NEOPLASIA
www.neoplasia.com

Volume 20 Number 11 November 2018 pp. 1095–1105 1095

Interaction of Abl Tyrosine Kinases with SOCS3 Impairs Its Suppressor Function in Tumorigenesis[1,2,3]

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Abstract

Suppressor of cytokine signaling 3 (SOCS3) is involved in Bcr-Abl–induced tumorigenesis. However, how SOCS3 interacts with Bcr-Abl and is regulated by Abl kinases remains largely unknown. Since c-Abl plays a critical role in tumorigenesis, we asked whether SOCS3 is regulated by c-Abl–dependent phosphorylation. Here, we found that SOCS3 interacted with all three Abl kinases (Bcr-Abl, v-Abl, and c-Abl), and SH1 domain of the Abl kinases was critically required for such interaction. Furthermore, the SH2 domain of SOCS3 was sufficient to pull down the SH1 domain but not the full length of Bcr-Abl. Importantly, SOCS3 was highly tyrosine phosphorylated by c-Abl, leading to impairment of its ability to suppress JAK8+72 activity. In addition, disrupting the tyrosine phosphorylation of SOCS3 promoted apoptosis of c-Abl–expressing cells and impeded xenograft growth of these tumor cells in nude mice. The results demonstrate that SOCS3 is highly tyrosine phosphorylated by c-Abl and that tyrosine phosphorylation of SOCS3 is required for the survival and tumorigenesis of certain cells. Our findings provide novel insights into complicated mechanisms underlying the oncogenic function of Abl kinases.

Neoplasia (2018) 20, 1095–1105

Introduction

The Abelson (Abl) family of nonreceptor tyrosine kinases, comprising Abl1 and Abl2, plays a critical role in the regulation of various cellular processes, including cell proliferation, migration, and survival [1–4]. The kinase activity of Abl is tightly regulated by intra- and intermolecular interactions as well as phosphorylation [2]. However, as a consequence of viral fusion or chromosomal translocation, structural alterations of the cellular Abl (c-Abl, Abl1) lead to generation of constitutively activated forms of the Abl1 tyrosine kinase that drive leukemia development in mice and humans [2]. *Gag-Abl* (*v-Abl*), the oncogene of Abelson murine leukemia virus (A-MuLV), induces pre–B-cell transformation through constitutively activated Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) signaling in mice [5,6]. In humans, chromosomal translocations generate *Bcr-Abl* hybrid gene that mediates the pathogenesis of chronic myelogenous leukemia [7,8].

Abbreviations: CML, chronic myeloid leukemia; EGFR, Epidermal growth factor receptor; FUS1, Fused in sarcoma-1; GST, Glutathione S-transferase; IGF-1, Insulin-like growth factor-1; JAK/STAT, Janus tyrosine kinase/signal transducer and activator of transcription; MMP-1, Matrix metalloproteinases-1; mTOR, mammalian/mechanistic target of rapamycin; PI, Propidium iodode; Pim, proviral insertion in murine; SOCS, Suppressors of cytokine signaling.

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[1] Funding: This study was supported by Natural Science Foundation of China (81472611, 81502397) and National Basic Research Program (973) of China (2014CB541804, 2015CB910502).
[2] Competing Interests: No competing financial interests to declare.
[3] Conflicts of Interest Statement: None.
[4] These authors contributed equally to this work.
Received 9 May 2018; Revised 5 September 2018; Accepted 7 September 2018

1476-5586
https://doi.org/10.1016/j.neo.2018.09.002

Besides, accumulating evidence has implicated that Abl kinases also contribute to the development of solid tumors characterized by enhanced expression or hyperactivation of Abl kinases [2,9–11]. It is well known that c-Abl plays a critical role in multiple cellular processes and tumorigenesis, and several c-Abl inhibitors have been tested for the treatment of numerous solid tumors [9]. However, the function of c-Abl in different cell types may be opposite. For example, c-Abl inhibits cell migration and enhances apoptosis via phosphorylating MDM2 in human lung carcinoma cells [12–14] but promotes melanoma cell invasion via distinct pathways [15]. Thus, the molecular mechanisms underlying the involvement of c-Abl in the progression of tumors are not fully understood.

Suppressor of cytokine signaling (SOCS) proteins have been identified as key negative regulators of JAK/STAT signaling, which are vital in many immunologic and pathologic processes [16,17]. Of the eight family members, SOCS-1 and SOCS-3 are the most potent inhibitors of JAK/STAT signaling pathway. Since activation of JAK/STAT signaling is required for cellular transformation mediated by several oncogenes, the suppressor function of SOCS proteins needs to be overcome during the tumorigenesis of particular cells [18]. For example, a previous study has revealed that v-Abl could bypass SOCS1 inhibition through phosphorylation of SOCS1 and reduce its ability to inhibit JAK1 activation [18]. In addition, myeloproliferative disorder-associated JAK2 mutant (JAK2 V617F) can escape negative regulation of SOCS3 through tyrosine phosphorylation of SOCS3 [19]. Interestingly, a recent report has shown that c-Abl can also activate JAK2 in response to IL-3 through their direct interaction in hematopoietic cells [20]. Furthermore, signal transducer and activator of transcription 3 (STAT3) can be activated by c-Abl in human primary melanomas, and c-Abl promotes melanoma cell invasion via STAT3-dependent upregulation of matrix metalloproteinase-1 [15]. Together, these observations demonstrate that c-Abl can activate JAK/STAT signaling. However, how c-Abl bypasses the inhibitory effects of SOCS proteins remains to be determined.

Our previous study has shown that SOCS3 is tyrosine-phosphorylated by Bcr-Abl, which is associated with Bcr-Abl–mediated cellular transformation [21]. These data prompted us to further investigate the interactions between SOCS3 and various Abl tyrosine kinases including Bcr-Abl, v-Abl, and c-Abl and explore the functional involvement of SOCS3 phosphorylation in c-Abl–mediated cellular processes.

Materials and Methods

Ethics Approval and Consent to Participate

The animal experimental design and protocols used in this study were approved by the Regulation of the Institute of Microbiology, Chinese Academy of Sciences of Research Ethics Committee (Permit Number: PZIMCAS2015008). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

Cell Lines, Cell Culture, and Western Blotting

Cell lines 293T, K562, HL-60, HepG2, and Huh-7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 or Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (Gibco) and antibiotics (penicillin and streptomycin; Invitrogen, Carlsbad, CA) as described previously [22]. The v-Abl–transformed mouse pre–B-cell lines NS2 and W44 were generated and cultured as previously described [1]. Western blotting was performed as described previously [22,23]. Briefly, cell lysates were separated on SDS polyacrylamide gel, transferred onto a nitrocellulose membrane, and probed with indicated antibodies.

