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**Synaptotagmin-like protein 1 interacts with the GTPase-activating protein Rap1GAP2 and regulates dense granule secretion in platelets**

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Short title: Slp1 regulates platelet secretion

## **Abstract**

The small guanine-nucleotide-binding protein Rap1 plays a key role in platelet aggregation and hemostasis and we recently identified Rap1GAP2 as the only GTPase-activating protein of Rap1 in platelets. In search of Rap1GAP2-associated proteins we performed yeast-two-hybrid screening and found synaptotagmin-like protein 1 (Slp1) as new binding partner. We confirmed the interaction of Rap1GAP2 and Slp1 in transfected COS-1 and HeLa cells and at endogenous level in human platelets. Mapping studies showed that Rap1GAP2 binds through amino acids T524-K525-X-T527 within its C-terminus to the C2A domain of Slp1. Slp1 contains a Rab27-binding domain and we demonstrate that Rap1GAP2, Slp1 and Rab27 form a trimeric complex in transfected cells and in platelets. Purified Slp1 dose-dependently decreased dense granule secretion in streptolysin-O permeabilized platelets stimulated with calcium or GTPgammaS. The isolated C2A domain of Slp1 had a stimulatory effect on granule secretion and reversed the inhibitory effect of full-length Slp1. Purified Rap1GAP2 augmented dense granule secretion of permeabilized platelets, whereas deletion of the Slp1-binding TKXT motif abolished the effect of Rap1GAP2. We conclude that Slp1 inhibits dense granule secretion in platelets and that Rap1GAP2 modulates secretion by binding to Slp1.

## Introduction

Blood platelets are essential for hemostasis and play an important role in the development of thrombosis in the vasculature <sup>1</sup>. During primary hemostasis platelets adhere to sites of endothelial damage and the initial platelet coat is soon reinforced by additional platelets forming a stable aggregate. At the same time platelets secrete their intracellular granules containing substances that further activate platelets in an autocrine loop and affect local coagulation and endothelial and smooth muscle cell functions <sup>2</sup>.

Two main types of secretory granules have been described in platelets, dense granules and alpha-granules. Dense granules contain small molecules including ADP, ATP and 5-hydroxytryptamine (5HT, serotonin), whereas alpha-granules store many proteins such as fibrinogen, von Willebrand factor, various cytokines and growth factors <sup>3,4</sup>. Dense granule secretion is regulated by the small GTPase Rab27 that is localized at the granule membrane <sup>5</sup>. Rab27 probably mediates its action by promoting granule motility or by enhancing tethering and fusion of granules with the plasma membrane <sup>6,7</sup>. For these functions Rab27 needs to interact with effector proteins and Munc13-4 was identified as the first Rab27-binding protein in platelets <sup>8</sup>. Other proteins involved in dense granule secretion are the small GTPase Ral <sup>9</sup> and membrane proteins of the soluble NSF attachment protein receptor family (SNARE) either bound to the granule such as VAMP-8 <sup>10</sup> or to the plasma membrane such as SNAP-23 and syntaxin 2 <sup>11</sup>. Granule secretion is accompanied by the development of tight cellular interactions between platelets. This aggregation of platelets is mediated by integrins and other adhesion receptors <sup>2</sup>. Integrin activation is tightly regulated by the small guanine-nucleotide-binding protein Rap1 and deletion of the platelet isoform Rap1b in mouse platelets results in a bleeding phenotype <sup>12</sup>. Rap1 activity is controlled by GTPase-activating proteins (GAP) and guanine-nucleotide exchange factors (GEF). We recently discovered Rap1GAP2 as the only GTPase-activating protein of Rap1 in platelets <sup>13</sup>. Rap1GAP2 contains a conserved central catalytic GAP domain, an N-terminal 14-3-3-binding site and a large C-terminal region of unknown function. Platelet activation results in phosphorylation of Rap1GAP2 on serine 9, binding of 14-3-3 protein and probably inhibition of GAP function <sup>14</sup>. In parallel, activation of platelet specific GEFs such as CD-GEF I and III and PDZ-GEF1 results in increased Rap1-GTP levels and thus adhesion and aggregation <sup>13,15</sup>.

In this article we demonstrate that the Rab27-binding protein Slp1 is a new direct binding partner of Rap1GAP2 in human platelets. We show that Rap1GAP2 binding to Slp1 is mediated through a short sequence within the C-terminal region of Rap1GAP2, and *vice versa* the C2A domain of Slp1 is sufficient for Slp1 binding to Rap1GAP2. Furthermore, we prove the existence of a trimeric complex composed of Rap1GAP2, Slp1 and Rab27 in human platelets, and provide evidence for a regulatory role of Slp1 in platelet dense granule secretion.

## **Materials and Methods**

### *Antibodies, Constructs, Materials*

To detect Rap1GAP2a a previously described polyclonal antibody was used <sup>13</sup>. To detect Slp1 a polyclonal antibody against Slp1 was produced using full-length recombinant glutathione-S-transferase-tagged Slp1 purified from *Escherichia coli* BL21 as antigen. Immunization of rabbits and subsequent purification were performed by ImmunoGlobe Antikörpertechnik (Himmelstadt, Germany). Commercially available antibodies were used for detection of Rab27a (M02, Abnova), FLAG tag (M2, Sigma), myc tag (A-14, 9E10, Santa Cruz), VSV tag (P5D4, Sigma), and GST tag (GST-2, Sigma). Horseradish peroxidase-coupled goat anti-rabbit and goat anti-mouse were from Dianova (Hamburg, Germany) and were used as secondary antibodies for immunoblot analysis visualized by enhanced chemiluminescence method (Millipore, Amersham Biosciences). Cy3- and Cy5-labelled secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA). Rap1GAP2 wild-type peptide with the sequence HNSMEVTKTTFSPPV (amino acids 518-532 of Rap1GAP2a) and Rap1GAP2ΔEVTKTT peptide with the sequence GISHNSMFSPPVVAA (amino acids 515-535 of Rap1GAP2a lacking amino acids 522-527) were obtained from Schafer-N (Copenhagen, Denmark).

Rap1GAP2a <sup>13</sup> was FLAG-tagged or hexahistidine-tagged at the C-terminus and expressed using mammalian expression vector pcDNA4/TO (Invitrogen). Site-directed mutagenesis was performed as described <sup>14</sup>. Full-length Slp1 cDNA (clone IRATp970G0456D, GenBank™ accession number BC035725) was obtained from RZPD (Berlin, Germany) and subcloned into EcoRI and XhoI sites of the mammalian expression vector pcDNA3.1™/myc-His (Invitrogen). To produce recombinant glutathione-S-transferase fusion protein of Slp1 full-size cDNA was subcloned into

pGEX-4T3 vector (GE Healthcare). For hexahistidine-tagged recombinant protein production cDNA of Slp1 was subcloned into pET28 (Merck). Rab27a-containing plasmid pEGFP-C3, a kind gift from M.C. Seabra (Imperial College London, United Kingdom), was used to subclone Rab27a VSV-tagged at the C-terminus into HindIII and XhoI sites of pcDNA4/TO (Invitrogen). Hexahistidine-tagged Rap1b cloned in pET28 and glutathione-S-transferase-tagged 14-3-3 $\beta$  cloned in pGEX-4T3 were described before<sup>14</sup>. All constructs were sequence-verified.

Unless otherwise specified, all chemicals were obtained from Sigma (Taufkirchen, Germany), except for streptolysin-O which was kindly provided by S. Bhakdi (Johannes Gutenberg University, Mainz, Germany).

### *Protein Purification*

GST-Slp1, GST-C2A, GST-14-3-3 $\beta$  and His<sub>6</sub>-Slp1 were expressed in *Escherichia coli* BL21 and affinity purified using glutathione-sepharose 4B beads (GE Healthcare) or nickel-nitrotriacetic acid-agarose (Qiagen), respectively. His<sub>6</sub>-tagged recombinant Rap1GAP2 wild-type and Rap1GAP2 $\Delta$ 522-527 mutant were affinity purified from COS-1 cells. Briefly, one hundred 10 cm dishes of COS-1 were transiently transfected with the appropriate plasmids. 48 h post-transfection, cells were lysed with lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>/NaOH, pH 8, 300 mM NaCl, 1% (w/v) Triton-X 100) containing protease inhibitors, and His<sub>6</sub>-tagged proteins were affinity purified using Ni-NTA agarose (Qiagen). His<sub>6</sub>-Rap1b was purified from *Escherichia coli* BL21 via HisTrap HP column (GE Healthcare) using an ÄKTA-system and subsequently loaded with GTP as described before<sup>14</sup>. The purity of all proteins was examined by SDS-PAGE followed by Coomassie-Blue staining.

### *Yeast-two-hybrid Screening*

The MATCHMAKER Two-Hybrid System 3 (Clontech-Takara Bio, Saint-Germain-en-Laye, France) was used with full-length Rap1GAP2a as bait and human adult brain cDNA library as prey as described<sup>14</sup>.

### *Cell Preparation, Transfection, Lysis, Immunoprecipitation and Pull-down Experiments*

Culture and transfection of COS-1 and HeLa cells has been described previously<sup>14</sup>. Venous blood was drawn from healthy volunteers taking no medications who gave their informed consent according to the declaration of Helsinki. Washed platelets were obtained by sequential centrifugation as described<sup>16</sup>. Cell lysis, immunoprecipitation and pull-down assays were performed as described<sup>14</sup> using 5  $\mu$ l of ANTI-FLAG M2 Affinity Gel (Sigma) or 10  $\mu$ g of anti-Rab27a antibody (Abnova) or 1  $\mu$ l or 5  $\mu$ l of Gluthatione Sepharose<sup>TM</sup> 4B suspension (GE Healthcare) saturated with GST, GST-Slp1, or GST-14-3-3 $\beta$ , respectively.

