

SWAP-70 regulates mast cell FcεRI-mediated signaling and anaphylaxis

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Mast cells, perhaps best known by their ability to trigger allergic reactions after stimulation through the FcεRI, express the unusual phosphatidylinositol 3-kinase (PI3K)-dependent, Rac-binding protein SWAP-70. Here, we show that the IgE-mediated passive cutaneous and the systemic anaphylactic responses are strongly reduced in SWAP-70^{-/-} mice. Cultured SWAP-70^{-/-} immature bone marrow mast cells (BMMC) are also impaired in FcεRI-mediated degranulation, which can be restored by expression of exogenous wild-type SWAP-70, but less so if a phosphatidylinositol trisphosphate (PIP₃) binding mutant is expressed. SWAP-70 itself supports inositol-3-phosphate and PIP₃ production, the latter indicating a potential feedback from SWAP-70 towards PI3K. FcεRI-stimulated transcription and release of cytokines is controlled by SWAP-70. Key FcεRI signal transduction events like activation of LAT by phosphorylation, activation of Akt/PKB and of p38 MAP kinase are reduced in SWAP-70^{-/-} BMMC, but ERK is strongly hyperactivated. Some requirements for SWAP-70 were apparent only under limited-strength signaling conditions. We suggest that SWAP-70 defines a new element of efficient mast cell activation upon FcεRI signaling, important for the control of mast cell-dependent anaphylaxis.

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Introduction

Murine SWAP-70, originally isolated from activated, mature B lymphocytes [1], is strongly expressed in mast cells [2]. Molecularly, SWAP-70 loosely resembles proteins of the Dbl-family of guanine nucleotide exchange factors (GEF) for small Rho GTPases [3, 4].

SWAP-70 interacts specifically with the Rho GTPase Rac, which is a central molecular switch in a number of signal transduction pathways including FcεRI and c-kit signaling [5, 6], and supports accumulation of its activated form, to which it preferentially binds [7]. In mast cells, SWAP-70 is found in the cytoplasm and at the cytoplasmic membrane, but unlike in activated B cells is not observed in the nucleus [2, 8, 9]. A pleckstrin homology (PH) domain [10] exists in the center region of SWAP-70, binds phosphatidylinositol trisphosphate (PIP₃), the second messenger product generated by phosphatidylinositol 3-kinase (PI3K), and mediates membrane localization of the protein. As shown with a mutant (R230C) impaired in PIP₃ binding, PIP₃ binding is necessary for SWAP-70 to localize to membrane F-actin structures called membrane ruffles [7, 11], which represent sites of membrane F-actin

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Abbreviations: **BMMC:** bone marrow mast cell · **DH:** Dbl homology · **GEF:** guanine nucleotide exchange factors · **IP₃:** inositol-1,4,5-triphosphate · **PCA:** passive cutaneous anaphylaxis · **PH:** pleckstrin homology · **PIP₃:** phosphatidylinositol trisphosphate

remodeling, at which Rac activation was also reported to happen [12]. The Dbl homology (DH) domain of SWAP-70 displays only limited amino acid identity (ca. 20%), and overlaps with a tripartite coiled-coil region [1]. In SWAP-70, the DH domain is positioned not N-terminal but C-terminal to its PH domain, contrasting nearly all proteins of the Dbl family. SWAP-70's closest homolog, a protein called Def-6, SLAT or IBP [13–15], is the only known protein that also carries the PH and DH domains in the N-C arrangement. Thus, SWAP-70 and IBP may form a rather unique Rho GTPase-interacting protein family. IBP was reported to act as a GEF for Rac and Cdc42, and functions in T cell signaling, particularly of the Th2 subset [14].

Earlier, we determined a function for SWAP-70 in mast cells in signaling from the c-kit receptor [16]. SWAP-70^{-/-} bone marrow mast cells (BMMC) are impaired in a number of c-kit-triggered processes, indicating that SWAP-70 is an important regulator of specific c-kit effector pathways including mast cell activation, migration, and cell adhesion.

Mast cells contribute important and specific functions to the immune system [17–20]. One well-known function is in IgE-triggered allergic reactions. After binding IgE, the cells can be stimulated by cross-linking the FcεRI-IgE complex by either antigen or by anti-IgE antibody to release a variety of biologically active, preformed mediators such as histamine, proteoglycans, proteases, serotonin, and others. Cross-linking also induces transcription, synthesis and subsequent release of cytokines. Some reports suggest that binding of only IgE to the FcεRI without or with minimal cross-linking initiates limited signaling, which induces the production of certain cytokines [21–23].

In exocytosis assays that monitor release of hexoseaminidase as an indicator for degranulation, SWAP-70^{-/-} BMMC, stimulated by cross-linking the IgE/FcεRI complex with anti-IgE antibody, yield a signal of about one-fifth of that of wild-type (wt) BMMC. If cross-linked by antigen, the reduction is about 50% [2]. Triton X-100 lysis and ionomycin-triggered degranulation, however, generated the wt-like signal, indicating that SWAP-70^{-/-} BMMC contain functional granules, which upon ionomycin-triggered Ca⁺⁺ influx release their content. Thus, not degranulation *per se* but the IgE/FcεRI signaling pathway leading to exocytosis requires SWAP-70 [2]. Events downstream of the FcεRI required for mast cell degranulation include, among others, activation of LAT, inositol-1,4,5-trisphosphate (IP₃) and PIP₃ production, activation of kinases such as Akt, p38, and ERK, but whether SWAP-70 acts in any of these processes is unknown. Generally, the precise signaling pathways that lead to degranulation are not yet described in sufficient details, and more molecules important in this pathway such as

regulators or effectors need to be determined and characterized [24–29].

Our studies were done in wt or SWAP-70^{-/-} mice or with cultures of IL-3-dependent BMMC derived from them [2]. These cultures, homogenous after about 4–5 wk, reflect mostly the mucosal type of mast cells [30, 31]. FACS analysis of surface-bound IgE showed that more than 98% of wt or SWAP-70^{-/-} cells bind IgE. The equal intensity of the signals also indicates similar numbers of receptors on their surface. Wild-type or SWAP-70^{-/-} mast cells respond similarly to IL-3 with regard to proliferation, cell cycle, and apoptosis under starvation conditions [2].

Together, indications exist that SWAP-70 plays important role(s) in mast cell signaling. This study aimed at identifying specific contributions of SWAP-70 to FcεRI signaling *in vivo* and *in vitro*.

Results

Mast cell dependent anaphylaxis depends on SWAP-70

In order to assess the importance of SWAP-70 for mast cell function in the organism and to study mast cell FcεRI-mediated reactions *in vivo*, we induced passive cutaneous anaphylaxis (PCA) in mice by intravenous injections of the antigen DNP-BSA with 0.5% Evans blue into wt or SWAP-70-deficient mice that were sensitized by intradermal ear injections the day before with IgE in HBBS (left ear) or HBSS alone (right ear). Extravasation of the dye is caused by histamine and serotonin release from FcεRI-activated mast cells [32] and the dye accumulates in the tissue. At 30 min after antigen stimulation, the extravasated dye in the ears of mice was measured spectrophotometrically. SWAP-70^{-/-} mice showed 1.7-fold lower total levels of extravasated dye as compared to wt (Fig. 1A). However, the signal observed with SWAP-70^{-/-} mice (0.255 ± 0.019) is at background levels as derived from the control ear (0.253 ± 0.033), while a clear signal of 0.41 ± 0.048 was obtained from wt mice (control at 0.207 ± 0.041).

In addition, we carried out systemic anaphylaxis experiments by challenging IgE-primed mice with 500 µg DNP-KLH and measuring changes in body temperature caused by anaphylactic shock [33] (Fig. 1B). A decrease in basal body temperature from 38 to about 30°C was observed within 30 min in wt animals. Temperature returned to normal after about 2 h. No change in body temperature was observed in mice that were not IgE-primed prior to DNP-KLH stimulation. SWAP-70^{-/-} mice primed with IgE and challenged with DNP-KLH showed only a small decrease in body temperature to about 35°C and a quick recovery,

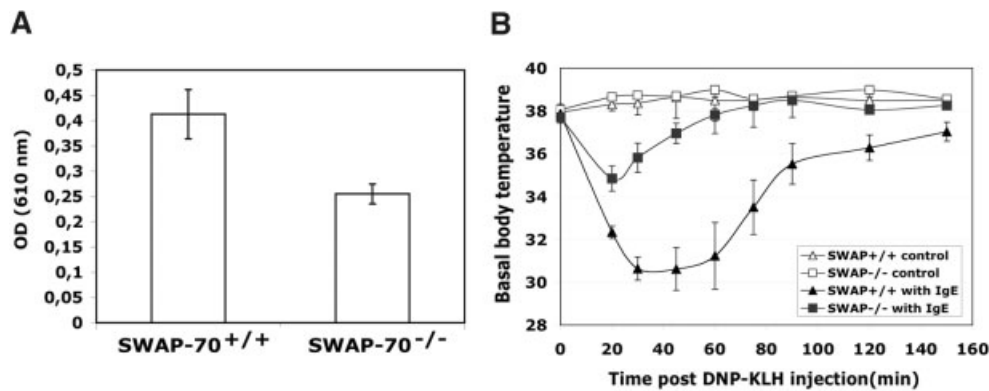


Figure 1. Quantification of IgE-induced passive cutaneous and active systemic anaphylaxis. SWAP-70^{+/+} or SWAP-70^{-/-} mice were primed intradermally in their left ears with 20 μ g IgE (anti-DNP antibody clone SPE-7) and injected with HBSS in their right ears for internal controls, followed after 24 h by intravenous injections of 100 μ g antigen DNP-BSA together with 0.5% Evans blue in HBSS to induce PCA reactions. Control mice received HBSS with Evans blue alone without DNP-BSA. After 30 min, extracted dye from minced ear tissues was measured spectrophotometrically at OD 610 nm and normalized against the appropriate controls. Each group under study comprised five mice. The average difference in OD of sensitized left ears versus the HBSS-injected right ear controls was 0.41 ± 0.048 versus 0.207 ± 0.041 for SWAP-70^{+/+} and 0.255 ± 0.019 versus 0.253 ± 0.033 for SWAP-70^{-/-} mice (A). SWAP-70^{+/+} or SWAP-70^{-/-} mice priorly sensitized intravenously or not with 50 μ g IgE were challenged 48 h later with 500 μ g DNP-KLH. Changes in basal body temperature were monitored at 20-min intervals for up to 2.5 h. Ten mice were analyzed in each group. Values are means $\pm$ SEM (B).

indicating a strong reduction in the systemic anaphylactic response induced by Fc ϵ RI.

