

JPET#249706

**Title**

Distinct Roles of Small GTPases Rac1 and Rac2 in Histamine H<sub>4</sub>  
Receptor-Mediated Chemotaxis of Mast Cells

Atsuo Kuramasu, Mie Wakabayashi, Makoto Inui, and Kazuhiko Yanai

Department of Pharmacology, Yamaguchi University Graduate School of  
Medicine, Ube, Yamaguchi, Japan (A.K., M.I.); Department of Pharmacology,  
Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan (M.W.,  
K.Y.)

JPET#249706

## Running Title Page

Running title: Rac GTPases downstream of H<sub>4</sub> Receptor

Corresponding author: Atsuo Kuramasu, Department of Pharmacology,  
Yamaguchi University Graduate School of Medicine, 1-1-1 Minami-Kogushi, Ube,  
Yamaguchi 755-8505, Japan. E-mail: [atsuok@yamaguchi-u.ac.jp](mailto:atsuok@yamaguchi-u.ac.jp)

Number of text pages: 43

Number of tables: 1

Number of figures: 8

Number of references: 45

Number of words in the Abstract: 203

Number of words in the Introduction: 519

Number of words in the Discussion: 1499

Nonstandard abbreviations:

ANOVA	analysis of variance
BMMC	bone marrow-derived mast cell
ERK	extracellular signal-regulated kinase
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GST	glutathione S-transferase
IL	interleukin

JPET#249706

IP <sub>3</sub>	1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MBP	maltose binding protein
MEK	Mitogen-activated protein kinase kinase
PAK1	p21-activated kinase
PBD	p21 binding domain
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PLC	phospholipase C
shRNA	short-hairpin RNA

Recommended section assignment: Cellular and Molecular

JPET#249706

## Abstract

Histamine induces chemotaxis of mast cells through the H<sub>4</sub> receptor. However, little is known about the precise intracellular signaling pathway that mediates this process. In this study, we identified small GTPases Rac1 and Rac2 as intracellular binding partners of the H<sub>4</sub> receptor and characterized their roles in H<sub>4</sub> receptor signaling. We showed that histamine induced Rac GTPase activation via the H<sub>4</sub> receptor. A Rac inhibitor NSC23766 attenuated chemotaxis of mast cells toward histamine, as well as histamine-induced calcium mobilization and extracellular signal-regulated kinase (ERK) activation. Histamine-induced migration of mast cells was also sensitive to PD98059, an inhibitor of the mitogen-activated protein kinase kinase (MEK), indicating that the Rac-ERK pathway was involved in chemotaxis through the H<sub>4</sub> receptor. Inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) by LY294002 suppressed the histamine-induced chemotaxis and activation of Rac GTPases, suggesting that PI3K regulates chemotaxis upstream of Rac activation. Specific knockdown of Rac1 and Rac2 by short hairpin RNA revealed that both Rac GTPases are necessary for histamine-induced migration. Downregulation of Rac1 and Rac2 led to attenuated response in calcium mobilization and ERK activation, respectively. These observations suggested that Rac1 and Rac2 have distinct and essential roles in intracellular signaling downstream of H<sub>4</sub> receptor-PI3K in histamine-induced chemotaxis of mast cells.

JPET#249706

## Introduction

Directed migration or chemotaxis of mast cells is an important process for their recruitment to target tissues in various pathophysiological conditions such as inflammation and allergy. Many chemoattractants including eicosanoids, antigens, growth factors, chemokines, and others are known to induce chemotaxis of mast cells (Halova et al., 2012). Additionally, mast cells themselves produce and release various attractants such as histamine to attract other mast cells and/or their progenitor cells, resulting in mast cell accumulation in local tissues (Hofstra et al., 2003).

The histamine H<sub>4</sub> receptor is a G protein-coupled receptor (GPCR) that is predominantly expressed in immune cells such as mast cells, eosinophils, dendritic cells, monocytes, and T lymphocytes, and plays various roles including migration, shape change, actin polymerization, expression of surface molecules, and regulation of cytokine production (Zampeli and Tiligada, 2009; Thurmond, 2015). In mouse bone marrow-derived mast cells (BMMCs), H<sub>4</sub> receptor stimulation induces calcium mobilization from intracellular storage and chemotaxis toward histamine, both of which are dependent on pertussis toxin-sensitive G protein and phospholipase C (PLC) (Hofstra et al., 2003). Histamine also induces interleukin (IL)-6 production in mast cells, which requires both the PI3 kinase and extracellular signal-regulated kinase (ERK) pathways downstream of H<sub>4</sub> receptor (Desai and Thurmond, 2011). Although many H<sub>4</sub> receptor-related functions have been reported in various cell types, little is known on the functional relevance of intracellular signaling pathway downstream of H<sub>4</sub> receptor activation.

JPET#249706

Rac GTPases belong to the Rho subfamily of the Ras small G protein superfamily. While Rac1 is widely expressed, Rac2 expression is restricted to hematopoietic cells. Rac2-deficient mice show functional abnormality in various cell types including mast cells, neutrophils, macrophages, and lymphocytes, suggesting that Rac1 and Rac2 have distinct roles (Roberts et al., 1999; Yang et al., 2000; Pradip et al., 2003; Croker et al., 2002). In mast cells, activation of Rac GTPases is involved in various cell functions such as migration, polarity, adhesion, cell cycle, and transcriptional regulation (Samayawardhena et al., 2007; Yang et al., 2000; Timokhina et al., 1998; Massol et al., 1998; Gu et al., 2002). Typically, Rac activation in these events occurs downstream of cytokine receptor or IgE receptor. However, little is known on Rac GTPase activation downstream of GPCR such as histamine H<sub>4</sub> receptor in mast cells.

Accumulating evidence indicated that the carboxy-terminal cytoplasmic domain of GPCR interacts with various intracellular proteins that may have important functions, such as receptor targeting, clustering of receptor with various effector molecules, and modulation of signaling efficiency (Bockaert et al., 2003). Our previous work also showed that proteins that interact with the carboxy-terminal domain of histamine H<sub>2</sub> and H<sub>3</sub> receptors have essential roles in receptor trafficking and signal transduction (Xu et al., 2008; Maeda et al., 2008).

In this study, to further characterize the H<sub>4</sub> receptor signaling, we determined proteins that interacted with the carboxy-terminal domain of H<sub>4</sub> receptor. We showed that Rac1 and Rac2 GTPases physically associated with H<sub>4</sub> receptor. Furthermore, these proteins were also functionally coupled with H<sub>4</sub>

JPET#249706

receptor regulation of histamine-induced chemotaxis of mast cells. Thus, our results indicated that Rac1 and Rac2 have distinct intracellular roles: Rac1 regulates histamine-induced calcium mobilization and Rac2 controls ERK activation.

## **Materials and Methods**

### **Reagents and Antibodies**

Histamine dihydrochloride, pyrilamine maleate, and cimetidine were from Sigma (St. Louis, MO). JNJ7777120 and NSC23766 were from Tocris Bioscience (Bristol, UK). PD98059, LY294002, and phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody were from Cell Signaling Technology (Tokyo, Japan). SP600125 was from Abcam (Tokyo, Japan). Fluo-3 AM was from Dojindo (Kumamoto, Japan). Anti-Rac1 mouse monoclonal antibody (clone 23A8) and anti-Rac2 rabbit polyclonal antibody were from Millipore (Billerica, MA). Anti-maltose binding protein (MBP) monoclonal antibody was from New England Biolabs (Ipswich, MA). ERK1/2 polyclonal antibody was from Enzo Life Sciences (Farmingdale, NY). PE-Cy7 anti-mouse CD117 (c-Kit) antibody (clone 2B8) and PE anti-mouse FcεR1α antibody (clone MAR-1) were from BioLegend (San Diego, CA). Peroxidase-conjugated anti-GAPDH monoclonal antibody was from Wako (Osaka, Japan). Horseradish peroxidase-conjugated anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA). Peroxidase-labeled

JPET#249706

anti-mouse IgG (H+L) antibody was from KPL (Gaithersburg MD).

### **Plasmids**

Plasmids encoding glutathione S-transferase (GST)-fused mouse histamine H<sub>4</sub> receptor and MBP-fused Rac were constructed from pGEX-5X2 (GE Healthcare, Tokyo, Japan) and pMAL-c2x (New England Biolabs), respectively. The coding sequence for the full carboxy terminus (CT; amino acids 363–391), the proximal half of carboxy terminus (CTΔ; amino acids 363–376), and the third intracellular loop (i3; amino acids 195–306) of mouse H<sub>4</sub> receptor, and the full-length mouse Rac1 and Rac2 were amplified by PCR. The cDNA for p21-activated kinase 1 (PAK1) p21 binding domain (PBD) (amino acids 199–450) was cloned into pGEX-5X2 to generate the GST-PAK1-PBD expression plasmid.

### **BMMC Culture and Flow Cytometry**

Bone marrow cells were isolated from the femur bones of a female 4–6-week-old C57BL/6 mouse. The use of animals was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and approved by the Institutional Animal Care and Use Committee of Tohoku University and Yamaguchi University. Cells were suspended in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-100 µg/ml streptomycin, and 5 ng/ml recombinant mouse IL-3 (BioLegend). After 24 h of culture, floating cells were

JPET#249706

transferred to a new dish, and cell medium was replaced every 5–7 days.

Differentiation into mast cells was confirmed by the expression of FcεRI and CD117 (c-kit) as assessed by flow cytometry. After 4 to 12 weeks in culture, the BMMCs were used for experiments.

### **Affinity Purification and Nano LC-MS/MS**

Cleared lysate of *Escherichia coli* expressing GST or GST-fused mouse H<sub>4</sub> receptor CT was bound to Glutathione Sepharose 4B beads (GE Healthcare). BMMCs ( $6 \times 10^7$  cells) were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM EGTA, 1 mM EDTA, 0.5% Triton X-100, with Roche Complete protease inhibitor cocktail (Basel, Switzerland)) at 4°C on a rotator overnight and centrifuged at 15,000 *g* for 15 min at 4°C. The supernatant was applied to the GST column and the flow-through was then applied to the GST-fused mouse H<sub>4</sub> receptor CT column. Both columns were washed extensively and bound proteins were eluted with elution buffer (50 mM Tris-HCl pH 7.4, 1M NaCl, 10% glycerol, 1 mM DTT, 1 mM EGTA, 1 mM EDTA, 0.5% Triton X-100). The proteins were precipitated with ice-cold trichloroacetic acid (final concentration of 10%) and centrifuged at 15,000 *g* for 10 min at 4°C. The pellet was washed with ether/ethanol (1:1) and dissolved in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were resolved on a 10–20% gradient SDS-PAGE gel and stained using

JPET#249706

the silver stain MS kit (Wako). Excised gel bands were reduced with 100 mM DTT and alkylated with 100 mM iodoacetamide. After washing, the gel pieces were incubated with trypsin overnight at 30°C. Recovered peptides were desalted with Ziptip c18 (Millipore). Samples were analyzed by nano LC-MS/MS systems (DiNa HPLC, KYA TECH Corporation (Tokyo, Japan) and QSTAR XL (Applied Biosystems, Foster City, CA)). Mass data acquisitions were piloted by the Mascot software (Kanno et al., 2007).

### ***In Vitro* Pull-Down Experiment**

Cleared lysates from *E. coli* expressing GST- or MBP-fusion proteins were mixed together and incubated for 1 h. The mixture was further incubated with Glutathione Sepharose 4B for 1 h, and proteins bound to the beads were separated on SDS-PAGE and subjected to immunoblot with anti-MBP antibody. Signals were visualized by horseradish peroxidase-conjugated secondary antibody and Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan).

### **Rac Activation Assay**

BMMCs ( $3 \times 10^7$  cells) were starved of IL-3 for at least 24 h. The cells were resuspended in Hank's balanced salt buffer with calcium and magnesium (Wako) containing 0.1% bovine serum albumin (BSA, Sigma Aldrich), and preincubated for 10 min in the presence of an inhibitor or antagonist at the indicated concentrations at 37°C. The BMMCs were stimulated with 10  $\mu$ M histamine for 5

JPET#249706

min and immediately placed on an ice-water bath. The cells were washed once with ice-cold Tris-buffered saline containing 0.1% BSA and lysed with lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP-40, 1 mM DTT, 5% glycerol, 1.5× Roche Complete protease inhibitor cocktail) for 5 min at 4°C. The cleared lysate was incubated with GST-PAK1-PBD-bound beads at 4°C for 1 h. The beads were washed and bound proteins were extracted with Laemmli sample buffer (Sigma Aldrich). A portion of total lysate was mixed with SDS sample buffer to detect the total Rac input. The samples were separated on 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. Rac GTPases were detected by immunoblotting using mouse Rac1- and Rac2-specific antibodies.

### **Chemotaxis Assay**

Chemotaxis cell well inserts with a pore size of 5 µm (KURABO, Osaka, Japan) were coated with 10 µg/ml of human plasma fibronectin (Roche) for 30 min at 37°C. After removing the fibronectin solution, cells ( $1 \times 10^5$ ) in an assay medium (0.1% BSA in RPMI1640) were added into the Chemotaxis cell inserts in a 200 µL volume with or without inhibitor. After incubation for 30 min at 37°C, 600 µL of assay medium with 10 µM histamine was added into the lower chamber. The plate was further incubated for 1 h at 37°C and the number of migrated cells in the lower chamber was counted by flow cytometry. A suspension of

JPET#249706

fluorospheres of known counts (Flow-Count; Beckman Coulter, Brea, CA) was included to determine the absolute cell number.

