

The phosphatidylinositol transfer protein, RdgB β binds 14-3-3 via its unstructured C-terminus whereas its lipid binding domain interacts with the integral membrane protein, AGTRAP (Angiotensin II Type I Receptor-associated Protein)

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Short Title: RdgB β -sp1 forms a complex with 14-3-3 and AGTRAP

Abbreviations: ATRAP (alt. name AGTRAP), angiotensin II receptor-associated protein; BIM-I, bisindolylmaleimide I; CHX, cyclohexamide; DIG, digoxigenin; PC, phosphatidylcholine; PI, phosphatidylinositol; PI(4,5)P₂, phosphatidylinositol (4,5) bisphosphate; PITP, phosphatidylinositol transfer protein; RdgB, retinal degeneration type B; DCC, deleted in colorectal cancer; PMA, phorbol myristate acetate;

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SYNOPSIS

Phosphatidylinositol transfer proteins (PITPs) bind and transfer phosphatidylinositol between intracellular membranes and participate in many cellular processes including signalling, lipid metabolism and membrane traffic. The largely uncharacterised PITP, RdgB β (PITPNC1), contains a long C-terminal disordered region following its defining N-terminal PITP domain. We report that the C-terminus contains two tandem phosphorylated binding sites (Ser274 and Ser299) for 14-3-3. The C-terminus also contains PEST sequences which are shielded by 14-3-3 binding. Like many proteins containing PEST sequences, the levels of RdgB β are regulated by proteolysis. RdgB β is degraded with a half-life of 4hrs following ubiquitination via the proteasome. A mutant RdgB β which is unable to bind 14-3-3 is degraded even faster with a half-life of 2hrs. *In vitro*, RdgB β is 100-fold less active than PITP α for PI transfer and RdgB β proteins (wild-type and a mutant that cannot bind 14-3-3) either expressed in COS-7 cells or endogenous proteins from heart cytosol do not exhibit transfer activity. When cells are treated with PMA, the PITP domain of RdgB β interacts with the integral membrane protein, ATRAP (Angiotensin II receptor-associated protein; Alt name: AGTRAP) causing membrane recruitment. We suggest that RdgB β executes its function following recruitment to membranes via its PITP domain and the C-terminal end of the protein could regulate entry to the hydrophobic cavity.

INTRODUCTION

Phosphoinositides play diverse roles in cells and participate in many aspects including signalling, cytoskeletal regulation, ion channel regulation and membrane traffic. Phosphatidylinositol transfer proteins (PITPs) have emerged as key regulators that interface the spatial distribution of phosphatidylinositol coupled with its conversion to seven phosphorylated derivatives [1,2]. PITPs are a family of lipid-binding proteins that transfer phosphatidylinositol (PI) between membrane compartments. Originally identified as soluble proteins of approximately 35 kDa [3], the family of PITP-related proteins has subsequently grown to five members subdivided into the Class I PITPs, α and β (35kDa) and the Class II RdgB proteins based on the sequence of the PITP domain [4-6]. The defining feature of a member of the PITP family is the presence of an N-terminal PITP domain. The class I PITPs, PITP α and PITP β , are best characterised; PITP α is enriched in neurons and is required for axonal outgrowth [7,8] whilst PITP β functions in retrograde transport from the Golgi to the endoplasmic reticulum by COPI-containing vesicles [9]. The founding member of the Class II proteins is the *Drosophila melanogaster* (Dm)-RdgB α [10]. The RdgB proteins are named retinal degeneration type B, after the phenotype observed upon disruption of this gene in flies: impairment of visual transduction coupled with retinal degeneration [11]. There are two RdgB α isoforms in humans, RdgB α I (alt. name Nir2/PITPNM1) and RdgB α II (alt name Nir3/PITPNM2). RdgB α proteins are multi-domain proteins that, in addition to the PITP domain, possess a FFAT motif that anchors the protein to the endoplasmic reticulum by binding to VAP, a DDHD metal-binding domain of 180 residues and a LSN2 domain of 130 amino acids, both of unknown function. Individual members of the PITP family are likely to be involved in specific functions as distinctive phenotypes are observed when their genes are ablated in model organisms including mice. In *vibrator* mice, the 80% reduction in PITP α levels leads to neurodegeneration followed by juvenile death, whilst ablation of the PITP α gene results in a more severe phenotype where death occurs within days after birth [12,13]. In contrast, ablation of the gene for PITP β or for RdgB α I results in embryonic lethality [14,15].

Comparatively little is known about the smaller, soluble RdgB β protein. Two splice variants of RdgB β have been described; a long splice variant, referred throughout this paper as RdgB β (-sp1) (332 amino acids, 38kDa) translated from an mRNA comprised of exons 1-8 and 10 and the short splice variant, sp2 (268 amino acids, 32kDa) translated from an mRNA comprised of exons 1-9 [16,17]. Expression of RdgB β has been examined at the mRNA level and transcripts are enriched in the heart, muscle, kidney, liver, and peripheral blood leukocytes,

brain and testes [16,17]. However, the presence of the endogenous protein has mainly been described from high-throughput proteomic screens [18-22].

The PITP domain of human RdgB β is 41% identical to PITP α and retains all the key residues that are essential for PI binding [23]. The Class I PITPs are thought to interact with and dock onto cell membranes via residues following the G-helix at the C-terminus of the protein [24,25]. Interestingly, the main source of variation between the PITPs occurs at this C-terminal region. The C-terminus of PITP α interacts with DCC (deleted in colorectal cancer), the receptor for netrin-1 whilst the C-terminus of PITP β determines Golgi localisation [7,26]. We therefore set out to examine whether the C-terminus of RdgB β could interact with other proteins. It had been previously reported that RdgB β could interact with the angiotensin II type I receptor-associated protein (AGTRAP, alt. name ATRAP), in a genome wide yeast two-hybrid screen for interacting partners [27,28].

In this study we identify that RdgB β binds directly to 14-3-3 proteins and to ATRAP. 14-3-3 binds to two phosphorylated serine residues in the C-terminal region whilst ATRAP binds to the PITP domain of RdgB β , but only following treatment of cells with PMA. We also demonstrate that RdgB β is subject to rapid turnover in cells, is ubiquitinated and degraded at the proteasome. Comparison of the *in vitro* phosphatidylinositol transfer activity between recombinant PITP α and RdgB β proteins show that the concentration required for RdgB β proteins to show activity is 100-fold greater than that for PITP α . Thus examination of the phosphatidylinositol transfer activity of endogenous or over-expressed RdgB β (wild-type and mutants that cannot bind 14-3-3) from cellular extracts demonstrates no activity. We propose that RdgB β has to be recruited to membranes by ATRAP via its PITP domain to enable lipid exchange to take place at the adjacent membrane at membrane contact sites.

EXPERIMENTAL

Antibodies

The following antibodies were obtained from Santa Cruz Biotechnology: pAb: pan 14-3-3 (K-19) (sc-629); pAb: FLAG (OctA-Probe (D-8)) (sc-807); and mAb: HA (HA-Probe (F-7)) (sc-7392). The mAb: FLAG (DDK) was obtained from OriGene Technologies, (TA50011) and pAb: Ubiquitin (U5379) from Sigma-Aldrich. PITPNC1 polyclonal antibody was obtained from ORBIGN (PAB-02250) and ATRAP (also referred to as AGTRAP) monoclonal antibody (ab57555) was obtained from Abcam. Antibodies to ARF1 (pAb:678), PITP β (mAb:1C1) and PITP α (pAb:674) were made in-house and described previously [29-31].

Mammalian and bacterial expression plasmids

pEFPLink2-FLAG-RdgB β (human) was a generous gift from J. Hsuan [16], and was used as a template to clone RdgB β into pRSET-C-His expression vector (Invitrogen) for bacterial expression using the *Xho*I/*Eco*RI restriction sites, and into pIRES2 EGFP bicistronic vector (BD Bioscience Clontech) for mammalian expression of the untagged protein. The RdgB β -sp2 IMAGE clone cDNA (IMAGE: 4299595) (human) was obtained from MRC geneservice and was cloned into the pRSET-C-His vector for bacterial expression using the *Xho*I/*Eco*RI restriction sites. The human sequences of both RdgB β and RdgB β -sp2 were amplified by PCR from the pRSET-C vectors with the addition of an N-terminal FLAG tag, introduced from the forward primer, and cloned into the pcDNA3.1(-) vector for mammalian expression. RdgB β -sp2 was also cloned into pcDNA3.1(-) without the addition of a protein tag, using the *Xho*I/*Hind*III restriction sites. Point mutations were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene): pRSET-C-RdgB β -sp1 1-263 (mutation of L264 to a stop codon, L264_STOP), pcDNA3.1-FLAG-RdgB β S274A, S299A and SS274/299AA, pcDNA3.1-FLAG-RdgB β 1-251 (I252_STOP). All constructs were sequenced for verification. pcDNA3-HA-ATRAP (mouse) was a generous gift from M. Lopez-Illasaca [32].

Purification of recombinant proteins expressed in *Escherichia coli*

His-tagged RdgB β , RdgB β -sp2, truncated RdgB β (residues 1-263), PITP α and PITP β proteins were expressed in the *E. coli* strain, BL21(DE3)pLysS and purified using HIS-Select Nickel Affinity gel as described previously [33]. The recombinant proteins were desalted into PIPES buffer (20mM PIPES, 137mM NaCl, 3mM KCl, pH 6.8), analysed by SDS-PAGE for purity; the protein concentration was adjusted accordingly and the proteins stored at -80°C.

Production and validation of RdgB β -specific polyclonal antisera (Ab:101)

Antibodies were raised in two rabbits using two internal peptides (TKYEDNKGSNDTIFD (residues 110-125) and ACDETIPERYK (residues 140-151) common to both rodent and human RdgB β splice variants, sp1 and sp2. Peptide synthesis and immunization was performed by Eurogentech. For validation, three different siRNAs (1 plus 5, and 6 alone) were used in two independent combinations exactly as described except that a single round of knockdown was carried out [9]. The siRNA sequences against human and monkey RdgB β were: oligonucleotide no. 1: 5'-GGAUUUGGAGCCUAAUUAATT-3'; oligonucleotide no. 5: 5'-CACCGUAGACGAGUACAATT-3'; oligonucleotide no. 6: 5'-GAGCGCUACUACAAAGAAUTT-3' and were obtained from QIAGEN.

Trypsin digest of RdgB β proteins

100 μ g RdgB β or RdgB β -sp2 recombinant protein (in 500 μ l) was incubated with 5 μ l trypsin-EDTA solution (Sigma T3924) at 37°C. 50 μ l aliquots were removed directly into SDS-PAGE sample buffer at each time point. For the Coomassie-stained gel, 2 μ g protein was loaded per lane; for the western blot 30ng was loaded per lane. Ab:101 was used to detect the trypsin-digested proteins. Transfer activity was measured using 200 μ g protein /ml as described below.

Culture and electroporation of COS-7 cells, and fractionation of cytosol by size exclusion chromatography

COS-7 cells, cultured in DMEM medium supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 0.5 iu/ml penicillin, 50 μ g/ml streptomycin, were electroporated with the appropriate plasmid as described previously [25]. 48 hrs later the cells were trypsinised, washed and re-suspended in 240 μ l SET buffer (0.25M sucrose, 1mM EDTA, 10mM Tris-HCl pH 7.4) with protease inhibitor cocktail (Sigma-Aldrich, P8340). To harvest the cytosol, the cells were sonicated 3 x 15s followed by ultracentrifugation at 110,000 x g for 60 mins at 4°C. The cytosol was decanted, re-centrifuged at 15,000 x g for 10 min and 200 μ l (16mg protein) was immediately loaded on a Superose 12 10/300 column; bed volume 24ml (GE Healthcare). The column was calibrated using a kit containing proteins of molecular weight 200, 67, 43, 25 and 13.7 kDa and their elution profile is indicated in Fig. 5A. For the recombinant proteins, 200 μ g (diluted in SET buffer to 200 μ l) was loaded onto the column. The column was eluted with PIPES buffer (20mM PIPES, 137mM NaCl and 3.7mM KCl, pH 6.8) and 0.5ml fractions were collected. 50 μ l of each fraction for the COS-7 cytosol, or 3 μ l of each fraction for the recombinant protein was used for the western blot using Ab:101. To obtain rat heart cytosol, a heart was perfused with saline to remove the red blood cells and the tissue chopped finely in 600 μ l SET buffer and homogenised with a dounce homogeniser. After 10 strokes, the homogenate was transferred into a 15 ml tube, sonicated 3x 15s and cytosol was obtained as described above.