Since mature peritoneal mast cells do not express SWAP-70 [2], we asked which types of mature tissue mast cells express SWAP-70. Analyses of fixed, paraffin-embedded sections from back skin and sections from frozen stomach mucosa by immunohistochemistry, using FITC-conjugated avidin to stain mast cells [34] and polyclonal rabbit anti-SWAP-70 followed by secondary anti-rabbit-Cy3 antibody, showed that mast cells from back skin as well as mucosal mast cells strongly express SWAP-70 (not shown). Back skin sections from SWAP-70^{-/-} mice showed fewer numbers of mast cells, in agreement with an earlier report on twofold reduced skin mast cells in these mice [2], while mucosal stomach mast cell numbers are unaltered compared to wt. Gastrointestinal tract mast cells were stained with toluidine blue and no difference in numbers between wt and SWAP-70^{-/-} mice were found.

SWAP-70 reconstitution restores the degranulation efficiency of SWAP-70^{-/-} BMMC

Earlier, we reported that SWAP-70^{-/-} BMMC are defective *in vitro* in Fc ϵ RI-mediated degranulation of preformed mediators such as hexoseaminidase [2]. To confirm a direct role of SWAP-70 and to get insights into the molecular mechanisms of its function, we transfected mutant BMMC with pEGFP constructs to transiently express SWAP-70, or with the site-specific mutant R230C which is reduced in PIP₃ binding [7], or with vector alone for control. Expression of SWAP-70 in

transfected SWAP-70^{-/-} and control wt BMMC was assessed by performing Western blot analysis (Fig. 2A). The transfected cells were starved for 2 h, primed with anti-DNP-KLH IgE for 1 h, followed by cross-linking using 100 ng of the antigen DNP-KLH. The results are as shown in Fig. 2B as the average of three experiments. Compared to wt cells, SWAP-70^{-/-} BMMC show ~1.4-fold reduced degranulation after cross-linking with antigen, similar to the twofold reduction reported earlier [2]. Infection of wt or SWAP-70^{-/-} cells with control vector had only marginal effects. Wild-type BMMC that were transfected with SWAP-70 showed an increase of about 1.8-fold in degranulation upon stimulation with antigen. If background levels are deducted, the stimulation is 2.5-fold. Reconstitution of SWAP-70^{-/-} BMMC with SWAP-70 restored degranulation to levels similar to that seen in non-transfected wt BMMC. Expression of the SWAP-70 R230C mutant, which is compromised in PIP₃ binding [35], in wt or SWAP-70^{-/-} cells stimulated degranulation to a lesser degree than expression of the unaltered SWAP-70. The failure of R230C to efficiently restore degranulation despite its quite strong expression indicates a requirement for PIP₃ binding of SWAP-70 in signaling to degranulation.

SWAP-70 controls cytokine gene expression

Activation of mast cells induces synthesis and secretion of cytokines, rendering mast cells important contributors to the cytokine repertoire of an organism. Earlier, we obtained indications for reduced secretion of IL-6 or TNF- α by Fc ϵ RI-stimulated SWAP-70^{-/-} BMMC [2]. To

assay not only for degranulation of preformed mediators as above, but also for induction of new synthesis of mediators, we asked whether SWAP-70 is involved in regulating transcription of cytokine genes. Using RT-PCR with RNA prepared at various time points after either cross-linking of the IgE-loaded FcεRI or after IgE loading only without cross-linking, we show here that

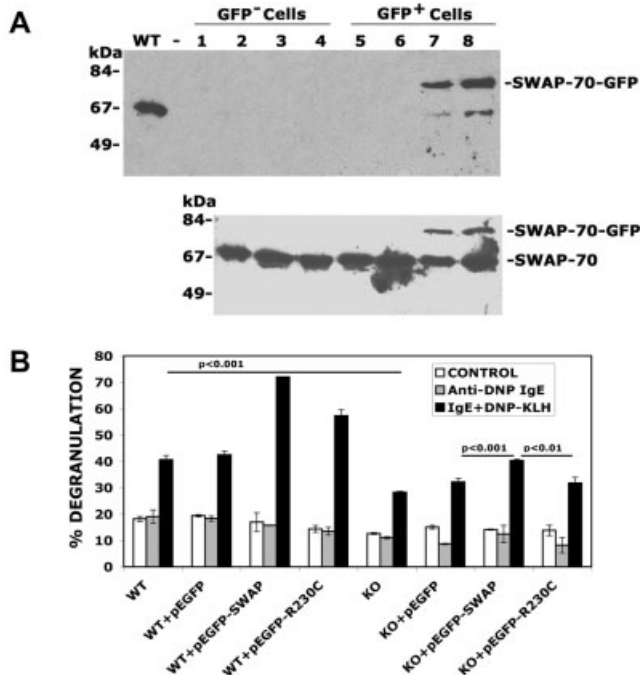


Figure 2. In vitro reconstitution of SWAP-70 expression and degranulation in SWAP-70^{-/-} BMMC. Immunoblots probed with anti-SWAP-70 antibody showing expression of SWAP-70 or SWAP-70-EGFP in transfected BMMC. SWAP-70^{-/-} (top) or wt (lower panel) BMMC transiently transfected with pEGFP vector alone, pEGFP-SWAP-70, or pEGFP-R230C were sorted 48 h post transfection for GFP expression using a MoFlo cell sorter. RIPA extracts from GFP⁻ (nos. 1–4) or GFP⁺ cells (nos. 5–8) of 1×10^5 wt and 5×10^5 SWAP-70^{-/-} cells were subjected to SDS-PAGE followed by immunoblot with anti-SWAP-70 antibody. The upper panel shows extracts from mock transfected wt (no. 1) as a control followed by mock transfected SWAP-70^{-/-} BMMC extract (no. 5). Extracts from wt or SWAP-70^{-/-} BMMC are shown in the upper and lower panels, respectively, from cells that were transfected with pEGFP vector alone (nos. 2, 6), pEGFP-SWAP-70 (nos. 3, 7), and pEGFP-R230C (nos. 4, 8) (A). Hexoseaminidase assay for degranulation in reconstituted SWAP-70^{-/-} BMMC (B). Cells (10^5) from each batch of wt or SWAP-70^{-/-} BMMC after transfection and sorting for expression of GFP as described above for (A) were starved for 4 h, sensitized with 0.5 μ g anti-DNP IgE for 1 h, and stimulated with the antigen DNP-KLH (100 ng) for 15 min. Supernatants were subjected to spectrophotometric quantification of the released hexoseaminidase using fluorescent N-acetyl- β -D-glucosaminidase as a substrate, as a measure for degranulation. Degranulation was calculated and plotted as % of total hexoseaminidase from the respective Triton X-100-lysed cell pellets. Values shown are the averages from three experiments $\pm$ SEM (A).

transcription of several interleukins is altered in SWAP-70^{-/-} BMMC (Fig. 3A, B). Upon cross-linking, induction of transcription of IL-1, IL-2 or IL-10 largely fails in SWAP-70^{-/-} cells, and transcription of IL-6 and TNF- α is mildly reduced if compared to wt cells. IL-5 transcription is up-regulated as in wt cells, IL-4 expression is mildly increased, and IL-3 transcription may be slightly reduced. Transcription of β -microglobulin is unchanged as compared to the HPRT control.

To test whether additional requirements for SWAP-70 would become apparent under limiting signaling conditions, *i.e.* by sensitization with IgE alone, we tested cytokine gene expression at different time points after IgE binding (Fig. 3B). Expression of IL-6, IL-10 or TNF- α under these conditions is readily observed in wt but very weak in SWAP-70^{-/-} cells, where IFN- γ is also clearly reduced. IL-5 expression is similarly induced in both genotypes, and IL-3 (not shown) is not detectable in either. Remarkably, expression of IL-4 is strongly up-regulated in mutant BMMC, while it is not induced in wt cells.

Since IL-4 released by mast cells upon activation through the IgE receptor has been previously shown to provide an initial pulse to trigger T cells for sustained release of IL-4 [36] and to contribute to B cell activation [37], and thus is biologically relevant, we investigated basal levels of IL-4 in the serum of SWAP-70^{-/-} mice, compared to wt (Fig. 4A). Analysis by ELISA revealed twofold higher levels of IL-4 in SWAP-70-deficient mice independent of the strain background, *i.e.* in both, the 129SvEMS and C57BL/6 wt or SWAP-70^{-/-} strains. To further evaluate the effect of IgE on the IL-4 levels in SWAP-70^{-/-} mice compared to wt, we injected different concentrations of IgE and 24 h later analyzed the serum by ELISA for IL-4 (Fig. 4B). Both in control mice and in mice injected with 50 or 100 μ g IgE, SWAP-70^{-/-} mice showed higher serum IL-4 levels compared to wt, even though at 100 μ g IgE an inhibitory effect was observed in all strains.

Activation of Akt, ERK, and p38

Degranulation as well as cytokine gene expression triggered by FcεRI stimulation is mediated by activation of kinases such as Akt, ERK and p38. Therefore, we analyzed activation of these kinases in SWAP-70-deficient BMMC. Western blotting using antibodies specific for phosphorylated Akt (P-Ser 473) revealed differences between wt and SWAP-70^{-/-} BMMC, both upon IgE sensitization only and after FcεRI cross-linking. In total cell lysates of wt cells, Akt phosphorylation is higher than in lysates from SWAP-70^{-/-} cells (Fig. 5A, B). Phosphorylation was further mildly increased for a few minutes after cross-linking. SWAP-70^{-/-} cells showed only weak Ser 473 phosphor-

ylation of Akt, visible often only upon prolonged exposure of the film. Total amounts of Akt protein were the same in both cell types.

Since phosphorylation of Akt is affected in SWAP-70^{-/-} BMMC, we performed Akt kinase assays using GSK3- α fusion protein as substrate in the presence of ATP with BMMC lysates from SWAP-70^{-/-} or wt BMMC (Fig. 5C). About tenfold less phosphorylated GSK3- α was noted when SWAP-70^{-/-} BMMC lysates were used as compared to wt lysates. This confirms the requirement of SWAP-70 for proper activation of Akt in Fc ϵ RI-stimulated BMMC.

