

Proteomic analysis of the SLP76 interactome in resting and activated primary mast cells

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Running title: The SLP76 interactome in mast cells.

Abbreviations

ADAP: Adhesion- and Degranulation-promoting Adapter Protein
Bcr: Breakpoint Cluster Region protein
BMMC: Bone marrow-derived mast cells
DAG: diacyl-glycerol
DNP: Dinitrophenyl
ES: Embryonic stem
FcεRI: high-affinity receptors for the Fc portion of IgE
FDR: false discovery rate
Fyb: Fyn-binding protein
Grap2: Grb2-related adapter protein 2
Grb2: growth factor receptor-bound protein 2
HSA: human serum albumin
iBAQ: intensity-based absolute quantification
IP3: inositol tris-phosphate
ITAM: Immunoreceptor Tyrosine-based Activation Motif
Itk : IL-2-inducible T-cell kinase
KI: knock-in
LAT: Linker of Activation of T cells
LCP2: Lymphocyte Cytosolic Protein 2
LM: Laurylmaltoside
mAb: monoclonal antibody
MAP: Mitogen-activated protein
MS/MS: tandem mass spectrometry
nanoLC: nano liquid chromatography
Nck: non-catalytic region of tyrosine kinase adapter protein
NTAL: non-T cell activation linker
OST: One-Strep-Tag
PAG: Phosphoprotein associated with glycosphingolipid-enriched microdomains
PLC: Phospholipase C
SH: Src Homology
SHIP: SH2 domain-containing inositol 5'-phosphatase
SKAP: Src kinase-associated phosphoproteins
SLP76: SH2 domain-containing Leukocyte Protein of 76 kDa
SDS: Sodium dodecyl sulfate
TCA: Trichloroacetic acid
TCR: T Cell Receptor
WT: wild-type
ZAP70: Zeta-Associated Protein of 70 kDa

Summary.

We report the first proteomic analysis of the SLP76 interactome in resting and activated primary mouse mast cells. This was made possible by a novel genetic approach used for the first time here. It consists in generating knock-in mice that express signaling molecules bearing a C-terminal tag that has a high affinity for a streptavidin analogue. Tagged molecules can be used as molecular baits to affinity-purify the molecular complex in which they are engaged, which can then be studied by mass spectrometry. We examined first SLP76 because, although this cytosolic adapter is critical for both T cell and mast cell activation, its role is well known in T cells but not in mast cells. Tagged SLP76 was expressed in physiological amounts and fully functional in mast cells. We unexpectedly found that SLP76 is exquisitely sensitive to mast cell granular proteases, that Zn^{2+} -dependent metalloproteases are especially abundant in mast cells and that they were responsible for SLP76 degradation. Adding a Zn^{2+} chelator fully protected SLP76 in mast cell lysates, thereby enabling an efficient affinity-purification of this adapter with its partners. Label-free quantitative mass spectrometry analysis of affinity-purified SLP76 interactomes uncovered both partners already described in T cells and novel partners seen in mast cells only. Noticeably, molecules inducibly recruited in both cell types primarily concur to activation signals, whereas molecules recruited in activated mast cells only are associated with inhibition signals. The transmembrane adapter LAT2, and the serine/threonine kinase with an exchange factor activity Bcr were the most recruited molecules. Biochemical and functional validations established the unexpected finding that Bcr is recruited by SLP76 and positively regulates antigen-induced mast cell activation. Knock-in mice expressing tagged molecules with a normal tissue distribution and expression therefore provide potent novel tools to investigate signalosomes and to uncover novel signaling molecules in mast cells.

Introduction

Mast cells play critical roles in the initiation of IgE-dependent allergic inflammation (1). They express high-affinity receptors for the Fc portion of IgE (FcεRI), which are prototypic immunoreceptors (2). Mast cell FcεRI are composed of an IgE-binding subunit, FcεRIα, and of two Immunoreceptor Tyrosine-based Activation Motif (ITAM)-containing subunits, FcRβ and FcRγ (3). FcεRI activate mast cells when receptor-bound IgE antibodies are aggregated by a multivalent specific antigen (4). FcεRI aggregation triggers the constitution of signalosomes in which positive and negative signals are generated, the integration of which determines quantitatively and qualitatively the biological responses of the mast cell. The composition of signalosomes is likely to evolve rapidly, as molecules are sequentially recruited and as enzymes act on their substrates. Determining the composition and describing the dynamics of FcεRI signalosomes is a major challenge for who aims at understanding fundamental mechanisms of allergy and at developing therapeutic tools for controlling allergic reactions.

Mass spectrometry (MS)-based proteomics has emerged as a powerful tool to study signaling networks. Indeed, it enables large-scale analysis of stimulus-induced post-translational modifications (5-7). Thus, MS-based proteomics has been used to identify molecular partners that, at any given time, are associated with a molecular bait of interest (8). This bait carries an affinity-purification tag that markedly enhances the efficiency with which it can be purified from a cell lysate (9). The experimental conditions used may, however, limit the significance of the result. Classically, baits are over-expressed in cells that already express an untagged endogenous version of the bait. Unbalanced expression of corresponding molecules may profoundly alter biological

responses. In some cases, baits are expressed in cells that do not normally express the molecule, where they may generate artefactual signalosomes. Finally, baits are often expressed in transformed cells that can be grown in high numbers, so that affinity-purified molecules are obtained in amounts amenable to MS analysis (10, 11). Signaling pathways can be constitutively activated in these cells, due to the expression of transforming oncogenes.

To overcome these problems, we generated a series of knock-in (KI) mice expressing each a key signaling molecule with a C-terminal One-Strep-Tag (OST) (12). OST-tagged molecules, as well as the molecules with which they interact can be affinity-purified using beads coated with Strep-Tactin. Strep-Tactin is a streptavidin derivative that has a high affinity for OST (12). Affinity-purified molecules can then be identified by MS. As documented here, this novel approach enables one to study the interactome of endogenous OST-tagged molecules that are present in physiological amounts, in non-transformed cells by which they are normally expressed. Combining the interactomes of each tagged molecule analyzed before and at different times after FcεRI engagement should ultimately make it possible to obtain a comprehensive dynamic functional map of the FcεRI signalosome. As a proof-of-concept, we studied the interactome of the Src Homology (SH)2 domain-containing leukocyte protein of 76 kDa (SLP76) (a.k.a. Lymphocyte cytosolic protein 2 or LCP2) in primary mouse mast cells.

SLP76 is a cytosolic adapter that nucleates signaling complexes generated by immunoreceptors (13). It contains a N-terminal leucine Zip motif, a tyrosine-rich domain, a proline-rich domain and a C-terminal SH2 domain. It is constitutively associated with the growth factor receptor-bound protein 2 (Grb2)-related adapter protein 2 (Grap2, a.k.a. GADS) through the interaction of its proline residues with one of

the SH3 domains of Grap2 (14). SLP76 was mostly studied in T lymphocytes. Following T Cell Receptor (TCR) engagement, Grap2 binds to phosphorylated residues in the raft-associated transmembrane adapter Linker of Activation of T cells LAT1. SLP76-Grap2 complexes bind to Phospholipase C (PLC) γ -1 (15), which stabilizes the recruitment of this enzyme by LAT1. SLP76 tyrosines are phosphorylated by the cytosolic kinase zeta-associated protein of 70 kDa (ZAP70) (16, 17), which provides binding sites for the SH2 domains of Tec kinases such as the IL-2-inducible T-cell kinase (Itk)(18), for the guanine nucleotide exchange factors Vav-1 (17) and for adapters such as the non-catalytic region of tyrosine kinase adapter protein (Nck) (19). Molecules recruited by SLP76 launch the intracellular propagation of signals leading to calcium mobilization, the activation of Mitogen-activated protein (MAP) kinases and, ultimately, full-blown T cell responses.

SLP76 is also expressed by mast cells, and mast cell activation was markedly impaired in SLP76-deficient mice (20). Surprisingly, signaling molecules that interact with SLP76 in mast cells are not well characterized, and only a few papers have been published on the subject. It was however shown that the function of SLP76 in mast cells may not depend entirely on LAT1, as it does in T cells. Indeed, LAT1 deficiency could be, at least in part, compensated by the presence of LAT2 in mast cells (21). LAT2 (a.k.a. non-T cell activation linker or NTAL) is a raft-associated tyrosine-rich transmembrane adapter related to LAT1. LAT2 is absent in resting T cells, but present in B cells where it fulfills some of the functions fulfilled by LAT1 in T cells. In mast cells, LAT1 and LAT2 function as a pair of antagonistic molecules (22). The role of SLP76 in mast cells cannot therefore be deduced from that of SLP76 in T cells, and the mechanism(s) by which it contributes to mast cell activation needs to be established.

We report here the first description of the SLP76 interactome in resting and activated primary cultured mast cells. This was done by combining high-affinity purification and label-free quantitative proteomics in mast cells derived from KI mice expressing SLP-76 with a C-terminal OST (*Slp76^{OST/OST}* mice). To reach this goal, we first unraveled and solved an unexpected problem due to proteases contained in high amounts in mast cell granules. We found that, compared to the T cell SLP76 interactome, the mast cell SLP76 interactome is enriched in molecules involved in negative signaling. We also found that the *Breakpoint Cluster Region protein* Bcr is inducibly recruited by SLP76 and we could validate this unexpected finding by showing that mast cells from Bcr-deficient mice released less granular mediators than mast cells from wt mice. This is the first evidence that Bcr contributes to FcεRI signaling.

Experimental Procedures

Generation of *Slp76*^{OST} mice. A 6.2-kb genomic fragment containing exons 19-21 of the *Slp76* gene was isolated from a BAC clone (clone # RP23-216O16A; <http://www.lifesciences.sourcebioscience>) of C57BL/6J origin. A OST-(Stop)₂loxP-tACE-CRE-PGK-gb2-neo-loxP cassette (23) was introduced at the 3' end of the *Slp76* coding sequence found in exon 21 using homologous recombination (24). The targeting construct was then abutted to a thymidine kinase expression cassette and linearized with *FseI*. JM8.F6 C57BL/6N ES cells (25) were electroporated with the *Slp76*^{OST} targeting vector and selected in G418 and gancyclovir. Colonies were screened for homologous recombination by Southern blot. A neomycin-specific probe was used to ensure that adventitious non-homologous recombination events had not occurred in the selected ES clones. Appropriately targeted ES cells were injected into FVB blastocysts. After germline transmission, screening for the deletion of the neo cassette and the presence of the intended modification was performed by PCR and Southern blot. All experiments were done in accordance with French and European guidelines for animal care.

Cells: *Bone marrow-derived mast cells (BMMC):* BMMC were generated from femoral bone marrow harvested from *Slp76*^{OST/OST} and C57BL/6 (*Slp76*^{+/+}) control mice (Charles River, France), from *Bcr*^{-/-} and littermate control (*Bcr*^{+/+}) mice (a gift from Dr. Eunjoon Kim, Korea Advanced Institute of Science and Technology, Daejeon, South Korea), and from *Dok-3*^{-/-} and littermate control (*Dok-3*^{+/+}) mice (a gift from Dr. Kong-Peng Lam, Singapore Immunology Network, Singapore). BMMC were propagated and cultured in OptiMEM (Gibco, St Aubin, France) supplemented with 10% FBS (PAA Laboratories, Austria), 50 μ M 2-mercaptoethanol (Gibco), 100 μ g/ml Penicillin-Streptomycin (Gibco),

and 2 ng/ml recombinant murine IL-3 (Biolegend, San Diego, CA). Homogeneous populations of FcεRI⁺, Kit⁺ cells were obtained after 3 weeks of culture. *Thymocytes*: Thymi were collected from 6-week old C57BL/6 mice. Red cells were lysed in 10 mM Tris-HCL pH 7.5 and 142 mM NH₄Cl.

Gene expression analysis: Three independent cultures were seeded from the bone marrow of three individual mice and BMMC were generated as described. Total RNA was extracted from resting BMMC from the three replicate cultures using TRIzol reagent (Life Technologies, Gibco), and RNeasy mini kit from Qiagen (Courtaboeuf, France). Purified total RNA was amplified by RT-PCR, and transcripts were analyzed using the Applied Biosystems AB1700 microarrays (Carlsbad, California). We estimated the number of proteases expressed in BMMC, according to the procedure described in *Supplementary Materials*. 539 out of the 685 annotated protease-coding transcripts were found to be represented in the AB1700 microarrays. Among them, 400 genes were found expressed in BMMC with a confidence level greater than 90% (Figure 3A). Table S2 reports the results for the expression of the 539 protease-coding genes covered by the AB1700 microarrays.

Mast cell stimulation: BMMC were sensitized overnight with IgE anti-DNP (mAb 2682-I) supernatant in complete culture medium (100 ng/ml IgE, final concentration). Cells were extensively washed with HEPES-containing RPMI (Gibco), and challenged for various periods of time at 37 °C with 100 ng/ml DNP-HSA (Sigma-Aldrich, St. Louis, MO) in the same medium.

β-hexosaminidase release: IgE anti-DNP-sensitized cells were challenged for 20 min at 37°C with the indicated concentrations of DNP-HSA. β-hexosaminidase released in cell supernatants was measured using an enzymatic assay (26).

Cell lysates: *Laurylmaltoside (LM) lysates:* BMMC or thymocytes were solubilized on ice for 10 min in lysis buffer consisting of 100 mM Tris-HCL, pH 7.4 or pH 8.0, 150 mM NaCl, 2 mM MgCl₂, 5% glycerol, 0.2% LM (Thermo Scientific, Courtaboeuf, France), and 25 U/ml Benzonase (Merck, Darmstadt, Germany), supplemented with specific protease inhibitors (Table S4) or without. Protease inhibitor cocktails were from Roche (Mannheim, Germany, Cat. #11 697 498 001), and from Sigma-Aldrich (Cat. #P8340. Phosphoramidon (Cat. #R7385), EDTA and EGTA were from Sigma-Aldrich. *SDS lysates:* BMMC or thymocytes were lysed by boiling at 90°C for 5 min in 1% SDS and 100 mM Tris-HCL pH 8.0. 25 U/ml Benzonase and 2 mM MgCl₂ was added to SDS lysate that was left on ice for 10 min.