Preparation of membranes and cytosol

COS-7 cells were electroporated with the appropriate plasmids. After 24 hrs, the cells were treated with PMA (100nM) as indicated. 16 hrs later, the cells were trypsinised, washed and re-suspended in 300 μ l PBS in the presence of a protease inhibitor cocktail (Sigma). To prepare membranes and cytosol, the cells were sonicated on ice (50 microns, 3 x 15sec) and centrifuged for 10 min (1,800 x g, 4°C) to pellet the nuclei and unbroken cells. The lysate was centrifuged for 60 min at 110,000 x g at 4°C to pellet the membranes and the supernatant consisting of the cytosolic fraction was retained for further analysis. To remove contaminating

cytosolic proteins, the membranes were resuspended in 1 ml of SET buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4), and centrifuged at 110,000 x g (4°C, 60 min). The membranes were resuspended in 100 µl of SET buffer or in PBS. Protein concentrations were determined for both the membrane and cytosolic fractions. The distribution of proteins between membranes and the cytosol was analysed through separation of the proteins by SDS-PAGE followed by Western blot analysis.

Immunoprecipitation

COS-7 cells were seeded in 10cm cell culture dishes in fresh media at $1.0\text{-}2.0 \times 10^5$ cells/ml. Transfection was carried out 6hrs after seeding using FuGENE HD reagent (Roche Diagnostics) following the manufacturer's instructions (DNA:FuGENE HD ratio 2:6). Cells were harvested on ice 48hr after seeding in 1ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 1% Triton X-100) with protease and phosphatase I and II inhibitors (Sigma-Aldrich, P8340, P2850 and P5726, respectively). The cells were sonicated and lysates obtained after centrifugation (15,000 x g, 15min 4°C). The BCA assay was used to determine lysate protein concentration. For immunoprecipitation, 300µg of protein was incubated with 40µl equilibrated Anti-FLAG M2 affinity gel (Sigma-Aldrich, A2220) on a rotating wheel for 2hr at 4°C. The tubes were centrifuged at 15,000 X g for 2min and the supernatant discarded. The beads were washed with lysis buffer three times; the final wash was carried out in a fresh Eppendorf tube. After the final centrifugation the supernatant was aspirated, replaced with 20µl 2x SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE gel electrophoresis and then transferred to nitrocellulose membrane for western blot analysis using appropriate primary antibodies. For the protein kinase inhibitor treatments, the culture media was replaced with fresh media containing the appropriate inhibitor at the concentrations indicated 16 hrs prior to cell harvest: H-89 (PKA inhibitor), LY294002 (PI3K inhibitor), BIM-1 (Bisindolylmaleimide, alt. name GF 109203X) (PKC inhibitor) (Calbiochem, 371963, 440206 and 203290, respectively), and PMA (Sigma-Aldrich, P8139). For treatment with the proteasome inhibitor, MG-132 (Z-leu-leu-leu-al) (Sigma C2211) cells were treated with 20µM MG-132 for 4 hrs.

14-3-3 far western assay for direct binding

This was carried out exactly as described [34]. In brief, FLAG-immunoprecipitates and recombinant RdgBβ proteins were subjected to SDS-PAGE gel electrophoresis. The proteins were transferred to nitrocellulose membrane which was then blocked for 1hr in 5% BSA TBS-T. The membrane was washed and incubated with *in vitro* DIG-labelled BMH1 and BMH2 *S. cerevisiae* 14-3-3 isoforms in 5% BSA TBS-T for 2hrs RT. The membrane was washed again then finally incubated with anti-DIG-horseradish peroxidase for 45mins before detection of the bound 14-3-3 protein with ECL (GE Healthcare). 14-3-3 far western reagents were a generous gift from C. MacKintosh (MRC Protein Phosphorylation Unit, Dundee).

Cyclohexamide treatment

COS-7 cells were electroporated with pcDNA3.1-FLAG-RdgBβ WT or S299A mutant plasmids and divided into 16 x 3.5cm cell culture dishes (2ml per dish). 48hrs after transfection the media was removed and replaced with fresh media containing 100µg/ml cyclohexamide (CHX). After 0, 2, 4 or 6 hrs the media was removed and the cells washed twice in PBS. The cells were harvested in 300µl RIPA buffer (150mM NaCl, 1% (v/v) Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50mM Tris-HCl pH 7.5) and incubated on ice for 30mins. The tubes were centrifuged 15,000 x g for 15mins at 4°C. The supernatant was transferred to a fresh tube. The protein concentration of the lysate was determined using the BCA assay. 50µg protein was boiled with SDS-PAGE sample buffer and loaded per lane of the gel. Proteins were transferred to nitrocellulose membrane and probed with anti-FLAG mAb DDK. Membranes were stripped and re-probed for endogenous PITPβ.

Assay for PI transfer activity

PI transfer activity was assayed by measuring the transfer of [³H]PI from radiolabelled rat liver microsomes to unlabelled synthetic liposomes (PC:PI 98:2 molar ratio) by recombinant proteins or fractions (100 μ l), as described previously [35]. Transfer activity was calculated as a percentage of the total radioactivity present in the assay after subtraction of the number of counts transferred in the absence of a PITP source. For the recombinant proteins, transfer activity was monitored in duplicate samples; for fractions obtained after size exclusion chromatography individual fractions were analysed singly. All data presented are representative of at least three independent experiments, and for the recombinant proteins, at least two protein preparations.

Localisation of RdgB β and ATRAP by immunofluorescence

COS-7 cells were co-transfected with FLAG-tagged RdgB β and HA-tagged ATRAP using Fugene-HD and were cultured on glass cover slips for 48 hrs. Cells were treated with 100nM PMA for 4 hrs. The cells were washed twice with PBS and were fixed with 4% paraformaldehyde and subsequently permeabilised with digitonin (40 μ g/ml) for 10 min on ice and washed with cold PBS. The cells were incubated with primary antibodies (MAb: HA for ATRAP and pAb: OctA (FLAG) for RdgB β as indicated followed by fluorescent (Alexa fluor 488 (ATRAP) or 546 (RdgB β)) conjugated secondary antibodies (Molecular Probes). Fluorescence was recorded by excitation at 488 nm or 546 nm with a light source (Excite 120) using an Olympus IX80 microscope fitted with a x100 oil immersion objective. Images were acquired with a charge-coupled device camera (ORCA) cooled to -35°C and controlled with the CellF software (Olympus).

RESULTS

Characterisation of a polyclonal antibody (Ab:101) to RdgB β

A polyclonal antibody made against two internal peptides of RdgB β detects both splice variants including a C-terminal truncated protein of the long splice variant (residues 1-263). The antibody does not cross-react with PITP α , PITP β or to RdgB α PITP domain (Fig. 1A, *left panel*). When COS-7 cell lysates are probed with the antibody, no band corresponding to either splice variant of RdgB β is seen (Fig. 1A, *middle panel*). However, the antibody detects RdgB β when over-expressed (indicated by an arrow). The antibody also recognises an endogenous protein present as a doublet band at 35 kDa; this protein is not susceptible to RNAi indicating that it is a cross-reactive band. We also examined the detection level by titration of recombinant RdgB β proteins and show that the antibody could just detect \sim 5ng and above (Fig. 1A, *right panel*).

Bioinformatic analysis shows that the RdgB β C-terminus is a low complexity region susceptible to proteolytic cleavage.

Analysis of the sequence of RdgB β indicates that it has an N-terminal PITP domain (Pfam: IP_trans), which shares 41% identity with PITP α . PITP α , RdgB β and RdgB β -sp2 differ mainly at their C-termini (Fig. 1B). The N-terminal PITP domain of PITP α and PITP β is a compact structure containing a lipid-binding cavity. It consists of eight β -strands which form a large concave sheet, flanked by two long α -helices [24,25]. This cavity is closed by a 'lid' composed of a C-terminal α -helix (G-helix) and an 11 amino acid extension in PITP α and PITP β (Fig. 1B). Movement of the 'lid' is required for lipid exchange [25]. The putative G-helix in RdgB β is indicated by a dotted line (Fig. 1B). Whilst the RdgB β -sp2 C-terminal extension is similar in length to that of PITP α , the RdgB β C-terminus is extended by \sim 60 amino acid residues (Fig. 1B).

To understand more about the C-terminus of RdgB β , the human sequence (accession number: Q9UKF7) was uploaded to the DISOPRED2 Disorder Prediction Server [36]. This identified a region of disorder beginning at residue 252 in RdgB β and continuing to the extreme C-terminus of the protein (332aa). To confirm this experimentally the recombinant RdgB β protein was incubated with trypsin alongside RdgB β -sp2 as a control, and aliquots of the

recombinant protein removed at specified time points for analysis by Coomassie-stained SDS-PAGE gel and by western blot (Fig. 1C). RdgB β -sp2 has a much shorter C-terminus than RdgB β (16 residues compared to 80 residues following the compact domain predicted by DISOPRED2). Consistent with the absence of trypsin cleavage sites in the C-terminus of RdgB β -sp2 after residue 252 (Fig. 1B), sp2 is resistant to trypsin cleavage (Fig. 1C). This, together with earlier observations of trypsin digestion of PITP α where the compact PITP domain was found to be resistant to proteolytic cleavage [37,38], indicates that the stable product (~37kDa) reached by 10 minutes in the trypsin digestions shown in Fig. 1C is the compact PITP domain. In support of this, the trypsinized protein exhibits PI transfer activity comparable to the untreated protein in the *in vitro* assay (Fig. 1C).

Further bioinformatic analysis of the C-terminus of RdgB β indicates the presence of two 'PEST' sequences (Fig. 1B, denoted by ***). PEST sequence analysis was performed using the pestfind algorithm on the Emboss server (<http://emboss.bioinformatics.nl/>). The PEST sequence motif was first identified in 1986 as a proteolytic signal region [39,40]. Such regions are rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues and are most common in rapidly-degraded proteins.

RdgB β contains a 14-3-3 protein binding site at its C-terminus

Large scale phosphoproteomic studies have reported that the C-terminus of RdgB β is phosphorylated *in vivo* at Ser274 and Ser299 (Fig. 1B). Phosphoproteomic analysis of the developing mouse brain (fore-brain and mid-brain from E16.5) [18] and HeLa cells in G1 phase of the mitotic cycle identified Ser274 as being phosphorylated [19]. Ser299 was found to be phosphorylated in human embryonic stem cells [20], following receptor tyrosine kinase stimulation of cancer cell lines (H1703, H3255 and MKN45 cells) by EGF, PDGF and c-Met [21], after stimulation of signalling by the oncogenic mutant of Flt3 [22], as well as in Jurkat cells treated with phosphatase inhibitors calyculin and pervanadate (observed on more than 25 separate occasions) (PhosphoSitePlus, Cell Signalling Technology) [41].

A survey of the sequence surrounding pSer299 using the cell signalling interaction prediction program Scansite at high stringency [42], and the PhosphoMotif Finder tool at the Human Protein Reference Database [43], indicate that this region is a consensus binding site for 14-3-3 proteins. Two modes of 14-3-3 binding have previously been identified [44,45]; the region surrounding Ser299 conforms to the mode I site RSX(pS)XP (where pS is the phosphorylated serine residue), with a lysine residue in place of the first serine in RdgB β (Fig. 1B). Interestingly this is very similar to the region surrounding Ser274, which conforms to the sequence RSXXX(pS)XP. 14-3-3 proteins form dimers, with each monomer providing one phospho-serine-binding site. In this way 14-3-3 proteins have been shown to bind two tandem sites on a single target protein resulting in strong 'bidentate' binding [44-46]. Ser274 and Ser299 could form two such 14-3-3-binding sites on RdgB β . RdgB β -sp2 lacks these residues and so is not predicted to bind 14-3-3 proteins.

To examine whether RdgB β is present as a complex with 14-3-3 proteins *in vivo*, COS-7 cells were transfected with FLAG-tagged RdgB β . RdgB β was immunoprecipitated via its FLAG tag and western blotted using a pan 14-3-3 antibody (Fig. 2A, *top right*). In addition, the immunoprecipitate was probed with 14-3-3 by far western blotting in which recombinant 14-3-3 proteins with a DIG tag were incubated with the membrane and 14-3-3-binding revealed using anti-DIG Fab fragments conjugated with horse-radish peroxidase (Fig. 2A, *middle right*). Both membranes were stripped and re-probed with anti-FLAG antibody to confirm the presence of FLAG-RdgB β . Only one is shown (Fig. 2A, *bottom right*). These experiments show that 14-3-3 is present in the FLAG-RdgB β immunoprecipitate, and that binding to 14-3-3 is a direct interaction.

