의학박사 학위논문

Novel finding for screening Small G proteins influencing transient receptor potential canonical 4 (TRPC4) channels

TRPC4 이온통로에 영향을 미치는 Small G protein 의 스크리닝을 통한 새로운 발견

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ABSTRACT

Canonical transient receptor potential 4 (TRPC4) channels are calcium-permeable, non-selective cation channels that are widely distributed in mammalian cells. It is generally speculated that TRPC4 channels are activated by $G_{q/11}$-PLC pathway or directly activated by $G_{i/o}$ proteins. Although many mechanistic studies regarding TRPC4 have dealt with heterotrimeric G-proteins, here, we first report the functional relationship between TRPC4 and small GTPase. We performed patch clamp, western blotting, and FRET technique. Rab proteins, Rasd1, Rasd2, and Rit protein increased without GTPγS. Rasd1 selectively activated TRPC4 channels and it was the Ras protein among small G protein families that can potently activate TRPC4 channels. For this to occur, it was found that certain population of functional $G\alpha_{i1}$ protein is essential. Meanwhile, dexamethasone, a synthetic gluco-corticoid and anti-inflammatory drug were known to increase mRNA level of Rasd1 in pancreatic β-cells. We have found that dexamethasone triggers TRPC4-like cationic current in INS-1 cells *via* increasing protein expression level of Rasd1. This relationship among dexamethasone, Rasd1 and TRPC4 could suggest a new therapeutic agent for hospitalized diabetes mellitus (DM) patients with prolonged dexamethasone prescription.

Keywords: TRPC4, Small G protein, Rasd1, GPCR, Dexamethasone, Insulin

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LIST OF ABBREVIATIONS

TRP channel: transient receptor potential channel

IP_3: inositol triphosphate

DAG: di-glyceride

PIP_2: phosphatidylinositol 4, 5-bisphosphate

PLC: phospholipase C

PTX: pertussis toxin

GPCR: g protein-coupled receptors

M_2R: muscarinic acetylcholine receptor 2

INTRODUCTION

The transient receptor potential (TRP) protein superfamily consists of a diverse group of cation channels that bear structural similarities to the Drosophila TRP. TRP superfamily is distinct from other groups of ion channels in displaying a daunting diversity in ion selectivity, modes of activation, and physiological functions [**25**]. Nevertheless, they all share the common feature of six transmembrane domains, varying degrees of sequence similarity, and permeability to cations. The fourth transmembrane domain lacks the complete set of positively charged residues necessary for the voltage sensor in many voltage-gated ion channels [**27**]. Recently, the structure of a TRPV1 channel has been solved. Mammalian TRP channel proteins may be grouped into six subfamilies on the basis of amino acid sequence homology (TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML) [**41**].

Functional characterization of recombinant TRP homologues and gene knockout mouse studies revealed a plethora of stimuli that activate TRP channels [**46**]. Respective TRP homologues are susceptible to multiple activation triggers and can function as multimodal sensors. The activation triggers for mammalian TRP channels may be subdivided into three groups [**35**]. First, receptor activation is associated with TRP channels. Receptor tyrosine kinases and G protein coupled receptors (GPCRs) that activate phospholipase C (PLC) can modulate the TRP channel activity in at least three ways. The receptor stimulation at the plasma membrane (PM) activates PLC via tyrosine kinases and GTP-binding G proteins, which generates the

following messengers, (a) inositol 1,4,5-trisphosphate (IP_3) and (b) diacylglycerol (DAG) from (c) phosphatidylinositol-4,5-bisphosphate (PIP_2). This induces an increment of cytosolic Ca^{2+} concentration, IP_3-induced Ca^{2+} release via IP_3 receptors (IP_3Rs) from the intracellular Ca^{2+} store, the endoplasmic reticulum (ER), and Ca^{2+} influx across the PM. Evidence is stronger for the second (b) and third (c), but many fundamental mechanistic questions remain unanswered. Second, ligand activation is associated with TRP channels. Ligand that activate TRP channels may be classified as (a) external small organic molecules, synthetic compounds and natural products (I cilin, 2-APB, and capsaicin), (b) endogenous products from the lipid metabolism or lipids (anandamide, phosphoinositides, diacylglycerols and eicosanoids), (c) inorganic ions with Mg^{+2} and Ca^{+2} or (d) purine nucleotides and their metabolites [βNAD+, and adenosine diphosphoribose (ADP-ribose)]. Lastly, TRP channels can be activated directly. Changes in temperature strongly modulate the opening of TRPV and TRPM by poorly understood mechanisms. Other direct activators involve channel phosphorylation, conformational coupling to IP_3 receptors, and mechanical stimuli [**35**].

