RESEARCH ARTICLE

The Regulation of SPRY4 Intronic Transcript 1 (SPRY4-IT1) on KIT Signaling and Imatinib Resistance of Gastrointestinal Stromal Tumor (GIST) Cells

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Abstract

BACKGROUND: SPRY4 intronic transcript 1 (SPRY4-IT1), is a long non-coding RNA coded by the intron of SPRY4. SPRY4 is highly expressed in gastrointestinal stromal tumor (GIST) and inhibits the tumorigenesis of GIST, but whether SPRY4-IT1 regulates the tumorigenesis of GIST or not remains unclear. Therefore, in this study, the regulation of SPRY4-IT1 expression and its role in GIST will be investigated.

METHODS: GIST-T1 cells, and Ba/F3 cells which express KIT proto-oncogene (KIT) and SPRY4-IT1 were used as cell models. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to examine mRNA expression, while the protein expression and signal transduction were examined by western blot. The association between SPRY4-IT1 and KIT was examined by pull down of KIT and PCR. Cell proliferation, survival, and cell cycle progression were examined by cell counting kit-8 (CCK8) and flow cytometry.

RESULTS: KIT mutants increased the expression of SPRY4-IT1 in GIST. SPRY4-IT1 bound to KIT, also enhanced the activation and expression of both wild-type KIT and primary KIT mutants, therefore increasing the activation of downstream signaling proteins AKT and ERK of KIT, GIST cell survival, and proliferation. In addition, SPRY4-IT1 reduced the sensitivity of wild-type KIT, or primary KIT mutants to the first-line targeted therapeutic drug of GIST, imatinib, which can inhibit KIT activation. Gaining drug-resistant secondary KIT mutants might be one of the main reasons of GIST recurrence after targeted therapy. Similar to wild-type KIT and primary KIT mutants, the activation and expression of secondary KIT mutants and their resistance to imatinib were also increased by SPRY4-IT1.

CONCLUSION: The results indicated positive feedback between SPRY4-IT1 and wild-type KIT, primary KIT mutants or secondary KIT mutants, and the upregulation of AKT and ERK activation by SPRY4-IT1 in GIST cells, providing a new insight in the KIT signaling regulation in GIST, and the resistance of GIST to targeted therapy.

KEYWORDS: SPRY4-IT1, KIT, GIST, SPRY4, signaling

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Introduction

As a mesenchymal tumor, gastrointestinal stromal tumor (GIST) often arises in the stomach. It occurs in the rest parts of the digestive tract, but less frequent.(1) The results of genomic sequencing of GIST tissues shows that some mutations were identified in KIT proto-oncogene (KIT), platelet derived growth factor receptor alpha (PEGFRA), Raf oncogene (RAF), and others.(2-5) Among them, KIT is the most frequent mutated, accounting for 70-80% of all mutations in GIST cases.(4,6) Surgical resection of the tumor is widely used for the treatment of GIST in low risk and those without metastasis, while targeted therapy against KIT is recommended for those tumors in high risk and metastatic tumors.(7,8)

The predominantly mutated molecule KIT in GIST is a type III receptor tyrosine kinase. Under normal situations, KIT is expressed as a monomer on the cell surface. The KIT ligand, stem cell factor (SCF), binds to KIT and induces KIT dimerization and activation, which subsequently activates downstream signaling pathways. Unlike the wild-type KIT, that requires ligand stimulation for its activation, mutated KIT variants found in malignancies such as GIST, acute myeloid leukemia, and mastocytosis, are constitutively activated without ligand engagement.(9,10) As a KIT inhibitor, imatinib has been approved for the targeted therapeutic drug of GIST in the clinic setting (11), and has dramatically improved treatment outcomes. However, tumor relapse is common due to acquired drug-resistant secondary mutations of KIT (12,13) or due to the activation of alternative signaling pathways (14,15). Although the second-, third- and fourth-line targeted therapeutic drugs for GIST have been used clinically, they typically only marginally improve patient survival, often extending it by just a few months.(16-18)

SPRY4 is expressed in GIST at a high level (19), and it was previously found that SPRY4 inhibits KIT signaling (20). KIT can increase the expression of SPRY4-IT1 in GIST, and SPRY4-IT1 forms a positive feedback loop with KIT by increasing KIT signaling, which is in contrary to the negative role of SPRY4 in KIT signaling although the expression of both SPRY4 and SPRY4-IT1 is upregulated by KIT. It was known that SPRY4-IT1 is coded by SPRY4 gene as well (21); however, whether KIT signaling regulates SPRY4-IT1 expression, whether SPRY4-IT1 regulates KIT signaling, and whether SPRY4-IT1 plays a role in GIST in a similar way as SPRY4 remain unknown. Therefore, in this study, regulation of SPRY4-IT1 expression and its role in GIST was investigated.

Methods

Cells Culture

Ba/F3 cells were cultured in RPMI 1640 medium, 10% fetal bovine serum, 10 ng/mL recombinant murine interleukin (IL)-3, 100 units/mL penicillin, and 100 µg/mL streptomycin. The retroviral vector pMSCVpuro expressing wild-type KIT, the often KIT mutations in GISTs W557K558del and V560D were transfected into EcoPack cells. Supernatant containing the virus were used to infect Ba/F3 cells followed by selection with 1.2 µg/mL puromycin to establish Ba/F3 cells expressing wild-type KIT. GIST-T1 cells which carries KIT mutation in exon 11 endogenously and EcoPack cells were cultured in DMEM medium, 10% fetal bovine serum, 100 µg/mL streptomycin and 100 units/mL penicillin.(22)

Construction of Plasmid and Cell Lines Expressing SPRY4-IT1

Human SPRY4-IT1 cDNA was synthesized and inserted into the vector pMSCVneo followed by transfection into EcoPack cells by Lipofactamine 2000 (Thermofisher Scientific, Waltham, MA, USA). Supernatants containing virus were collected to infect Ba/F3 cells expressing wild-type KIT, or KIT mutants that were constructed before (23), and selected with 1 mg/mL neomycin, SPRY4-IT1 expression was detected by polymerase chain reaction (PCR).

Cell Treatment, Immunoprecipitation, and Western Blotting

Ba/F3 cells were washed with phosphate buffered saline (PBS), incubated in RPMI 1640 medium at 37°C for 4 hours, stimulated with 100 ng/mL SCF (ORF genetics, Kópavogur, Iceland) at 37°C for 2 minutes, washed with ice-cold PBS, and lysed as previously reported.(23) After centrifugation by centrifuge 5425R (Eppendorf, Hamburg, Germany), cell lysates were proceeded for western blot or immunoprecipitation. Meanwhile, the GIST-T1 cells were washed with PBS, incubated in DMEM medium at 37°C for 4 hours, lysed, centrifuged, and then processed for western blot or immunoprecipitation in the same way as the Ba/F3 cells.

For immunoprecipitation, 0.5 µg KIT antibody used (21) was mixed with cell lysate followed by incubation at 4°C for 1 hours, and then incubated with Dynabeads™ Protein G (ThermoFisher Scientific) at 4°C for 30 minutes. After washing with cell lysis buffer, the immunoprecipitates were examined by western blotting.

For western blotting, samples were separated by SDS-PAGE using PowerPac™ Basic Power Supply (Bio-Rad, Hercules, CA, USA), and electro-transferred to PVDF membranes (Millipore, Temecula, CA, USA) by Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were blocked in PBS with 0.2% TWWEN-20 (PBST) at room temperature for 2 hours, incubated with primary antibodies as indicated at room temperature for 2 hours, washed with PBST, incubated with secondary antibodies at room temperature for 1 hour and washed. The membrabes were developed with Immobilon ECL Ultra Western HRP Substrate (Millipore), and the photos were captured by the Bio-Rad imaging system (Bio-Rad).