We hypothesized that the recombinant RdgB β (purified after expression in *E. coli*) would not bind 14-3-3 proteins as it lacks the phosphorylation required for 14-3-3-binding. FLAG-RdgB β immunoprecipitate was used as a positive control; recombinant RdgB β , RdgB β -sp2 and RdgB β truncated (residues 1-263) were analysed alongside and were all subjected to 14-3-3 far western blotting (Fig. 2B). The recombinant 14-3-3 only binds the COS-7 cell-expressed RdgB β

which has had the opportunity to be phosphorylated, not to recombinant RdgB β lacking the phosphorylation nor RdgB β -sp2 or RdgB β (1-263) which lack the consensus 14-3-3-binding sites.

To confirm the residues responsible for binding to 14-3-3, serine-to-alanine point mutations of Ser274 and Ser299 were constructed (S274A, S299A and SS274/299AA double mutant) and expressed in COS-7 cells. Immunoprecipitation of wild-type and mutant RdgB β revealed that mutation of either Ser274 or Ser299 abolished binding to 14-3-3 (Fig. 3A). We conclude that 14-3-3 needs to bind both pSer274 and pSer299 for stable association.

The consensus sites for 14-3-3-binding show high similarity to the consensus sequences for phosphorylation by the basophilic kinases PKA, PKB/Akt and PKC [44]. To establish which kinase may be responsible for either of the serine phosphorylations we treated COS-7 cells expressing FLAG-RdgB β for 16 hrs with inhibitors of these kinases (H89 for PKA inhibition, LY294002 for inhibition of PKB indirectly through PI3K, and BIM-I for inhibition of PKC). Only BIM-I causes a reduction in 14-3-3 binding (Fig. 3B) suggesting that PKC could be responsible for one or both of the serine phosphorylations, pSer274 and pSer299.

A reduction of RdgB β expression is observed in the lysates of BIM-treated cells (Fig. 3B) suggesting that 14-3-3 binding could protect RdgB β from degradation. The C-terminal region of RdgB β is unstructured and contains two PEST sequences (Fig. 1B) suggesting that the protein could be subject to rapid turnover. Moreover, binding to 14-3-3 would shield the PEST sequence and may provide protection. We therefore examined the rate of wild type RdgB β and S299A mutant degradation after treatment with the protein synthesis inhibitor, cyclohexamide (Fig. 4A). Wild-type RdgB β is reduced by half within 4 hrs whilst the mutant protein that cannot bind 14-3-3 is reduced by half within 2 hrs. At the end of the six hour period, very little of the phospho-defective RdgB β mutant is present. We also compared the turnover rate of another soluble PITP, PITP β , which is not seen to degrade in the 6 hr experiment time.

A common mechanism by which intracellular proteins are degraded is via a ubiquitin-dependent mechanism involving the 26S proteasome. Treatment of COS-7 cells expressing wild type FLAG-RdgB β with the proteasome inhibitor, MG-132, revealed that RdgB β is ubiquitinated (Fig. 4B). Phosphorylation of substrate proteins may promote recruitment of the E3 ubiquitin ligase, which binds to the substrate and attaches ubiquitin to it. Phosphorylation is typically required on serine and threonine residues in a PEST sequence to activate the signal and promote degradation of the substrate protein [39]. We therefore examined whether phosphorylation of Ser274 or Ser299 on RdgB β was required for ubiquitination (Fig. 4C). FLAG-RdgB β and phospho-defective mutant immunoprecipitates were probed with an anti-ubiquitin antibody, which showed that RdgB β -sp1 was ubiquitinated regardless of its phosphorylation state.

Assessment of PI transfer activity of RdgB β when complexed with 14-3-3

To examine the consequences of 14-3-3 binding to RdgB β on lipid transfer activity, wild type and mutants of RdgB β that could not bind 14-3-3 were expressed in COS-7 cells. COS-7 cells express negligible levels of endogenous RdgB β (Fig 1A) but do express endogenous PITP α and PITP β [25] and therefore it was necessary to separate these PITPs from RdgB β . Cytosol prepared from cells over-expressing WT FLAG-RdgB β was fractionated by size exclusion chromatography, was examined for the PITP proteins by western blot, and individual fractions assessed for PI transfer activity. Endogenous PITP α and β co-elute in fractions 23-25 and are well-separated from RdgB β -containing fractions (17-19) (Fig. 5A). RdgB β containing fractions also contain 14-3-3 (Fig. 5A). Whilst the fractions (23-25) enriched in PITP α / β show robust PI transfer activity [25], the RdgB β -containing fractions (17-19) exhibit none (Fig. 5A). Next we considered the possibility that the complex of RdgB β with 14-3-3 is unable to transfer because the C-terminus is immobilised due to 14-3-3 binding. We therefore expressed the mutant S299A and S274A RdgB β proteins which are unable to bind to 14-3-3. After separation of the proteins by gel filtration, the fractions were blotted for PITP α and β as well as for the mutant RdgB β proteins. PITP α / β eluted in fractions 23-25; these fractions exhibited PI transfer

activity, whereas fractions (17-19) containing the mutant proteins lacked transfer activity. As a control, bacterially-expressed recombinant RdgB β (200 μ g) was similarly separated by gel filtration; recombinant RdgB β elutes in fractions 17-21 and is associated with PI transfer activity (Fig. 5A).

Over-expressed RdgB β proteins whether bound to 14-3-3 or not are devoid of lipid transfer activity, although the recombinant protein is active even after gel filtration. One possibility is that RdgB β proteins are less active compared to PITP α (and β) and that concentrations achieved by over-expression are insufficient. Comparison of the concentration dependence of transfer activity between PITP α and RdgB β confirmed that this was indeed the case (Fig. 5B). Unlike PITP α which shows transfer activity at even 200ng/ml, RdgB β just begins to show activity when its concentration exceeds 10 μ g/ml. Significant activity is only observed when RdgB β concentrations rise above 50 μ g/ml. In separate experiments, we calculated that ~300ng/ml of RdgB β was present in the RdgB β -enriched fractions, a level insufficient to exhibit lipid transfer. It is possible that the *in vitro* lipid transfer assay used here is not optimal for RdgB β and therefore permeabilised HL60 cells were tested as the donor (rather than microsomes) [47]. We also varied the composition of the acceptor liposomes. None of these modifications improved the efficiency of lipid transfer by RdgB β .

Endogenous RdgB β is present in rat heart

To investigate the function of RdgB β *in vivo*, we needed to identify cell-lines and tissues that express endogenous RdgB β proteins. Using the pAb:101 as well as a commercial antibody called PITPNC1 (Orbigen: PAB-02250), which was raised against amino acids 67-266 of RdgB β we screened lysates (50-100 μ g) prepared from several cell-lines and rat tissues by western blot. Cell-lines examined included COS-7, HEK-293, HKC-8 cells (kidney proximal tubule cell line), Jurkat (a T cell-line), HL60 cell (a promyelocytic leukemic cell line), HeLa cells, Hep-G2 (a liver cell line), PC12 cells (chromaffin cell line) and RBL-2H3 mast cells and rat tissues included liver, brain, heart, kidney and muscle. The western blot signal from the lysates was ambiguous as both antibodies recognised several weak bands at the appropriate size. RdgB β transcripts are highly enriched in the heart [16] and we therefore fractionated rat heart cytosol by size exclusion chromatography and western-blotted fractions with both RdgB β antibodies as well as for 14-3-3 (Fig. 6). As a positive control, we used fractions enriched in (over-expressed) untagged RdgB β prepared from COS-7 cell cytosol. Fractions 17-21 show immunoreactivity with both antibodies for a protein of the right size. Moreover, recombinant proteins and COS-7 cell-expressed proteins also elute in the same fractions (see Fig. 5). We suggest that rat heart does contain RdgB β and its elution on gel filtration partially overlaps with 14-3-3 proteins suggesting that some of the RdgB β proteins are likely to be in a complex with 14-3-3. The fractions were also analysed for PI transfer and a peak of activity was observed that could be accounted for by the presence of endogenous PITP α and PITP β (Fig. 6). No PI transfer activity was associated with RdgB β proteins.

RdgB β associates with Angiotensin II Receptor Associated Protein, ATRAP

The results described above regarding the absence of *in vitro* transfer activity for RdgB β reinforces our recent speculation that PITP proteins function by being recruited to specific membrane compartments potentially by interaction with integral membrane proteins [48]. The best example is that of PITP α which is recruited by DCC (deleted in colon cancer), the receptor for netrin-1 [7]. It has been reported that RdgB β interacts with an integral membrane protein, angiotensin II receptor-associated protein (ATRAP), in a high throughput yeast two hybrid screen where systematic mapping of protein-protein interactions of 8100 human proteins was undertaken [27,28]. To validate this interaction in mammalian cells, FLAG-tagged RdgB β was co-expressed with HA-tagged ATRAP. No interaction between the two proteins was observed, however. Since ATRAP is protective following chronic stimulation with angiotensin II *in vivo*, we reasoned that RdgB β association would be regulated by downstream signalling from the angiotensin II receptor. Angiotensin II is a G-protein-coupled receptor and stimulates

phospholipase C. Events downstream to phospholipase C is the activation of protein kinase C and to mimic chronic angiotensin II signalling we examined complex formation between RdgB β and ATRAP with PMA, an activator of protein kinase C. FLAG-RdgB β immunoprecipitates contained ATRAP only following treatment of the cells with PMA (Fig. 7A). BIM inhibited the interaction suggesting that events downstream of protein kinase C activation were required (Fig. 7B). A 4-16 hr PMA treatment was required for the interaction; conditions that would have down-regulated some isoforms of protein kinase C [49] but not PKD (PKC μ) [50]. In addition, HA-immunoprecipitates, in which ATRAP was immunoprecipitated via its HA tag, contained FLAG-RdgB β (data not shown).

To examine whether the binding of RdgB β to ATRAP was affected by 14-3-3 binding at the C-terminus, we engineered a C-terminal truncated RdgB β mutant (1-251), that lacks the 14-3-3 binding site. In addition we used the S299A mutant that does not bind 14-3-3 (Fig. 3A). The constructs were expressed in COS-7 cells and the cells were incubated with PMA for 16 hrs. It was noted that PMA treatment causes increased expression of RdgB β (Fig. 8). Immunoprecipitation of the RdgB β constructs revealed that binding to ATRAP was independent of 14-3-3 binding. Thus we can conclude that ATRAP binds to the PITP domain whilst 14-3-3 binds to the unstructured C-terminus. This also implies that RdgB β -sp2 should also interact with ATRAP (Fig. 1). (Expression of RdgB β -sp2 in COS-7 cells is extremely low and therefore the interaction was inconclusive). The interaction between ATRAP and the PITP domain of RdgB β is specific as neither PITP α nor PITP β was found to interact with ATRAP.

Recruitment of RdgB β to membranes upon PMA treatment.

ATRAP is an integral membrane protein [51] whereas RdgB β is a soluble protein. In previous studies, it was reported that RdgB β localised throughout the cytoplasm but excluded from the nucleus in COS-7 and in PC12 cells [16,17] whilst ATRAP was found to localise mainly at the endoplasmic reticulum and the Golgi in HEK cells [51]. We examined the co-localisation of ATRAP and RdgB β after co-expression in COS-7. RdgB β localised to the cytosol showing a uniform staining throughout but excluded from the nucleus whilst ATRAP shows perinuclear staining. Upon treatment with, increased expression of RdgB β is observed and co-localisation of RdgB β with ATRAP at the perinuclear region is also observed.

Since treatment with PMA does cause an interaction between the two proteins, we surmised that RdgB β would get recruited to membranes after PMA treatment and this could be monitored biochemically. COS-7 cells were co-transfected with RdgB β and ATRAP and treated with PMA. Membranes and cytosol were prepared and blotted for RdgB β and ATRAP. ATRAP was present in the membranes whilst RdgB β was mainly cytosolic. Upon PMA treatment, RdgB β was found to translocate to membranes (Fig. 9A). In the presence of PMA, RdgB β levels are much higher in cells and this could be due to an inhibition of its degradation (Fig. 8A). Therefore we calculated the percentage distribution of RdgB β between membranes and cytosol for the untreated- and for the PMA-treated cells. Membranes prepared from PMA-treated cells have more RdgB β (after accounting for increased expression) with a corresponding decrease in the cytosolic fractions.