Many diseases are associated with proteins embedded in cell membranes. Ion channels represent one group of such molecules. Channels form pores in cell membranes and allow particular ions to pass through them based on the concentration gradient. TRP channels are associated with Ca^{+2} signaling. Thus, TRP channel can strongly affect a variety of cellular and systemic processes. TRPV1 has been reported in the joint synovia and may be associated with the pathogenesis of the osteoarthritis. Cold hyperalgesia, a symptom of

inflammatory and neuropathic pain is associated with TRPA1. In posterior root ganglion (DRG) neuron, TRPA1 increases inflammation and nerve injury without affecting the abundance of the cold-sensing channel TRPM8 [**26**]. TRPC6 is reported in the late-onset of human proteinuric kidney disease, focal and segmental glomerulosclerosis (FSGS) [**28**]. Airway tracheal and bronchial smooth muscles express TRPC4 and TRPC5 that are recognized as possible candidates in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD) [**27**].

The TRPC subfamily consists of seven proteins designated as TRPC1 to 7, which can be further divided into four subgroups based on their sequence homology and functional similarities: (1) TRPC1, (2) TRPC4 and TRPC5, (3) TRPC3, TRPC6 and TRPC7, and (4) TRPC2 [**27, 31**]. The activation of TRPC channels involves $G\alpha_{q/11}$ proteins and PLC. Molecules downstream of PLC, such as IP_3 and DAG, and PIP_2 hydrolysis have been suggested as activators and as an activation mechanism for TRPC channels, respectively [**34**].

TRPC4 channel consist of six transmembrane domains, intracellulary located amino and carboxyl termini and a putative pore-forming region [**28**]. TRPC4 is expressed in diverse organs and cell types including the dendrites and soma of various types of neurons, smooth muscles, the cardiovascular system, including endothelial and cardiac cells, skeletal muscle cells, the myometrium, the kidney, and immune cells such as mast cells. Activation of TRPC4 channels by agonists induced Ca^{2+} entry directly or indirectly via depolarization and activation of voltage-gated Ca^{+2} channels. TRPC4 channels

interact with proteins in specific membrane microdomains (caveolin-1), scaffolding proteins (HNERF) and $G\alpha_i$-coupled receptors [**28**]. The diversity of TRPC4 channels interfere with the development of specific agonists or antagonists, but, recently, ML204 was identified as a blocker of both recombinant and endogenous TRPC4 channel that lacks activity on most voltage-gated channels and other TRPs except TRPC3 and TRPC5 [**23**]. The physiological role of these channels was recently established, demonstrating that TRPC4 and TRPC6 are the molecular candidates for non-selective cation channels activated by the muscarinic receptor stimulation (*mIcat*) in visceral smooth muscle cells. *mIcat* mediates the physiological action of acetylcholine in evoking smooth muscle contraction [**44**].

One of the largest and most various membrane protein families is the G protein coupled receptors (GPCRs) family. GPCRs are encoded by more than 800 genes in the human genome [**45**]. They play a role in mediating cellular response to extracellular signaling ranging from photons and small molecules to peptide and proteins and present a seven-transmembrane topology (7-TM). Ligand binding to GPCRs induces conformational changes that influence the activation of complex cytosolic signaling networks, resulting in a cellular response. Since 2007, the GPCR structure and function, which had been probed using biochemical and physical methods, is interpreted via rhodopsin-based modeling [**15**]

Muscarinic acetylcholine receptors are known to belong to the GPCR family. They are expressed in the central nervous system as well as in non-neural tissues. There are five muscarinic receptor (M_1R-M_5R) proteins that

present a rhodopsin-like architecture [**9**]. M_1R, M_3R and M_5R are coupled to $G\alpha_{q/11}$, and activate phospholipase C. M_2 and M_4 are coupled to the $G\alpha_{i/o}$ class of G protein, and abolish adenylyl cyclase activity. Activation of these G protein-gated potassium channels leads to membrane hyperpolarization in excitable cells. M_2 receptor inhibits adenylyl cyclase and prevents the adrenergic response in smooth muscle.

