

# Signal regulatory protein $\alpha$ negatively regulates mast-cell activation following Fc $\epsilon$ RI aggregation

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Mast cells elicit allergic reaction through degranulation and release of proinflammatory mediators after aggregation of the IgE receptor Fc $\epsilon$ RI. Here we provide evidence to show that signal regulatory protein  $\alpha$  (SIRP $\alpha$ ), an ITIM-containing receptor, is an endogenous regulator of IgE-Ag induced mast-cell activation. SIRP $\alpha$  expression is promptly reduced in mast cells in response to Fc $\epsilon$ RI aggregation. Impaired expression of SIRP $\alpha$  in mast cells facilitates Fc $\epsilon$ RI-evoked degranulation and de novo synthesis of cytokines (IL-4, IL-13, IL-6, and TNF- $\alpha$ ). We further demonstrate that SIRP $\alpha$  knockdown in mast cells accelerates calcium mobilization and affects cytoskeletal rearrangement (F-actin disassembly and polymeric tubulin formation) after Fc $\epsilon$ RI aggregation. Mechanistic studies highlight the prolonged activation of NF- $\kappa$ B and MAPKs as well as PLC- $\gamma$  after Fc $\epsilon$ RI stimulation as a consequence of the inhibition of SIRP $\alpha$  expression in mast cells. Immunoprecipitation analysis shows that SIRP $\alpha$  knockdown markedly increases IgE-induced SHP2 interaction with PI3K regulatory subunit PI3Kp85 or IKK- $\beta$  in mast cells, indicating that SIRP $\alpha$  may accomplish this through its association and sequestration of SHP2. Collectively, our results strongly indicate that SIRP $\alpha$  is a biological important regulator of Fc $\epsilon$ RI signaling.

**Keywords:** Allergy · Cytokine production · Degranulation · Mast cell · Signal regulatory protein  $\alpha$



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## Introduction

Mast cells are important immune effector cells and play a central role in IgE-dependent allergic disease. Fc $\epsilon$ RI aggregation contributes to inflammatory symptoms by inducing the release of proinflammatory mediators and the production of various cytokines. Upon crosslinking by IgE-allergen, the Fc $\epsilon$ RI  $\beta$  and  $\gamma$

subunits are phosphorylated at ITAMs by the receptor-proximal Src family protein tyrosine kinases (SFKs), such as Lyn, Fyn, and Hck [1, 2]. Activated Fc $\epsilon$ RI induces a large number of downstream molecules activation, such as phosphatidylinositol 3'-kinase (PI3K)-Akt kinase [3, 4], phospholipase C $\gamma$  (PLC- $\gamma$ ) [5], mitogen-activated protein kinases (MAPKs) [3], and nuclear factor  $\kappa$ B (NF- $\kappa$ B) [2], resulting in enhanced calcium flux, prominent

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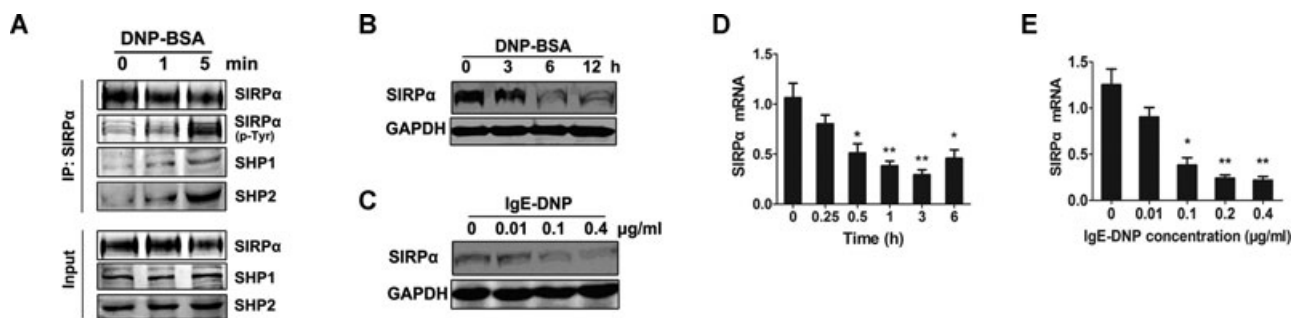


**Figure 1.** Ag stimulation induces SIRP $\alpha$  reduction in mast cells. (A) BM-derived mast cells (BMMCs) were sensitized with 0.4  $\mu$ g/mL anti-DNP IgE and then stimulated with 20 ng/mL DNP-BSA for the indicated times. Cell lysates were immunoprecipitated with anti-SIRP $\alpha$  Ab and blotted with pTyr, SIRP $\alpha$ , SHP1, and SHP2 Abs. Data shown are from one experiment representative of three independent experiments. (B) BMMCs were activated with IgE (0.4  $\mu$ g/mL) and DNP-BSA (20 ng/mL) stimulation for the indicated times, and SIRP $\alpha$  protein expression was determined by immunoblotting. GAPDH was detected as a loading control. One experiment representative of three independent experiments is shown. (C) BMMCs were incubated with different dosage of anti-DNP IgE for 2 h and then stimulated with DNP-BSA (20 ng/mL) for 12 h. The expression of SIRP $\alpha$  was detected. GAPDH was detected as a loading control. Data shown are from one experiment representative of four independent experiments. (D, E) BMMCs were activated (D) for the indicated times or (E) by the indicated dosage of IgE and SIRP $\alpha$  mRNA expression was determined by real-time PCR. Data are shown as mean + SEM of four independent experiments each measured triplicates. \* $p$  < 0.05; \*\* $p$  < 0.01, Mann-Whitney test.

degranulation and increased synthesis of various cytokines. However, the precise mechanism involved in the regulation of this process is still not fully understood.

Appropriate activation of mast cells is mediated by several factors, including the ability of the cells to distinguish activating or inhibitory stimuli as well as the strength and duration of a given stimulus [6, 7]. Several negative intracellular regulators modulate the signaling events initiated by Fc $\epsilon$ RI in mast cells, including the SH2-containing tyrosine phosphatases 1 (SHP1) [8], the lipid phosphatase SH2-containing inositol 5' phosphatase 1 (SHIP1) [9], and the E3 ubiquitin ligase c-Cbl [10]. In addition, once coaggregated with their ligands, cell surface receptors that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) provide inhibitory signals to dampen those Fc $\epsilon$ RI-initiated events [11]. Through their ITIMs, these receptors suppress cell activation by promoting dephosphorylation of key molecules via SHP1, SHP2, or the SHIP family [12].

Signal regulatory protein  $\alpha$  (SIRP $\alpha$ ), also known as Src homology-containing phosphotyrosine phosphatase substrate (SHPS), is a member of the Ig-like receptor superfamily proteins. SIRP $\alpha$  is especially abundant in innate immune cells including mast cells [13]. The putative extracellular region of SIRP $\alpha$  possesses three Ig-like domains with multiple N-linked glycosylation sites. The cytoplasmic region of SIRP $\alpha$  contains two ITIMs with four tyrosine residues, which are phosphorylated in response to a variety of growth factors and integrin-mediated cell adhesion [14]. This phosphorylation enables recruitment of SHP2 [15, 16]. SHP2, a widely expressed cytoplasmic tyrosine phosphatase with two SH2 domains, has been reported to be involved in growth factor-induced cell proliferation, probably through activation of the Ras-MAPK cascade [17]. The SIRP $\alpha$ /SHP2 complex could negatively or positively regulate intracellular signaling initiated either by tyrosine kinase-coupled receptors for growth factors or by cell adhesion to extracellular matrix proteins. Moreover, dominant-negative form of SIRP $\alpha$  makes cells resistant to TNF-induced apoptosis through activation of NF- $\kappa$ B [18]. A previous publi-

cation has demonstrated that coaggregation of the intracellular region of SIRP $\alpha$  with Fc $\epsilon$ RI inhibits immunoreceptor-dependent mast-cell activation [19]. However, the functional properties of native SIRP $\alpha$  molecule in mast cells have not been fully examined.

In the present study, we show that SIRP $\alpha$  negatively regulates Fc $\epsilon$ RI-evoked signaling in mast cells. We found that SIRP $\alpha$  expression was quickly reduced in mast cells upon Fc $\epsilon$ RI aggregation. Furthermore, SIRP $\alpha$  knockdown in mast cells promoted Fc $\epsilon$ RI-induced degranulation and cytokine production.