The extracellular signal-regulated kinases (ERK) constitute a subfamily of mitogen-activated protein

kinases (MAP kinases). ERK are involved in the control of cell proliferation, cell shape, cell mobility, and cell-cell interactions [38–40]. Since signaling through the Fc ϵ RI stimulates ERK [21, 41, 42], we asked whether ERK activation may be affected by SWAP-70 deficiency as well. We tested phosphorylation of ERK1 and ERK2 by a phospho-specific antibody. After triggering signal transduction by IgE sensitization alone (Fig. 6A) or by cross-linking with anti-IgE antibody (Fig. 6B), we found phosphorylation of both ERK1 and ERK2 – though under these conditions primarily ERK2 – up-regulated in wt cells. However, phosphorylation in SWAP-70^{-/-} cells is much stronger than in wt cells. This increased phosphorylation is independent of the mode of

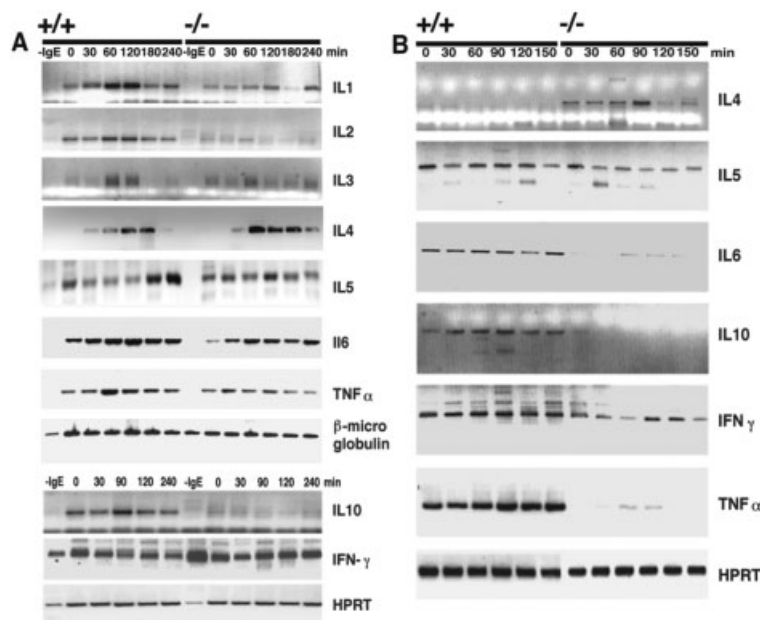


Figure 3. Cytokine gene expression of SWAP-70^{+/+} vs. SWAP-70^{-/-} BMMC. RNA from SWAP-70^{+/+} or SWAP-70^{-/-} BMMC upon stimulation by IgE binding for 4 h followed by anti-IgE antibody cross-linking was subjected to RT-PCR analysis to monitor cytokine gene expression after IgE binding followed by anti-IgE antibody cross-linking (A) or IgE binding alone (B), for the indicated times (min). Results are representative of at least four, in many cases six experiments for each of the cytokines shown.

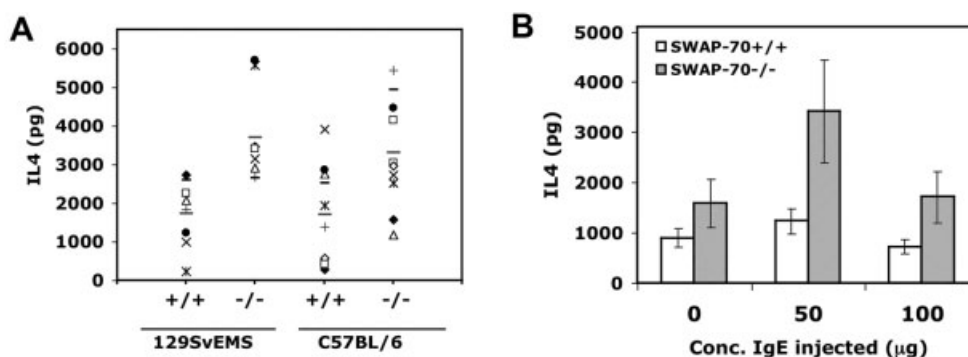


Figure 4. Effect of SWAP-70 on serum IL-4 levels: Serum IL-4 levels from SWAP-70^{+/+} or SWAP-70^{-/-} mice on the C57BL/6 and 129SvEMS background were quantified using ELISA. Each symbol represents one mouse (A). Effect of IgE stimulation on serum IL-4 levels (B). Groups of four each of SWAP-70^{-/-} or wt mice were sensitized intravenously with 50 or 100 μ g IgE (anti-DNP antibody) in HBSS or with HBSS alone. After 24 h, IL-4 levels from sera of these mice were quantified. Values are plotted as means $\pm$ SEM.

stimulation, although the difference between wt and SWAP-70^{-/-} cells was highest if the FcεRI was cross-linked. The total amount of ERK2 is similar in cells of both genotypes. In SWAP-70^{-/-} BMMC, an additional band appears that reacts with the anti-ERK1/2 antibody and migrates at a position between ERK1 and ERK2. A comparable polypeptide has been observed earlier [16] and seems to replace the ERK1 band in non-stimulated or briefly stimulated cells. After 1 h of IgE stimulation, the typical ERK1 signal appears. The identity of the SWAP-70^{-/-}-specific band is subject to future studies. Differentially regulated isoforms of ERK have been reported in various systems [43].

Kinase assays using an ELK-1 fusion protein as a substrate for ERK immunoprecipitated from BMMC lysates, which were prepared after stimulation with only IgE, revealed much higher levels of activated ERK in SWAP-70^{-/-} BMMC lysates as compared to wt lysates (Fig. 6C) (~12-fold higher at the 10-min time point). This supports our notion of hyperactivation of ERK in the absence of SWAP-70. Similarly increased ERK activity was found upon cross-linking of the FcεRI (data not shown).

As another key component, kinase p38 has been implicated in mast cell signaling to control migration, adhesion, cytokine production, and survival [21, 41, 44, 45]. We analyzed p38 phosphorylation and activity. Upon FcεRI stimulation through IgE binding only, we observed that SWAP-70^{-/-} BMMC show similar or only mildly lower phosphorylation of p38 compared to wt BMMC (Fig. 7A). The difference is much more pronounced when the FcεRI/IgE complex is cross-linked (Fig. 7B; there is no detectable signal after 1 h of pretreatment with IgE only). There also appears to be more p38 expressed in wt BMMC before and after stimulation, although slightly more lysate may have been loaded in Fig. 7B. To assess p38 kinase activity, we subjected lysates from wt or SWAP-70^{-/-} cells with or without FcεRI cross-linking to immunokinase assays with the ATF2-GST fusion protein as substrate (Fig. 7C). Very little p38 activity was observed in SWAP-70-deficient BMMC, indicating a reduction of ca. 10–25-fold (5-min time point) compared to wt.

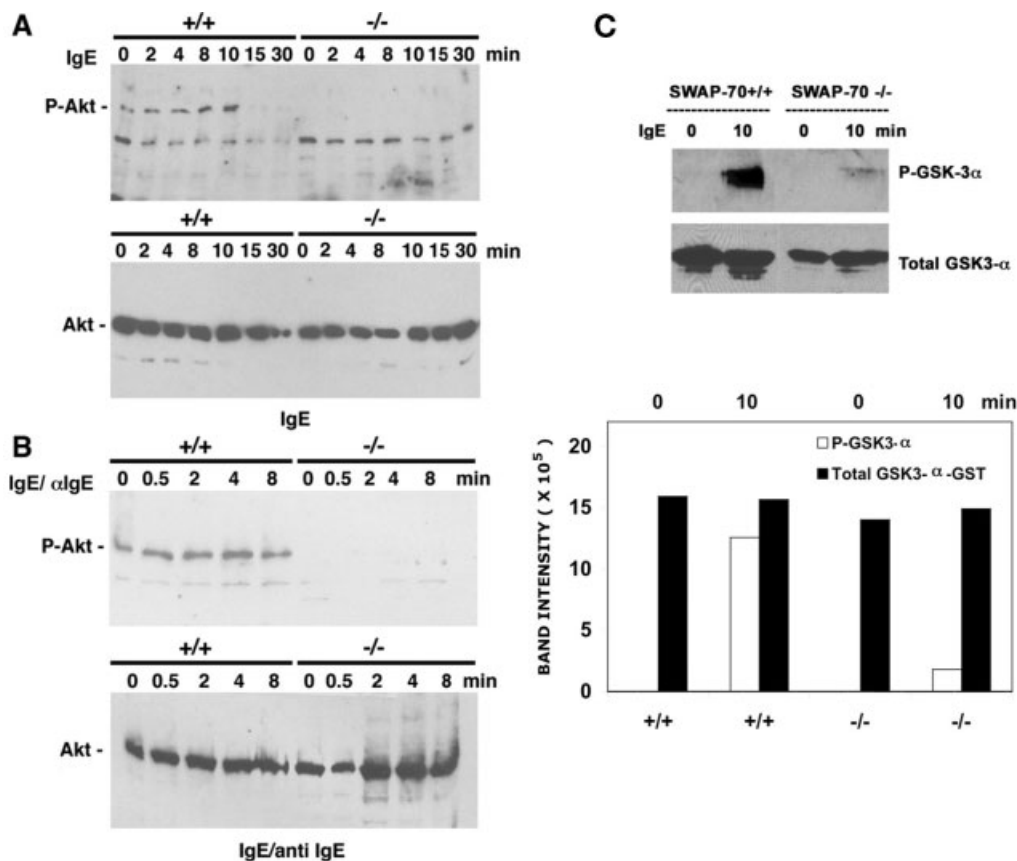


Figure 5. Akt activation upon FcεRI stimulation. Phosphorylation of Akt at Ser 473 was analyzed by immunoblotting extracts from SWAP-70^{+/+} or SWAP-70^{-/-} BMMC stimulated with IgE only (A) or with IgE followed by anti-IgE antibody (B). Extracts were probed with antibodies for either phosphorylated or total Akt. Representative of five experiments. (C) Akt activity was measured by immunokinase assays using GSK3-α-GST fusion protein as substrate for Akt immunoprecipitated from extracts from SWAP-70^{+/+} or SWAP-70^{-/-} BMMC. The p-GSK3-α generated was analyzed by immunoblotting (C) and quantitation is shown in the lower panel. The experiment was repeated three times.