### **ERK Activation Assay**

BMMCs ( $1 \times 10^5$  cells) were cultured in RPMI1640 containing 0.1% BSA for at least 24 h. The cells were preincubated for 5 min in the presence of an inhibitor or antagonist at the indicated concentrations at 37°C. The BMMCs were stimulated with 10  $\mu$ M histamine for 2 min and immediately placed on an ice-water bath. The cells were washed once with ice-cold phosphate-buffered saline containing 0.1% BSA and lysed with SDS-PAGE sample buffer. Cell lysates were separated on SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with anti-ERK1/2 or anti-phospho-p44/p42 MAPK antibody followed by a horseradish peroxidase-conjugated secondary antibody. Signals were detected with the Chemi-Lumi One L detection kit and Hyperfilm ECL (GE Healthcare), and band intensity was quantified by densitometry. The signal for activated ERK was normalized to that of total ERK. ERK activation was expressed as the fold increase over unstimulated cells.

### **Calcium Mobilization Assay**

Cells were suspended in assay medium (Hank's balanced salt buffer with calcium and magnesium containing 20 mM Hepes pH 7.4 and 0.1% BSA) and loaded with 4  $\mu$ M Fluo-3 AM for 30 min at 37°C in the presence of 2.5 mM

JPET#249706

probenecid (Wako) and 0.08% pluronic acid (Dojindo). The cells were washed three times with assay medium and plated in a black-wall 96-well plate. The plate was centrifuged for 3 min at 300g, and calcium mobilization was assayed with a FlexStation 3 microplate reader (Molecular Devices, San Jose, CA). The  $\Delta F/F$  was calculated according to the following formula:

$$\Delta F/F = (F - F_{\text{rest}}) / F_{\text{rest}}$$

where F is the dye fluorescence at any given time and  $F_{\text{rest}}$  is the average fluorescence signal prior to histamine addition.

### **Lentivirus Production**

To construct the shRNA expression plasmids, oligonucleotides corresponding to the sense target sequence, hairpin loop (5'-TTCAAGAGA-3'), and antisense target sequence were synthesized, annealed together, and inserted into the pLKO1.puro vector (#8453; Addgene, Cambridge, MA). The shRNA target sequences (sense) were as follows: mouse Rac1 sh1, 5'-GACGGAGCTGTTGGTAAA-3'; mouse Rac1 sh11, 5'-CTGGAACCTTTGTACGCT T-3'; mouse Rac2 sh14, 5'-ATGTGATGGTGGACAGTAA-3'; mouse Rac2 sh16, 5'-GGCCAAGGATATTGA TTC A-3'. Each shRNA expression vector were transfected into HEK293T cells along with a lentiviral packaging plasmid psPAX2 (#12260; Addgene) and envelope expression vector pVSV-G (Clontech, Fremont,

JPET#249706

CA). The virus supernatants were harvested 48 h after transfection and concentrated by ultracentrifugation (70,000 *g* for 2 h at 4°C). Virus particles from a 10-cm dish were resuspended in 0.1 ml of RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and stored at –80°C until use.

### **shRNA-Mediated Knockdown of Rac1 and Rac2 in Mast Cells**

RetroNectin, a recombinant human fibronectin fragment (Takara, Kusatsu, Japan), was used for the lentiviral transduction. Briefly, a polystyrene tube (BD Falcon 2058; BD Biosciences, Franklin Lakes, NJ) was coated with 30 µg/ml RetroNectin for 2 h at 20°C. Lentivirus (0.1 mL) was bound to the RetroNectin-coated tube by centrifugation at 1,350 *g* for 4 h at 32°C. After removing unbound lentivirus, lineage-negative bone marrow cells ( $1 \times 10^5$  cells), isolated using the mouse lineage cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) from a female 4–6-week-old Balb/c mouse, were added into the lentivirus-coated tube and centrifuged at 400*g* for 30 min to facilitate transduction. The cells were then cultured in complete RPMI1640 medium containing 50 ng/ml mouse stem cell factor (SCF, Biolegend) and 10 ng/ml mouse IL-3. Two days after transduction, puromycin was added to a final concentration of 2 µg/ml. Differentiation into mast cells was confirmed by the expression of FcεRI and CD117 (c-kit).

### **Statistical Analysis**

JPET#249706

Data are presented as the mean with S.E.M. Comparisons between two groups were assessed with unpaired t-test, and among three or more groups with one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. A P value of < 0.05 was considered statistically significant.

## Results

### Association of Rac1 and Rac2 with Histamine H<sub>4</sub> Receptor

To fully elucidate the signaling pathway downstream of H<sub>4</sub> receptor, we sought to identify binding partners interacting with the intracellular domain of mouse histamine H<sub>4</sub> receptor. For the pull-down assay, BMMC lysate was affinity-purified with bacterially expressed GST-fused carboxy-terminal tail of mouse H<sub>4</sub> receptor (GST-mH4RCT) as the bait. Figure 1A shows silver-staining of the affinity-purified proteins separated on SDS-PAGE. Major bands that were specific for binding to GST-mH4RCT were analyzed by nano LC-MS/MS (Kanno et al., 2007). Identified proteins included ERM proteins (ezrin and moesin), small G proteins (Rac1 and Rac2), and casein kinase 2 (Table 1). Further, immunoblotting using specific antibodies confirmed Rac1 and Rac2 as binding partners of mouse H<sub>4</sub> receptor (Fig. 1B).

Direct interaction of H<sub>4</sub> receptor carboxy-terminal tail with Rac1 or Rac2 was examined using bacterially expressed recombinant fusion proteins. The full carboxy-terminus (H4RCT; aa 363-391), proximal half of carboxy-terminus (H4RCTΔ; aa 363-376), and third intracellular loop (H4Ri3; aa 195-306) of mouse H<sub>4</sub> receptor expressed as GST-fusion proteins (Fig. 1C) were tested for

JPET#249706

binding with MBP-fused full-length mouse Rac1 or Rac2 *in vitro*. The result showed that the proximal half of carboxy-terminus or the third intracellular loop was sufficient for H<sub>4</sub> receptor binding to Rac1 and Rac2 (Fig. 1D). These results indicated that Rac1 and Rac2 physically interacted with H<sub>4</sub> receptor in mouse BMMC.

### **Rac Activation via H<sub>4</sub> receptor**

We next explored whether Rac1 and Rac2 were activated by histamine in BMMC. Rac activation was assessed by a pull-down assay using the PAK1-PBD to which the active GTP-bound form of Rac specifically binds. In BMMC, 10  $\mu$ M of histamine increased the activation of Rac1 and Rac2 by 2 to 3 folds, respectively (Fig. 2A and B). Because BMMC expresses histamine H<sub>1</sub>, H<sub>2</sub>, and H<sub>4</sub> receptors (Ito et al., 2012), we next examined the effect of specific receptor antagonists on histamine-induced Rac activation. In the presence of 1  $\mu$ M JNJ7777120, a specific H<sub>4</sub> receptor antagonist, histamine-induced activation of Rac1 and Rac2 was significantly attenuated (Fig. 2A). Pyrilamine and cimetidine, an H<sub>1</sub> and H<sub>2</sub> receptor antagonist, respectively, failed to block the effect of histamine (Fig. 2B). These results suggested that both Rac1 and Rac2 were activated by histamine via H<sub>4</sub> receptor in BMMC.

### **Rac Involvement in BMMC Chemotaxis Toward Histamine**

Because H<sub>4</sub> receptor mediates mast cell migration toward histamine (Hofstra et al., 2003), we subsequently investigated the role of Rac activation in this process. NSC23766 is a specific Rac inhibitor that effectively blocks Rac1 activation by Rac-specific guanine nucleotide exchange factor (GEF) Trio or Tiam1 (Gao et al., 2004). We found that NSC23766 inhibited BMMC chemotaxis

JPET#249706

toward histamine in a concentration-dependent manner with a 50%-inhibitory concentration of 51  $\mu$ M (Fig. 3), which was comparable to its inhibitory effect on Rac-Trion interaction *in vitro* or PDGF-induced Rac1 activation in NIH-3T3 cells (Gao et al., 2004). This result suggested that Rac activation was involved in H<sub>4</sub> receptor-mediated chemotaxis.

### **Involvement of Rac Activation in H<sub>4</sub> receptor-Mediated Signaling**

Because previous reports showed that histamine induces calcium mobilization from intracellular storage (Hofstra et al., 2003) and ERK activation (Desai and Thurmond, 2011) through H<sub>4</sub> receptor in BMMC, we evaluated the involvement of Rac activation in these signaling pathways. NSC23766 at 50  $\mu$ M had negligible effect on calcium mobilization. At 500  $\mu$ M, the inhibitor partially but significantly decreased the peak  $\Delta F/F$  value (Fig. 4A), indicating that calcium mobilization through H<sub>4</sub> receptor was partially mediated by Rac activation. We next evaluated whether Rac activation was involved in ERK activation. As shown in Figure 4B, histamine-induced ERK phosphorylation was inhibited by NSC23766 in a concentration-dependent fashion. At a concentration of 50  $\mu$ M or higher, ERK activation was nearly abolished (Fig. 4B), indicating that this process was fully dependent on Rac activation.

### **Histamine-Induced Chemotaxis was Dependent on MEK**

Next, we determined whether histamine-induced ERK activation mediated BMMC chemotaxis toward histamine. PD98059 is an inhibitor of MEK, a MAP kinase kinase that phosphorylates ERK. PD98059 significantly attenuated histamine-induced BMMC migration in a concentration-dependent manner (Fig. 5A), suggesting that ERK activation was involved in H<sub>4</sub> receptor-mediated

JPET#249706

BMMC chemotaxis, downstream of Rac activation. SP600125, an inhibitor of c-Jun N-terminal kinase (JNK), another MAP kinase downstream of Rac, had no inhibitory activity on histamine-induced chemotaxis of BMMCs, suggesting that JNK was not involved in this process (Fig. 5B).

### **PI3K-Dependent Rac Activation**

Numerous Rac-GEFs are known to be activated by PI3K (Welch et al., 2003). Furthermore, H<sub>4</sub> receptor activation in BMMC leads to cytokine production via the PI3K pathway (Desai and Thurmond, 2011). These findings led us to hypothesize that PI3K may be involved in H<sub>4</sub>R-mediated Rac activation and cell migration. Thus, we examined whether histamine-induced BMMC migration and Rac activation were mediated by the PI3K pathway. LY294002, a PI3K inhibitor, suppressed BMMC chemotaxis toward histamine in a concentration-dependent manner (Fig. 6A). Furthermore, this inhibitor attenuated histamine-induced activation of both Rac1 and Rac2 (Fig. 6B). These results suggested that PI3K regulated H<sub>4</sub> receptor-mediated chemotaxis of BMMC, upstream of Rac activation.

### **Both Rac1 and Rac2 are Required for BMMC Migration**

To define the specific role of Rac1 and Rac2 in histamine-induced chemotaxis of BMMCs, short-hairpin RNA (shRNA) was used to individually knockdown Rac1 or Rac2 expression. Lineage-negative bone marrow cells were transduced with lentiviral vector expressing the individual shRNA and induced to differentiate into mast cells. After successful differentiation as assessed by the expression of both c-kit and FcεRIα (Fig. 7A), specific downregulation of Rac1 or Rac2 was confirmed by immunoblot (Fig. 7B). When compared to vector-only

JPET#249706

transduction, knockdown of Rac1 and Rac2 significantly reduced the percentage of cells that migrated toward histamine (Fig. 7C). These results suggested that both Rac1 and Rac2 were required for mast cells to migrate toward histamine.

### **Distinct Roles of Rac1 and Rac2 in H<sub>4</sub> receptor-Mediated Signaling**

We subsequently explored whether each Rac GTPase played specific role in downstream signaling. While Rac1 downregulation had minimal effect on histamine-induced ERK phosphorylation, Rac2 knockdown had a significant inhibitory effect on ERK activation (Fig. 8A), indicating that Rac2 was mainly involved in histamine-induced phosphorylation of ERK. In a calcium mobilization assay, both Rac1 knockdowns (sh1 and sh11) resulted in significantly reduced peak calcium concentration by approximately 40% (Fig. 8B). In contrast, Rac2 knockdowns (sh14 and sh16) had minimal effect on calcium signaling; while sh16 resulted in a slight decrease in peak calcium concentration by 20%, sh14 had a negligible effect on calcium mobilization (Fig. 8B). These results suggested that Rac1 was primarily involved in calcium signaling.

### **Discussion**

In this study, we identified several intracellular binding partners of the histamine H<sub>4</sub> receptor by proteomics. Among these proteins, the ERM proteins and casein kinase 2 were previously implicated in GPCR signaling or trafficking (Stanasila et al., 2006; Torrecilla et al., 2007). However, we were not able to confirm their binding to the histamine H<sub>4</sub> receptor. In contrast, we demonstrated that Rac GTPases bound to the H<sub>4</sub> receptor, and further evaluated these small GTPases in our study.

JPET#249706

We showed that histamine activated small GTPases Rac1 and Rac2 through histamine H<sub>4</sub> receptor in mouse BMMCs. This is the first demonstration of small GTPase activation downstream of H<sub>4</sub> receptor. In addition to the functional coupling, we also demonstrated the physical association of H<sub>4</sub> receptor with both Rac1 and Rac2, suggesting that H<sub>4</sub> receptor forms a multiprotein complex to transduce signals efficiently and precisely. Attenuation of histamine-induced chemotaxis by a Rac inhibitor, which is known to suppress the activation of both Rac1 and Rac2 (Cancelas et al., 2005), suggested the importance of Rac1 and/or Rac2 activation in H<sub>4</sub> receptor-mediated BMMC migration. While Rac1 is ubiquitously expressed, Rac2 expression is specific to hematopoietic cells. Rac2-deficient mice, which have normal Rac1 expression, show functional abnormalities in multiple blood lineages, suggesting that Rac2 has specific roles that cannot be substituted by Rac1 (Roberts et al., 1999; Yang et al., 2000; Pradip et al., 2003; Croker et al., 2002). In agreement with this, specific knockdown of Rac1 or Rac2 in BMMC revealed that both proteins were necessary for histamine-induced chemotaxis, suggesting that the two Rac GTPases have nonredundant roles.