### *Peptide Binding Assay (PepSpot)*

Synthetic peptides with either wild-type Rap1GAP2a sequence HNSMEVTKTTFSPPV or with one amino acid mutated to alanine or key threonines phosphorylated were synthesized on cellulose membrane (ImmunoGlobe Antikörpertechnik) and incubated with 1  $\mu$ g/ml of purified recombinant GST-Slp1 in TBS with 0.1% Tween 20 and 5% BSA for 3 h at room temperature. Bound GST-Slp1 was visualized by immunoblotting using a monoclonal anti-GST antibody (Sigma).

### *Confocal Microscopy*

HeLa cells were grown on glass coverslips and transfected with expression vectors for EGFP-tagged Rab27a, VSV-tagged Rap1GAP2 and myc-tagged Slp1. 24 h post-transfection, cells were fixed with 3.7% paraformaldehyde in PBS for 15 min on ice, washed with PBS and then permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. To detect VSV-tagged Rap1GAP2 and myc-tagged Slp1 primary tag-specific antibodies diluted in PBS with 1% BSA were added and incubated for 1 h at 37°C followed by incubation with Cy5-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG as secondary antibodies. After further washing with PBS and water, samples were mounted in GEL/Mount<sup>TM</sup> (Biomedica, Foster City, CA). Staining was observed using a Zeiss LSM 510 confocal laser scanning microscope equipped with a Plan-Apochromat 63x/1.4 oil DIC objective lens and LSM 510 META software (Carl Zeiss, Göttingen, Germany).

### *Assay for Secretion of Platelet Dense Granules*

Freshly obtained washed platelets (approximately  $1 \times 10^8$  platelets/ml, counted with CASY Cell Counter, Innovatis AG, Bielefeld, Germany) were resuspended in 70  $\mu$ l of prewarmed buffer A (50 mM HEPES/KOH pH 7.4, 78 mM KCl, 4 mM MgCl<sub>2</sub>, 2 mM EGTA, 0.2 mM CaCl<sub>2</sub>, 5 mM DTT) containing 4 mg/ml BSA, 5 mM ATP (Roche), 8 mM creatine phosphate (Fluka), 50  $\mu$ g/ml creatine phosphokinase (Sigma). Then, platelets were permeabilized using 0.6  $\mu$ g/ml streptolysin-O in buffer A containing 4 mg/ml BSA at 30°C for 5 min. Permeabilized platelets were placed on ice and incubated with purified proteins or peptides to be tested for 40 min followed by further incubation at 30°C for 5 min. Finally, platelets were stimulated with either 10  $\mu$ l of prewarmed buffer A, where the free calcium ion concentration was calculated to  $\sim$ 20 nM, or 10  $\mu$ l of prewarmed stimulation buffer (50 mM HEPES/KOH pH 7.4, 78 mM KCl, 4 mM MgCl<sub>2</sub>, 2 mM EGTA, 20 mM CaCl<sub>2</sub>), which results in 20  $\mu$ M of free calcium, at 30°C for 1 min. In case of GTP $\gamma$ S-induced secretion of dense granules, platelets were stimulated with 100  $\mu$ M of GTP $\gamma$ S at 30°C for 5 min. The reaction was stopped by addition of 200  $\mu$ l of 2-fold concentrated ice-cold stop buffer (100 mM HEPES/KOH pH 7.4, 156 mM KCl, 8 mM MgCl<sub>2</sub>, 18 mM EGTA, 0.4 mM CaCl<sub>2</sub>) and incubation on ice for 5 min. Then, platelets were removed by centrifugation at 4°C 5000 x *g* for 5 min and released serotonin (5-hydroxytryptamine, 5HT) in the supernatant was measured using Walac Victor 1420 Multilabel Counter as described<sup>17</sup>. The secretion levels of serotonin were expressed as percentage of total serotonin of permeabilized platelets before the final centrifugation. Shown data represent the means  $\pm$  S.E.M. of at least three independent experiments performed in triplicate. The statistical significance of the means was analyzed by ANOVA and Bonferroni post test (95% confidence interval) using GraphPad Prism 4.0 software. *P*-values are expressed as follows \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.001 and considered as statistically significant. Protein concentrations were determined by Bradford method (Bio-Rad) and from intensities of the bands in Coomassie-Blue stained SDS-PAGE gels using bovine albumin serum as standard. Permeabilization was monitored by immunoblotting using anti-LDH antibody (Chemicon International).

### *In vitro GAP Assay*

*In vitro* GAP assay was performed as described previously<sup>14</sup>.

## Results

### *Rap1GAP2 interacts with Slp1.*

We were interested in identifying proteins that might be involved in the regulation of Rap1GAP2<sup>13</sup>. For this purpose, we performed yeast-two-hybrid screening using full-length wild-type Rap1GAP2a, the predominant splice isoform of Rap1GAP2 in platelets, as bait and human adult brain cDNA library as prey. Among the library clones that were found to interact strongly two clones were identified as synaptotagmin-like protein 1 (Slp1, also called JFC1)<sup>18,19</sup>. These clones contained the C-terminal part of Slp1 comprising two tandem C2 domains, C2A and C2B. To confirm direct binding of Slp1 to Rap1GAP2 we performed pull-down assays using COS-1 cell lysates overexpressing FLAG-tagged Rap1GAP2 and purified recombinant full-length Slp1 as GST fusion protein. GST-Slp1 was clearly able to pull down transfected Rap1GAP2 from cell lysates, whereas GST alone did not bind Rap1GAP2 (Fig.1A). To verify that Rap1GAP2 and Slp1 interact also in intact mammalian cells we performed co-immunoprecipitation experiments. COS-1 cells were transfected with epitope-tagged versions of Rap1GAP2 and Slp1 either alone or in combination, and Rap1GAP2 was immunoprecipitated with anti-FLAG antibody. Analysis of the precipitates by immunoblotting with anti-myc antibody revealed the presence of Slp1 only in precipitates from cells overexpressing both, Slp1 and Rap1GAP2 (Fig.1B, lane 3). To assess the Rap1GAP2/Slp1 interaction in a more physiological context we performed pull-down assays using human platelet lysates containing endogenous Rap1GAP2 protein. Only GST-Slp1, but not GST alone, bound to endogenous Rap1GAP2 (Fig.1C). To check if Slp1 is expressed in human platelets we generated an antibody against full-length human Slp1. The antibody specifically recognized the 66-kDa Slp1 protein in HeLa cells transfected with Slp1 but not in mock transfected cells (data not shown). Importantly, the antibody recognized a band of similar molecular weight in human platelet lysate, suggesting that Slp1 is endogenously expressed in human platelets. Neither our Rap1GAP2 antibody nor the newly generated anti-Slp1 antibody were able to immunoprecipitate their antigens efficiently from human platelet lysates. In order to obtain conclusive evidence that endogenous Rap1GAP2 and Slp1 interact we applied an alternative precipitation approach as previously described<sup>14</sup>. We exploited the ability of 14-3-3 proteins to bind to Rap1GAP2 at phosphorylated serine 9 within the N-terminus of Rap1GAP2. 14-3-3 precipitates Rap1GAP2 from platelet lysate, and thrombin

treatment was shown to enhance this interaction<sup>14</sup>. We also demonstrated that 14-3-3 and Slp1 do not interact directly with each other (Slp1 is designated Rip2 in figure 3D in<sup>14</sup>). To further exclude a possible direct interaction of Slp1 and 14-3-3 we performed an additional experiment using purified recombinant GST-14-3-3. In lysates of transfected cells 14-3-3 was able to bind to Slp1 only in the presence of Rap1GAP2 (Fig.1D). Finally, we used purified recombinant GST-14-3-3 to precipitate Rap1GAP2 from lysates of thrombin-treated platelets and were able to co-precipitate endogenous Slp1 (Fig.1E, lane 4). A control experiment showed that GST alone did not bind Rap1GAP2 or Slp1 (Fig.1E, lane 3). Taken together, these experiments confirm that the interaction between Rap1GAP2 and Slp1 is direct and that both proteins interact in transfected cells as well as at endogenous level in human platelets.

*C2A domain of Slp1 is sufficient for binding of Slp1 to Rap1GAP2.*

Slp1 is composed of an N-terminal Rab27-binding site and two C2 domains, C2A and C2B at the C-terminus<sup>18,19</sup>. To determine the Rap1GAP2-binding site within Slp1 we generated Slp1 mutants composed of C2A, C2B or C2AB domain fused to GST. From our yeast-two-hybrid result we assumed the binding site for Rap1GAP2 to be located within the two C2 domains of Slp1. As shown in Fig.2A, only GST-C2A and GST-C2AB were able to pull down transfected Rap1GAP2 from HeLa cell lysates indicating that the C2A domain of Slp1 is required and sufficient for binding of Slp1 to Rap1GAP2. This result was further confirmed in intact mammalian cells. In immunoprecipitation experiments using HeLa cell lysates overexpressing FLAG-tagged Rap1GAP2 and myc-tagged C2A we were able to co-immunoprecipitate C2A together with Rap1GAP2 (Fig.2B, lane 3). Binding activities of some C2 domains are modulated by calcium ions<sup>20</sup>, however, the Rap1GAP2/Slp1 interaction was not affected by calcium depletion in pull-down assays (data not shown).