Heterotrimeric G proteins are the molecular switch turning on intracellular signaling cascades in response to the activation of GPCRs by extracellular stimuli. G proteins are key players in defining the specificity and feature of the cellular responses and consist of three subunits (α, β, and γ). There are 21 Gα subunits encoded by 16 genes, 6 Gβ subunits encoded by 5 genes and 12 Gγ subunits in humans [**30**]. G proteins are inactive in the heterotrimeric conformation where Gα binds GDP and constitutive βγ [**29**]. Extracellular signals such as proteins, photon, small molecules and peptides induce a conformational change permitting G-protein binding activity and catalyze GDP release from Gα. Gα binds GTP, leading to a structural rearrangement of Gα-GTP and Gβγ. Gα-GTP and Gβγ regulate downstream effector proteins. This response is concluded when Gα hydrolyzes GTP to GDP and re-binds with Gβγ. Primary sequence similarity of the Gα subunits allowed their classification into four classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_{12}$, and $G\alpha_{q/11}$ [**30**]. Gα subunits have a molecular weight of 39-52 kDa and share 35-95% sequence homology. Gβγ subunits share 50-90% sequence homology and have a molecular weight of approximately 36 kDa [**29**]. Previously, we reported that $G\alpha_i$ proteins are

potent activators of the TRPC4β channel [**13, 16**]. Among the G proteins, $G\alpha_i$ proteins are shown to be activated by extracellular carbachol stimulating the type 2 muscarinic acetylcholine receptor (M_2R). The activation can be mimicked by expressing a constitutively active form of the $G\alpha_i$ proteins.

Small GTP binding proteins were first discovered as the v-Ha-Ras and v-Ki-ras oncogenes of sarcoma viruses around 1980 [**22**]. There are monomeric G proteins with molecular masses of 20-40kDa [**39**]. Small G proteins constitute a superfamily formed by more than 100 members and exist in eukaryotes from yeast to human. Small GTP binding proteins are key regulators not only in temporal, but also in spatial determination of specific cell functions. Multiple small G protein form signaling cascades that are involved in various cellular functions, such as budding processes of the yeast and regulation of the actin cytoskeleton in fibroblasts. In addition, two distinct small G proteins associate with specific cellular functions in a cooperative or antagonistic manner. These proteins are structurally classified into at least five families: Ras, Rab, Rho, Ran, and Sar/Arf families. Ras proteins regulate cell division, proliferation, and gene expression. Rho proteins handle cytoskeleton reorganization, and vesicle trafficking. Rab proteins controll intracellular vesicle trafficking. Sar/Arf proteins are involved in microtubule organization. Ran proteins regulate the nucleocytoplasmic transport and nuclear reassembly after mitosis [**22**].

These proteins also associate with channels. TRPV5 or TRPV6 interact with Rab11 that regulates TRPV5 and TRPV6 trafficking to the plasma membrane, requiring a direct interaction. ENaC channels, present on the

apical plasma membrane, are being exchanged with channels from the intracellular pool in a Rab11A dependent manner [**14**]. RhoA modulates Kv1.2 activity and represents a link in the process of GPCR-mediated tyrosine kinase suppression of a potassium channel. The relationship between small G-protein and TRPC5 channel has been reported. Bezzerides *et al.*, [**1**] reported that rapid translocation of TRPC5 channels to the plasma membrane is induced by epidermal growth factor (EGF) *via* Rac1 small G-protein-dependent signaling pathway. However, no relationship between TRPC4 channel and small G protein has been reported.

Considering previous reports indicating that TRPC4/TRPC5 can be activated by $G\alpha_i$ [**11, 12, 13**], we investigated whether small G proteins activates TRPC4 channels. We screened 105 small G proteins, including constitutively active forms. GTPγS was used as a positive control for the activation of the TRPC4 channel. Many small G proteins inhibited GTPγS-activated TRPC4 currents, but we focused on small G proteins that activated TRPC4 channels without GTPγS. Among small G proteins, Rasd1 potently increased TRPC4 currents without GTPγS. We investigated whether Rasd1 activates the TRPC4 channel. Currently, whether Rasd1 is involved in the activation process of TRPC4 remain unknown. Since both Rasd1 and TRPC4 were reported to be involved in insulin secretion in pancreatic β-cell [**27, 41**], *in vitro* mechanistic studies would help elucidating serum glucose level homeostasis.

