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The tyrosine kinase CSK associates with FLT3 and c-Kit receptors and regulates downstream signaling

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Abstract

Type III receptor tyrosine kinases (RTKs), FLT3 and c-Kit play important roles in a variety of cellular processes. A number of SH2-domain containing proteins interact with FLT3 and c-Kit and regulate downstream signaling. The SH2-domain containing non-receptor protein tyrosine kinase CSK is mainly studied in context of regulating Src family kinases. Here we present an additional role of this kinase in RTK signaling. We show that CSK interacts with FLT3 and c-Kit in a phosphorylation dependent manner. This interaction is facilitated through the SH2-domain of CSK. Under basal conditions CSK is mainly localized throughout the cytosolic compartment but upon ligand stimulation it is recruited to the inner side of cell membrane. CSK association did not alter receptor ubiquitination or phosphorylation but disrupted downstream signaling. Selective depletion of CSK using siRNA, or inhibition with CSK inhibitor, led to increased phosphorylation of Akt and Erk, but not p38, upon FLT3 ligand (FL) stimulation. Stem cell factor (SCF)-mediated Akt and Erk activation was also elevated by CSK inhibition. However, siRNA mediated CSK knockdown increased SCF stimulated Akt phosphorylation but decreased Erk phosphorylation. CSK depletion also significantly increased both FL- and SCF-induced SHC, Gab2 and SHP2 phosphorylation. Furthermore, CSK depletion contributed to oncogenic FLT3- and c-Kit-mediated cell proliferation, but not to cell survival. Thus, the results indicate that CSK association with type III RTKs, FLT3 and c-Kit can have differential impact on receptor downstream signaling.

Highlights

- ❖ CSK associates with type III receptor tyrosine kinases
- ❖ CSK SH2 domain and receptor activation are mandatory for this association
- ❖ CSK differentially regulates FLT3 and c-Kit downstream signaling
- ❖ CSK contributes in control of oncogenic FLT3 and c-Kit mediated cell proliferation

Keywords

FLT3, c-Kit, CSK.

1. Introduction

The protein tyrosine kinase is one of the largest groups of protein kinases in mammalian genome encoding by more than 90 different genes [1, 2]. This group is further divided into 20 families of receptor tyrosine kinases (RTKs) and 10 families of non-receptor tyrosine kinases (NRTKs). The type III receptor tyrosine kinase (also known as PDGFR family) is a family of RTKs consisting of five RTKs including CSF1R, FLT3, c-Kit, PDGFR α and PDGFR β . Members of this family of proteins play important roles in a number of physiological conditions and many of them are frequently mutated in variety of cancers [3-6]. While RTKs need ligand binding to the extracellular domains for dimerization and activation, oncogenic mutations allows RTKs to escape from the ligand dependency for activation [4].

Receptor activation triggers a plethora of downstream signaling pathways which are tightly regulated by a number of different interacting proteins. For example, association of Grb10 or SLAP with FLT3 enhances downstream signaling while SOCS2, SOCS6 or LNK negatively regulates signaling of the same receptor [7-11]. The c-Kit signaling has also been reported to be regulated by various interacting proteins such as Grb2, SHP2, Cbl, Crk etc. (for review see [3]). A number of interacting proteins modulate receptor signaling by altering its stability while others help the receptor to transduce information by recruiting signaling proteins.

The Src family tyrosine kinases (SFKs) are a major group of non-receptor tyrosine kinases abundantly expressed in variety of cells. This family is comprised of eight members including Src, Yes, Fyn, Fgr, Lyn, Hck, Lck and Blk. The C-terminal Src kinase (CSK; also known as C-terminal Src kinase) and CSK homologous kinase (CHK) are well-known negative regulators of SFKs which phosphorylate SFKs at the C-terminal tyrosine residue corresponding to Y530 in the human sequence of Src. This residue along with CSK recognition sequence is highly conserved within the SFKs [12]. Phosphorylation of the C-terminal tyrosine residue allows SFKs to fold in an inactive conformation. Thus, loss of CSK function results in an aberrant activation of SFKs as well as survival pathways. In this report we show that CSK associates with various type III receptor tyrosine kinases and differentially regulates receptor signaling.

2. Materials and Methods

2.1. Plasmids

The pcDNA3-FLT3-WT, pcDNA3-FLT3-ITD, pcDNA3-c-Kit-WT, pcDNA3-c-Kit-D816V, pMSCV-c-Kit-WT, pMSCV-c-Kit-D816V and pcDNA3-PDGFR β plasmids were described elsewhere [13-15]. The pMSCV-FLT3-WT and pMSCV-FLT3-ITD plasmids were a kind gift from Dr. D. Gary Gilliland. We generated a modified pcDNA3 vector (pcFLAG) by inserting double strand oligo containing M-BamHI-XhoI-FLAG-Stop. We used pcFLAG vector to sub-clone wild-type human CSK using BamHI and XhoI cloning sites. The CSK-R107E mutant was generated by site directed mutagenesis using the QuikChange mutagenesis XL kit (Stratagene, La Jolla, CA). The CSK- Δ SH3 (73 to 450 amino acids) and CSK-SH3-SH2 (2 to 190 amino acids) mutants were generated by sub-cloning respective parts of human CSK in pcFLAG vector.

2.2. Antibodies

Rabbit polyclonal anti-FLT3, anti-c-Kit, anti-PDGFR β and anti-Erk antibodies were described previously [13-15]. Mouse anti-FLAG and anti- β -Actin antibodies were purchased from Sigma. Phycoerythrin (PE)-conjugated anti-FLT3 and anti-c-Kit antibodies were from BD Biosciences. Rabbit polyclonal anti-phospho-Gab2, anti-phospho-SHP2 and Alexa Fluor 647 conjugated anti-FLAG antibodies were from Cell signaling. Rabbit polyclonal anti-CSK, anti-phospho-Erk1/2, anti-SHP2 and goat polyclonal anti-Akt antibodies were from Santa Cruz Biotechnology. Mouse anti-mono-ubiquitin antibody was from Covance Research Products. General phosphotyrosine antibody 4G10 was from Millipore. Rabbit polyclonal anti-Akt antibody was from Epitomics. Mouse monoclonal anti-phospho-p38 and anti-p38 antibodies, and rabbit anti-SHC antibody were from BD Transduction Laboratories.

2.3. Chemical reagents, ligands and siRNAs

The transfection reagents jetPEI and Lipofectamine 2000 were from Polyplus-transfection SA and Invitrogen respectively. Cycloheximide and MG132 were from Sigma. Chloroquine diphosphate

was from Sigma. FLT3 ligand (FL), stem cell factor (SCF), and PDGF-BB were purchased from Prospecc Tany.