*Rap1GAP2/Slp1 interaction is mediated through the TKXT motif within the C-terminus of Rap1GAP2.*

To map the binding site for Slp1 in Rap1GAP2 we generated truncation mutants of Rap1GAP2 and performed GST-Slp1 pull-down experiments. As shown in Fig.3A, Slp1 bound to wild-type Rap1GAP2 and Rap1GAP2 $\Delta$ Nterm mutant lacking the N-terminal part with equal potency, whereas no binding could be detected between Slp1

and a C-terminally truncated Rap1GAP2 mutant (Rap1GAP2 $\Delta$ Cterm). By further truncational analysis (data not shown) we narrowed down the interaction site to a short sequence of six residues in the C-terminal region of Rap1GAP2. To confirm the role of these residues for Slp1 binding we generated a Rap1GAP2 deletion mutant lacking the suspected sequence from position 522 to 527 and a control mutant, where an arbitrarily chosen sequence in proximity was deleted. We tested both mutants in GST-Slp1 pull-down experiments. The control mutant bound Slp1 comparable to wild-type Rap1GAP2 (Fig.3B, lane 1 and 2), whereas Rap1GAP2 $\Delta$ 522-527 mutant did not bind Slp1 (Fig.3B, lane 3). From this result we conclude that the EVTKTT sequence (amino acids 522-527 of Rap1GAP2) is required for binding of Slp1 to Rap1GAP2. To determine the role of each amino acid residue of this motif we generated consecutive alanine point mutants of the EVTKTT sequence and performed GST-Slp1 pull-down assays (Fig.3C). We observed that mutation of E522 to alanine did not affect binding of Slp1 to Rap1GAP2 (Fig.3C, lane 2 and 3), mutation of V523 or T526 to alanine only slightly reduced binding (Fig.3C, lane 4 and 7), whereas mutation of T524, K525 or T527 almost completely abolished binding of Slp1 to Rap1GAP2 (Fig.3C, lanes 5, 6 and 8). These results indicate that amino acids T524, K525 and T527, the so-called TKXT motif, are important for binding of Slp1 to Rap1GAP2. To confirm these data using a complementary approach we performed peptide binding assays. For this purpose, short peptides containing the key sequence EVTKTT in wild-type form or with one amino acid mutated to alanine were synthesized on a cellulose membrane and incubated with purified GST-Slp1. As shown in Fig.3D, if T524, K525 and T527 were mutated no binding of GST-Slp1 could be detected. Interestingly, mutation of E522 to alanine led to a stronger binding of GST-Slp1. We conclude that the residues T524, K525 and T527 in the C-terminal part of Rap1GAP2 constitute the binding site for Slp1. To answer the question whether Slp1 binding to Rap1GAP2 would involve phosphorylation of the threonine residues in the TKXT motif we tested peptides having the key threonines T524, T527 or both phosphorylated. No binding of GST-Slp1 to the phosphorylated versions of the TKXT motif could be observed (Fig. 3D, lanes 9-11), indicating that phosphorylation of the TKXT motif of Rap1GAP2 is not required and could even abolish Slp1 binding. Finally, we wanted to test if peptides containing the key sequence EVTKTT or peptides carrying a deletion of the EVTKTT motif could affect binding of Slp1 to Rap1GAP2. In a pull-down assay using GST-Slp1, addition of the

wild-type peptide to platelet lysate blocked the interaction of Slp1 and Rap1GAP2, whereas addition of the mutant peptide did not affect binding of Slp1 to Rap1GAP2 (Fig.3E).

*Rap1GAP2, Slp1 and Rab27 form a trimeric complex.*

Slp1 has been shown to bind to Rab27, a small GTPase involved in vesicle-regulated exocytosis of many cell types<sup>21</sup>. Rab27 is expressed in 2 isoforms, Rab27a and Rab27b that share about 71% identity at amino acid level. In platelets, both Rab27 isoforms are present<sup>22</sup>. To demonstrate that Slp1 interacts with endogenous Rab27 we performed GST-Slp1 pull-down experiments using platelet lysates (Fig.4A). The used monoclonal anti-Rab27a antibody recognized both Rab27 isoforms (data not shown) so that the band for Rab27 most likely represents a mixture of Rab27a and Rab27b. In reverse experiments using purified recombinant GST-Rab27a and GST-Rab27b binding of endogenous Slp1 from human platelet lysates could be observed to both isoforms of Rab27, and additionally, binding of Slp1 to Rab27 was independent of the nucleotide state of the Rab protein (data not shown). To determine if Slp1, Rab27 and Rap1GAP2 form a trimeric complex *in-vivo* we transfected HeLa cells with epitope-tagged Slp1, Rab27a and Rap1GAP2 and performed co-immunoprecipitation experiments using anti-FLAG antibody. Rab27a could be detected only in precipitates from cell lysates overexpressing all three proteins, indicating that Rab27a, Slp1 and Rap1GAP2 indeed form a complex in intact mammalian cells (Fig.4B). This result could be confirmed in a reverse experiment using anti-VSV antibody (data not shown). We were also able to co-immunoprecipitate endogenous Rab27, Slp1 and Rap1GAP2 from human platelet lysates using anti-Rab27 antibody (Fig.4C). To investigate the subcellular localization of Rab27, Slp1 and Rap1GAP2 HeLa cells were co-transfected with EGFP-tagged Rab27a, myc-tagged Slp1 and VSV-tagged Rap1GAP2. The cells were fixed, permeabilized and immunostained with tag-specific primary and dye-labelled secondary antibodies. Immunofluorescence analysis revealed a partial co-localization of all three proteins in the cytosol as well as at the plasma membrane (Fig.4D). Altogether, these data provide strong evidence for the existence of a trimeric complex composed of Slp1, Rab27 and Rap1GAP2 in transfected cells and in platelets.

*Involvement of Slp1 and Rap1GAP2 in platelet dense granule secretion.*

In platelets, Rab27 has been shown to regulate dense granule secretion<sup>8</sup>. Slp1 is a Rab27-binding protein and thus might be a Rab27 effector. In addition, here we show that Slp1 also binds to Rap1GAP2. To elucidate the roles of Slp1 and Rap1GAP2 in granule secretion we adopted and modified a previously described method for measuring serotonin release using streptolysin-O permeabilized platelets<sup>17,23,24</sup>. To induce granule release we used calcium ions or the non-hydrolysable GTP-analog guanosine 5'-O-[gamma-thio]triphosphate (GTP $\gamma$ S). Upon incubation of permeabilized platelets with purified recombinant Slp1 Ca<sup>2+</sup>-induced dense granule secretion of serotonin was significantly inhibited (Fig.5A). The inhibitory effect of Slp1 was dose-dependent, while baseline serotonin release was not affected by Slp1 (Fig.5A). Similar results were obtained in case of stimulation of permeabilized platelets with GTP $\gamma$ S (Fig.5B). Boiling of the protein abolished the inhibitory effect of Slp1. To analyze the inhibitory function of Slp1 in greater detail we studied the effects of the isolated C2A domain of Slp1 on dense granule release. Previous investigators have shown that the C2A domain competes with endogenous Slp1 and thus can be used as a dominant negative control<sup>25,26</sup>. As shown in Fig.5C, the isolated C2A domain of Slp1 fused to GST augmented serotonin secretion. Furthermore, a molar excess of C2A reversed the inhibitory function of GST-Slp1. We used GST-fused proteins in these experiments because hexahistidine-tagged and un-tagged C2A proteins were not stable. Taken together, our data strongly support the concept that Slp1 inhibits dense granule secretion in platelets.

Next, we investigated a potential influence of Rap1GAP2 on secretion. Addition of purified recombinant wild-type Rap1GAP2 to permeabilized platelets significantly augmented Ca<sup>2+</sup>- and GTP $\gamma$ S-induced dense granule secretion (Fig.6A). This effect was dose-dependent and baseline levels of serotonin release were not changed by Rap1GAP2 (Fig.6A). Of note, addition of Rap1GAP2 together with Slp1 did not affect the inhibitory effect of Slp1 on dense granule secretion in the permeabilized cell system (data not shown). To investigate the relevance of the Rap1GAP2/Slp1 interaction we prepared and purified a Rap1GAP2 $\Delta$ EVTKTT mutant which lacks the Slp1-binding site and therefore is not able to bind to Slp1 ( $\Delta$ 522-527 in Fig.3B). Incubation of permeabilized platelets with this mutant had no effect on Ca<sup>2+</sup>- and GTP $\gamma$ S-induced dense granule secretion (Fig.6B). To corroborate the Rap1GAP2

effect we also tested Rap1GAP2 peptides described in figure 3E in the secretion assay system. The wild-type peptide containing the Slp1-binding motif, but not the peptide lacking this motif, augmented serotonin secretion from platelet dense granules (Fig.6C). To assure Rap1GAP2 function in platelet secretion to be independent of its GAP activity we incubated permeabilized platelets with purified recombinant Rap1 either native or GTP-loaded. As expected, no effect on serotonin secretion of platelet dense granules could be observed (Fig.7A). Additionally, Slp1 binding to Rap1GAP2 had no effect on the catalytic GTPase-activating function of Rap1GAP2 in *in-vitro* GAP assays using purified proteins (Fig.7B). From these data we conclude that Slp1 inhibits platelet dense granule secretion, whereas Rap1GAP2 has a modulatory function in secretion which requires binding to Slp1 but is independent of the GAP activity of Rap1GAP2.

## Discussion

We have identified Slp1 as new direct binding partner of Rap1GAP2 in platelets and we have shown that Slp1 is involved in the regulation of platelet dense granule secretion.