Our results also demonstrated distinct roles of Rac1 and Rac2 in downstream signaling. Rac1 knockdown reduced histamine-induced calcium mobilization, whereas Rac2 downregulation suppressed ERK phosphorylation. These results suggested that H<sub>4</sub> receptor signaling diverge to at least two distinct pathways: the Rac1-calcium pathway and Rac2-ERK pathway. Because chemotaxis involves multiple events that include sensing of attractant, polarity formation, leading edge protrusion, cell body translocation, and posterior

JPET#249706

retraction, all of which need to be tightly regulated to cooperate, divergence in H<sub>4</sub> receptor signaling is necessary. In a previous study, inhibition of PLC completely suppressed histamine-mediated calcium mobilization and chemotaxis, suggesting that inositol 1,4,5-triphosphate (IP<sub>3</sub>)-mediated calcium mobilization is necessary for H<sub>4</sub> receptor-mediated chemotaxis (Hofstra et al., 2003). Our results showed that a MEK inhibitor can significantly suppress histamine-induced chemotaxis of BMMC, suggesting that the MEK-ERK pathway is also involved. In addition to the regulation of cell migration by various calcium-dependent proteins (Wei et al., 2012), ERK phosphorylation is also known to regulate several proteins in chemotaxis (Huang et al., 2004); both mechanisms may work cooperatively to regulate a complex series of events to establish chemotaxis in a spatiotemporal manner.

We demonstrated that a Rac inhibitor partially but significantly suppressed histamine-induced calcium mobilization. In addition, specific knockdown of Rac1, but not Rac2, significantly attenuated calcium mobilization induced by histamine, suggesting that Rac1 was upstream of PLC to mobilize calcium from intracellular storage. Several PLC isozymes are expressed in BMMC, including PLCβ, PLCγ, PLCδ, and PLCε (Ito et al., 2012). Among these, PLCβ and PLCγ are known to be activated by Rac GTPases (Kadamur and Ross, 2013). Rho family GTPases Rac1, Rac2, and Cdc42 can activate PLCβ2 by directly binding to the PH domain of the enzyme (Illenberger et al., 1997; Illenberger et al., 1998; Illenberger et al., 2003; Snyder et al., 2003). PLCβ is also activated directly by Gβγ subunit, which does not involve Rac activation (Kadamur and Ross, 2013); this may explain the finding that histamine-induced calcium mobilization was

JPET#249706

only partially sensitive to the Rac inhibitor. PLC $\gamma$  is generally considered to be downstream of tyrosine protein kinases; however, Piechlek et al. showed that PLC $\gamma$ 2, but not PLC $\gamma$ 1, is directly stimulated by Rac1 and Rac2 (Piechlek et al., 2005) independently of tyrosine phosphorylation. Rac regulates PLC $\gamma$ 2 through by interacting with the PH domain (Walliser et al., 2008). In B cells, Rac-mediated stimulation of PLC $\gamma$ 2 by direct protein-protein interaction amplifies B cell receptor-induced calcium signaling (Walliser et al., 2015). In platelets, Rac1 mediates ITAM-dependent PLC $\gamma$ 2 activation (Pleines et al., 2009). A previous study revealed that the PLC-IP $_3$  pathway downstream of H $_4$  receptor and pertussis toxin-sensitive G protein is involved in the chemotaxis of BMMC toward histamine (Hofstra et al., 2003). Our study adds Rac1 as an important signaling molecule in this pathway.

Histamine-induced calcium signaling comprises the acute and chronic sustained phases. The rapid increase of calcium in the acute phase occurs in the absence of extracellular calcium, while the sustained phase requires extracellular calcium (Hofstra et al., 2003), suggesting that the latter phase is initiated by store-operated calcium entry. In various cell types including mast cells, store-operated calcium channels are involved in cell migration (Lin et al., 2018; Prakriya and Lewis, 2015). In addition to the IP $_3$ -mediated calcium increase, the sustained phase might also participate in the calcium-mediated regulation of histamine-induced chemotaxis of mast cells. In Rac1-knockdown cells or BMMC treated with Rac inhibitor, both the acute and sustained phases appeared to be suppressed, suggesting possible involvement of Rac1 in both the acute and sustained phases of calcium signaling induced by histamine.

JPET#249706

Our results demonstrated that Rac inhibitor completely suppressed histamine-induced ERK phosphorylation in BMMC. Furthermore, specific knockdown of Rac2, but not Rac1, resulted in significant attenuation of histamine-induced ERK activation, suggesting that H<sub>4</sub> receptor-mediated ERK activation was entirely dependent on Rac2. These findings are in agreement with those of previous reports showing that Rac2 deficiency led to reduced ERK1/2 activation in fMLP-activated neutrophils or TCR-stimulated T cells (Kim and Dinauer, 2001; Yu et al., 2001). PAK1, a main downstream effector of Rac and Cdc42 GTPases, is known to phosphorylate Raf and MEK, which are necessary for ERK signaling (Zang et al., 2002; Frost et al., 1997). This may be the mechanism by which Rac2 activation promotes the MEK-ERK pathway. In contrast to MEK inhibition, which significantly suppressed mast cell chemotaxis toward histamine, inhibition of JNK, another MAP kinase downstream of Rac GTPase (Yu et al., 2006; Gu et al., 2002; Timokhina et al., 1998), had negligible effect on histamine-induced migration, suggesting that the Rac-JNK pathway was not involved in H<sub>4</sub> receptor-mediated mast cell migration. ERK has many roles in cell migration by phosphorylating various proteins such as the myosin light chain kinase, calpain, focal adhesion kinase, and paxillin (Huang et al., 2004). Because histamine-induced ERK activation in mast cells is transient, peaking at 5 minutes and declining by 60 minutes after stimulation (Desai and Thurmond, 2011), we hypothesized that ERK activation functions in the early phase of chemotaxis. Supporting this notion, Mendoza et al. suggested that ERK drives the initial lamellipodia protrusion by activating the WAVE2 regulatory complex (Mendoza et al., 2011).

JPET#249706

Results of this study raised a further question on the mechanism by which the two divergent pathways are specifically regulated, because Rac1 and Rac2 are highly homologous with 92% amino acid identity. One possible mechanism is through different subcellular localization. Rac2 is known to be predominantly localized in intracellular compartments, while Rac1 is localized to the plasma membrane especially upon activation (Tao et al., 2002; Michaelson et al., 2001). Several PLCs are also known to be associated with plasma membrane where they catalyze the formation of diacylglycerol and IP<sub>3</sub>. This different subcellular localization may be the mechanism by which calcium signaling is specifically regulated by Rac1 in histamine-stimulated BMMCs. Another possible mechanism for different Rac GTPases to mediate distinct signaling is through the action of scaffolding proteins that hold various signaling molecules to prevent promiscuous activation. There are several scaffold proteins that are known to be involved in the MEK-ERK signaling pathway, such as the kinase suppressor of Ras (KSR),  $\beta$ -arrestin, and paxillin (Shaul and Seger, 2007). Further study is required to clarify the mechanism of the specific regulation of Rac1 and Rac2.

Our results also demonstrated that histamine-induced migration could be blocked by a PI3K inhibitor, suggesting that PI3K is involved in the signaling pathway downstream of H<sub>4</sub> receptor. A previous report showed that histamine-induced IL-6 production in BMMCs is blocked by a PI3K inhibitor, which is consistent with our result (Desai and Thurmond, 2011). Furthermore, histamine-induced activation of both Rac1 and Rac2 was attenuated by PI3K inhibition, suggesting that PI3K was an upstream regulator of Rac GTPases. PI3K downstream of GPCRs is known to be activated by G $\beta\gamma$  subunits. A lipid

JPET#249706

messenger phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P<sub>3</sub>) produced by PI3K is known to activate many GEFs for Rac GTPases by binding to the PH domain and exerting autoinhibition (Welch et al., 2003; Weiss-Haljiti et al., 2004). The GEF responsible for activating Rac1 and Rac2 in histamine-stimulated mast cell is yet to be determined and requires further investigation.

In conclusion, we demonstrated that small GTPases Rac1 and Rac2 physically and functionally coupled with the histamine H<sub>4</sub> receptor. The Rac GTPases have an essential role to diverge signal from H<sub>4</sub> receptor to at least two distinct arms, the Rac1-PLC-calcium and Rac2-MEK-ERK pathways. Thus, various events of chemotaxis can be coordinately regulated spatially and temporally in mast cells migrating toward histamine.

JPET#249706

## Acknowledgement

The authors would like to acknowledge the technical expertise of The DNA Core facility of the Center for Gene Research, Yamaguchi University, supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan. We also thank Shin-ichiro Kanno for technical assistance in nano LC-MS/MS analysis and Editage ([www.editage.jp](http://www.editage.jp)) for English language editing.

JPET#249706

### **Authorship contribution**

Participated in research design: Kuramasu, Yanai

Conducted experiments: Kuramasu, Wakabayashi

Contributed new reagents or analytic tools: Kuramasu, Wakabayashi

Performed data analysis: Kuramasu, Wakabayashi, Inui

Wrote or contributed to the writing of the manuscript: Kuramasu, Inui, Yanai

JPET#249706

## References

- Bockaert J, Marin P, Dumuis A, and Fagni L (2003) The 'magic tail' of G protein-coupled receptors: an anchorage for functional protein networks. *FEBS Lett* **546**: 65-72.
- Cancelas JA, Lee AW, Prabhakar R, Stringer KF, Zheng Y, and Williams DA (2005) Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. *Nat Med* **11**: 886-891.
- Crocker BA, Tarlinton DM, Cluse LA, Tuxen AJ, Light A, Yang FC, Williams DA, and Roberts AW (2002) The Rac2 guanosine triphosphatase regulates B lymphocyte antigen receptor responses and chemotaxis and is required for establishment of B-1a and marginal zone B lymphocytes. *J Immunol* **168**: 3376-3386.
- Desai P and Thurmond RL (2011) Histamine H<sub>4</sub> receptor activation enhances LPS-induced IL-6 production in mast cells via ERK and PI3K activation. *Eur J Immunol* **41**: 1764-1773.
- Frost JA, Steen H, Shapiro P, Lewis T, Ahn N, Shaw PE, and Cobb MH (1997) Cross-cascade activation of ERKs and ternary complex factors by Rho family proteins. *EMBO J* **16**: 6426-6438.
- Gao Y, Dickerson JB, Guo F, Zheng J, and Zheng Y (2004) Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proc*

JPET#249706

*Natl Acad Sci U S A* **101**: 7618-7623.

Gu Y, Byrne MC, Paranaivitana NC, Aronow B, Siefring JE, D'Souza M, Horton

HF, Quilliam LA, and Williams DA (2002) Rac2, a hematopoiesis-specific

Rho GTPase, specifically regulates mast cell protease gene expression in

bone marrow-derived mast cells. *Mol Cell Biol* **22**: 7645-7657.

Halova I, Draberova L, and Draber P (2012) Mast cell chemotaxis -

chemoattractants and signaling pathways. *Front Immunol* **3**: 119.

Hofstra CL, Desai PJ, Thurmond RL, and Fung-Leung WP (2003) Histamine H<sub>4</sub>

receptor mediates chemotaxis and calcium mobilization of mast cells. *J*

*Pharmacol Exp Ther* **305**: 1212-1221.

Huang C, Jacobson K, and Schaller MD (2004) MAP kinases and cell migration.

*J Cell Sci* **117**: 4619-4628.

Illenberger D, Schwald F, and Gierschik P (1997) Characterization and

purification from bovine neutrophils of a soluble guanine-nucleotide-binding

protein that mediates isozyme-specific stimulation of phospholipase C  $\beta_2$ .

*Eur J Biochem* **246**: 71-77.

Illenberger D, Schwald F, Pimmer D, Binder W, Maier G, Dietrich A, and

Gierschik P (1998) Stimulation of phospholipase C- $\beta_2$  by the Rho GTPases

Cdc42Hs and Rac1. *EMBO J* **17**: 6241-6249.

Illenberger D, Walliser C, Strobel J, Gutman O, Niv H, Gaidzik V, Kloog Y,

JPET#249706

- Gierschik P, and Henis YI (2003) Rac2 regulation of phospholipase C- $\beta_2$  activity and mode of membrane interactions in intact cells. *J Biol Chem* **278**: 8645-8652.
- Ito T, Smrž D, Jung MY, Bandara G, Desai A, Smržová Š, Kuehn HS, Beaven MA, Metcalfe DD, and Gilfillan AM (2012) Stem cell factor programs the mast cell activation phenotype. *J Immunol* **188**: 5428-5437.
- Kadamur G and Ross EM (2013) Mammalian phospholipase C. *Annu Rev Physiol* **75**: 127-154.
- Kanno S, Kuzuoka H, Sasao S, Hong Z, Lan L, Nakajima S, and Yasui A (2007) A novel human AP endonuclease with conserved zinc-finger-like motifs involved in DNA strand break responses. *EMBO J* **26**: 2094-2103.
- Kim C and Dinanuer MC (2001) Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. *J Immunol* **166**: 1223-1232.
- Lin YP, Nelson C, Kramer H, and Parekh AB (2018) The allergen Der p3 from house dust mite stimulates store-operated Ca<sup>2+</sup> channels and mast cell migration through PAR4 receptors. *Mol Cell* **70**: 228-241.
- Maeda K, Haraguchi M, Kuramasu A, Sato T, Ariake K, Sakagami H, Kondo H, Yanai K, Fukunaga K, Yanagisawa T, and Sukegawa J (2008) CLIC4 interacts with histamine H3 receptor and enhances the receptor cell surface

JPET#249706

expression. *Biochem Biophys Res Commun* **369**: 603-608.