## Results

### Fc $\epsilon$ RI aggregation induces SIRP $\alpha$ reduction in mast cells

To examine whether SIRP $\alpha$  is involved in the regulation of Fc $\epsilon$ RI-mediated mast cells activation, WT BM-derived mast cells (BMMCs) were sensitized with anti-DNP IgE and then stimulated with DNP-BSA. This stimulation led to rapid tyrosine phosphorylation of SIRP $\alpha$  within 5 min. Meanwhile, the SH2-containing tyrosine phosphatases, SHP1 and SHP2, were found to be recruited to SIRP $\alpha$  (Fig. 1A). As shown in Figure 1B, Fc $\epsilon$ RI-mediated BMMCs activation induced a time-dependent reduction of SIRP $\alpha$  expression. This reduction persisted for at least 12 h. Moreover, anti-DNP IgE sensitization at 0.1  $\mu$ g/mL resulted in a significant decrease of SIRP $\alpha$  expression in response to DNP-BSA stimulation (Fig. 1C). The rapid reduction of SIRP $\alpha$  expression was further confirmed by real-time PCR analysis (Fig. 1D and E). Interestingly, the SIRP $\alpha$  mRNA level quickly diminished during mast-cell activation, occurring within 30 min (Fig. 1D). Taken together, the Fc $\epsilon$ RI-induced decrease in SIRP $\alpha$  expression suggests its role as a regulator of Fc $\epsilon$ RI signaling.

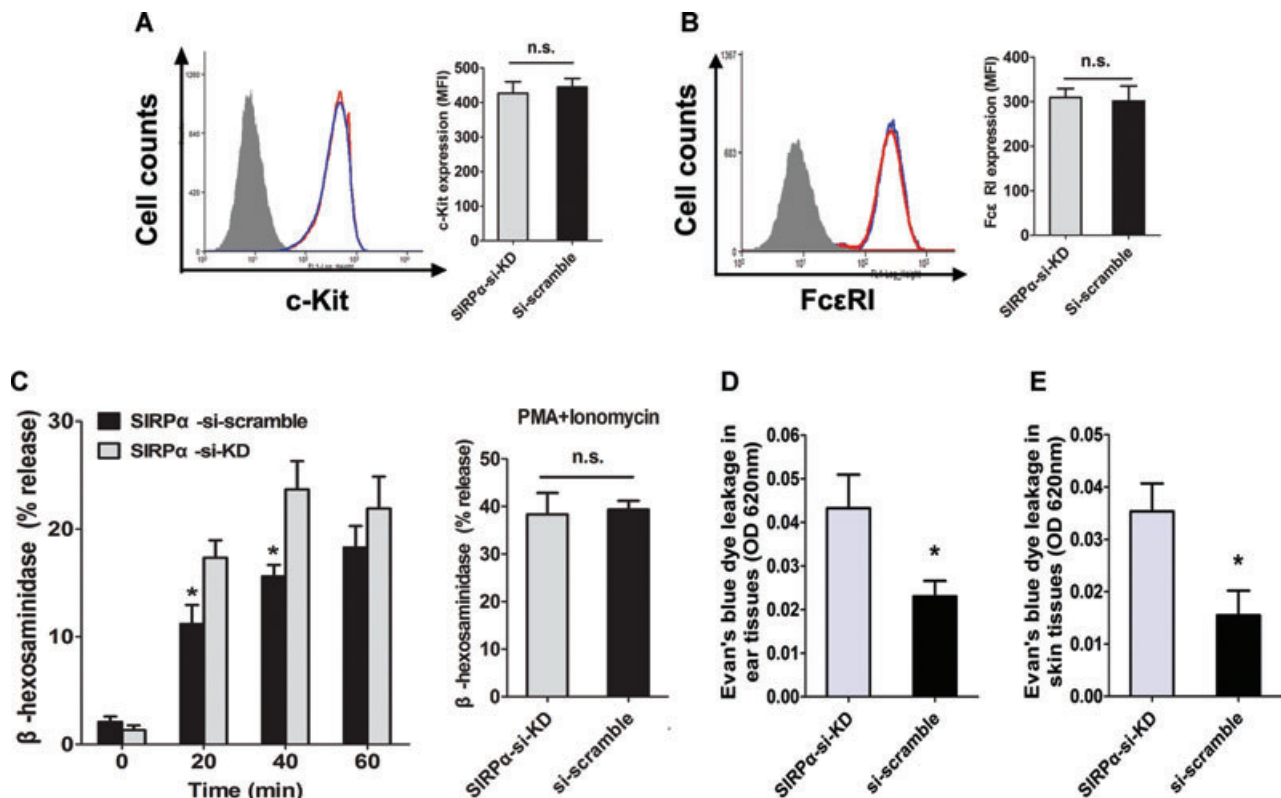


**Figure 2.** SIRP $\alpha$  negatively regulates Fc $\epsilon$ RI-mediated mast-cell degranulation. (A) c-Kit and (B) Fc $\epsilon$ RI expression on SIRP $\alpha$ -si-KD (blue line) and si-scramble (red line) mast cells was detected by flow cytometry with specific Abs or isotype control (gray). All the viable cells were gated as shown in Supporting Information Fig. 1B. The MFI of c-Kit and Fc $\epsilon$ RI expression is shown as mean + SEM of three samples pooled from four independent experiments. (C) Degranulation assay. SIRP $\alpha$ -si-KD and si-scramble BM-derived mast cells (BMMCs) were stimulated with 20 ng/mL DNP (left) or PMA plus ionomycin (right) and the capacity of degranulation was measured by  $\beta$ -hexosaminidase release. Data are shown as mean + SEM of four samples pooled from three experiments. (D, E)  $W^{w-sh}/W^{w-sh}$  mice were reconstituted with IgE-sensitized SIRP $\alpha$ -KD or control BMMCs by intradermally injecting into (D) ear and (E) dorsal skin tissue for 24 h. The mice were then challenged with an intravenous injection of DNP-BSA in PBS containing 1% Evans blue; 30 min later, the mice were killed and the dye was extracted and measured at 620 nm. Data are shown as mean + SEM of four to six mice per group pooled from two experiments. The results were analyzed by Mann-Whitney test; \* $p < 0.05$ ; n.s. no significant difference.

### SIRP $\alpha$ negatively regulates Fc $\epsilon$ RI-mediated mast-cell degranulation

To test the role of SIRP $\alpha$  in Fc $\epsilon$ RI signaling, BMMCs were transfected with SIRP $\alpha$ -siRNA (SIRP $\alpha$ -si-KD) or scramble-siRNA (si-scramble), the cells were collected and assayed for SIRP $\alpha$  expression. A significant reduction of SIRP $\alpha$  expression was observed (Supporting Information Fig. 1A and B). To assess whether SIRP $\alpha$  could impair c-Kit or Fc $\epsilon$ RI expression on mast cells, flow cytometry analysis was performed on SIRP $\alpha$ -si-KD and SIRP $\alpha$ -si-scramble cells. No obvious difference was observed between the two cells (Fig. 2A and B). To examine whether SIRP $\alpha$  is involved in Fc $\epsilon$ RI-mediated mast-cell degranulation, SIRP $\alpha$ -si-KD and si-scramble BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-BSA for 1 h. Mast-cell degranulation was measured by the release of  $\beta$ -hexosaminidase. As shown in Figure 2C,  $\beta$ -hexosaminidase release levels were elevated by 68% and 42% in SIRP $\alpha$ -KD BMMCs at 20 and 40 min compared with those in control group, respectively. In contrast, degranulation induced by 12-O-tetradecanoylphorbol-13-acetate (PMA)

plus ionomycin remained at a similar level between the two cells (Fig. 2C). This result implies that the secretion machinery is normal in SIRP $\alpha$  knockdown cells and that the enhanced degranulation is attributable to the inhibition of SIRP $\alpha$  expression. Moreover, rat basophil RBL-2H3 cells with SIRP $\alpha$  stable overexpression (SIRP $\alpha$ -OV) were established (Supporting Information Fig. 1C and D) and diminished  $\beta$ -hexosaminidase release was found in these SIRP $\alpha$ -OV cells (Supporting Information Fig. 2A). These data indicate that SIRP $\alpha$  plays a negative role in Fc $\epsilon$ RI-mediated mast-cell degranulation in vitro. To understand whether SIRP $\alpha$  plays any roles in Fc $\epsilon$ RI-evoked degranulation in vivo, a passive cutaneous anaphylaxis (PCA) mouse model was employed. In this model, SIRP $\alpha$ -si-KD or si-scramble BMMCs were sensitized with anti-DNP IgE and intradermally injected into ear tissues or dorsal skin of mast-cell-deficient mice ( $W^{w-sh}/W^{w-sh}$ ) for 24 h, following an i.v. challenge of DNP-BSA in 1% Evans blue dye solution. Thirty minutes later, the tissues were collected and the Evans blue dye was extracted and measured to access the vascular permeability, a process dependent on histamine and serotonin secreted by mast cell. As expected, the amount of dye in the ear or skin tissues

was dramatically elevated in the mice receiving SIRP $\alpha$ -KD BMMCs compared with mice injected with si-scramble BMMCs, indicating an important role of SIRP $\alpha$  in mast-cell-mediated immediate-phase PCA reaction (Fig. 2D and E). Taken together, these results demonstrate that SIRP $\alpha$  is critical in regulating IgE-mediated mast-cell degranulation.