ATRAP is highly enriched in the heart and the kidney [32] and we confirmed that COS-7 cells, a kidney fibroblast-like cell line, do contain endogenous ATRAP (Fig. 9B). Translocation of RdgB β to membranes by PMA treatment was also observed in COS-7 cells. Finally, we examined whether a mutant RdgB β that did not bind 14-3-3 would also translocate to membranes. Because of the increased expression of RdgB β by PMA, the results are expressed as a ratio of PMA-treated membranes to control membranes. There is an 8-fold increase in the membranes compared to a 2-fold increase in the cytosol for WT RdgB β . For the mutant that does not bind 14-3-3, there is a 14-fold increase suggesting that 14-3-3 binding hinders membrane translocation. These changes were specific for RdgB β as changes in the distribution of 14-3-3, PITP α or PITP β was not observed (Fig. 9B).

DISCUSSION

Proteins belonging to the phosphatidylinositol transfer protein family have a defining PITP domain at their N-terminus but differ markedly in their C-terminal residues. Of the three soluble PITPs, splice variant 1 of RdgB β (referred throughout as RdgB β) has the most extensive C-terminal region, 80 amino acids long. This region contains two residues, Ser274 and Ser299, which are phosphorylated *in vivo* [18-20]. These residues lie in consensus sequences for 14-3-3-binding [44] and in this study we provide compelling evidence that RdgB β directly binds to 14-3-3 proteins via its C-terminus and that phosphorylation of Ser274 and Ser299 is required to mediate this interaction. 14-3-3 proteins are cup-shaped dimeric proteins and each subunit of the dimer is able to bind one discrete phospho-serine- (or phospho-threonine)-containing ligand. In RdgB β there are two 14-3-3 binding sites, which are separated by 25 amino acids. A minimal distance of 10 residues is sufficient for 14-3-3 proteins to bind to a single polypeptide [46], suggesting that the 14-3-3 dimer engages a single molecule of RdgB β . Mutation of either serine residue to alanine is sufficient to inhibit 14-3-3 binding indicating that both residues are essential for optimal binding. Owing to the dimeric nature of 14-3-3 proteins, a phosphopeptide with two binding motifs can bind 14-3-3 proteins with a 30-fold greater affinity than a phosphopeptide containing a single motif [44]. This suggests that the high affinity of 14-3-3 binding to RdgB β only occurs when both residues are phosphorylated.

In addition to the presence of 14-3-3 binding sites on the C-terminus of RdgB β , this region also contains two PEST sequences which in many other proteins act as signals for degradation [39]. Binding to 14-3-3 proteins would mask the PEST sequence and therefore prevent the degradation. Nonetheless, RdgB β is rapidly degraded in resting cells with a half-life of 4 hrs suggesting that binding of 14-3-3 proteins to RdgB β is highly dynamic. 14-3-3-binding does afford some protection since a mutant of RdgB β that is unable to bind 14-3-3 is degraded even faster; its half-life is reduced to 2 hrs. The recognition of the PEST sequence and the mechanism of subsequent degradation of the substrate protein has been shown in many studies to be mediated by ubiquitin and requires a properly-functioning proteasome [39]. The PEST sequence by its very nature contains a high proportion of serine and threonine residues. In many studies phosphorylation of serine and threonine residues in the PEST sequence is a prerequisite for degradation of the protein [52,53]. However in our studies, mutation of the phosphorylated serine residues to alanine had no effect on ubiquitination of RdgB β indicating that phosphorylation is not required for activation of the PEST sequence and protein degradation in this case. In addition, bioinformatic analysis shows that the C-terminus of RdgB β is largely disordered and consequently is susceptible to proteolytic cleavage by trypsin. Such disordered sequences have also been shown to be necessary for the degradation of proteins at the proteasome, effectively giving the proteasome a point from which to unravel the target protein to allow access of proteases [54]. Transplantation of a disordered region from one protein to another can increase the rate of degradation of a protein, although the disordered region itself is not sufficient for proteasomal degradation: ubiquitination is also required [54]. These results suggest that there exists an E3 ubiquitin ligase or ligases that are responsible for targeting RdgB β -sp1 for degradation and there are >600 E3 ligases which are sub-divided by the presence of RING, HECT or U-box domains that could be responsible [55].

RdgB β is a member of the PITP family and is able to bind and transfer PI *in vitro* [16]. The entrance to the hydrophobic cavity in PITP α is controlled by the C-terminus (see Fig. 1). Thus our prediction was that 14-3-3 binding to the C-terminus of RdgB β is likely to immobilize the C-terminus and restrict lipid exchange. To test this we expressed wild-type RdgB β and mutants that could not bind 14-3-3 in COS-7 cells and separated these proteins from endogenous PITPs. No transfer activity was detected with either construct. RdgB β is highly expressed in the heart, but we were unable to detect any *in vitro* transfer activity associated with the endogenous protein. The lack of activity is attributed to insufficient protein and this was confirmed by examination of the concentration dependence for transfer activity. Although RdgB β proteins are active *in vitro*, the concentration required is 50-100 μ g/ml. In comparison, PITP α requires 300-500ng/ml, a difference of 100-fold. Thus we would suggest that RdgB β proteins

would need to be concentrated at the membrane by interaction with membrane proteins for lipid exchange. Despite their inability to transfer PI in the *in vitro* assay, nonetheless, RdgB β binds PI in its hydrophobic cavity (Fig. 5C).

We have identified that RdgB β interacts with ATRAP (Angiotensin II receptor-associated protein), an 18 kDa transmembrane protein that contains three hydrophobic domains and an extended hydrophilic cytoplasmic carboxyl-terminal tail (Fig. 10). This interaction between RdgB β and ATRAP is observed after treatment with PMA. Binding of RdgB β to ATRAP occurs via the PITP domain, a site distinct from the 14-3-3 binding site on RdgB β (Fig. 10). We suggest that ATRAP may prefer to bind RdgB β that is not bound to 14-3-3 – conditions that may allow interaction of the C-terminus with an adjacent membrane initiating the process of lipid exchange. This conclusion is supported by the observation that the RdgB β S274 mutant, translocates to the membrane at a higher level (Fig. 9B) than wild type RdgB β and the cytosolic pool is depleted to a greater extent when results are calculated as the distribution between membrane and cytosol as in Fig. 9A.

ATRAP was originally identified in a yeast two-hybrid screen as an interacting protein for the Angiotensin II Type I receptor. Residues 110-120 of ATRAP were found to interact with the C-terminus of the receptor [32,51]. Interaction between ATRAP and the receptor is observed in the basal state and is enhanced upon stimulation with angiotensin II. It is generally thought that ATRAP increases internalization of the AT1 receptor leading to a suppression of angiotensin II-mediated signalling including phospholipase C activation [51,56,57]. In the heart, angiotensin II stimulation promotes cardiac hypertrophy, concomitant with a decrease in cardiac ATRAP expression, and transgenic mice over-expressing ATRAP are protected from cardiac hypertrophy provoked by chronic angiotensin II infusion [58]. The highest expression of ATRAP is found in the kidney, where it is localised in the proximal tubule, particularly the brush border. Blood pressure and plasma volume are raised in mice lacking ATRAP. Thus ATRAP has a protective effect on the deleterious effects of Angiotensin II. Since both ATRAP and RdgB β are highly expressed in the heart and the kidney, it would suggest that RdgB β could modulate the function of ATRAP. Angiotensin II stimulates phospholipase C via Gq and this results in the downstream activation of protein kinase C. The effects of angiotensin II on cardiac hypertrophy require a chronic stimulation with angiotensin II, and mice are infused with angiotensin II for 14 days to induce cardiac hypertrophy [58]. Thus ATRAP interactions with RdgB β most likely occur following a chronic stimulation with angiotensin II. We suggest that PMA mimics this chronic stimulation by angiotensin II. In addition to interacting with the angiotensin II Type I receptor, ATRAP can bind a number of other proteins which are independent of Angiotensin II signalling. This includes CAML [59] and RACK1 [60]. Thus the consequences of RdgB β interaction with ATRAP may go beyond Angiotensin II signal transduction. Since RdgB β possesses phosphatidylinositol transfer activity, binding of RdgB β to ATRAP at the membrane could promote PI exchange at the opposing membrane (Fig. 10, see inset).

Our data indicate that RdgB β -sp1 is an unusual protein whose expression and activity is tightly-regulated in cells. The C-terminus of RdgB β -sp1 is highly disordered and contains PEST sequences that were originally identified as degradation signals due to their abundance in rapidly-degraded proteins [39,40]. Proteins with unstructured regions predominantly have signalling or regulatory roles and are often reused in multiple pathways to produce different physiological outcomes [61]. The C-terminus of RdgB β has all the properties found in unstructured proteins where proteolytic degradation contributes to controlling its abundance. We suggest that RdgB β plays a central role in angiotensin II-mediated cell signaling both in the heart and the kidney, two organs where RdgB β proteins are enriched together with ATRAP.

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FIGURE LEGENDS

Figure 1. The C-terminus of RdgB β is disordered and contains putative 14-3-3-binding sites. **(A)** Characterisation of polyclonal antibody 101 to RdgB β . *Left panel.* Ab:101 recognises both splice variants of RdgB β and the C-terminal truncated RdgB β but not other PITP proteins: 50ng of recombinant proteins expressed in E.Coli per lane. *Middle panel.* A band at ~46 kDa is recognised by Ab:101 when RdgB β (untagged) is expressed in COS-7 cells. siRNA confirms that the prominent band observed at 35 kDa is a cross-reactive protein. *Right panel.* Titration of recombinant RdgB β proteins. **(B)** Domain organisation of PITP α , RdgB β and RdgB β -sp2. All three proteins contain a PITP domain (Pfam: IP_trans) at their N-termini (1-254) but have C-terminal extensions of varying lengths. Closed (PDB: 1uw5) and open (PDB: 1kcm) structures of PITP α demonstrate the position of the G-helix and C-terminal extension that form a 'lid' in PITP α . Sequences of the C-termini of splice forms, RdgB β (Q9UKF7.2) and RdgB β -sp2 (NP_858057.1) (residues 181 to end) with the region identical in the two splice variants boxed in grey. Phosphorylated residues Ser274 and Ser299 are indicated and the potential 14-3-3-binding sequences are underlined with key residues in bold. Residue 252 marks the beginning of disorder predicted by DISOPRED2 (see text). Predicted trypsin cleavage sites with >80% cleavage probability are indicated (PeptideCutter tool at the ExPASy Proteomics Server). PEST sequences are indicated. **(C)** Trypsin digest (0-10min) of RdgB β and RdgB β -sp2 recombinant proteins. *Left panel:* Coomassie stained SDS-PAGE: 2 μ g protein per lane. *Middle panel:* Western blot: 30ng protein per lane. Experiment reproduced on four separate occasions. PI transfer activity of RdgB β (200 μ g/ml) before and after trypsin treatment (10min).

Figure 2. RdgB β binds directly to endogenous 14-3-3 proteins. **(A)** COS-7 cells were transfected with empty vector or FLAG-RdgB β and lysates probed for expression (left panels). FLAG-RdgB β immunoprecipitates were separated by SDS-PAGE and immunoblotted with pan 14-3-3 polyclonal antibody (right, top) or probed by far western blotting using 14-3-3-DIG recombinant proteins (right, middle) as described in Experimental Procedures. Membranes were re-probed with anti-FLAG mAb. **(B)** FLAG-RdgB β immunoprecipitate (IP) was obtained as in **(A)** Recombinant RdgB β (-sp1), RdgB β -sp2 and RdgB β (-sp1) truncated protein (residues 1-263) (50 and 100ng) were subjected to far western blotting using 14-3-3-DIG recombinant proteins.

Figure 3. Phosphorylation of both Ser274 and Ser299 of RdgB β is required for 14-3-3 binding **(A)** COS-7 cells were transfected with WT, S274A, S299A and S274A/S299A FLAG-RdgB β as indicated. Cell lysates were immunoprecipitated with anti-FLAG M2 Agarose beads and immunoblotted for 14-3-3. The membrane was re-probed with mAb:FLAG. Cell lysates (12 μ g) were probed for FLAG-RdgB β expression and for ARF1 (loading control). Cell lysates (3 μ g) were probed for 14-3-3 proteins. **(B)** COS-7 cells were transfected with FLAG-RdgB β and incubated with 10 μ M H89 (for inhibition of PKA), 10 μ M LY294002 (for inhibition of PKB via PI3K), or 5 μ M BIM-1 (for inhibition of PKC) for 16 hrs. Cell lysates were processed as in **(A)**.

