed the size and the numbers of colonies in soft-agar medium (Figure 4D). Furthermore, to better verify the function of Csk SUMOylation rather than by mutation of amino acids (K to R) method, we made a SUMOylation construct (Figure 5A), of which was described before [42]. The measurements as cell proliferation, plate colony formation and soft-agar anchorage-independent growth were carried out again. In contrast, SUMO1-Csk stable cells could rescue the phenotype of CskK53R cells with faster growth, more colonies formation compared with Csk WT. However, it should be noted that it appears that SUMO1-Csk still has inhibitory effect on cell growth and survival as compared to the vector control, which means SUMOylation modification has partial negative regulation ability for Csk anti-oncogenic function (Figure 5B-D). Collectively, these data from both CskK53R (SUMO-deficient form) and fused SUMO1-Csk (high -SUMO form) stable
cells demonstrate that SUMO modification negatively regulates anti-oncogenic function of Csk.

**Csk SUMOylation Weakens its Binding with Cbp Leading to Activation of Src**

Our above results suggested that SUMOylation represented a negatively regulatory mechanism for controlling the anti-oncogenic function of Csk, but the details were still unknown. Since there was no correlation between its SUMOylation and phosphorylation at Ser364, the active form of Csk (Figure 3C), it didn’t like that Csk SUMOylation could directly affect its kinase activity. We have noticed that the SUMO site, lysine 53, is located in the SH2 domain (amino acid 16–65) of Csk, it was reasoned that this modification could interfere with the SH2 domain-dependent function. It is well

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**Figure 4.** SUMOylation of Csk may suppress tumor cell growth and colony formation. (A) The prostate cancer cell line DU145 were infected with a lentiviral vector CD513B containing HA-Csk or HA-Csk\textsuperscript{K53R}. Western Blotting showed the expression of Csk or Csk\textsuperscript{K53R} in these cells. GAPDH is a loading control. (B) Proliferation of stable Du145 cell lines was examined by Cell Counting Kit-8. (C) Colony formation assay. 2 × 10\textsuperscript{2} /well DU145 cells were seeded and cultured in DMEM medium containing 10% FBS in 6-well plates for 2–3 weeks until counting. (D) Soft-agar colony assay. DU145 cells were seeded at a density of 2 × 10\textsuperscript{3} cells /well in 2 ml of 0.35% agar gel with 5% FBS, and were layered on the base gel in 6-well plates. The photographs of the colonies developed in soft agar were taken and the number of colonies were scored by Photoshop about 2–3 weeks after seeding. All data are mean ± S.D. Differences in colonies were analyzed by t-test (*P < .05, **P < .01, ***P < .001). (C and D).
established that Cbp (Csk binding protein), a transmembrane phosphoprotein that is ubiquitously expressed and binds specifically to the SH2 domain of Csk, is involved in the membrane localization of Csk as well as in the Csk-mediated inhibition of Src [43]. Thus, the binding pattern between Csk and Cbp regulated by SUMOylation was analyzed using coimmunoprecipitation (co-IP). As shown in Figure 6A, immunoprecipitation with anti-Flag antibody followed by immunoblotting with anti-Myc antibody revealed that Cbp WT, but

Figure 5. Fused SUMO1-Csk may suppress tumor cell growth and colony formation. (A) The prostate cancer cell line DU145 were infected with a lentiviral vector CD513B containing Flag-Csk or fused Flag-SUMO1-Csk. Western Blotting showed the expression of Csk or SUMO1-Csk in these cells. GAPDH is a loading control.(B) Proliferation of stable Du145 cell lines was examined by Cell Counting Kit-8.(C) Colony formation assay. 3 × 10^5 /well DU145 cells were seeded and cultured in DMEM medium containing 10% FBS in 6-well plates for 2–3 weeks until counting.(D) Soft-agar colony assay. DU145 cells were seeded at a density of 2 × 10^5 cells /well in 2 ml of 0.35% agar gel with 5% FBS, and were layered on the base gel in 6-well plates. The photographs of the colonies developed in soft agar were taken and the number of colonies were scored by Photoshop about 2–3 weeks after seeding. All data are mean ± S.D. Differences in colonies were analyzed by t-test (*P < .05, **P < .01, ***P < .001). (C and D).
not Cbp Y317F, the unphosphorylated form, could bind to Csk strongly (lane 2 and 3, Figure 6A), which is consistent with the rule that SH2 domain recognizes specific phosphopeptide sequences that bind to tyrosine sites. However, under high-SUMO condition by cotransfected with SUMO1, the binding between Cbp and Csk dramatically decreased (lane 4, Figure 6A). Conversely, Csk K53R, the
SUMO-deficient mutant, had a same binding intensity with Cbp whether or not under SUMO condition (lane 2 and 4, Figure 6B). Both results suggested that the SUMOylation status of Src regulated by Csk SUMOylation. HEK293T cells were transiently transfected with HA-Csk or/and His-SUMO1, and anti-Src (pY416 and pY527) antibodies were used to monitor the Src activation. As shown in Figure 6C, compared with the mock cells, phosphorylation level of Src Y527 was moderately higher in Csk-transfected cells (lane 3), but this phenomenon was reversed along with pY416 increased when co-transfected with SUMO1 (lane 4). It was noticed that the level of either pY416 or pY527 had hardly change by Src SUMOylation reported by our group previously [31] or by transfected with SUMO1 alone (lane 2), so the Src activation (pY527 decreased and pY416 increased) was probably dependent on Csk SUMOylation. In addition, the moderate increased pY416 was also observed in fused SUMO1-Csk transfected cells (lane 3, Figure 6D). Notably, it is well accepted that SUMO can act as a ubiquitin antagonist since SUMO conjugation could block other lysine dependent modification such as ubiquitination [44]. Therefore, it should be excluded that Csk SUMOylation function was attributed to its ubiquitination. Indeed, the pattern of Csk ubiquitination was unchanged when coexpressed with SUMO1 (Figure 6E). All the results suggest that SUMOylated Csk weakens its binding with Cbp leading to activation of Src.