Massol P, Montcourrier P, Guillemot JC, and Chavrier P (1998) Fc

receptor-mediated phagocytosis requires CDC42 and Rac1. *EMBO J* **17**:  
6219-6229.

Mendoza MC, Er EE, Zhang W, Ballif BA, Elliott HL, Danuser G, and Blenis J

(2011) ERK-MAPK drives lamellipodia protrusion by activating the WAVE2  
regulatory complex. *Mol Cell* **41**: 661-671.

Michaelson D, Silletti J, Murphy G, D'Eustachio P, Rush M, and Philips MR

(2001) Differential localization of Rho GTPases in live cells: regulation by  
hypervariable regions and RhoGDI binding. *J Cell Biol* **152**: 111-126.

Pleines I, Elvers M, Strehl A, Pozgajova M, Varga-Szabo D, May F,

Chrostek-Grashoff A, Brakebusch C, and Nieswandt B (2009) Rac1 is  
essential for phospholipase C- $\gamma$ 2 activation in platelets. *Pflugers Arch* **457**:  
1173-1185.

Pradip D, Peng X, and Durden DL (2003) Rac2 specificity in macrophage

integrin signaling: potential role for Syk kinase. *J Biol Chem* **278**:  
41661-41669.

Prakriya M and Lewis RS (2015) Store-operated calcium channels. *Physiol Rev*

**95**: 1383-1436.

Roberts AW, Kim C, Zhen L, Lowe JB, Kapur R, Petryniak B, Spaetti A, Pollock

JPET#249706

JD, Borneo JB, Bradford GB, Atkinson SJ, Dinauer MC, and Williams DA  
(1999) Deficiency of the hematopoietic cell-specific Rho family GTPase  
Rac2 is characterized by abnormalities in neutrophil function and host  
defense. *Immunity* **10**: 183-196.

Samayawardhena LA, Kapur R, and Craig AW (2007) Involvement of Fyn kinase  
in Kit and integrin-mediated Rac activation, cytoskeletal reorganization, and  
chemotaxis of mast cells. *Blood* **109**: 3679-3686.

Shaul YD and Seger R (2007) The MEK/ERK cascade: from signaling specificity  
to diverse functions. *Biochim Biophys Acta* **1773**: 1213-1226.

Snyder JT, Singer AU, Wing MR, Harden TK, and Sondek J (2003) The  
pleckstrin homology domain of phospholipase C- $\beta_2$  as an effector site for  
Rac. *J Biol Chem* **278**: 21099-21104.

Stanasila L, Abuin L, Diviani D, and Cotecchia S (2006) Ezrin directly interacts  
with the alpha1b-adrenergic receptor and plays a role in receptor recycling.  
*J Biol Chem* **281**: 4354-4363.

Tao W, Filippi MD, Bailey JR, Atkinson SJ, Connors B, Evan A, and Williams DA  
(2002) The TRQQKRP motif located near the C-terminus of Rac2 is  
essential for Rac2 biologic functions and intracellular localization. *Blood* **100**:  
1679-1688.

Thurmond RL (2015) The histamine H<sub>4</sub> receptor: from orphan to the clinic. *Front*

JPET#249706

*Pharmacol* **6**: 65.

Timokhina I, Kissel H, Stella G, and Besmer P (1998) Kit signaling through PI 3-kinase and Src kinase pathways: an essential role for Rac1 and JNK activation in mast cell proliferation. *EMBO J* **17**: 6250-6262.

Torrecilla I, Spragg EJ, Poulin B, McWilliams PJ, Mistry SC, Blaukat A, and Tobin AB (2007) Phosphorylation and regulation of a G protein-coupled receptor by protein kinase CK2. *J Cell Biol* **177**: 127-137.

Walliser C, Retlich M, Harris R, Everett KL, Josephs MB, Vatter P, Esposito D, Driscoll PC, Katan M, Gierschik P, and Bunney TD (2008) Rac regulates its effector phospholipase  $Cy_2$  through interaction with a split pleckstrin homology domain. *J Biol Chem* **283**: 30351-30362.

Walliser C, Tron K, Clauss K, Gutman O, Kobitski AY, Retlich M, Schade A, Röcker C, Henis YI, Nienhaus GU, and Gierschik P (2015) Rac-mediated Stimulation of Phospholipase  $Cy_2$  Amplifies B Cell Receptor-induced Calcium Signaling. *J Biol Chem* **290**: 17056-17072.

Wei C, Wang X, Zheng M, and Cheng H (2012) Calcium gradients underlying cell migration. *Curr Opin Cell Biol* **24**: 254-261.

Weiss-Haljiti C, Pasquali C, Ji H, Gillieron C, Chabert C, Curchod ML, Hirsch E, Ridley AJ, Hooft van Huijsduijnen R, Camps M, and Rommel C (2004) Involvement of phosphoinositide 3-kinase gamma, Rac, and PAK signaling

JPET#249706

in chemokine-induced macrophage migration. *J Biol Chem* **279**:  
43273-43284.

Welch HC, Coadwell WJ, Stephens LR, and Hawkins PT (2003)

Phosphoinositide 3-kinase-dependent activation of Rac. *FEBS Lett* **546**:  
93-97.

Xu AJ, Kuramasu A, Maeda K, Kinoshita K, Takayanagi S, Fukushima Y,

Watanabe T, Yanagisawa T, Sukegawa J, and Yanai K (2008)

Agonist-induced internalization of histamine H2 receptor and activation of  
extracellular signal-regulated kinases are dynamin-dependent. *J*

*Neurochem* **107**: 208-217.

Yang FC, Kapur R, King AJ, Tao W, Kim C, Borneo J, Breese R, Marshall M,

Dinauer MC, and Williams DA (2000) Rac2 stimulates Akt activation

affecting BAD/Bcl-XL expression while mediating survival and actin function  
in primary mast cells. *Immunity* **12**: 557-568.

Yu H, Leitenberg D, Li B, and Flavell RA (2001) Deficiency of small GTPase

Rac2 affects T cell activation. *J Exp Med* **194**: 915-926.

Yu M, Luo J, Yang W, Wang Y, Mizuki M, Kanakura Y, Besmer P, Neel BG, and

Gu H (2006) The scaffolding adapter Gab2, via Shp-2, regulates kit-evoked  
mast cell proliferation by activating the Rac/JNK pathway. *J Biol Chem* **281**:  
28615-28626.

JPET#249706

Zampeli E and Tiligada E (2009) The role of histamine H<sub>4</sub> receptor in immune and inflammatory disorders. *Br J Pharmacol* **157**: 24-33.

Zang M, Hayne C, and Luo Z (2002) Interaction between active Pak1 and Raf-1 is necessary for phosphorylation and activation of Raf-1. *J Biol Chem* **277**: 4395-4405.

JPET#249706

## Footnotes

This work was supported in part by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science [Grant #19390061].

JPET#249706

## Figure Legends

**Figure 1.** Rac1 and Rac2 physically associate with H<sub>4</sub> receptor. (A) Silver staining of affinity-purified H<sub>4</sub> receptor-binding proteins separated on SDS-PAGE. BMMC lysate was first negatively purified with GST-bound beads and the flow-through was further purified with GST-fused carboxy-terminal tail of mouse H<sub>4</sub> receptor (GST-mH4RCT)-bound beads. Seven bands indicated by arrowheads were isolated and subjected to LC-MS/MS. M, marker; lane 1, proteins bound to GST; lane 2, proteins bound to GST-mH4RCT. Numbers on the left indicate molecular weight in kDa. (B) Affinity-purified proteins were separated on SDS-PAGE and immunoblotted with anti-Rac1 and anti-Rac2 antibodies to confirm the LC-MS/MS result. (C) Schematic illustration of GST-fused full carboxy-terminus (GST-mH4RCT), proximal half of carboxy-terminus (GST-mH4RΔ), and third intracellular loop (GST-mH4Ri3) of mouse H<sub>4</sub> receptor. Numbers indicate the amino acid position. (D) Rac1 and Rac2 bind directly to the third intracellular loop and carboxy-terminus of histamine H<sub>4</sub> receptor. Bacterially expressed GST fusion proteins were incubated with MBP-fused Rac1 or Rac2 *in vitro* and pulled down with glutathione beads. Proteins were separated on SDS-PAGE and immunoblotted with anti-MBP antibody.

JPET#249706

**Figure 2.** H<sub>4</sub> receptor-mediated activation of Rac1 and Rac2. (A) BMMCs were stimulated with 10  $\mu$ M histamine alone or with an H<sub>4</sub> receptor antagonist, JNJ7777120 (1  $\mu$ M). Active and total Rac1 and Rac2 were detected by immunoblotting. Data are expressed as fold activation over unstimulated, and are presented as the mean with S.E.M. from five independent experiments. \*P < 0.05 by unpaired t-test. A representative blot is shown on the right panel. (B) BMMCs were stimulated with 10  $\mu$ M histamine alone or with an H<sub>1</sub> receptor antagonist pyrilamine (10  $\mu$ M) or H<sub>2</sub> receptor antagonist cimetidine (10  $\mu$ M). Active and total Rac1 and Rac2 were detected by immunoblotting. Data are expressed as fold activation over unstimulated, and are presented as the mean with S.E.M. from three independent experiments. The activation of Rac1 and Rac2 in the presence of H<sub>1</sub> or H<sub>2</sub> receptor antagonist was not significantly different than that of the control by one-way ANOVA with post-hoc Dunnett's test. A representative blot is shown on the right panel.

**Figure 3.** Effect of Rac inhibitor NSC23766 on chemotaxis of BMMCs toward histamine. BMMCs were preincubated in the absence or presence of NSC23766 at the indicated concentrations and subjected to chemotaxis assay. Cells migrated into lower chamber were mixed with fluorospheres and analyzed on a

JPET#249706

flow cytometer. Representative dot plots are shown at the bottom. The fluorospheres and BMMCs appear in the upper-left rectangle gate and lower oval gate, respectively. Data are expressed as the mean percentage of control (without inhibitor) with S.E.M. from three independent experiments. \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$  by one-way ANOVA with post-hoc Dunnett's test compared to the control without inhibitor.

**Figure 4.** Histamine-induced calcium mobilization and ERK activation are Rac-dependent. (A) Effect of Rac inhibitor NSC23766 on histamine-induced calcium mobilization in BMMCs. Histamine (10  $\mu$ M) was added at 30 sec. The left panel shows a representative trace (solid black, no inhibitor; solid gray, 50  $\mu$ M NSC23766; dashed, 500  $\mu$ M NSC23766). Data are expressed as mean percentage of the control without inhibitor with S.E.M. from at least five independent experiments. \*\*\* $P < 0.001$  by one-way ANOVA with post-hoc Dunnett's test compared to the control without inhibitor. (B) Effect of Rac inhibitor NSC23766 on histamine-induced ERK activation in BMMCs. Phosphorylated and total ERKs were detected by immunoblotting, and a representative blot is shown on the left panel. Data are expressed as mean percentage of the control without inhibitor with S.E.M. from three independent experiments. \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$  by one-way ANOVA with post-hoc Dunnett's test compared to the

JPET#249706

control without inhibitor.

**Figure 5.** Histamine-induced migration of BMMCs is MEK dependent. Effect of MEK inhibitor PD98059 and JNK inhibitor SP600125 on chemotaxis of BMMCs toward histamine BMMCs were preincubated in the absence or presence of PD98059 (A) or SP600125 (B) at the indicated concentrations and subjected to chemotaxis assay. Data are expressed as mean percentage of the control without inhibitor with S.E.M. from three independent experiments. \*\*P < 0.01, \*\*\*P < 0.001 and \*\*\*\*P < 0.0001 by one-way ANOVA with post-hoc Dunnett's test compared to the control without inhibitor.

**Figure 6.** Histamine-induced chemotaxis and Rac activation of BMMCs are PI3K dependent. (A) Effect of PI3K inhibitor LY294002 on chemotaxis of BMMCs toward histamine. BMMCs were preincubated in the absence or presence of inhibitor at the indicated concentrations and subjected to chemotaxis assay. Data are expressed as mean percentage of the control without inhibitor with S.E.M. from three independent experiments \*\*\*P < 0.001 by one-way ANOVA with post-hoc Dunnett's test compared to the control without inhibitor. (B) Effect of PI3K inhibitor LY294002 on histamine-induced activation of Rac1 and Rac2 in BMMCs. BMMCs were stimulated with 10  $\mu$ M histamine alone or with PI3K

JPET#249706

inhibitor LY294002 (50  $\mu$ M). Active and total Rac1 and Rac2 were detected by immunoblotting. Data are expressed as fold activation over unstimulated cells, and are presented as the mean with S.E.M. from four independent experiments. \*P < 0.05 and \*\*P < 0.01 by unpaired t-test. A representative blot is shown on the right panel.

**Figure 7.** Rac1 and Rac2 are not redundant in histamine-induced chemotaxis.

(A) Lineage-negative (Lin (-)) bone marrow cells were transduced with a lentiviral vector to express shRNA. Transduced cells were selected by puromycin and differentiated into mast cells with 50 ng/ml SCF and 10 ng/mL IL-3 for 4 weeks. The cells were stained with PE-Cy7-conjugated anti-mouse c-kit antibody and PE-conjugated anti-mouse Fc $\epsilon$ R1 $\alpha$  and analyzed by flow cytometry. Numbers in the upper-right quadrant indicate the percentage of double positive cells. KD, knockdown. (B) Lysate of transduced cells was subjected to immunoblotting to confirm knockdown. (C) Effect of Rac1 or Rac2-specific shRNA expression on histamine-induced chemotaxis. Lin (-) mast cells expressing shRNA were subjected to chemotaxis assay. Data are expressed as mean percentage of migrated cells over input cells with S.E.M. from 4–6 independent experiments. \*\*P < 0.01 and \*\*\*P < 0.001 by one-way ANOVA with post-hoc Dunnett's test compared to control cells.