Examination of The Association of SPRY4-IT1 and KIT

Cells were lysed as aforementioned plus RNase Inhibitor (Beyotime, Shanghai, China). After immunoprecipitation using KIT antibody (21), the immunoprecipitates were incubated with 1 mg/mL proteinase K (Beyotime) at 55°C for 30 minutes, and 95°C for 10 minutes consecutively, and proceeded to qRT-PCR analysis of SPRY4-IT1 as described in the section of RNA purification and qRT-PCR.

siRNA Transfection

The KIT or SPRY4-IT1 expression was knocked down in GIST-T1 cells by treatment with 50 nM KIT siRNA (GGAUGGCACCUGAAAGCAUTT, Genepharma, Shanghai, China) or 50 nM SPRY4-IT1 siRNA (CCTAGACTGTTGGCCCAGATGTTGA, Genepharma) using Lipofectamine 2000 (Thermofisher Scientific) according to the manufacturer's instructions. Briefly, the siRNAs or Lipofectamine 2000 were mixed with Opti-MEM, respectively, and then incubated for 5 minutes at the room temperature, and followed by mixture and incubation for 15 minutes at room temperature and load to the cells.

RNA Purification and qRT-PCR

RNA simple Total RNA kit (Tiangen Biotech, Beijing, China) was used to purify total RNAs, and Reverse Transcription Kit (Takara Biomedical Technology, Beijing, China) was used for reverse transcription. TB Green was used as the dye in the qPCR by qTOWER³G (Analytik Jena, Jena, Germany) to examine mRNA expression using program: 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, and 60°C for 34 seconds. The target gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. RPL19 was used as a control. RPL19 primers were as follow: forward: ACATGGGCATAGGTAAGCGGAAG, and reverse: TTCACCTTCAGGTACAGGCTGTG; KIT primers were as follow: forward: GCGTTCTGCTCCTACTGCTTCG, and reverse: TGGATGGATGGTGGAGACGGTTC; while SPRY4-IT1 primers were as follow: forward: GCCTACTGCCTGAACTGCTTCTG, and reverse: GTGCCACCAAGTCCGCTGATG.

Detection of KIT Expression Using Flow Cytometry

PBS was used to wash Ba/F3 cells, cells were stained with PE-anti-KIT antibody (Cat #983304; Biolegend, San Diego, CA, USA) for 30 minutes in the dark, after washing with PBS, the cells were centrifuged and resuspended in PBS, and examined by NovoCyte Flow Cytometer (Agilent, Santa Clara, CA, USA).

Cell Survival, Proliferation, and Cell Cycle

Cell survival, cell proliferation, and cell cycle were examined as previously reported.(21) Briefly, Ba/F3 cells were washed with PBS and grown in Ba/F3 growth medium without IL-3 but with 100 ng/mL SCF or no cytokine as control. Cell survival was analyzed by flow cytometry after staining with PE annexin V apoptosis detection kit (Cat #559763; BD Biosciences, Franklin Lakes, NJ, USA). Cell proliferation was examined using CCK8 kit (Cat #M4839; AbMole Bioscience, Houston, TX, USA) according to the manufacturer's instruction, the absorbance at 450 nm was read by Multiskan GO Spectrophotometer (Thermofisher Scientific). And cell cycle was examined by flow cytometry after staining with propidium iodate (PI) reagent (Keygen Biotech, Nanjing, China). The cell survival, proliferation and cell cycle of GIST-T1 cells were examined as aforementioned except that GIST-T1 cells were trypsinized before cell cycle and survival assay.

Statistical Analysis

Statistical analysis was performed after three experiments by GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The results were presented as mean±standard deviation (SD). Comparisons between two groups were analyzed by the t-test, with $p<0.05$ was considered as statistically significant.

Results

KIT Promoted SPRY4-IT1 Expression in GIST Cells

In the current work, the regulation of KIT on SPRY4-IT1 expression in GIST was studied. Similar to that of SPRY4, in GIST-T1 cells, SPRY4-IT1 expression was decreased by around 80% after inhibition of KIT activation by imatinib, and SPRY4-IT1 expression was decreased by 58% after knockdown of KIT expression by siRNA (Figure 1), indicating that KIT could promote SPRY4-IT1 expression in a similar manner as its regulation on SPRY4 expression.

SPRY4-IT1 Enhanced Wild-type KIT and Primary KIT Mutants' Expression

Knockdown of SPRY4-IT1 expression in GIST-T1 cells resulted in reduced expression of KIT protein but not KIT mRNA, indicating that SPRY4-IT1 might enhance KIT expression in GIST cells without changing KIT transcription, and KIT and SPRY4-IT1 might form a positive feedback loop. As a consequence of reduced KIT expression by SPRY4-IT1 siRNA, the activation of downstream proteins

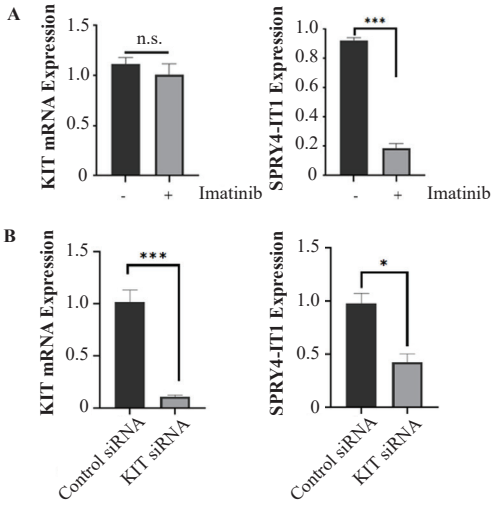

Figure 1. KIT promoted SPRY4-IT1 expression in GIST-T1. A: qRT-PCR results of KIT and SPRY4-IT1 mRNA expression in GIST-T1 cells based on the imatinib treatment. B: qRT-PCR results of KIT and SPRY4-IT1 mRNA expression in GIST-T1 cells based on the type of siRNA. The statistical difference was calculated by t-test; *p<0.05, ***p<0.001.

of KIT, AKT and ERK, were inhibited by SPRY4-IT1 siRNA as well (Figure 2A). To examine KIT expression regulation by SPRY4-IT1, Ba/F3 cells that express KIT and SPRY4-IT1 were constructed. Examination of KIT expression showed that SPRY4-IT1 increases wild-type KIT and primary KIT mutants' expression (Figure 2B), further suggesting that SPRY4-IT1 enhances KIT expression.

SPRY4-IT1 Enhanced Wild-type KIT and Primary KIT Mutant Activation, and The Activation of Downstream Signaling in GIST

To examine whether there was any association between SPRY4-IT1 and KIT, KIT was immunoprecipitated in GIST-T1 cells. Amplification of the immunoprecipitants using primers for SPRY4-IT1 showed that SPRY4-IT1 was pulled down together with KIT. Examination of Ba/F3 cells that express SPRY4-IT1 and wild-type KIT or primary KIT mutants showed similar results (Figure 3A), suggesting that SPRY4-IT1 bound with KIT, including wild-type KIT and primary KIT mutants. Furthermore, in GIST-T1 cells, knockdown of SPRY4-IT1 expression inhibited KIT activation, and the activation of downstream signaling molecules ERK and AKT (Figure 2A), which play an important role in KIT mediated cell proliferation and survival. While the presence of SPRY4-IT1 in Ba/