2.4. Cell culture

The COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FBS and 10 ng/ml recombinant murine interleukin-3 (IL-3). Since IL3-withdrawal interferes functional redundancy of oncogenic FLT3 and c-Kit signaling [16], we used IL-3 containing medium for the culture of all transfected Ba/F3 cells.

2.5. Transient transfection

The JetPEI transfection reagent (Polyplus Transfection) was used for transient transfections. Cells were seeded in the evening for next morning transfections. For co-transfections, 5 µg of total plasmids (2 µg receptor plasmid, 3 µg CSK plasmid) and 15 µl of JetPEI were used. Transfection was carried out in a 10 cm petri-dish with 5 ml of complete medium. Six hours after transfection cells washed with PBS for three times and cultured in serum free medium for 18 hours before stimulation and/or lysis.

2.6. Retroviral transduction

Retroviral transfection procedure was described previously [8, 9]. In brief, packaging EcoPack cells were transfected with respective plasmids using Lipofectamine 2000 (Life Technologies) as per the manufacturers' protocol. Six hours after transfection medium was replaced with fresh complete growth medium. Cells were then grown for 48 hours to produce viral particles. Then Ba/F3 cells were cultured in medium containing viral particles for 24 hours and were grown for an additional 48 hours in complete Ba/F3 medium before starting puromycin selection.

2.7. Electroporation of siRNA

For siRNA transfection to Ba/F3 cells, we used 4D-Nucleofector system from Lonza. Cells were washed with PBS before transfection. Five million cells were used to transfect 6 µg of siRNA in 100 µl of SG solution (Lonza) using CM150 program.

2.8. Immunoprecipitation, SDS-PAGE and Western blotting

Ba/F3 cells were kept in IL-3 and serum-free medium for 4 hours before stimulation. After stimulation cells were washed with ice-cold PBS. Cells were then lysed in 1% Triton X100 on ice. Lysates were processed for immunoprecipitation, SDS-PAGE and Western blotting [8].

2.9. Subcellular localization:

Cells transfected with respective CSK and FLT3/c-Kit plasmids were fixed using 4% para-formaldehyde. Cells were then permeabilized with 0.5% Triton X100 and blocked with 5% goat serum. Cells were stained with fluorophore-conjugated anti-FLAG and anti-FLT3/anti-c-KIT antibodies. Nuclei were stained with DAPI and subcellular localization was visualized with a Carl Zeiss LSM 710 Laser Scanning Microscope.

2.10. Receptor Ubiquitination

Cells were serum starved for 4 hours and then incubated with MG132 and chloroquine diphosphate for 30 minutes. Cells were then stimulated with FL or SCF for the indicated period of time in presence of MG132 and chloroquine diphosphate and processed for lysis. Cell lysates were immunoprecipitated with an anti-FLT3 or anti-c-Kit antibody followed by Western blotting analysis.

2.11. Cell proliferation

Transfected Ba/F3 cells were washed three times with PBS to remove IL-3 and seeded in 96-well plates (10,000 cells/well). Cells were then incubated with or without 100 ng/ml FL or SCF for 46 hours. Then 10 µl of PrestoBlue ((Molecular Probes) was added to each well, followed by 2 h of incubation. Absorbance at 570 nm and 600 nm was measured using a 96-well plate reader and cell viability was calculated according to the manufacturer's protocol.

2.12. Cell survival

The annexin V/7-aminoactinomycin D kit (Pharmingen) was used to measure cell survival. Double-negative (annexin V/7-aminoactinomycin D) cells were counted using BD FACSArray (BD Biosciences) for cell survival.

3. Results

3.1. CSK associates with FLT3 and c-Kit in a phosphorylation dependent manner

Besides association with SFKs, CSK interacts with several receptors including insulin receptor (IR), insulin-like growth factor-I receptor (IGF-IR) [17] and T cell antigen receptor (TCR) zeta [18]. Phosphorylation on receptors' tyrosine residues was essential for interaction. Type III receptors also become phosphorylated on multiple tyrosine residues upon ligand stimulation. Thus we tested whether type III receptor tyrosine kinases also interact with CSK. We used transient transfection to detect interactions. COS-1 cells were transfected with FLAG-tagged CSK and FLT3, c-Kit or PDGFR β constructs. We observed that CSK associates with FLT3 (Fig. 1A), c-Kit (Fig. 1B) and PDGFR β (Fig. 1C). This association was dependent on ligand stimulation and oncogenic mutants of FLT3 (FLT3-ITD) and c-Kit (c-Kit-D816V) displayed constitutive association with CSK (Fig. 1A-B). These data indicate that CSK interacts with receptor tyrosine kinases FLT3, c-Kit and PDGFR β , and that receptor phosphorylation is required for association. Although, we demonstrated interaction between CSK and multiple type III RTKs by overexpressing both proteins, we failed to detect an endogenous interactions probably due to the poor specificity of the antibodies used.

3.2. CSK-SH2 domain is sufficient for the interaction with FLT3 and c-Kit

The IGF-IR and IR interact with CSK through the CSK-SH2 domain [17]. Since we observed that receptor phosphorylation is required for association of CSK with FLT3 and c-Kit we hypothesized that CSK associates with FLT3 and c-Kit through the CSK SH2 domain. To verify our hypothesis we generated multiple CSK mutants (Fig. 1D). The CSK-R107E mutant lacks a critical arginine residue in the phosphotyrosine binding pocket of the SH2 domain which is replaced by

negatively charged glutamic acid. This mutation abrogates the ability to the SH2 domain to interact with phosphotyrosine residues. Indeed, we observed that neither FLT3 (Fig. 1E, 3rd panel) nor c-Kit (Fig. 1F, 3rd panel) was able to associate with CSK-R107E mutant indicating that a functional CSK-SH2 domain is required for interaction. Conversely, either the SH3 domain deletion mutant (CSK- Δ SH3) or a kinase domain deletion mutant (CSK-SH3-SH2) associated fairly with both FLT3 (Fig. 1E) and c-Kit (Fig. 1F). Thus we assert that CSK interacts with receptor tyrosine kinases through its SH2 domain and that the SH3 domain is not required for interaction.

3.3. CSK is recruited to the cell membrane in response to ligand stimulation

A previous study showed that CHK translocates from the cytosol to the membrane vicinity upon heregulin stimulation and associates with the ERBB2 receptor [19]. CSK displays considerable structural homology as well as functional similarity to CHK. Thus it is of relevance to check whether CSK also localizes to the cytoplasmic side of the cell membrane in response to FL and SCF. We stained CSK-FLAG and FLT3-WT or c-Kit-WT transfected cells with fluorophore-conjugated anti-FLAG and anti-FLT3 or anti-c-Kit antibodies. We observed that under basal conditions CSK is predominantly localized within the cytosolic compartment, but upon ligand stimulation it is recruited to the plasma membrane (Fig. 2A and 2B). Similar localization patterns were displayed by the CSK- Δ SH3 (Fig. 2C and 2D) and CSK-SH3-SH2 (Fig. 2E and 2F) mutants. On the contrary, the non-functional SH2-domain mutant (CSK-R107E) did not show a membrane translocation (Fig. 2G and 2H). Furthermore, colocalization in between receptors and CSK was significantly increased in all functional SH2 domain containing CSK mutants (Fig. 2I and 2J). These results suggest that a functional SH2 domain is required for the interaction with the receptor and thus for the localization of CSK to the inner side of the plasma membrane.