JPET#249706

**Figure 8.** Differential downstream signaling by Rac1 and Rac2. (A) Effect of Rac1 or Rac2-specific knockdown on histamine-induced ERK activation in lineage-negative (Lin (-)) mast cells. Serum-starved cells were stimulated with 10  $\mu$ M histamine for 2 min and subjected to immunoblotting. Phosphorylated and total ERK bands were quantified, and data are expressed as mean percentage of the control with S.E.M. from four independent experiments. \*\*P < 0.01 and \*\*\*P < 0.001 by one-way ANOVA with post-hoc Dunnett's test compared to the control. Representative blots are shown on the right panel. KD, knockdown. (B) Effect of Rac1 or Rac2-specific knockdown on histamine-induced calcium mobilization in Lin (-) mast cells. Data are expressed as mean percentage of the control with S.E.M. from 5–7 independent experiments. \*\*P < 0.01 by one-way ANOVA with post-hoc Dunnett's test compared to the control. Representative traces are shown on the right panel; arrows indicate the addition of 10  $\mu$ M histamine.

JPET#249706

**Table 1.** H<sub>4</sub> receptor-binding proteins identified by affinity purification and nano LC-MS/MS.

Bands identified by SDS-PAGE were subjected to nano LC-MS/MS analysis. Protein names and the Mascot score with number of matched peptides are shown.

Band	Protein name	Score (peptides)
p50	Moesin	271 (6)
p40	Moesin	322 (6)
	Fructose-bisphosphate aldolase	224 (4)
	Ezrin	179 (4)
	Casein kinase 2 alpha	58 (1)
p22	GTP binding protein Rac2	33 (2)
p11	GTP binding protein Rac1	274 (7)

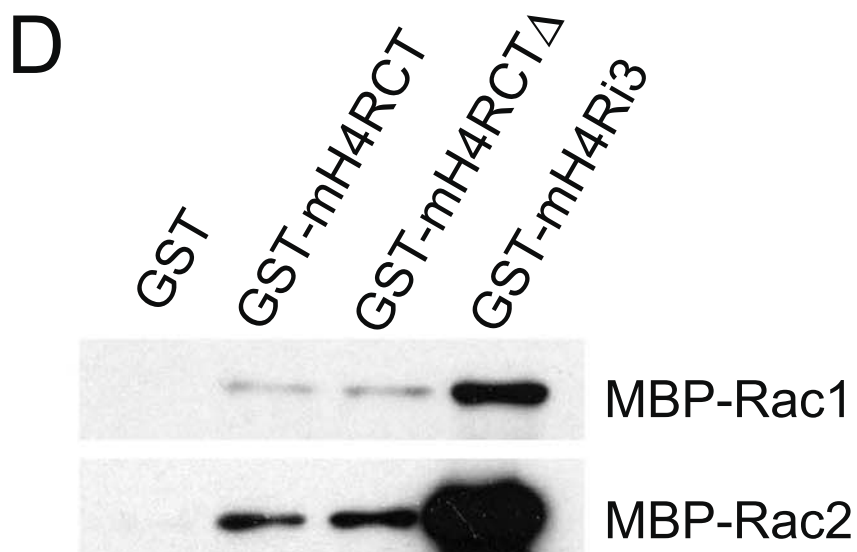
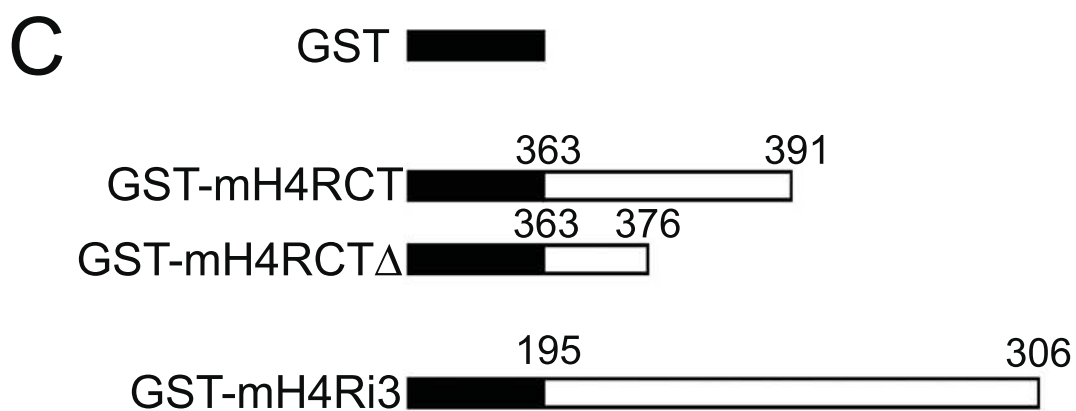
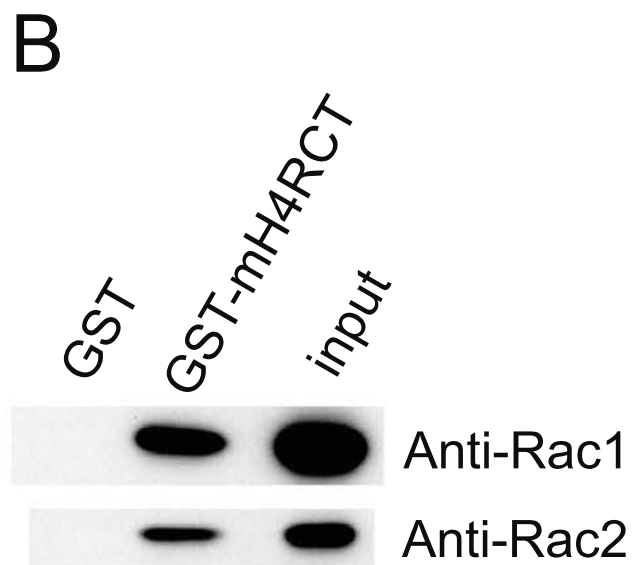
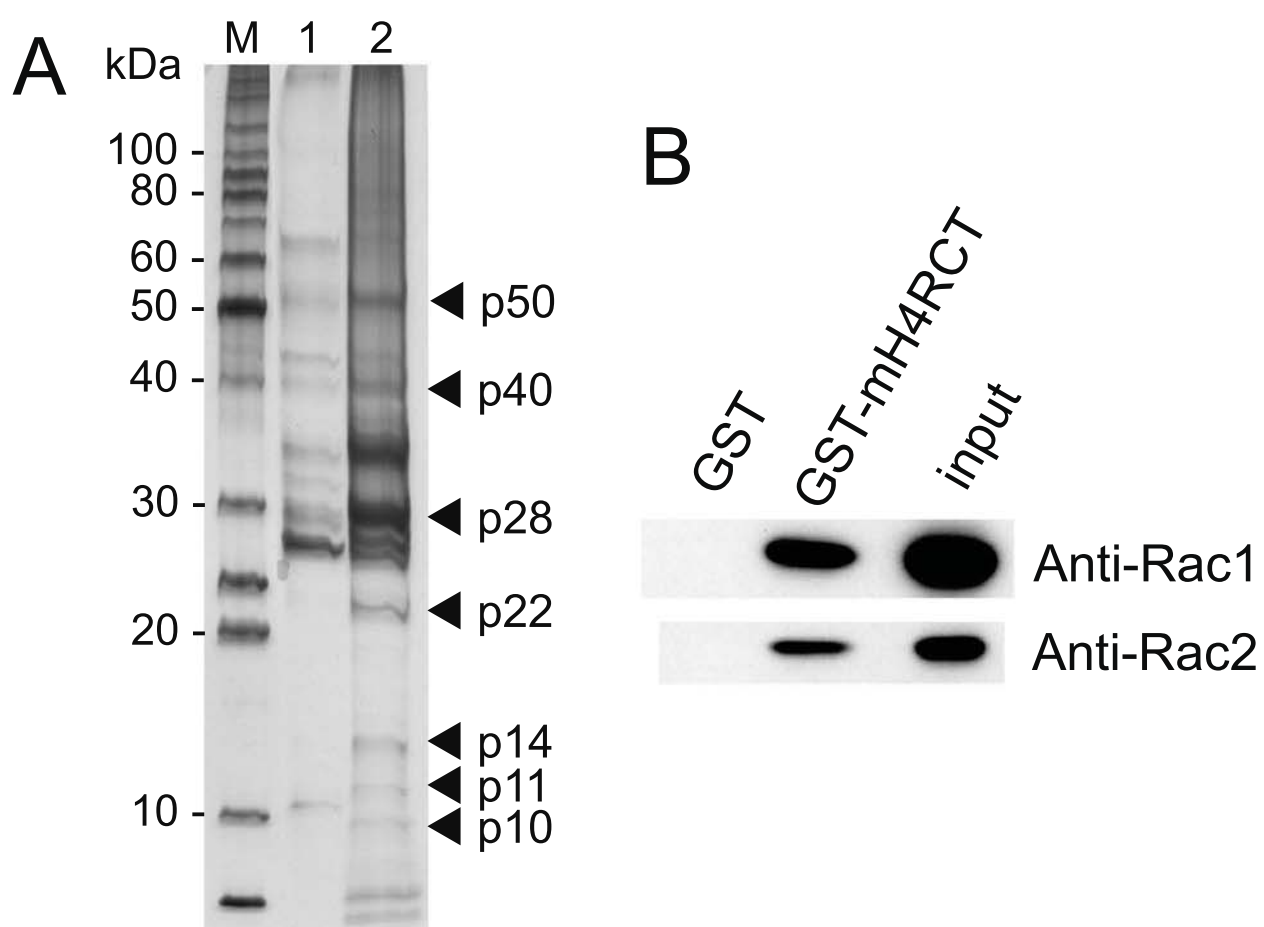


Figure 1

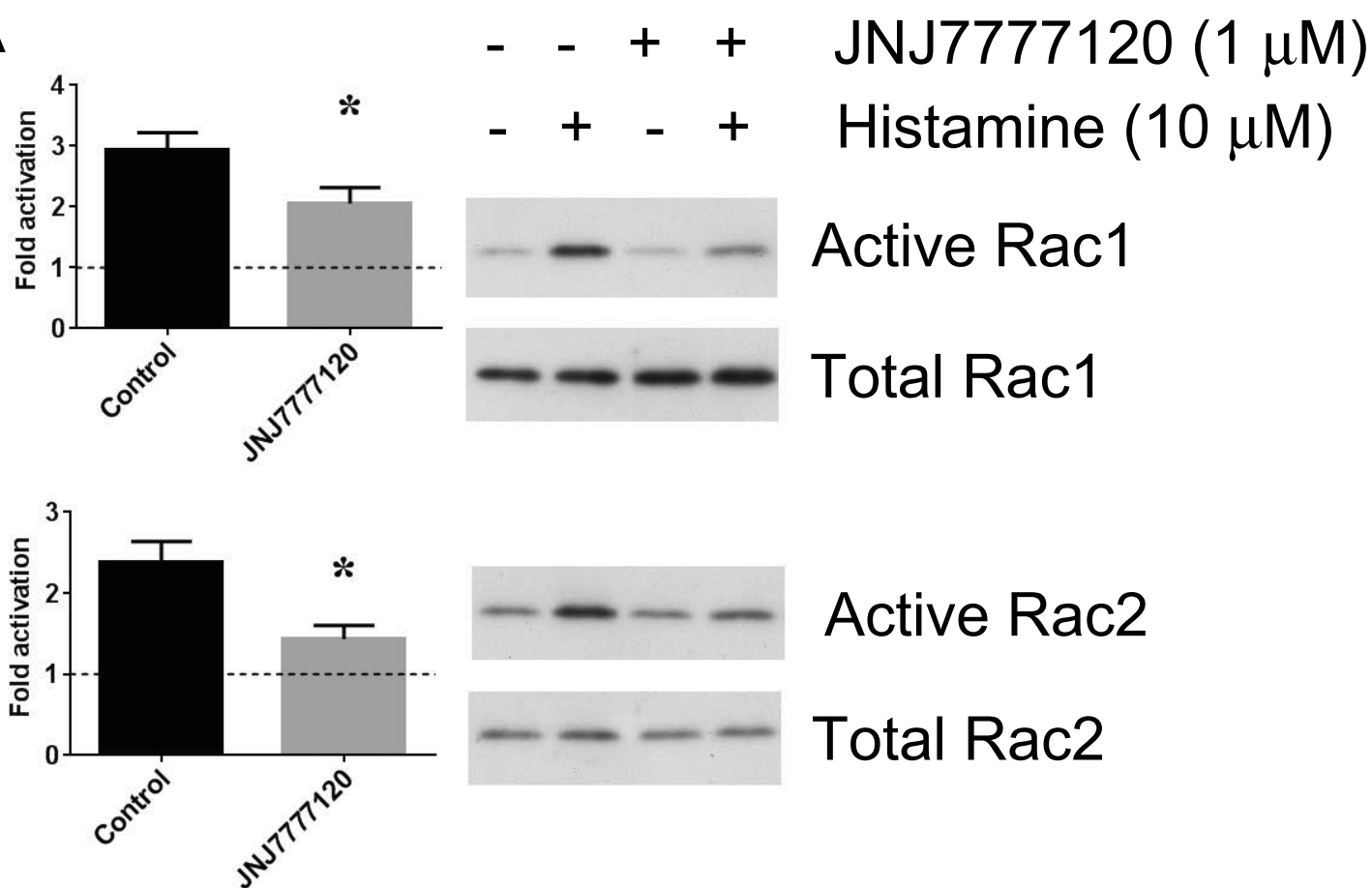
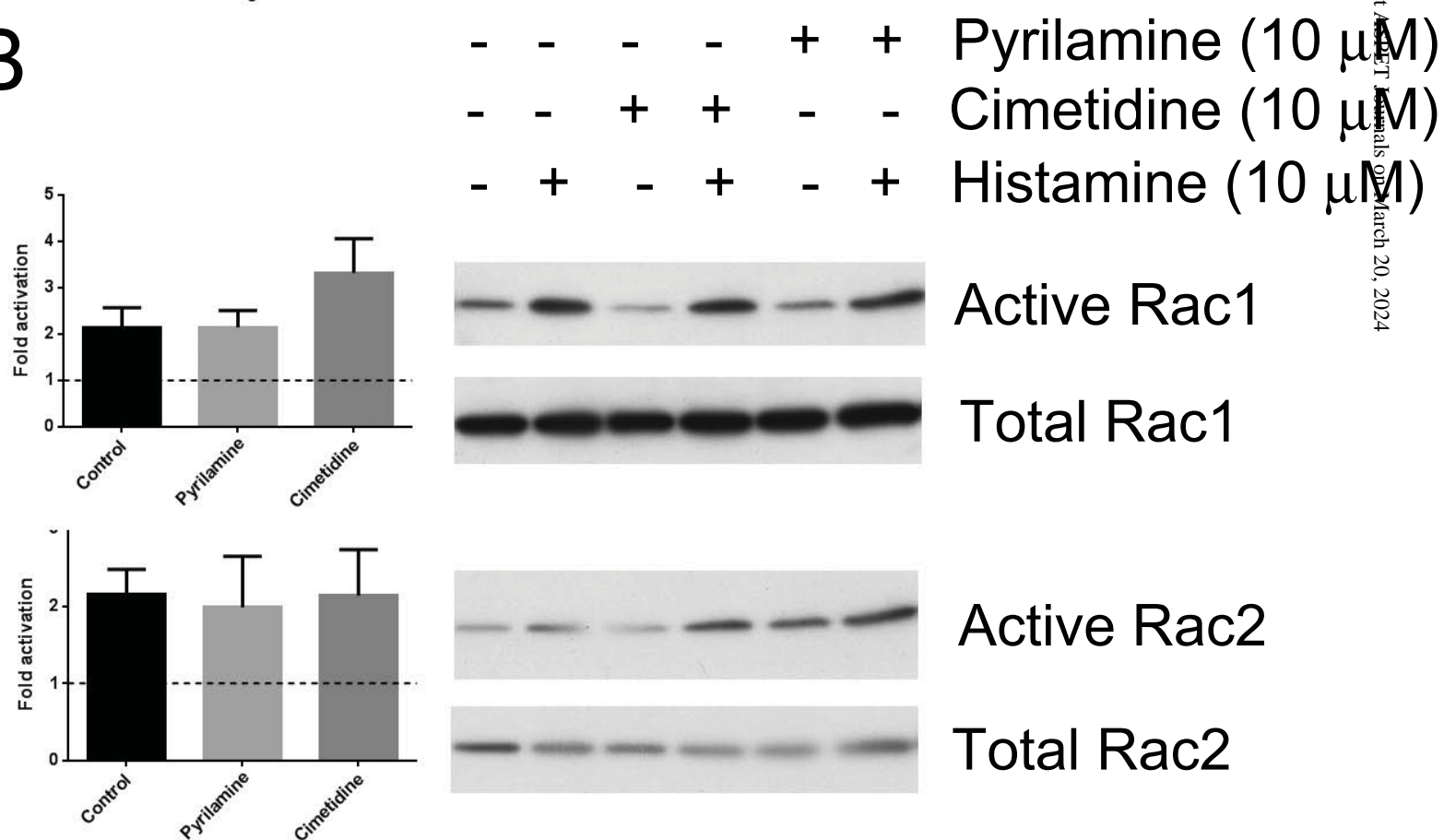
**A****B**