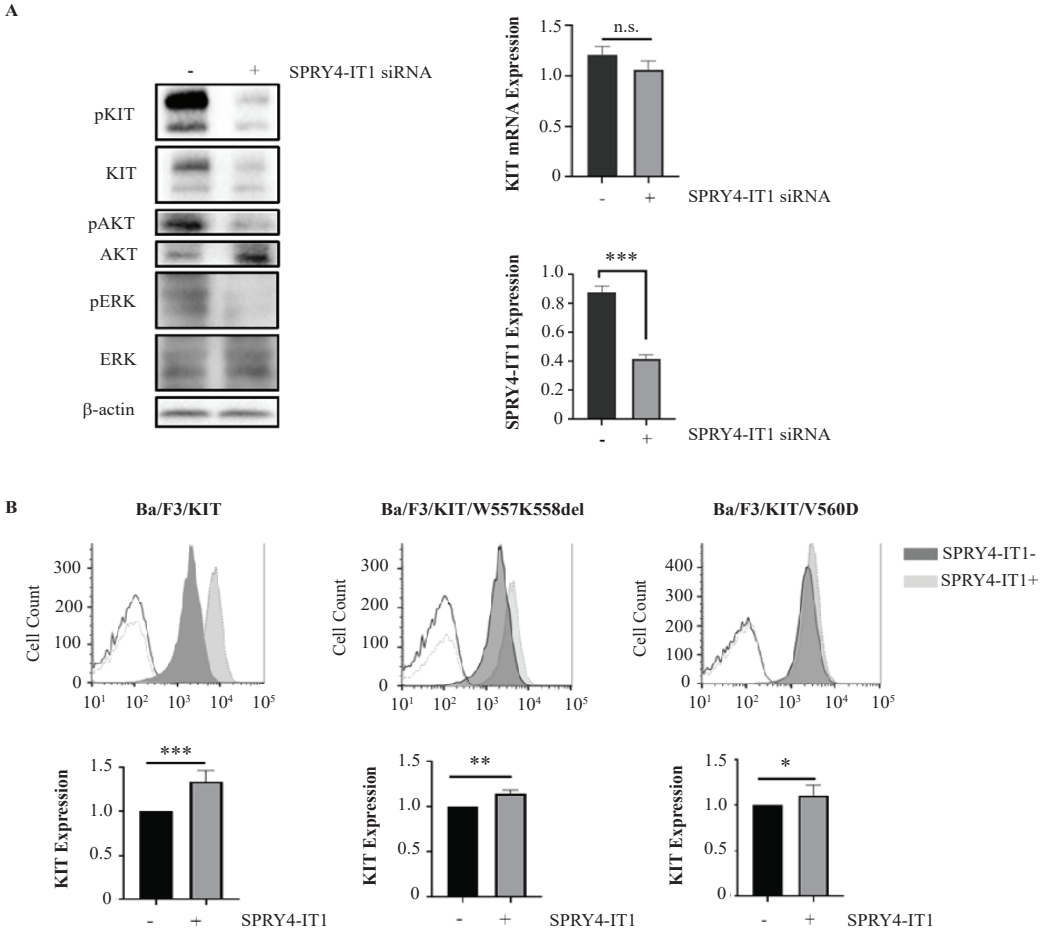

Figure 2. The SPRY4-IT1 enhanced KIT expression including wild-type KIT, and primary KIT mutants. A: Western blot results of GIST-T1 cells using 4G10, KIT, pAKT, AKT, pERK, ERK, and β-actin antibodies, and qRT-PCR results of KIT and SPRY4-IT1 mRNA expression in GIST-T1 cells based of SPRY4-IT1 siRNA. B: KIT expression in Ba/F3 cells by flow cytometry. Dark grey: No SPRY4-IT1 expression, light grey: SPRY4 expression, empty area: control, grey area: KIT. Expression of KIT was quantified and analyzed. The statistical difference was calculated by t-test; *p<0.05, **p<0.01, ***p<0.001.

F3 cells that express wild-type KIT or primary KIT mutants increased KIT activation, and the activation of its downstream signaling molecules AKT and ERK (Figure 3B), indicating that SPRY4 could enhance KIT signaling, which was in contrary to the fact that SPRY4 inhibited KIT signaling.

SPRY4-IT1 Increased KIT-mediated Cell Proliferation and Survival

Considering the critical role of SPRY4-IT1 in KIT expression and signaling, the SPRY4-IT1's regulation on cell proliferation and survival mediated by KIT *in vitro* was also examined. In agreement with the signaling results, in GIST-T1 cells, inhibition of SPRY4-IT1 expression decreased cell survival, cell proliferation, and cell cycle progression (Figure 4A), while the overexpression of SPRY4-IT1 in Ba/F3 cells that express wild-type KIT, or primary KIT mutants increased cell survival, cell proliferation, and cell cycle progression (Figure 4B, 4C, and 4D), suggesting the crucial role of SPRY4-IT1 in the cell response mediated by KIT.

SPRY4-IT1 Reduced Wild-type KIT and Primary KIT Mutants' Sensitivity to Imatinib

Due to the important role of SPRY4-IT1 in KIT signaling, in this study was also studied whether SPRY4-IT1 regulates

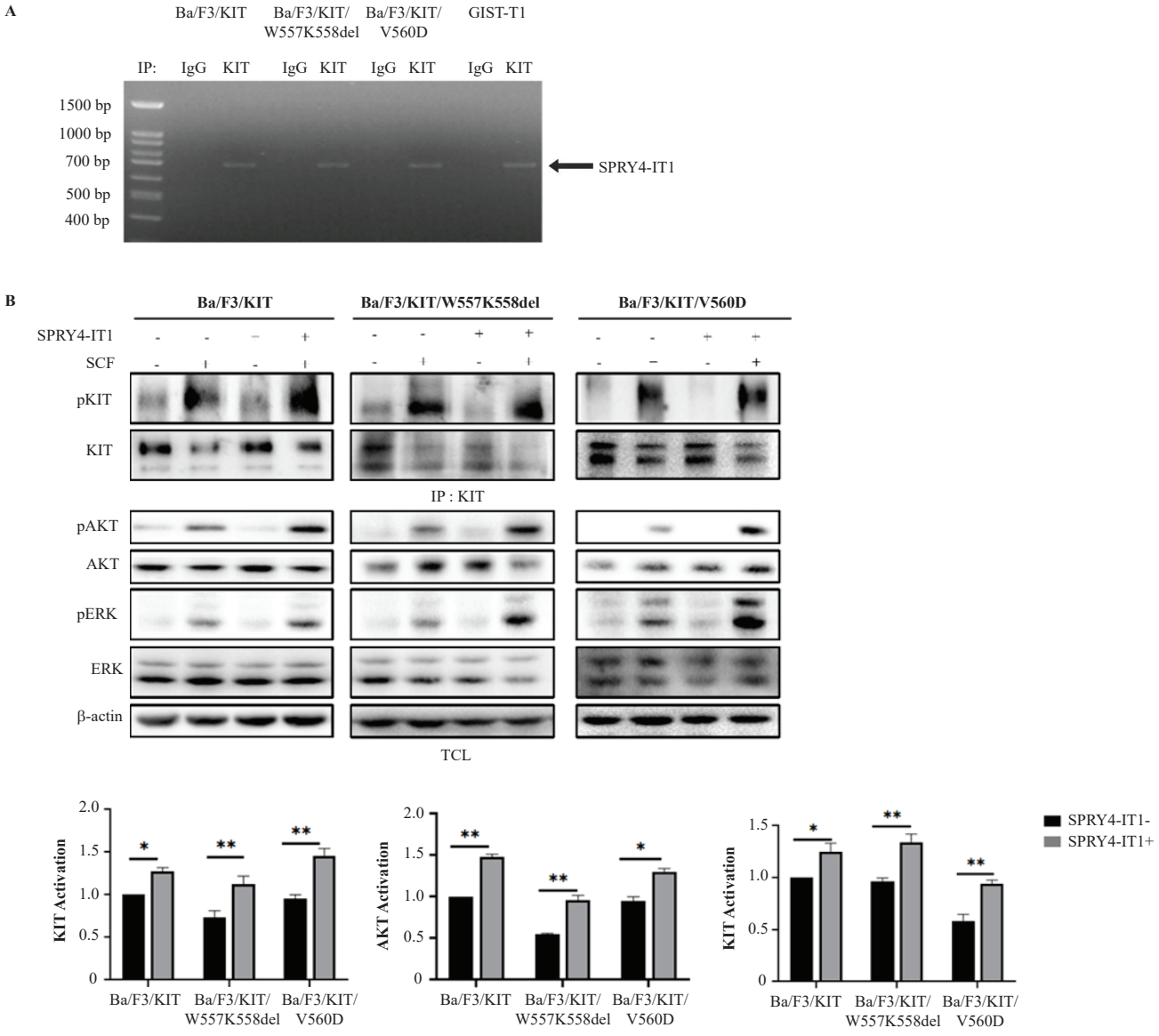

Figure 3. SPRY4-IT1 enhanced the activation of wild-type KIT and primary KIT mutants in GIST. A: KIT associated SPRY4-IT1 in GIST-T1 cells and Ba/F3 cells by PCR. B: Activation of KIT, AKT, and ERK in Ba/F3 cells by western blot. IP: Immunoprecipitation. The statistical difference was calculated by t-test; *p<0.05, **p<0.01.