Construction of Plasmids and Generation of Stable Cell Lines

The mutants SOCS3 (Y204F), SOCS3 (Y221F), and SOCS3 (Y204F, 221F) were generated by site-directed mutagenesis with the QuickChange XL system (Stratagene, La Jolla, CA) as previously described [21]. SOCS3 and their mutants were subcloned into pFLAG-CMV-5 vector and retroviral vector pMIG-IRES-GFP (gifts from Dr. Richard Van Etten, Tufts University, Boston, MA). Cell lines overexpressing SOCS3 and their mutants were generated as previously described [1]. Briefly, retroviruses encoding SOCS3 and their mutants were produced in 293T cells. These retroviruses were then collected, filtered through a 0.22-μm MCE membrane (Millipore), and used to infect indicated cells. c-Abl knockdown cell lines were generated by infecting cells with lentiviruses expressing specific short hairpin RNAs (shRNAs) in pSIH-H1-GFP vector (System Biosciences, Palo Alto, CA) as described previously [24]. Two pairs of shRNA sequences targeting c-Abl are shown as follows: sh-c-Abl-1: 5′-GGGTGTAC CATTACAGGATCA-3′ and sh-c-Abl-2: 5′-GGAAGAGTTCTT GAAAGAAGC-3′.

Antibodies

The following antibodies were used in this study: anti–c-Abl, anti-phosphotyrosine clone 4G10 (Millipore, Billerica, MA); anti-SOCS3, anti–phospho-c-Abl (Y412), anti-STAT3, anti–phospho-STAT3 (Y705), anti-JAK2, anti–phospho-JAK2 (Y1007/1008) (Cell Signaling Technology, Danvers, MA); and anti-Bcr and anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA).

Glutathione S-transferase (GST) Pull-Down and In Vitro Binding

GST fusion proteins were expressed in the bacteria BL21 (DE3) and purified, and pull-down experiments were performed as previously described [1,21]. Briefly, pull-down experiments were performed by incubating glutathione beads with cell extracts, and the beads were washed extensively followed by Western blotting.

Apoptosis Assay

Apoptosis assay was performed as previously described [22,23]. Briefly, cells were treated with etoposide (Sigma, St. Louis, MO) for the indicated time. Then, the cells were stained with 2.5 μg/ml Annexin V-FITC and 1 μg/ml propidium iodide. Samples were examined by fluorescence-activated cell sorter (BD Bioscience, San Jose, CA), and data were analyzed by FCS Express V3 Software (De Novo Software, Glendale, CA).

Proliferative Activity and Cell Cycle Analysis

Proliferative activity was examined using Ki67 cell proliferation Detection Kit (KeyGEN BioTECH, Nanjing, China) according to the manufacturer's instructions. Briefly, cells were fixed in 70% ethanol at 4°C for 1 hour and incubated with Ki67 antibody at room temperature for 1 hour, followed by incubation with second antibody for 30 minutes. The samples were then analyzed with a fluorescence-activated cell sorter (BD Bioscience).

Cell cycle progression was investigated as previously described [25]. Briefly, cells were synchronized at the G1/S with 2 mM of thymidine for 13.5 hours and released for 9 hours, followed by treatment with thymidine for 13.5 hours and release for 2.5 hours or 5 hours. For flow cytometry analysis, cells were collected at the indicated time, fixed in 75% ethanol at −20°C overnight, then washed with PBS and incubated with propidium iodide (5 mg/ml with 0.1% RNase A) for 30 minutes. The samples were analyzed with a fluorescence-activated cell sorter (BD Bioscience).

Xenograft Study in Nude Mice

Cells (1×10^7) were injected subcutaneously into female nude mice (5-6 weeks old). Tumor growth was monitored and measured in volume (length × height × width) at the indicated time points during the 21-day period after inoculation. Bioluminescent imaging was performed to detect the growth of tumors from GFP-expressing cells. Images were quantified as photons/s using the indigo software (Berthold Technologies, Bad Wildbad, Germany).

Statistical Analysis

All data represented the mean values ± standard error of at least three independent experiments. Statistical analysis was determined by Student's t test, and the P values < 0.05 were considered to be significant.

Results

Identification of the Domain in Bcr-Abl That Interacts with SOCS3

Our previous study has demonstrated that Bcr-Abl regulates tyrosine phosphorylation of SOCS3, which is critically associated with cellular transformation mediated by Bcr-Abl oncogene [21]. However, an interaction of SOCS with Bcr-Abl and the essential domain(s) responsible for such interaction remain largely unknown. In this study, we constructed several truncations of Bcr-Abl including Bcr, $Abl^{SH1\text{-}SH2\text{-}SH3}$ ($Abl^{SH1\text{-}3}$), $Abl^{C\text{-}Terminal}$ (Abl^{CT}), Abl^{SH1}, and $Abl^{SH2\text{-}SH3}$ ($Abl^{SH2\text{-}3}$) (Figure 1*A*) and utilized these truncations to test the interaction between Bcr-Abl and SOCS3 through GST pull-down experiments. As shown in Figure 1*B*, SOCS3 bound to the full length of Bcr-Abl but not its Bcr domain, indicating that Bcr-Abl might interact with SOCS3 via its Abl domain. To address this possibility, we used GST-SOCS3 to pull down $Abl^{SH1\text{–}3}$ and Abl^{CT} truncated proteins. Our results showed a specific association of SOCS3 with $Abl^{SH1\text{–}3}$ rather than Abl^{CT} (Figure 1*C*).

In addition, when GST-SOCS3 was used to pull down Abl^{SH1} and $Abl^{SH2\text{–}3}$ truncations, SOCS3 was found to strikingly bind to Abl^{SH1} but not $Abl^{SH2\text{–}3}$ (Figure 1*D*). This finding was further confirmed by additional independent experiments (Figure 1*E*). Conversely, we also employed GST-Abl^{SH1} to pull down SOCS3. Similarly, a strong interaction between Abl^{SH1} and SOCS3 was observed in the pull-down complex (Figure 1*F*). Together, our results indicate that SH1 domain of Bcr-Abl is the exact domain responsible for the binding with SOCS3.

SH2 Domain of SOCS3 Binds to SH1 Domain But Not the Full-Length Protein of Bcr-Abl

Next, we determined which domain of SOCS3 is responsible for its interaction with Bcr-Abl. To this end, we generated a series of SOCS3 truncation mutants for GST pull-down experiments. As shown in Figure 2*A*, SOCS3-T1, SOCS3-T2, and SOCS3-T4 truncations all contain the SH2 domain except for SOCS3-T3 mutant. We observed the associations of Abl^{SH1} with SOCS3 wild type, SOCS3-T1, SOCS3-T2, and SOCS3-T4 (Figure 2, *B* and *C*). In contrast, no interaction was detected between Abl^{SH1} and SOCS3-T3 (having no SH2 domain), suggesting that the SH2 domain of SOCS3 is essential for its interaction with Abl^{SH1} (Figure 2*B*). However, we found that the SH2 domain of SOCS3 (SOCS3-T4) failed to pull down the full length of Bcr-Abl using either Bcr-Abl overexpression system (Figure 2*D*) or endogenous expression system (Figure 2*E*). Similar results were observed in SOCS3-T1, SOCS3-T2, or SSOCS3-T3 as well (Figure 2*F*). These data indicate that the SH2 domain of SOCS3 is essential for SOCS3 interaction with SH1 domain of Bcr-Abl but is not sufficient to bind to the full-length protein of Bcr-Abl, suggesting that an intact SOCS3 protein may be required for its interaction with Bcr-Abl kinase.