Figure 2

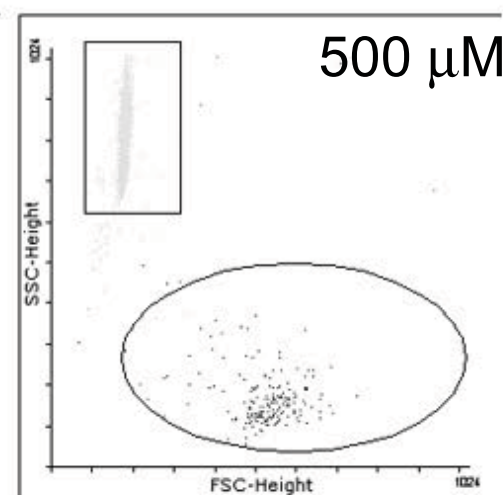
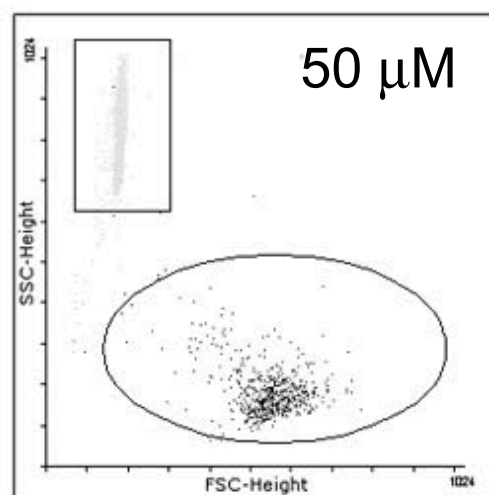
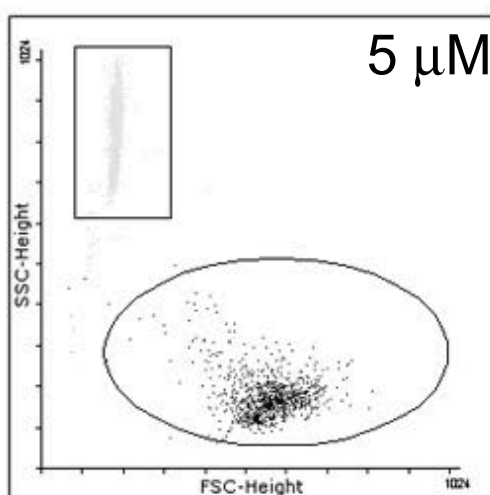
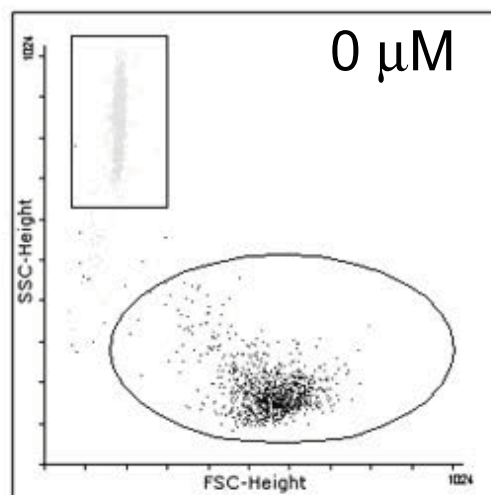
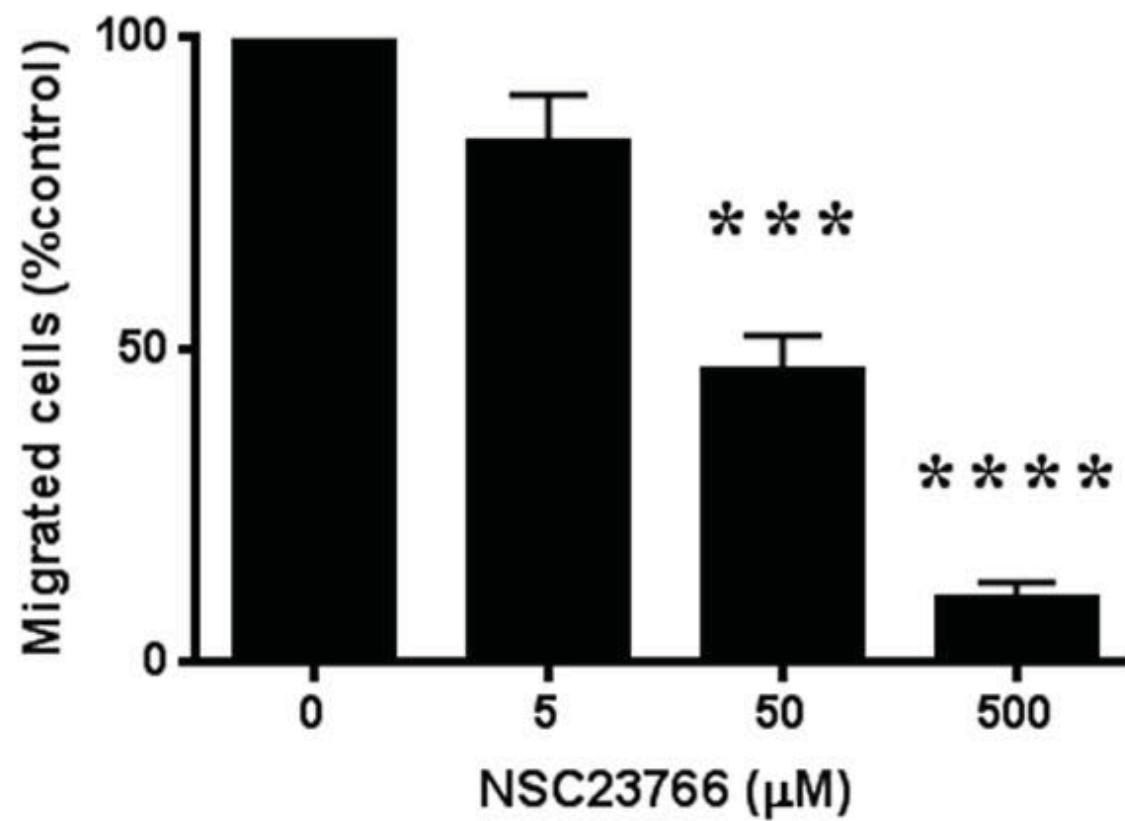


Figure 3

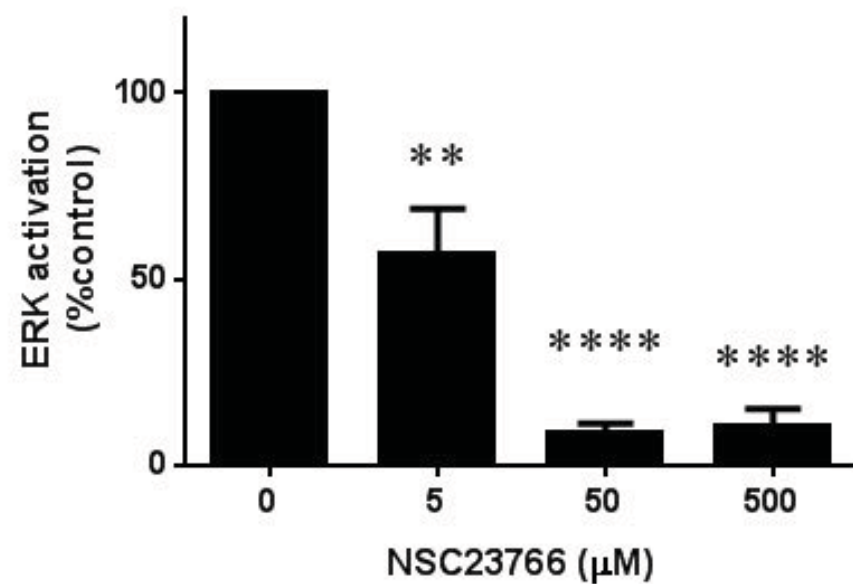
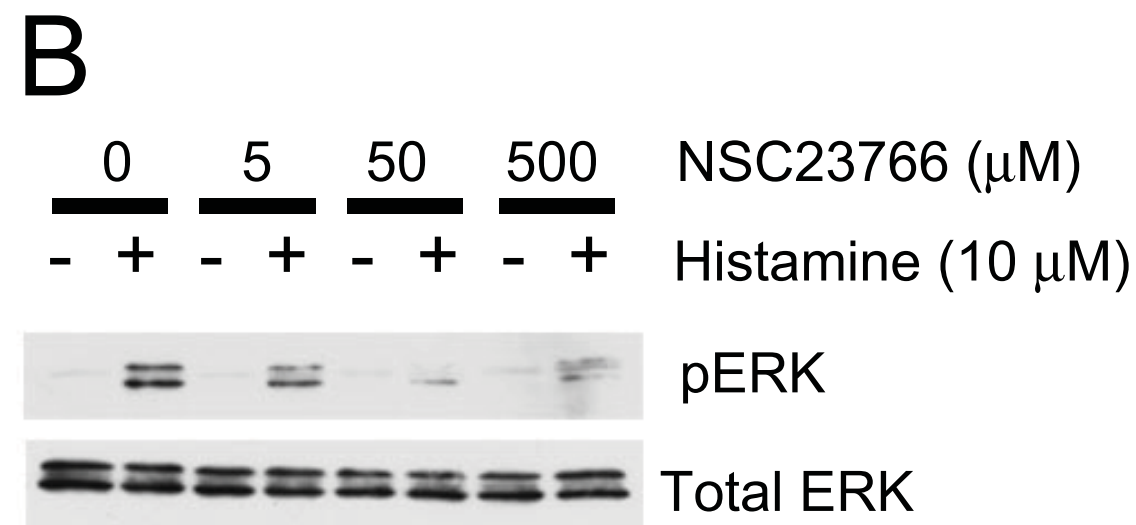
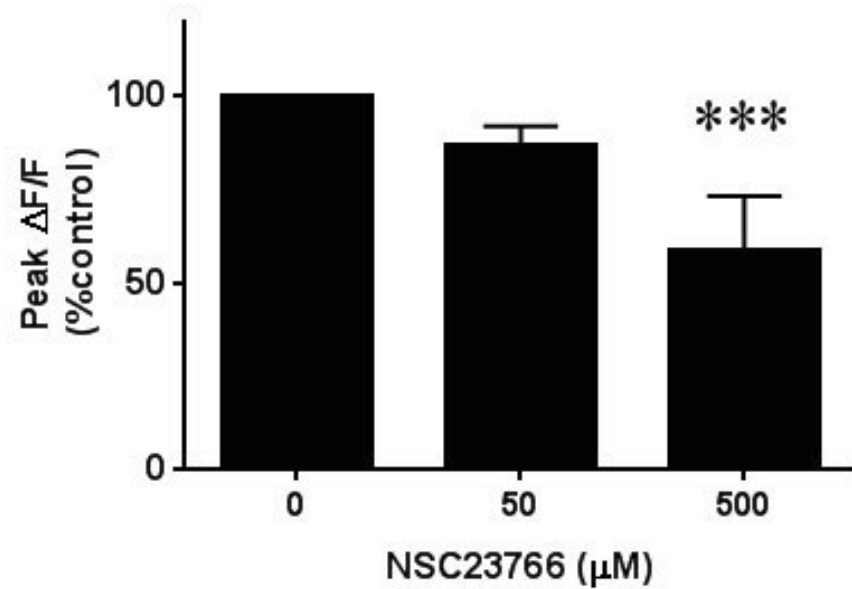
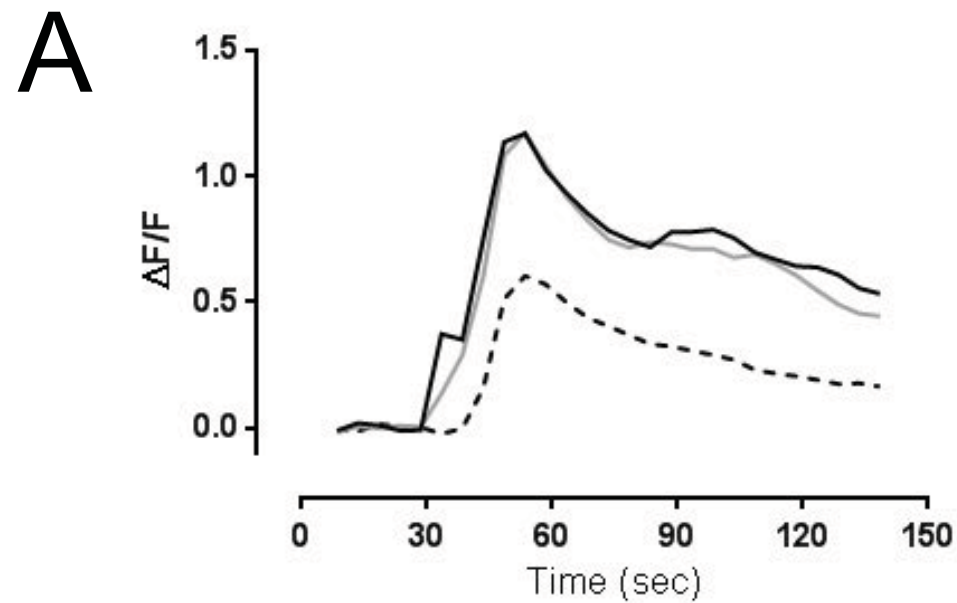