3.4. CSK depletion did not alter neither receptor ubiquitination nor phosphorylation

Several receptor interacting proteins alter receptor stability and/or phosphorylation. For instance, SOCS6 and SLAP accelerate receptor degradation by increasing ubiquitination without interfering with phosphorylation [8, 10], while LNK associates with FLT3 and decreases FLT3

phosphorylation [7]. To test whether CSK disrupts receptor stability or phosphorylation, we generated stably transfected Ba/F3 cells by transfecting either FLT3 or c-Kit. Ba/F3 cells express a decent level of endogenous CSK. To study the effects of CSK on FLT3 and c-Kit receptors, we attempted to silence CSK by means of CSK specific siRNA achieving up to 76% of knockdown of endogenous CSK (Fig. 3A). CSK depletion did not significantly affect neither FLT3 ubiquitination nor phosphorylation (Fig. 3B and 3C). Similarly to FLT3, ubiquitination or phosphorylation of c-Kit was also not altered by CSK siRNA (Fig. 3D and 3E).

3.5. CSK negatively regulates ligand-induced Akt phosphorylation

CSK regulates integrin-mediated signaling through Akt activation [20] and overexpression of CHK potentiates Akt phosphorylation in breast cancer MCF-7 cell line [21]. Furthermore, CSK overexpression in PC12 cells leads to increased Akt phosphorylation in response to nerve growth factor [22]. These observations suggest that CSK and its homologous kinase differentially regulate Akt activation in different biological contexts, although both proteins exhibit similar function in SFKs regulation. Thus we tested whether CSK plays a role in FL- or SCF-mediated Akt activation. Ba/F3-FLT3-WT or Ba/F3-c-Kit-WT cells were transfected with control siRNA or CSK siRNA. Two days after transfections cells were serum-starved for four hours before ligand stimulation. We observed that CSK depletion led to an increase in Akt activation in both Ba/F3-FLT3-WT (Fig. 4A) and Ba/F3-c-Kit-WT (Fig. 4B) cells suggesting that CSK acts as a negative regulator of Akt signaling downstream to type III receptor tyrosine kinases. In addition, a selective CSK inhibitor (ASN2324598) [23, 24] also augmented Akt phosphorylation in response to both FL (Fig. 4C) or SCF (Fig. 4D) stimulation indicating that functional CSK is required to maintain basal Akt phosphorylation.

3.6. CSK differentially regulates FL- and SCF-induced Erk1/2 phosphorylation

Type III receptor tyrosine kinases transduce mitogenic signaling through activation of the Erk mitogen-activated protein kinase [3-5]. Overexpression of CSK decreases Erk1/2 phosphorylation in response to different stimuli [20, 22]. We observed that while CSK knockdown enhanced Erk1/2 phosphorylation in response to FL stimulation (Fig. 5A), it decreased Erk1/2 phosphorylation in

response to SCF (Fig. 5B). The ASN2324598-mediated CSK inhibition led to a prolonged Erk1/2 activation in response to both FL (Fig. 5C) and SCF (Fig. 5D). On the whole, these results indicate that CSK differentially regulates MAPK signaling downstream of FLT3 and c-Kit receptors and that intact CSK is required for c-Kit-mediated Erk1/2 signaling.

3.7. CSK depletion has no significant effect on p38 activation

Since both FL and SCF can activate p38, we then tested whether CSK also controls ligand-stimulated p38 phosphorylation. CSK depletion slightly decreased FL-stimulated p38 phosphorylation at the 2 minute time point but slightly increased at the 5 minute time point (Fig. 6A). Similar to FL, SCF induced p38 activation was slightly attenuated in CSK knockdown cells at the 2 minute time point (Fig. 6B). Although knockdown of CSK led to a slight deregulation of p38 phosphorylation induced by either receptor, differences were not statistically significant.

3.8. CSK depletion increases SHC, Gab2 and SHP2 tyrosine phosphorylation

Activation of Akt and Erk1/2 by receptor tyrosine kinases FLT3 and c-Kit is mediated through activation of various signaling proteins such as SHP2, SHC and Gab2 [3, 4]. Therefore we tested which intermediate regulators are involved in transducing signals from FLT3 and c-Kit receptors through CSK. Ba/F3-FLT3-WT and Ba/F3-c-Kit-WT cells were transfected with control and CSK siRNA. Two days after transfection cells were serum-starved for four hours followed by two minutes and five minutes ligand stimulation. We observed that phosphorylation on tyrosine residues of SHC was increased in CSK siRNA transfected cells in response to both FL (Fig. 7A) and SCF (Fig. 7B). Using phospho-specific antibodies against phosphotyrosine residues in Gab2 we showed that both FL (Fig. 7C) and SCF (Fig. 7D) stimulation enhanced Gab2 phosphorylation in cells where CSK expression had been knocked down. Similarly to SHC and Gab2, we also observed an increase in SHP2 phosphorylation following knockdown of CSK in Ba/F3-FLT3-WT (Fig. 7E) and Ba/F3-c-Kit-WT (Fig. 7F) cells upon respective ligand stimulation.

3.9. CSK depletion increases oncogenic FLT3- and c-Kit-mediated cell proliferation but not cell survival

Since we observed that CSK differentially regulates Akt and Erk1/2 signaling in response to the FL and SCF stimulation, we investigated whether CSK depletion resulted in differential biological outcomes. We tested cell proliferation and survival using constitutively active mutants of FLT3 and c-Kit which are known to induce IL-3-independent Ba/F3 cell survival [16]. We observed that CSK depletion resulted in an increased proliferation of Ba/F3-FLT3-ITD (Fig. 8A) and Ba/F3-c-Kit-D816V cells (Fig. 8B), while cell survival remained unchanged (Fig. 8C and 8D).

4. Discussion

The C-terminal Src kinase (CSK) contributes to regulation of SFKs activity. Thus, loss of CSK function mediates aberrant SFK signaling leading to abnormal cell growth. Apart from its catalytic activity CSK is known to interact with variety of signaling proteins. CSK associates with TCR complex through CSK-SH2 domain and this interaction brings CSK closer to SFKs resulting in negative regulation of TCR signaling [18]. In this study, we demonstrate that CSK associates with multiple type III receptor tyrosine kinases. Moreover, we showed that a functional CSK SH2 domain is necessary for interactions to the activated receptors. Furthermore, our results indicate that ligand activation triggers CSK membrane localization and that CSK regulates receptor downstream signaling eventually leading to receptors-mediated cell proliferation.