In our previous study, we have observed that Bcr-Abl–dependent phosphorylation of SOCS3 occurs mainly on Y221 and slightly on Y204, which are within the conserved SOCS box [21]. Here, these two tyrosine residues were mutated to phenylalanine either individually or in combination, i.e., GST-SOCS3 (Y204F), GST-SOCS3 (Y221F), and GST-SOCS3 (Y204F, Y221F), and used to pull down Bcr-Abl (Figure 2*G*). The results revealed that there still existed the interaction between SOCS3 (Y204F) and Bcr-Abl. Remarkably, SOCS3 (Y221F) or SOCS3 (Y204F, Y221F) mutation totally disrupted their binding to Bcr-Abl (Figure 2*G*). These observations demonstrate that tyrosine residue Y221 of SOCS3 is critical for SOCS3 interaction with Bcr-Abl.

C-Abl–Dependent Phosphorylation of SOCS3 Impairs Its Ability to Negatively Regulate JAK2/STAT3 Activation

Having demonstrated that Abl domain but not the Bcr of Bcr-Abl protein was responsible for Bcr-Abl interaction with SOCS3, we asked whether other Abl kinases such as c-Abl and v-Abl that contain the Abl domain could also interact with SOCS3. To test this possibility, we examined c-Abl expression in several cell lines and selected c-Abl highly expressing cancer cell lines, including human promyelocytic leukemia HL-60 and human hepatocarcinoma Huh7 cell lines, for further studies (Figure 3*A*). HL-60 and Huh7 are Bcr-Abl–negative cell lines. Indeed, endogenous c-Abl in HL-60 cells was successfully pulled down by GST-SOCS3 (Figure 3*B*). Moreover, association of SOCS3 with v-Abl was also observed in both NS2 and W44 cell lines, which are v-Abl–transformed murine pre–B-cell lines (Figure 3, *C* and *D*). Hence, the results support that SOCS3 is able to directly bind to all three Abl kinases.

SOCS3 is known to be tyrosine-phosphorylated on Y204 and Y221 by Bcr-Abl, and this phosphorylation diminishes its inhibitory effects on the activation of JAK and STAT5, thereby enhancing JAK/STAT5 signaling [21]. Having observed that SOCS3 was able to directly bind to c-Abl, which can activate JAK/STAT signaling [15,20], we hypothesized that SOCS3 might also be phosphorylated by c-Abl, resulting in loss of its inhibitory effects on JAK/STAT signaling. To this end, we used either SOCS3 wild type or SOCS3 (Y204F), SOCS3 (Y221F), and SOCS3 (Y204F/Y221F) mutants to analyze the phosphorylation status of SOCS3 in the presence of c-Abl. We found that wild-type SOCS3 was highly tyrosine-phosphorylated when coexpressed with c-Abl. In contrast, no tyrosine phosphorylation was detectable in SOCS3 (Y204F/Y221F) mutant under the same condition (Figure 3*E*). Tyrosine phosphorylation levels of SOCS3

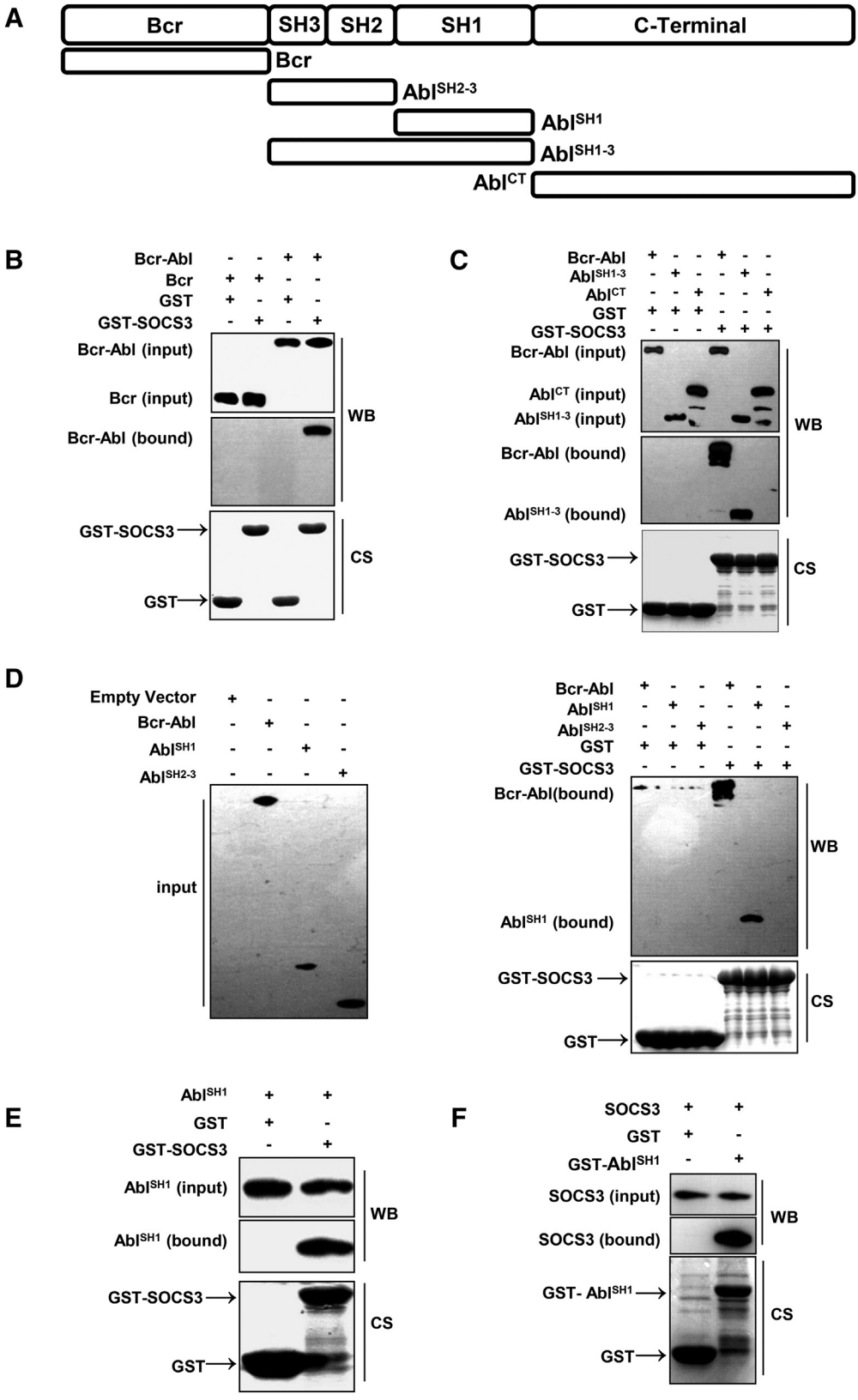


Figure 1. Identification of the domain in Bcr-Abl that interacts with SOCS3. (A) Diagram of the Bcr-Abl protein and its truncations used in the GST pull-down experiments, including Bcr, Abl$^{SH1\text{-}SH2\text{-}SH3}$ (Abl$^{SH1\text{–}3}$), Abl$^{C\text{-}Terminal}$ (Abl^{CT}), Abl^{SH1}, and Abl$^{SH2\text{-}SH3}$ (Abl$^{SH2\text{–}3}$). (B-D) The interaction between SOCS3 and Bcr-Abl was examined by GST pull-down experiments. Glutathione beads coupled to either GST alone or GST-SOCS3 were incubated with the lysates of 293T cells transfected with Bcr-Abl or the truncations. Cell lysates were either mock or λ phosphatase treated. The bound proteins were detected by Western blotting (WB) and Coomassie blue staining (CS). (E and F) The SH1 domain of Bcr-Abl was identified to be critically required for its interaction with SOCS3. (E) Lysates from 293T cells with Abl^{SH1} transfection were incubated with purified GST or GST-SOCS3 coupled beads. (F) Lysates from 293T cells with SOCS3 transfection were incubated with purified GST or GST-Abl^{SH1} coupled beads. The bound proteins were detected by WB and CS.