PI3K activity and IP₃ production are impaired in SWAP-70^{-/-} BMMC

Phospholipids are produced during FcεRI and serve as key second messengers. PI3K is activated and catalyzes the synthesis of IP₃ [46]. This is considered a key reaction, for several signaling proteins like serine/threonine kinases, protein tyrosine kinases, GEF and GTPases depend on IP₃, which they bind for their translocation from the cytosol to the membrane [47]. SWAP-70, which also binds IP₃, is considered to act

downstream of PI3K, but we wondered whether its absence nevertheless affects IP₃ production, since IL-4 production is increased and known to down-regulate PI3K by recruiting SHP-1 [48]. We assayed IP₃ production by PI3K, which was immunoprecipitated from wt or SWAP-70^{-/-} cells (Fig. 8A). Upon IgE binding, IP₃ production by PI3K was found to be ~1.5-fold lower in SWAP-70^{-/-} cells than in wt. By further cross-linking of the IgE-loaded FcεRI, IP₃ production remained consistently lower (average wt 48 ± 5 pmol; pmol; SWAP-70^{-/-} 40 ± 4.5 pmol). Immunoprecipita-

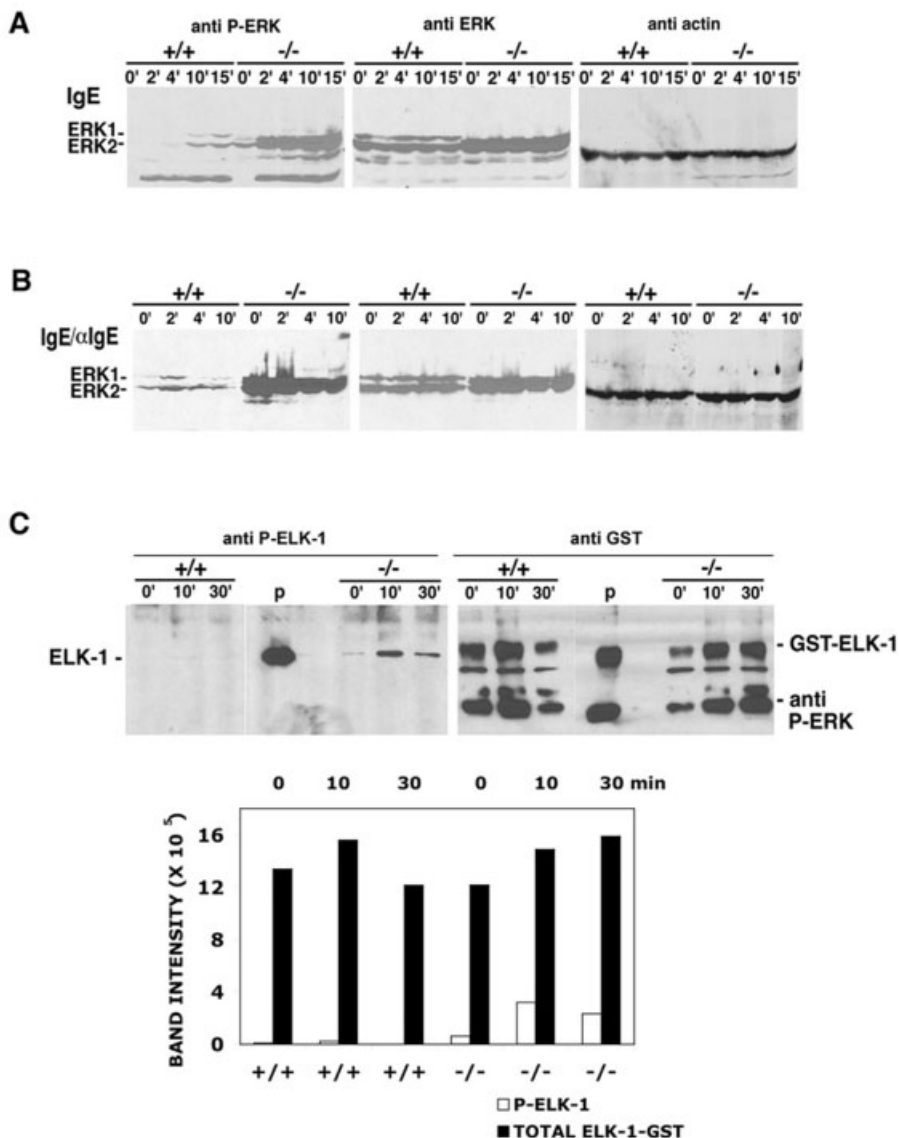


Figure 6. ERK phosphorylation and activity in SWAP-70^{+/+} or SWAP-70^{-/-} BMMC. Extracts from BMMC stimulated with IgE alone (A) or with IgE followed by anti-IgE antibody (B) were probed by immunoblotting with antibodies specific for phosphorylated or total ERK. Antibody specific against actin was used as the loading control. The experiment was repeated four or five times, respectively. (C) ERK activity was measured in immunokinase assays of SWAP-70^{+/+} or SWAP-70^{-/-} BMMC extracts, using immunoprecipitated phosphorylated ERK and ELK-1-GST fusion protein as substrate; p stands for the control protein (GST-ELK-1). Immunoblots probed with phospho-specific ELK-1 (left) or GST control antibodies (right) are shown in the upper panel. The GST-ELK-1 band and the antibody L-chain of the anti-phospho-ERK antibody are indicated. The lower panel shows quantification of the kinase activity by Imagequant analysis of immunoblots.

tion experiments from RIPA lysates of IgE/anti-IgE antibody-activated BMMC with anti-phosphotyrosine antibody (4G10) and subsequent probing of the precipitate by immunoblotting with anti-p85 antibody indicated reduced phosphorylation of the p85 regulatory subunit of PI3K (not shown).

Phospholipase C γ (PLC γ) converts phosphatidylinositol-4,5-bisphosphate (PIP₂) to IP₃, which through binding to IP₃ receptors on the endoplasmic reticulum allows Ca⁺⁺ channels to be opened [49]. This process may be at least partially dependent on PIP₃ production and is required for Fc ϵ RI-triggered mast cell degranulation [49–52]. Ca⁺⁺ flux is mildly impaired upon Fc ϵ RI

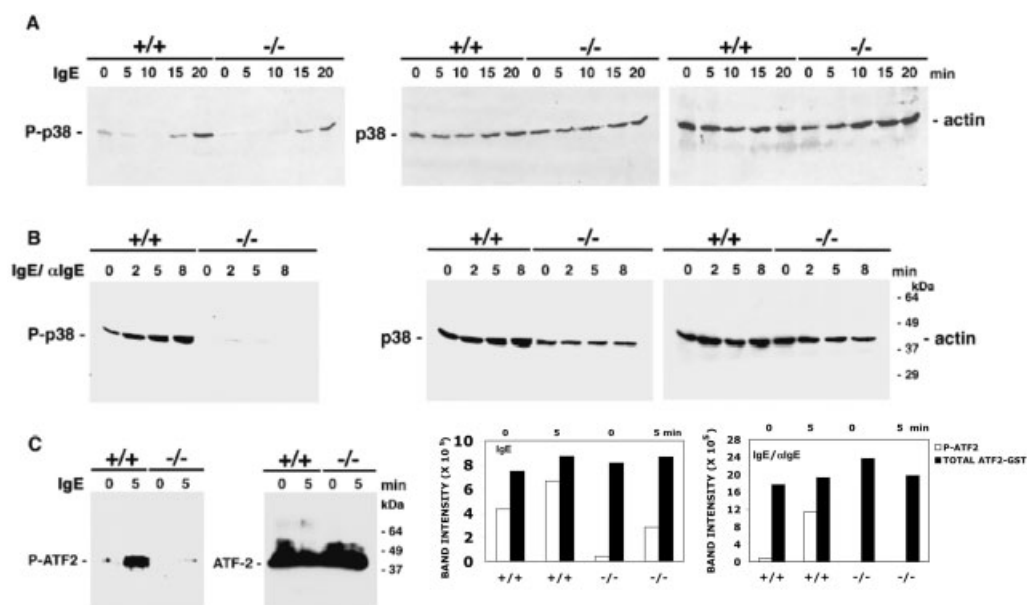


Figure 7. Phosphorylation and activity of p38 in SWAP-70^{+/+} or SWAP-70^{-/-} BMMC. Phosphorylation of p38 was analyzed by immunoblotting of cells stimulated with IgE alone (A) or IgE followed by anti-IgE antibody (B). Antibodies specific for phosphorylated or for total p38 were used. Antibody specific against actin was used as the loading control. These experiments were repeated three times. (C) p38 immunoprecipitated from extracts of IgE/anti-IgE antibody-treated SWAP-70^{+/+} or SWAP-70^{-/-} BMMC was assayed in kinase assays with ATF2-GST fusion protein as substrate. Products were analyzed by immunoblotting with antibodies specific for phosphorylated ATF2 and GST (detects all ATF-GST, indicated by ATF-2, right hand blot) for control as shown in the left two panels. The right two panels show quantitation of p38 activity for either BMMC treated with IgE only or with IgE/anti-IgE antibody. Representative of three experiments.