Both CSK and CHK deficient mice have been generated. Although CHK deficient mice survived and were apparently healthy [25], CSK deficient mice died due to defects in the neural tissues [26, 27] suggesting that CSK plays additional roles in embryogenesis other than regulation of SFKs. Differential subcellular localization in response to different physiological stimulant might regulate downstream signaling differentially. Many kinases display differential localization patterns and thereby differentially regulate downstream signaling [28-31]. CSK probably also elicit different signaling whether it is cytosolic or membrane-localized. While cytosolic CSK interacts with adapters or scaffold proteins [12], membrane localized CSK associates with receptors and transduces or blocks receptor signaling.

CSK is predominantly present in cytosol due to lack of a transmembrane domain or fatty-acyl modifications, but its substrate SFKs are mainly anchored inner surface of the cell membrane. Association of CSK with ligand-stimulated receptors brings CSK in close proximity to the cell membrane where it phosphorylates SFKs [12]. Since, both FLT3 and c-Kit transduce signal through SFKs [4], CSK association with the receptors might have negative impact on receptor downstream signaling. Elevated SHC phosphorylation in CSK depleted cells further supports this hypothesis. SFKs induce tyrosine phosphorylation of several signaling proteins including Gab2 and SHP2 resulting in activation of downstream serine/threonine kinases Akt and Erk1/2 [4]. Our data also suggests that loss of CSK function contributes to increased Gab2 and SHP2 tyrosine phosphorylation as well as to increased Akt serine phosphorylation. Since activation of the Akt pathway facilitates cell survival and proliferation through transcriptional activation of cell cycle activators and phosphorylation-dependent inactivation of cell cycle repressors [4, 32], it is anticipated that depletion of CSK will further accelerate oncogenic receptor-mediated cell proliferation.

The observation that CSK depletion or inhibition elevated FL-induced Erk1/2 phosphorylation could also be explained by the fact that loss of CSK function reduces control of SFKs activation. However, while CSK inhibition potentiated SCF-induced Erk1/2 phosphorylation which is in line with the FLT3 downstream signaling, siRNA-mediated CSK depletion decreased SCF-mediated phosphorylation of same proteins. This observation cannot be explained by an influence on SFKs regulation and it is of interest how CSK association to the c-Kit induces prolonged Erk1/2 phosphorylation. One possible explanation could be adaptor activity. The presence of both SH3 and SH2 domains helps many proteins to link multiple signaling proteins [33]. Thus, CSK might link c-Kit with other signaling proteins resulting in activation of Erk1/2 which is independent of the regulations of SFKs.

5. Conclusions

In this study we demonstrate that CSK associates with multiple type III receptor tyrosine kinases including FLT3 and c-Kit. While this association is mediated through CSK SH2 domain and tyrosine phosphorylated receptors, SH3 domain is not required for the interaction. Association of CSK

with receptors increases the membrane localization of CSK resulting in extended SFKs regulation, which further controls of downstream PI3K-Akt pathway as well as cell proliferation. Although FLT3-CSK association negatively regulates Erk1/2 signaling, c-Kit-CSK interaction enables prolonged Erk1/2 activation. Thus our results suggest a novel role of CSK in downstream signaling of type III receptor tyrosine kinases.

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Competing interests

The authors declare no conflict of interest.

Authors' contributions

JUK conceived, designed and performed the experiments, analyzed the data and wrote the manuscript. MV and SP contributed in the localization experiments. SA contributed in the cell proliferation and survival assays. EB conceived the experiments and wrote the manuscript. LR conceived and designed the experiments, discussed data and wrote the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1. CSK associates with phosphorylated FLT3 and c-Kit receptors through the CSK-SH2 domain

Plasmids encoding wild-type CSK and either of FLT3-WT, FLT3-ITD (A), c-Kit-WT, c-Kit-D816V (B) or PDGFR β (C) were transfected into COS-1 cells. Six hours after transfections cells were serum-starved for 18 hours and then stimulated with 100 ng/ml with either FL, SCF or PDGF-BB, respectively, followed by lysis. Cell lysates were immunoprecipitated with anti-FLAG antibody and then subjected to Western blotting analysis. (D) Schematic representation of CSK mutants generated for this study. Plasmids expressing different mutants of CSK and FLT3-WT (E) or c-Kit-WT (F) were transfected in COS-1 cells and analyzed as described above.

Figure 2. Ligand stimulation increases CSK membrane abundance

(A-H) Cells were transfected with plasmids encoding FLAG tagged-CSK mutants and FLT3 or c-Kit. Six hours after transfection cells were serum starved for 18 hours and then stimulated or not with the respective ligand for 10 minutes before fixing. Fixed cells were permeabilized and stained with fluorophore-conjugated anti-FLAG and anti-FLT3/anti-c-Kit antibodies. Nuclei were stained with DAPI and subcellular localization was visualized with a Carl Zeiss LSM 710 Laser Scanning Microscope. (I, J) Colocalization was measured using CoLocalizer Pro 2.7.1.

Figure 3. CSK depletion did not alter receptor ubiquitination or phosphorylation:

(A) Ba/F3 cells were transfected with control siRNA or three different CSK siRNAs. Two days after transfection cells were lysed and cell lysates were subjected to Western blotting analysis. (B) Ba/F3-FLT3-WT cells were transfected with control siRNA or CSK siRNA. Two days after transfection cells were serum-starved for four hours before stimulation. Cells were then lysed and subjected to immunoprecipitation and Western blotting analysis. (C) Signal intensities from multiple blots of experiment "B" were quantified using Multi-Gauge software. (D) Ba/F3-c-Kit-WT cells were transfected with control siRNA or CSK siRNA. Two days after transfection cells were serum starved for four hours before stimulation. Cells were then lysed and subjected to immunoprecipitation and Western blotting analysis. (E) Signal intensities from multiple blots of experiment "D" were quantified using Multi-Gauge software. Data were analyzed with GraphPad Prism 5.0. ns, not significant.

Figure 4. CSK depletion increases Akt phosphorylation

Ba/F3-FLT3-WT (A) or Ba/F3-c-Kit-WT (B) cells were transfected with control siRNA or CSK siRNA. Two days after transfection cells were serum-starved for four hours before stimulation with the respective ligand. Ba/F3-FLT3-WT (C) or Ba/F3-c-kit-WT (D) cells were serum-starved for four hours and then treated with 10 μ M CSK inhibitor for 1 hour before ligand stimulation. Cells were then lysed and lysates were subjected to Western blotting analysis. Signal intensities from multiple blots of experiment were quantified using Multi-Gauge software and analyzed with GraphPad Prism 5.0.