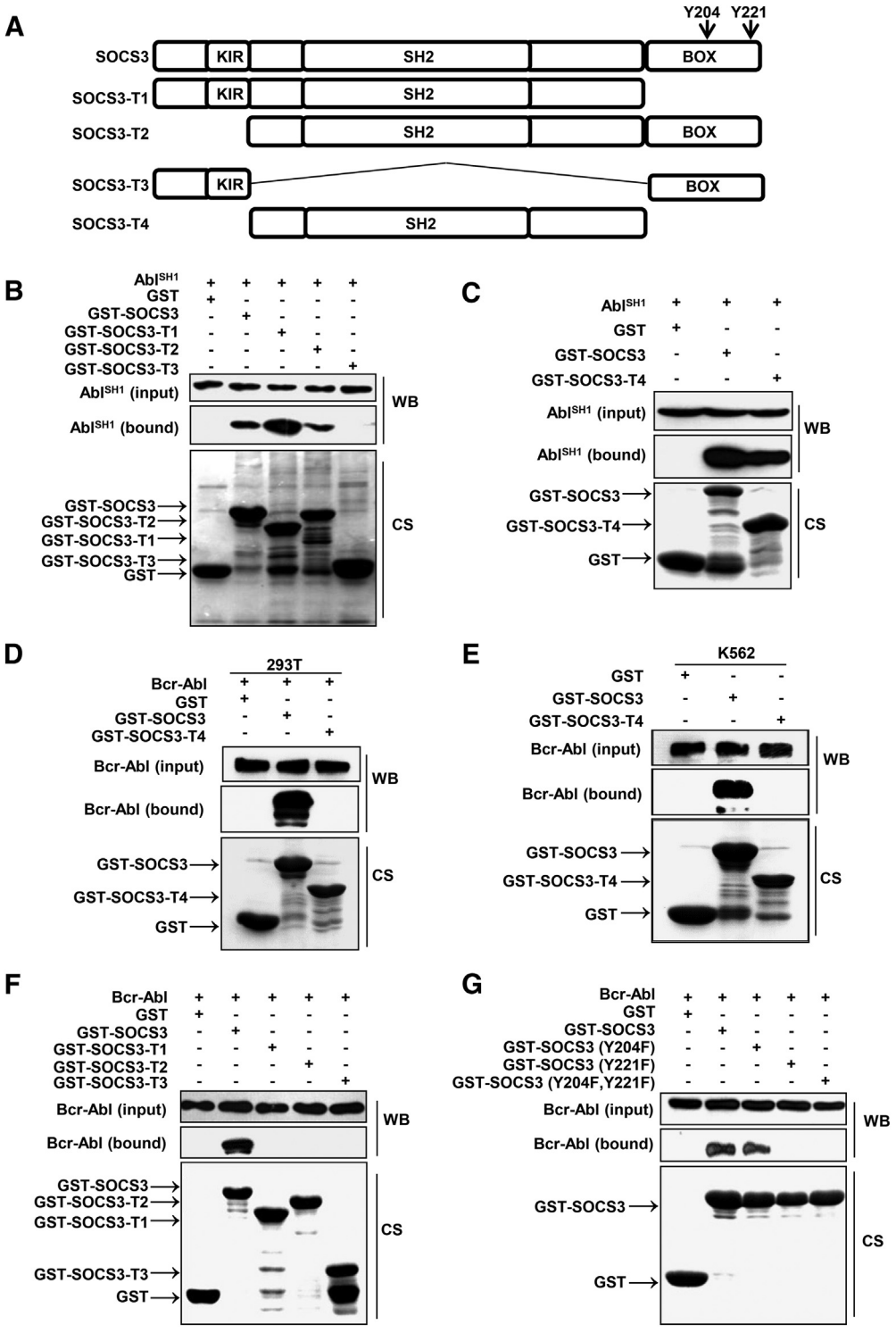


Figure 2. SH2 domain of SOCS3 can bind to SH1 domain of Bcr-Abl but not its full-length protein. (A) Diagram of the SOCS3 protein and its truncations used in the GST pull-down experiments, named SOCS3-T1, SOCS3-T2, SOCS3-T3, and SOCS3-T4. In particular, all of the truncations contain the SH2 domain except for SOCS3-T3 truncation. The Bcr-Abl–dependent phosphorylation of SOCS3 on Y204 and Y221 is conserved in the SOCS box. (B and C) The SH2 domain of SOCS3 can bind to SH1 domain of Bcr-Abl. Glutathione beads coupled to either GST alone, GST-SOCS3, GST-SOCS3-T1, GST-SOCS3-T2, GST-SOCS3-T3, or GST-SOCS3-T4 were incubated with extracts derived from 293T cells transfected with Abl^{SH1}. Cell lysates were either mock or λ phosphatase treated. Bound Abl^{SH1} was detected by WB and CS. (D-F) SH2 domain of SOCS3 alone was not sufficient to bind to the full length of Bcr-Abl. (D) Lysates from 293T cells with Bcr-Abl transfection were incubated with purified GST, GST-SOCS3, or GST-SOCS3-T4 coupled beads. (E) Lysates from K562 cells with endogenous Bcr-Abl were incubated with purified GST, GST-SOCS3, or GST-SOCS3-T4 coupled beads. (F) Lysates from 293T cells with Bcr-Abl transfection were incubated with purified GST, GST-SOCS3, GST-SOCS3-T1, GST-SOCS3-T2, or GST-SOCS3-T3 coupled beads. Bound Bcr-Abl was detected by WB and CS. (G) Tyrosine residue Y221 of SOCS3 was critical for the interaction between SOCS3 and Bcr-Abl. GST alone, GST-SOCS3, GST-SOCS3 (Y204F), GST-SOCS3 (Y221F), or GST-SOCS3 (Y204F, Y221F) mutants were used to pull down Bcr-Abl from the lysates of 293T transfectants. Experiments were performed as described in (F).