MATERIALS AND METHODS

1. Cell culture and transient transfection

Human embryonic kidney (HEK293) cells (ATCC, Manassas, VA, USA) were maintained according to supplier's recommendations. The insulin–secreting cell line INS-1 was kindly supplied by Dr. Won-Kyung Ho (Seoul National University). For transient transfection, the cells were seeded in 12-well plates. The following day, 0.5 μg/well pcDNA3 vector containing the cDNA was mixed and transfected using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals, USA), as detailed in manufacturer's protocol. Mouse TRPC4α-EGFP and mouse TRPC4β-EGFP were kindly donated by Dr. Michael Schaefer (University of Leipzig). Human TRPC5-EGFP was kindly donated by Dr. Shuji Kaneko (Kyoto University). Mouse TRPC6-GFP was kindly donated by Dr. Yasuo Mori (Kyoto University). Human Rasd1-ECFP, human Rasd1^{G31V}-ECFP, human Rasd1^{G81A}-ECFP, human Rasd1^{A178V}-ECFP, human Rasd1^{S33V}-ECFP, K-Ras^{Q61L}-ECFP, H-Ras^{Q61L}-ECFP, N-Ras^{Q61L}-ECFP, R-Ras1^{Q87L}-ECFP, R-Ras2^{Q71L}-ECFP, Di-Ras2^{G16V}-ECFP, RasL^{Q62L}-ECFP, and RasL11B^{S42V}-ECFP were kindly donated by Dr. Won Do Heo (Korea Advanced Institute of Science and Technology) [**12**]. $G\beta_1$, $G\beta^{W99A}$, $G\beta^{I80A}$, βARK, $G\gamma_1$, $G\gamma_7$ was kindly donated by Dr. Seong Woo Jeong (Sogang University) [**13**]. Dominant negative forms $G\alpha_{i1}{}^{G202T}$, $G\alpha_{i2}{}^{G203T}$ and $G\alpha_{i3}{}^{G202T}$ were kindly donated by Dr. Yong-Sung Juhnn (Seoul National University) [**2**]. Type 2 muscarinic acetylcholine receptor ($mAChR_2$ or M_2R) was purchased from Missouri S&T cDNA resource center (Missouri, USA). The co-

expression of TRPC channels with small G proteins or receptors was achieved through a channel-to-G-protein transfection ratio of 1:1:1. After 24 to 40 h, the cells were trypsinized and transferred to a small recording chamber (RC-11; Warner Instruments, USA) for whole-cell recording.

2. Electrophysiology

The whole-cell configuration was used to measure the TRPC channel current in HEK cells as described previously **[13, 24**]. The cells were transferred to a small chamber on the stage of an inverted microscope (TE2000S, Nikon Japan) and attached to coverslips in the small chamber for 10 min prior to patch recording. The currents were recorded using an Axopatch 200B patch clamp amplifier (Axon Instrument, USA). The bath solutions were constantly perfused with a physiological salt solution at a rate of 1–2 ml/min. Glass microelectrodes with 2–4 megaohm resistance were used to obtain gigaohm seals. After establishing the whole-cell configuration, the external solution was changed from normal Tyrode (NT) to Cs^+-rich external solution for measuring TRPC4 current in HEK293 cells. Measuring the activity of TRPC4 channel can be manipulated by altering the extracellular ion composition, especially for cations with high permeability [**19, 25**]. Voltage ramp pulse was applied from +100 mV to -100 mV for 500 ms at -60 mV holding potential. The pCLAMP (version 10.2) and Digidata 1440A (Axon Instruments, USA) software programs were used for data acquisition and the application of command pulses. The data were filtered at 5 kHz and displayed on a computer monitor. The data were analyzed using