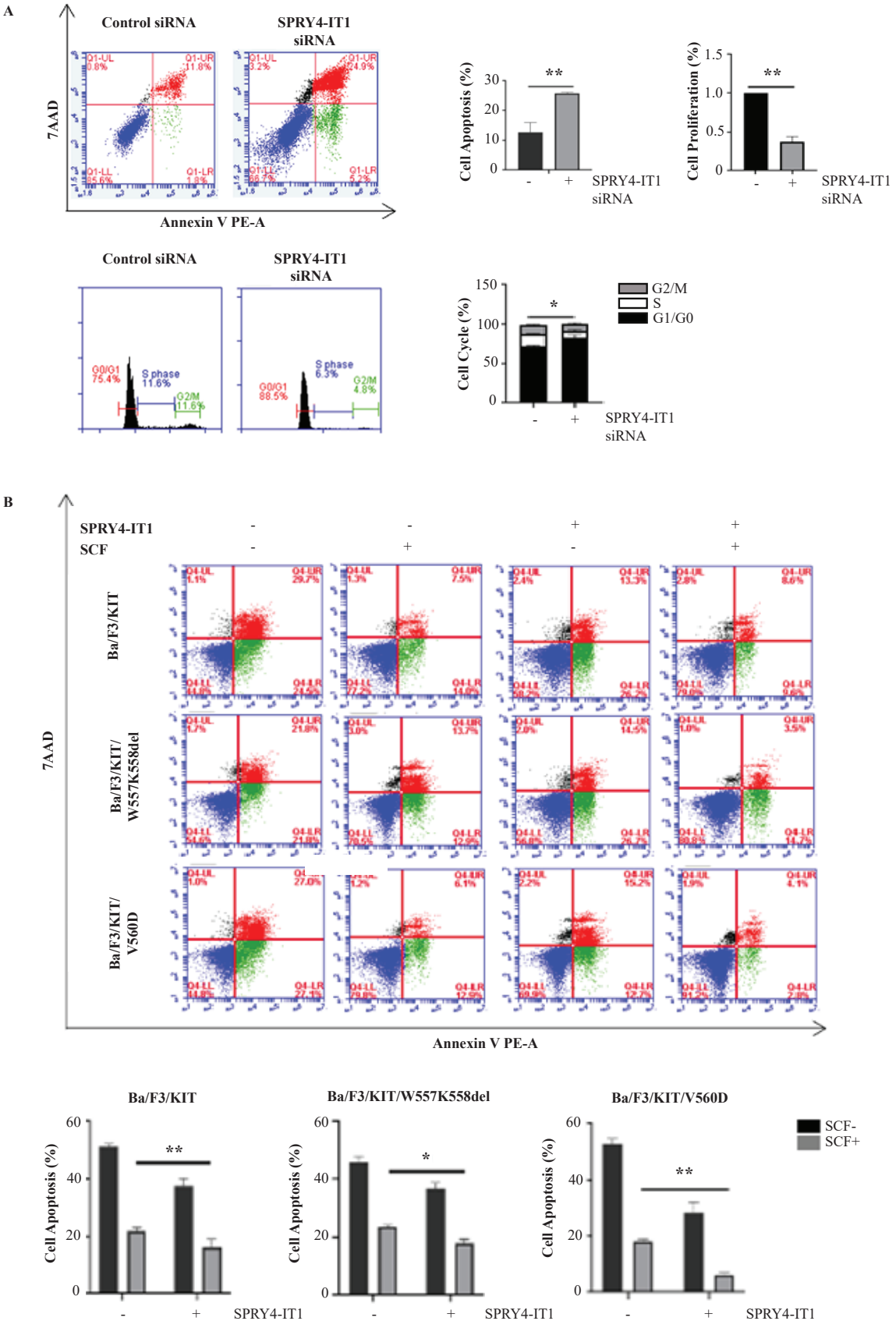

Figure 4. SPRY4-IT1 increased cell proliferation and survival. A: Apoptosis, proliferation and cell cycle of GIST-T1 cells. B: Apoptosis of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D with or without SPRY4-IT1.(continue)

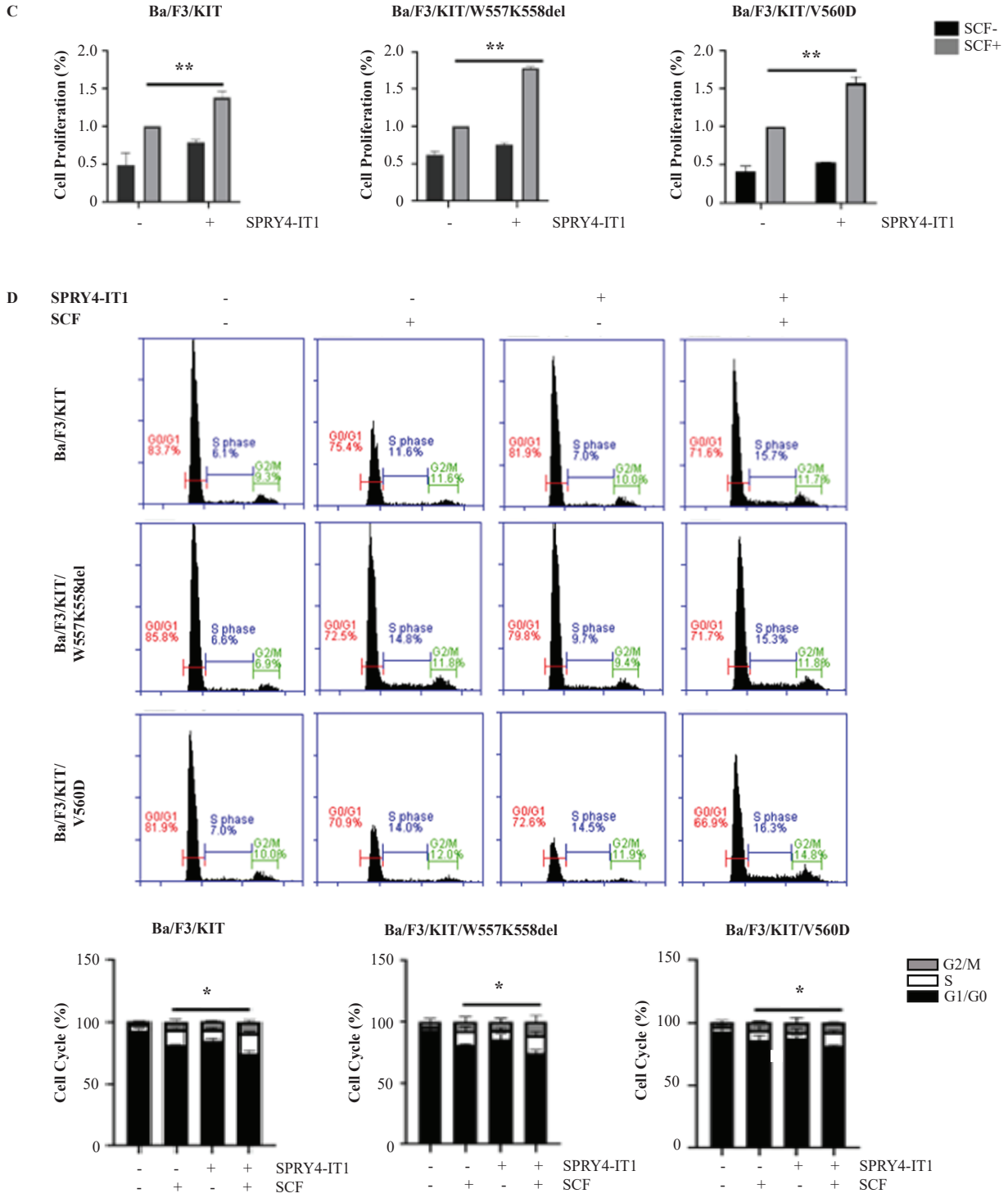

Figure 4. SPRY4-IT1 increased cell proliferation and survival.(continue) C: Proliferation of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D with or without SPRY4-IT1. Proliferation of each cells without SPRY4-IT1 in the presence of SCF was normalized as 1. D: Cell cycle of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D with or without SPRY4-IT1. The statistical difference was calculated by t-test; *p<0.05. **p<0.01.