Figure 4. RdgB β undergoes rapid turnover in COS-7 cells. **(A)** COS-7 cells expressing the indicated plasmids were treated with 100 μ g/ml cyclohexamide for 0, 2, 4 and 6 hrs. The lysates were immunoblotted using mAb:FLAG, then re-probed with anti-PITP β Ab:1C1. Experiments were carried out in quadruplet. Western blots were subjected to densitometry, the background intensity subtracted and protein concentration calculated relative to expression at zero time. **(B)** COS-7 cells transfected with WT FLAG-RdgB β were treated with the proteasome inhibitor, MG-

132, 20 μ M for 4 hrs. FLAG-RdgB β immunoprecipitates were probed using anti-ubiquitin antibody and re-probed with mAb: FLAG. Cell lysates were immunoblotted for FLAG-RdgB β , and then re-probed for ARF (loading control). **(C)** COS-7 cells were transfected with the indicated plasmids. FLAG-RdgB β immunoprecipitates were probed with pAb:Ubiquitin and re-probed with mAb: FLAG. Cell lysates were probed with mAb:FLAG and re-probed with pAb:ARF.

Figure 5. PI transfer activity of COS-7 cell-derived RdgB β -sp1 and recombinant RdgB β proteins. **(A)** COS-7 cell cytosol (16mg) was harvested from cells expressing WT, S274A and S299A FLAG-RdgB β and fractionated by size exclusion chromatography. Recombinant RdgB β (200 μ g) was also fractionated alongside. Fractions were probed for RdgB β , 14-3-3, PITP β and PITP α and also assessed for PI transfer activity. **(B)** Concentration dependence of transfer activity of the recombinant proteins, RdgB β and PITP α . The data in both panels are the same except that the right panel includes additional data at higher PITP α and RdgB β concentrations. Results are representative of three independent experiments.

Figure 6. RdgB β is present in rat heart cytosol. Rat heart was perfused with saline to remove red blood cells prior to homogenisation. Cytosol (30mg) was fractionated by size exclusion chromatography. All fractions were probed for RdgB β using two separate antibodies Ab:101 (made in-house) and Ab:PITPNC1 (Orbigen). Both antibodies identify a protein of appropriate size. The control sample used was untagged RdgB β -sp1 expressed in COS-7 cells from the pIRES vector. Fractions were also probed for PITP α , PITP β and for 14-3-3 proteins. PI transfer activity was also assayed using 100 μ l of the fractions.

Figure 7. RdgB β interacts with ATRAP following treatment with PMA. **(A)** COS-7 cells transfected with the indicated plasmids were incubated with 100nM PMA for 16 hrs. Cell lysates (12 μ g) were immunoblotted for HA-ATRAP and re-probed for FLAG-RdgB β (anti-FLAG antibody DDK). Cell lysates were incubated with FLAG-beads and immunoprecipitates were blotted with anti-HA antibody for ATRAP and re-probed with anti-FLAG mAb DDK for RdgB β -sp1. **(B)** COS-7 cells were co-transfected with HA-ATRAP and FLAG-RdgB β and treated with 100nM PMA or 100nM PMA plus BIM-I (5 μ M) for 4 hrs. The cells were processed as in **(A)**.

Figure 8. ATRAP interacts with the PITP domain of RdgB β . COS-7 cells were co-transfected with HA-ATRAP and either WT-FLAG-RdgB β , a truncated FLAG-RdgB β (1-251aa), or the FLAG-RdgB β S299A mutant. The cells were treated with 100nM PMA for 16 hrs prior. FLAG-immunoprecipitates were probed for HA-ATRAP and re-probed with mAb: FLAG. Cell lysates were immunoblotted for HA-ATRAP and re-probed using mAb: FLAG. This result is representative of two independent experiments.

Figure 9. Translocation of RdgB β to membranes upon PMA treatment. **(A)** COS-7 cells co-expressing RdgB β and ATRAP were treated with 100nM PMA for 16 hrs. The cells were sonicated and the membranes and cytosols prepared, and were probed for RdgB β , ATRAP, 14-3-3, PITP α and PITP β . The percentage distribution of RdgB β between membranes and cytosol was calculated from three independent experiments. Control and PMA-treated cells were plated on glass cover slips and stained for RdgB β and for ATRAP. **(B)** COS-7 were electroporated as indicated and membranes and cytosol probed for RdgB β , endogenous and expressed ATRAP, 14-3-3, PITP α and PITP β . RdgB β levels were expressed as a ratio of PMA-treated membranes versus control membranes and compared to PMA-treated cytosol versus control cytosol.

Figure 10. Molecular interactions between RdgB β , 14-3-3 proteins and ATRAP leading to lipid exchange. RdgB β is phosphorylated at Ser274 and Ser299 and bound by 14-3-3 proteins in cells under basal conditions. 14-3-3-binding stabilizes RdgB β -sp1 against degradation but there is a dynamic equilibrium between 14-3-3-bound and -unbound RdgB β ; RdgB β not bound

to 14-3-3 has two unshielded PEST sequences which signal rapid degradation at the proteasome. Stimulation of COS-7 cells with PMA promotes association of RdgB β with membranes. For lipid exchange, we predict that RdgB β functions *in trans* at membrane contact sites (see inset). The PITP domain of RdgB β was modelled on the template with PDB 1t27 (rat PITP β complexed with PC) using MODELLER.