Figure 5. CSK depletion differentially regulates Erk1/2 phosphorylation:

Ba/F3-FLT3-WT (A) or Ba/F3-c-Kit-WT (B) cells were transfected with scramble control siRNA or CSK siRNA. Two days after transfection cells were serum-starved for four hours before stimulation. Ba/F3-FLT3-WT (C) or Ba/F3-c-Kit-WT (D) cells were serum-starved for four hours and then treated with 10 μ M CSK inhibitor for 1 hour before stimulation. Cells were then lysed and lysates were subjected to Western blotting analysis with phospho-Erk and Erk antibodies. Signal intensities from multiple blots of experiment were quantified using Multi-Gauge software and analyzed with GraphPad Prism 5.0.

Figure 6. CSK depletion did not affect p38 phosphorylation

Ba/F3-FLT3-WT (A) or Ba/F3-c-Kit-WT (B) cells were transfected with control siRNA or CSK siRNA. Two days after transfection cells were serum-starved for four hours before stimulation with FL and SCF, respectively. Cells were then lysed and whole cell lysates were subjected to Western blotting analysis with phosphor-p38 and p38 antibodies. Signal intensities from multiple blots of experiment were quantified using Multi-Gauge software and analyzed with GraphPad Prism 5.0

Figure 7. CSK depletion increases SHC, Gab2 and SHP2 phosphorylation:

Ba/F3-FLT3-WT or Ba/F3-c-Kit-WT cells were transfected with control siRNA or CSK siRNA. Two days after transfection cells were serum-starved for four hours before stimulation. Cells were then lysed and subjected to immunoprecipitation and Western blotting analysis. Signal intensities from multiple blots of experiment were quantified using Multi-Gauge software and analyzed with GraphPad Prism 5.0. (A and B) Cell lysates were immunoprecipitated using anti-SHC antibody and membranes were probed with 4G10 and anti-SHC antibodies. Whole cell lysates were used for analysis and membranes were probed with (C and D) phospho-Gab2 and Gab2, and (E and F) phospho-SHP2 and SHP2.

Figure 8. CSK depletion increases cell proliferation but not cell survival:

Ba/F3-FLT3-ITD (A) or Ba/F3-c-Kit-D816V (B) cells were transfected with control siRNA or CSK siRNA and then cells were seeded in a 96-well plate. Cells were cultured with or without FL or SCF, respectively, for 46 hours followed by PrestoBlue cell proliferation assay. Ba/F3-FLT3-ITD cells (C) or Ba/F3-c-Kit-D816V cells (D) were transfected with control siRNA or CSK siRNA and then seeded in a 12-well plate. Cells were cultured with or without FL or SCF, respectively, for 48 hours followed by Annexin V/7AAD apoptosis assay.

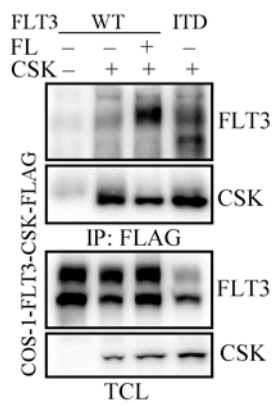
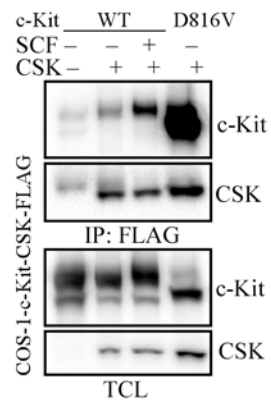
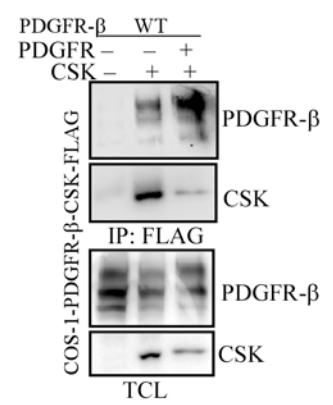
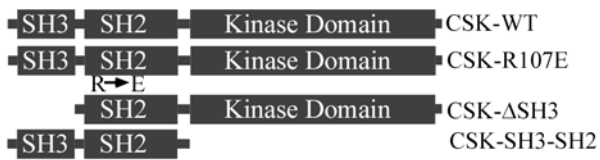
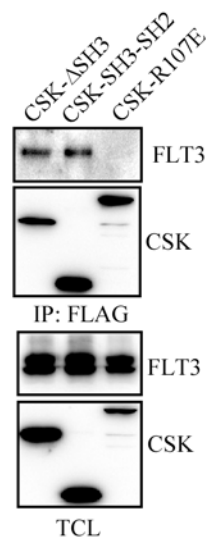
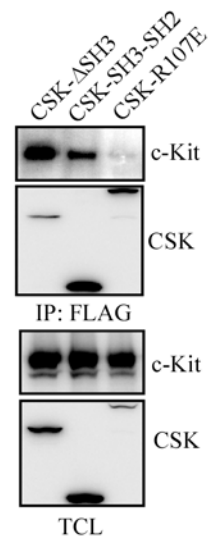
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Figure 1