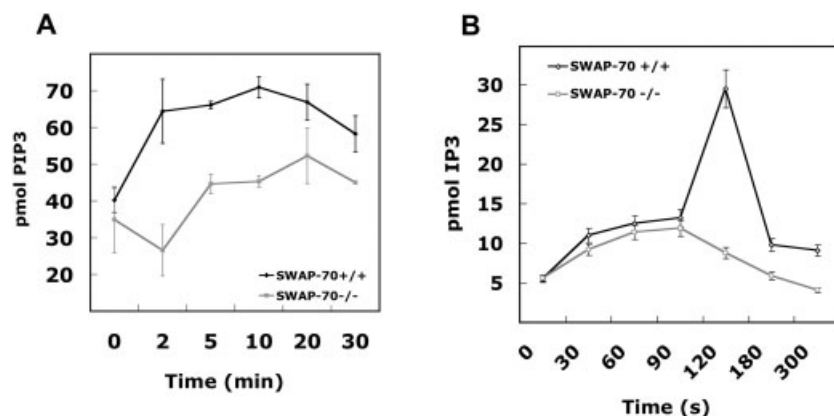


Figure 8. PI3K activity and IP₃ production in SWAP-70^{+/+} or SWAP-70^{-/-} BMMC. ELISA measuring PI3K activity (A). PI3K activity was measured in kinase reactions using SWAP-70^{+/+} or SWAP-70^{-/-} BMMC extracts with the amount of PIP₃ generated, plotted against the indicated time intervals of activation with IgE, as the read-out. One representative experiment of three is shown. Generation of the second messenger IP₃ upon stimulation by IgE binding followed by anti-IgE antibody cross-linking (B). IP₃ generated upon stimulation of SWAP-70^{+/+} or SWAP-70^{-/-} BMMC was measured using the BIOTRAK TRK-1000 kit. The concentration of IP₃ (pmol) is plotted against the time intervals of stimulation.

in SWAP-70^{-/-} BMMC (data not shown), similar to our observation of reduced Ca⁺⁺ flux upon c-kit activation of these cells [16]. PLC γ 1 and PLC γ 2 phosphorylation in Fc ϵ RI-triggered SWAP-70^{-/-} BMMC are similar to wt, but their distribution between NP40-soluble and -insoluble fractions differs, since we find much lower amounts of PLC γ in the soluble fraction, but more in the cytoskeletal fraction, prepared from SWAP-70^{-/-} cells as compared to wt fractions (not shown). We analyzed levels of IP₃ in Fc ϵ RI-stimulated SWAP-70^{-/-} or wt BMMC. Kinetic analysis in 30 s intervals of IP₃ levels in defined numbers of cells revealed that the spike of IP₃ production in wt cells, seen within 1–3 min after Fc ϵ RI cross-linking, is missing in mutant cells where only a mild increase was observed (Fig. 8B). In wt cells, levels of IP₃ increase from an average of 8 to 24 pmol per 10⁷ cells. Analyzing IP₃ levels at 15 s intervals confirmed the absence of the spike in IP₃ production in SWAP-70-deficient cells (not shown).

LAT phosphorylation is reduced in SWAP-70^{-/-} BMMC

The transmembrane adaptor molecule LAT is another key element in early steps of Fc ϵ RI signaling [53]. Upon its phosphorylation and activation by tyrosine kinases, it

binds to adaptors and other signaling proteins to drive several transduction pathways, including PLC γ , Grb2, Gads, and SLP-76, and forms membrane-bound signaling complexes including many of these proteins [26]. To determine whether SWAP-70 is involved in a step as early as LAT phosphorylation, we analyzed SDS-solubilized total extracts (RIPA extracts) from SWAP-70^{-/-} and wt BMMC for LAT phosphorylation using phospho-specific antibodies to LAT (pTyr 171). SWAP-70^{-/-} BMMC showed reduced phosphorylation of LAT as compared with wt BMMC, both upon and prior to stimulation with IgE/anti-IgE antibody, while total LAT expression remained unaltered in both (Fig. 9A). We further performed immunoprecipitation experiments using anti-SWAP-70 antibody with BMMC lysates prepared in less stringent RIPA buffer that only solubilized a fraction of LAT. Still, we observed some IgE-dependent coprecipitation of SWAP-70 and phosphorylated LAT, which disappeared after anti-IgE antibody cross-linking (Fig. 9B). However, the reverse precipitation, *i.e.* using anti-LAT antibody, did not precipitate LAT with any antibody tried. Together, these experiments indicate that SWAP-70 also affects LAT and thus an early step in Fc ϵ RI signaling.

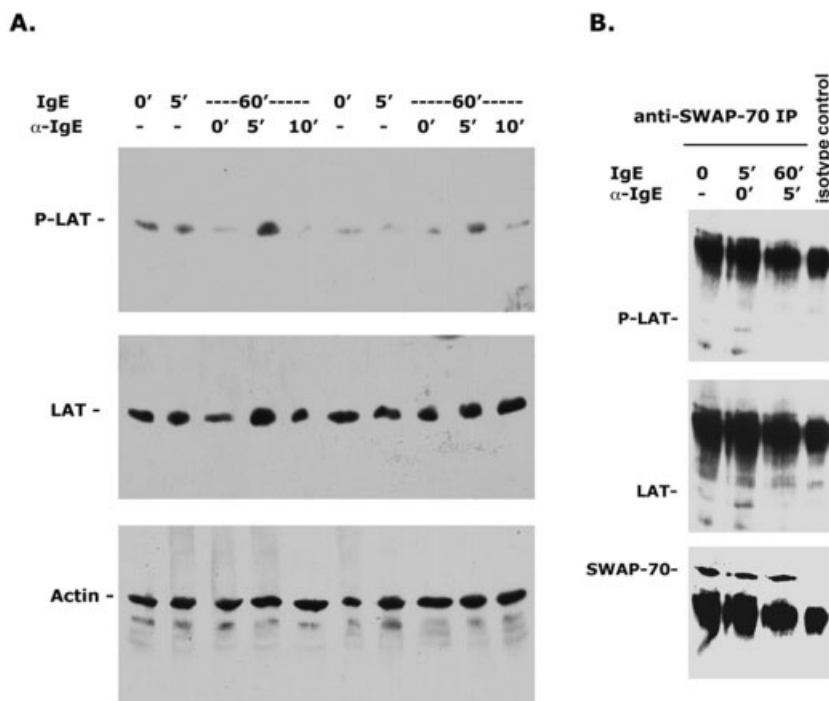


Figure 9. Activation of LAT upon Fc ϵ RI stimulation. SDS extracts from BMMC sensitized with IgE followed by anti-IgE antibody cross-linking (A) were subjected to SDS-PAGE followed by immunoblotting with antibodies specific for P-LAT (Tyr 171), total LAT and actin for control. The results shown are representative of three experiments. (B) Co-immunoprecipitation of LAT with SWAP-70. RIPA extracts from BMMC upon stimulation by anti-IgE antibody cross-linking following IgE presensitization were subjected to immunoprecipitation with anti-SWAP-70 antibody and immunoblotted with anti-P-LAT, anti-LAT followed by anti-SWAP-70 antibody.

Discussion

Initial data from a previous study suggested a role for SWAP-70 in mast cell Fc ϵ RI signaling [2], and we set out to identify specific requirements for SWAP-70 in the Fc ϵ RI signaling pathway. Our data reveal roles for SWAP-70 in the activation of LAT, Akt, ERK, and p38, in the production of PIP₃ and IP₃, and in the regulation of cytokine gene expression as illustrated in a working model (Fig. 10). In addition, IgE-dependent local and systemic anaphylaxis are impaired in SWAP-70^{-/-} mice, correlating with impaired Fc ϵ RI signaling and degranulation seen *in vitro*.

The key contribution of mast cells to anaphylactic reactions has been widely demonstrated. For example, anti-IgE antibody-induced mast cell degranulation is involved in anaphylactic reactions that result in cardiopulmonary changes and mortality in mice [54]. Mast cell-deficient W/W^v as well as Sl/Sl^d mice were shown to exhibit negligible anaphylactic responses upon anti-IgE antibody stimulation as compared to wt mice. Upon mast cell reconstitution, cardiopulmonary alterations and mortality were restored to wt levels, indicating the essential role for mast cell Fc ϵ RI signaling in anaphylaxis. Further, functional elimination of certain effectors of Fc ϵ RI signaling, like LAT [55], SLP-76 [56], Btk [57], or vav [42], causes diminished responses in mast cell-mediated anaphylaxis. In contrast, mice

lacking the signal transducer PIPKI α , which negatively regulates mast cell degranulation, exhibit enhanced anaphylactic responses upon Fc ϵ RI stimulation [58]. However, only a limited number of Fc ϵ RI signaling components are known, and only few have been shown to be involved in the anaphylactic reaction. Here, we demonstrate SWAP-70 as an important factor in passive cutaneous and active systemic anaphylaxis. The reduction in anaphylactic responses is likely a direct consequence of impaired Fc ϵ RI signaling as observed in SWAP-70^{-/-} BMMC; however, not all types of mature mast cells express SWAP-70, which is absent in mature peritoneal mast cells [2]. We have now determined that skin mast cells and stomach mucosal mast cells express SWAP-70. As reported earlier [2], there are somewhat fewer skin mast cells present in SWAP-70^{-/-} animals, but the number of mucosal mast cells, at least in the stomach, is like in wt mice. The strong reduction of systemic anaphylaxis indicates that SWAP-70 is particularly important in the mucosal cells, since they express SWAP-70, show an unaltered frequency in SWAP-70^{-/-} mice, and are similar to BMMC, which are impaired in degranulation in the absence of SWAP-70. The cutaneous anaphylactic response cannot be explained by the twofold reduced number of skin mast cells in SWAP-70^{-/-} mice since, if background signal is deducted, SWAP-70^{-/-} mice show essentially no cutaneous anaphylaxis signal at all. Thus, SWAP-70 is of key

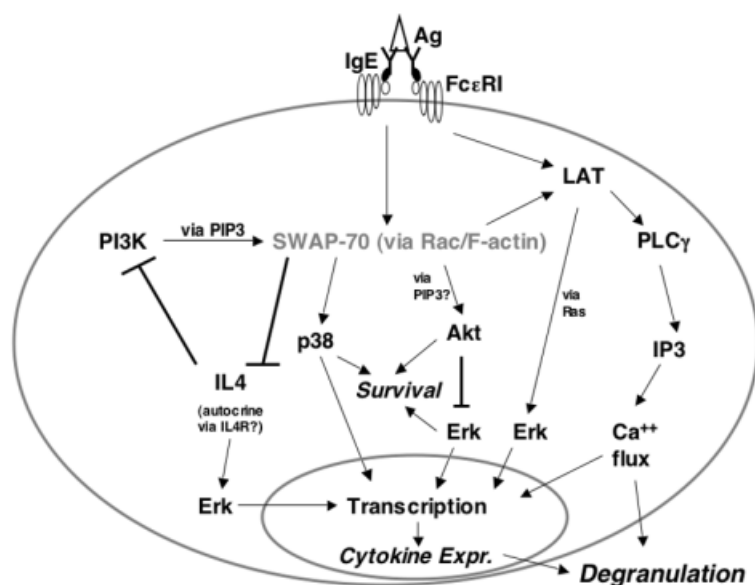


Figure 10. Hypothetical model for SWAP-70 function in mast cell Fc ϵ RI signaling (see Discussion section). Several of the processes indicated here occur at the cytoplasmic membrane, and there are certainly levels of crosstalk between these processes that are not illustrated. The LAT-Ras-ERK pathway is adopted from Gu et al. [71]. The Akt-mediated inhibitory effect of SWAP-70 on ERK cannot be less potent than the stimulatory effect of SWAP-70 through LAT on ERK, for ERK becomes hyperactivated in the absence of SWAP-70. It cannot be excluded that SWAP-70 also acts directly on PLC γ , ERK, Akt, and/or PI3K. SWAP-70 inhibition of the negative effect of IL-4 on PI3K supports PI3K activity. Based on our earlier reports, we hypothesize that many or all of the effects of SWAP-70 shown here are exercised through control of Rho GTPases and F-actin rearrangements of membrane-proximal cytoskeletal structures and their associated signaling molecules.

importance to the anaphylactic reaction. In any case, the two explanations for the reduced anaphylactic response in the skin – impaired Fc ϵ RI signaling in mature mast cells or developmentally decreased numbers of mature mast cells – are not mutually exclusive.