### Knockdown of SIRP $\alpha$ alters cytoskeletal rearrangement and calcium mobilization

Cytoskeletal rearrangements, including cortical F-actin disassembly and polymeric tubulin formation, are important events in Fc $\epsilon$ RI-mediated mast-cell degranulation [20, 21]. As shown in Figure 3A, Fc $\epsilon$ RI-induced formation of polymeric tubulin was enhanced in SIRP $\alpha$ -si-KD BMMCs. In accordance with this, overexpression of SIRP $\alpha$  in RBL-2H3 cells retarded the microtubule polymerization (Supporting Information Fig. 2B). To test whether SIRP $\alpha$  affects F-actin cytoskeletal dynamics, confocal microscope was used to visualize changes of cortical F-actin in BMMCs. IgE sensitization induced similar organization of cortical F-actin ring in SIRP $\alpha$ -si-KD and si-scramble BMMCs, while DNP-BSA stimulation destroyed this structure. However, SIRP $\alpha$  knockdown accelerated this process (Fig. 3B). These results indicate that SIRP $\alpha$  modulates IgE-induced mast-cell cytoskeletal rearrangement.

Since intracellular calcium is essential in mast-cell cytoskeleton rearrangement and degranulation [20], we then examined whether SIRP $\alpha$  modulates the calcium release from intracellular stores in allergen-activated mast cells. IgE-primed SIRP $\alpha$ -si-KD and si-scramble BMMCs were seeded in the calcium-free medium and preloaded with Fluo-3 AM, followed by stimulation with DNP-BSA. Compared with the control BMMCs, impaired expression of SIRP $\alpha$  displayed relative higher intracellular Ca<sup>2+</sup> in response to Fc $\epsilon$ RI aggregation (Fig. 3C). Furthermore, the peak and duration of intracellular Ca<sup>2+</sup> was significantly diminished in SIRP $\alpha$ -OV RBL-2H3 cells after activation in calcium-free medium (Supporting Information Fig. 2C). These results demonstrate that SIRP $\alpha$  negatively regulates Fc $\epsilon$ RI-mediated intracellular calcium release. Consistent with these data, DNP-BSA induced PLC- $\gamma$  (PLC- $\gamma$ 1 and PLC- $\gamma$ 2) activation, which is responsible for IP3-dependent calcium release from the endoplasmic reticulum, was dramatically enhanced in SIRP $\alpha$ -si-KD BMMCs than control (Fig. 3D). Meanwhile, Rac1 activity was increased in SIRP $\alpha$ -si-KD cells (Fig. 3E). Since Rac1 exerts its effect on calcium mobilization through regulation of PLC- $\gamma$  activity and calcium release from the intracellular stores, the above results indicate that SIRP $\alpha$  regulates the calcium mobilization partly through altering the PLC- $\gamma$  and Rac1 activation.

### SIRP $\alpha$ knockdown alters Fc $\epsilon$ RI-mediated cytokine production pattern in mast cell

Mast-cell-derived cytokines contribute to Fc $\epsilon$ RI-mediated late-phase allergic reactions. To evaluate the effect of SIRP $\alpha$  on

cytokine production in BMMCs, SIRP $\alpha$ -si-KD as well as si-scramble mast cells were immunologic activated and the supernatants were collected at various time points. As expected, IL-4, IL-13, IL-6, and TNF- $\alpha$  production were significantly elevated in scramble-BMMCs in response to DNP-BSA stimulation. However, knockdown of SIRP $\alpha$  expression resulted in 0.5- to 1.2-fold higher levels of these cytokines compared with control (Fig. 4A–D). Moreover, overexpression of SIRP $\alpha$  impaired synthesis of cytokine transcripts in RBL-2H3 cells during Fc $\epsilon$ RI-aggregation (Supporting Information Fig. 3A–C). Collectively, these data demonstrate that SIRP $\alpha$  negatively regulates cytokine production in mast cells.

### SIRP $\alpha$ negatively regulates IgE induced NF- $\kappa$ B and NFAT activation as well as MAPK pathway

NF- $\kappa$ B and NFAT are two important mediators for mast-cell production and remodeling the cytokine milieu at the inflammation sites [22, 23]. An immunoblotting assay showed that knockdown of SIRP $\alpha$  expression in BMMCs notably facilitated IgE-induced phosphorylation of I $\kappa$ B- $\alpha$  and NF- $\kappa$ B p65 (Fig. 5A). The dephosphorylation status of NFAT subunit NFATc2 (the activation form) was also enhanced in SIRP $\alpha$ -KD BMMCs after Fc $\epsilon$ RI stimulation, consistent with a more quick aggregation of NFATc2 in the nuclear fraction (Fig. 5A and B). Moreover, a luciferase-reporter assay showed that the activities of NF- $\kappa$ B and NFAT were reduced in SIRP $\alpha$ -OV RBL-2H3 cells (Supporting Information Fig. 4A and B). These results demonstrate a critical role of SIRP $\alpha$  in regulating NF- $\kappa$ B and NFAT activation during Fc $\epsilon$ RI-aggregation. Given that NF- $\kappa$ B promotes IL-6 and TNF- $\alpha$  expression [23] and NFAT enhances IL-13 production [24] in mast cells, the increased activation of NF- $\kappa$ B and NFAT in SIRP $\alpha$ -KD BMMCs may contribute to the excessive production of these cytokines. The activation of MAPKs is a remarkable event responsible for production of many types of cytokines [25]. Our results showed that impaired expression of SIRP $\alpha$  dramatically enhanced activation of ERK, JNK, and p38 in response to Fc $\epsilon$ RI crosslinking (Fig. 5C).

### SIRP $\alpha$ negatively regulates Fc $\epsilon$ RI signaling through sequestration of SHP2 from PI3Kp85 and IKK $\beta$

To evaluate whether activation of Fc $\epsilon$ RI is affected by SIRP $\alpha$ , immunoprecipitation assay was performed on the lysates of activated SIRP $\alpha$ -si-KD and si-scramble mast cells with the Ab against phosphotyrosine (4G10). Immunoblotting assay showed that the levels of tyrosine phosphorylation of Fc $\epsilon$ RI  $\gamma$  chains were similar between the two cells (Supporting Information Fig. 5A). The kinase Lyn is reported to involve in tyrosine phosphorylation of Fc $\epsilon$ RI. Further work showed that the activation of Lyn in KD cells was equivalent to that in control cells (Supporting Information Fig. 5B). The above-mentioned results reveal that SIRP $\alpha$  does not impair Fc $\epsilon$ RI activation at least during the early stage.

The results had revealed a negative role of SIRP $\alpha$  in regulating PLC- $\gamma$  activation, which inspired us to further detect the