Western blotting analysis: LM and SDS cell lysates were centrifuged at 14,000 rpm for 15 min at 4°C and supernatants were electrophoresed on 4-12% Criterion XT precast gel (Bio-Rad, Marnes-la-Coquette, France) using XT MOPS (Bio-Rad) running buffer, and transferred onto nitrocellulose Hybond-P membranes (Amersham, Velizy-Villacoublay, France). Membranes were saturated either with 5% BSA (Sigma-Aldrich) or 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France) for 1h at room temperature and blotted overnight at 4°C with the indicated antibodies. Rabbit anti-SLP76, anti-Akt, anti-Erk1/2, anti-phospho-Erk1/2, anti-phospho-p38 and mouse anti-phospho-Tyrosine (P-Tyr-100) antibodies were from Cell Signaling Technology (St-Quentin-en-Yvelines, France). Rabbit anti-LAT1 and anti-phospho-LAT1, mouse anti-Vav, rabbit anti-Sos antibodies were from Upstate Biotechnology (Lake placid, NY). Goat anti-actin, mouse anti-PLC γ -1, anti-Dok-3 and anti-SHIP1, Rabbit anti-Fyn, anti-Bcr, anti-phopsho-Bcr (pY177), anti-Lyn and anti-phospho-PLC γ -1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-LAT2 antibodies were from Alexis

Biochemical (Lausanne, Switzerland). Rabbit anti-PAG antibodies were from ExBio (Praha, Czech Republic). Mouse anti-Grb2 and anti-phospho SLP76 antibodies were from BD Bioscience (Le-Pont-de-Claix, France). Goat anti-Dok-3 antibody was from Abcam (Paris, France). HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Mouse anti-phosphotyrosine antibodies (mAb 4G10) were a gift from Dr. S. Latour (Inserm U.768, Hôpital Necker, Paris, France). HRP was detected using an ECL kit (Thermo Scientific).

Strep-Tactin affinity-purification: Four independent affinity purifications were performed on each set of samples (*Slp76^{+/+}* and *Slp76^{OST/OST}* BMMC challenged with antigen or without). One $\times 10^8$ *Slp76^{+/+}* or *Slp76^{OST/OST}* BMMC were lysed for 10 min at 0°C with buffer containing 100 mM Tris-HCL, pH 8, 150 mM NaCl, 2 mM MgCl₂, 5% glycerol, 0.2% LM, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 25 U/ml Benzonase and mast cells complete inhibitor cocktail (Table S4). Cell extracts were centrifuged at 14,000 rpm for 15 min at 4°C. Cleared lysates were incubated with 200 μ l prewashed Strep-Tactin sepharose beads (Iba BioTagnologies, Goettingen, Germany) on a wheel for 1 h at 4°C. Beads were washed twice in 1 ml LM lysis buffer containing protease and phosphatase inhibitors and three times with 1 ml LM buffer without inhibitors. Beads were eluted 4 times with 150 μ l of 2.5 mM D-Biotin (Sigma-Aldrich). Eluates were pooled and precipitated with TCA/Acetone.

Co-immunoprecipitation: LM lysates of *Slp76^{+/+}* BMMC were incubated for 2 hr at 4°C with protein-A sepharose beads (GE Healthcare BioScience, Sweden) coated with anti-Bcr or anti-Dok-3 antibodies. After washing with lysis buffer, the beads were boiled at 90°C with loading buffer for 5 min. Eluates were electrophoresed, and Western blotted with anti-SLP76, anti-Bcr, anti-Dok-3, or anti-pY antibodies.

Proteomic analyses: 1) *TCA precipitation.* After elution, 125 µl of ice-cold TCA (Sigma-Aldrich) was added (final concentration 20%) to each sample and the mixture was incubated 30 min on ice and centrifuged at 20,000 g for 20 min at 4°C. The supernatant was removed and 600 µl of TCA 10 % was added to wash the pellet. The samples were centrifuged as above. The supernatant was removed and 1ml of ice-cold acetone (Sigma-Aldrich) was added to wash the pellet. The samples were centrifuged as above. The acetone-containing supernatant was removed and the pellet was air-dried. The pellet was suspended in 30 µl of sample buffer, consisting of 25 mM Tris-HCl, 4 % glycerol, 2% SDS, 5 % β-mercaptoethanol and 0.01 % bromophenol blue, and boiled for 5 min. Proteins were stacked by SDS-PAGE in a 6 mm-thick band visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad). 2) *Protein digestion.* The gel band was manually excised and cut in pieces. The following sample incubations were performed automatically using a Freedom EVO150 robot, (Tecan Trading AG, Switzerland). Gel pieces were washed by 6 successive incubations of 15 min in 25 mM NH₄HCO₃ and in 25mM NH₄HCO₃ (Sigma-Aldrich) containing 50% (v/v) acetonitrile. Gel pieces were then dehydrated with 100 % acetonitrile and incubated for 45 min at 53°C with 10 mM DTT (Sigma-Aldrich) in 25mM NH₄HCO₃ and for 35 min in the dark with 55 mM iodoacetamide (Sigma-Aldrich) in 25mM NH₄HCO₃. Alkylation was stopped by adding 10 mM DTT in 25 mM NH₄HCO₃ and mixing for 10 min. Gel pieces were then washed again by incubation in 25 mM NH₄HCO₃ before dehydration with 100% acetonitrile. 0.2 µg of modified trypsin (Promega, sequencing grade) in 25 mM NH₄HCO₃ was added to the dehydrated gel pieces for an overnight incubation at 37 °C. Peptides were then extracted from gel pieces in three 15 min sequential extraction steps in 30 µL of 50% acetonitrile, 30 µL of 5% formic acid (Aristar, VWR International, UK) and finally 30 µL of 100% acetonitrile. The pooled supernatants were then dried under vacuum. 3) *Nano-*

LC-MS/MS analyses. The dried extracted peptides were resuspended in a minimal volume of 5 % acetonitrile and 0.1 % trifluoroacetic acid (Sigma-Aldrich) and analysed twice by online nanoLC-MS/MS (Ultimate 3000, Dionex and LTQ-Orbitrap Velos pro, Thermofisher, CA, USA). The nanoLC method consisted in a 115-minutes gradient ranging from 4 % to 45 % acetonitrile in 0.1 % formic acid at a flow rate of 300 nL/min. Peptides were sampled on a 300 µm x 5 mm PepMap C18 precolumn and separated on a 75 µm x 250 mm C18 PepMap column (Thermofisher). MS and MS/MS data were acquired using Xcalibur (Thermofisher). Spray voltage and heated capillary were respectively set at 1.4 kV and 200°C. Survey full-scan MS spectra ($m/z = 400-1600$) were acquired in the Orbitrap with a resolution of 60,000 after accumulation of 10^6 ions (maximum filling time: 500 ms). The 20 most intense ions from the preview survey scan delivered by the Orbitrap were fragmented by collision induced dissociation (collision energy 35%) in the LTQ after accumulation of 10^4 ions (maximum filling time: 100 ms).

4) Data analyses. RAW files were processed using MaxQuant (27) version 1.3.0.5. Spectra were searched against the Uniprot database (*Mus musculus* taxonomy 10090, 78810 sequences, July 2012 version) and the frequently observed contaminants database embedded in MaxQuant. Trypsin was chosen as the enzyme and 2 missed cleavages were allowed. Precursor mass error tolerances were set respectively at 20 ppm and 6 ppm for first and main searches. Fragment mass error tolerance was set to 0.5 Da. Peptide modifications allowed during the search were: carbamidomethylation (C, fixed), acetyl (Protein N-ter, variable), oxidation (M, variable) and deamidation (NQ, variable). Minimum peptide length was set to 7 amino acids. Minimum number of peptides, razor + unique peptides and unique peptides were set to 1. Maximum false discovery rates - calculated by employing a reverse database strategy - were set to 0.01 at peptide and protein levels. Raw MS data files, unfiltered protein groups and peptides tables have

been uploaded in ProteomeXchange server (www.proteomexchange.org accession PXD000052). Identification and quantification data are also provided for proteins in Table S7 and for peptides in Table S8. Proteins identified as “contaminants”, “reverse” and “only identified by site” were discarded from the list of identified proteins. iBAQ values were calculated using MaxQuant as previously described (28), from MS intensity of unique peptides. Data coming from technical MS replicates were summed automatically. 5) *Statistical analyses*. Normalization of protein iBAQ values was performed on each sample using median calculated from proteins with values in all 16 samples. Only proteins quantified with a minimum of 2 unique peptides and for which a quantitative value could be measured for the four affinity purifications replicates in at least one condition were considered for the statistical analysis. The following steps were performed using the Perseus toolbox (version 1.2.0.16) available in the MaxQuant environment. After log2 transformation of iBAQ values and data imputation (replacing missing values by normal distribution as described in (29), protein expression differences were identified using t-testing with a Permutation-based FDR to correct for multiple hypothesis testing, S0 value set to 1 (30) and p-value threshold value set to 10^{-3} . Only proteins with a significant t-test result and log2 ratio above 2 for enrichment in respectively non-activated (-) and activated (+) mast cells from *Slp76^{OST/OST}* vs *Slp76^{+/+}* mice were validated in resting and stimulated complex, respectively. Taking into account that contaminant proteins may be not reproducible between non activated and activated mast cells from *Slp76^{+/+}*, we additionally filtered proteins enriched in resting complex (with respect to stimulated complex) if protein iBAQ in activated (+) mast cells from *Slp76^{+/+}* mice was above protein iBAQ in non-activated (-) mast cells from *Slp76^{OST/OST}* mice (respectively if protein iBAQ in non-activated (-) mast cells from *Slp76^{+/+}* mice was above protein iBAQ in activated (+) mast cells from *Slp76^{OST/OST}* mice).

The enrichment existing between activated (OST+) and non-activated (OST-) BMMC was also t-tested with S0 value set to 1 (30), p-value threshold value set to 10^{-2} . Only proteins from resting or stimulated complex with a significant t-test result and log2 ratio above 2 were further considered.

Sources of information and software used for protein network analyses: Information on protein interactions was extracted from the STRING (<http://string-db.org>) and PINA (<http://cbg.garvan.unsw.edu.au/pina/>) databases. The software Cytoscape was used to construct and visualize the SLP76 network (<http://www.cytoscape.org>). The LAT2 and Bcr protein-protein interaction networks were obtained from STRING database. We integrated experimentally verified interactions and those described in databases. Information regarding protease-coding genes has been extracted from Expasy web service (<http://expasy.org>).

Results

SLP76^{OST} is normally expressed and functional in mast cells.

Using gene KI in ES cells, a sequence corresponding to the OST MS tag was introduced at the 3' end of the *Slp76* gene. Upon germline transmission of the modified allele (denoted as *Slp76^{OST}*), KI mice homozygous for the *Slp76^{OST}* allele were established. Bone Marrow-derived Mast Cells (BMMC) were generated from homozygous *Slp76^{OST/OST}* KI mice and from wild-type (wt) controls (*Slp76^{+/+}* mice) (Fig. 1A&B).

As judged by Western blotting of SDS lysates, *Slp76^{OST/OST}* BMMC contained similar amounts of SLP76 molecules as *Slp76^{+/+}* BMMC (Fig. 1C), indicating that the presence of the OST did not affect the level of SLP76 expression. When sensitized with mouse IgE anti-DNP and challenged with DNP-HSA, *Slp76^{OST/OST}* BMMC released similar percentages of the granular mediator β -hexosaminidase as *Slp76^{+/+}* BMMC (Fig. 1D). Moreover, SLP76 and key signaling molecules, including LAT1, PLC γ -1, and the MAPK kinases Erk1/2 and p38, which are terminally activated upon Fc ϵ RI engagement, were similarly phosphorylated in *Slp76^{OST/OST}* and *Slp76^{+/+}* BMMC (Fig. 1E). SLP76^{OST} molecules are thus expressed at the same levels as wt SLP76 molecules in BMMC, and the presence of the OST has no effect on antigen-induced IgE-dependent Fc ϵ RI signaling and subsequent mast cell degranulation.

SLP76 is degraded in BMMC lysates by mast cell-specific proteases.

Since we aimed at performing affinity purification of the SLP76^{OST} bait under native conditions, BMMC were solubilized with lysis buffer containing a mild non-denaturing

detergent, laurylmaltoside (LM), and a standard protease inhibitor cocktail (Roche, Mannheim, Germany). Unexpectedly, SLP76, which was readily detected by Western blotting in denaturing SDS lysates, could not be detected in non-denaturing LM lysates. In contrast, all the other signaling molecules examined were found in comparable amounts in SDS and LM lysates (Fig. 2A). SLP76 was similarly undetectable in LM lysates of *Slp76^{OST/OST}* BMMC (not shown) and in *Slp76^{+/+}* BMMC lysates obtained with other mild non-denaturing detergents such as octylglycoside, IGEPAL, Brij35 or Triton X100 supplemented with standard protease inhibitors (Fig. S1). Similar amounts of SLP76 were, however, detected in both SDS and LM lysates from thymocytes (Fig. 2B). The inability to detect SLP76 in LM lysates is therefore specific to mast cells.

Surprisingly, although SLP76 was undetectable in LM lysates from non-stimulated BMMC sensitized with IgE antibodies, it became increasingly detectable in LM lysates from the same cells challenged with specific antigen for increasing periods of time before lysis (Fig. 2C). As stimulated mast cells degranulate within minutes, we hypothesized that granular proteases might be responsible for SLP76 degradation. Indeed, proteases contained in intact granules in non-stimulated cells, but not in activated, degranulated mast cells, could be freed upon lysis and degrade SLP76 in mast cell lysates. To test this hypothesis, we examined SLP76 in a non-activated BMMC LM lysate, in a thymocyte LM lysate and in a 1 to 1 mixture of both. As expected from the above experiments, SLP76 was observed in thymocyte LM lysate, but not in BMMC LM lysate. Strikingly, SLP76 was also undetectable in the mixture (Fig. 2D). Mast cell-specific proteases present in BMMC could thus degrade thymocyte-derived exogenous SLP76 molecules and, therefore, mast cell-derived endogenous SLP76 molecules following lysis in LM-containing buffer. We attempted to overcome this problem by using other protease inhibitor cocktails and by increasing their concentrations. SLP76

was protected neither by increasing 10 times the concentration of Roche protease inhibitors nor by using other mixtures of inhibitors, whether commercially available (Sigma-Aldrich) or custom-made (21) (Fig. 2E). Altogether, the above set of data indicates that, when mast cells are lysed with mild non-denaturing detergents, SLP76 is degraded by endogenous granular proteases that are resistant to conventional protease inhibitors.