To unravel the specific contribution of SWAP-70 to Fc ϵ RI signaling, we tested the SWAP-70 dependence of a number of events associated with degranulation and Fc ϵ RI signaling and demonstrate a series of hitherto unknown functions of SWAP-70. Some functions, such as regulation of cytokine expression, are apparently downstream of PI3K. The supportive role of SWAP-70 in PI3K activation and PIP₃ production, however, is seemingly upstream of PI3K. Rather than classifying SWAP-70 as an upstream or downstream factor, we hypothesize that SWAP-70 plays a complex role in regulating and perhaps coordinating different signaling events within a signaling pathway such as the Fc ϵ RI pathway. Once activated by binding PIP₃, and likely by other events such as phosphorylation (Pearce and Jessberger, manuscript in preparation), SWAP-70 may – as our data indicate – in a feedback loop directly or indirectly (see Results section on PI3K activity and Fig. 8A) further increase or maintain the generation of PIP₃ itself. Thus, SWAP-70 may have a signal multiplier role. Although SWAP-70 may serve as a feedback multiplier for PI3K activation, e.g. by down-regulating IL-4 production, which has been shown to inhibit PI3K activity in an autocrine fashion [48], the loss of this feedback loop still allows production of some PIP₃.

The impairment in PIP₃ production seen in SWAP-70^{-/-} BMMC may be caused by increased IL-4 expression in these mutants, and therefore be an indirect effect. Interestingly, in c-fos-deficient mast cells, expression of many cytokines is reduced but IL-4 expression is increased [59], similar to SWAP-70^{-/-} BMMC. Since Lee *et al.* [59] also showed that c-fos^{-/-} mast cells express much less SWAP-70, our results suggest that the effect on cytokine expression seen in c-fos^{-/-} mast cells may be a consequence of reduced SWAP-70 expression. Mast cells contribute IL-4 especially in the early phase of an immune response, where their IL-4 supports expression of IL-4 in T cells [19, 60].

The cytokines that are only weakly expressed in SWAP-70^{-/-} BMMC belong to various classes, among them pro-inflammatory cytokines such as IL-1, IL-2, or TNF- α and the anti-inflammatory IL-10, rendering the biological consequences complex. While IL-4 may down-regulate expression of the Fc ϵ RI [61], IL-6 is thought to up-regulate it [62]. There is induction of IL-6 expression upon Fc ϵ RI cross-linking to almost wt levels in SWAP-70^{-/-} cells, but when only IgE is bound, SWAP-70^{-/-} BMMC only weakly express IL-6. The parallel up-regulation of IL-4 may suggest that expression of the Fc ϵ RI is no longer as much supported in the

SWAP-70^{-/-} cells as it is in wt cells if treated with IgE only. However, RT-PCR and FACS indicated equal levels of Fc ϵ RI expression in wt and SWAP-70^{-/-} BMMC before and after stimulation [2].

Cytokine gene expression depends on upstream signaling events that include molecules like ERK (IL-4) [63–65] or Akt (IL-2) [66]. Impaired Akt activation and ERK hyperactivation in SWAP-70^{-/-} BMMC may contribute to the observed changes in cytokine expression, which, however, may also be generated through other pathways, e.g. altered activation of transcription factors or translocation of factors into the nucleus through altered Rac activation and F-actin rearrangements. Nevertheless, the increase in IL-4 production correlates with increased ERK activation, and positive stimulatory crosstalk between IL-4 and ERK was reported [63].

Akt kinase acts in multiple processes including apoptosis and cell survival, proliferation, cell motility, gene expression and angiogenesis [67]. Akt is activated in signaling from several receptors including the Fc ϵ RI and c-kit and its activation depends among others on PIP₃ [68, 69]. Akt binds PIP₃ in the cytoplasmic membrane *via* its PH domain, becomes phosphorylated, and then dissociates from the membrane to act on various substrates. Phenotypic consequences of impaired Akt activation in BMMC may include reduced exocytosis, gene expression, or compromised cell survival [66]. Indeed, SWAP-70^{-/-} BMMC were found to be more sensitive to γ -irradiation [2], impaired in degranulation and to display altered cytokine gene expression patterns. Since activated Akt may inhibit ERK [70], hyperactivation of ERK observed in stimulated SWAP-70^{-/-} BMMC may be a result of impaired Akt activation. It remains to be shown, however, through which mechanism ERK becomes hyperactivated. ERK is known to be activated *via* the Ras/Raf/MEK pathway independently of PI3K/Akt [71, 72]. The up-regulation of ERK activity may constitute a compensatory mechanism for the loss of Akt activation to support cell survival. However, we cannot exclude that SWAP-70 acts through an Akt-independent pathway as a negative regulator of ERK. This pathway is also likely not dependent on the LAT/PLC γ /IP₃ pathway, for LAT and IP₃ are reduced in the absence of SWAP-70. Experiments to pharmacologically inhibit PI3K activity by the broad-spectrum PI3K inhibitor LY294002 abolished Akt and ERK activation in wt BMMC with and without Fc ϵ RI cross-linking, but did not abolish most of the activated ERK in SWAP-70^{-/-} BMMC, pointing to a PI3K-independent ERK activation pathway (not shown). IL-4 gene expression was not affected by the LY294002 treatment, indicating that regulation of cytokine gene expression occurs through a PI3K/Akt-independent pathway.

There are several lines of evidence indicating that the importance of SWAP-70 depends on the strength of the

signal originating at the Fc ϵ RI. Cross-linking IgE bound to Fc ϵ RI sometimes triggers similar responses in wt or mutant cells, *e.g.* in expression of IL-4, IL-5, IFN- γ , IL-6, or TNF- α . Binding of IgE, which weakly cross-links due to its partially oligomeric form to the Fc ϵ RI without further cross-linking – thus triggering a weaker signal – reveals a requirement for SWAP-70 for proper transcription of IL-4, IL-6 or TNF- α . Similarly, some phenotypes seen after Fc ϵ RI cross-linking are barely observable upon binding of IgE only, *e.g.* low p38 phosphorylation. Thus, for full amplification of a signal of limiting strength, SWAP-70 is often required. Upon cross-linking of the receptor, SWAP-70 deficiency can sometimes be overcome, probably by complementing factors or alternate converging pathways. The importance of the degree of activation of the Fc ϵ RI for the plasticity of mast cell responses has been described [21, 73]. SWAP-70, with its roles in low-strength signaling and as a regulator and perhaps signal multiplier, likely constitutes an important factor in determining Fc ϵ RI-dependent mast cell plasticity. Given the known role of SWAP-70 in rearrangement of cytoskeletal, cytoplasmic membrane-proximal F-actin structures, we hypothesize that SWAP-70 affects the Fc ϵ RI pathway and the specific components identified here through intracellular recruitment, assembly into proper signaling complexes at the right time and the right location. This hypothesis can now be tested.

Materials and methods

Bone marrow-derived murine mast cell (BMMC) cultures were derived from wt or SWAP-70 $^{-/-}$ mice on the 129SvEMS genetic background. Cell culture procedures, reagents and assays for phosphorylation and activity of Akt and ERK are described in Sivalenka and Jessberger [16]. pEGFP vectors expressing SWAP-70 or the PIP $_3$ binding-defective SWAP-70 mutant R230C have been described earlier [7], as were degranulation assays [2]. The IL-4 ELISA kit was obtained from Peprotech, NJ, USA. p38 phosphorylation and activity assays and RT-PCR for cytokine expression are described in the Supporting Information.

Passive cutaneous anaphylaxis

To induce PCA, SWAP-70 $^{-/-}$ or wt control mice were injected intradermally with 20 μ g IgE (anti-DNP antibody, clone SPE-7) in HBSS into their left ears and HBSS as control into the right ears. After 24 h, the mice were challenged intravenously with 100 μ g DNP-BSA in HBSS containing 1% Evans blue. Control mice received HBSS with Evans blue alone without DNP-BSA. At 30 min after antigen challenge, quantification of extravasated Evans blue dye was performed. Briefly, ear tissues were minced in formamide and incubated at 80°C for 2 h. The optical density of the dye in the extract was measured at 610 nm using a spectrophotometer. The optical density of

samples extracted from the right ears (IgE-primed) was normalized to the internal control samples from the respective left ears (non-primed).

Active systemic anaphylaxis

Wild-type or SWAP-70 $^{-/-}$ mice were sensitized by intravenous injection of 50 μ g anti-DNP IgE, and were challenged with 500 μ g DNP-KLH per mouse intravenously after 48 h. Respective controls were performed without IgE sensitization. Changes in body temperature associated with anaphylaxis [33] were monitored by measuring the rectal temperature before and up to 2.5 h after antigen challenge using a rectal probe (Yellow Springs Instrument Co., Yellow Springs, OH).