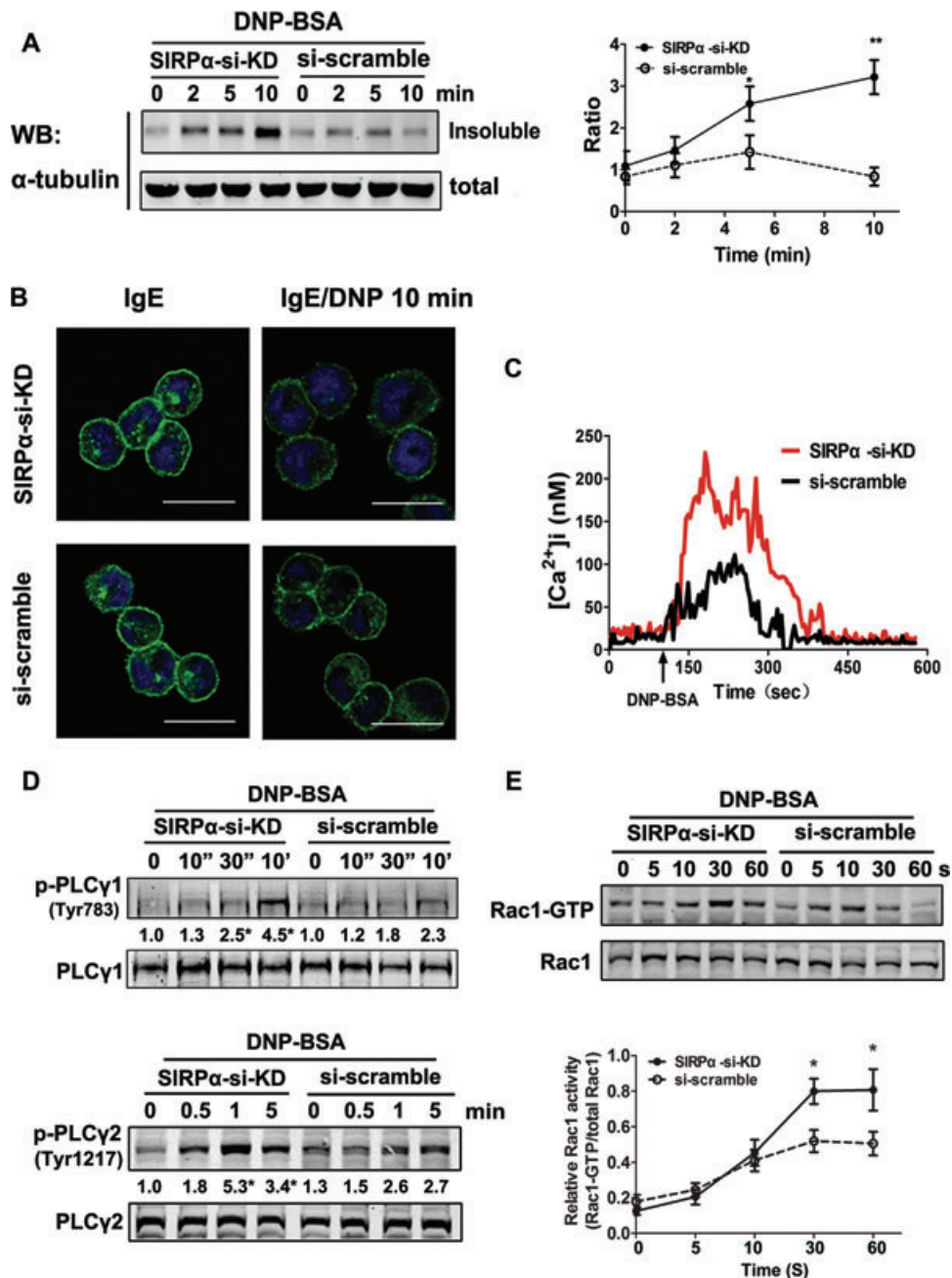


**Figure 3.** Impaired expression of SIRPα alters cytoskeletal rearrangement and intracellular calcium release. (A) SIRPα-KD or si-scramble BM-derived mast cells (BMMCs) were sensitized with IgE and stimulated with 20 ng/mL DNP-BSA for the indicated times. The cells were lysed in 0.1% Triton X-100 and the insoluble fraction was subjected to SDS-PAGE with anti-α-tubulin Ab. Images shown are from one experiment representative of three individual experiments (left). The data of the relative ratio (intensity of poly-MT versus intensity of total tubulin in unstimulated cells) are collected from the same experiments and showed as mean ± SEM (right). (B) IgE-sensitized SIRPα-KD and si-scramble BMMCs were stimulated with DNP-BSA (20 ng/mL) for 10 min. The cells were then fixed and stained with Alexa Fluor 488 phalloidin and DAPI. Representative images by confocal microscopy of four experiments are shown (magnification 60×; scale bar 10 μm). (C) BMMCs were stained with Fluo-3 AM in the calcium-free HBSS. The kinetics of intracellular Ca<sup>2+</sup> after treated with DNP-BSA was continuously monitored with a laser scanning confocal microscope. Results presented by the mean values of more than 20 cells in every experiment are shown. Each panel is representative of two independent experiments. (D) IgE-primed SIRPα-si-KD and si-scramble BMMCs were activated with DNP-BSA for the indicated times. Cell lysates were subjected to immunoblotting with anti-phospho-PLC-γ1 and anti-phospho-PLC-γ2 Abs. Total amounts of PLC-γ1 and PLC-γ2 were detected as the loading controls. The relative intensity of each sample is shown as the mean values of three experiments. (E) Activated SIRPα-si-KD or si-scramble BMMCs were lysed and centrifuged. The activation form of Rac1 (GTP-bound) was detected. Total amounts of Rac1 were measured as the loading control. One representative of four experiments is shown. The relative ratio (Rac1-GTP versus total Rac1 in unstimulated cells) of the experiments is shown as mean ± SEM. \**p* < 0.05; \*\**p* < 0.01, Mann-Whitney test.

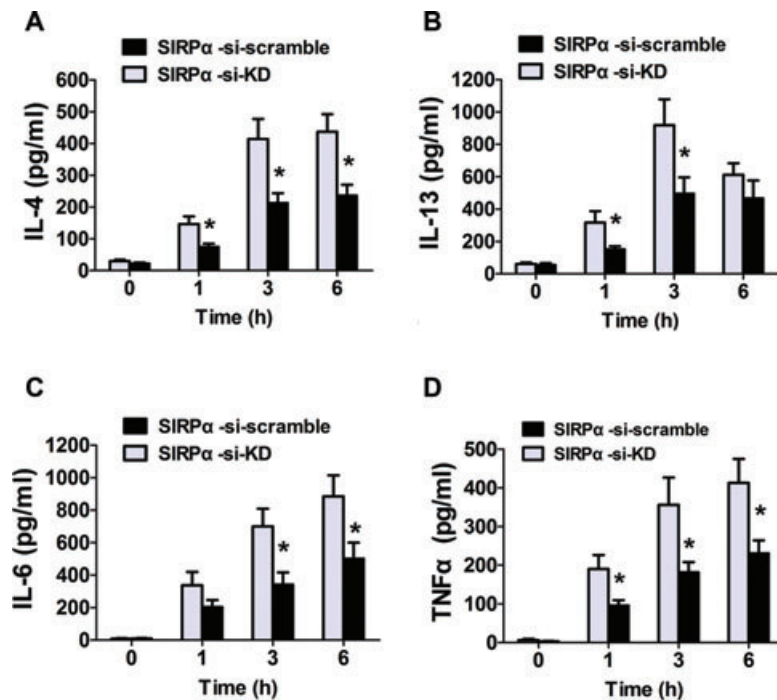


**Figure 4.** SIRP $\alpha$  is involved in the regulation of cytokine production in mast cells. (A–D) The production of (A) IL-4, (B) IL-13, (C) IL-6, and (D) TNF- $\alpha$  by SIRP $\alpha$ -si-KD or si-scramble BM-derived mast cells (BMMCs) was measured by ELISA in the cell-free supernatant after Fc $\epsilon$ RI-stimulation. Data are shown as mean + SEM of three samples pooled from five separate experiments. \* $p < 0.05$ , Mann-Whitney test.

activity of another PI3K downstream key molecule, Akt. Compared with the control BMMCs, SIRP $\alpha$  knockdown significantly increased Akt activation in response to allergen stimulation (Fig. 6A). In addition, GSK-3 $\beta$ , whose activity was regulated by Akt and could modulate NFAT activation, also displayed an inactivation form (p-GSK-3 $\beta$ ) in SIRP $\alpha$ -KD BMMCs (Fig. 6A). Taken together, these findings indicate that SIRP $\alpha$  negatively regulates PI3K-Akt signaling pathway in mast cells upon Fc $\epsilon$ RI aggregation. Moreover, we found that tyrosine phosphorylation of SIRP $\alpha$  increased its association with PI3Kp85 as well as SHP2 (Fig. 6B). To determine whether SHP2 is involved in Fc $\epsilon$ RI signaling pathway in mast cells, immunoprecipitation assay was performed. DNP-BSA treatment markedly increased association of SHP2 with PI3Kp85 in SIRP $\alpha$ -KD BMMCs but not in the control cells (Fig. 6C). Although SIRP $\alpha$  did not interact with IKK $\beta$  during mast-cell activation (Supporting Information Fig. 5C), SIRP $\alpha$  knockdown drastically enhanced IgE-induced SHP2-IKK $\beta$  association (Fig. 6C). In line with this, mast cells with SHP2 expression transiently knockdown displayed reduced activation of NF- $\kappa$ B pathway upon Fc $\epsilon$ RI aggregation (Supporting Information Fig. 5D). These results strongly suggest that SIRP $\alpha$  competes with PI3Kp85 and IKK- $\beta$  to bind SHP2, thus indicating the essential role of SHP2 in Fc $\epsilon$ RI signaling activation.

## Discussion

Our studies identify that SIRP $\alpha$  functions as a negative regulator of IgE-induced mast-cell activation and allergic reaction. The physiological importance of SIRP $\alpha$  as a negative regulator of IgE-mediated mast-cell activation was suggested by the enhancement

of degranulation and cytokine production of SIRP $\alpha$ -KD BMMCs and more severe PCA response in mice reconstructed with SIRP $\alpha$ -KD mast cells. Mechanistic analysis revealed that activation of SIRP $\alpha$  resulted in the sequestration of SHP2 from PI3Kp85 and IKK- $\beta$ , leading to disrupt the interacting of SHP2 with PI3Kp85 and IKKs complex.