Rap1GAP2 is a modular protein containing a central GAP domain that is required to confer GTPase activity towards Rap1, a protein in control of platelet aggregation. However, the large C-terminal region of Rap1GAP2 has so far been of unknown function. In our present study we demonstrated that at least part of this C-terminal region of Rap1GAP2 is involved in protein-protein interactions. We mapped the Slp1-binding site to a very small motif comprising three essential residues in the C-terminus of Rap1GAP2. This TKXT motif interacts with the C2A domain of Slp1 that has been shown to mediate binding of Slp1 to the plasma membrane<sup>27</sup>. C2 domains are generally considered to mediate phospholipid binding<sup>20</sup>, however, certain C2 domains have also been observed to be involved in protein-protein interactions. These interactions usually occur intramolecularly, such as the interaction of the C1 and C2 domains of PKC<sup>28</sup> or the binding of the C2 domain to the catalytic domain in SynGAP<sup>29</sup>. The C2 domain of PKC $\delta$  was recently shown to bind to phosphotyrosine residues<sup>30</sup>. Although the TKXT motif of Rap1GAP2 contains two threonines that could be subject to phosphorylation, our data suggest that phosphorylation is not required for binding of the C2A domain to the TKXT motif in Rap1GAP2 (Fig.3D).

The TKXT motif could be involved in subcellular targeting of Rap1GAP2 to the plasma membrane via Slp1 and we indeed observed a co-localization of Rap1GAP2 and Slp1 at the plasma membrane (Fig.4D).

Slp1 was previously shown to stimulate secretion of prostate-specific antigen by prostate cells<sup>25</sup> and secretion of azurophilic granules by granulocytes<sup>26</sup>. Furthermore, Slp1 regulates exocytosis of secretory lysosomes by cytotoxic T-lymphocytes<sup>31</sup> and blocks amylase secretion by pancreatic acinar cells<sup>32</sup>. For the first time, we show that Slp1 is also expressed in human platelets. Slp1 is a member of a family of Rab27-binding proteins and is known to interact with Rab27 via an N-terminal Rab27-binding domain<sup>33,34</sup>. We could verify binding of Slp1 to Rab27 in human platelets and showed that Rap1GAP2 can join this complex (Fig.4C). However, possible regulatory mechanisms involved in the formation of the Slp1/Rap1GAP2/Rab27 protein complex in platelets will be the subject of future studies.

To elucidate the functional role of the Slp1/Rap1GAP2 interaction in platelets we performed secretion assays using streptolysin-O permeabilized platelets. Addition of recombinant Slp1 strongly reduced serotonin secretion. Experiments using the isolated membrane-binding C2A domain of Slp1 as dominant negative control<sup>25,26</sup> showed the opposite effect thus confirming the inhibitory role of full-length Slp1. Interestingly, the Slp1 effect was independent of the stimulus, both  $Ca^{2+}$ - and GTP $\gamma$ S-induced secretion were inhibited.  $Ca^{2+}$  probably triggers the final fusion event of granules with the plasma membrane, whereas the GTP $\gamma$ S effect might be mediated by small GTPases such as Rab27 or Ral<sup>9,35</sup>. Thus, Slp1 appears to be involved in a phase of the secretion process common to both stimuli. Studies of other Slp family members suggest that Slps can have dual, stimulatory and inhibitory, roles in granule secretion through diverse interactions with other proteins or the membrane<sup>36-40</sup>. Testing of the Slp1-interacting protein Rap1GAP2 in secretion assays with permeabilized platelets revealed that Rap1GAP2 augments dense granule release. This effect was dependent on the binding of Rap1GAP2 to Slp1 and could be mimicked by a small peptide containing the Slp1-binding motif. Although Rap1GAP2 is required for GTPase activity of Rap1, Rap1 itself does not have any direct effect on platelet dense granule secretion.

We conclude that Slp1 inhibits dense granule secretion in platelets. Furthermore, Slp1 interacts with Rap1GAP2 and binding of Rap1GAP2 to Slp1 augments dense granule

release. The effect of Rap1GAP2 on secretion requires the new Slp1-binding TKXT protein motif in the C-terminal part of Rap1GAP2. Our data provide new insights into the control of granule secretion and suggest possible connections between the regulation of granule secretion and aggregation in platelets.

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### **Authorship**

Contribution: O.N., M.H., J.B., C.P., K.G., and A.P.S. performed experiments and analyzed data; O.N., M.H., and A.P.S. designed the research; O.N. and A.P.S. wrote the paper.

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### Figure legends

Figure 1: Rap1GAP2 interacts with Slp1.

A: Pull-down of transfected Rap1GAP2 with GST-Slp1.

COS-1 cells were transfected with FLAG-tagged Rap1GAP2 (RG2-FLAG). Equal amounts of GST as control and GST-Slp1 coupled to GSH-Sepharose beads were used for precipitation (*lower panel*). Bound Rap1GAP2 protein was visualized by immunoblot using anti-FLAG antibody (*upper panel*). Expression level of Rap1GAP2 is shown as 2% input of total RG2-FLAG. The broad band of Rap1GAP2 is probably due to extensive post-translational modifications.

B: Co-immunoprecipitation of transfected Rap1GAP2 and Slp1.

COS-1 cells were transfected with FLAG-tagged Rap1GAP2, myc-tagged Slp1 or FLAG-tagged Rap1GAP2 together with myc-tagged Slp1. After lysis Rap1GAP2 was precipitated with anti-FLAG antibody. The precipitates were analyzed for the presence of bound Slp1 by immunoblot using anti-myc antibody. The *upper panel* shows precipitation results and the *two lower panels* show transfection levels of Rap1GAP2 (total RG2-FLAG, 2% input) and Slp1 (total Slp1-myc, 2% input).

C: Pull-down of endogenous Rap1GAP2 with GST-Slp1 from human platelets.

Equal amounts of GST as control and GST-Slp1 coupled to GSH-Sepharose beads were incubated with human platelet lysate. Bound endogenous Rap1GAP2 protein was visualized with anti-Rap1GAP2 antibody (RG2). Expression level of Rap1GAP2 is shown as 2% input of total RG2.

D: Pull-down of transfected Rap1GAP2 and Slp1 with GST-14-3-3.

COS-1 cells were transfected with myc-tagged Slp1 and without or with FLAG-tagged Rap1GAP2 (RG2-FLAG). Cells were lysed, and GST-14-3-3 $\beta$  was used to pull down Rap1GAP2 and indirectly Slp1 bound to Rap1GAP2. Precipitated Slp1 was visualized by immunoblotting using anti-myc antibody (*top panel*). Precipitation of Rap1GAP2 was controlled by immunoblot with anti-FLAG antibody (*second panel from top*). In parallel, total cell lysates were analyzed for the expression of Slp1 (total Slp1-myc, 2% input) and Rap1GAP2 (total RG2-FLAG, 2% input).

E: Pull-down of endogenous Rap1GAP2 and Slp1 from human platelets.

Lysates from thrombin-treated platelets were subjected to pull-down assays using either GST as control or GST-14-3-3 $\beta$ . The precipitates were analyzed for the presence of endogenous Rap1GAP2 (RG2) and Slp1 using specific anti-Rap1GAP2 and anti-Slp1 antibodies (*first and second panel from top*). Expression levels of Rap1GAP2 and Slp1 are shown as 2% input of total protein amounts. The *lower panel*

shows the amounts of GST and GST-14-3-3 $\beta$  used for precipitation. Unspecific band is marked with asterisk (\*).

Figure 2: C2A domain of Slp1 is sufficient for binding of Slp1 to Rap1GAP2.

A: Pull-down of transfected Rap1GAP2 with GST-Slp1 mutants.

Lysates of HeLa cells overexpressing FLAG-tagged Rap1GAP2 (RG2-FLAG) were subjected to pull-down experiments using equal amounts of GST as control and GST fusion proteins of C2A (amino acids 292-393 of Slp1), C2B (amino acids 433-580 of Slp1) and C2AB (amino acids 292-580 of Slp1). The precipitates were analyzed for the presence of bound Rap1GAP2 by immunoblot with anti-FLAG antibody (*upper panel*). The *lower panel* shows expression levels of Rap1GAP2 (total RG2-FLAG, 2% input).

B: Co-immunoprecipitation of transfected Rap1GAP2 and Slp1-C2A.

HeLa cells were transfected with FLAG-tagged Rap1GAP2, myc-tagged C2A domain of Slp1 or FLAG-tagged Rap1GAP2 together with myc-tagged Slp1-C2A. After lysis Rap1GAP2 was precipitated with anti-FLAG antibody. The precipitates were analyzed for the presence of bound Slp1-C2A by immunoblot using anti-myc antibody (*upper panel*). The *two lower panels* show expression levels of Slp1-C2A (total C2A-myc, 2% input) and Rap1GAP2 (total RG2-FLAG, 2% input).

Figure 3: Binding of Rap1GAP2 to Slp1 is mediated through the TKXT motif within the C-terminus of Rap1GAP2.

A: Pull-down of transfected Rap1GAP2 truncation mutants with GST-Slp1.

HeLa cells were transfected with either FLAG-tagged Rap1GAP2 (RG2-FLAG) wild-type or different Rap1GAP2 truncation mutants. Rap1GAP2 $\Delta$ Cterm lacks amino acids 467-715, whereas Rap1GAP2 $\Delta$ Nterm lacks amino acids 1-121. Cells were lysed and pull-down assays using GST or GST-Slp1 were performed. Precipitates were analyzed for the presence of FLAG-tag containing proteins by immunoblot with anti-FLAG antibody. The *upper panel* shows precipitation results, and the *lower panel* expression levels of RG2 proteins (total RG2-FLAG, 2% input).