Nucleofection of BMMC

Cultured wt or SWAP-70 $^{-/-}$ BMMC (1×10^6 /sample) were nucleofected with 2.5 μ g pEGFP-C1 vector alone, pEGFP containing SWAP-70, pEGFP containing the R230C PIP $_3$ binding-defective mutant of SWAP-70, or were mock transfected without DNA for control. An AMAXA nucleofection kit (macrophage kit) from AMAXA Biosystems was used with the Y01 program as per the manufacturer's instructions. After 48 h, BMMC were harvested and transfection efficiency was measured by FACS analysis. An average of about 20–30% BMMC were positive for GFP. In control nucleotransfections, viability and proliferation of transfected BMMC and their responsiveness to IL-3 or SCF were found to be normal.

Statistics

Where indicated, statistics were calculated using Student's *t*-test.

PI3K activity assay

PI3K activity was measured using an activity ELISA kit obtained from Echelon Biosciences Inc., USA, as per the manufacturer's instructions. Briefly, PI3K was immunoprecipitated (rabbit anti-p85 antibody; Cell Signaling Technology Inc.) from 10^6 BMMC stimulated with 0.5 μ g/mL IgE and 0.5 μ g/mL anti-IgE antibody for various time intervals. Subsequently, immunoprecipitated PI3K was used in kinase assays, with 100 pmol (2.4 μ g) diC $_8$ PI(4,5)P $_2$ as substrate. Reactions were terminated by addition of EDTA to 5 mM, and the reaction mixtures were transferred to an incubation plate. Alternatively, standards were prepared in separate wells of the same incubation plate containing PIP $_3$ at concentrations ranging from 0 to 100 pmol per 50 μ L standard solution. Standards and samples were run in triplicate. An equal volume of PIP $_3$ detector (1 : 200) was added to each of the wells and incubated at room temperature for 60 min in the dark. Of the secondary detection reagent (1 : 40 dilution), 100 μ L was added and incubated for another 60 min. Solutions were then discarded and wells washed with 300 μ L Tris-buffered saline (TBS) or TBS containing 0.05% Tween-20. 100 μ L TMB was added to each well, and reactions were stopped after color development (within 20–30 min) by addition of 100 μ L 0.5 M H $_2$ SO $_4$. Absorbance was measured at 450 nm.

Measurement of IP₃ production

IP₃ production was measured using the commercially available assay kit BIOTRAK TRK1000 (Amersham Pharmacia Biotech, NJ). BMMC (10⁷) were sensitized with anti-DNP IgE and stimulated in PBS containing protease and phosphatase inhibitors in the absence or presence of 0.5 µg/mL anti-IgE antibody. Stimulation was terminated at the desired time intervals by adding 1 : 10 v/v ice-cold 100% trichloroacetic acid. Samples were then neutralized and IP₃ partitioned into the aqueous phase using water-saturated diethyl ether. The amount of IP₃ in each sample was quantified by employing competitive binding assays of IP₃ to an IP₃-binding protein, competed for by a tracer of D-myo-H3 inositol 1,4,5-trisphosphate. Standard IP₃ provided with the kit was assayed simultaneously in concentrations ranging between 0.19 and 25 pmol; pmol IP₃ in a sample was derived from the standard curve.

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

References

- Borggreffe, T., Wabl, M., Akhmedov, A. T. and Jessberger, R., A B-cell-specific DNA recombination complex. *J. Biol. Chem.* 1998. **273**: 17025–17035.
- Gross, B., Borggreffe, T., Wabl, M., Sivalenka, R. R., Bennett, M., Rossi, A. B. and Jessberger, R., SWAP-70-deficient mast cells are impaired in development and IgE-mediated degranulation. *Eur. J. Immunol.* 2002. **32**: 1121–1128.
- Zheng, Y., Dbl family guanine nucleotide exchange factors. *Trends Biochem. Sci.* 2001. **26**: 724–732.
- Rossman, K. L., Der, C. J. and Sodek, J., GEF means go: Turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* 2005. **6**: 167–180.
- Takai, Y., Sasaki, T. and Matozaki, T., Small GTP-binding proteins. *Physiol. Rev.* 2001. **81**: 153–208.
- Etienne-Manneville, S. and Hall, A., Rho GTPases in cell biology. *Nature* 2002. **420**: 629–635.
- Shinohara, M., Terada, Y., Iwamatsu, A., Shinohara, A., Mochizuki, N., Higuchi, M., Gotoh, Y. et al., SWAP-70 is a guanine-nucleotide-exchange factor that mediates signalling of membrane ruffling. *Nature* 2002. **416**: 759–763.
- Borggreffe, T., Keshavarzi, S., Gross, B., Wabl, M. and Jessberger, R., Impaired IgE response in SWAP-70-deficient mice. *Eur. J. Immunol.* 2001. **31**: 2467–2475.
- Masat, L., Caldwell, J., Armstrong, R., Khoshnevisan, H., Jessberger, R., Herndier, B., Wabl, M. and Ferrick, D., Association of SWAP-70 with the B cell antigen receptor complex. *Proc. Natl. Acad. Sci. USA* 2000. **97**: 2180–2184.
- Lemmon, M. A., Ferguson, K. M. and Abrams, C. S., Pleckstrin homology domains and the cytoskeleton. *FEBS Lett.* 2002. **513**: 71–76.
- Fukui, Y., Wakamatsu, I., Tachikawa, H., Okamura, Y., Tanaka, T. and Ihara, S., Activity of beta3-beta4 loop of the PH domain is required for the membrane targeting of SWAP-70. *J. Biol. Chem.* 2007. **282**: 99–103.
- Kraynov, V. S., Chamberlain, C., Bokoch, G. M., Schwartz, M. A., Slabaugh, S. and Hahn, K. M., Localized Rac activation dynamics visualized in living cells. *Science* 2000. **290**: 333–337.
- Hotfilder, M., Baxendale, S., Cross, M. A. and Sablitzky, F., Def-2, -3, -6 and -8, novel mouse genes differentially expressed in the haemopoietic system. *Br. J. Haematol.* 1999. **106**: 335–344.
- Tanaka, Y., Bi, K., Kitamura, R., Hong, S., Altman, Y., Matsumoto, A., Tabata, H., Lebedeva, S. et al., SWAP-70-like adapter of T cells, an adapter protein that regulates early TCR-initiated signaling in Th2 lineage cells. *Immunity* 2003. **18**: 403–414.
- Gupta, S., Lee, A., Hu, C., Fanzo, J., Goldberg, I., Cattoretti, G. and Pernis, A. B., Molecular cloning of IBP, a SWAP-70 homologous GEF, which is highly expressed in the immune system. *Hum. Immunol.* 2003. **64**: 389–401.
- Sivalenka, R. R. and Jessberger, R., SWAP-70 regulates c-kit-induced mast cell activation, cell-cell adhesion, and migration. *Mol. Cell. Biol.* 2004. **24**: 10277–10288.
- Metcalfe, D. D., Baram, D. and Mekori, Y. A., Mast cells. *Physiol. Rev.* 1997. **77**: 1033–1079.
- Li, L. and Krilis, S. A., Mast-cell growth and differentiation. *Allergy* 1999. **54**: 306–312.
- Galli, S. J., Nakae, S. and Tsai, M., Mast cells in the development of adaptive immune responses. *Nat. Immunol.* 2005. **6**: 135–142.
- Metz, M. and Maurer, M., Mast cells – key effector cells in immune responses. *Trends Immunol.* 2007. **28**: 234–241.
- Kalesnikoff, J., Huber, M., Lam, V., Damen, J. E., Zhang, J., Siraganian, R. P. and Krystal, G., Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity* 2001. **14**: 801–811.
- Kitaura, J., Song, J., Tsai, M., Asai, K., Maeda-Yamamoto, M., Mocsai, A., Kawakami, Y. et al., Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the FcεRI. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 12911–12916.
- Kitaura, J., Kinoshita, T., Matsumoto, M., Chung, S., Kawakami, Y., Leitges, M., Wu, D. et al., IgE- and IgE+Ag-mediated mast cell migration in an autocrine/paracrine fashion. *Blood* 2005. **105**: 3222–3229.
- Daeron, M., Fc receptor biology. *Annu. Rev. Immunol.* 1997. **15**: 203–234.
- Tkaczyk, C. and Gilfillan, A. M., Fc(ε)RI-dependent signaling pathways in human mast cells. *Clin. Immunol.* 2001. **99**: 198–210.
- Rivera, J., Arudchandran, R., Gonzalez-Espinosa, C., Manetz, T. S. and Xirasagar, S., A perspective: Regulation of IgE receptor-mediated mast cell responses by a LAT-organized plasma membrane-localized signaling complex. *Int. Arch. Allergy Immunol.* 2001. **124**: 137–141.
- Iwaki, S., Tkaczyk, C., Metcalfe, D. D. and Gilfillan, A. M., Roles of adaptor molecules in mast cell activation. *Chem. Immunol. Allergy* 2005. **87**: 43–58.
- Gilfillan, A. M. and Tkaczyk, C., Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 2006. **6**: 218–230.
- Kopec, A., Panaszek, B. and Fal, A. M., Intracellular signaling pathways in IgE-dependent mast cell activation. *Arch. Immunol. Ther. Exp. (Warsz.)* 2006. **54**: 393–401.
- Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H. and Kitamura, Y., Nerve growth factor induces development of connective tissue-type mast cells *in vitro* from murine bone marrow cells. *J. Exp. Med.* 1991. **174**: 7–14.
- Smith, T. J., Ducharme, L. A. and Weis, J. H., Preferential expression of interleukin-12 or interleukin-4 by murine bone marrow mast cells derived in mast cell growth factor or interleukin-3. *Eur. J. Immunol.* 1994. **24**: 822–826.
- Inagaki, N., Goto, S., Yamasaki, M., Nagai, H. and Koda, A., Studies on vascular permeability increasing factors involved in 48-hour homologous PCA in the mouse ear. *Int. Arch. Allergy Appl. Immunol.* 1986. **80**: 285–290.
- Dombrowicz, D., Flamand, V., Brigman, K. K., Koller, B. H. and Kinet, J. P., Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. *Cell* 1993. **75**: 969–976.
- Tharp, M. D., Seelig, L. L., Jr., Tigelaar, R. E. and Bergstresser, P. R., Conjugated avidin binds to mast cell granules. *J. Histochem. Cytochem.* 1985. **33**: 27–32.