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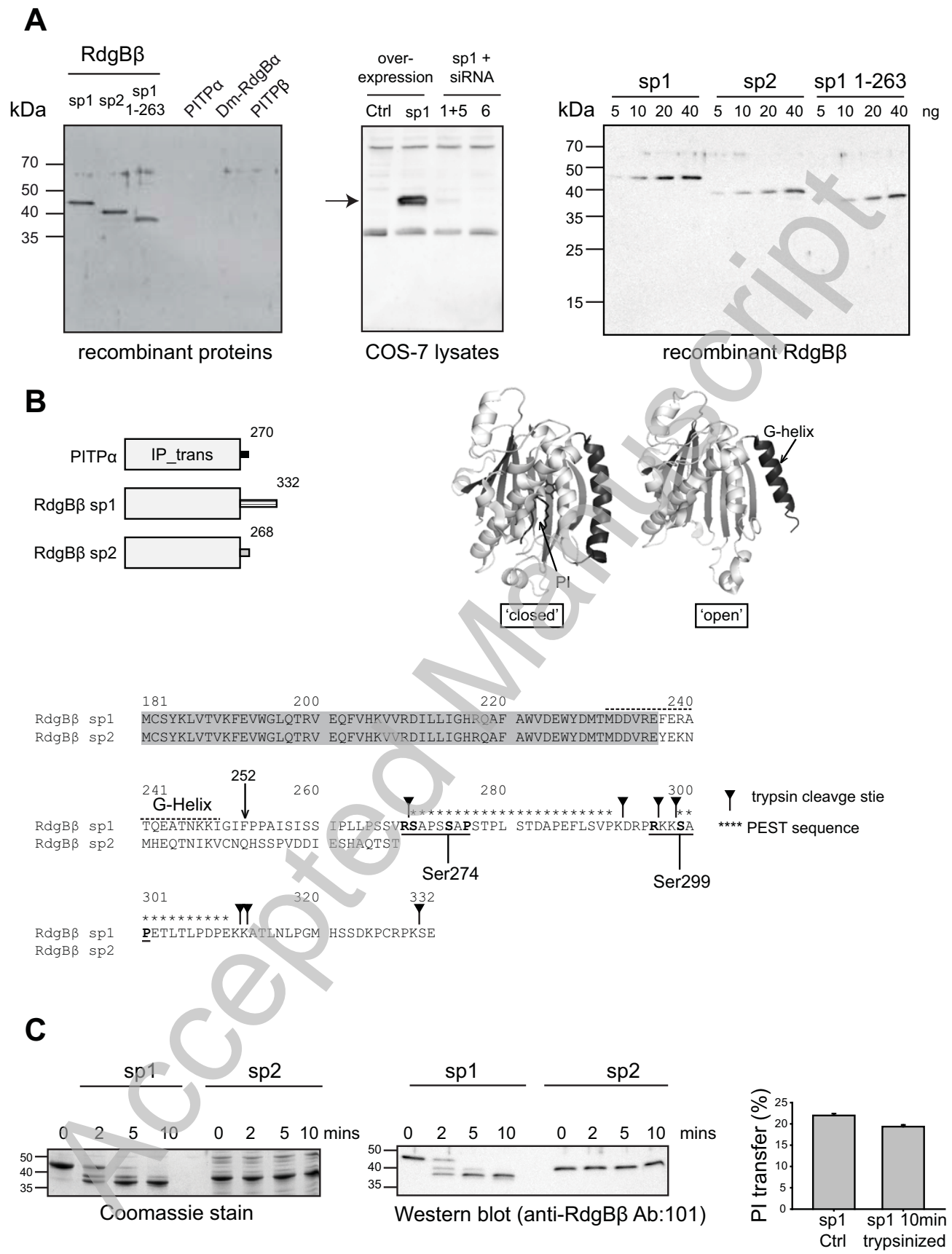
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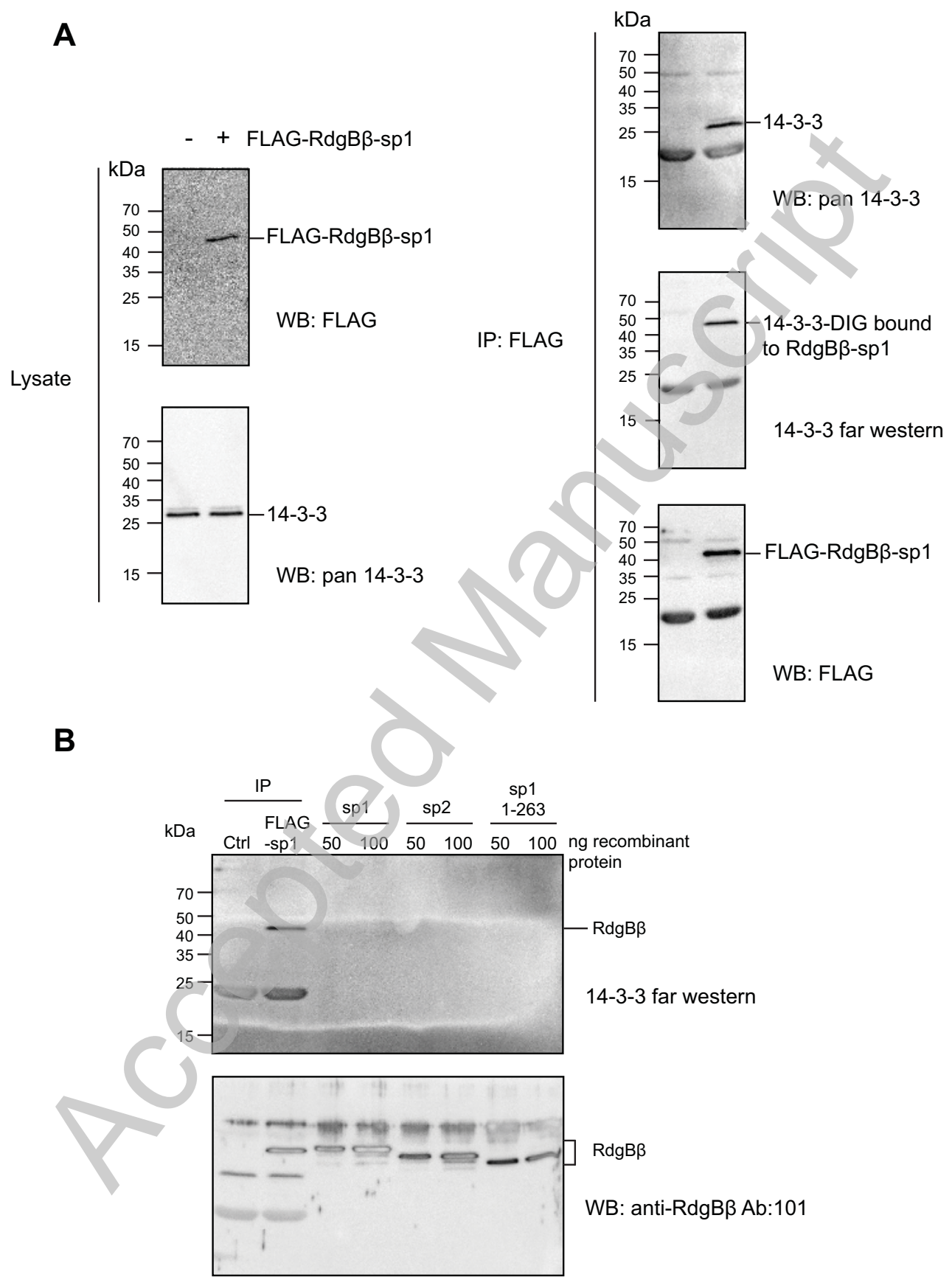
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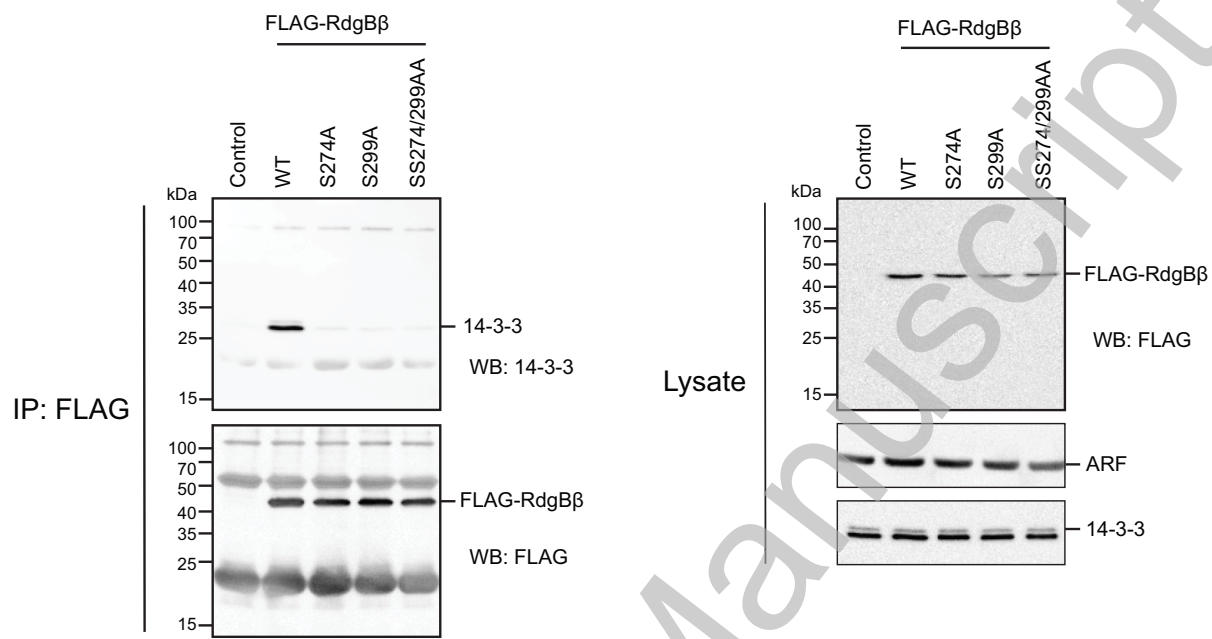
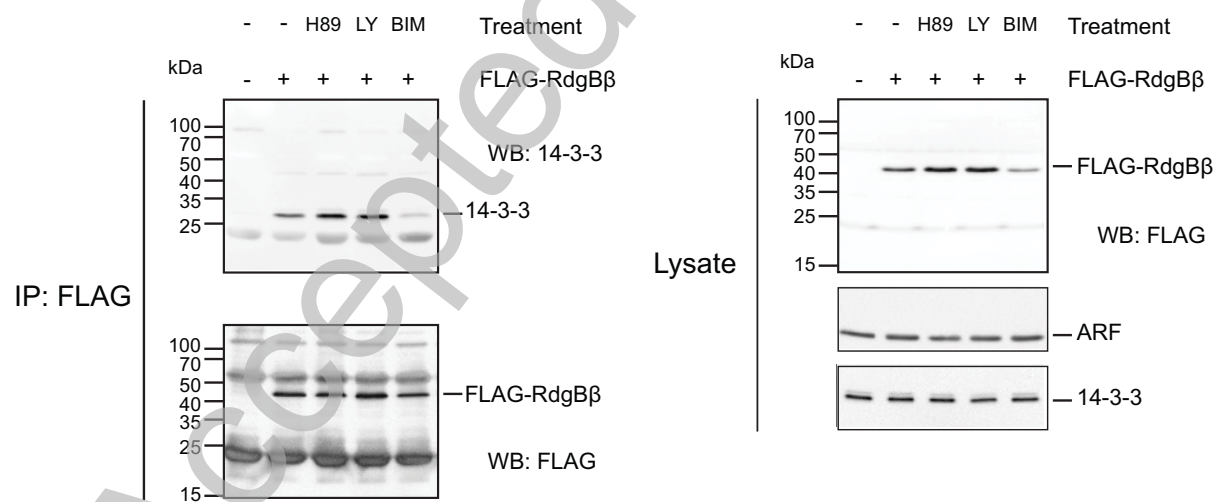