KIT's response to imatinib. The results showed that, when SPRY4-IT1 was expressed, KIT activation, including wild-type KIT and primary KIT mutants, and the activation of their downstream signaling molecules AKT and ERK were stronger than that in the absence of SPRY4-IT1 when cells were treated with imatinib (Figure 5A). These data suggested that SPRY4-IT1 reduced the sensitivity of KIT to imatinib, including the wild-type KIT and also the

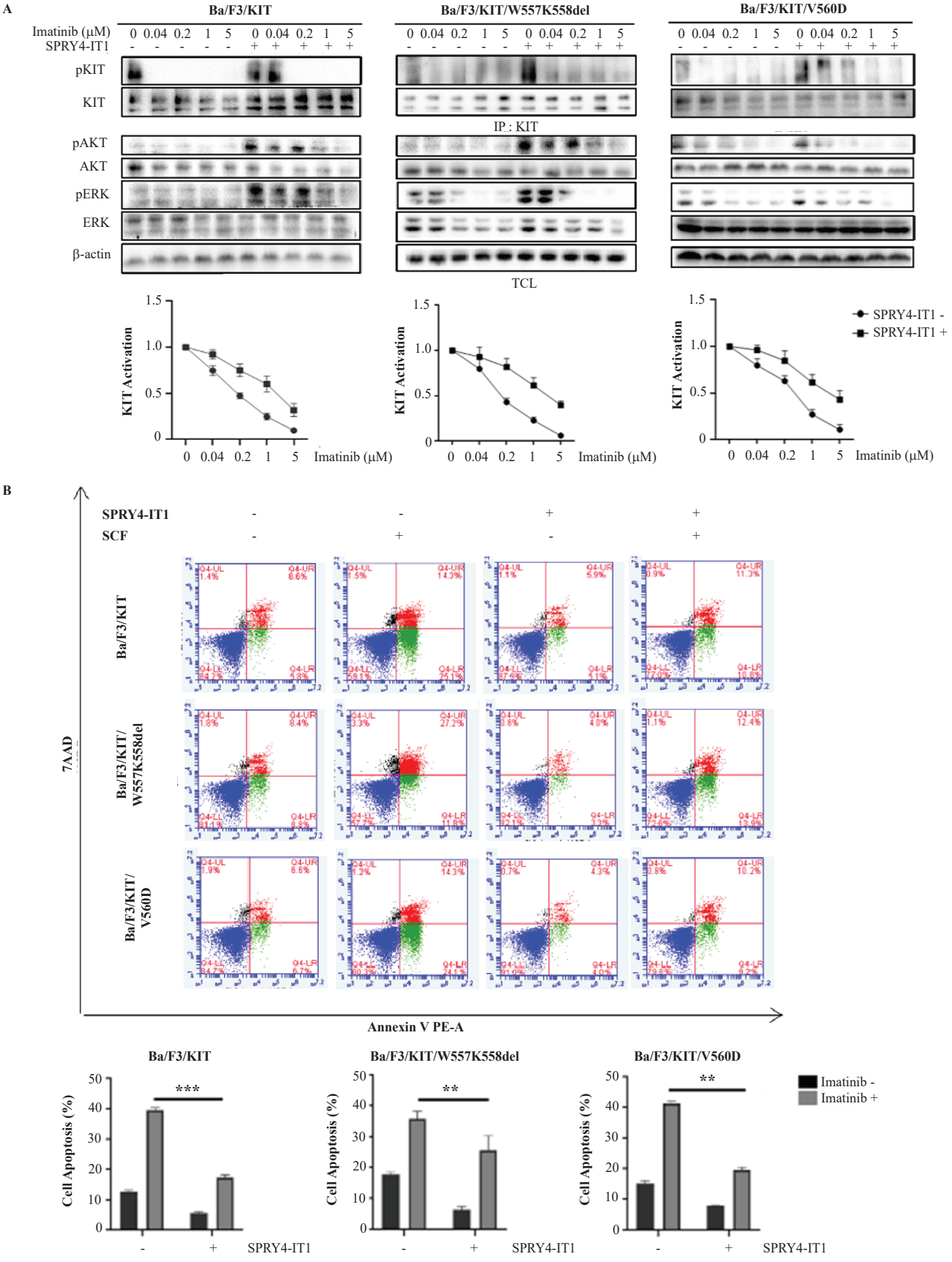

Figure 5. SPRY4-IT1 reduced wild-type KIT and primary KIT mutants' sensitivity to imatinib. A: Western blot results of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del, or KIT/V560D with or without SPRY4-IT1 using pAKT, AKT, pERK, ERK, β-actin, 4G10, and KIT antibodies. The activation of KIT was quantified. B: Apoptosis of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del or KIT/V560D with or without SPRY4-IT1.(continue)

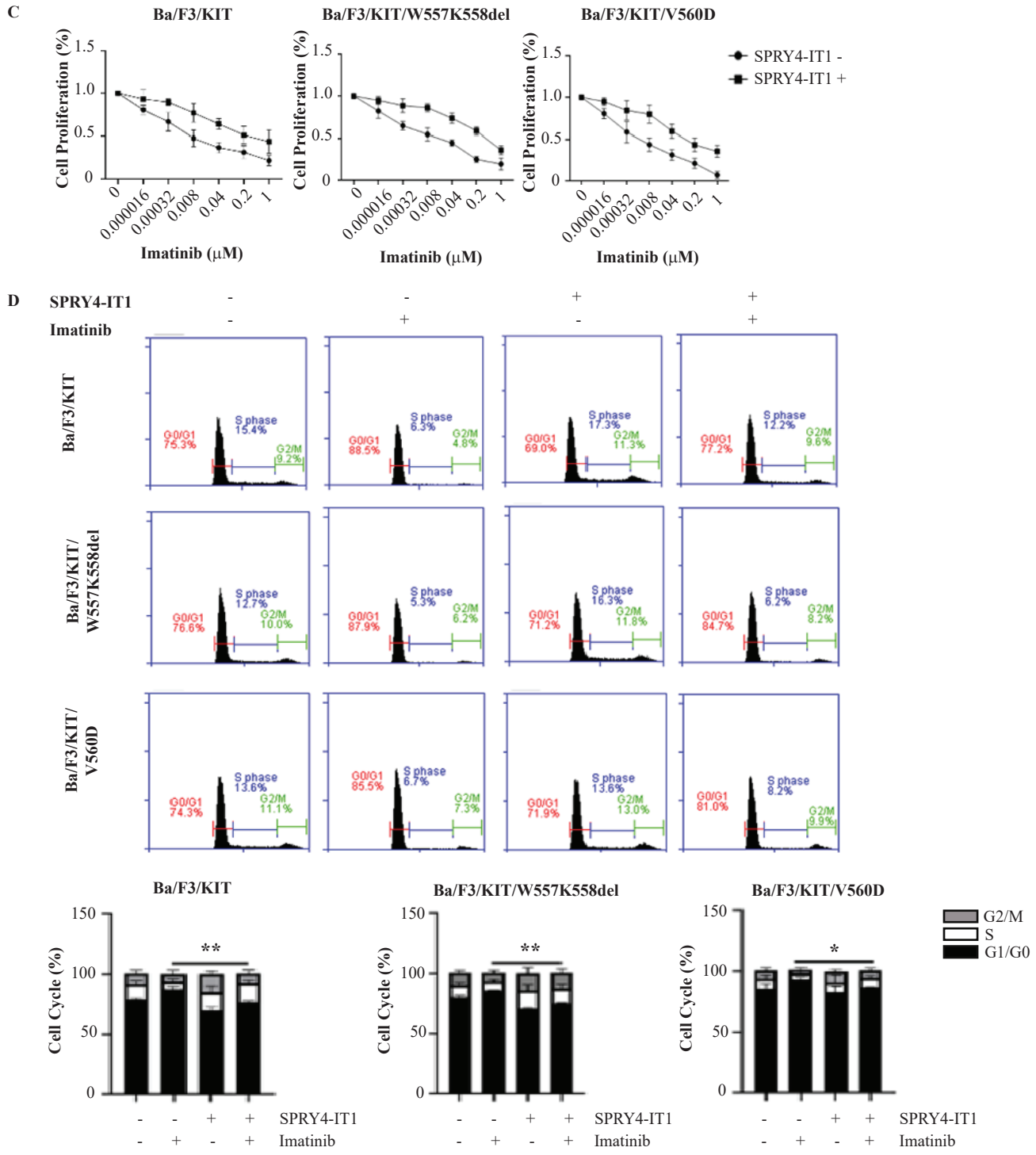

Figure 5. SPRY4-IT1 reduced wild-type KIT and primary KIT mutants' sensitivity to imatinib.(continue) C: Proliferation of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del or KIT/V560D with or without SPRY4-IT1. D: Cell cycle of Ba/F3 cells expressing wild-type KIT, KIT/W557K558del or KIT/V560D with or without SPRY4-IT1. The statistical difference was calculated by t-test; *p<0.05, **p<0.01, ***p<0.001.