Zinc-dependent metalloproteases account for SLP76 degradation in mast cell lysates.

To investigate the candidate protease(s) responsible for SLP76 degradation, we performed a transcriptomic analysis of BMMC. A total of 400 protease transcripts were identified, belonging to the four main groups of proteases. Serine proteases, cysteine proteases and metalloproteases were the most abundant (Fig. 3A and Table S1). The metalloprotease genes expressed in BMMC encode 176 metalloproteases (Fig. 3B and Table S2), 73% of which are zinc-binding proteins (Fig. 3B and Table S3). We noticed that, although most protease inhibitor cocktails contain inhibitors of proteases that depend on divalent cations such as Ca^{2+} (EGTA) or $\text{Mg}^{2+}/\text{Mn}^{2+}$ (EDTA), they do not contain specific inhibitors of Zn^{2+} -dependent metalloproteases (30). We therefore investigated whether a Zn^{2+} chelator would protect SLP76 from degradation in mast cell lysates. We found that, indeed, phenanthroline reduced the proteolysis of SLP76 when added to LM lysis buffer. Benzamidine, an inhibitor of aspartic and serine proteases, also protected SLP76, albeit less efficiently (Fig. 3C). The protection of SLP76 by phenanthroline was much more efficient at pH 8.0 (Fig. S2B) than at pH 7.4 (Fig. S2A). An increased protection by the Roche cocktail was also found at high pH (Fig. S2C).

When used at pH 8.0, phenanthroline protected SLP76 more efficiently than any other inhibitor.

On the basis of these results, we devised a mast cell protease inhibitor cocktail suitable for the biochemical analysis of signaling molecules in mast cells. This "MC cocktail" contained E-64, AEBSF, chymostatin, antipain, bestatin, phenanthroline and benzamidine (Table S4). SLP76 was dose-dependently protected when cells were lysed in LM buffer containing increasing concentrations of MC cocktail at pH 8.0. Protection was complete when using the highest concentration of MC cocktail (Table S4) since, under these conditions, comparable amounts of SLP76 were recovered in LM lysates and in SDS lysates. Importantly, the level of protection observed at 10 min was fully retained after 90 min at 0°C (Fig. 3D). The establishment of these optimized lysis conditions was an absolute requirement for an MS-based proteomic analysis of the SLP76 interactome in mast cells, since it is based on the prior affinity purification of tagged SLP76 molecules from LM lysates.

SLP76 and interacting proteins can be affinity-purified from $Slp76^{OST/OST}$ BMMC.

$Slp76^{OST/OST}$ and $Slp76^{+/+}$ BMMC were lysed in LM buffer pH 8.0 supplemented with MC cocktail, and SLP76 was affinity-purified from lysates using Strep-Tactin-coated Sepharose beads. Similar amounts of lysates from $Slp76^{OST/OST}$ and $Slp76^{+/+}$ BMMC were applied to the Strep-Tactin column (Fig. 4A, input). As expected, no SLP76 was recovered from $Slp76^{+/+}$ BMMC, whereas most of the SLP76 molecules detected in lysate of $Slp76^{OST/OST}$ BMMC were recovered from Strep-Tactin-coated beads using 4 elutions steps with 2.5 mM D-Biotin, a ligand that binds to Strep-Tactin with a higher affinity

than the OST (Fig. 4A). Our protocol therefore enables the recovery of most of the SLP76 species present in mast cell lysates.

To examine which proteins associate with SLP76 upon mast cell activation, *Slp76^{OST/OST}* and *Slp76^{+/+}* BMMC were sensitized with IgE antibodies and challenged with antigen or without for 2 min. SLP76 was affinity-purified on Strep-Tactin-coated beads, and eluates were analyzed by Western blotting. Similar amounts of SLP76 were recovered in lysates from challenged and unchallenged *Slp76^{OST/OST}* BMMC, whereas no SLP76 was recovered from *Slp76^{+/+}* BMMC. As expected, the constitutively associated adapter protein Grap2 co-purified with SLP76^{OST}, prior to and after antigen challenge. SLP76^{OST} was inducibly tyrosyl-phosphorylated upon antigen challenge, and two well-known SLP76 partners, Grb2 and PLCγ-1, co-purified with phosphorylated, but not with non-phosphorylated SLP76^{OST} (Fig. 4B). Well-known activation-inducible SLP-76 interactors could therefore be identified by immunoblotting, following Strep-Tactin affinity purification of LM lysates, provided that SLP76 degradation was prevented by the protease inhibitor cocktail that we specifically designed. Biochemical conditions having been optimized, the SLP76 interactome was investigated next by label-free quantitative proteomic analysis in *Slp76^{OST/OST}* BMMC.

The SLP76 interactome of resting mast cells.

Unlike classical biochemistry approaches, Strep-Tactin-based affinity purification of SLP76^{OST} enabled us to study proteins that interact with SLP76 in resting cells. Indeed, molecules that were co-purified from resting *Slp76^{OST/OST}* BMMC, but not from resting *Slp76^{+/+}* BMMC or in much lower amounts, could be considered as being specifically and

constitutively associated with SLP76 in non-stimulated cells. The constitutive SLP76 interactome is largely unknown, especially in mast cells. The statistical treatment of iBAQ values coming from MaxQuant processing of LC-MS/MS data corresponding to four independent affinity purifications involving two MS replicates revealed three proteins significantly enriched in eluates from resting *Slp76^{OST/OST}* BMMC compared to eluates from resting *Slp76^{+/+}* BMMC (Fig. 5A, Table 1 and Table S5, p-value threshold set to 10^{-3} with $s_0=1$ and $\log_2$ ratio OST-/WT- >2). Among them, Grap2, a well-known binding partner of SLP76, that could be seen by Western blotting (Fig. 4B), was 500-fold more abundant in eluates from *Slp76^{OST/OST}* BMMC than in eluates from *Slp76^{+/+}* BMMC. The Rho GTPase-activating proteins RABE1 and RHG27 were enriched to a lesser extent in eluates from *Slp76^{OST/OST}* BMMC. RABE1 and RHG27 have never been described before as SLP76 interactors.

The SLP76 interactome is markedly enriched in activated mast cells.

To determine the activation-induced SLP76 interactome in mast cells, we performed two sets of comparative MS analyses of proteins co-purified with SLP76. First, we compared the SLP76 interactome in *Slp76^{OST/OST}* BMMC and in *Slp76^{+/+}* BMMC, sensitized with IgE antibodies and challenged with antigen. The statistical treatment of iBAQ values coming from MaxQuant processing of LC - MS/MS data corresponding to four independent affinity purifications involving two MS replicates revealed 35 proteins significantly enriched in eluates from activated *Slp76^{OST/OST}* BMMC compared to eluates from activated *Slp76^{+/+}* BMMC (p-value threshold set to 10^{-3} with $s_0=1$ and $\log_2$ ratio OST+/WT+ >2 , Table 2 and Table S5). This comparison aimed at discriminating molecules that bound specifically to SLP76 from molecules that bound nonspecifically to

Strep-Tactin sepharose beads in activated mast cells. We then compared the SLP76 interactome in *Slp76^{OST/OST}* BMMC sensitized with IgE antibodies and challenged with antigen or without for 2 min. This comparison aimed at identifying molecules that were recruited specifically by SLP76 upon FcεRI engagement (as assessed by the OST+/OST- ratio in Table 2 and Table S5).

Eighteen proteins were significantly (p-value threshold set to 10^{-2} with $s_0=1$ and $\log_2$ ratio OST+/OST- >2) and specifically associated with SLP76 in activated *Slp76^{OST/OST}* BMMC (compared to non-activated *Slp76^{OST/OST}* BMMC) (red nodes in Fig. 5A). In addition, the three SLP76 partners identified in resting BMMC (white nodes in Fig. 5A) also co-purified with SLP76 in activated BMMC. Grap2 remained associated in comparable amounts, RHG27 was enriched 2-fold, and RABE1 decreased more than half. Among the 21 proteins that constitute the SLP76 interactome of activated BMMC (Figure 5B), nine molecules were inducibly recruited with a high statistical significance ($p<10^{-3}$). Surprisingly, the transmembrane adapter LAT2 (NTAL), which was not detected in non-activated *Slp76^{OST/OST}* BMMC, was dramatically enriched in activated *Slp76^{OST/OST}* BMMC, much more than any other molecule. In comparison, LAT1, which was also not detected in non-activated *Slp76^{OST/OST}* BMMC, was detected in activated *Slp76^{OST/OST}* BMMC, but it was much less enriched ($p<10^{-2}$) than LAT2. The molecular basis of this dramatic difference remains to be determined. A limited number of proteins are known to interact with LAT2 (Fig. 5C). Two unexpected molecules, the Breakpoint Cluster Region protein (Bcr) and HIG1A, were also highly enriched. Bcr is a protein with compound functions which interacts with numerous molecules (Fig. 5D). HIG1A is a HIG1-domain family member whose function is not known. Other partners recruited with a high statistical significance were also enriched but to a lower extent. They included molecules

known to be recruited by SLP76 in activated T cells such as PLC- γ 1, Shc1 and Grb2, but also molecules that were not previously identified as SLP76 partners, such as the Src kinase-associated phosphoproteins SKAP1/2 and the cytosolic adapter Dok-3. Other molecules that were statistically significantly recruited ($p < 10^{-2}$) included LAT1, as already mentioned, a member of the 14-3-3 family, 14-3-3 η , the Fyn-binding protein Fyb, and the Tec kinase BTK.

The cytosolic adapter Dok-3 interacts with several signaling molecules among which the SH2 domain-containing inositol 5-phosphatase SHIP1 (INPP5D) (Fig. 5D). It belongs to the same family as Dok-1, which is a major player in SHIP1-dependent negative regulation of both B cell (31) and mast cell (32) activation. Dok-3 was found in lysates of non-activated and activated BMMC, from *Slp76^{OST/OST}* (Fig. 6A, upper panel) and from *Slp76^{+/+}* (Fig. 6B, upper panel) mice. Confirming our AP-MS analysis, Dok-3 co-purified with SLP76 in activated cells in higher amount than in non-activated cells (Fig. 6A, lower panel). Conversely, SLP76 co-precipitated with Dok-3 in activated cells more than in resting cells, and Dok-3 was markedly phosphorylated upon antigen challenge. Noticeably, SHIP1 also co-precipitated with phosphorylated Dok-3 in activated cells (Fig. 6B, lower panel). To further investigate the involvement of Dok-3 in Fc ϵ RI signaling, we compared β -hexosaminidase released by BMMC from Dok-3^{-/-} mice and by BMMC from Dok-3^{+/+} mice (Fig. 6C). Antigen-induced mediator release was of the same magnitudes in Bcr^{-/-} and Bcr^{+/+} BMMC sensitized with IgE (Fig. 6D). The deletion of Dok-3 therefore did not detectably affect mast cell activation.

Because Bcr was an unexpected partner of SLP76, because it was inducibly recruited in high amounts upon Fc ϵ RI engagement, because it was not previously linked to Fc ϵ RI signaling, because it interacts with numerous molecules and because it can exert

multiple functions that may have a major impact in FcεRI signaling, we validated biochemically the interaction between SLP76 and Bcr. We indeed found that Bcr was similarly observed in lysates of non-activated and activated BMMC, whether from *Slp76^{OST/OST}* (Fig. 7A, upper panel) or from *Slp76^{+/+}* (Fig. 7B, upper panel & Fig. 7C) mice. Bcr was co-purified with phosphorylated SLP76^{OST} in activated, but not in non-activated *Slp76^{OST/OST}* BMMC (Fig. 7A, lower panel). Conversely, SLP76 was co-immunoprecipitated with Bcr in *Slp76^{+/+}* BMMC, and in higher amount in activated cells than in non-activated cells (Fig. 7B, lower panel). Also, Bcr was inducibly phosphorylated at Y178 upon antigen challenge (Fig. 7C). To further investigate the involvement of Bcr in FcεRI signaling, we compared β-hexosaminidase released by BMMC from *Bcr^{-/-}* mice and by BMMC from *Bcr^{+/+}* mice (Fig. 7D). Antigen-induced mediator release was decreased in *Bcr^{-/-}* BMMC, compared to *Bcr^{+/+}* BMMC, sensitized with IgE (Fig. 7E). The deletion of Bcr therefore impaired mast cell activation.

Discussion

We report here the first proteomic analysis of FcεRI signaling in primary mouse mast cells. Specifically, we describe the SLP76 interactome in resting and in activated cultured mast cells, using affinity purification coupled to label-free quantitative proteomics. Data presented in this paper are the first results of a large-scale program aiming at assembling a comprehensive dynamic map of signaling events triggered by FcεRI engagement in mast cells.

SLP76 has been extensively investigated following TCR engagement in T cells, but much less following FcεRI engagement in mast cells. SLP76 deletion was however found to

profoundly affect IgE-induced mast cell activation (20). As uncovered in the present study, one reason of this poor knowledge on an adapter molecule that is essential for FcεRI signaling may be linked to its unexpected sensitivity to proteolysis by mast cell granular proteases. We indeed found that SLP76 was undetectable in LM lysates of non-activated mast cells, whereas it was readily detected in SDS lysates of the same cells. No other intracellular molecule among the 14 that we tested behaved like SLP76. This reminded us that, under similar lysis conditions, SHIP1 was degraded by unknown proteases in Peritoneal Cell-derived Mast Cells, a model of mature serosal-type mast cells that are especially rich in granular proteases, but not in BMMC (33). We noticed in the present study that similar amounts of SLP76 were detected in LM and SDS lysates of thymocytes and in LM lysates of activated but not of non-activated mast cells. To explain these differences, we hypothesized that proteases contained in the granules might digest SLP76 in lysates of non-activated mast cells, if granular membranes are disrupted by detergents. This hypothesis was confirmed by experiments showing that thymocyte-derived SLP76 was also degraded in a 1:1 mixture of LM lysates of thymocytes and of non-activated BMMC. Even if restricted to SLP76, this proteolysis was a major obstacle for our project. The SLP76 molecule is indeed functioning as a scaffold protein, and its degradation is likely to dramatically alter the signaling complex to be investigated. To circumvent this problem, we undertook an extensive analysis of proteases in BMMC.