Figure 4

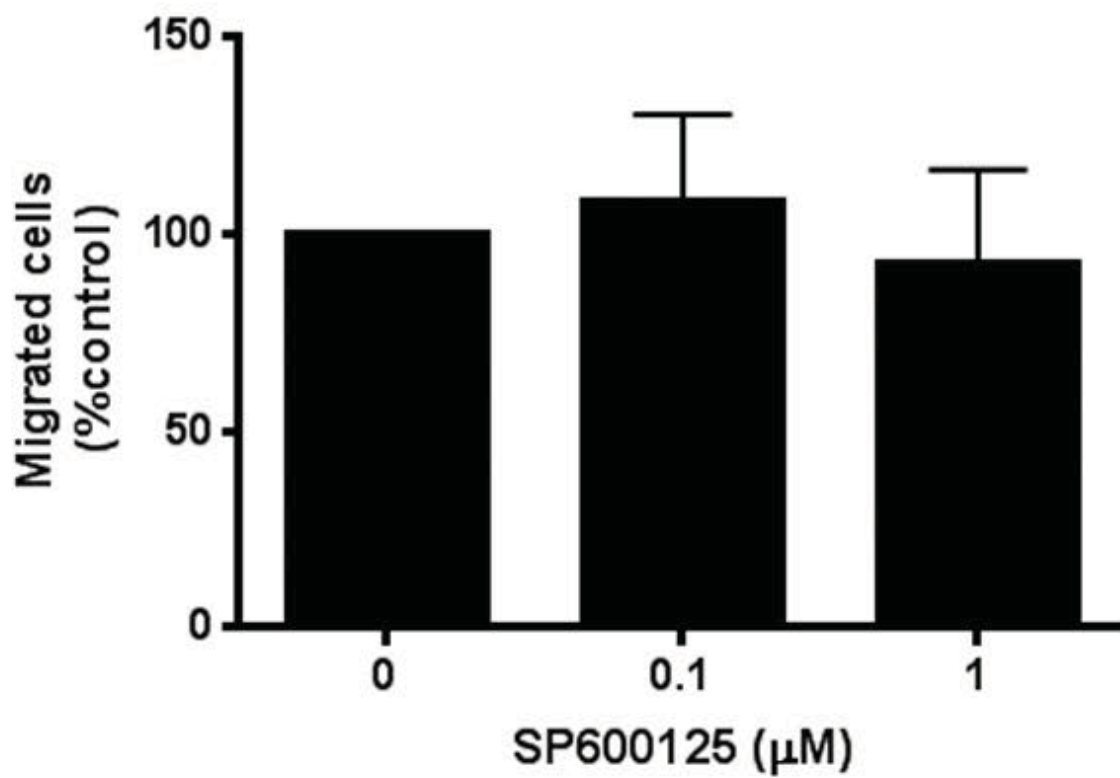
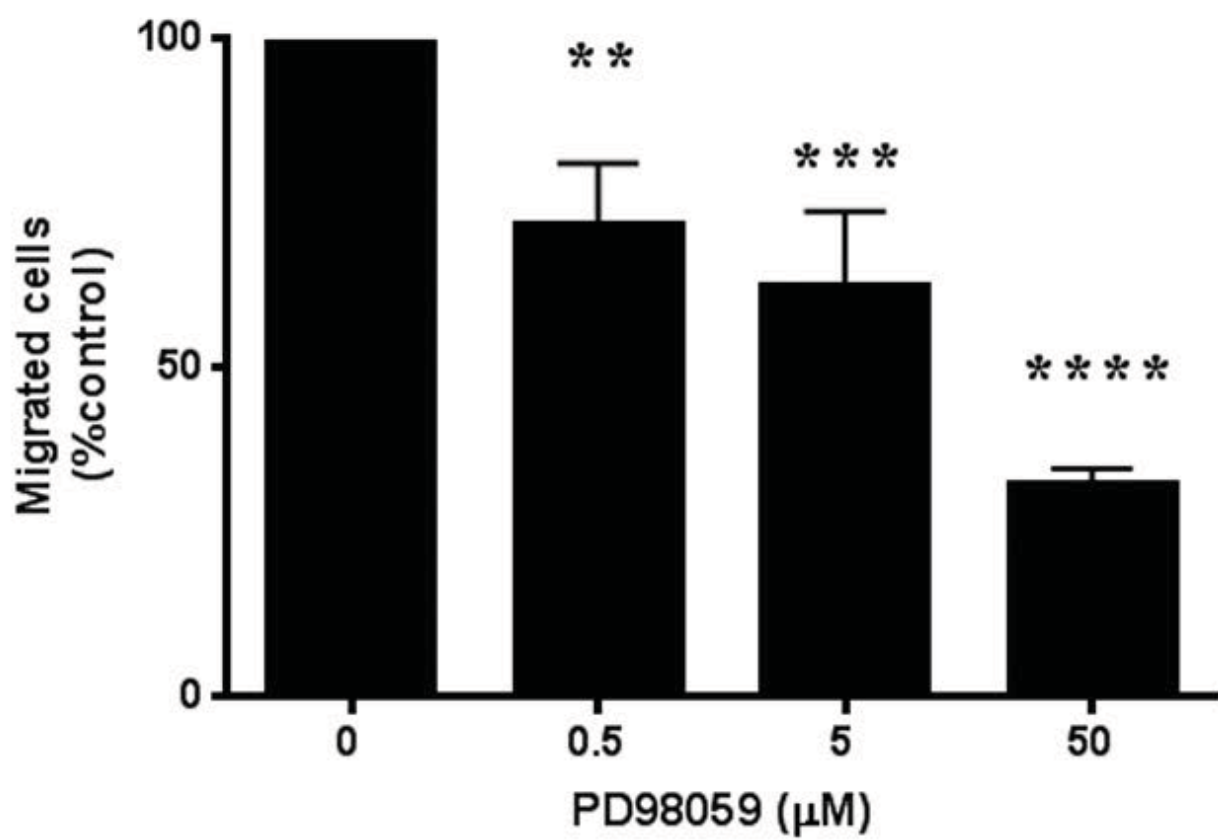
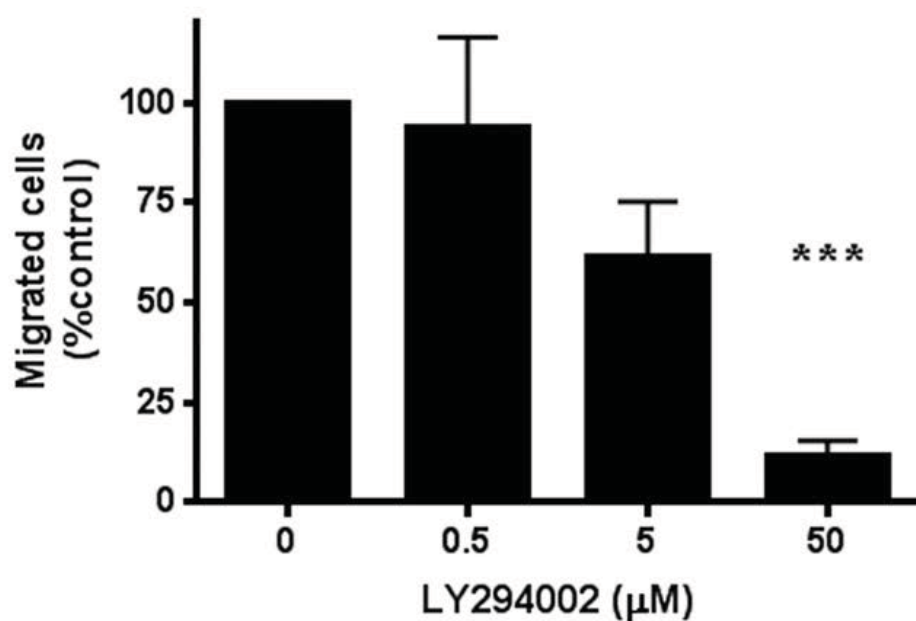
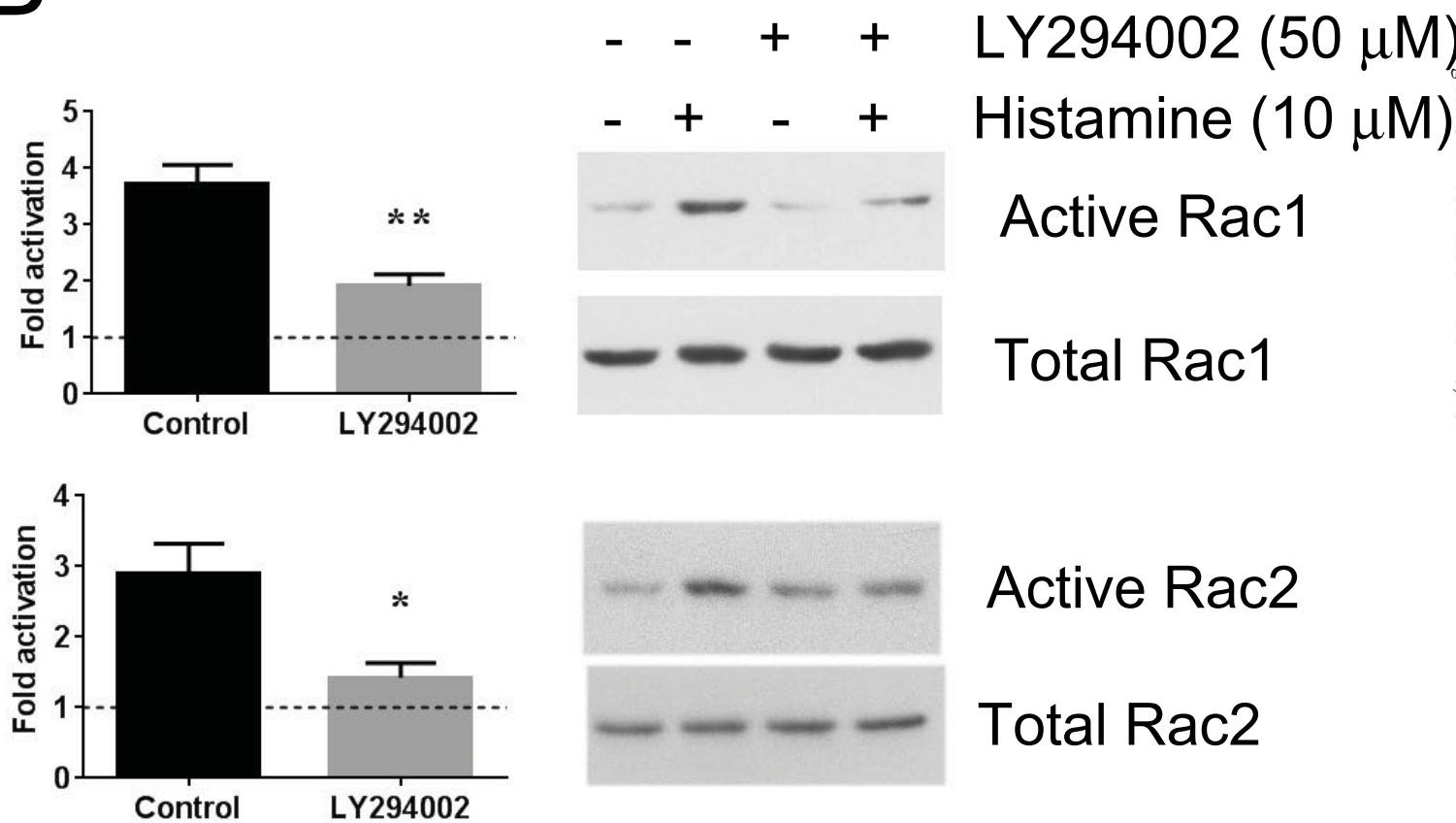