Mast-cell degranulation and release of the inflammatory mediators is the major cause of immediate-phase of allergic reaction. We find that SIRP $\alpha$  negatively regulates mast-cell degranulation after Fc $\epsilon$ RI crosslinking. Several studies have demonstrated the importance of cytoskeletal rearrangements in mast-cell degranulation. Both the calcium-dependent F-actin disassembly and the calcium-independent polymeric microtubule formation are detected upon DNP-BSA stimulation in mast cells [20]. In our studies, SIRP $\alpha$  not only regulates F-actin rearrangement but also affects polymeric microtubule formation upon Fc $\epsilon$ RI engagement. Recent studies have provided evidence that at least two complementary signaling pathways are involved in Fc $\epsilon$ RI-induced calcium mobilization, one of which activates PLC- $\gamma$  and the other of which activates PI3K, using specific transmembrane and cytosolic adaptor molecules [26]. In our study, we found that phosphorylation of PLC- $\gamma$ 1 and PLC- $\gamma$ 2 were both enhanced in SIRP $\alpha$ -si-KD mast cells upon Fc $\epsilon$ RI aggregation, together with elevated degranulation capacity. Moreover, PI3K activation might increase calcium mobilization by activation of sphingosine kinase (SK) through phospholipase D (PLD), resulting in the formation of sphingosine 1-phosphate (S1P) [27]. We have found that SIRP $\alpha$  sequesters SHP2 from PI3K regulatory subunit PI3Kp85, leading to reduced activation of PI3K. These results indicate that SIRP $\alpha$  may involve in S1P-mediated signaling, but it remains further evidences to prove.

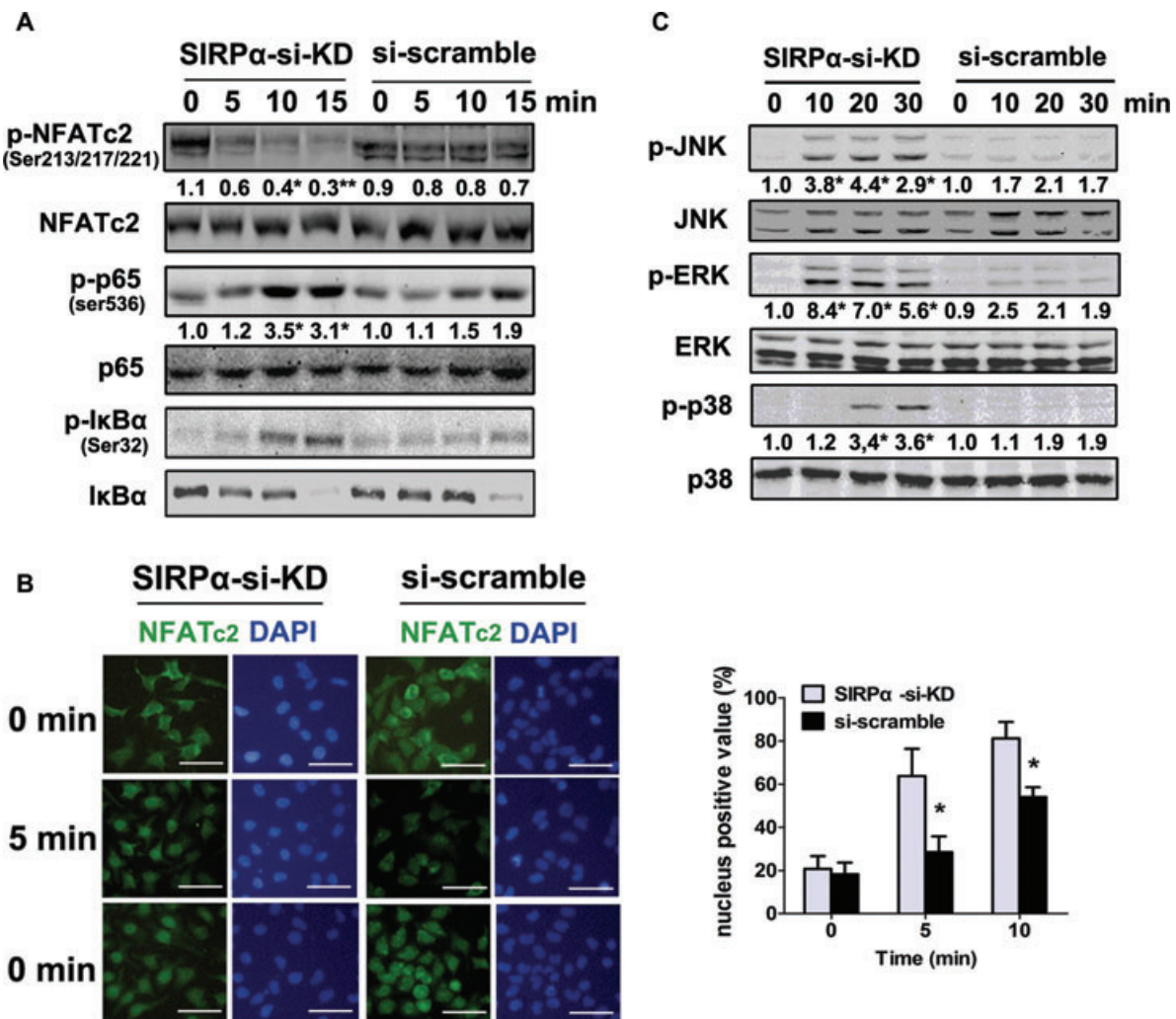


**Figure 5.** SIRP $\alpha$  negatively regulates IgE induced NF- $\kappa$ B and NFAT activation as well as MAPK signaling pathway in mast cells. (A) Cell lysates from activated BM-derived mast cells (BMMCs) were subjected to SDS-PAGE and immunoblotted by the indicated Abs. Total amounts of NFATc2 and p65 were detected as loading controls. The relative intensity of NFATc2 and NF- $\kappa$ B phosphorylation is shown as the mean value of three independent experiments. (B) Immunostaining of NFATc2 (green) and nucleus (DAPI, blue) of activated SIRP $\alpha$ -si-KD or si-scramble BMMCs. Images shown are from one experiment representative of three experiments (magnification 40 $\times$ , scale bar 50  $\mu$ m). The proportion of cells with NFATc2 nuclear-positive staining is shown as mean  $\pm$  SEM from the same three experiments (right). (C) ERK, JNK, and p38 activation was analyzed by immunoblotting following IgE-induced activation. The relative intensity of p-JNK, p-ERK, and p-p38 is quantified and shown as the mean value of three separate experiments. Total amounts of JNK, ERK, and p38 were measured as loading controls. \* $p$  < 0.05, Mann-Whitney test.

The increased production of cytokines such as IL-6, TNF- $\alpha$ , IL-4, and IL-13 in SIRP $\alpha$ -KD mast cells is in agreement with the observed enhanced activation of NF- $\kappa$ B and NFAT. It is reported that SHP2 is an integral component of the IKK complex and contributes to I $\kappa$ B- $\alpha$  phosphorylation [4, 28]. To our surprise, we found that SIRP $\alpha$  knockdown increased the association of SHP2 with IKK- $\beta$  upon DNP-BSA stimulation. Our previous study showed that SHP2, but not SHP1, formed an inducible complex with IKK- $\beta$  upon LPS stimulation [15]. Because SIRP $\alpha$  preferentially associates with, and most probably sequesters, SHP2 from the downstream molecules of Fc $\epsilon$ RI, such as PI3K and IKKs, it is likely that SIRP $\alpha$  acts its regulatory role mainly through SHP2.

We also noted an altered activation and nuclear localization of NFAT in SIRP $\alpha$ -KD mast cells. It is known that NFAT acti-

vation is critically dependent on the intracellular Ca<sup>2+</sup> [29]. In this study, impaired SIRP $\alpha$  expression was found to significantly enhance IgE-induced Ca<sup>2+</sup> release from the intracellular stores in BMMCs, which might facilitate NFAT localized into the nucleus. Nuclear export of NFAT is mediated by NFAT kinases both located in nuclear and cytosolic fraction. One of the first identified NFAT kinases is GSK3 $\beta$  [30]. In this regard, a direct link among PI3K-Akt, GSK-3 $\beta$ , and NFAT is established by the fact that GSK-3 $\beta$  is an Akt substrate and NFAT is regulated by GSK-3 $\beta$ . According to this, enhanced activation of Akt and phosphorylation of GSK-3 $\beta$  may contribute to the increased activation of NFAT in SIRP $\alpha$ -si-KD cells.