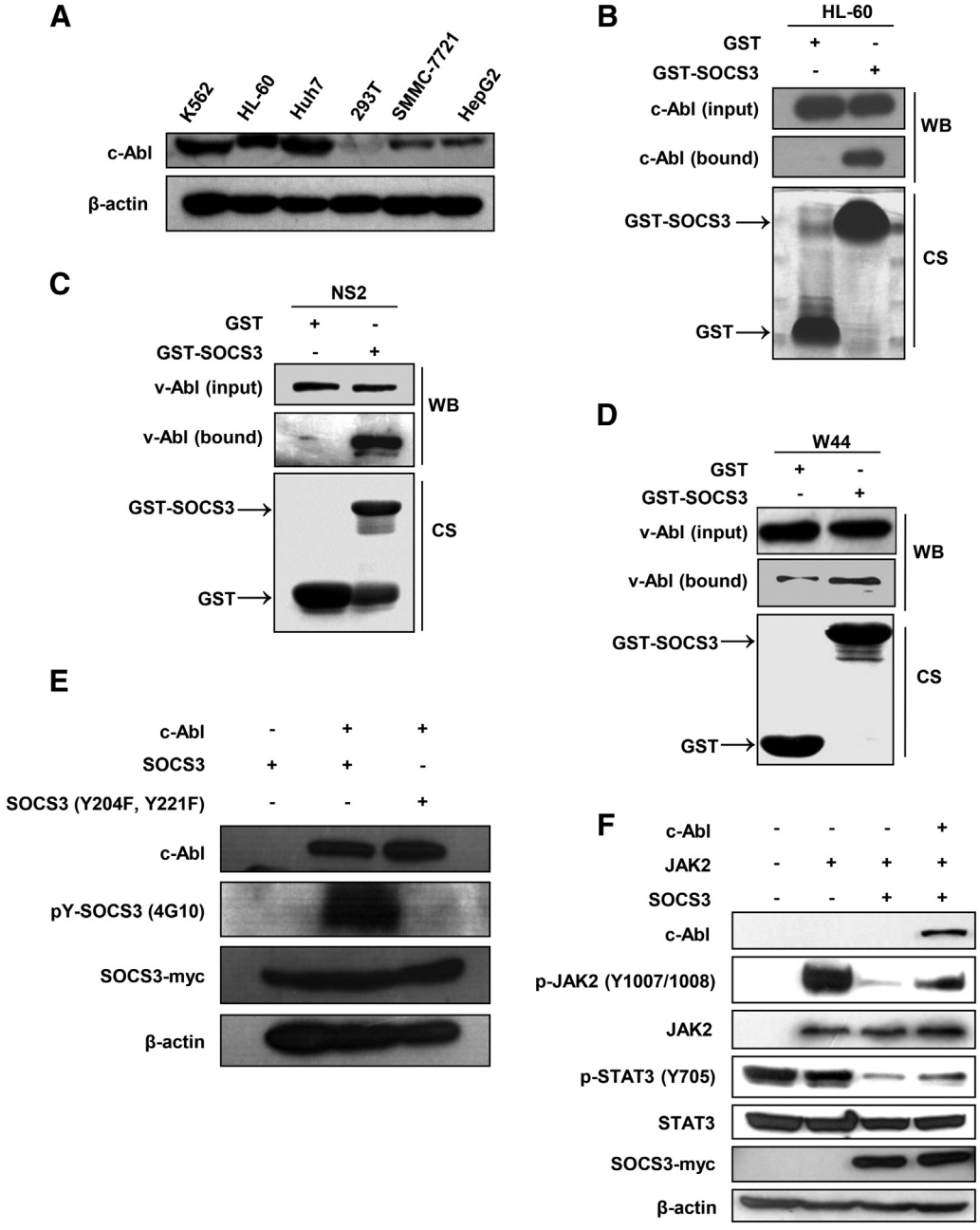


Figure 3. C-Abl–dependent phosphorylation of SOCS3 impairs its ability to negatively regulate JAK2/STAT3 activation. (A) The expression levels of c-Abl were examined by WB in human cancer cell lines, including human chronic myeloid leukemia cell line K562, human promyelocytic leukemia cells line HL-60, human hepatocellular carcinoma cell line SMMC-7721, HepG2 and Huh7, and human embryonic kidney cell line 293T. c-Abl was highly expressed in HL-60 and Huh7 cell lines, but less so in HepG2 cell line. (B-D) SOCS3 can bind to both c-Abl and v-Abl. (B) Glutathione beads coupled to either GST alone or GST-SOCS3 were incubated with endogenous c-Abl extracted from HL-60 cells, either treated with λ phosphatase or mock. Bound c-Abl was detected by WB and CS. (C and D) Glutathione beads coupled to either GST alone or GST-SOCS3 were incubated with endogenous v-Abl extracted from NS2 or W44 cells. Bound v-Abl was detected as above.(E) SOCS3 can be highly tyrosine-phosphorylated by c-Abl. c-Abl was cotransfected with Myc-tagged SOCS3 or SOCS3 (Y204F, Y221F) in 293T cells. After 24 hours of culture, the cells were harvested and extracted. The levels of protein expression and phosphorylated SOCS3 were examined by WB using indicated antibodies. (F) The ability of SOCS3 to inhibit JAK2/STAT3 activation was regulated by c-Abl. JAK2 was cotransfected with SOCS3 with or without c-Abl in 293T cells. Protein levels and phosphorylated JAK2 and STAT3 were analyzed by WB using antibodies as indicated.

(Y204F) and SOCS-3(Y221F) mutants were markedly reduced compared with that of SOCS3 wild type (Supplementary Figure S1). Together, these results reveal that c-Abl signaling mediates tyrosine phosphorylation of SOCS3 on both Y204 and Y221 residues.

Furthermore, we investigated whether c-Abl affects the ability of SOCS3 to negatively regulate JAK activation. To address this possibility, SOCS3 and JAK2 were coexpressed with or without c-Abl in 293T cells. As shown in Figure 3*F*, the phosphorylation level of