B: Pull-down of transfected Rap1GAP2 deletion mutants with GST-Slp1.

Lysates of HeLa cells overexpressing either FLAG-tagged Rap1GAP2 wild-type (RG2-FLAGwt) or deletion mutants Rap1GAP2 $\Delta$ 536-542 as control and

Rap1GAP2 $\Delta$ 522-527 were subjected to GST-Slp1 pull-down assays followed by immunoblot analysis using anti-FLAG antibody. The *upper panel* shows precipitation results, and the *lower panel* expression levels of RG2 proteins (total RG2-FLAG, 2% input).

C: Pull-down of transfected Rap1GAP2 alanine point mutants with GST-Slp1.

HeLa cells were transfected with FLAG-tagged Rap1GAP2 wild-type (RG2-FLAGwt) or different Rap1GAP2 point mutants having each of the amino acids within the EVTKTT sequence (amino acids 522-527) changed to alanine as indicated. Cells were lysed, and lysates were subjected to GST-Slp1 pull-down assays followed by immunoblot analysis with anti-FLAG antibody. The *upper panel* shows precipitation results, and the *lower panel* expression levels of RG2 proteins (total RG2-FLAG, 2% input).

D: Peptide binding assay (PepSpot).

Synthetic Rap1GAP2 (RG2) peptides covalently bound to cellulose membrane containing either wild-type EVTKTT sequence of Rap1GAP2 or with consecutive amino acid changed to alanine (A) or phosphorylated threonine residues (pT) as indicated were subjected to GST-Slp1 overlay assay followed by immunoblot analysis using anti-GST antibody.

E: Pull-down of endogenous Rap1GAP2 with GST-Slp1 from human platelet lysates in absence or presence of Rap1GAP2 peptides.

Human platelet lysate or lysate supplemented with Rap1GAP2 wild-type (RG2wt) peptide (amino acids 518-532 of Rap1GAP2) or Rap1GAP2 $\Delta$ EVTKTT (RG2 $\Delta$ EVTKTT) peptide (amino acids 515-535 of Rap1GAP2 lacking amino acids 522-527) in DMSO 100 $\mu$ M each were subjected to GST-Slp1 pull-down assays. The presence of endogenous Rap1GAP2 protein (RG2) was analyzed by immunoblot using anti-Rap1GAP2 antibody. The *upper panel* shows precipitation results, and the *lower panel* expression levels of endogenous Rap1GAP2 protein (total RG2, 2% input).

Figure 4: Rap1GAP2, Slp1 and Rab27 form a trimeric complex *in-vivo*.

A: Pull-down of endogenous Rab27 with GST-Slp1 from human platelets.

Human platelet lysate was subjected to GST-Slp1 pull-down assay followed by immunoblot analysis using anti-Rab27 antibody which recognizes both isoforms,

Rab27a and Rab27b. The *upper panel* shows precipitation results, and the *lower panel* expression levels of endogenous Rab27 protein (total Rab27, 2% input).

B: Co-immunoprecipitation of transfected Rap1GAP2 in complex with Slp1 and Rab27a.

HeLa cells were transfected either with VSV-tagged Rab27a alone, together with FLAG-tagged Rap1GAP2, or with FLAG-tagged Rap1GAP2 and myc-tagged Slp1. 24h post-transfection, cells were lysed, and Rap1GAP2 was immunoprecipitated using anti-FLAG antibody. The precipitates were analyzed for the presence of Rab27a using anti-VSV antibody. The *upper panel* shows precipitation results. Immunoglobulin heavy and light chains are marked with asterisks (\*). The *three lower panels* demonstrate expression levels of transfected Rab27a-VSV, Slp1-myc and Rap1GAP2-FLAG, each as 2% input.

C: Co-immunoprecipitation of endogenous Rap1GAP2 in complex with Slp1 and Rab27 from human platelets.

Human platelet lysate containing endogenous Rab27, Rap1GAP2 and Slp1 was subjected to co-immunoprecipitation using anti-Rab27a antibody. The precipitates were examined for the presence of Rap1GAP2 and Slp1 by immunoblot using specific anti-Rap1GAP2 and anti-Slp1 antibodies (*first and second panels from top*). Amounts of precipitated Rab27 were controlled with anti-Rab27a antibody (*third panel from top*). As indicated in A, the Rab27 band most likely consists of both isoforms, Rab27a and Rab27b. Precipitated Rab27 is marked by arrowhead, while asterisk (\*) indicates the immunoglobulin light chain. The *three lower panels* show expression levels of endogenous Rap1GAP2 (total RG2), Slp1 (total Slp1) and Rab27 (total Rab27), each as 2% input. Vertical lines have been inserted to indicate a repositioned gel lane in the first and second panels from top as well as in the bottom panel.

D: Co-localization of transfected Rap1GAP2, Slp1 and Rab27a.

Co-localization of EGFP-tagged Rab27a, VSV-tagged Rap1GAP2 and myc-tagged Slp1 overexpressed in HeLa cells was analyzed by immunofluorescence as described in Materials and Methods. Arrows indicate co-localization of all three proteins.

Figure 5: Slp1 inhibits platelet dense granule secretion in a dose-dependent manner.

A: Ca<sup>2+</sup>-induced dense granule secretion after incubation of permeabilized platelets with Slp1.

Permeabilized platelets were incubated with the indicated concentrations of purified recombinant His<sub>6</sub>-tagged Slp1 or 1 μM of boiled Slp1 and then stimulated with Ca<sup>2+</sup> for 1 min. For baseline serotonin secretion platelets were left unstimulated in absence or presence of Slp1. Baseline and Ca<sup>2+</sup>-induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and Methods. The results shown are expressed as means ± S.E.M. of three independent experiments performed in triplicate. *P* values are expressed as follows \* *p* < 0.05 and \*\*\* *p* < 0.001 and considered as statistically significant.

B: GTPγS-induced dense granule secretion after incubation of permeabilized platelets with Slp1.

Permeabilized platelets were incubated with the indicated concentrations of purified recombinant His<sub>6</sub>-tagged Slp1 or 1 μM of boiled Slp1 and then stimulated with GTPγS for 5 min. Baseline and GTPγS-induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and Methods. The results shown are expressed as means ± S.E.M. of three independent experiments performed in triplicate. *P* values are expressed as follows \* *p* < 0.05 and \*\*\* *p* < 0.001 and considered as statistically significant.

C: Ca<sup>2+</sup>-induced dense granule secretion after incubation of permeabilized platelets with the C2A domain of Slp1.

Permeabilized platelets were incubated with 1 μM of either GST as control, GST-Slp1, GST-C2A or with a combination of 1μM GST-Slp1 and 5μM GST-C2A and then incubated without or with Ca<sup>2+</sup> for 1 min. Baseline and Ca<sup>2+</sup>-induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and Methods. The results shown are expressed as means ± S.E.M. of six independent experiments performed in triplicate. *P* values are expressed as follows \* *p* < 0.05 and \*\* *p* < 0.01 and considered as statistically significant.

Figure 6: Rap1GAP2 enhances platelet dense granule secretion by binding to Slp1.

A: Ca<sup>2+</sup>- and GTPγS-induced dense granule secretion after incubation of permeabilized platelets with Rap1GAP2.

Permeabilized platelets were incubated without or with the indicated concentrations of purified recombinant His<sub>6</sub>-tagged Rap1GAP2 and then stimulated with Ca<sup>2+</sup> for 1 min or with GTPγS for 5 min. For baseline serotonin secretion platelets were left

unstimulated (-stim) in absence or presence of Rap1GAP2. Baseline and induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and Methods. The results shown are expressed as means  $\pm$  S.E.M. of three independent experiments performed in triplicate. *P* values are expressed as \*  $p < 0.05$  and considered as statistically significant.

B:  $\text{Ca}^{2+}$ - and GTP $\gamma$ S-induced dense granule secretion after incubation of permeabilized platelets with mutant Rap1GAP2 that is deficient in Slp1 binding.

Permeabilized platelets were incubated without or with 1 $\mu$ M of purified recombinant His<sub>6</sub>-tagged Rap1GAP2 $\Delta$ EVTKTT mutant which does not bind to Slp1. Then, platelets were stimulated with  $\text{Ca}^{2+}$  for 1 min or with GTP $\gamma$ S for 5 min. For baseline serotonin secretion platelets were left unstimulated (-stim) in absence or presence of Rap1GAP2 $\Delta$ EVTKTT. Baseline and induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and Methods. The results shown are expressed as means  $\pm$  S.E.M. of seven independent experiments performed in triplicate.

C:  $\text{Ca}^{2+}$ -induced dense granule secretion after incubation of permeabilized platelets with Rap1GAP2 peptides.

Permeabilized platelets were incubated with Rap1GAP2 wild-type peptide (RG2wt peptide) or Rap1GAP2 peptide lacking the Slp1-binding TKXT motif (RG2 $\Delta$ EVTKTT) at 100 $\mu$ M each. The solvent DMSO was used as control. Baseline and  $\text{Ca}^{2+}$ -induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and Methods. The results shown are expressed as means  $\pm$  S.E.M. of five independent experiments performed in triplicate. *P* value is expressed as \*  $p < 0.05$  and considered as statistically significant.

Figure 7: Slp1 and Rap1GAP2 effects on platelet secretion are not mediated by Rap1.

A:  $\text{Ca}^{2+}$ -induced dense granule secretion after incubation of permeabilized platelets with Rap1.