primary KIT mutants. Similarly, in Ba/F3 cells that express wild-type KIT, or in the primary KIT mutants, SPRY4-IT1 increased the cell survival, cell proliferation, and cell cycle progression when treated with imatinib (Figure 5B, 5C, and 5D).

SPRY4-IT1 Increased The Activation and Expression of Secondary KIT Mutants, and Cell Survival and Proliferation Mediated by Secondary KIT Mutants

Similar to wild-type KIT, and primary KIT mutants, the results of this study showed that the presence of SPRY4-

IT1 increased KIT expression in Ba/F3 cells that express the often occurred secondary KIT mutants (Figure 6A). When stimulated with SCF constitutively, and the reduction of KIT expression in the presence of SPRY4-IT1 was less than that in the absence of SPRY4-IT1 (Figure 6B), indicating that SPRY4-IT1 increased secondary KIT mutant expression

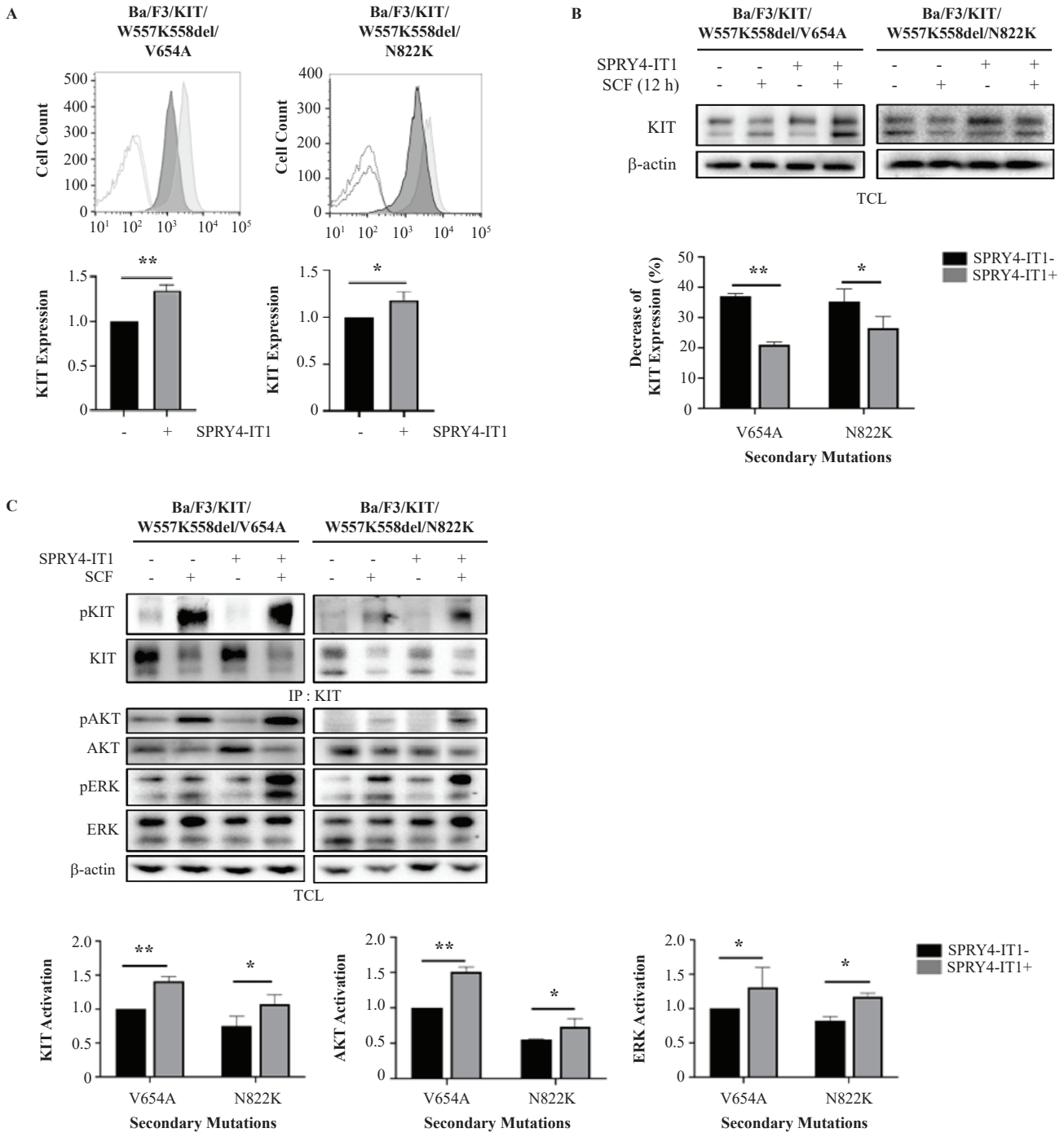

Figure 6. SPRY4-IT1 increased the activation and expression of secondary KIT mutants. A: KIT expression in Ba/F3 cells expressing KIT/W557K558del/V654A or KIT/W557K558del/N822K with or without SPRY4-IT1. Dark grey: cells without SPRY4-IT1 expression, light grey: cells with SPRY4 expression, empty area: isotype control, grey area: PE-anti-KIT antibody. Expression of KIT was quantified and analyzed. B: Western blot results of Ba/F3 cells expressing KIT/W557K558del/V654A or KIT/W557K558del/N822K with or without SPRY4-IT1 using KIT and β-actin antibodies. KIT expression was quantified, and the decrease in KIT expression after SCF stimulation was calculated. C: Western blot results of Ba/F3 cells expressing KIT/W557K558del/V654A or KIT/W557K558del/N822K with or without SPRY4-IT1 using pAKT, AKT, pERK, ERK, β-actin, 4G10 and KIT antibodies.(continue)