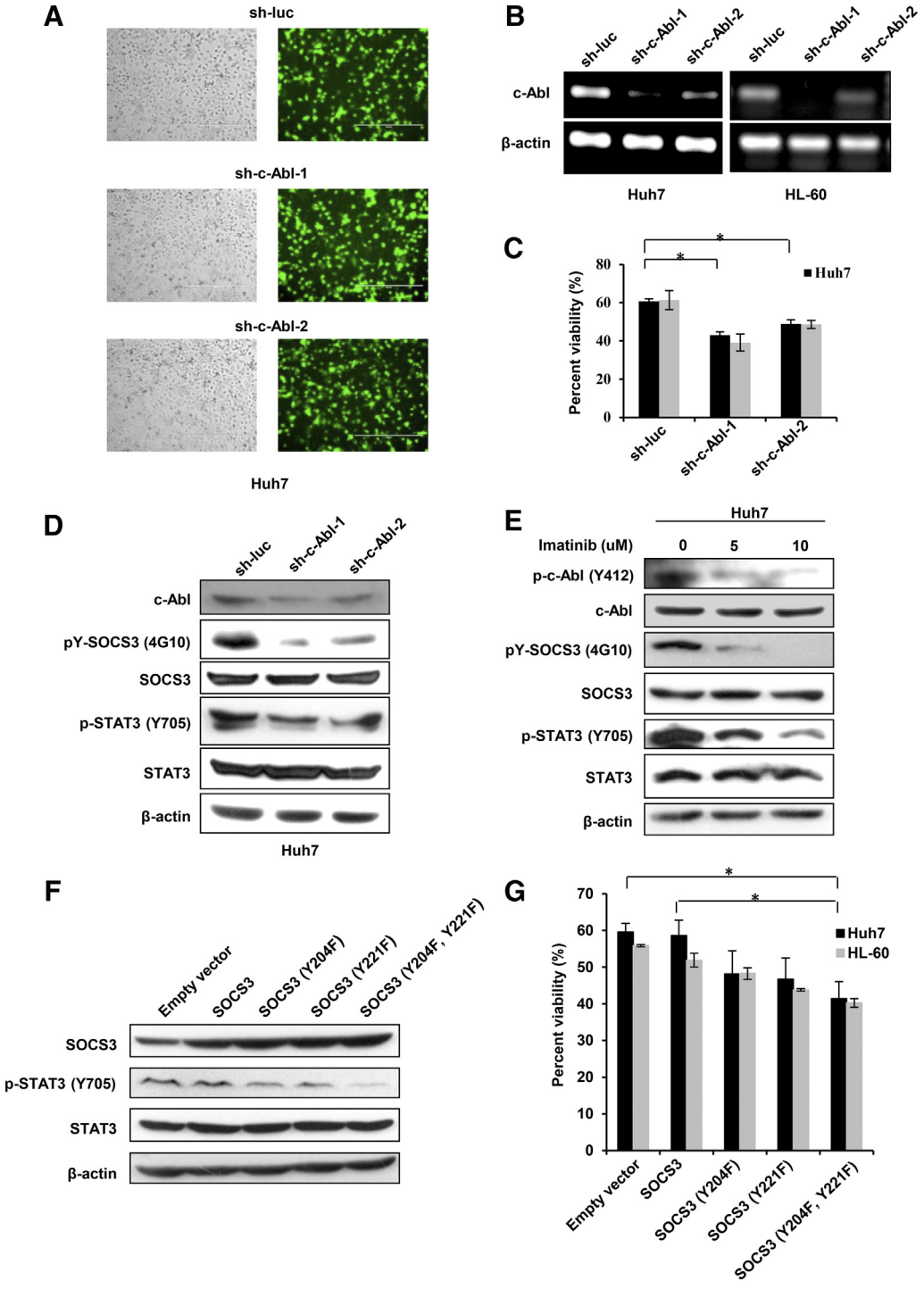
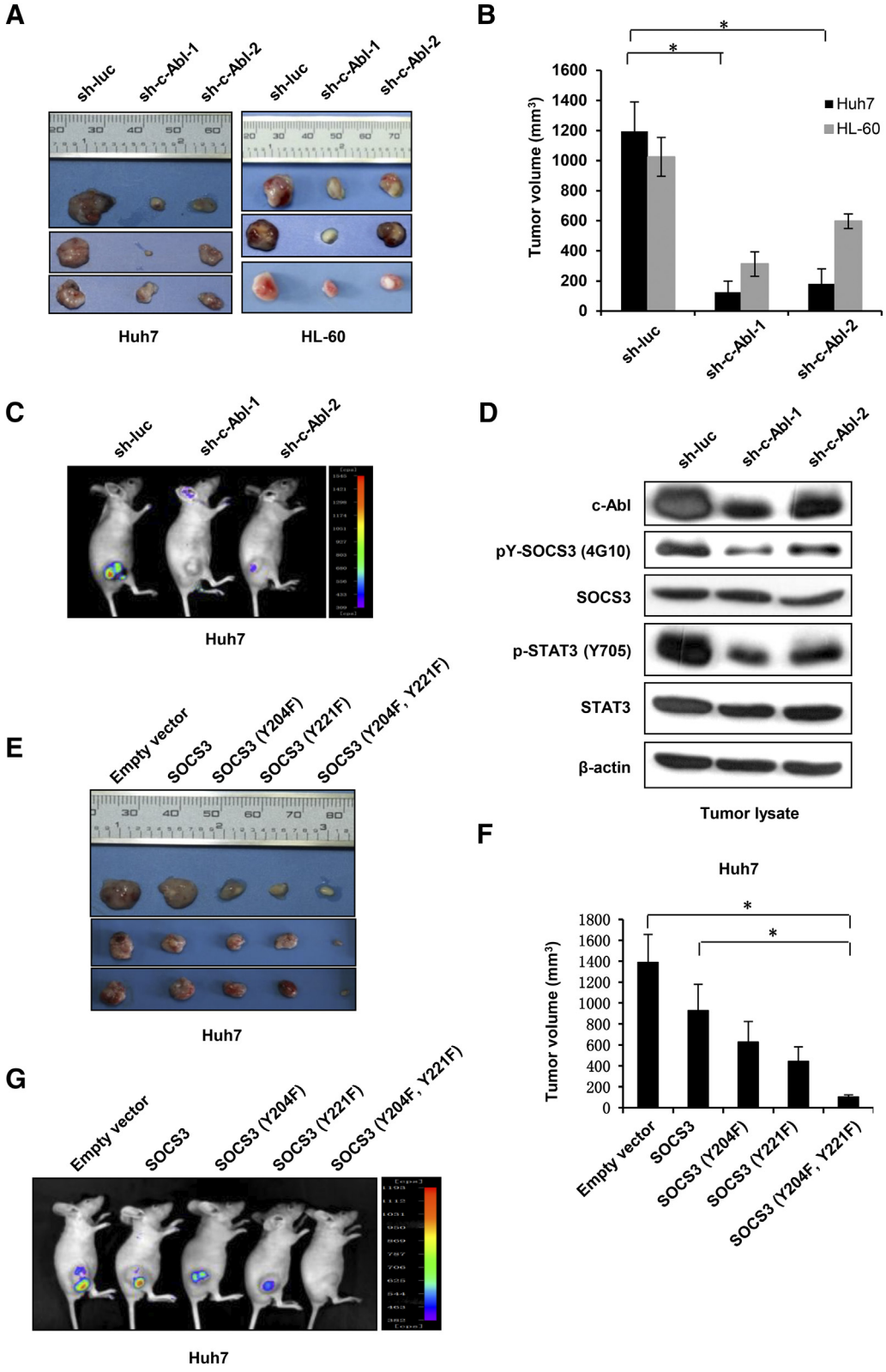


Figure 4. Disrupting tyrosine phosphorylation of SOCS3 sensitizes c-Abl–expressing cells to undergo apoptosis. (A-C) Depletion of c-Abl promoted etoposide-induced apoptosis of Huh7 or HL-60 cells. Huh7 or HL-60 cells stably expressing luciferase shRNA (sh-luc) or c-Abl shRNAs (sh-c-Abl-1 and sh-c-Abl-2) were generated as described in Methods. (A) The micrographs of c-Abl knockdown Huh7 cells or control cells were obtained under a fluorescent microscope equipped with a digital camera. (B) RT-PCR was performed to examine the c-Abl expression in indicated cell lines. Both shRNAs targeting c-Abl efficiently knocked down c-Abl expression, especially for the sh-c-Abl-1. (C) Survival of Huh7 or HL-60 cells stably expressing sh-c-Abl-1, sh-c-Abl-2, or sh-luc was analyzed by flow cytometry after treatment with etoposide. Results in panel (C) are presented as mean ± S.E.M. ($n = 3$), $^*P < .05$. (D and E) Inhibition of c-Abl kinase activity by shRNA (D) or imatinib treatment (E) reduced the tyrosine phosphorylation of SOCS3 and STAT3 in Huh7 cells. Cell extracts were prepared, and phosphorylation levels of SOCS3 and STAT3 were analyzed by WB and quantitated by densitometry normalized to actin expression levels. The tyrosine phosphorylation levels of SOCS3 are 100% in Huh7 cells expressing sh-luc. Plotted are results from three independent experiments. (F and G) Disrupting the tyrosine phosphorylation of SOCS3 on Y204 and Y221 promoted etoposide-induced apoptosis of Huh7 or HL-60 cells. Huh7 or HL-60 cells stably overexpressing empty vector, SOCS3, SOCS3 (Y204F), SOCS3 (Y221F), or SOCS3 (Y204F, Y221F) mutants were generated. (F) The expression of SOCS3 and phosphorylation levels of STAT3 in Huh7 cells were examined by WB. (G) Survival of Huh7 or HL-60 cells ectopically expressing empty vector, SOCS3, or the mutants was analyzed by flow cytometry after etoposide treatment. At least three independent experiments were performed, and results were presented as mean ± S.E.M. ($n = 3$), $^*P < .05$.