Proteases were reported to account for up to 25% of total proteins in mast cells (34). They are stored in granules under a catalytically active form. They are released in the extracellular medium under physiological conditions during antigen-induced degranulation and they can have both pro-inflammatory effects in IgE-induced allergic inflammation, and anti-inflammatory effects in infection-associated inflammation (35). They can also be released under non-physiological conditions, when granular

membranes are solubilized by mild detergents during cell lysis. Intracellular molecules are thus suddenly exposed to large amounts of biologically active proteases that they normally never meet.

According to our transcriptomic study, more than a quarter (28%) of proteases expressed in BMMC are metalloproteases. Among these, almost three quarters (73%) are Zn²⁺-dependent. Commercially available protease inhibitor cocktails, however, do not or poorly inhibit Zn²⁺-dependent metalloproteases. We found that adding a specific Zn²⁺ chelator to other inhibitors fully protected SLP76, even for prolonged periods of times (90 min at 0°C) that are required for affinity purification.

The lysis conditions defined in the present study enabled us to analyze the SLP76 interactome, not only in activated mast cells, but also in non-activated mast cells. The unique tagged SLP76 molecules that we developed *via* a KI approach and used here enabled us to compare mast cells from *Slp76*^{+/+} and from *Slp76*^{OST/OST} mice. Under these conditions, molecules eluted from Strep-Tactin-coated beads in *Slp76*^{+/+} BMMC were considered as binding nonspecifically, *i.e.* as background, whereas molecules eluted from Strep-Tactin-coated beads in *Slp76*^{OST/OST} BMMC, but not in *Slp76*^{+/+} BMMC, could be considered as binding specifically to SLP76 in resting mast cells. Three molecules were affinity-purified along with SLP76^{OST} from non-stimulated *Slp76*^{OST/OST} BMMC but not, or in much lower amounts, from non-stimulated *Slp76*^{+/+} BMMC. These molecules corresponded to the adapter protein Grap2 and to the two GTPase-activating proteins, RABE1 and RHG27.

Among the molecules that displayed a statistically significant enrichment ($p < 10^{-2}$) upon FcεRI engagement, one can distinguish molecules that have been already described as

SLP76 partners in T cells and novel SLP76 partners that are identified for the first time here in mast cells.

Six molecules interacted with SLP76 in both activated T cells and activated mast cells. These were mostly adapter molecules (LAT1, Grap2, Grb2, Shc1, Fyb) and one enzyme (PLC γ -1). One notices that all these molecules concur to intracellular pathways that lead to cell activation. LAT1 is an essential scaffold protein that organizes the signalosomes generated upon TCR or Fc ϵ RI engagement. The cytosolic adapters Shc, Grb2 and Grap2 are widely used by activating receptors and are involved in numerous signaling pathways. Phospholipase C γ -1 (PLC γ -1) is a widely expressed major signaling molecule which, when activated upon tyrosyl-phosphorylation, generates inositol tris-phosphate (IP3) and diacyl-glycerol (DAG). IP3 triggers the efflux of Ca²⁺ from endothelial reticulum, leading to the influx of extracellular Ca²⁺, while DAG activates Protein Kinase C. By phosphorylating PLC γ -1, the Bruton's Tyrosine Kinase of the Tec family BTK plays a critical role in the activation of this phospholipase. SLP76 also associates with Clnk, a cytosolic adapter of the SLP76 family which promotes TCR-dependent activation of T cells (36, 37) and Fc ϵ RI-dependent responses of mast cells (38). Clnk deficiency, however, impaired neither TCR signaling in T cells nor Fc ϵ RI signaling in mast cells (39). Clnk interacts with two phosphoproteins expressed in mast cell lines, Fyb, a.k.a. SLP76-associated protein of 130 kDa (SLAP-130) or Adhesion- and Degranulation-promoting Adapter Protein (ADAP), and SKAP1. These adapters can interact with the SH2 domain of Src kinases to form a Clnk-Fyb-SKAP1 complex in mast cells (40). Fyb and SKAP1 were among the molecules that were recruited by SLP76 in mast cells, following Fc ϵ RI engagement ($p < 10^{-3}$ and $p < 10^{-2}$, respectively). Some proteins previously reported to associate with SLP76 in T cells were not found with a high enough statistical significance

in our MS-based proteomic analysis of the SLP76 interactome in mast cells. Lyn and Nck-1 could however be detected by Western blot analysis among molecules that were affinity-purified with SLP^{OST}(not shown). The reason for the discrepancy lies in the severe filters used for establishing statistical significance in our proteomic analysis (MS data and statistical analysis of these results are shown in Table S6).

Fifteen molecules that were inducibly recruited by SLP76 upon FcεRI engagement in mast cells have not been reported to be recruited by SLP76 upon TCR engagement in T cells. These comprised adapter molecules (LAT2, 14-3-3F, CSTF1 and Dok-3), a Tec family protein tyrosine kinase (Btk), Src kinase regulators (SKAP1 and SKAP2), GTPase-activating molecules (RABE1 and RHG37), a serine/threonine kinase with a guanine-nucleotide exchange factor activity for Rho GTPases (Bcr) (41), and molecules with poorly defined or unknown functions (HIG1A, SUCA, MSH2, PACS1 and DC1I2). One notices that most molecules with a known function that were inducibly recruited in high amounts by SLP76 in mast cells, but not in T cells, are associated with negative regulation.

The transmembrane adapter LAT2 was inducibly recruited by SLP76 exceedingly more than any other molecules in antigen-stimulated *Slp76^{OST/OST}* BMMC. LAT2 is a well-known antagonist of LAT1 in mouse mast cells, and LAT2-deficient mice exhibited enhanced secretory responses upon FcεRI engagement (42). The exact mechanisms by which LAT2 inhibits mast cell activation is, however, largely unknown (22), and it has been reported to interact with 6 proteins only.

Dok-3, a cytosolic adapter of the Dok family is also associated with negative regulation and was inducibly recruited in the SLP76 interactome. This interaction of Dok-3 with SLP76 could be confirmed biochemically as Dok-3 co-purified with SLP76^{OST}. Conversely,

SLP76 co-precipitated with Dok-3 in wt cells. Noticeably, Dok-3 was dramatically phosphorylated in activated mast cells. Dok-1 is well known to become phosphorylated upon immunoreceptor engagement in both B cells (31) and mast cells (32), and to mediate the recruitment of RasGAP which, by increasing the autocatalytic activity of Ras-GTP, inhibits the Ras pathway. Dok-2 plays a similar role as Dok-1 in T cells (43), but Dok-1 and Dok-3 were found to have non-redundant roles in B cells (44). Dok-3 was found among the proteins that are phosphorylated upon FcεRI engagement in mouse mast cells (45). When tyrosyl-phosphorylated in B cells and macrophages, Dok-3 mediated the membrane recruitment of SHIP1 and Csk, two molecules that have inhibitory properties. Dok-3 over-expression reduced B Cell Receptor-dependent B cell activation whereas the expression of Dok-3 the four C-terminal tyrosines of which were mutated, *i.e.* rendered unable to bind to the SH2 domain of SHIP1, enhanced B cell activation (46). We show here that Dok-3 can also inducibly recruit SHIP1 upon FcεRI engagement in mast cells as SHIP1 co-precipitated with phosphorylated Dok-3 in activated mast cells. SHIP1 is a major negative regulator of immunoreceptor signaling, and especially of FcεRI signaling (reviewed in (47)). The deletion of Dok-3, however, had no detectable effect on mast cell degranulation, probably because Dok-1, and possibly Dok-2, were sufficient to recruit SHIP1.

The second most inducibly recruited molecule was the Breakpoint Cluster Region protein Bcr. It was enriched almost 150 fold in eluates from antigen-stimulated *Slp76^{OST/OST}* BMMC, as compared to eluates from resting *Slp76^{OST/OST}* BMMC. Importantly, we could confirm the inducible interaction of Bcr with SLP76 with biochemical techniques. Bcr was indeed co-affinity-purified with SLP76^{OST} in lysates of activated *Slp76^{OST/OST}* BMMC, and SLP76 was co-immunoprecipitated with Bcr in lysates of activated *Slp76^{+/+}* BMMC. Supporting a functional role of Bcr in FcεRI signaling, we found

that it was inducibly phosphorylated at Y178 in activated mast cells. Bcr was similarly found to be phosphorylated at Y177 upon TCR engagement in human Jurkat cells (48). Finally, we obtained a compelling evidence that Bcr positively regulates IgE-dependent mast cell activation as mediator release was reduced in BMMC from Bcr-deficient mice. To our knowledge, this is the first demonstration that Bcr is involved in and contributes to FcεRI signaling. Bcr binds to multiple adapter molecules, including Grb2, Hck, Shc1 and Cbl as well as Dok-1, Dok-2 and therefore, possibly Dok-3. One notices several other interesting molecular partners in the Bcr interactome that can be constructed from published databases (Fig. 5D). These include Kit, which critically determines the proliferation and differentiation of mast cells, molecules that control cytokine receptor signaling such as Jak2 and STAT5A/B, the exchange factors Sos and Vav and, strikingly, a whole cluster of small G proteins of the Rho and Rac families that exert major cytoskeletal effects by controlling the dynamics of actin. Bcr has itself a serine-threonine kinase activity. Bcr is also a GTPase-activating protein for Rac1 and cdc42 and an exchange factor for Rho GTPases. Normally, the Bcr exchange factor activity is auto-inhibited by flanking sequences (49). It is constitutively activated in the Bcr-Abl fusion protein (41) that accounts for the transformation of myeloid cells in chronic myeloid leukemia. The GTPase activity of Bcr is positively regulated by a Protein Tyrosine Phosphatase Receptor in neurons. Bcr is indeed highly expressed in the central nervous system where it decreases dendrite formation by negatively regulating actin polymerization in neurons (50). Along this line, the genetic ablation of Bcr and Abr, another Rac/cdc42-specific GTPase-activating exchange factor, increased cytoskeleton-dependent motility and phagocytosis in primary macrophages (51).

In conclusion, the present work first describes the SLP76 interactome in primary mast cells and unravels that it markedly differs from the SLP76 interactome previously

described in T cells. Our work also provides the first evidence that Bcr is involved in FcεRI-dependent mast cell activation. It also validates OST-KI mice as powerful tools for the proteomic analysis of cell signaling. As SLP76^{OST} was expressed with a normal tissue distribution, it could be studied in non-transformed mast cells. SLP76^{OST} was expressed in mast cells from *Slp76^{OST/OST}* mice in similar amounts as SLP76^{wt} in mast cells from *Slp76^{+/+}* mice. A unique advantage of the present approach is that, unlike in cells transfected with cDNA encoding a tagged molecule, no competition and no dilution with endogenous non-tagged molecules occurs in cells from OST-KI mice as all molecules of interest are tagged. Importantly, OST-SLP76 was also functionally similar as wt-SLP76 since β-hexosaminidase release and intracellular phosphorylations of a comparable magnitude were induced upon antigen challenge in *Slp76^{OST/OST}* and *Slp76^{+/+}* mast cells sensitized with IgE antibodies. On the basis of these results, one can apply the same approach to study the interactomes of other key signaling molecules in mouse mast cells. One expects not only to order known molecules in space and time, and to untangle the complex network of their interactions, but also to discover molecules not previously known as being involved in mast cell activation and, ultimately, to build up a comprehensive molecular map of the FcεRI signalosome.

References

1. Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. *Annu Rev Immunol.* 1999;17:931-72. Epub 1999/06/08.
2. Reth M. Antigen receptor tail clue. *Nature.* 1989;338(6214):383-4. Epub 1989/03/30.
3. Blank U, Ra C, Miller L, White K, Metzger H, Kinet JP. Complete structure and expression in transfected cells of high affinity IgE receptor. *Nature.* 1989;337(6203):187-9. Epub 1989/01/12.
4. Turner H, Kinet JP. Signalling through the high-affinity IgE receptor Fc epsilonRI. *Nature.* 1999;402(6760 Suppl):B24-30. Epub 1999/12/10.
5. Sardiù ME, Washburn MP. Building protein-protein interaction networks with proteomics and informatics tools. *The Journal of biological chemistry.* 2011;286(27):23645-51. Epub 2011/05/14.
6. Bensimon A, Heck AJ, Aebersold R. Mass spectrometry-based proteomics and network biology. *Annual review of biochemistry.* 2012;81:379-405. Epub 2012/03/24.
7. Choudhary C, Mann M. Decoding signalling networks by mass spectrometry-based proteomics. *Nature reviews Molecular cell biology.* 2010;11(6):427-39. Epub 2010/05/13.
8. Collins MO, Choudhary JS. Mapping multiprotein complexes by affinity purification and mass spectrometry. *Curr Opin Biotechnol.* 2008;19(4):324-30. Epub 2008/07/05.
9. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. A generic protein purification method for protein complex characterization and proteome exploration. *Nature biotechnology.* 1999;17(10):1030-2. Epub 1999/10/03.
10. Daulat AM, Maurice P, Froment C, Guillaume JL, Broussard C, Monsarrat B, et al. Purification and identification of G protein-coupled receptor protein complexes under native conditions. *Molecular & cellular proteomics : MCP.* 2007;6(5):835-44. Epub 2007/01/12.
11. Oellerich T, Bremes V, Neumann K, Bohnenberger H, Dittmann K, Hsiao HH, et al. The B-cell antigen receptor signals through a preformed transducer module of SLP65 and CIN85. *EMBO J.* 2011;30(17):3620-34. Epub 2011/08/09.
12. Schmidt TG, Skerra A. The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat Protoc.* 2007;2(6):1528-35. Epub 2007/06/16.
13. Jackman JK, Motto DG, Sun Q, Tanemoto M, Turck CW, Peltz GA, et al. Molecular cloning of SLP-76, a 76-kDa tyrosine phosphoprotein associated with Grb2 in T cells. *The Journal of biological chemistry.* 1995;270(13):7029-32. Epub 1995/03/31.
14. Liu SK, Fang N, Koretzky GA, McGlade CJ. The hematopoietic-specific adaptor protein gads functions in T-cell signaling via interactions with the SLP-76 and LAT adaptors. *Curr Biol.* 1999;9(2):67-75. Epub 1999/02/18.