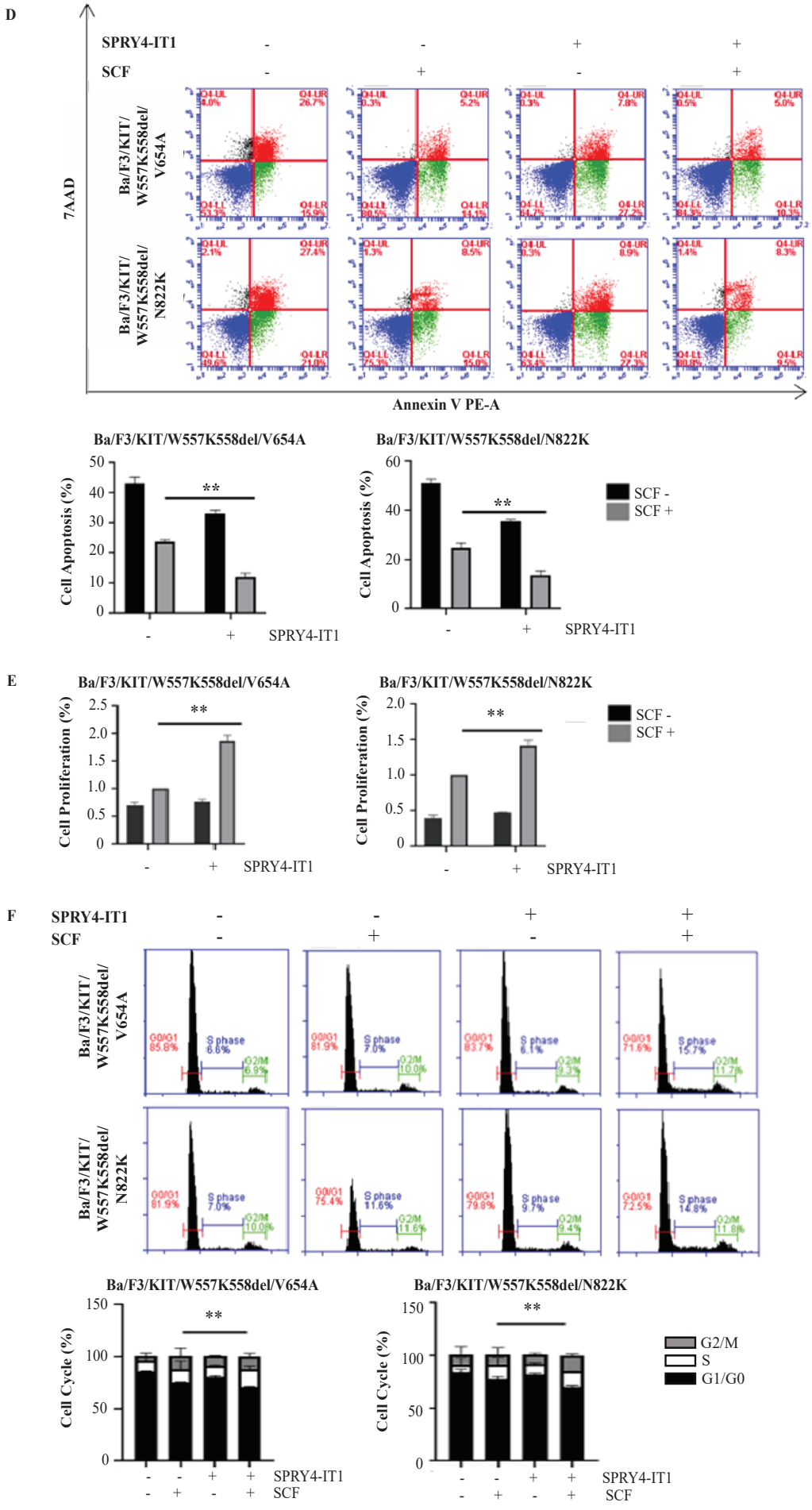

Figure 6. SPRY4-IT1 increased the activation and expression of secondary KIT mutants. (continue) D: Apoptosis of Ba/F3 cells expressing KIT/ W557K558del/V654A or KIT/W557K558del/N822K. E: Cell proliferation by CCK8 assay. F: Cell cycle by flow cytometry. The statistical difference was calculated by t-test; *p<0.05, **p<0.01.

regardless of ligand stimulation. Accordingly, SPRY4-IT1 promoted the activation of downstream signaling molecules ERK and AKT (Figure 6C). In addition, the presence of SPRY4-IT1 increased cell survival, proliferation, and cell cycle progression of Ba/F3 cells that express secondary KIT mutants (Figure 6D, 6E, and 6F), indicating that SPRY4-IT1 could increase the signaling of secondary KIT mutants.

SPRY4-IT1 Reduced Secondary KIT Mutants' Sensitivity to Imatinib

The secondary KIT mutants were resistant to imatinib. Therefore, imatinib inhibited the activation of secondary KIT mutants at a much higher concentration than that of primary KIT mutants. To know whether SPRY4-IT1 regulates secondary KIT mutants' sensitivity to imatinib, the Ba/F3 cells that express the often-occurred secondary KIT mutants were treated with imatinib in the presence or absence of SPRY4-IT1. SPRY4-IT1 increased KIT activation, and the activation of its downstream molecules ERK and AKT in Ba/F3 cells that express the often-occurred secondary KIT mutants when treated with imatinib (Figure 7A), suggesting that SPRY4-IT1 reduced both secondary KIT mutants' sensitivity to imatinib. Accordingly, SPRY4-IT1 increased cell survival, proliferation, and cell cycle progression of Ba/F3 cells expressing secondary KIT mutants when treated with imatinib (Figure 7B, 7C, and 7D).

Discussion

In this current work, it was found that KIT regulates SPRY4-IT1 expression in a similar pattern as the regulation of SPRY4 expression in GIST, which is probably due to the fact that they are transcribed from the same gene, and their transcription is regulated in a similar pattern. However, unlike that SPRY4 inhibits KIT signaling (20), we found that SPRY4-IT1 increases KIT signaling, showing that SPRY4 and SPRY4-IT1 have opposite functions in GIST: SPRY4 serves as a negative feedback molecule of KIT, while SPRY4-IT1 serves as positive feedback molecule of KIT. Interestingly, the two transcripts from the same gene have opposite functions in the same tissue (Figure 8).

miRNA is present in the circulation as important regulator of molecules in cells oncogenesis.(24,25) SPRY4-IT1 has been widely studied, and it is reported that SPRY4-IT1 acts as either an oncogene or tumor suppressor, depending on the host tissues.(26) SPRY4-IT1 regulates tumorigenesis through sequestering miRNAs including miR-6882-3p, miR-101-3p, and miR-22-3p.(26-29) It can interact with HIF-1a, AMPK, NF-kB/p65, MAPK, ZEB1, and PI3K/AKT signaling.(26) In GIST, it was recently reported that SPRY4-IT1 serves as a competing endogenous RNA to downregulate miR-101-5p and upregulate ZEB1, leading to increased ERK activation and, therefore contributing to the oncogenesis of GIST.(30) In addition to regulating miRNAs, our results identified a new pathway in GIST that SPRY4-IT1 increases KIT signaling.

Around 70-80% of GIST carry KIT mutants that need no ligand stimulation for their activation, leading to cell transformation.(4,6) The KIT activation regulation has been studied in GIST and other malignancies such as mastocytosis which is dominated by D816V mutation in the exon 17 of KIT. In addition to that KIT mutants's activation is independent of SCF binding, we have previously showed that the SRC family kinases, which contribute to wild-type KIT activation, are not necessary for the activation of KIT mutants that occur in both GIST and mastocytosis. In contrast, the direct association of PI3 kinase with KIT is necessary the constitutive activation of KIT mutants (31-33), showing the different characteristics of wild-type KIT and the oncogenic KIT mutants in their activation regulation. Unlike that, our results found that SPRY4-IT1 regulates wild-type KIT in a similar pattern as its regulation on KIT mutants.

Gaining secondary mutations of KIT is a main reason of GIST relapse (12,13), while primary KIT mutants and secondary KIT mutants have different characteristics in their activation and regulation. For example, although the primary KIT mutants are constitutively activated in the absence of ligand stimulation, binding with the ligand can increase their activation.(23,32) Unlike that, the constitutive activation of secondary KIT mutants is strong, and ligand stimulation can barely increase their activation. (23) In our previous study, we found that SPRY4 inhibits the signaling of primary KIT mutants, but not secondary KIT mutants.(20) In contrary to that, SPRY4-IT1 can regulate both primary KIT mutants and secondary KIT mutants, further indicating the different characteristics of primary and secondary KIT mutants in their activation and regulation, while the detailed mechanism of how SPRY4-IT1 regulates KIT signaling needs further studies to be discovered in the future. As targeted therapy becomes more favored lately (34), studies regarding those mechanism should be conducted.