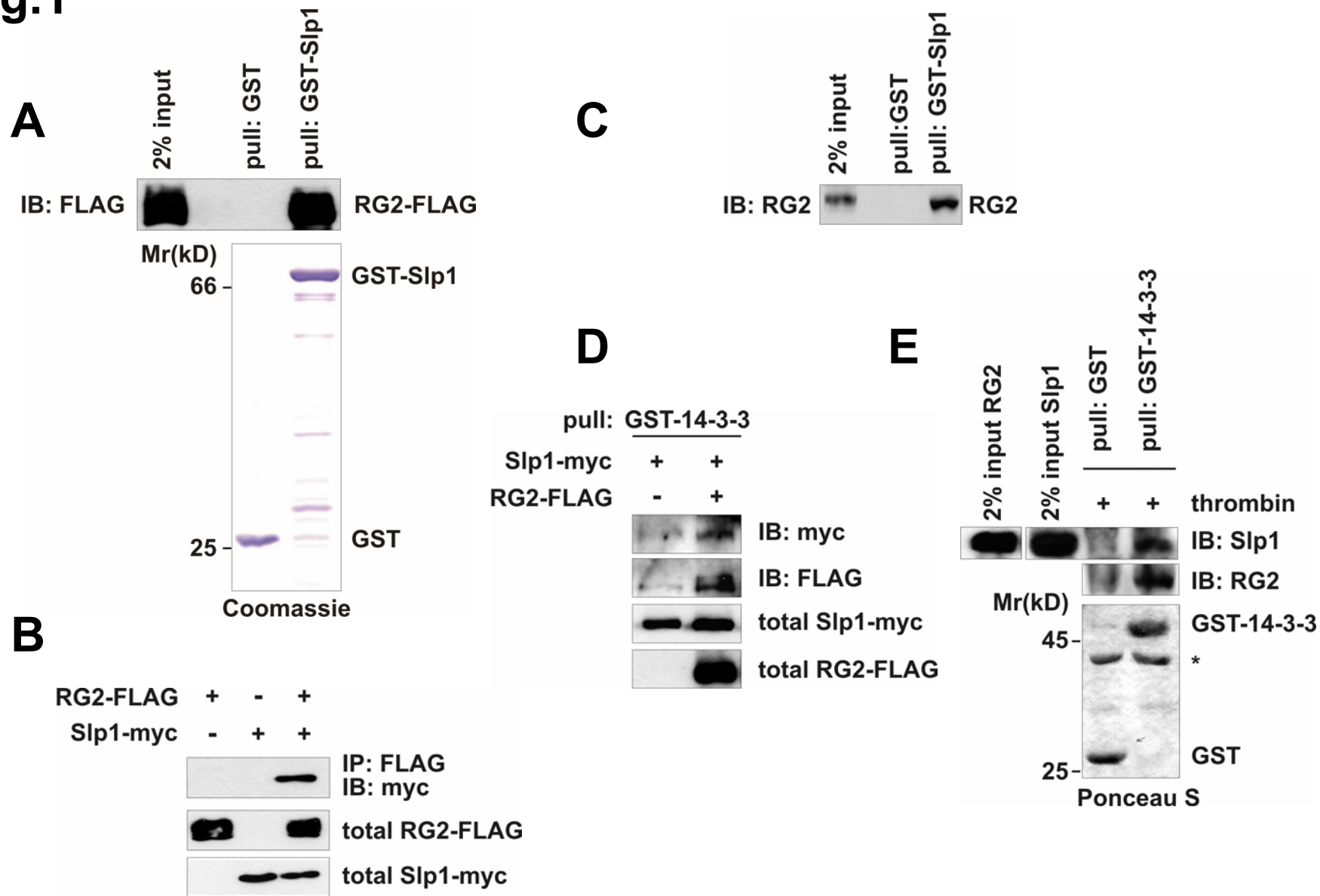
Permeabilized platelets were incubated with 1 $\mu$ M of either BSA as control or purified recombinant native Rap1b or Rap1b loaded with GTP. Then, platelets were stimulated with  $\text{Ca}^{2+}$  for 1 min. Baseline and  $\text{Ca}^{2+}$ -induced secretion of dense granules were analyzed by measuring released serotonin (5HT) as described in Materials and

Methods. The results shown are expressed as means  $\pm$  S.E.M. of five independent experiments performed in triplicate.

B: *In vitro* GAP assay

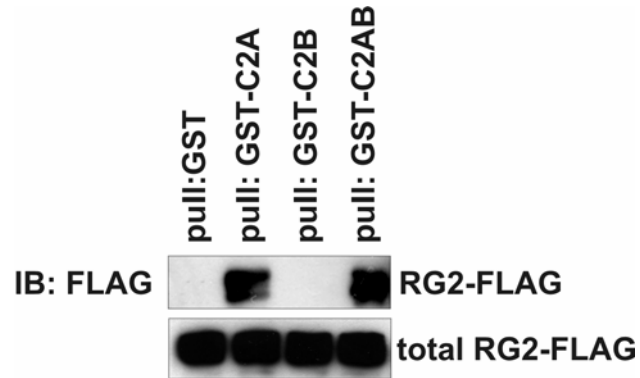
Epitope-tagged Slp1, Rap1GAP2 and Rap1GAP2 in complex with Slp1 were expressed in HeLa cells and purified using tag-specific affinity agarose. In parallel, His<sub>6</sub>-tagged Rap1b was purified from *E.coli* and loaded with [<sup>32</sup>P]-GTP as described in Materials and Methods. Precipitated Slp1 and Rap1GAP2 proteins were added to the GTP-loaded Rap1b and reactions were incubated at 25°C. Aliquots were removed at indicated time points, amounts of released [<sup>32</sup>P] were determined by liquid scintillation counting and plotted as percentage of input Rap1b-bound [<sup>32</sup>P]-GTP counts. Shown data represent the means  $\pm$  S.E. of three independent experiments performed in triplicate.