15. Yablonski D, Kadlec T, Weiss A. Identification of a phospholipase C-gamma1 (PLC-gamma1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-gamma1 and NFAT. *Molecular and cellular biology*. 2001;21(13):4208-18. Epub 2001/06/08.
16. Bubeck-Wardenburg J, Fu C, Jackman JK, Flotow H, Wilkinson SE, Williams DH, et al. Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. *The Journal of biological chemistry*. 1996;271(33):19641-4. Epub 1996/08/16.
17. Raab M, da Silva AJ, Findell PR, Rudd CE. Regulation of Vav-SLP-76 binding by ZAP-70 and its relevance to TCR zeta/CD3 induction of interleukin-2. *Immunity*. 1997;6(2):155-64. Epub 1997/02/01.
18. Su YW, Zhang Y, Schweikert J, Koretzky GA, Reth M, Wienands J. Interaction of SLP adaptors with the SH2 domain of Tec family kinases. *Eur J Immunol*. 1999;29(11):3702-11. Epub 1999/11/11.
19. Wunderlich L, Farago A, Downward J, Buday L. Association of Nck with tyrosine-phosphorylated SLP-76 in activated T lymphocytes. *Eur J Immunol*. 1999;29(4):1068-75. Epub 1999/05/06.
20. Pivniouk VI, Martin TR, Lu-Kuo JM, Katz HR, Oettgen HC, Geha RS. SLP-76 deficiency impairs signaling via the high-affinity IgE receptor in mast cells. *J Clin Invest*. 1999;103(12):1737-43. Epub 1999/06/22.
21. Kambayashi T, Okumura M, Baker RG, Hsu CJ, Baumgart T, Zhang W, et al. Independent and cooperative roles of adaptor molecules in proximal signaling during FcepsilonRI-mediated mast cell activation. *Molecular and cellular biology*. 2010;30(17):4188-96. Epub 2010/07/08.
22. Roget K, Malissen M, Malbec O, Malissen B, Daëron M. Non-T cell activation linker promotes mast cell survival by dampening the recruitment of SHIP1 by linker for activation of T cells. *J Immunol*. 2008;180(6):3689-98. Epub 2008/03/07.
23. Mingueneau M, Roncagalli R, Gregoire C, Kissenpfennig A, Miazeck A, Archambaud C, et al. Loss of the LAT adaptor converts antigen-responsive T cells into pathogenic effectors that function independently of the T cell receptor. *Immunity*. 2009;31(2):197-208. Epub 2009/08/18.
24. Zhang Y, Muirers JP, Testa G, Stewart AF. DNA cloning by homologous recombination in *Escherichia coli*. *Nature biotechnology*. 2000;18(12):1314-7. Epub 2000/12/02.
25. Pettitt SJ, Liang Q, Rairdan XY, Moran JL, Prosser HM, Beier DR, et al. Agouti C57BL/6N embryonic stem cells for mouse genetic resources. *Nat Methods*. 2009;6(7):493-5. Epub 2009/06/16.
26. Malbec O, Malissen M, Isnardi I, Lesourne R, Mura AM, Fridman WH, et al. Linker for activation of T cells integrates positive and negative signaling in mast cells. *J Immunol*. 2004;173(8):5086-94. Epub 2004/10/08.
27. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nature biotechnology*. 2008;26(12):1367-72. Epub 2008/11/26.

28. Schwanhaussner B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. *Nature*. 2011;473(7347):337-42. Epub 2011/05/20.
29. Deeb SJ, D'Souza RC, Cox J, Schmidt-Supprian M, Mann M. Super-SILAC allows classification of diffuse large B-cell lymphoma subtypes by their protein expression profiles. *Molecular & cellular proteomics : MCP*. 2012;11(5):77-89. Epub 2012/03/24.
30. Felber JP, Coombs TL, Vallee BL. The mechanism of inhibition of carboxypeptidase A by 1,10-phenanthroline. *Biochemistry*. 1962;1:231-8. Epub 1962/03/01.
31. Tamir I, Stolpa JC, Helgason CD, Nakamura K, Bruhns P, Daëron M, et al. The RasGAP-binding protein p62dok is a mediator of inhibitory FcγRIIB signals in B cells. *Immunity*. 2000;12(3):347-58. Epub 2001/02/07.
32. Ott VL, Tamir I, Niki M, Pandolfi PP, Cambier JC. Downstream of kinase, p62(dok), is a mediator of Fc γRIIB inhibition of Fc εRI signaling. *J Immunol*. 2002;168(9):4430-9. Epub 2002/04/24.
33. Malbec O, Roget K, Schiffer C, Iannascoli B, Dumas AR, Arock M, et al. Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. *J Immunol*. 2007;178(10):6465-75. Epub 2007/05/04.
34. Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood*. 2010;115(24):4981-90. Epub 2010/03/18.
35. Caughey GH. Mast cell proteases as protective and inflammatory mediators. *Advances in experimental medicine and biology*. 2011;716:212-34. Epub 2011/06/30.
36. Cao MY, Davidson D, Yu J, Latour S, Veillette A. Clnk, a novel SLP-76-related adaptor molecule expressed in cytokine-stimulated hemopoietic cells. *The Journal of experimental medicine*. 1999;190(10):1527-34. Epub 1999/11/24.
37. Yu J, Riou C, Davidson D, Minhas R, Robson JD, Julius M, et al. Synergistic regulation of immunoreceptor signaling by SLP-76-related adaptor Clnk and serine/threonine protein kinase HPK-1. *Molecular and cellular biology*. 2001;21(18):6102-12. Epub 2001/08/18.
38. Goitsuka R, Kanazashi H, Sasanuma H, Fujimura Y, Hidaka Y, Tatsuno A, et al. A BASH/SLP-76-related adaptor protein MIST/Clnk involved in IgE receptor-mediated mast cell degranulation. *International immunology*. 2000;12(4):573-80. Epub 2000/04/01.
39. Utting O, Sedgmen BJ, Watts TH, Shi X, Rottapel R, Iulianella A, et al. Immune functions in mice lacking Clnk, an SLP-76-related adaptor expressed in a subset of immune cells. *Molecular and cellular biology*. 2004;24(13):6067-75. Epub 2004/06/17.
40. Fujii Y, Wakahara S, Nakao T, Hara T, Ohtake H, Komurasaki T, et al. Targeting of MIST to Src-family kinases via SKAP55-SLAP-130 adaptor complex in mast cells. *FEBS letters*. 2003;540(1-3):111-6. Epub 2003/04/12.
41. Sahay S, Pannucci NL, Mahon GM, Rodriguez PL, Megjugorac NJ, Kostenko EV, et al. The RhoGEF domain of p210 Bcr-Abl activates RhoA and is required for transformation. *Oncogene*. 2008;27(14):2064-71. Epub 2007/10/09.

42. Volna P, Lebduška P, Drabero娃 L, Simova S, Heneberg P, Boubelik M, et al. Negative regulation of mast cell signaling and function by the adaptor LAB/NTAL. *The Journal of experimental medicine*. 2004;200(8):1001-13. Epub 2004/10/13.
43. Dong S, Corre B, Foulon E, Dufour E, Veillette A, Acuto O, et al. T cell receptor for antigen induces linker for activation of T cell-dependent activation of a negative signaling complex involving Dok-2, SHIP-1, and Grb-2. *The Journal of experimental medicine*. 2006;203(11):2509-18. Epub 2006/10/18.
44. Ng CH, Xu S, Lam KP. Dok-3 plays a nonredundant role in negative regulation of B-cell activation. *Blood*. 2007;110(1):259-66. Epub 2007/03/17.
45. Cao L, Yu K, Banh C, Nguyen V, Ritz A, Raphael BJ, et al. Quantitative time-resolved phosphoproteomic analysis of mast cell signaling. *J Immunol*. 2007;179(9):5864-76. Epub 2007/10/20.
46. Lemay S, Davidson D, Latour S, Veillette A. Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. *Molecular and cellular biology*. 2000;20(8):2743-54. Epub 2000/03/25.
47. Daëron M, Lesourne R. Negative signaling in Fc receptor complexes. *Advances in immunology*. 2006;89:39-86. Epub 2006/05/10.
48. Mayya V, Lundgren DH, Hwang SI, Rezaul K, Wu L, Eng JK, et al. Quantitative phosphoproteomic analysis of T cell receptor signaling reveals system-wide modulation of protein-protein interactions. *Science signaling*. 2009;2(84):ra46. Epub 2009/08/20.
49. Korus M, Mahon GM, Cheng L, Whitehead IP. p38 MAPK-mediated activation of NF-kappaB by the RhoGEF domain of Bcr. *Oncogene*. 2002;21(30):4601-12. Epub 2002/07/04.
50. Park AR, Oh D, Lim SH, Choi J, Moon J, Yu DY, et al. Regulation of dendritic arborization by BCR Rac1 GTPase-activating protein, a new substrate of protein tyrosine phosphatase receptor T. *Journal of cell science*. 2012. Epub 2012/07/07.
51. Cho YJ, Cunnick JM, Yi SJ, Kaartinen V, Groffen J, Heisterkamp N. Abr and Bcr, two homologous Rac GTPase-activating proteins, control multiple cellular functions of murine macrophages. *Molecular and cellular biology*. 2007;27(3):899-911. Epub 2006/11/23.

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Figure Legends

Fig. 1. Expression and function of SLP76^{OST} in BMMC. (A) *Diagram of the generation of Slp76^{OST/OST} BMMC.* (B) *Schematic representation of SLP76^{OST} molecules.* The One-Strep-tag (OST) is composed of two 8-aminoacid peptides (in red) separated by a 12-aminoacid linker. (C) *SLP76 expression in Slp76^{+/+} and Slp76^{OST/OST} BMMC.* BMMC were solubilized in SDS lysis buffer. Increasing amounts (1X, 2X and 3X) of lysates were electrophoresed and Western blotted with anti-SLP76 and anti-actin antibodies. (D) *β -hexosaminidase release.* IgE anti-DNP sensitized Slp76^{+/+} and Slp76^{OST/OST} BMMC were challenged with the indicated concentrations of DNP-HSA. β -hexosaminidase was measured in supernatant by an enzymatic assay. (E) *Intracellular signaling.* IgE anti-DNP-sensitized Slp76^{+/+} and Slp76^{OST/OST} BMMC were challenged with or without DNP-HSA for 2 min and lysed in SDS lysis buffer. Equal amounts of proteins were electrophoresed and Western blotted with indicated antibodies.

Fig. 2. Proteolysis of SLP76 molecules in BMMC. (A, B) BMMC (A) and thymocytes (B) were solubilized in LM lysis buffer or in SDS lysis buffer. Equal amounts of cell lysates were electrophoresed and Western blotted with the indicated antibodies. (C) IgE anti-DNP-sensitized BMMC were challenged with DNP-HSA for the indicated times and solubilized in LM lysis buffer. Equal amounts of BMMC lysates were electrophoresed and Western blotted with anti-SLP76 and anti-actin antibodies. (D) Equal amounts of proteins from a thymocyte LM lysate, a BMMC LM lysate or a mixture of both were incubated for 10 min on ice, electrophoresed and Western blotted with anti-SLP76 antibodies. (E) BMMC were lysed in SDS lysis buffer or in LM lysis buffer containing no inhibitors (1), Roche cocktail inhibitors 1X (2), Roche cocktail inhibitors 10X (3), Sigma-

Adlrch cocktail 1X (4) or custom-made cocktail inhibitors (21) (5). Lysates were electrophoresed and Western blotted with anti-SLP76 and anti-actin antibodies.

Fig. 3. A new protease inhibitor cocktail prevents SLP76 degradation in mast cells.

(A) *Families of proteases detected in BMMC by transcriptomic analysis.* **(B)** *Families of metal-binding protease encoded by BMMC metalloprotease genes.* **(C)** *Screening protease inhibitors that can protect SLP76 in BMMC lysates.* BMMC were solubilized in LM lysis buffer containing specific protease inhibitors. Equal amounts of lysates were electrophoresed and Western blotted with anti-SLP76 and anti-actin antibodies. **(D)** *Total protection of SLP76 degradation.* BMMC were solubilized in LM lysis buffer supplemented with a new custom-made inhibitor cocktail for 10 min or 90 min. Equal amounts of lysates were electrophoresed and Western blotted with anti-SLP76 and anti-actin antibodies.

Fig. 4. Strep-Tactin affinity purification of functional SLP76 protein complexes from BMMC lysates.

(A) *StrepTactin affinity-purification of SLP76^{OST} in LM lysates of BMMC from Slp76^{+/+} (upper panel) and Slp76^{OST/OST} (lower panel) mice.* Aliquots from each step of the purification procedure were Western blotted with anti-SLP76 antibodies (Input 1 %, FT; flow-through 1%, washes 1%; eluates 10%). The upper panels show an overview of the Strep-Tactin affinity-purification from each cell. **(B)** *Copurification of known interacting proteins of SLP76.* IgE anti-DNP-sensitized Slp76^{+/+} and Slp76^{OST/OST} BMMC were challenged with DNP-HSA or without for 2 min and lysed. Lysates were subjected to affinity-purification with Strep-Tactin. Eluates were electrophoresed and Western blotted with anti-phosphotyrosine (pY), anti-SLP76, anti-PLCγ-1, anti-Grap2, and anti-Grb2 antibodies.

Fig. 5. SLP76 protein-protein interaction network in resting and activated mast cells. **(A)** *Network view of the SLP76 interactome in mast cells.* White nodes: partners identified in resting cells; red nodes: additional partners identified in activated cells. Only proteins significantly associated with SLP76 were included in the network. **(B)** *Venn comparison diagram of SLP76 partners identified in mast cells in the study and of previously published SLP76 partners.* **(C-E)** *Interactome networks of LAT2 (C), Bcr (D) and Dok-3 (E).* Networks were obtained from the STRING database.