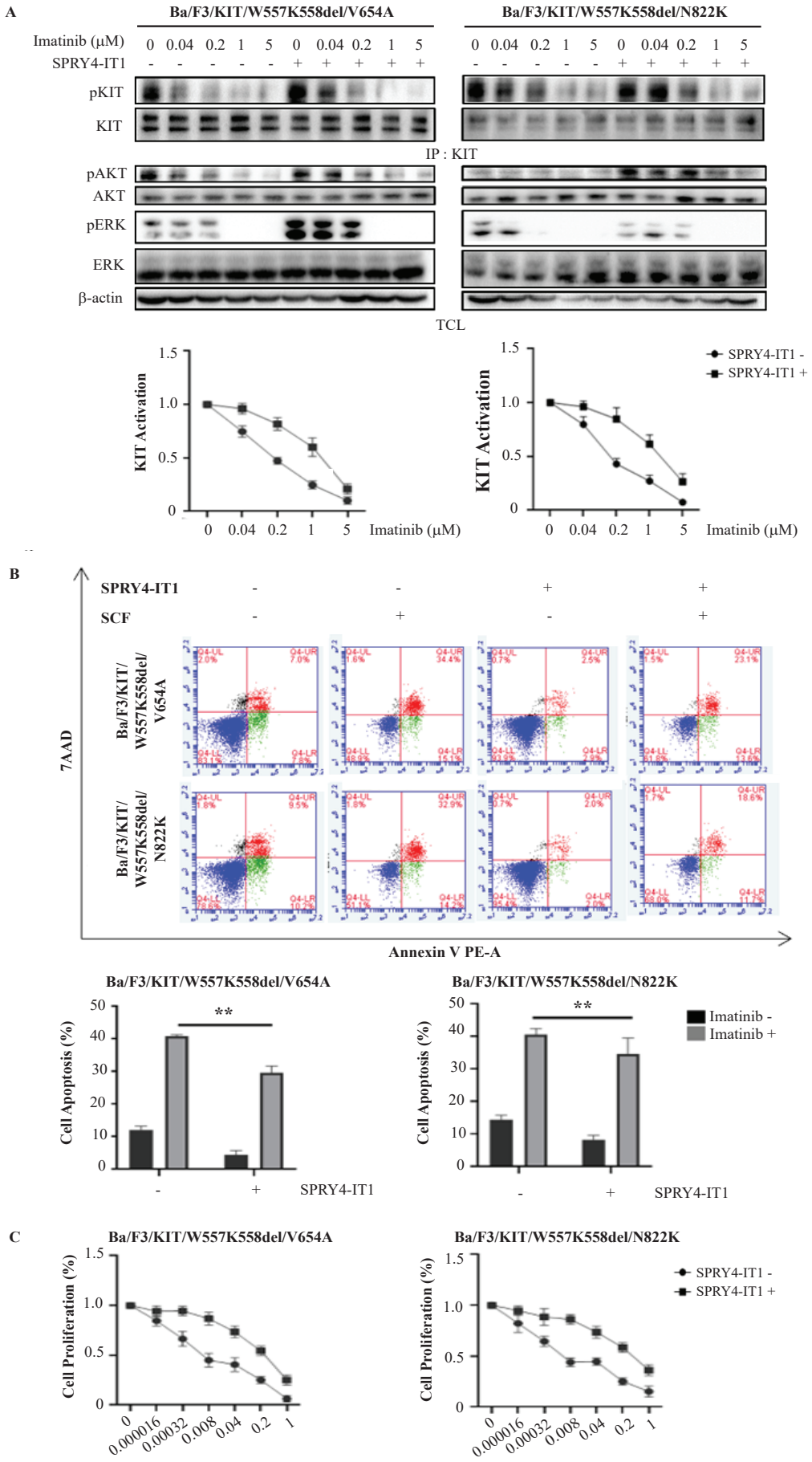

Figure 7. SPRY4-IT1 reduced secondary KIT mutants' sensitivity to imatinib. A: Western blot results of Ba/F3 cells expressing KIT/W557K558del/V654A or KIT/W557K558del/N822K with or without SPRY4-IT1 using pAKT, AKT, pERK, ERK, β-actin, 4G10 and KIT antibodies. B: Apoptosis of Ba/F3 cells expressing KIT/W557K558del/V654A or KIT/W557K558del/N822K with or without SPRY4-IT1. C: Cell proliferation by CCK8 assay.(continue)

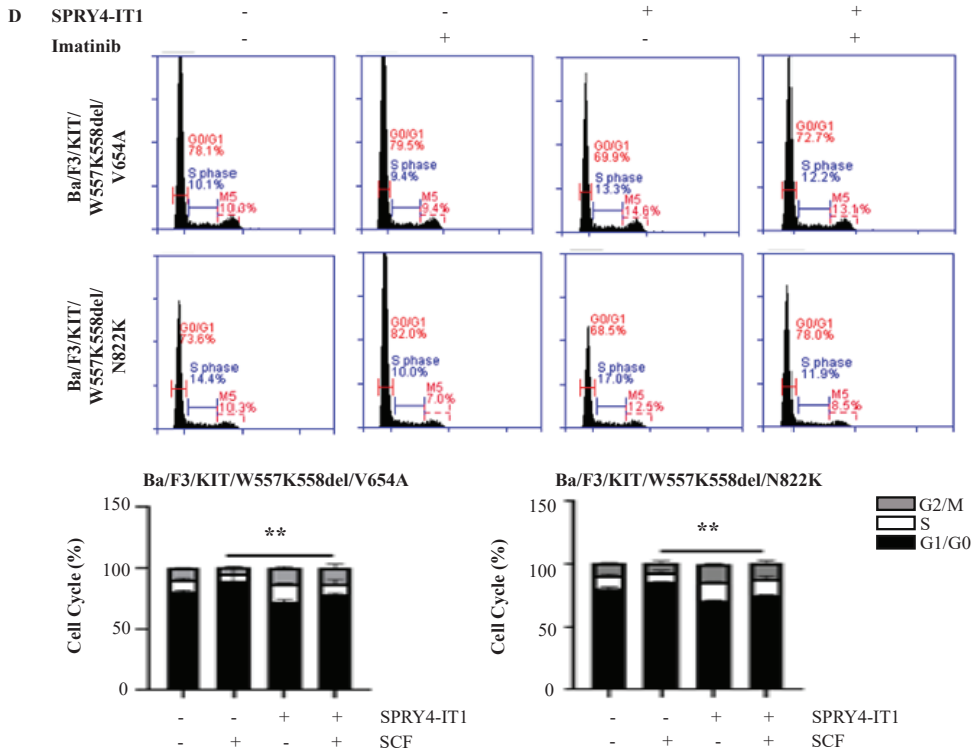

Figure 7. SPRY4-IT1 reduced secondary KIT mutants' sensitivity to imatinib.(continue) D: Cell cycle by flow cytometry. The statistical difference was calculated by t-test. $^{**}p<0.01$.

Conclusion

Our results found that KIT can increase SPRY4-IT1 expression and SPRY4-IT1, in return, SPRY4-IT1 increases the activation of wild-type KIT, primary KIT mutants and secondary KIT mutants, and the activation of their downstream signaling molecules AKT and ERK. The positive feedback between KIT and SPRY4-IT1 contributes to the tumorigenesis of GIST, and reduces the sensitivity of wild-type KIT, primary KIT mutants and secondary KIT mutants to imatinib.

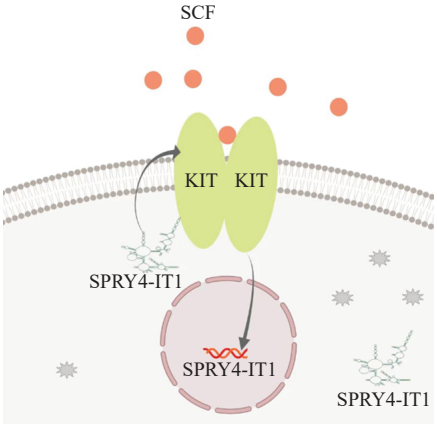

Figure 8. The schematic diagram about the regulation between KIT and SPRY4-IT1 in GISTs. KIT increase SPRY4-IT1 expression and SPRY4-IT1, in return, SPRY4-IT1 increases the activation of wild-type KIT, primary KIT mutants and secondary KIT mutants, and the activation of their downstream signaling molecules AKT and ERK.

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Authors Contribution

SL was involved in the study conception. SL and MA were involved in the study designing. YY, ZJ, SZ, and CL were involved in the data collection. YY analyzed and interpreted the results. YY and SL drafted the original manuscript. All authors reviewed the results and approved the final version of the manuscript.

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