both JAK2 and STAT3 was greatly suppressed by SOCS3 but clearly enhanced by c-Abl (Figure 3*F*). Thus, the results suggest that the inhibitory effect of SOCS3 on JAK2 activation was regulated by c-Abl–dependent tyrosine phosphorylation.

Disrupting Tyrosine Phosphorylation of SOCS3 Sensitizes c-Abl–Expressing Cells to Undergo Apoptosis

Next, we investigated the functional relevance of SOCS3 tyrosine phosphorylation in the cell survival. For this, first, c-Abl was knocked



down in Huh7 and HL-60 cell lines highly expressing c-Abl by using two shRNAs (sh-c-Abl-1 and sh-c-Abl-2) specially targeting c-Abl (Figure 4*A*, Supplementary Figure S3*A*). We found that sh-c-Abl-1 had better knockdown efficiency than sh-c-Abl-2 in both cell lines (Figure 4*B*). The viability of c-Abl–silenced Huh7 cells or c-Abl–silenced HL-60 cells was significantly lower than that of the control groups after treatment with etoposide (Figure 4*C*). However, no significant difference in proliferative activity and cell cycle progression was observed between Huh7 cell line expressing sh-c-Abl-1 and control cell (Supplementary Figure S4). Notably, knockdown of c-Abl reduced the tyrosine phosphorylation of SOCS3 (Figure 4*D*, Supplementary Figures S2 and S3*B*) and also resulted in a decrease in STAT3 phosphorylation in Huh7 and HL-60 cells (Figure 4*D*, Supplementary Figure S3*B*). Similar results were observed in the cells after inhibition of c-Abl kinase by imatinib, an inhibitor of Abl family kinases [26,27] (Figure 4*E*). These results indicate that c-Abl plays an important role in the survival of both Huh7 and HL-60 cell lines, probably by regulating SOCS3 tyrosine phosphorylation and thereby affecting STAT3 activation. Interestingly, silencing c-Abl expression could not influence apoptosis in HegG2 cells (Supplementary Figure S5), suggesting that c-Abl might have functional diversity in different cell types.

To substantiate the above finding, we generated Huh7 and HL-60 cell lines stably expressing empty vector, SOCS3, or their mutants (Figure 4*F*, Supplementary Figure S3*C*). As shown in Figure 4*F*, the phosphorylation of STAT3 was decreased in Huh7 cell lines overexpressing SOCS3 (Y204F) and SOCS3 (Y221F) as compared with Huh7 cell lines overexpressing SOCS3 wild type or empty vector. Particularly, the phosphorylation level of STAT3 was markedly reduced in Huh7 cell lines overexpressing SOCS3 (Y204F/Y221F) mutants. The viability of Huh7 cells expressing SOCS3 (Y204F) or SOCS-3 (Y221F) mutant decreased significantly, compared to that of Huh7 cells expressing SOCS3 or empty vector after etoposide treatment (Figure 4*G*). Furthermore, the survival of Huh7 cells expressing SOCS3 (Y204F/Y221F) mutant was more significantly reduced under the same condition (Figure 4*G*). Similar results were observed in HL-60 cells (Figure 4*G*). Collectively, these data suggest that tyrosine phosphorylation of SOCS3 on Y204 and Y221 is required for the survival of Huh7 and HL-60 cells, and disrupting such phosphorylation of SOCS3 promotes the apoptosis of these c-Abl–expressing cells through JAK2/STAT3 signaling pathway.

Tyrosine Phosphorylation of SOCS3 Is Required for Efficient Tumorigenesis Caused by c-Abl–Expressing Cells in a Mouse Model

We further investigated the requirement for tyrosine phosphorylation of SOCS3 in the tumor growth of Huh7 and HL-60 xenografts in nude mice. First, c-Abl–silenced Huh7 cells, c-Abl–silenced HL-60 cells, or control cells expressing shRNA targeting luciferase were injected into nude mice subcutaneously. Tumor growth was examined every week after inoculation. As shown in Figure 5*A*, tumors excised from nude mice injected with either c-Abl–silenced Huh7 cells or c-Abl–silenced HL-60 cells were significantly smaller than the control group, especially for the cell lines expressing sh-c-Abl-1. These observations were further confirmed by tumor volume measurement and bioluminescent imaging analysis (Figure 5, *B* and *C*). Importantly, we found that tyrosine phosphorylation levels of SOCS3 and STAT3 in the extracts derived from tumors of c-Abl–silenced Huh7 or c-Abl–silenced HL-60 cells were significantly reduced compared to that in the control group (Figure 5*D*). Thus, these data indicated that silencing c-Abl expression inhibited Huh7 or HL-60 xenograft growth in nude mice, which may be related to decreased tyrosine phosphorylation of SOCS3 and JAK2/STAT3 signaling pathway in the xenografts.

To confirm the role of SCOS3 tyrosine phosphorylation in the tumor growth, next, we inoculated nude mice subcutaneously with Huh7 or HL-60 cells stably expressing either empty vector, SOCS3, or SOCS3 mutants. Overexpression of SOCS3 wild type slightly inhibited the tumor growth. Interestingly, ectopic expression of either SOCS3 (Y204F) or SOCS3 (Y221F) significantly suppressed the tumor growth compared to the control (Figure 5*E*, Supplementary Figure S3*D*). Particularly, the Y204F/Y221F double mutation of SOCS3 had much stronger inhibitory effect on the tumor growth (Figure 5*E*, Supplementary Figure S3*D*). Consistent results were observed from tumor volume measurement and bioluminescent imaging analysis (Figure 5, *F* and *G*). Together, the results suggest that disruption of c-Abl–dependent tyrosine phosphorylation of SOCS3 is able to inhibit the tumorigenesis caused by c-Abl–expressing cells in vivo.

Discussion

Abl kinases (Bcr-Abl, v-Abl, and c-Abl) participate in multiple signaling pathways involved in cell proliferation and survival, including PI3K/AKT/mTOR (mammalian target of rapamycin) and JAK/STAT/PIM (proviral insertion in murine) [1,22,23]. Our previous studies have shown that Bcr-Abl–induced phosphorylation of SOCS1 and SOCS3 diminishes their inhibitory effects on the activation of JAK/STAT signaling, which depends on the interaction between SOCS proteins and Bcr-Abl [21]. In addition, phosphorylation of SOCS1 by v-Abl has also been reported to reduce its ability to inhibit JAK1 activation [18]. However, it is still unclear whether SOCS proteins interact with and are phosphorylated by c-Abl, and which domain is essentially responsible for such interaction.