Figure 5

**A****B****Figure 6**

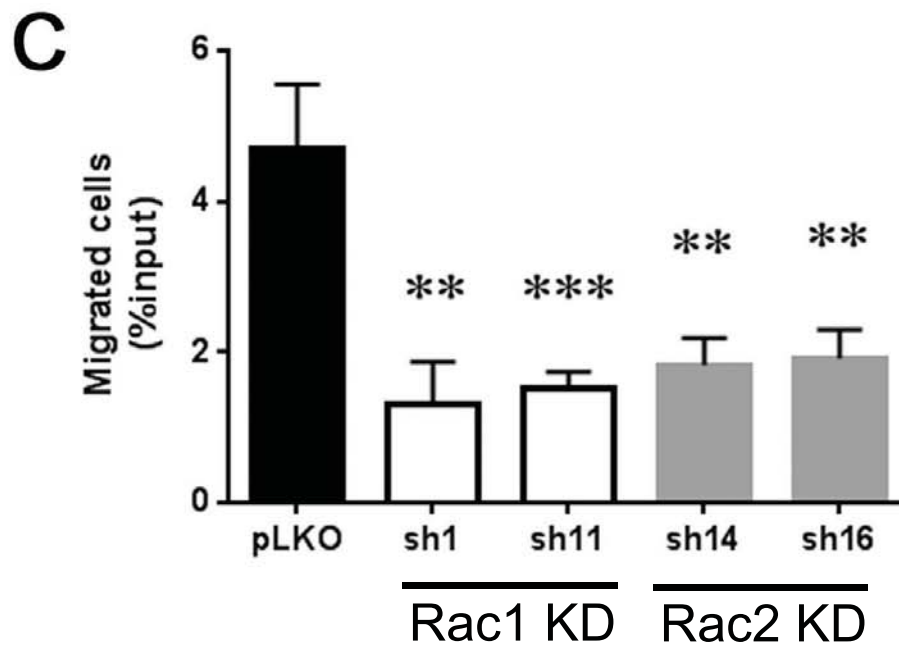
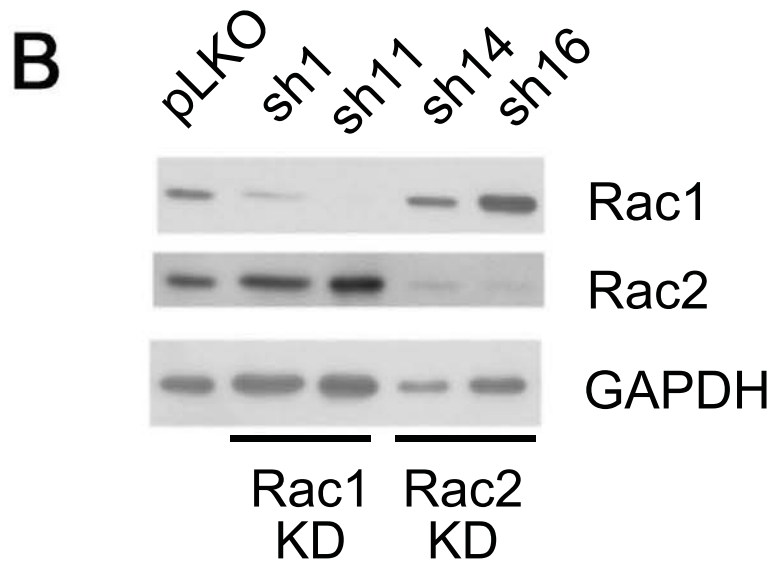
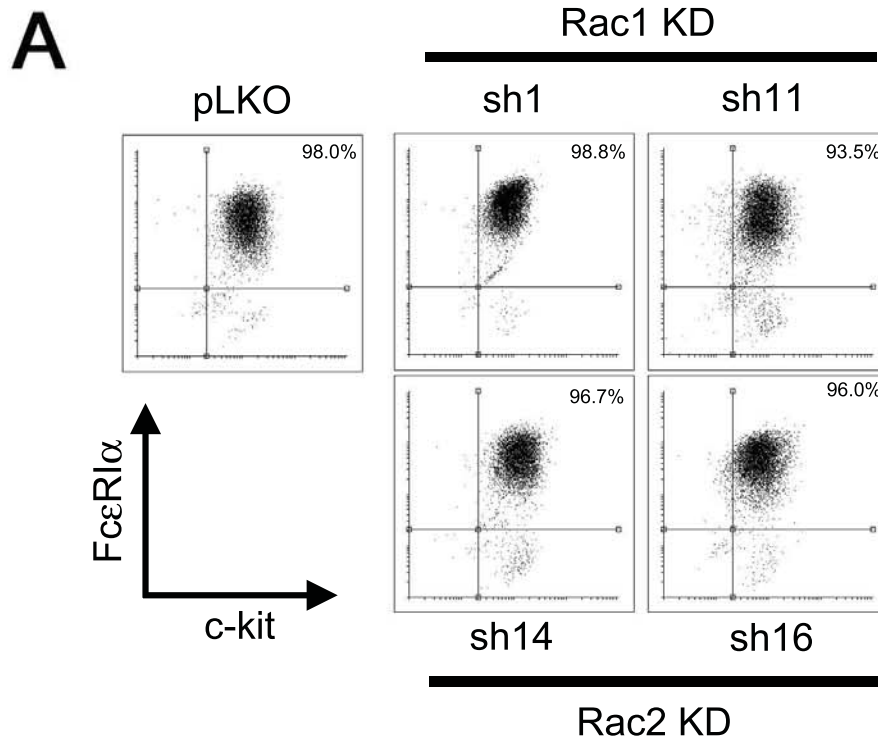


Figure 7

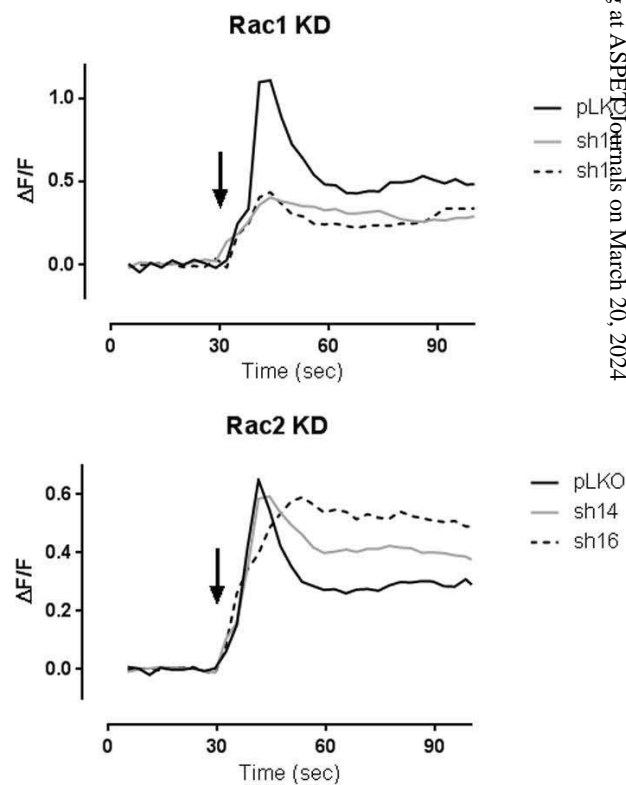
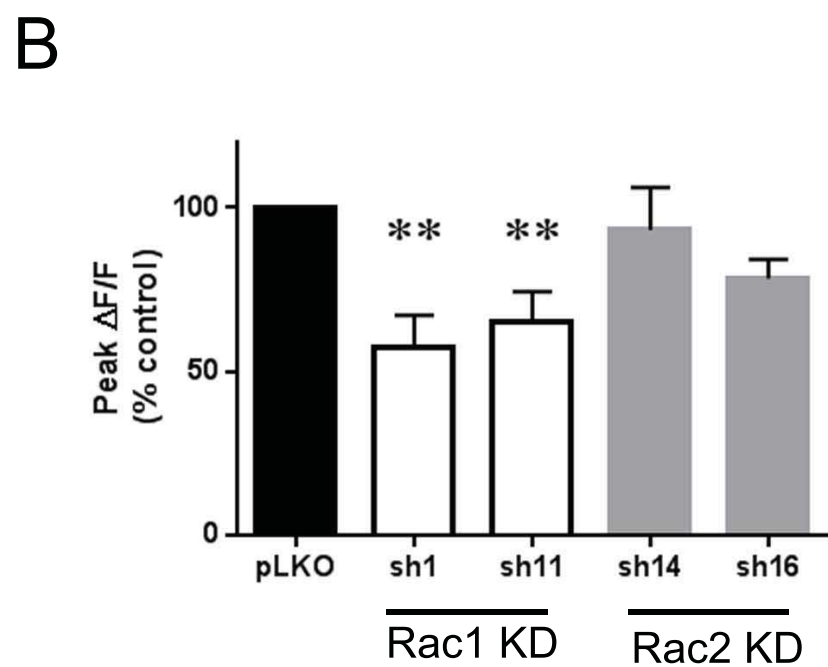
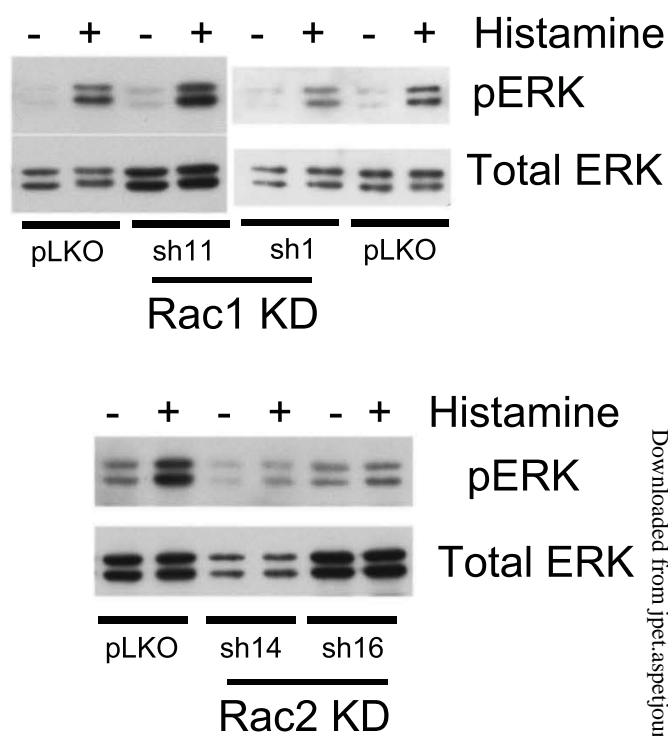
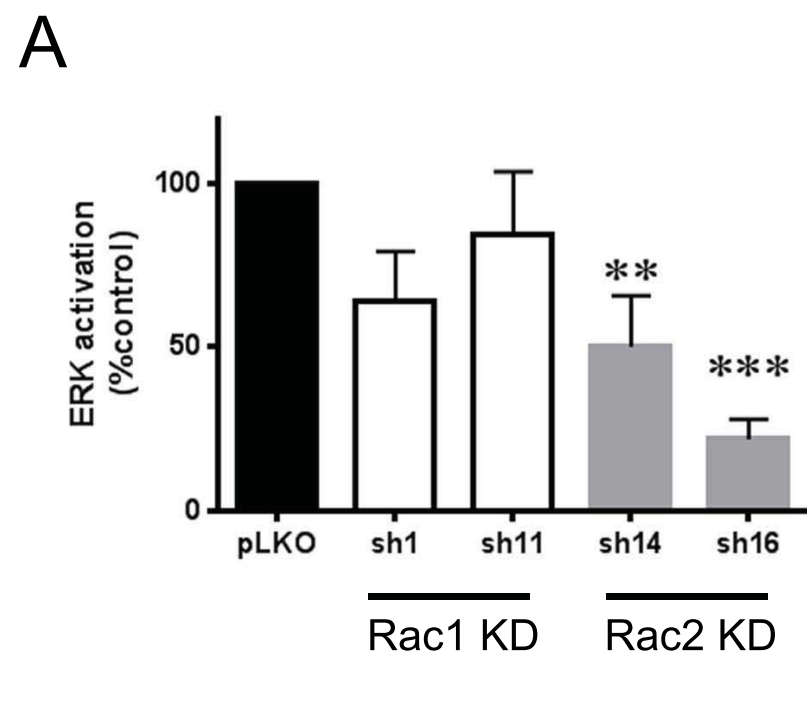


Figure 8