In summary, we have shown that SIRP $\alpha$  plays a negative role in mast-cell activation. IgE-induced SIRP $\alpha$  phosphorylation is

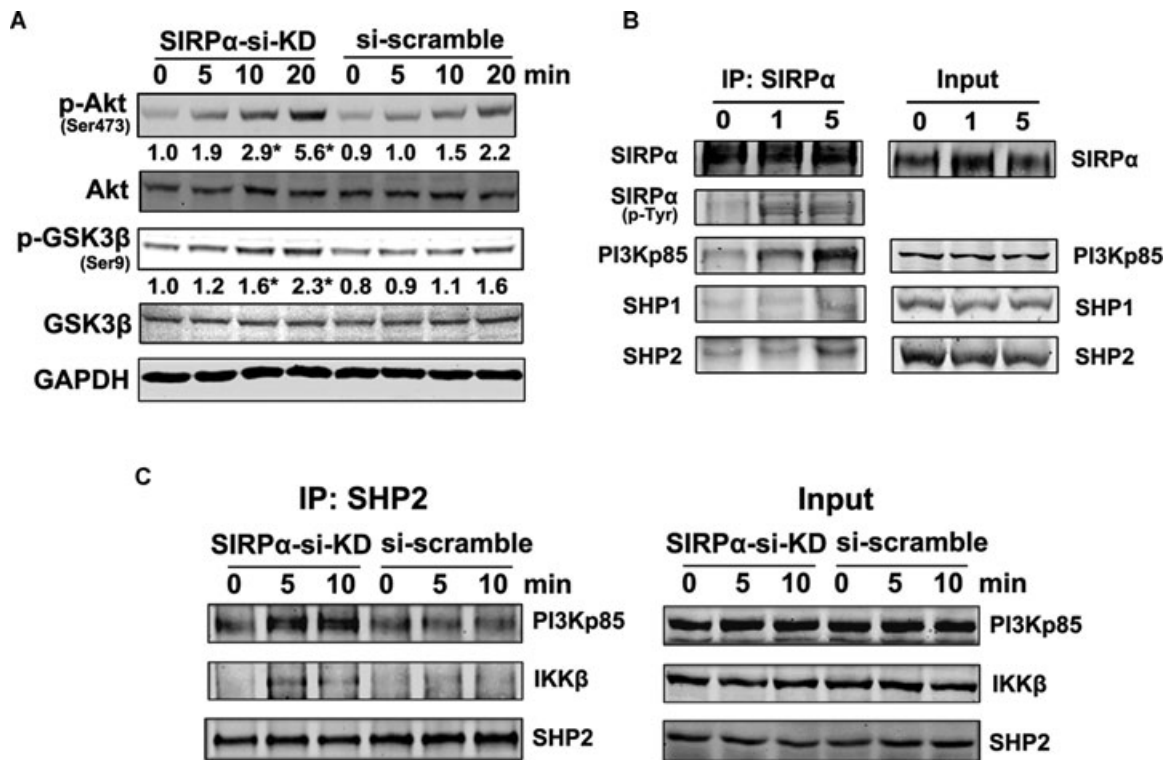


**Figure 6.** SIRP $\alpha$  negatively regulates Fc $\epsilon$ RI signaling events through sequestration of SHP2 from PI3Kp85 and IKK $\beta$ . (A) SIRP $\alpha$ -si-KD or si-scramble BM-derived mast cells (BMMCs) were activated for the indicated times and the cell lysates were subjected to SDS-PAGE. Akt as well as GSK3 $\beta$  phosphorylation was detected. Data are shown as the mean values of three independent experiments. \* $p < 0.05$ , Mann–Whitney test. (B) BMMCs were stimulated for the indicated times and the cell lysates were immunoprecipitated by anti-SIRP $\alpha$  Ab. Precipitated proteins were blotted with pTyr, SIRP $\alpha$ , SHP1, SHP2, and PI3Kp85 Abs. Data shown are from one experiment representative of three independent experiments. (C) SIRP $\alpha$ -si-KD or si-scramble BMMCs were activated and immunoprecipitated by anti-SHP2 Ab. Precipitated proteins were blotted with SHP2, PI3Kp85, or IKK $\beta$  (left). The input levels of these proteins are also shown (right). Data shown are from one experiment representative of four independent experiments.

required for inducing the optimal strength and duration of Fc $\epsilon$ RI signaling. SIRP $\alpha$  therefore functions as a homeostatic effector in mast-cell activation and this protein is likely to become a rational pharmacological target for treatment of patients with allergic diseases. Therefore, seeking for chemical agents to control the stability of SIRP $\alpha$  protein may be important. Multiple negative regulators are needed for mast cells to attenuate their release of potent inflammatory mediators. SIRP $\alpha$  is one of the ITIM-containing inhibitory receptors expressed on murine mast cells, a group including Fc $\gamma$ RIIB and gp49B1 [31,32]. Given that human basophils and mast cells express at least four ITIM-containing inhibitory receptors, a proper regulation of mast-cell activation by these negative regulators could prove to be important in allergic disorders treatment.

## Materials and methods

### Abs and reagents

Abs specific for p-JNK, p-p38, and p-ERK, as well as total ERK, JNK, p38; p-p65 (Ser536), p65, p-Akt (Ser473), Akt, p-PLC- $\gamma$ 2

(Tyr1217), PLC- $\gamma$ 2, p-I $\kappa$ B- $\alpha$  (Ser32), and I $\kappa$ B- $\alpha$ , were all purchased from Cell Signaling Technology; SHP1, SHP2,  $\alpha$ -tubulin, Rac1, p-NFATc2, NFATc2 Abs were obtained from Santa Cruz Biotechnology; p-GSK3 $\beta$  (Ser9), GSK3 $\beta$ , p-PLC- $\gamma$ 1 (Tyr783), PLC- $\gamma$ 1 Abs were obtained from Signalway Antibody Co., Ltd. Rabbit polyclonal SIRP $\alpha$  Ab was generated in our laboratory against residues in the cytoplasmic domain as described [15]. The Abs used for flow cytometry were purchased from eBioscience.

### Cells

Mice BMMCs were generated from BM cells from 6- to 8-week-old C57BL/6 mice, the cells were cultured in DMEM containing 10% FBS, 10 ng/mL IL-3, 20 ng/mL SCF (PeproTech, Rocky Hill, NJ, USA), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Fresh medium was changed twice every week. After 4–8 weeks the cells were examined by FACS assay with Fc $\epsilon$ RI and c-kit Abs.

### Gene silencing

Predesigned phosphorothioate-modified stealth<sup>TM</sup> RNA was provided by Invitrogen and the sequences was SIRP $\alpha$ :

5'-AAGUGAAGGUGACUCAGCCUGAGAA-3'; Specific-targeting siRNAs as well as scramble were transfected into cells by INTERFERin (Polyplus) and the protein levels were monitored. SIRP $\alpha$  protein expression was reduced at 24 h and could last for at least 3 days.

## Animals

Male C57BL/6 mice (6–8 weeks old) were obtained from the Chinese Academy of Science, Shanghai, China, and maintained at animal facility under pathogen-free conditions. The Kit<sup>w-sh</sup>/HNIhrJaeBsmJ mice were obtained from the Jackson Laboratory. The protocols of animal experiments were approved by the Committee for the Care and Use of Laboratory Animals in the Second Military Medical University.

## Immunologic activation

Cells were treated with 0.4  $\mu$ g/mL or the indicated dosage of anti-DNP IgE (Sigma-Aldrich, St Louis, MO, USA) for 2 h or overnight, then the cells were washed three times in serum-free medium and activated with 20 ng/mL DNP-BSA (Biosearchtech). Cells were then collected and analyzed for the following procedures.

## Degranulation and intracellular calcium

Release of  $\beta$ -hexosaminidase was measured as described [28]. Cells were grown overnight in the absence of IL-3. After washed with Tyrode's buffer, cells were stimulated with 20 ng/mL DNP-BSA or PMA plus ionomycin. Reactions were terminated and 200  $\mu$ L of the supernatant was collected for  $\beta$ -hexosaminidase assay. For intracellular Ca<sup>2+</sup> measurement, mast cells were incubated with 5  $\mu$ M Fluo-3 AM for 30 min. The cells were washed and seeded in Ca<sup>2+</sup>-free HBSS. Changes in fluorescence intensity were monitored on a confocal microscope (Zess LSM510META).

## Induction of mast-cell-mediated PCA in mice

IgE-sensitized (1  $\mu$ g/mL) SIRP $\alpha$ -si-KD/-scramble BMMCs were injected into ear tissues or dorsal skin of male mast-cell-deficient Kit<sup>w-sh</sup>/HNIhrJaeBsmJ mice. Twenty-four hours later, the mice were challenged with an intravenous injection of 200  $\mu$ g DNP-BSA in 200  $\mu$ L of PBS containing 1% Evans blue. The mice were then killed 30 min after challenge. The dye was extracted from the removed tissues at 80°C for 4 h in 500  $\mu$ L of formamide. The absorbance was measured at 620 nm.

## Microtubules (polymeric tubulin) assay and Rac1 activation assay

Microtubules assay was performed as described [20]. Briefly, immunologic activated mast cells were suspended in extraction

buffer (0.1 M Pipes, pH 7.1, 1 mM EGTA, 2 M glycerol, 1 mM MgSO<sub>4</sub>, 0.1% Triton X-100, and protease inhibitors) and centrifuged at 12 000  $\times$  g for 10 min. The pellets (Triton-insoluble fraction) were resuspended in SDS buffer and boiled for 10 min. The samples were then centrifuged at 12 000  $\times$  g for 10 min and the polymeric tubulin-containing supernatants were subjected to SDS-PAGE and detected by immunoblotting with anti- $\alpha$ -tubulin Ab. For Rac1 activation assay, activated mast cells were lysed and centrifuged. Equal amounts of proteins in the supernatants were incubated with PAK1-PBD agarose (Millipore) at 4°C for 1 h. The samples were washed two times and the pellets were boiled in 2  $\times$  SDS sample buffer for 10 min. Activation form of Rac1 (GTP-bound) was detected by immunoblotting using anti-Rac1 mAb.