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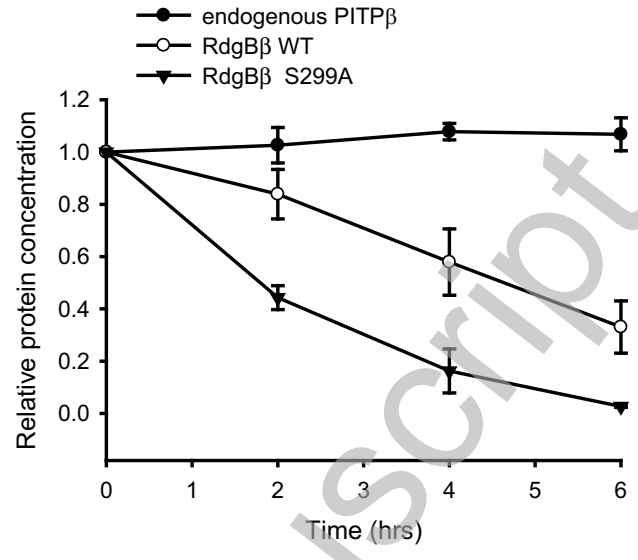
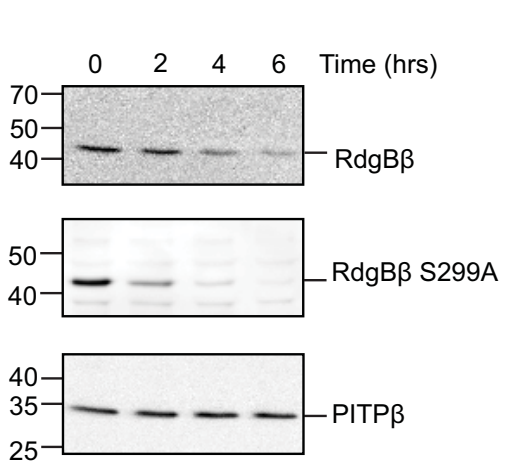
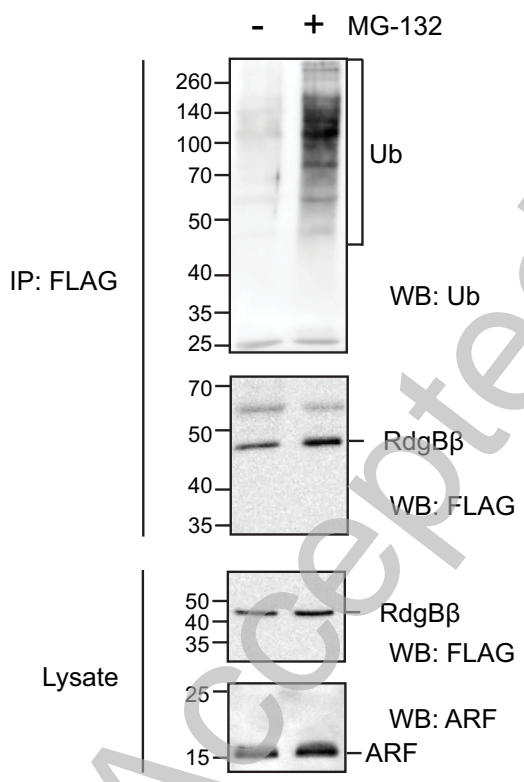
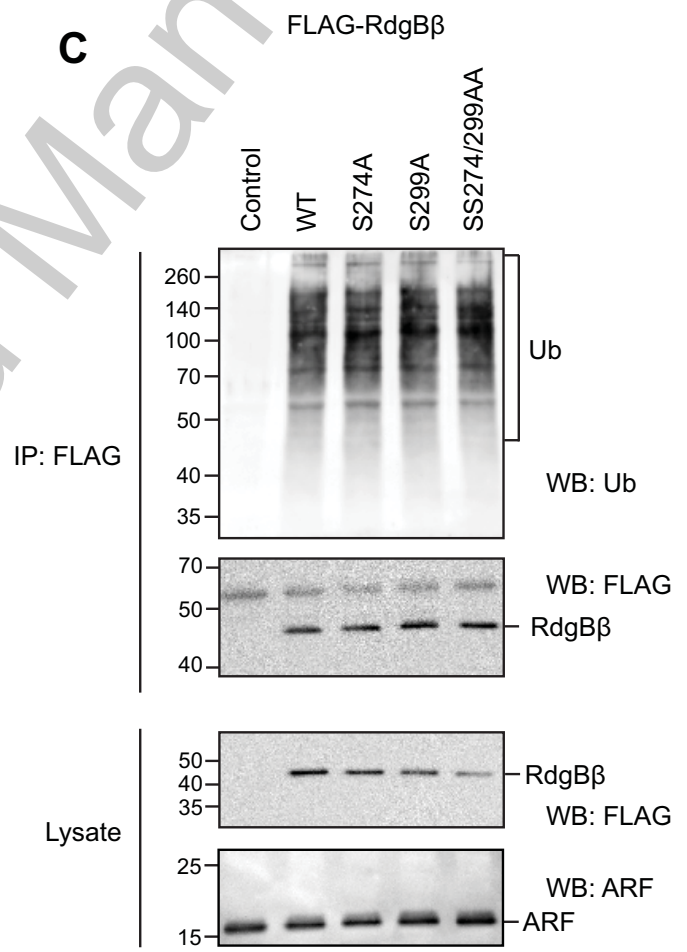
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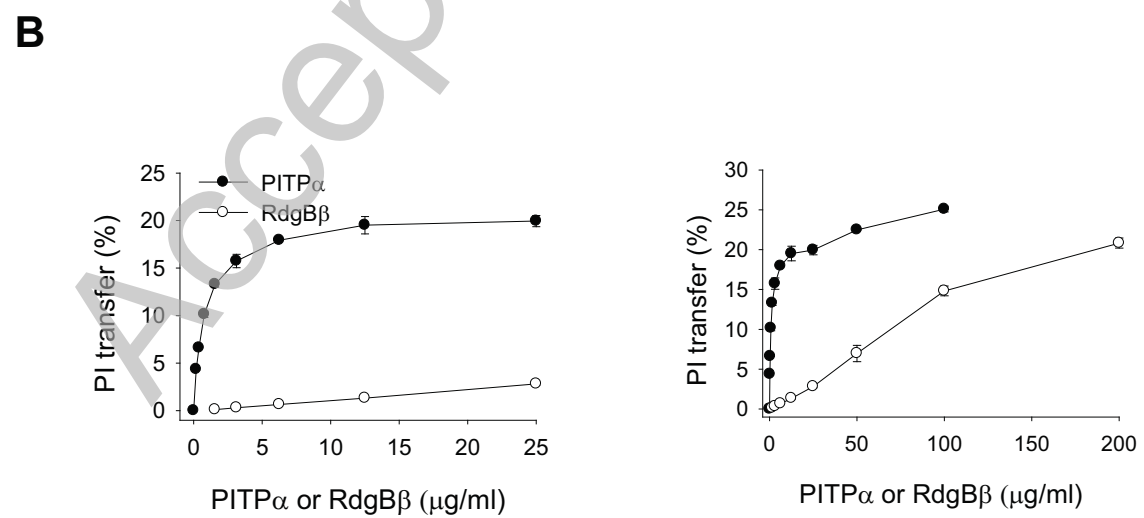
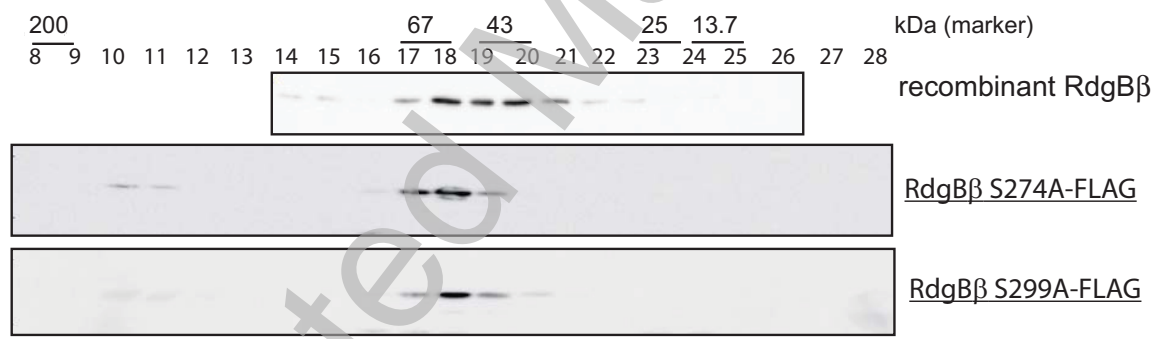
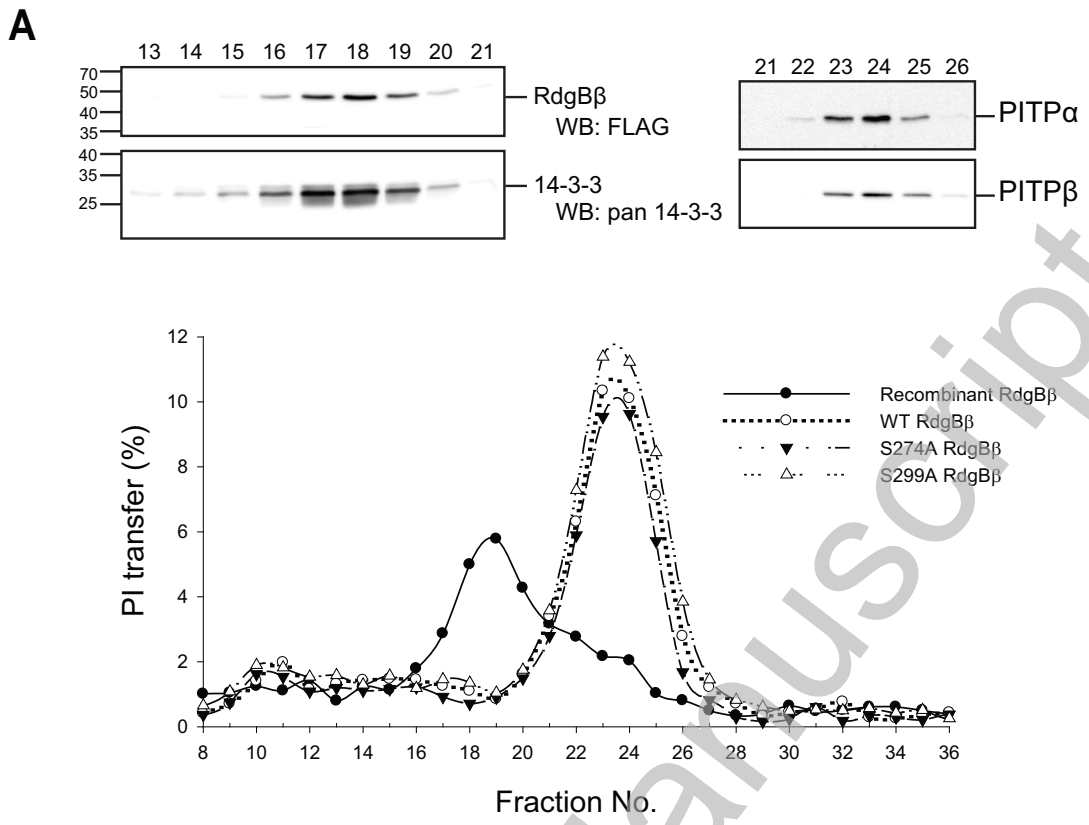
**Figure 1**