Figure 5. Tyrosine phosphorylation of SOCS3 significantly impacts tumor growth of c-Abl–expressing cells in vivo. (A-C) Silencing c-Abl expression inhibited xenograft growth of Huh7 or HL-60 cells in nude mice. c-Abl knockdown cells expressing sh-c-Abl-1 or sh-c-Abl-2 or control cells expressing sh-luc were injected into nude mice subcutaneously as described in Methods. (A) Tumors were excised from nude mice. Shown are representative images from three independent experiments with similar results. (B) The tumor volumes were measured at indicated time points. Plotted are results from three independent experiments. Error bars represent S.E.M. ($n = 10$), $^*P < 0.05$. (C) Bioluminescent imaging of injected nude mice was performed to detect tumor growth. Shown are representative images from at least three independent experiments with similar results. (D) Tyrosine phosphorylation of SOCS3 and STAT3 decreased remarkably in the xenografts of c-Abl knockdown Huh7 or HL-60 cell lines in nude mice. Tumors were excised from mice, and tumor extracts were prepared and analyzed by WB. Plotted are results from three independent experiments. (E-G) Tyrosine phosphorylation deficiency of SOCS3 on Y204 and Y221 impeded xenograft growth of Huh7 cells in nude mice. Nude mice were subcutaneously injected with Huh7 cells overexpressing empty vector, SOCS3, or the mutants. (E) Tumors were excised from nude mice, and shown are from three independent experiments with similar results. (F) Tumor volumes were measured at the indicated time points as described in (B). Plotted are results from three independent experiments. Error bars represent S.E.M. ($n = 10$), $^*P < 0.05$. (G) Bioluminescent imaging of injected nude mice was performed to detect tumor growth. Shown are representative images from at least three independent experiments with similar results.

In this study, we identified the Abl but not Bcr domain of Bcr-Abl responsible for its interaction with SOCS3. We further defined that SH1 domain of Bcr-Abl was required for the interaction between SOCS3 and Bcr-Abl. Moreover, SH2 domain of SOCS3 was sufficient to pull down SH1 domain of Bcr-Abl but not the full length of the protein. These results implied that the integrity of SOCS3 is crucial for its interaction with Bcr-Abl. Since Bcr-Abl, v-Abl, and c-Abl contain identical SH1 domain, we examined whether SOCS3 could also bind with v-Abl and c-Abl tyrosine kinases. Indeed, our results confirmed that SOCS3 did interact with all three Abl kinases. Importantly, c-Abl–dependent tyrosine phosphorylation of SOCS3 greatly impaired its inhibitory effect on JAK2 activation. However, further studies are needed to provide new insights into the function of Abl kinases-dependent phosphorylation of SOCS3. The crystal structure of their conjugation would precisely display the direct interaction sites and may reveal the molecular basis for development of therapeutic inhibitors of Abl-mediated tumorigenesis [28,29].

The c-Abl protein, different from Bcr-Abl and v-Abl, is a tightly regulated nonreceptor tyrosine kinase. Shuttling between nucleus and cytoplasm, c-Abl plays different roles in multiple cellular activities [2,30–32]. Although it has been described that c-Abl mediates apoptosis in some tumor cell lines through phosphorylating different targets [12–14,33,34], other studies have revealed that c-Abl promotes tumorigenesis in certain cell contexts [15,20,35,36]. Moreover, c-Abl also plays an important role in organismal development [27], DNA damage response [37,38], and neurodegeneration [39]. In addition, activation of c-Abl tyrosine kinase is involved in non–small cell lung cancer caused by loss of fused in sarcoma-1 gene expression [40], and active c-Abl tyrosine kinase lies downstream of deregulated epidermal growth factor receptor and/or the insulin-like growth factor-1 receptor in breast cancer [41,42]. All these lines of evidence indicate that the function of c-Abl is multiple, and c-Abl may switch its functions through unique mechanisms in different cancer cell types.

Here, we selected various cancer cell lines and found that c-Abl was highly expressed in human promyelocytic leukemia HL-60 cell line and human hepatocarcinoma Huh7 cell line but less expressed in human hepatocellular carcinoma HepG2 cell line. Depletion of c-Abl sensitized Huh7 and HL-60 cells to undergo apoptosis and impeded the tumor growth of these cells *in vivo*, suggesting that c-Abl plays an oncogenic role in these two cell lines. However, altering c-Abl expression in HepG2 cells had little effect on cell apoptosis (Supplementary Figure S5). It has been studied that there existed differences of protein expression and signaling pathway between Huh7 and HepG2 cell lines, and our results might imply that the function of c-Abl in Huh7 cells was more critical than that in HepG2 cells [43,44].

Since our results revealed that SOCS3 was highly tyrosine phosphorylated by c-Abl, we wondered if SOCS3 participates in c-Abl–mediated cell survival and tumor growth. As expected, we found that the tyrosine phosphorylation of SOCS3 in the xenografts of c-Abl–silenced Huh-7 and HL-60 cells decreased significantly. Importantly, disrupting the tyrosine phosphorylation of SOCS3 (especially both Y204 and Y221 sites) profoundly promoted the apoptosis of Huh7 and HL-60 cells in response to etoposide *in vitro* and inhibited the tumor growth of these cell lines *in vivo*. Together, these data indicate that c-Abl–dependent tyrosine phosphorylation of SOCS3 has a biological significance in c-Abl–mediated cell survival and tumor growth in particular cells. However, further research is still required to determine the relationship between SOCS3 and c-Abl in clinical samples from cancer patients.

Our previous studies have demonstrated the requirement for SOCS1 phosphorylation in Bcr-Abl–induced tumorigenesis, which occurs mainly on Y155 and Y204 residues [21]. Moreover, we have observed that SOCS1 was highly tyrosine phosphorylated in patients with Bcr-Abl–expressing chronic myeloid leukemia (CML), but SOCS1 tyrosine phosphorylation level was varied among different CML samples [21]. Interestingly, robust activation of JAK2 was detected in the CML samples containing highly tyrosine phosphorylated SOCS1 [21]. The data implied the significance of SOCS1 phosphorylation in Bcr-Abl–expressing CML clinical samples. Thus, the status of tyrosine phosphorylation of SOCS3 also needs to be detected in Bcr-Abl– or c-Abl–expressing clinical samples. In addition, it is worth studying whether SOCS1 participates in c-Abl–mediated solid tumorigenesis.

Conclusions

Here, for the first time, we demonstrated the interaction between SOCS3 and Abl kinases. Specifically, the SH1 domain of Bcr-Abl was critical for its interaction with SOCS3; SH2 domain of SOCS3 was able to bind to SH1 domain of Bcr-Abl but was not sufficient to bind to the full length of Bcr-Abl. We also confirmed that c-Abl–dependent phosphorylation of SOCS3 impaired its inhibition to the JAK/STAT signaling. Moreover, we revealed the requirement for tyrosine phosphorylation of SOCS3 in c-Abl–mediated cell survival and tumorigenesis in particular cancer cell lines.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.09.002.

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