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**Conflict of interest:** The authors declare no financial or commercial conflict of interest.

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**Abbreviations:** BMMC: BM-derived mast cell · PCA: passive cutaneous anaphylaxis · SIRPα: signal regulatory protein α

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**Supporting Information  
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Bo Zhang, Chun Yang, Zhong Li, Ye-xiong Tan and Hong-yang Wang

**Signal regulatory protein  $\alpha$  negatively regulates mast-cell activation following  
Fc $\epsilon$ RI aggregation**

## **Supporting Information**

### **Supporting Information Materials and Methods**

#### **Antibodies**

FcεRI  $\gamma$ , p-Tyr (4G10) antibodies were purchased from Millipore. Lyn, p-Lyn (Tyr396) antibodies were purchased from Abcam. Antibodies used for flow cytometry were purchased from eBioscience.

#### **Cells**

Rat basophile cell line RBL-2H3 was purchased from Chinese Academy of Science, Shanghai, China. Plasmid pcDNA3.1-Myc-SIRP $\alpha$  constructs and empty vector were introduced into RBL-2H3 cells using jetPEI (Polyplus) according to the manufacturer's instructions. Stable RBL-2H3 cell transformants were selected with 300  $\mu$ g/ml G418.

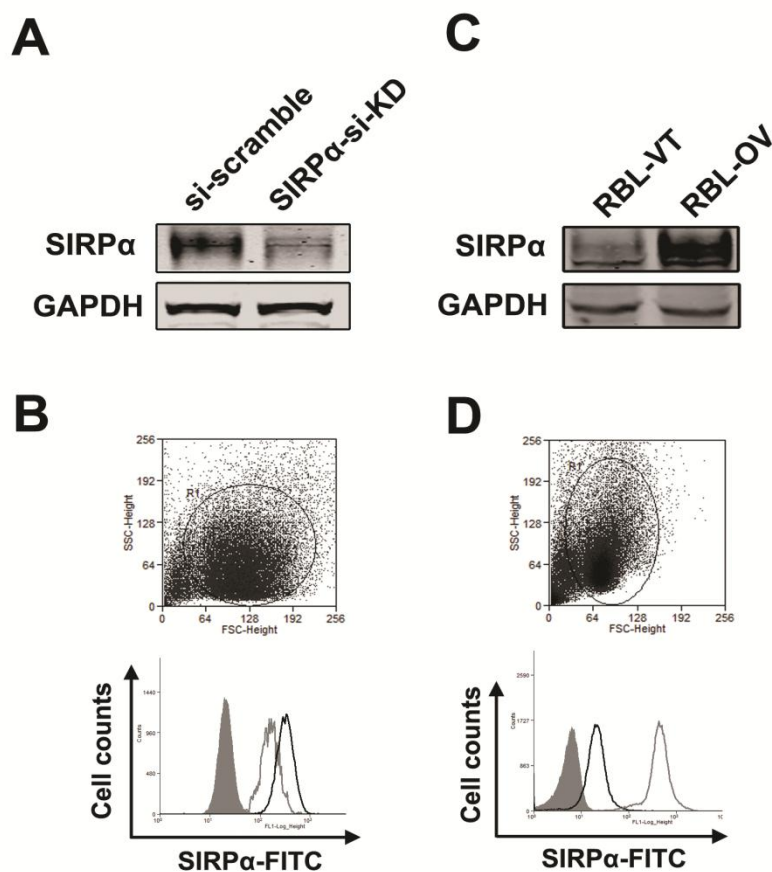
#### **Gene silencing**

Predesigned phosphorothioate-modified stealth<sup>TM</sup> RNA was provided by Invitrogen and the sequences was SHP2: 5'-AAGUAAUCCUUGGUGGACCAGACAA-3'. Specific-targeting siRNAs as well as scramble were transfected into cells by INTERFERin (Polyplus) and the protein levels were monitored.

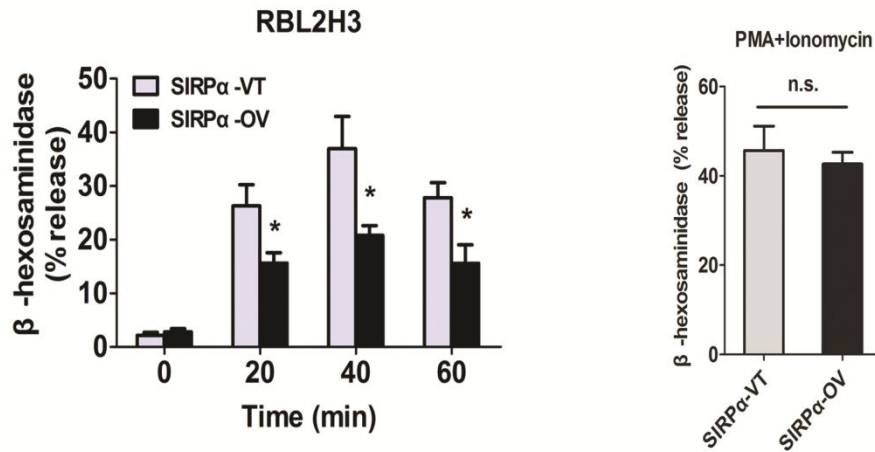
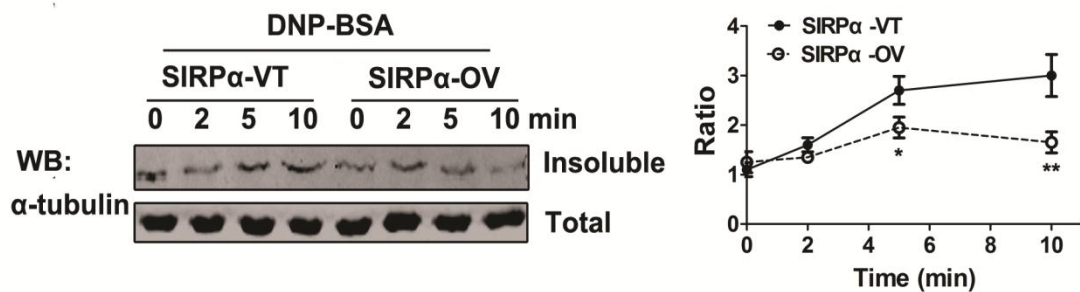
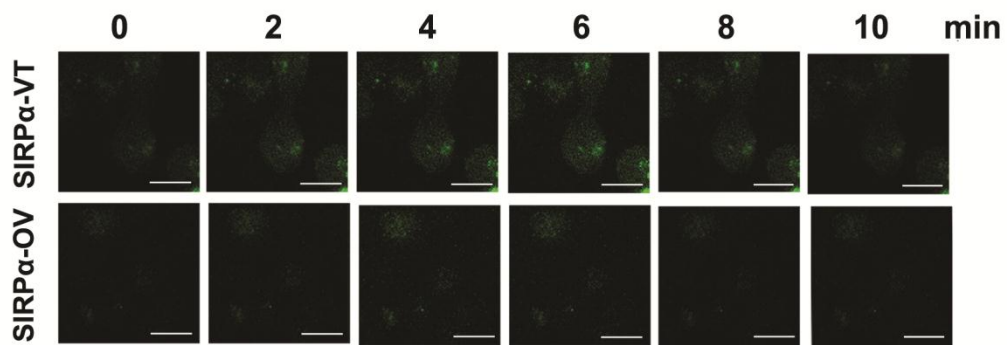
#### **PCR and real-time PCR**

Total RNA was extracted from activated cells using Trizol reagent (Invitrogen, CA). The cDNA was obtained by reverse transcription using M-MLV reverse transcription kit (Promega, Madison, Wis). Real-time PCR assay was carried out using SYBR-GREEN Kit (TaKaRa) on ABI 7900HT.

## Supporting Information Figures

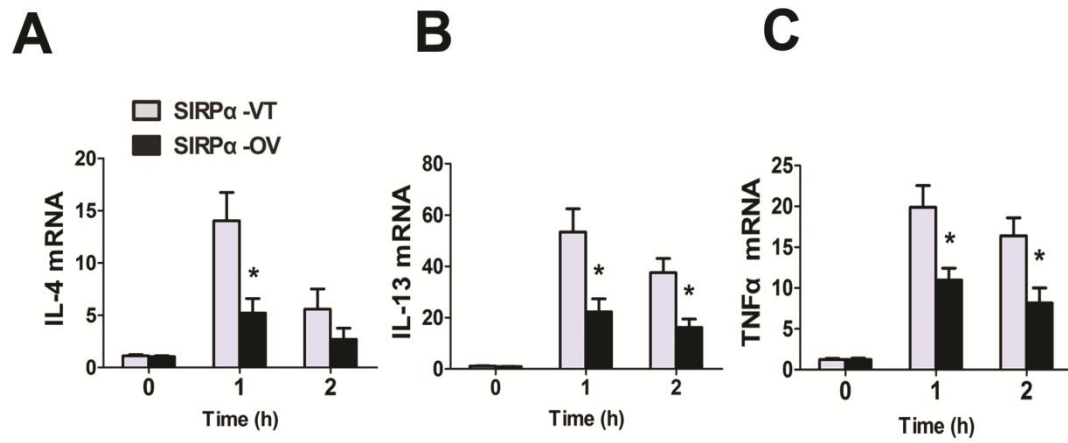


**Supporting Information Figure 1.** (A, B) Mice BMMCs were transiently transfected with SIRPα-siRNA as well as scramble-siRNA for 48 hours, the cells were collected and the expression level of SIRPα was detected by (A) immunoblotting and (B) flow cytometry. Data shown are from one experiment representative of four independent experiments. Isotype control, gray filled; SIRPα-si-KD, gray line; scramble, black line. GAPDH was detected as a loading control. (C, D) RBL-2H3 cells with SIRPα overexpression were established and examined by (C) immunoblotting and (D) flow cytometry. Data shown are from one experiment representative of three independent experiments. Isotype control, gray filled; RBL-OV, gray line; RBL-VT, black line. GAPDH was detected as a loading control.