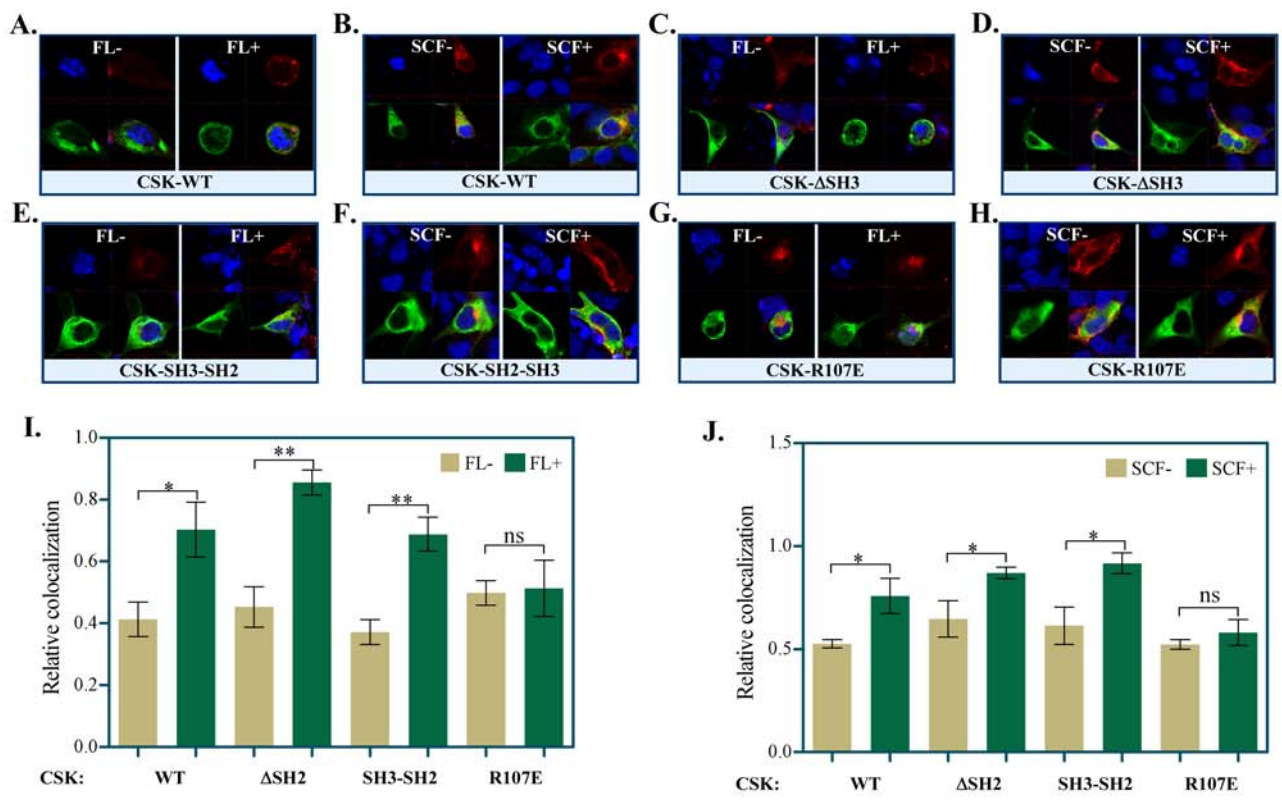


Figure 2

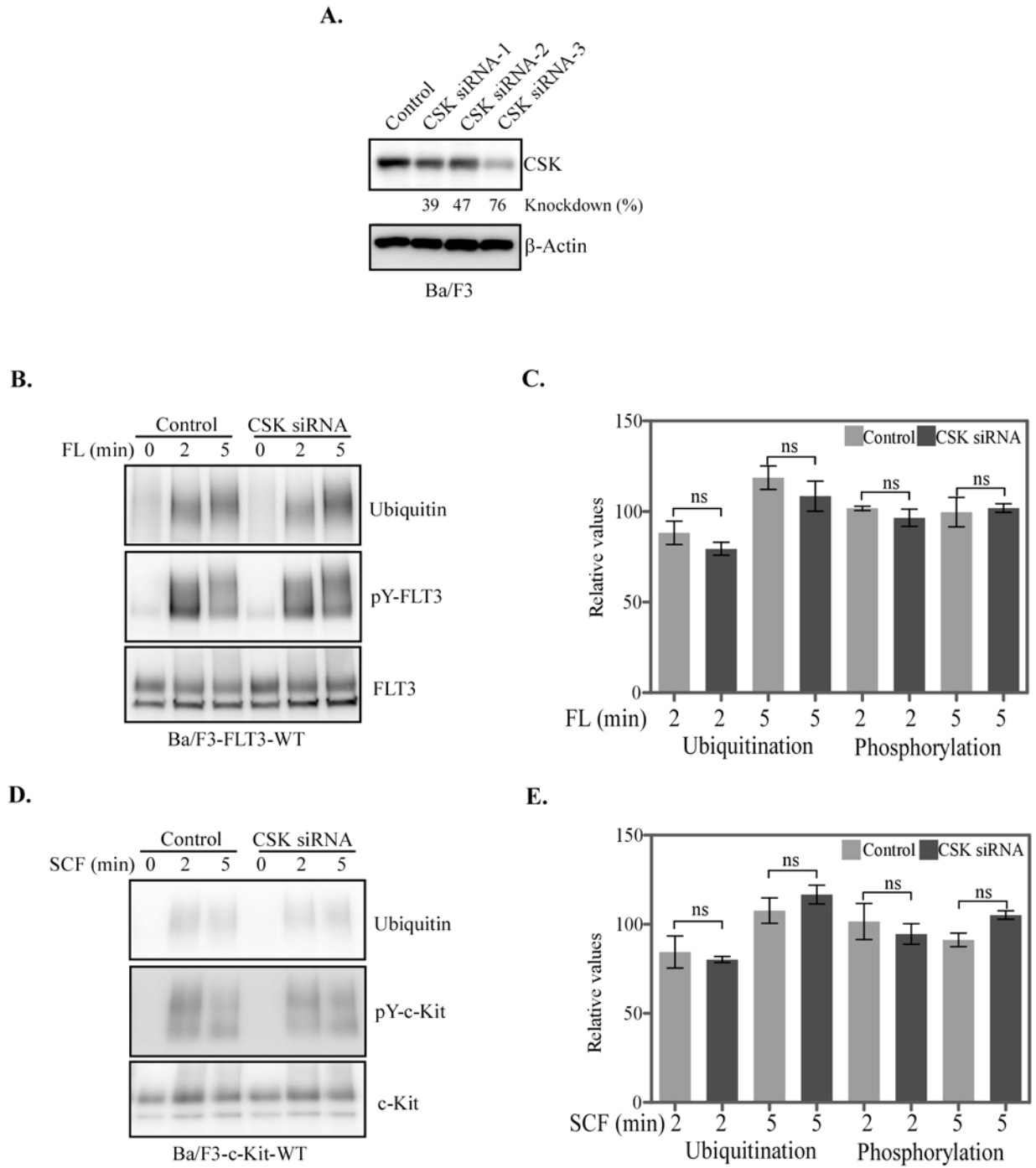


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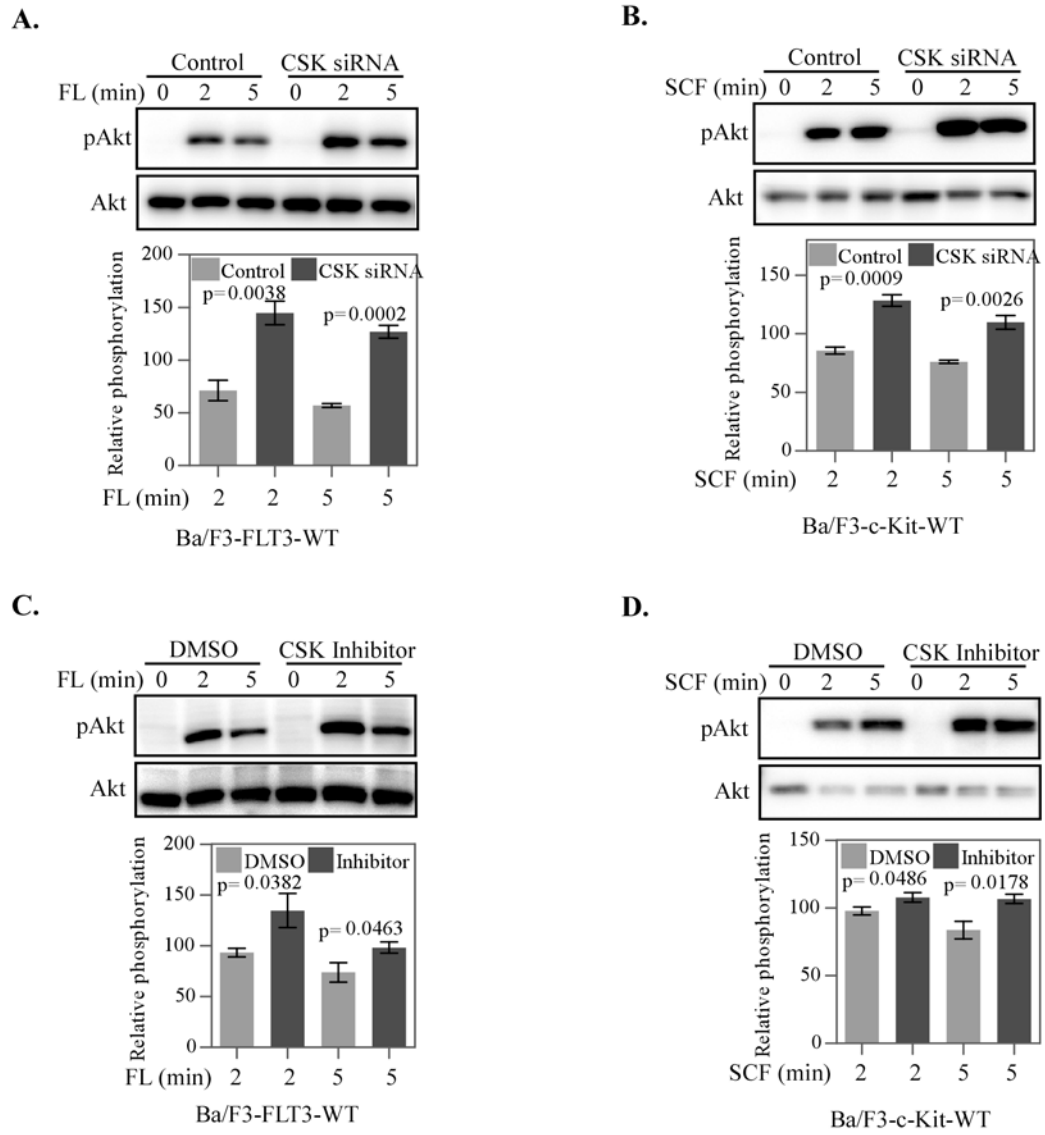


Figure 4

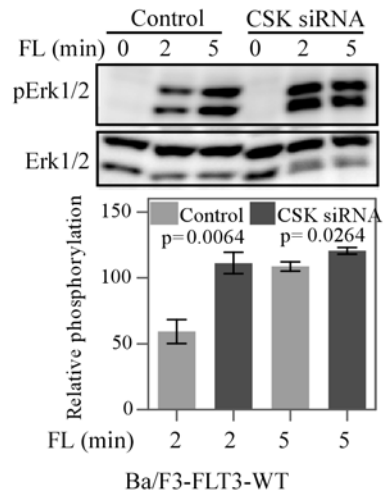
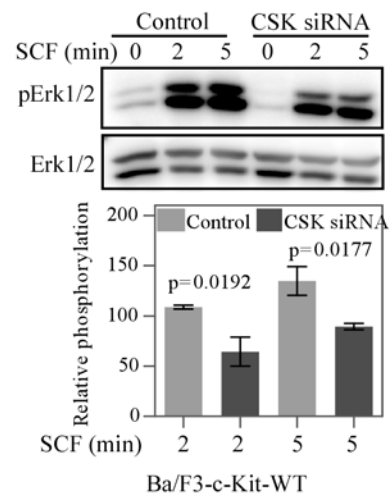