# Fig.1

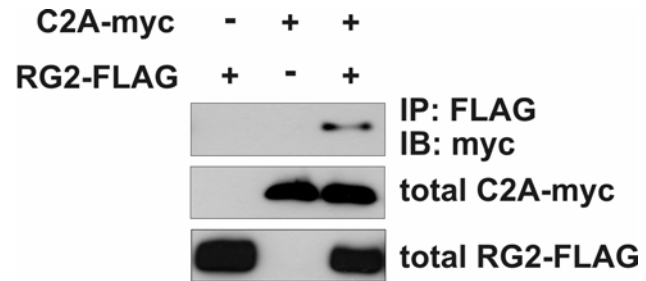


# Fig.2

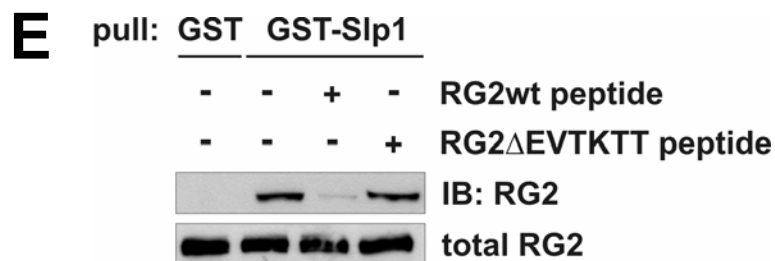
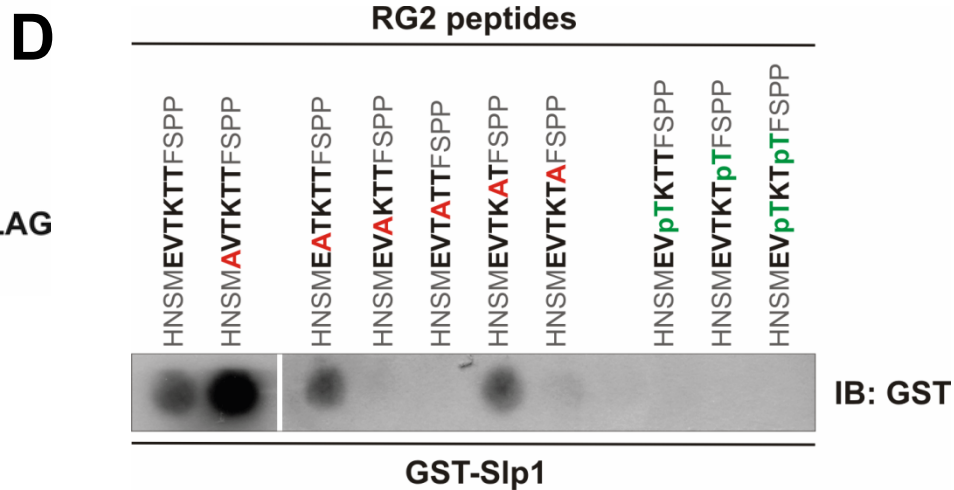
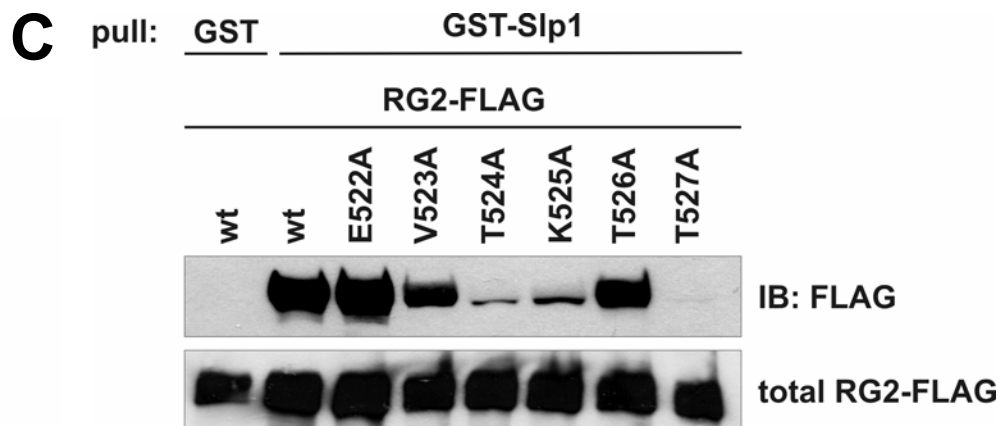
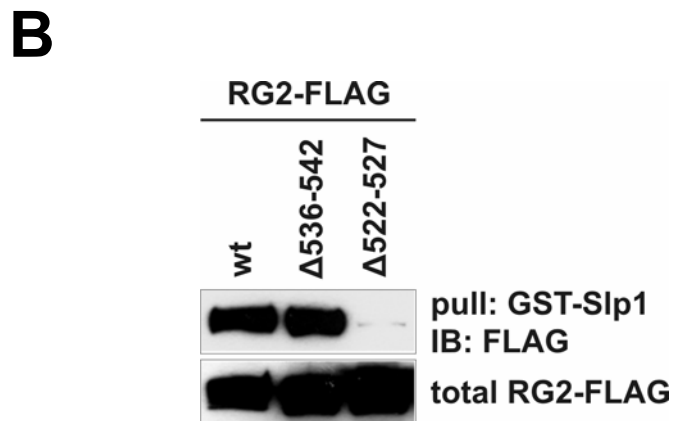
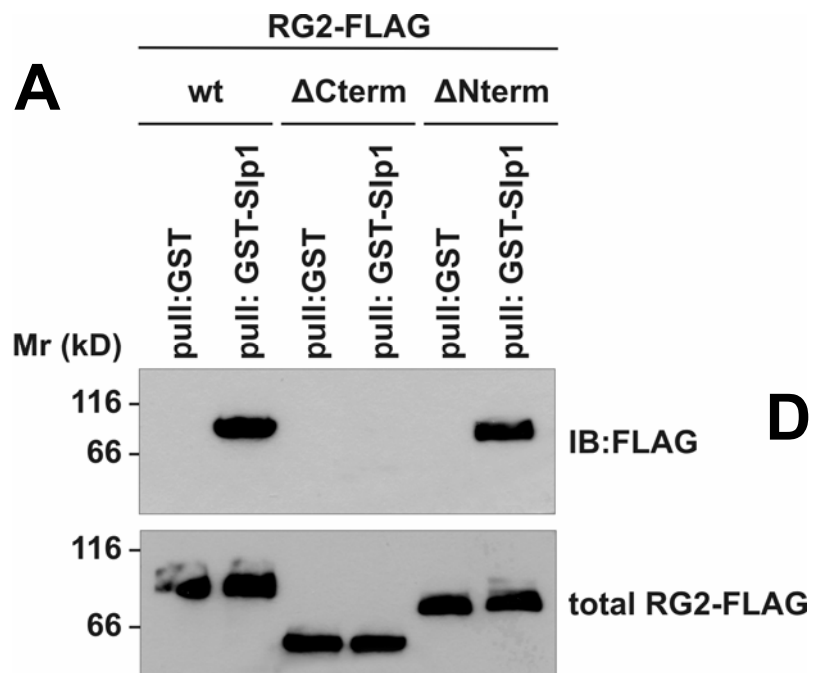
## A