Fig. 6. Validation of the SLP76-Dok-3 interaction in activated mast cells. **(A)** *Co-purification of Bcr with SLP76^{OST}.* *Slp76^{OST/OST}* BMMC sensitized with IgE anti-DNP were challenged with DNP-HSA (+) or without (-) for 2 min at 37°C and lysed. Samples of whole cell lysates (WCL) were Western blotted with anti-Dok-3 antibodies (upper panel). Remaining lysates were subjected to affinity-purification with Strep-Tactin. Eluates were electrophoresed and Western blotted with anti-SLP76, anti-phosphotyrosine (pY), and anti-Dok-3 antibodies (lower panel). **(B)** *Co-immunoprecipitation of SLP76 and SHIP1 with phosphorylated Dok-3.* *Slp76^{+/+}* BMMC sensitized with IgE anti-DNP were challenged with DNP-HSA (+) or without (-) for 2 min at 37°C and lysed. Samples of whole cell lysates (WCL) were Western blotted with anti-Dok-3 or anti-phospho-PLCγ-1 antibodies (upper panel). Remaining lysates were immunoprecipitated with anti-Dok-3 antibody. Euates were electrophoresed and Western blotted with anti-Dok-3, anti-phosphotyrosine (pY), anti-SLP76 or anti-SHIP1 antibodies (lower panel). **(C, D)** *Lack of genetic evidence that Dok-3 is involved in FcεRI signaling.* Aliquots of BMMC from *Dok-3^{-/-}* and from *Dok-3^{+/+}* mice were lysed in SDS. whole cell lysates (WCL) were Western blotted with anti-Dok-3 antibodies and, as

positive controls, with anti-Bcr or anti-Grb2 antibodies (C). Aliquots of the same cells were sensitized with IgE anti-DNP, and challenged with the indicated concentrations of DNP-HSA. β -hexosaminidase was measured in supernatant 10 min later (D).

Fig. 7. Validation of the SLP76-Bcr interaction in activated mast cells. (A) *Co-purification of Bcr with SLP76^{OST}. SLP76^{OST/OST} BMMC sensitized with IgE anti-DNP were challenged with DNP-HSA (+) or without (-) for 2 min at 37°C and lysed. Samples of whole cell lysates (WCL) were Western blotted with anti-Bcr antibodies. Remaining lysates were subjected to affinity-purification with Strep-Tactin. Eluates were electrophoresed and Western blotted with anti-SLP76, anti-phosphotyrosine (pY), and anti-Bcr antibodies. (B)* *Co-immunoprecipitation of SLP76 with Bcr. SLP76^{+/+} BMMC sensitized with IgE anti-DNP were challenged with DNP-HSA (+) or without (-) for 2 min at 37°C and lysed. Samples of whole cell lysates (WCL) were Western blotted with anti-Bcr or anti-phospho-PLC γ -1 antibodies. Remaining lysates were immunoprecipitated with anti-Bcr antibody. Eluates were electrophoresed and Western blotted with anti-Bcr, or anti-SLP76 antibodies. (C)* *Inducible Bcr phosphorylation in activated mast cells. BMMC sensitized with IgE anti-DNP were challenged with DNP-HSA (+) or without (-) for 2 min at 37°C and lysed. Samples of whole cell lysates (WCL) were Western blotted with anti-Bcr, anti-pY178 Bcr, anti-phospho-SLP76 or anti-Grb2 antibodies. (D,E)* *Genetic evidence that Bcr is involved in Fc ϵ RI signaling. Aliquots of BMMC from Bcr^{-/-} and from Bcr^{+/+} littermate control mice were lysed in SDS. whole cell lysates (WCL) were Western blotted with anti-Bcr antibodies and, as positive controls, with anti-Dok-3 or anti-Grb2 antibodies (D). Aliquots of the same cells were sensitized with IgE anti-DNP, and challenged with the indicated concentrations of DNP-HSA. β -hexosaminidase was measured in supernatant 10 min later (E).*

Table 1
SLP76 interactome in resting BMMC

Protein Symbol	Protein name	UniProt Acc	Function	ratio
Resting BMMC				OST-/WT-
GRAP2	GRB2-related adaptor protein 2	O89100	Adapter Protein	513
RABE1	Rab GTPase-binding effector protein 1	O35551	GTPase activation	192
RHG27	Rho GTPase-activating protein 27	A2AB59	GTPase activation	72

Proteins were identified in 4 affinity purifications of SLP76^{OST} complexes in resting cells. Listed molecules were enriched at least 4-fold in non-activated *Slp*^{OST/OST} compared to *Slp*^{+/+} BMMC (OST- vs WT-). The significance ($p < 10^{-3}$) of enrichment (OST-/WT- ratio) was determined using student's t-test. UniProt Accession numbers, protein symbols and molecular function are also shown

Table 2
SLP76 interactome in activated BMMC

Protein Symbol	Protein name	UniProt Acc	Function	ratio	
Activated BMMC				OST+/WT+	OST+/OST-
LAT2	Linker for activation of T-cells family member 2	Q9JHL0	Adapter Protein	7146	4820***
BCR	Breakpoint cluster region protein	Q6PAJ1	Kinase/GTPase activation	168	148**
HIG1A	HIG1 domain family member 1A	Q9JLR9	Unknown	144	104***
CDC23	Cell division cycle protein 23 homolog	Q8BGZ4	Cell division	245	55
DC1I2	Cytoplasmic dynein 1 intermediate chain 2	O88487	Cytoskeleton regulation	15	38**
LAT1	Linker for activation of T-cells family member 1	O54957	Adapter Protein	36	37**
PLC γ 1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1	Q62077	Phospholipase	36	36***
SHC1	SHC-transforming protein 1	P98083	Adapter Protein	22	30***
DOK3	Docking protein 3	Q9QZK7	Adapter Protein	8,6	29***
SKAP2	Src kinase-associated phosphoprotein 2	Q3UND0	Src Kinase regulator	34	22***
SUCA	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	Q9WUM5	Ligase	20	22**
GRB2	Growth factor receptor-bound protein 2	Q60631	Adapter Protein	18	16***
SKAP1	Src kinase-associated phosphoprotein 1	Q3UUU5	Src Kinase regulator	22	14***
HECD3	E3 ubiquitin-protein ligase HECTD3	Q3U487	Ligase	47	12
MSH2	DNA mismatch repair protein Msh2	P43247	DNA-binding	12	11**
CSTF1	Cleavage stimulation factor subunit 1	Q99LC2	mRNA processing	10	11**
FYB	FYN-binding protein	O35601	Adapter Protein	17	9.5**
BTK	Tyrosine-protein kinase BTK	P35991	Kinase	6,1	6.7**
PACS1	Phosphofurin acidic cluster sorting protein 1	Q8K212	Coat protein	13	5.4**
1433 η	14-3-3 protein eta	P68510	Adapter Protein	26	4**
1433 θ	14-3-3 protein theta	P68254	Adapter Protein	13	3,8
Q99M59	Interferon gamma inducible protein 47	Q99M59	Hydrolase activity	6,9	3,7
CLNK	Cytokine-dependent hematopoietic cell linker	Q9QZE2	Adapter Protein	40	3,7
VAV	Proto-oncogene vav	P27870	Guanine-nucleotide releasing factor	10	3,3
MA2C1	Alpha-mannosidase 2C1	Q91W89	Mannosidase	13	3,1
1433 γ	14-3-3 protein gamma	P61982	Adapter Protein	45	3,0
1433 ϵ	14-3-3 protein epsilon	P62259	Adapter Protein	20	2,8
E9Q4N7	Protein Arid1b	E9Q4N7	Unknown	28	2,4
1433 β	14-3-3 protein beta/alpha	Q9CQV8	Adapter Protein	19	2,3
1433 ζ	14-3-3 protein zeta/delta	P63101	Adapter Protein	15	2,0
RHG27	Rho GTPase-activating protein 27	A2AB59	GTPase activation	123	1,8
TP53B	Tumor suppressor p53-binding protein 1	Q99LC2	mRNA processing	10	1,3
FA49B	Protein FAM49B	Q921M7	Unknown	29	1,2
GRAP2	GRB2-related adaptor protein 2	O89100	Adapter Protein	1086	0,9
RABE1	Rab GTPase-binding effector protein 1	O35551	GTPase activation	20	0,4

The proteins listed were recurrently identified in 4 affinity purifications of SLP76^{OST} complexes in activated cells. Listed molecules were enriched at least 4-fold in activated *Slp*^{OST/OST} (OST+) compared to *Slp*^{+/+} BMMC (WT+). The enrichment was measured by the OST+/WT+ ratio and its significance ($p < 10^{-3}$) determined using student's t-test. The enrichment existing between activated (OST+) and non activated (OST-) BMMC is also shown and its significance was determined using student's t-test (at least 4-fold enrichment: ** $p < 10^{-2}$, *** $p < 10^{-3}$). UniProt Accession numbers, protein symbols and molecular function are also shown.

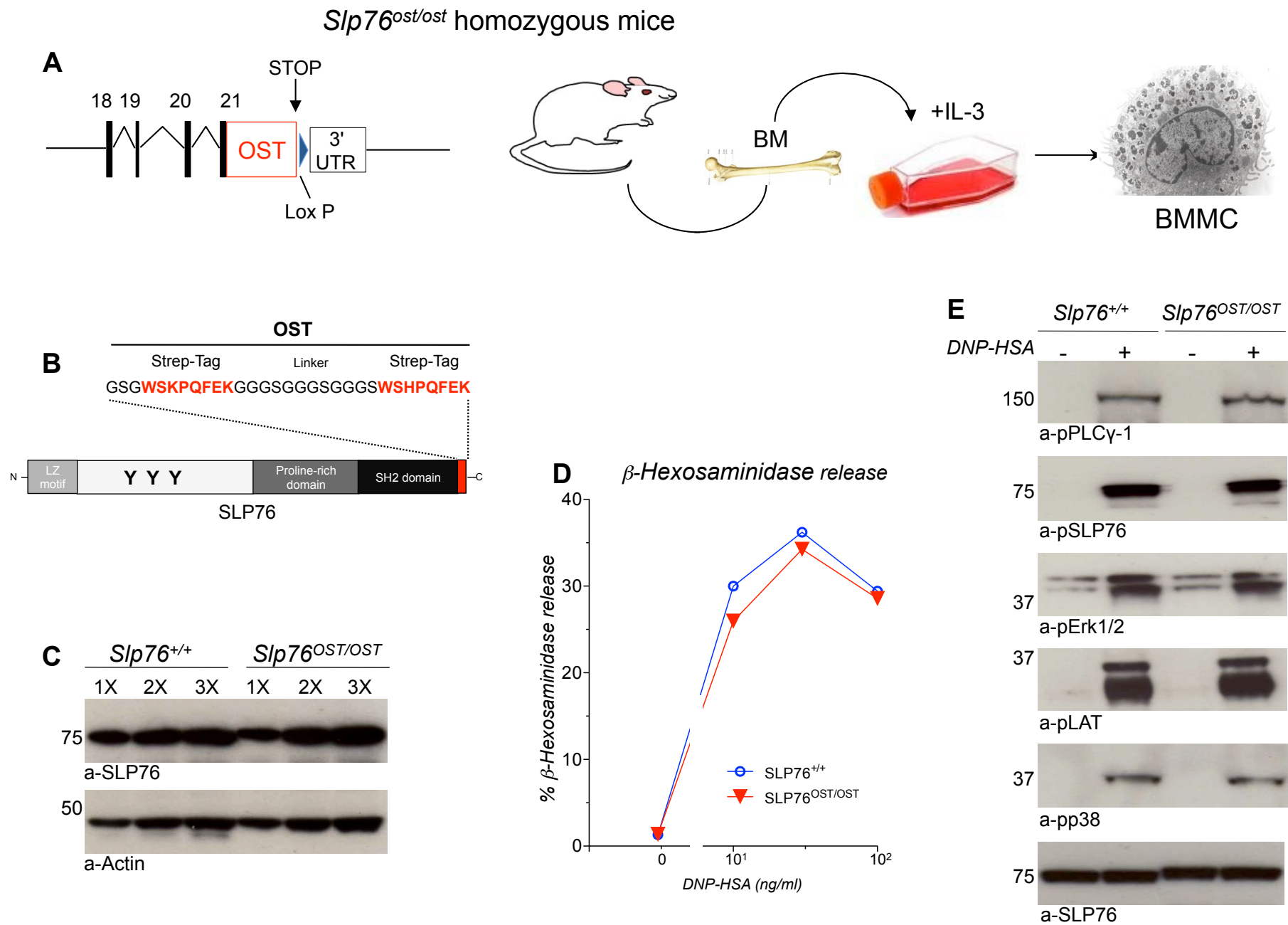
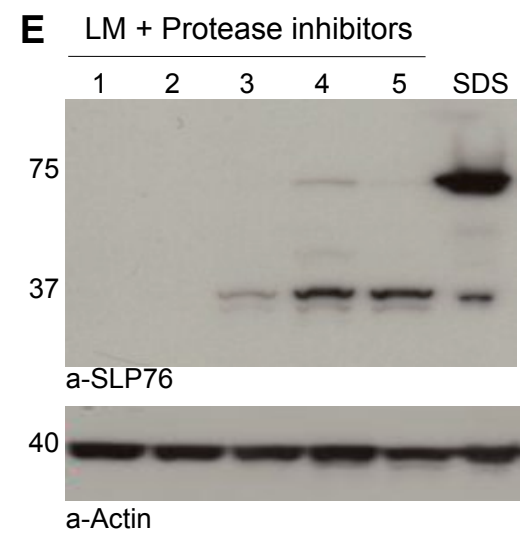
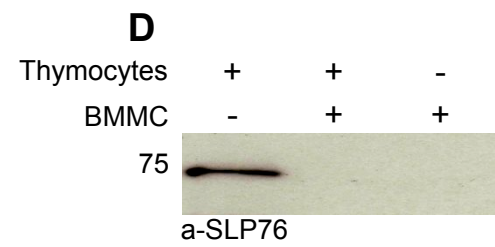
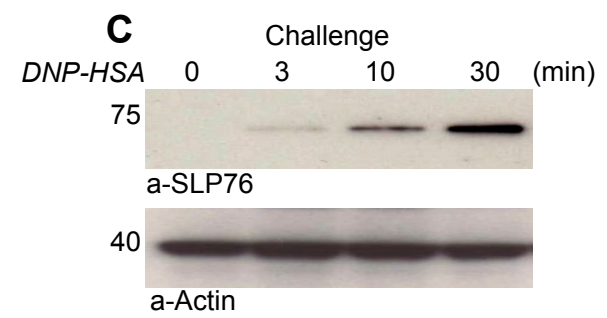
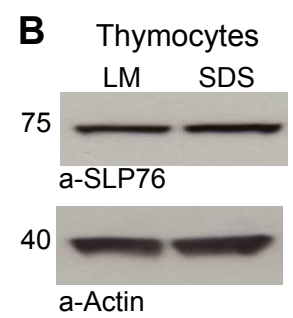
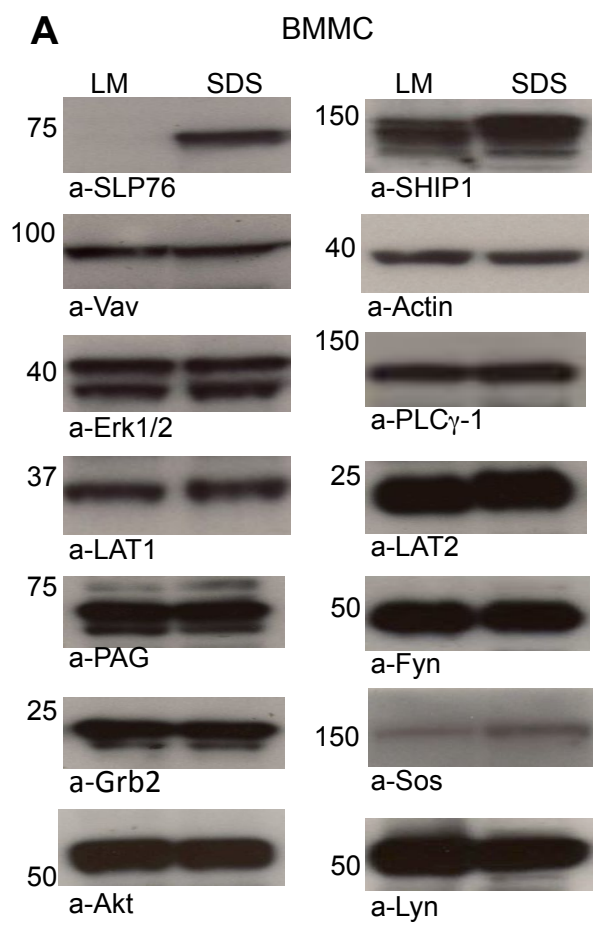