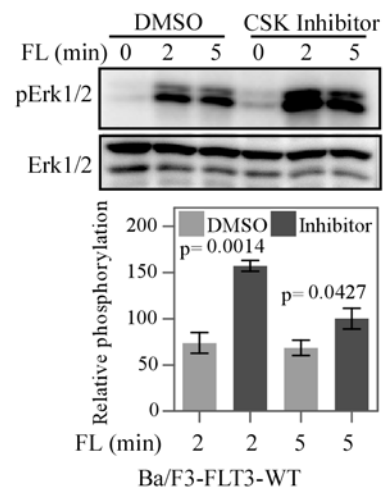
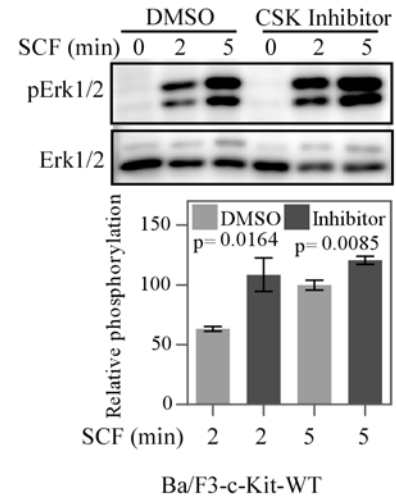
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Figure 5

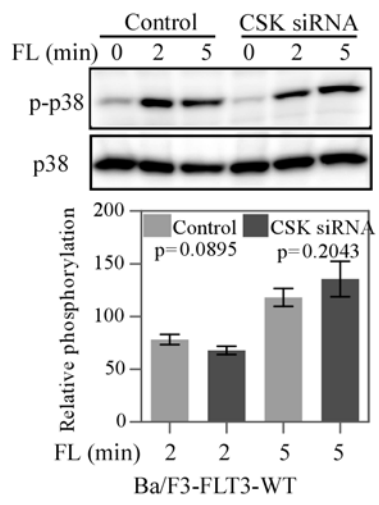
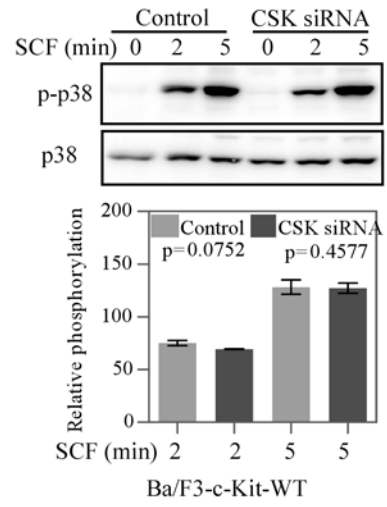
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Figure 6