Discussion

Since the initial link between SUMOylation and cancer originated from the discovery of the tumor suppressor PML (promyelocytic leukemia protein) as among the first known SUMO proteins [45,46], SUMO conjugation has become a widely recognized posttranslational modification in all cancer hallmark functions. In this study, we have confirmed that Csk, one tumor suppressor as a negative regulator of SFKs, can be modified by SUMO1 in HEK293T cells (Figure 1A and B) using His-tagged SUMO1 conjugates bound to Ni2+ -NTA beads. Indeed, co-transfection with Csk and SUMO1 but without Ubc9, the SUMO1-Csk band can be detected clearly, which is different from our previous reports, of which Ubc9 is essential to detect PTEN or Grb2 SUMOylation [34,39]. Importantly, we also used a high effective immunoprecipitation method and western blotting to show the endogenous SUMOylated Csk both in SENP1−/− HeLa cells (Figure 1E) and SENP1−/− HEK293T cells (Figure 1F). Moreover, during our project, it was reported that Csk can be SUMOylated in HEK293 cells by mass spectrometry method [36] although the identified SUMO site, lysine 196, has been denied by our experiments (Figures 2B and S3). All the evidence support that Csk is a bona fide SUMOylated protein.

It has been reported that low ROS level (<1 mM H2O2) are sufficient for favoring the creation of oxidative disulfide crosslink between the active site cysteine residues of SAE2 (E1) and Ubc9 (E2) enzyme, thereby inhibiting SUMOylation while leaving SENP activity intact [47], which means SUMOylation may function as an important redox sensing mechanism. Intriguingly, our present and previous studies showed that the level of SUMO1- Csk was down-regulated whereas SUMO1- Src was up-regulated in a time-dependent manner when treated with 100 μM H2O2 (Figure 3A and B). Thus, hyperoxia may have a dual role in Csk-Src signaling. In hyperoxia, deSUMO1-Csk promotes freed Csk binding with Cbp, and SUMO1-Src weakens its kinase activity on FAK Y925, both processes strongly imply that hyperoxia can inhibit Src activity.

Notably, we have characterized PIAS3 as a main E3 ligase to Csk SUMOylation meanwhile Csk also has a weak binding with PIAS1 and PIAS4 (Figure 3D). In considering of high salt concentration (350 mM NaCl) used in Co-IP experiment, it is likely that PIAS1or PIAS4 has a possibility to be an alternative E3 ligase for Csk in uncertain conditions. One reasonable explanation is PIAS family share 40% of homology and domain organization [28]. A similar conclusion is from the studies on p53 SUMOylation. It concludes that all PIAS members physically interact with p53 in vitro assays using both cell-free and cell culture-based assays [30], however, it is still unclear whether different ligase-dependent SUMOylation either activates or represses p53 activity. Further examination should be performed which PIAS member is the physiological partner of Csk probably depending on different cell types or tumor environment.

There is no significant change in the phosphorylation pattern of Src following Csk over-expression in our experiment (lane 1 and 3, Figure 6C). We are in agreement with the view from Arbet-Engels et al [48], in which it suggested that since Src is already phosphorylated on Tyr527 in un-stimulated cells, and only a fraction of Csk is involved in binding with Src resulting in phosphorylation of Src on Tyr527, the lack of an observable change is not unexpected.

In addition to Cbp, other SH2-binding scaffolding proteins of Csk have been identified, such as Paxillin [49] and caveorin-1 [50]. It was also proposed that the caveorin-1-Csk interaction mediated a feedback loop to regulate Src kinase activity in microdomains [51]. So it cannot be excluded that Csk SUMOylation may also weaken its binding with these proteins other than Cbp. Nevertheless, both
evidences support that Cbp is most likely to be the molecule correlated to Csk SUMOylation. One is the high stability of Cbk-Cbp complex still remained even in the presence of increased NaCl (1 M) concentration, but other known Csk-interacting proteins such as FAK and Paxillin were dissociated [18]. The other is the binding between Cbp and Csk, but not Csk K53R, the SUMO-deficient mutant, dramatically decreased under SUMO condition (lane 4, Figure 6A).

Generally, since there have been no reports of mutation in the Csk gene, our findings might give a new clue for understanding the biological significance of correlation between Csk and Src (also SFKs).

Conclusions

In summary, our results revealed an unexpected mechanism underlying specific regulation of Cbp-Csk-Src feedback loop by Csk SUMOylation (model in Figure 6F). SUMOylation of Csk mainly at K53, located at SH2 domain, is up-regulated by SUMO E3 ligase PIAS3 and down-regulated under hyperoxia. SUMOylated Csk could not bind to Cbp, which disturbs the Cbp-Csk-Src feedback leading to the sustained activation of Src.

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Author Contributions

J.H. and J.Y. supervised the project. J.H. and N.C. designed the experiments. N.C. performed most experiments. T.L., Y.G., J.D., Q. PG, and Ryu SH (2017). Intestinal Epithelial Cell-Specific Deletion of PLD2 Alleviates DSS-Induced Colitis by Regulating Occludin. Sci Rep 7, 1573.

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