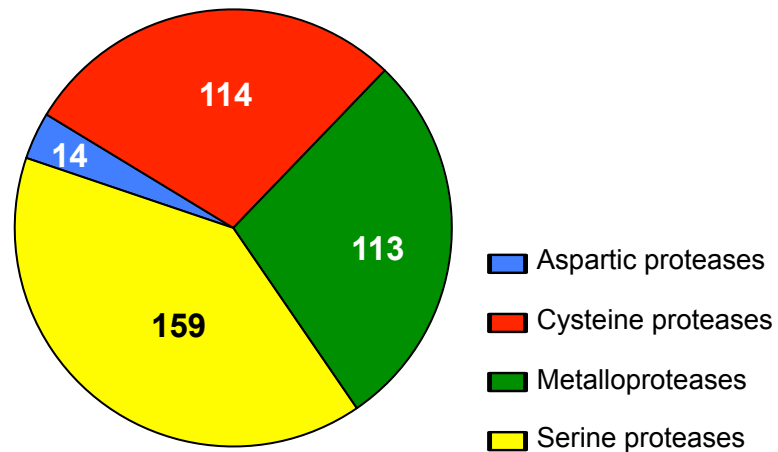
Fig. 1



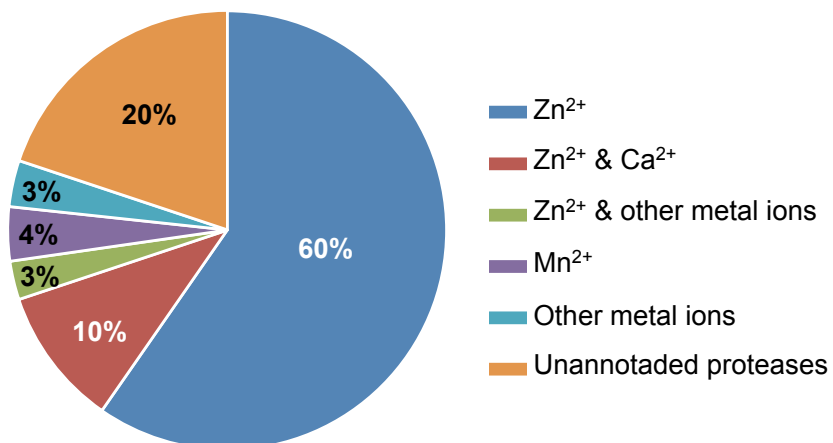
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 3: Roche 10X
 4: Sigma 1X
 5: Kambayashi *et al.*

Fig. 2

A Protease-encoding genes in BMMC

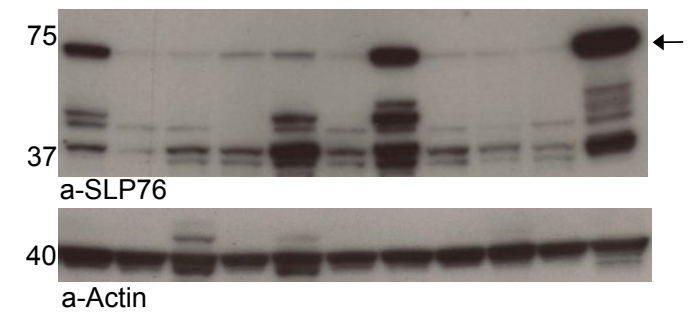


B BMMC metalloproteases



C

	Benzamidine	E-64	AEBSF	Chymostatin	Antipain	Bestatin	1,10 Phenanthroline	EGTA	EDTA	No inhibitor
Aspartic proteases										
Cysteine proteases										
Metalloproteases							Zn ²⁺	Ca ²⁺	Mg ²⁺	
Serine proteases									Mn ²⁺	