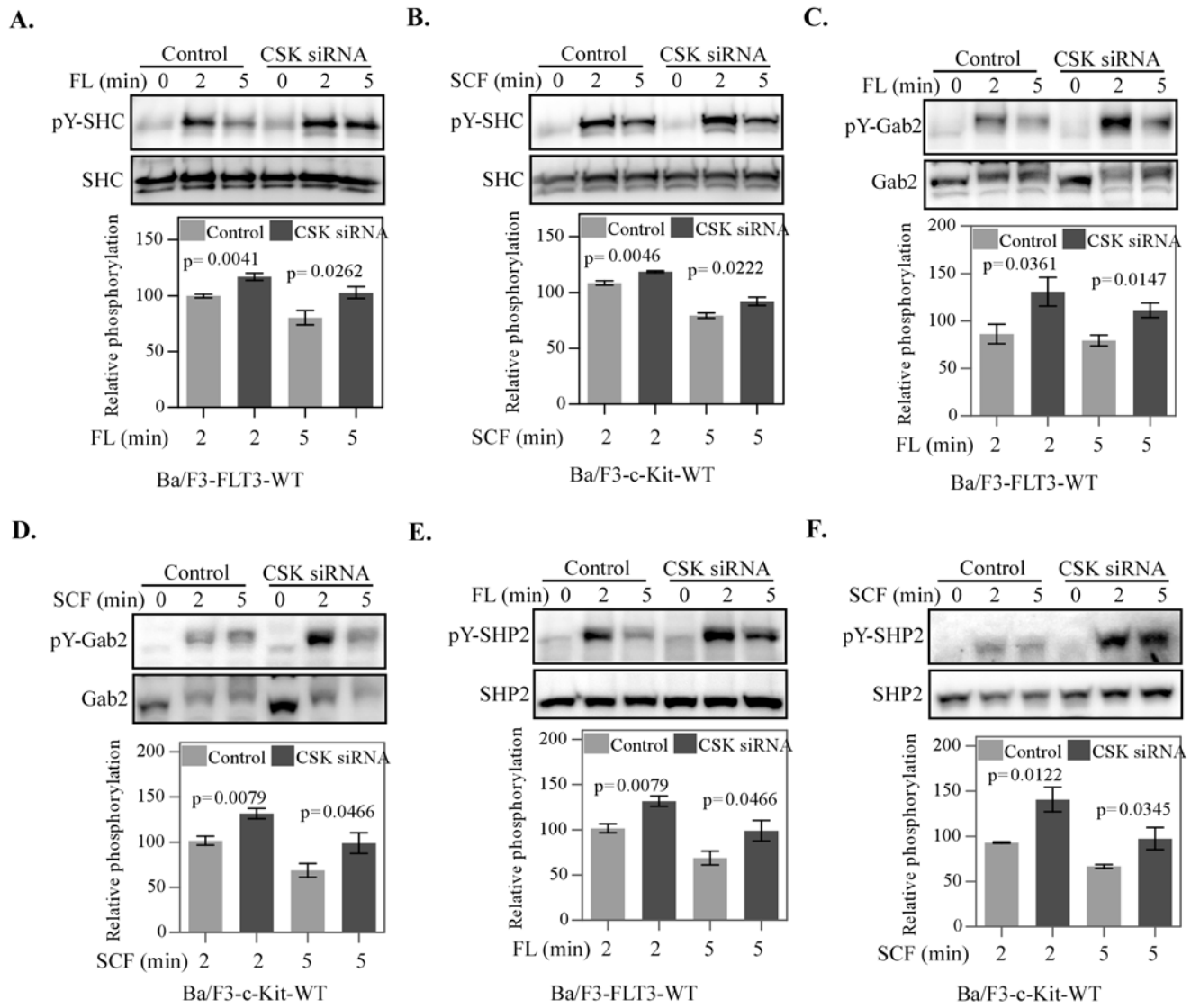


Figure 7

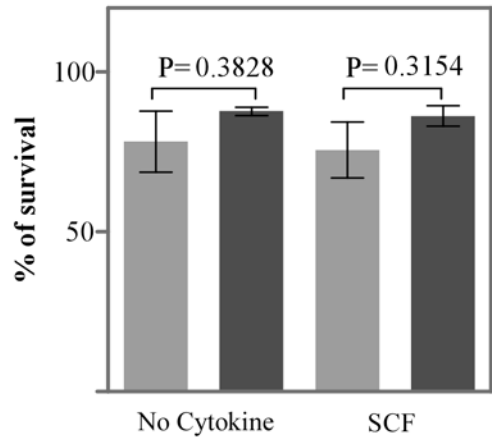
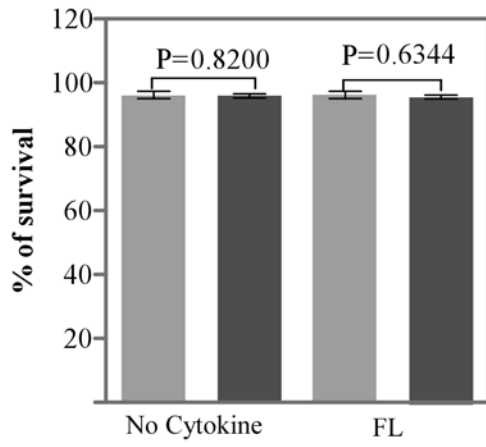
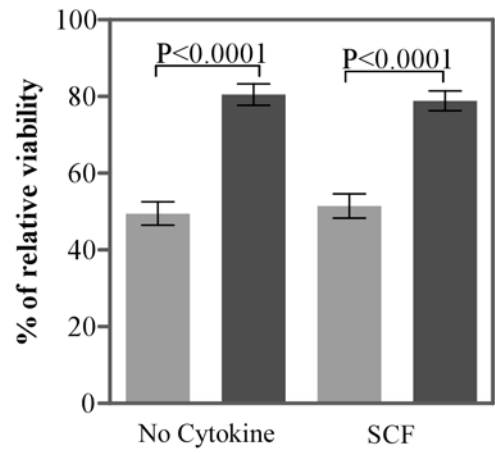
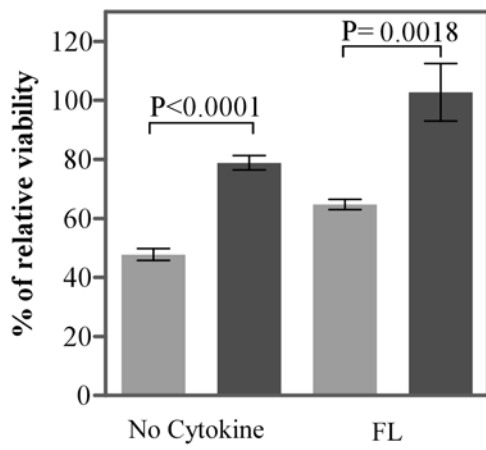


Figure 8