**Figure 2**

A**B****Figure 3**

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A**B****C****Figure 4**

**Figure 5**

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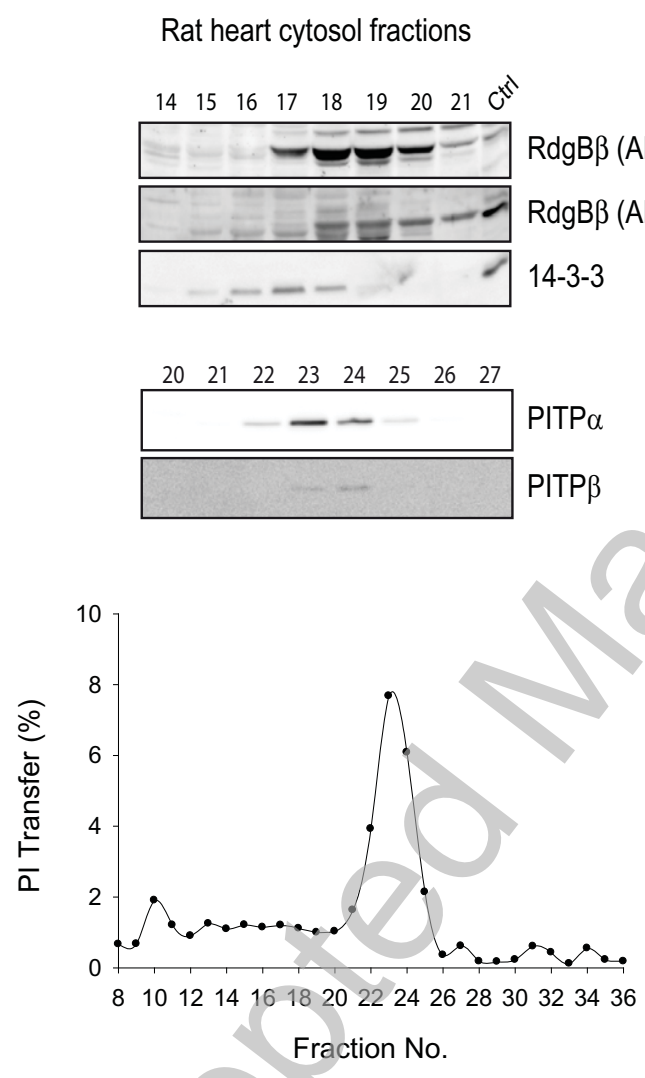
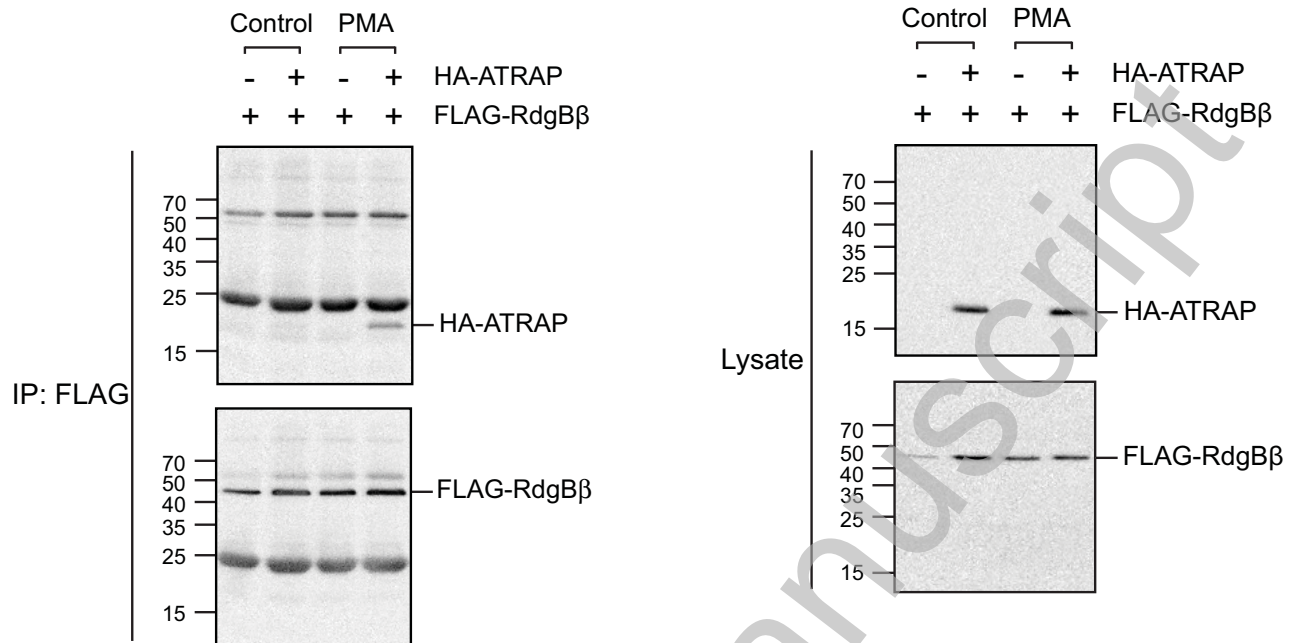
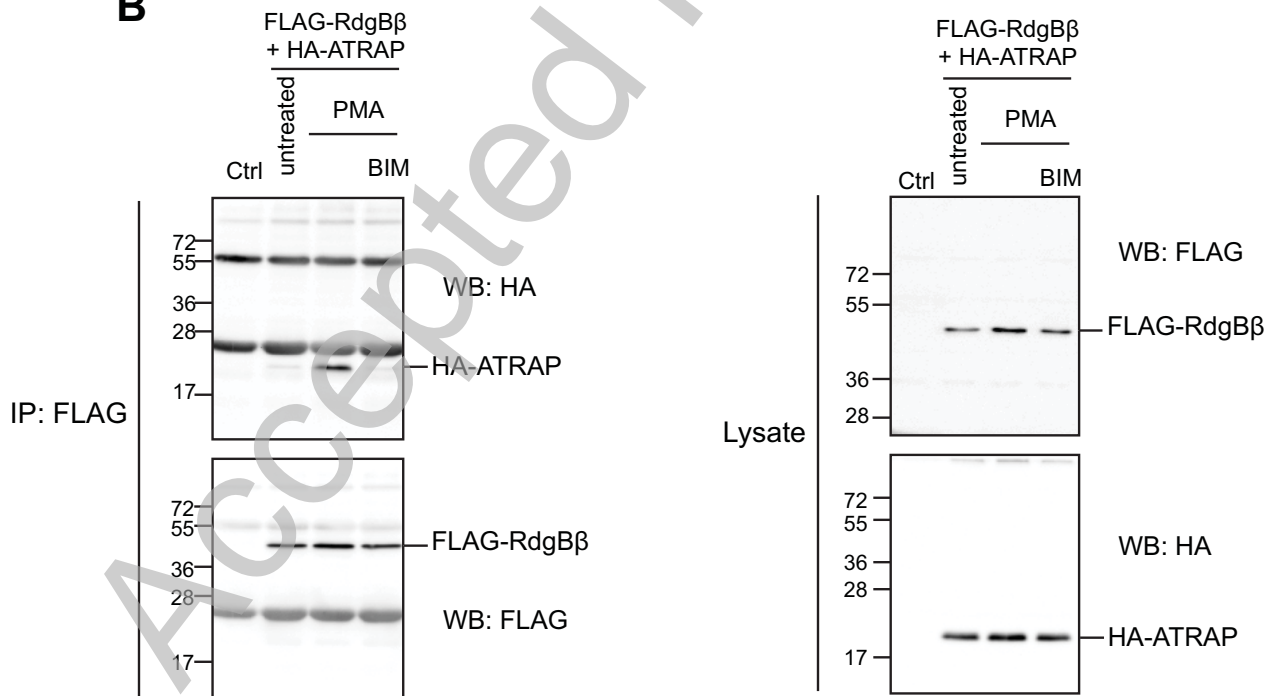
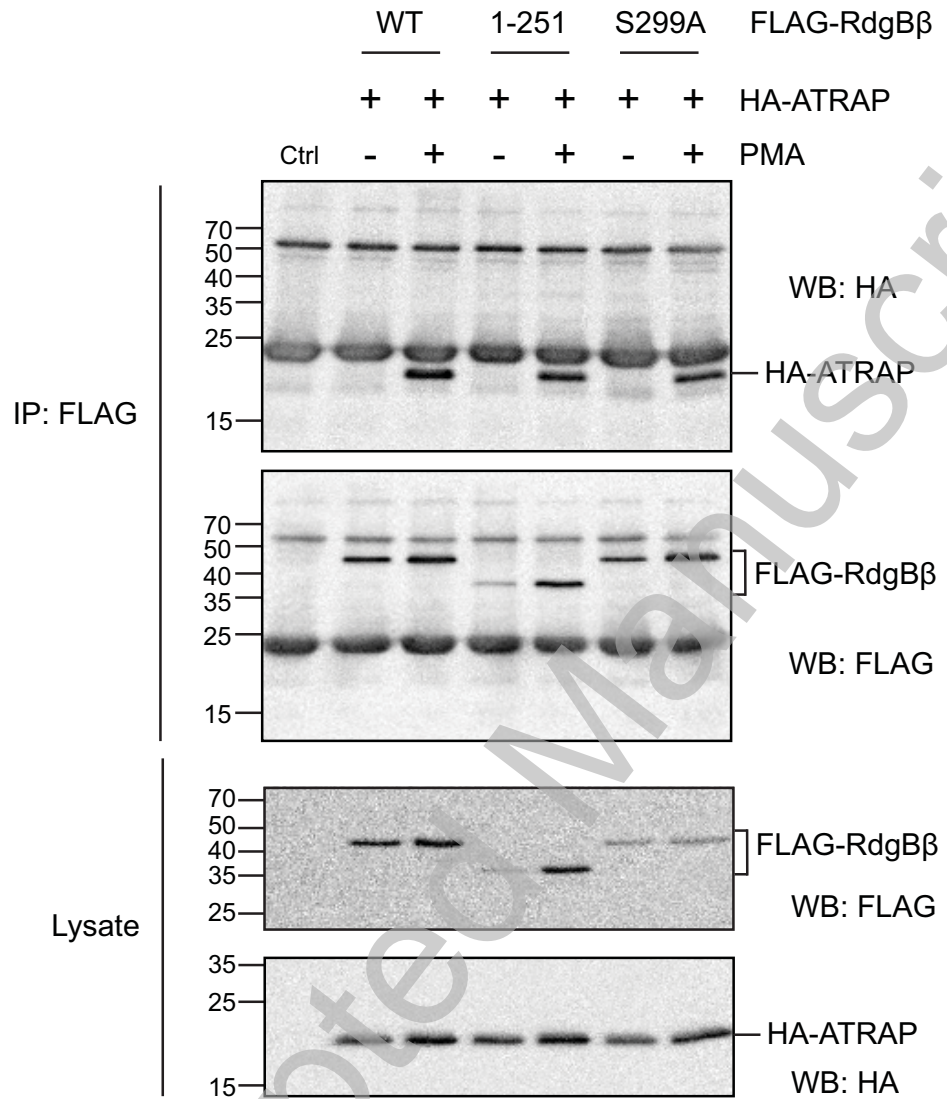
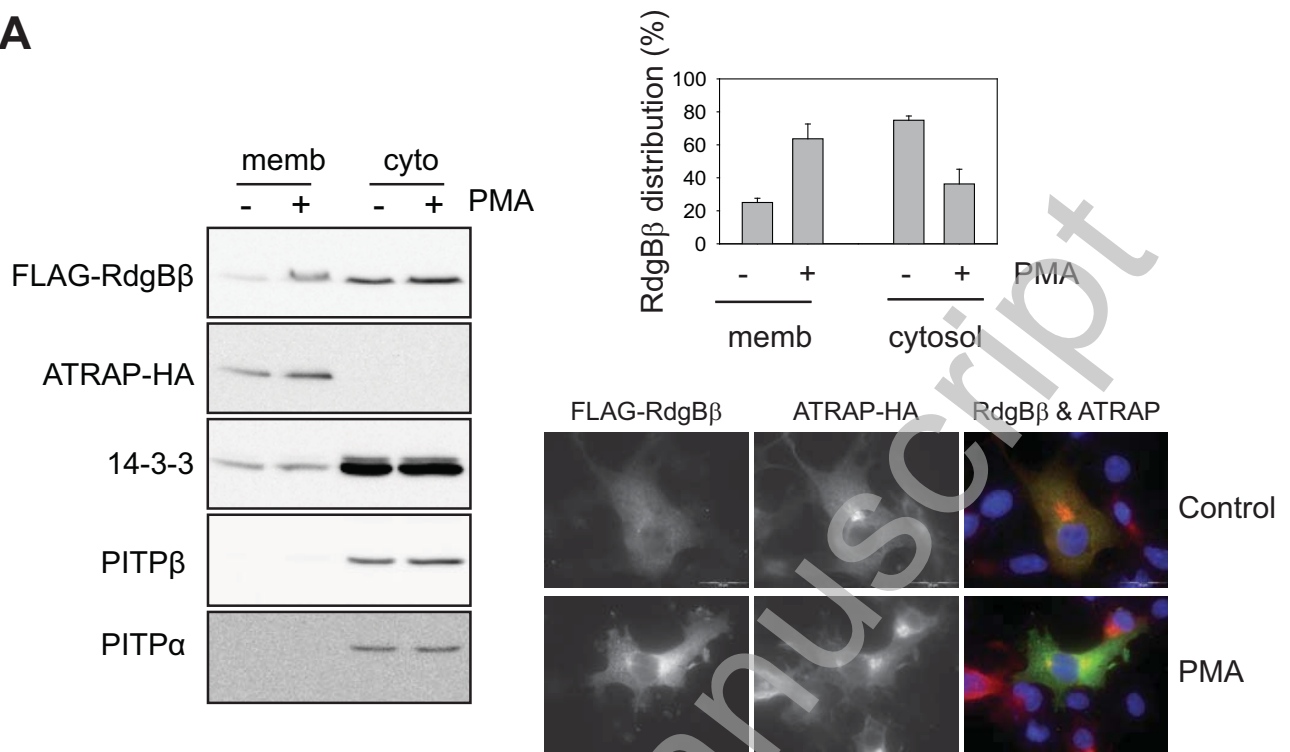
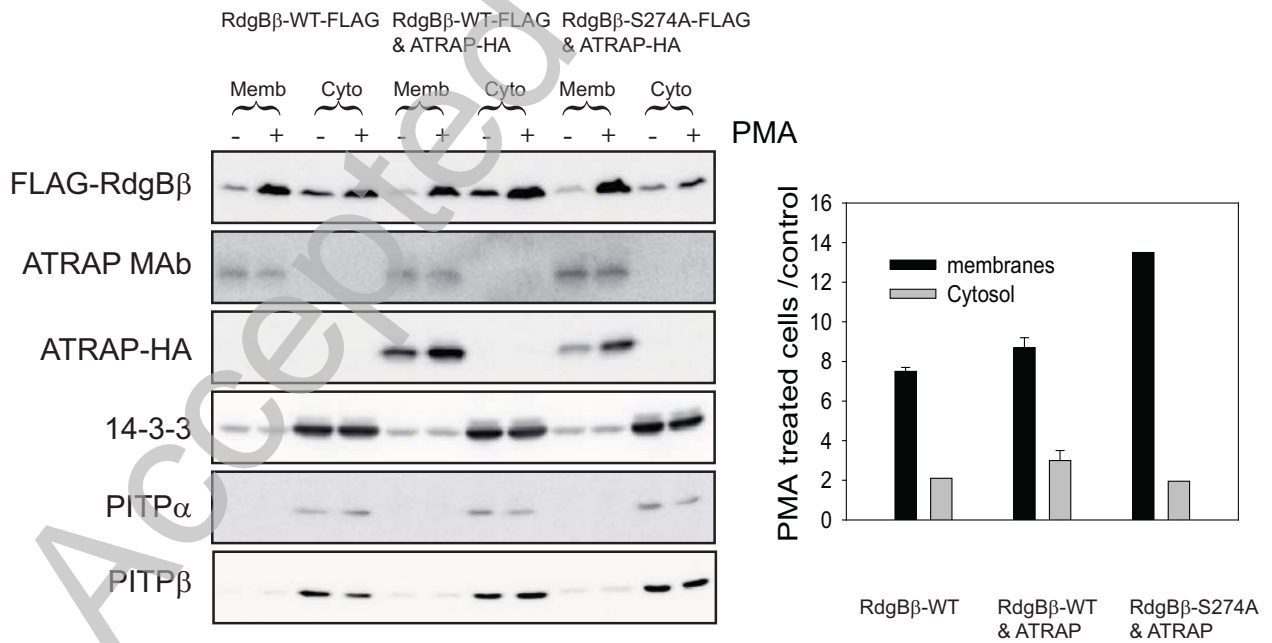


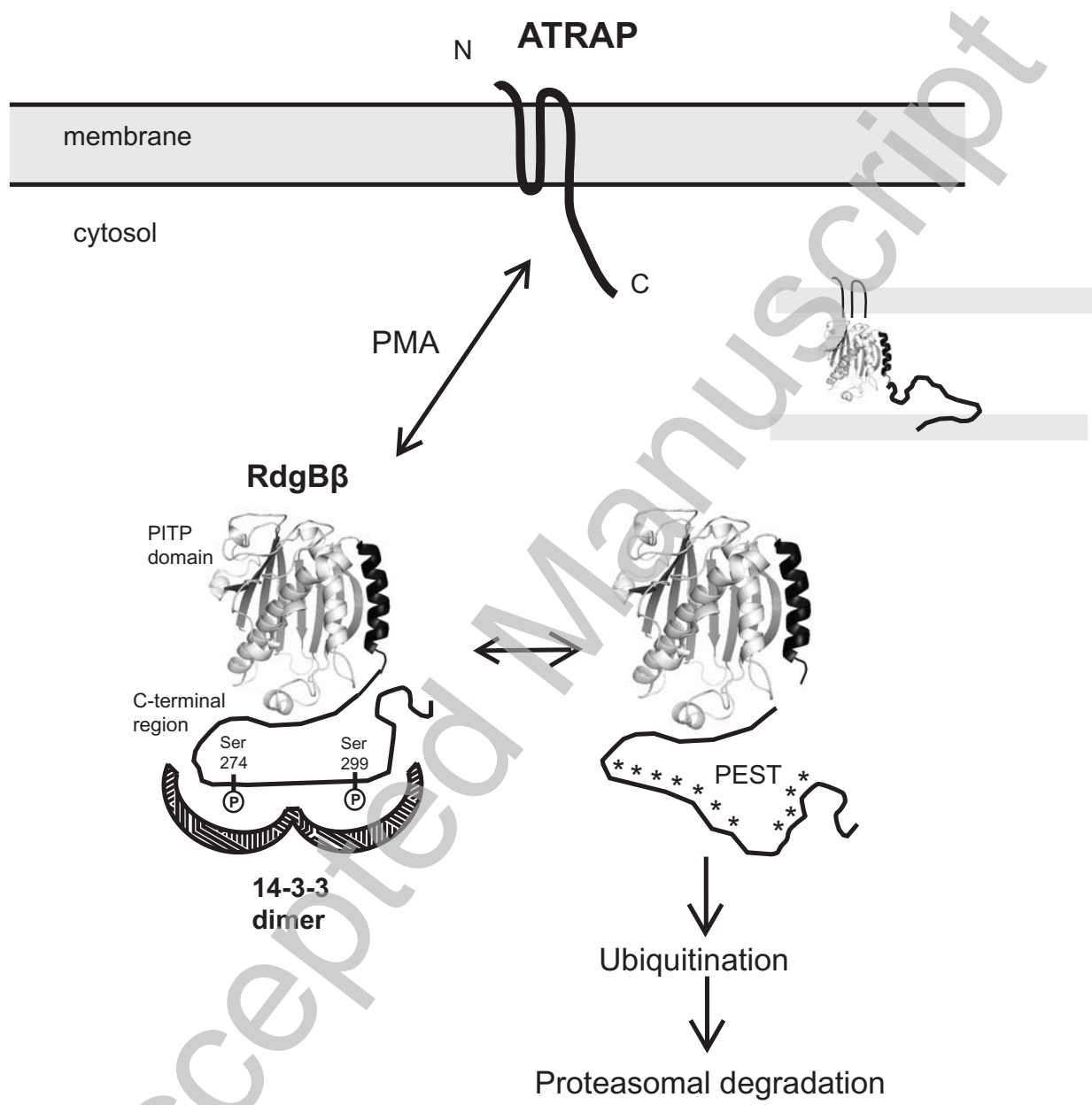
Figure 6

A**B****Figure 7**

**Figure 8**

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A**B****Figure 9**



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Figure 10