D

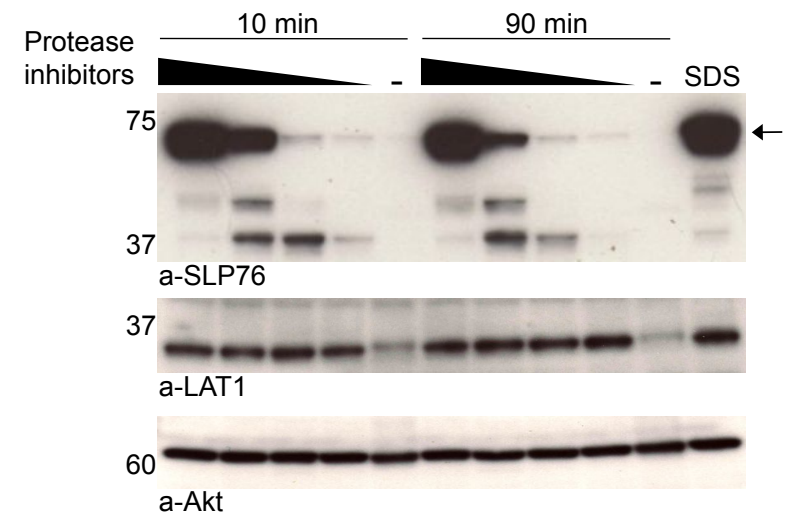


Fig. 3

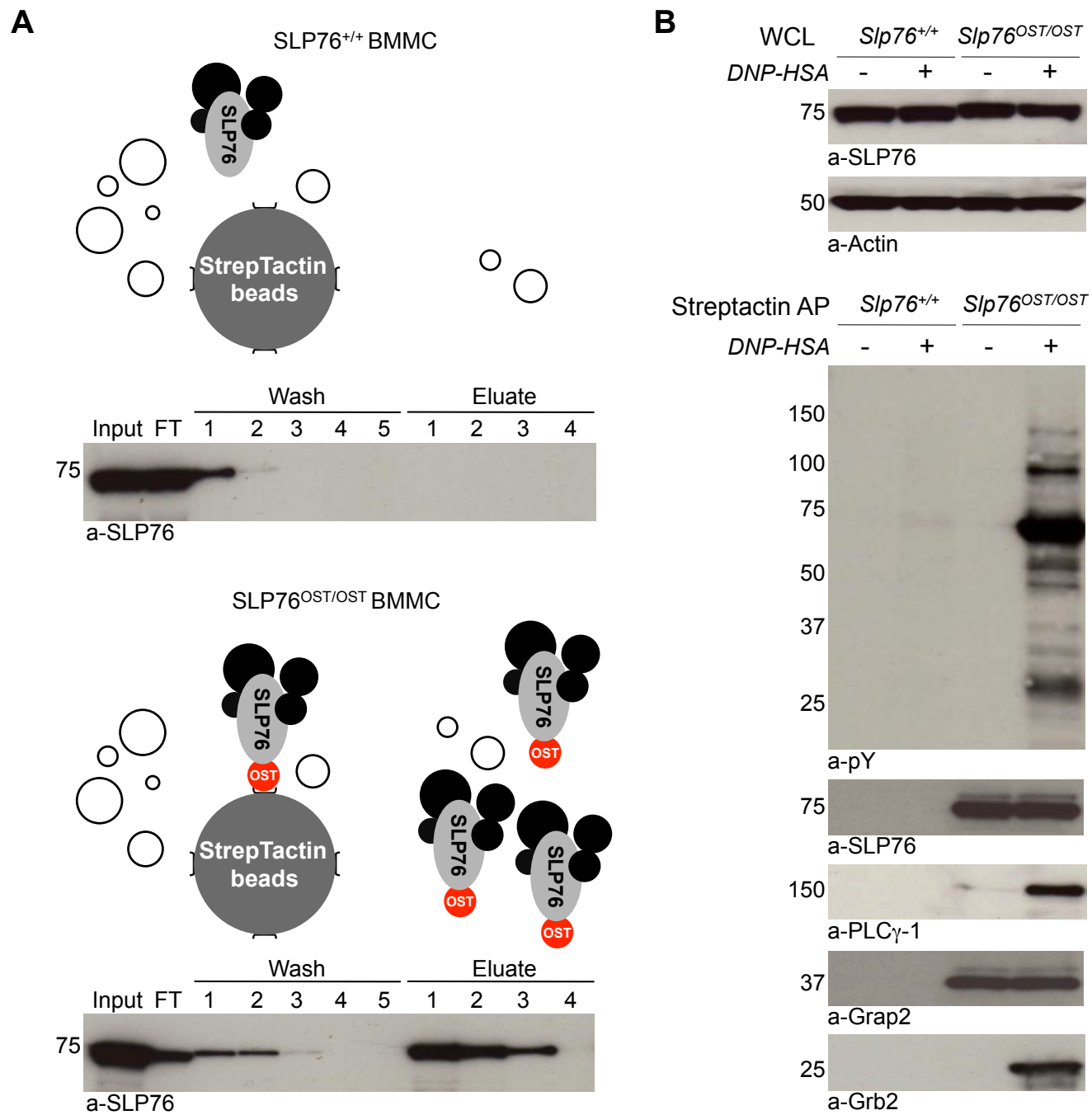


Fig. 4

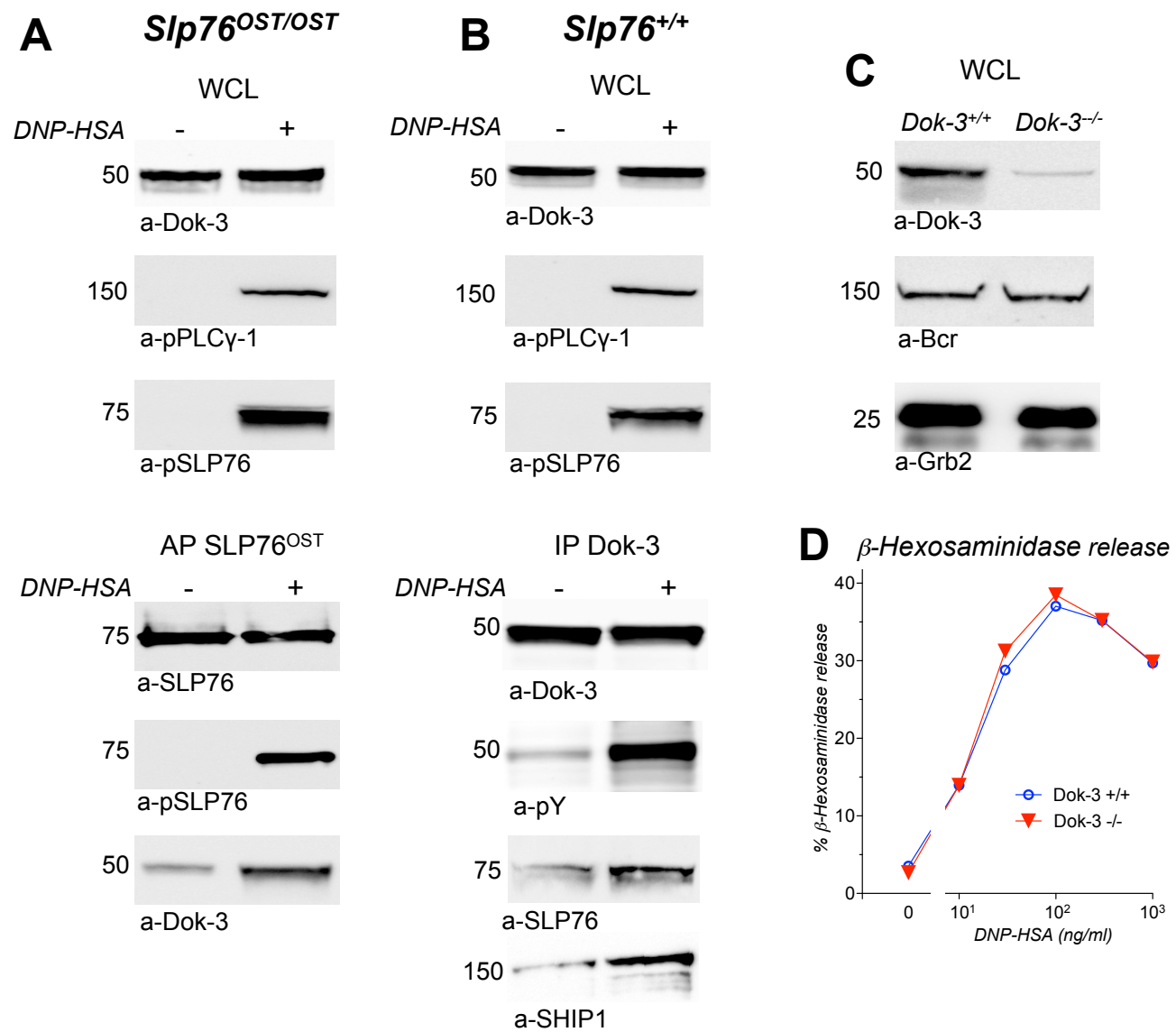


Fig. 6

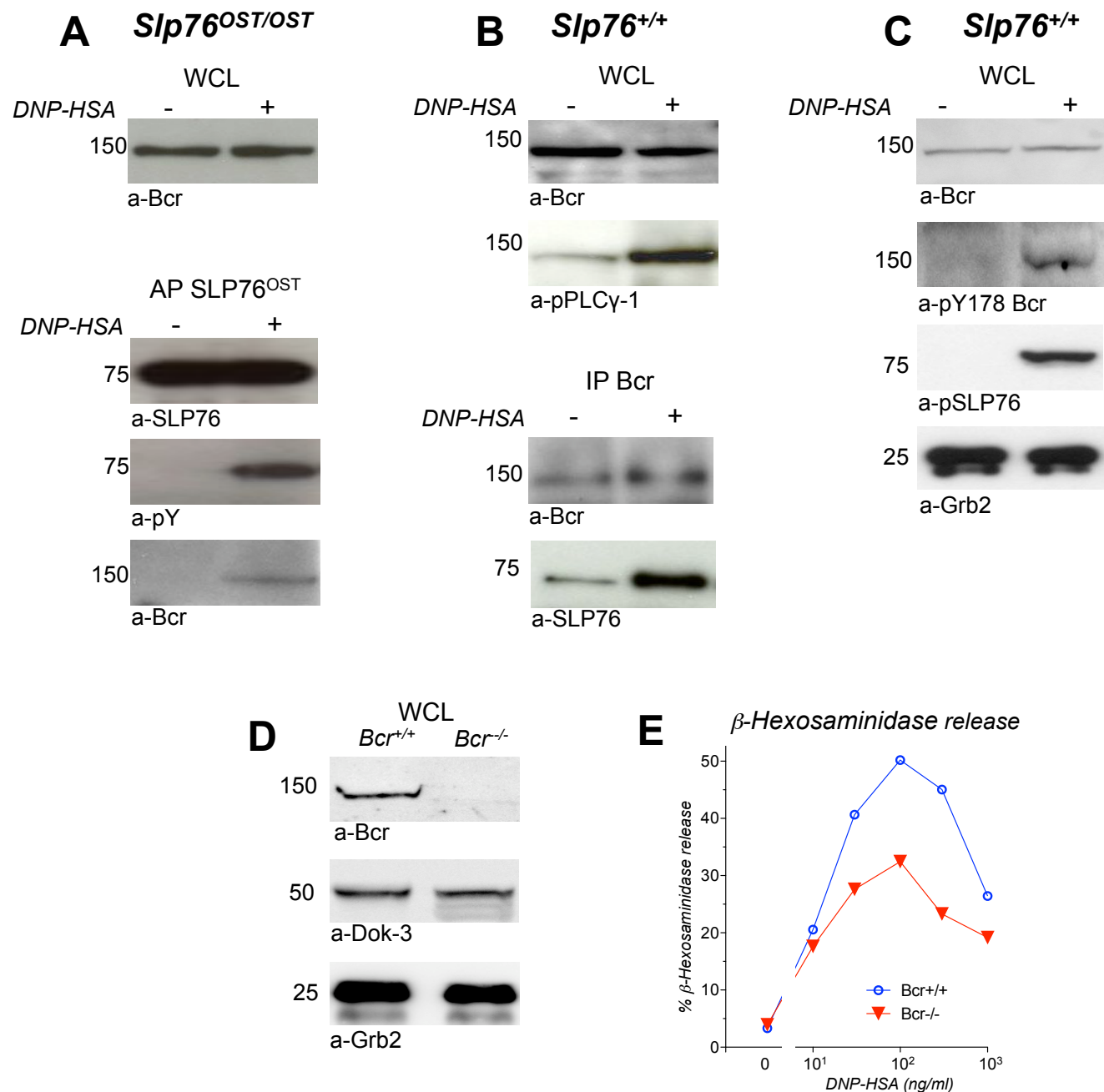


Fig. 7

SUPPLEMENTARY MATERIALS

Supplementary Figures

Fig. S1. SLP76 is lost in mast cell lysates prepared with a panel of mild non-denaturing detergents. BMMC were solubilized in lysis buffers containing laurylmaltoside (LM), octylglycoside (OG), IGEPAL, Brij35 or TX100, supplemented with standard protease inhibitor cocktail (Roche). Equal amounts of cell lysates were electrophoresed and Western blotted with anti-SLP76, anti-LAT1, anti-PAG or anti-actin antibodies.

Fig. S2. Phenanthroline protects SLP76 from BMMC proteases in LM lysis buffer pH. 8.0. (A, B) BMMC were solubilized in LM lysis buffer pH 7.4 (A) or pH 8.0 (B) supplemented with specific metalloprotease inhibitors or in SDS lysis buffer. Equal amounts of cell lysates were electrophoresed and Western blotted with anti-SLP76 antibodies. **(C)** BMMC were solubilized in LM lysis buffer pH 7.4 or pH 8.0 supplemented with Roche cocktail inhibitors or not. Equal amounts of cell lysates were electrophoresed and Western blotted with anti-SLP76 antibodies.

Supplementary Tables

Table S1: Genes expressed in BMMC. Known BMMC-specific genes are shown, along with their detection confidence level.

Table S2: Proteases expressed in BMMC. All the proteases retrieved on the AB1700 DNA-microarray platform are shown, along with their associated detection p-value. Protease families are also specified.

Table S3: Metal-binding proteases expressed in BMMC. Zn²⁺-dependent proteases are highlighted.

Table S4: Mast cells protease inhibitors cocktail.

Table S5: SLP76 interactome in resting and activated BMMC. Tables 1 and 2 have been completed with p-value of student's t-tests and iBAQ/MS intensity from all samples.

Table S6: AP-MS data from some known SLP76 partners in T-cells. Student's t-tests results (p-value and ratio) are gathered with raw iBAQ data.

Table S7: Proteins identification and quantification data. Number of total peptides, unique peptides, sequence coverage, MS intensity and iBAQ value in each sample are provided for each protein identified in the whole dataset.

Table S8: Peptides identification and quantification data. Sequence, best score, uniqueness of all peptides identified are provided with MS intensity in each sample.

Supplementary Materials and Methods :

DNA-microarray data analysis: The transcriptome of three biological replicates of resting BMMC cells were analyzed using AB1700 DNA-microarrays. An expression confidence level was computed for the genes covered by the platform, using the following fields from the hybridization output files (raw data):

- The **S/N field** is a computed test statistics of each probe's estimated signal-to-noise ratio. Thus, a detection confidence level can be computed according to a convenient probability distribution. As suggested by the manufacturer (*cf.* AB1700 platform

documentation downloaded by <http://www.appliedbiosystems.com>), we derived p-values directly from the Standard Normal probability distribution.

- The **Flag field** indicates, for each probe, the possible occurrence of quality issues. Values lower than or equal to 100 correspond to good quality probes. Before applying the statistical test, we set to zero all the S/N values corresponding to associated Flag greater than 100. In this way, all the poor quality measurements obtained a weak detection confidence level (*i.e.* p-value = 0.5).

Finally, in order to avoid multiple gene values (as we have three sample replicates, and as several probes may correspond to the same gene for a given replicate), we only kept the highest p-value obtained for each gene across all the corresponding probes in the dataset. We considered the genes showing a p-value lower than an arbitrarily chosen threshold (*i.e.* p-value < 0.1) as *expressed*. We tested this procedure by verifying the computed detection confidence level of known specific BMMC genes (Table S2). All these genes were declared expressed by our test.

Then, we estimated the number of protease-coding genes expressed in BMMC. From the MEROPS database (<http://merops.sanger.ac.uk>), we downloaded a list of 685 reported *Mus musculus* proteases genes annotated with their main family names (Serine, Metallo, Aspartic and Cysteine proteases). 539 out of the 685 annotated protease-coding genes were found to be represented in the DNA-microarray platform. Among them, 400 genes were declared expressed by the statistical test with a confidence level greater than 90% (Figure 3A). Table S2 reports the results for all the 539 protease-coding genes.

All data analyses were carried out using R programming language (<http://www.r-project.org>).

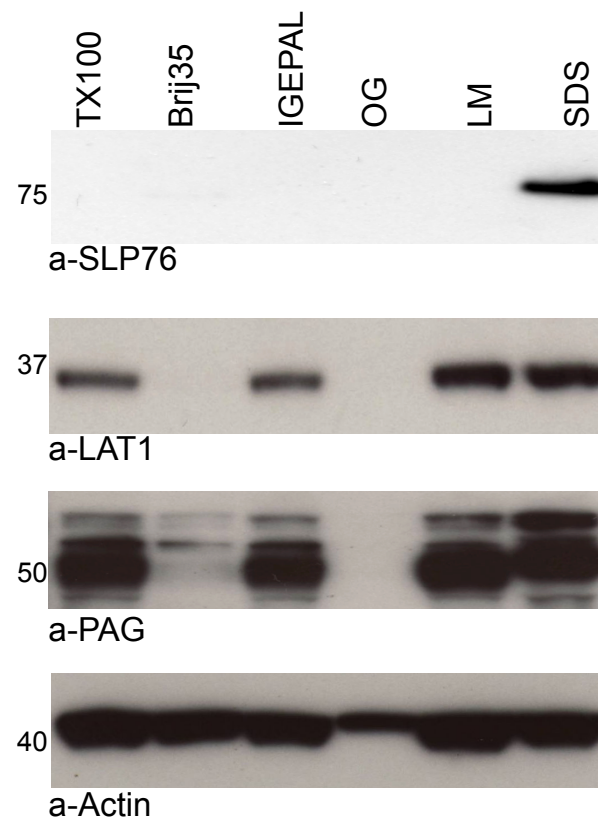


Fig. S1

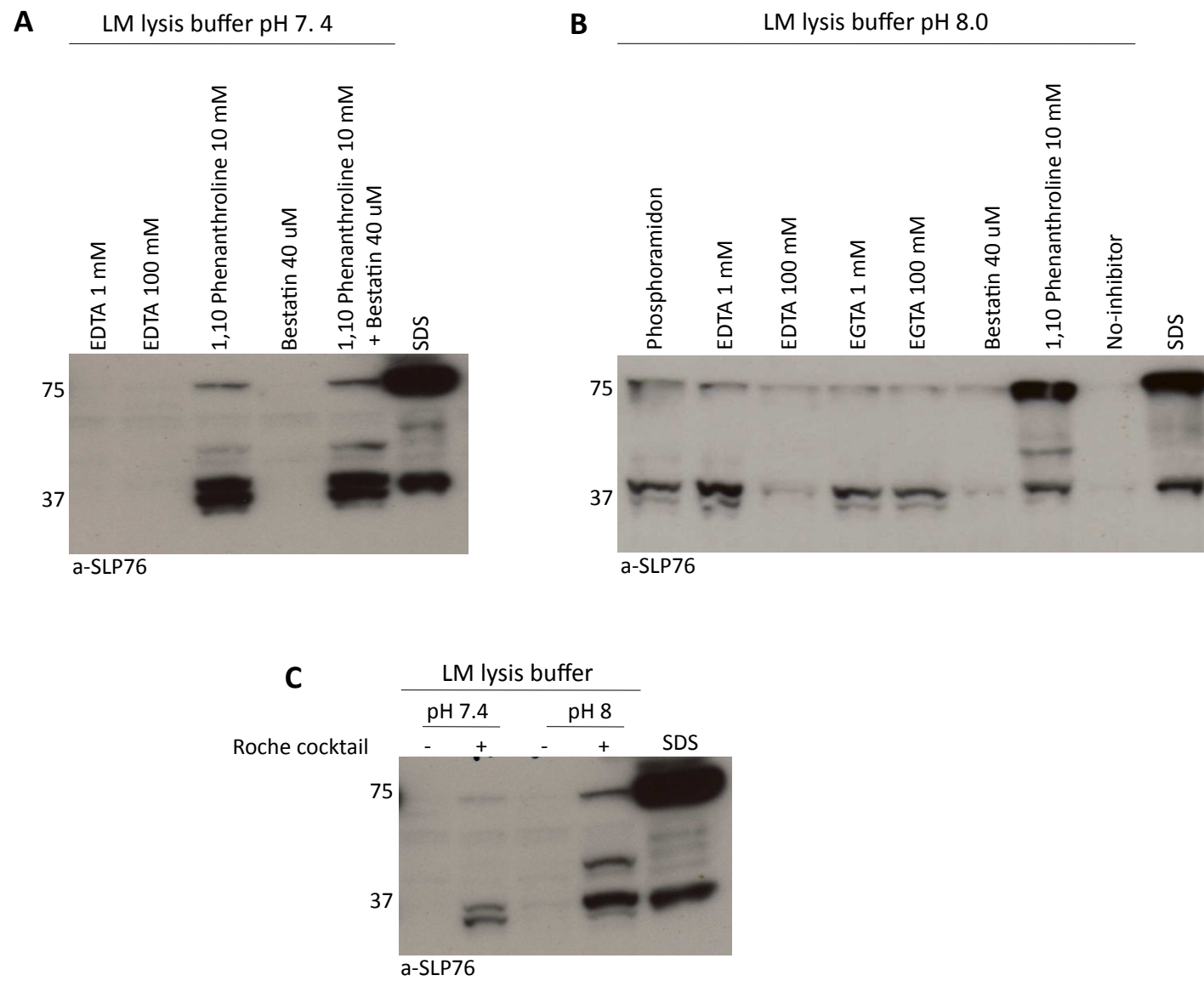


Fig. S2