- 35 Wakamatsu, I., Ihara, S. and Fukui, Y., Mutational analysis on the function of the SWAP-70 PH domain. *Mol. Cell. Biochem.* 2006. **293**: 137–145.
- 36 Bradding, P., Feather, I. H., Howarth, P. H., Mueller, R., Roberts, J. A., Britten, K., Bewes, J. P. et al., Interleukin 4 is localized to and released by human mast cells. *J. Exp. Med.* 1992. **176**: 1381–1386.
- 37 Tkaczyk, C., Villa, I., Peronet, R., David, B., Chouaib, S. and Mecheri, S., *In vitro* and *in vivo* immunostimulatory potential of bone marrow-derived mast cells on B- and T-lymphocyte activation. *J. Allergy Clin. Immunol.* 2000. **105**: 134–142.
- 38 Howe, A. K., Aplin, A. E. and Juliano, R. L., Anchorage-dependent ERK signaling – mechanisms and consequences. *Curr. Opin. Genet. Dev.* 2002. **12**: 30–35.
- 39 Johnson, G. L. and Lapadat, R., Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002. **298**: 1911–1912.
- 40 Yamasaki, S., Ishikawa, E., Kohno, M. and Saito, T., The quantity and duration of Fc γ signals determine mast cell degranulation and survival. *Blood* 2004. **103**: 3093–3101.
- 41 Ishizuka, T., Chayama, K., Takeda, K., Hamelmann, E., Terada, N., Keller, G. M., Johnson, G. L. and Gelfand, E. W., Mitogen-activated protein kinase activation through Fc epsilon receptor I and stem cell factor receptor is differentially regulated by phosphatidylinositol 3-kinase and calcineurin in mouse bone marrow-derived mast cells. *J. Immunol.* 1999. **162**: 2087–2094.
- 42 Manetz, T. S., Gonzalez-Espinosa, C., Arudchandran, R., Xirasagar, S., Tybulewicz, V. and Rivera, J., Vav1 regulates phospholipase C gamma activation and calcium responses in mast cells. *Mol. Cell. Biol.* 2001. **21**: 3763–3774.
- 43 Yung, Y., Yao, Z., Hanoch, T. and Seger, R., ERK1b, a 46-kDa ERK isoform that is differentially regulated by MEK. *J. Biol. Chem.* 2000. **275**: 15799–15808.
- 44 Boudreau, R. T., Hoskin, D. W. and Lin, T. J., Phosphatase inhibition potentiates IL-6 production by mast cells in response to Fc epsilon RI-mediated activation: Involvement of p38 MAPK. *J. Leukoc. Biol.* 2004. **76**: 1075–1081.
- 45 Lam, V., Kalesnikoff, J., Lee, C. W., Hernandez-Hansen, V., Wilson, B. S., Oliver, J. M. and Krystal, G., IgE alone stimulates mast cell adhesion to fibronectin via pathways similar to those used by IgE + antigen but distinct from those used by Steel factor. *Blood* 2003. **102**: 1405–1413.
- 46 Fukao, T., Terauchi, Y., Kadowaki, T. and Koyasu, S., Role of phosphoinositide 3-kinase signaling in mast cells: New insights from knockout mouse studies. *J. Mol. Med.* 2003. **81**: 524–535.
- 47 Cantley, L. C., The phosphoinositide 3-kinase pathway. *Science* 2002. **296**: 1655–1657.
- 48 Imani, F., Rager, K. J., Catipovic, B. and Marsh, D. G., Interleukin-4 (IL-4) induces phosphatidylinositol 3-kinase (p85) dephosphorylation. Implications for the role of SHP-1 in the IL-4-induced signals in human B cells. *J. Biol. Chem.* 1997. **272**: 7927–7931.
- 49 Kurosaki, T., Maeda, A., Ishiai, M., Hashimoto, A., Inabe, K. and Takata, M., Regulation of the phospholipase C-gamma2 pathway in B cells. *Immunol. Rev.* 2000. **176**: 19–29.
- 50 Wilde, J. I. and Watson, S. P., Regulation of phospholipase C gamma isoforms in haematopoietic cells: Why one, not the other? *Cell Signal.* 2001. **13**: 691–701.
- 51 Tkaczyk, C., Beaven, M. A., Brachman, S. M., Metcalfe, D. D. and Gilfillan, A. M., The phospholipase C gamma 1-dependent pathway of Fc epsilon RI-mediated mast cell activation is regulated independently of phosphatidylinositol 3-kinase. *J. Biol. Chem.* 2003. **278**: 48474–48484.
- 52 Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F. and Beaven, M. A., Ca(2+)-dependent and Ca(2+)-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca²⁺ and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* 1993. **268**: 1749–1756.
- 53 Saitoh, S., Arudchandran, R., Manetz, T. S., Zhang, W., Sommers, C. L., Love, P. E., Rivera, J. and Samelson, L. E., LAT is essential for Fc(epsilon)RI-mediated mast cell activation. *Immunity* 2000. **12**: 525–535.
- 54 Martin, T. R., Galli, S. J., Katona, I. M. and Drazen, J. M., Role of mast cells in anaphylaxis. Evidence for the importance of mast cells in the cardiopulmonary alterations and death induced by anti-IgE in mice. *J. Clin. Invest.* 1989. **83**: 1375–1383.
- 55 Saitoh, S., Odom, S., Gomez, G., Sommers, C. L., Young, H. A., Rivera, J. and Samelson, L. E., The four distal tyrosines are required for LAT-dependent signaling in Fc epsilon RI-mediated mast cell activation. *J. Exp. Med.* 2003. **198**: 831–843.
- 56 Pivniouk, V. I., Martin, T. R., Lu-Kuo, J. M., Katz, H. R., Oettgen, H. C. and Geha, R. S., SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. *J. Clin. Invest.* 1999. **103**: 1737–1743.
- 57 Hata, D., Kawakami, Y., Inagaki, N., Lantz, C. S., Kitamura, T., Khan, W. N., Maeda-Yamamoto, M. et al., Involvement of Bruton's tyrosine kinase in Fc epsilon RI-dependent mast cell degranulation and cytokine production. *J. Exp. Med.* 1998. **187**: 1235–1247.
- 58 Sasaki, J., Sasaki, T., Yamazaki, M., Matsuoka, K., Taya, C., Shitara, H., Takasuga, S. et al., Regulation of anaphylactic responses by phosphatidylinositol phosphate kinase type I alpha. *J. Exp. Med.* 2005. **201**: 859–870.
- 59 Lee, Y. N., Tuckerman, J., Nechushtan, H., Schutz, G., Razin, E. and Angel, P., c-Fos as a regulator of degranulation and cytokine production in Fc epsilon RI-activated mast cells. *J. Immunol.* 2004. **173**: 2571–2577.
- 60 Gessner, A., Mohrs, K. and Mohrs, M., Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. *J. Immunol.* 2005. **174**: 1063–1072.
- 61 Ryan, J. J., DeSimone, S., Klisch, G., Shelburne, C., McReynolds, L. J., Han, K., Kovacs, R. et al., IL-4 inhibits mouse mast cell Fc epsilon RI expression through a STAT6-dependent mechanism. *J. Immunol.* 1998. **161**: 6915–6923.
- 62 Conti, P., Kempuraj, D., Di Gioacchino, M., Boucher, W., Letourneau, R., Kandere, K., Barbacane, R. C. et al., Interleukin-6 and mast cells. *Allergy Asthma Proc.* 2002. **23**: 331–335.
- 63 So, E. Y., Oh, J., Jang, J. Y., Kim, J. H. and Lee, C. E., Ras/Erk pathway positively regulates Jak1/STAT6 activity and IL-4 gene expression in Jurkat T cells. *Mol. Immunol.* 2007. **44**: 3416–3426.
- 64 Lin, D. A. and Boyce, J. A., IL-4 regulates MEK expression required for lysophosphatidic acid-mediated chemokine generation by human mast cells. *J. Immunol.* 2005. **175**: 5430–5438.
- 65 Turner, H. and Cantrell, D. A., Distinct Ras effector pathways are involved in Fc epsilon RI regulation of the transcriptional activity of Elk-1 and NFAT in mast cells. *J. Exp. Med.* 1997. **185**: 43–53.
- 66 Kitaura, J., Asai, K., Maeda-Yamamoto, M., Kawakami, Y., Kikkawa, U. and Kawakami, T., Akt-dependent cytokine production in mast cells. *J. Exp. Med.* 2000. **192**: 729–740.
- 67 Brazil, D. P., Park, J. and Hemmings, B. A., PKB binding proteins. Getting in on the Akt. *Cell* 2002. **111**: 293–303.
- 68 Yang, F. C., Kapur, R., King, A. J., Tao, W., Kim, C., Borneo, J., Breese, R. et al., Rac2 stimulates Akt activation affecting BAD/Bcl-xL expression while mediating survival and actin function in primary mast cells. *Immunity* 2000. **12**: 557–568.
- 69 Djouder, N., Schmidt, G., Frings, M., Cavalie, A., Thelen, M. and Aktories, K., Rac and phosphatidylinositol 3-kinase regulate the protein kinase B in Fc epsilon RI signaling in RBL 2H3 mast cells. *J. Immunol.* 2001. **166**: 1627–1634.
- 70 Friedman, A. and Perrimon, N., A functional RNAi screen for regulators of receptor tyrosine kinase and ERK signalling. *Nature* 2006. **444**: 230–234.
- 71 Gu, H., Saito, K., Klamann, L. D., Shen, J., Fleming, T., Wang, Y., Pratt, J. C. et al., Essential role for Gab2 in the allergic response. *Nature* 2001. **412**: 186–190.
- 72 Ali, K., Bilancio, A., Thomas, M., Pearce, W., Gilfillan, A. M., Tkaczyk, C., Kuehn, N. et al., Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. *Nature* 2004. **431**: 1007–1011.
- 73 Gonzalez-Espinosa, C., Odom, S., Olivera, A., Hobson, J. P., Martinez, M. E., Oliveira-Dos-Santos, A., Barra, L. et al., Preferential signaling and induction of allergy-promoting lymphokines upon weak stimulation of the high affinity IgE receptor on mast cells. *J. Exp. Med.* 2003. **197**: 1453–1465.