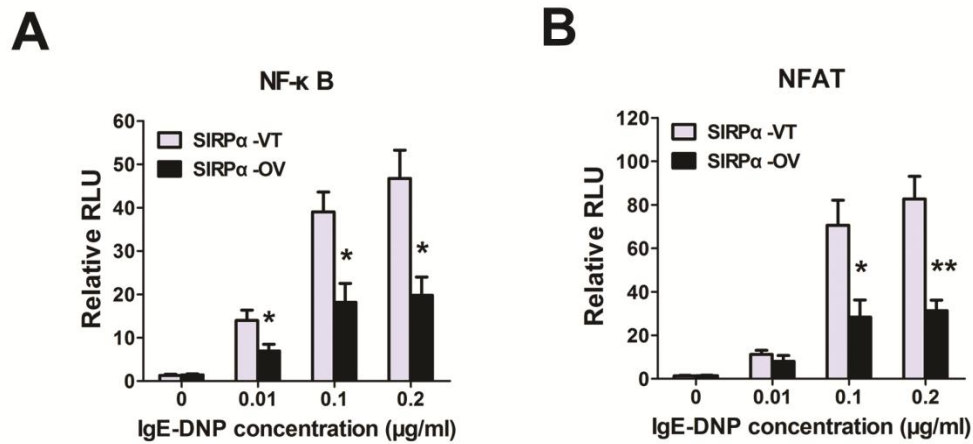
**A****B****C**

**Supporting Information Figure 2. SIRPα negatively regulates allergen-induced cytoskeletal rearrangement and calcium release in RBL-2H3 cells.** (A) SIRPα-OV and -VT RBL-2H3 cells were sensitized with 0.4 μg/ml anti-DNP IgE and stimulated with 20 ng/ml DNP-BSA (left) or PMA plus ionomycin (right). Cell degranulation was detected by β-hexosaminidase release. Data are shown as mean ± SEM of three samples pooled from four independent experiments. (B) Polymeric microtubule formation was analyzed in activated SIRPα-OV and -VT RBL-2H3 cells. Images are

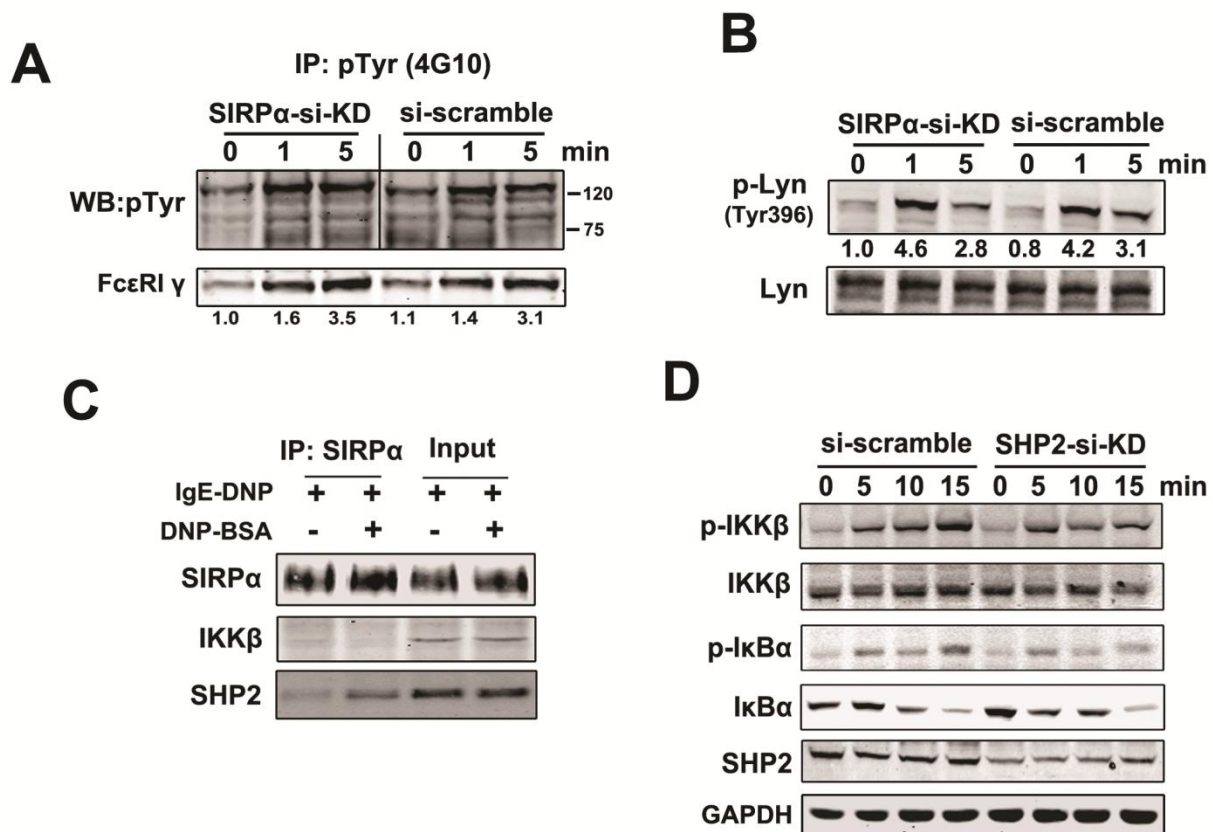
shown from one experiment representative of three experiments. The relative ratio (intensity of poly-MT vs intensity of total tubulin in unstimulated cells) is shown as mean  $\pm$  SEM of the same experiments. (C) SIRP $\alpha$ -OV and -VT RBL2H3 cells were sensitized with IgE (0.4  $\mu$ g/ml) and stained with Fluo-3 AM. The kinetics of calcium flux was detected as the dynamic changes of green fluorescence intensity and monitored by confocal microscopy Zess LSM510META (magnification 60 $\times$ ; scale bar 10 $\mu$ m). The results are analyzed by Mann-Whitney test. \* $p$ <0.05, n.s. no significant difference.



**Supporting Information Figure 3.** (A-C) SIRPα-VT, -OV RBL-2H3 cells were activated, (A) IL-4, (B) IL-13 and (C) TNFα mRNA levels were detected by real-time PCR. Data are shown as means  $\pm$  SEM of four individual experiments and analyzed by Mann-Whitney test. \* $p < 0.05$ .



**Supporting Information Figure 4.** Overexpression of SIRP $\alpha$  inhibits NF- $\kappa$ B and NFAT activation in response to Fc $\epsilon$ RI-aggregation. (A, B) RBL2H3-VT and -OV cells were transfected with 200 ng/ml (A) NF- $\kappa$ B or (B) NFAT reporter plasmids together with the control plasmid pRL-TK (2 ng/ml) for 24 hours. Cells were activated for the indicated times and the relative luciferase units (RLU) were measured. Data are shown as mean  $\pm$  SEM of four samples pooled from three individual experiments. Statistical significance determined by Mann-Whitney test. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Supporting Information Figure 5.** (A) SIRPα-si-KD and si-scramble BMMCs were activated and immunoprecipitated by the antibody against phosphotyrosine (4G10). The FcεRI γ subunit was detected. Data shown are from one experiment representative of three independent experiments. (B) The activity of Lyn kinase (Tyr396) in activated SIRPα-si-KD and si-scramble BMMCs was detected by immunoblotting. The relative intensity is shown as the mean values of three independent experiments. (C) Immunoprecipitation assay was performed on BMMCs by anti-SIRPα antibody. The interaction of SIRPα with IKKβ and SHP2 was detected by immunoblotting. Data shown are from one experiment representative of three experiments. (D) BMMCs were transiently transfected with SHP2-siRNA and scramble for at least 36 hours and then activated by IgE-DNP, the cells were collected at the indicated times and the phosphorylation bands of IKKβ and IκBα were detected by immunoblotting. Data shown are from one experiment representative of three independent experiments. GAPDH was detected as a loading control.