## B

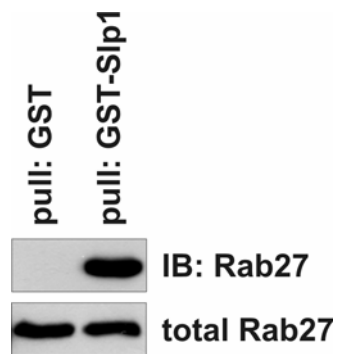


# Fig.3

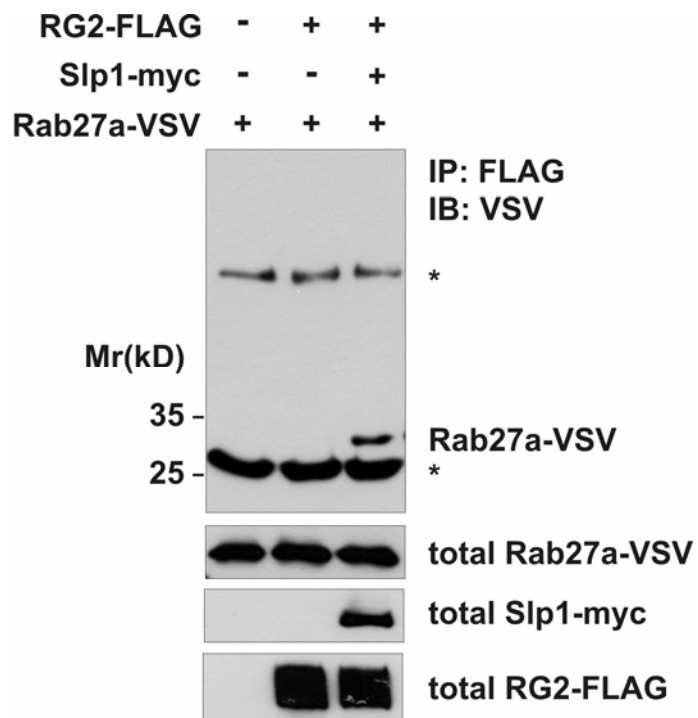


# Fig.4

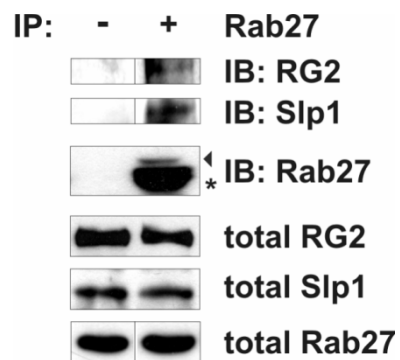
## A



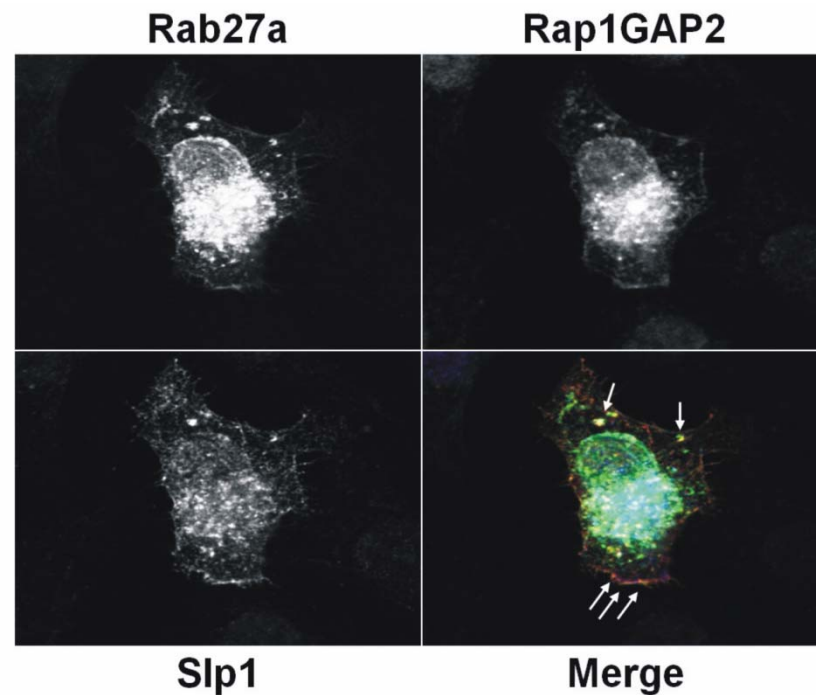
## B



## C

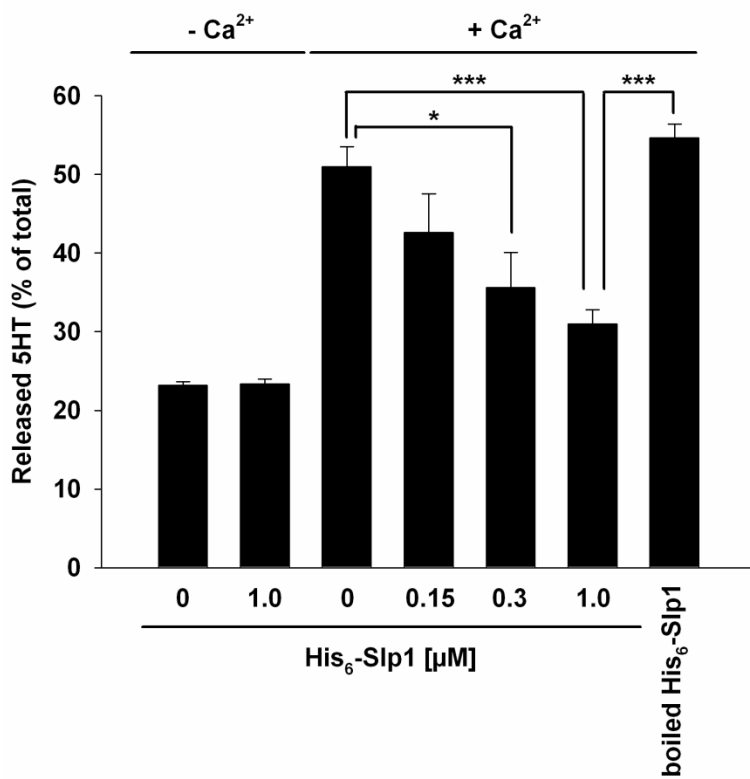


## D



# Fig.5

## A



## B

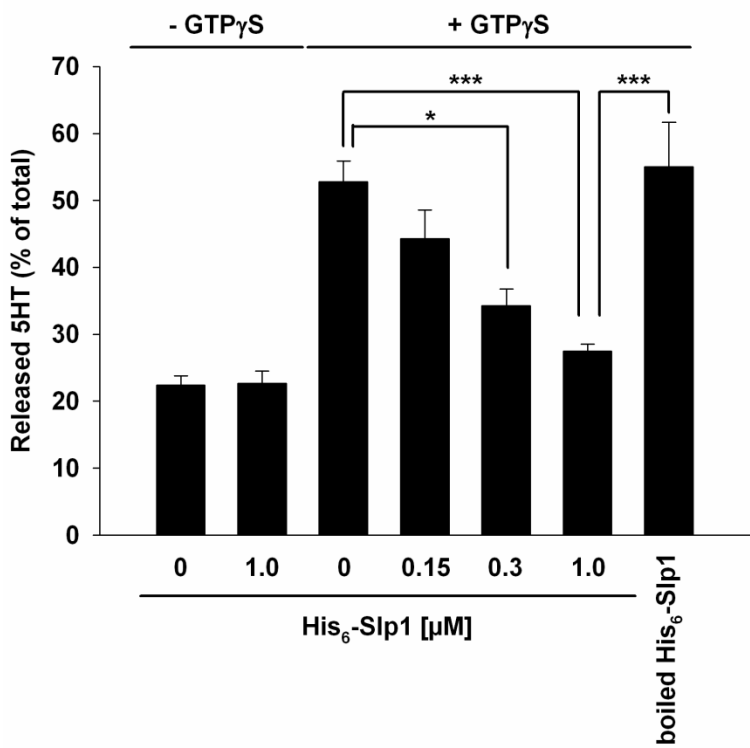
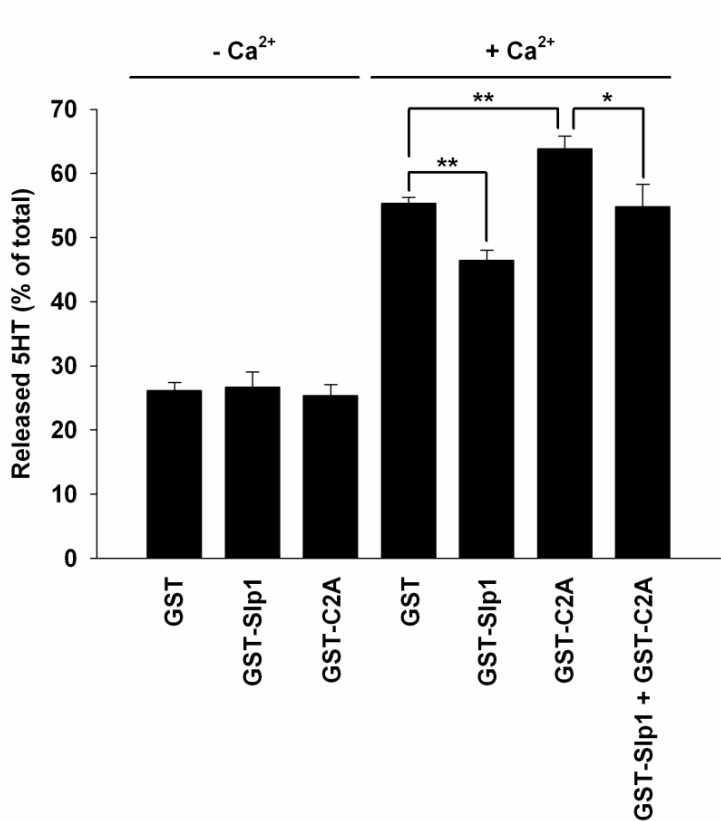


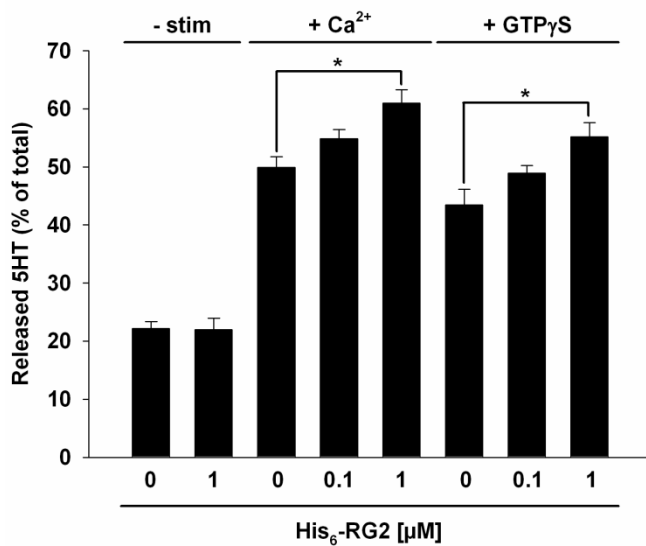
Fig.5

C

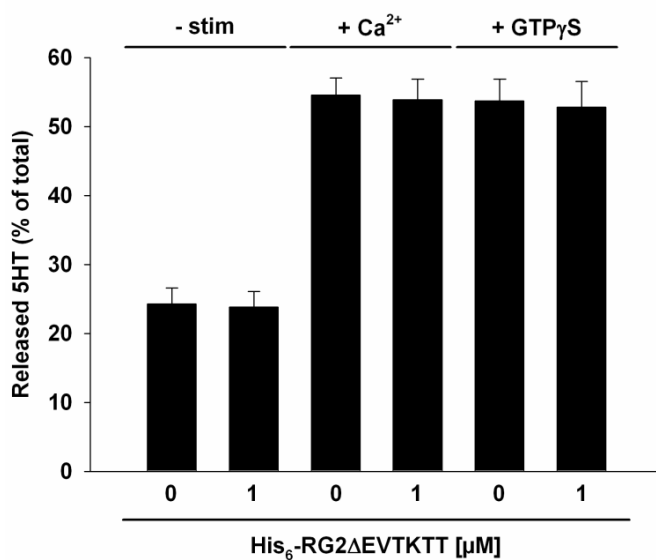


# Fig.6

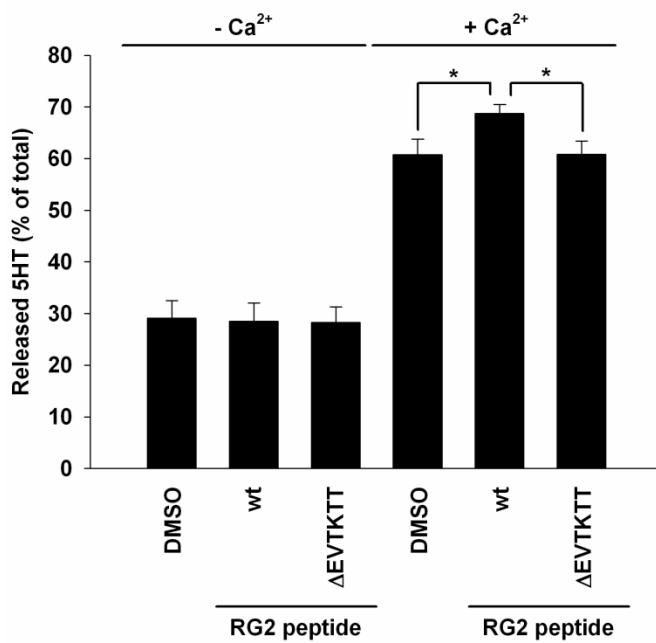
## A



## B

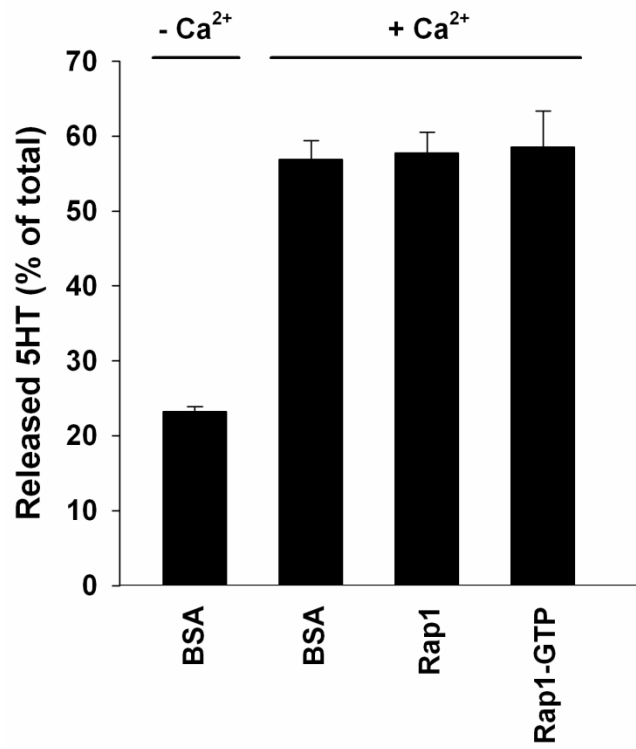


## C



**Fig.7**

**A**



**B**