pCLAMP (version 10.2) and Origin software (Microcal Origin, version 8; USA). For recordings of the TRPC channels, we used normal Tyrode solution (NT) unless otherwise mentioned and occasionally Cs^+-rich solution. The normal Tyrode solution contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES with a pH of 7.4 adjusted with NaOH. The Cs^+-rich external solution contained equimolar CsCl rather than NaCl and KCl. pH was adjusted to 7.4 using CsOH. For receptor stimulation, 100 μM carbachol (carbamoylcholine chloride) was added to each extracellular bath solution. The internal solution contained 140 mM CsCl, 10 mM HEPES, 0.2 mM Tris-GTP (Tris-guanosine 5'-triphsosphate) with a pH of 7.3 adjusted with CsOH. For pertussis toxin experiment, 100 ng/ml of the toxin was pretreated in 12-well plate 16 hours before trypsinization. For dexamethasone experiment, 100 μM of dexamethasone was pretreated in 12-well plate 48 hours before trypsinization.

Perforated patch clamp configuration was used for the electrophysiological recording of INS-1 cells. External and intracellular solution was identical to whole-cell configuration except for 10 μM nifedipine in extracellular solution and 200 μM nystatin in intracellular solution. One hundred nanomolar of dexamethasone was pretreated to INS-1 cells for corresponding experimental conditions.

Pertussis toxin was purchased from Calbiochem (USA), and all other reagents were purchased from Sigma (Sigma-Aldrich, USA)

3. Western blot analysis

The crude extracts were collected and incubated by RIPA buffer (120 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$, 50 mM HEPES, pH 7.4, phosphatase inhibitor mixture tablet, complete protease inhibitor mixture tablet (Roche Applied Science, USA), and 0.5% Triton X-100). The protein concentration was determined by BCA assay kit (Pierce biotechnology, USA). The samples were resolved using 10% SDS-PAGE and probed for indicated antibodies. Anti-Rasd1 was supplied by Abcam (Abcam, UK). Anti-β tubulin was purchased from Santa Cruz (Santa Cruz biotechnology, USA).

4. Image Quantification and FRET Measurements

HEK293 cells were cultured in 35-mm coverslip bottom dish to obtain image and to measure FRET efficiency. To obtain the image and FRET efficiency of a cell, we used an inverted microscope (IX70, Olympus, Japan) with a 60× oil objective lens and the 3 cube FRET calculation controlled by MetaMorph 7.6 (Molecular Devices). We mainly used 3 cube FRET. 3 cube FRET efficiency (cube settings for CFP, YFP, and Raw FRET) were acquired from a pE-1 Main Unit to 3 cube FRET (excitation, dichroic mirror, filter) through a fixed collimator: CFP (ET 435/20 nm, ET CFP/YFP, ET 470/24 nm, chroma); YFP (ET500/20m, ET CFP/YFP, ET535/30 nm, chroma); and Raw FRET (ET435/20 nm, ET CFP/YFP, ET535/30 nm, Chroma). The excitation LED and filter were sequentially rotated, rotation period for each of filter cubes was ～0.5 s, and all images (three for CFP/YFP/Raw FRET, respectively) were obtained within 1.5 s. Each of the images was acquired on a cooled 10 MHz (14 bit) CCD camera (DR-328G-C01-SIL: Clara, ANDOR Technology)

with an exposure time of 100 ms with 2 × 2 or 3 × 3 binning under the control of MetaMorph 7.6 software. Our FRET recording of the fluorophores was restricted in a range of CFP/YFP ratio being 0.5 to 2.0.

5. Statistics

Results are expressed as means ± S.E.M. Results were compared using Student's t test between two groups. $P < 0.05$ was considered statistically significant and indicated as *. All statistical analyses were done with Origin Pro 8 software (OriginLab, MA, USA).

RESULTS

Activation of mTRPC4β channel by small G protein families

Small G proteins are key regulators of various cellular and developmental events, including differentiation, vesicle transport, control of cytoskeleton and cell division. These proteins are known to associate with diverse channels. Activators of TRPC4 channels have already been reported, but selective activators have not. We were interested in TRPC4 currents increased by small G proteins without GTPγS. GTPγS was used as a positive control for the activation. Each small G proteins were co-transfected with mTRPC4β channels in HEK 293 cells. First, the whole-cell current was measured in HEK293 cells expressing 48 different Rab proteins and the mTRPC4β channel with or without GTPγS (**Fig. 1–5 and Table 1-5**). Rab3D, Rab5B, Rab5C, Rab20, Rab30 and Rab33A increased TRPC4 currents without GTPγS. Rab3D mediates diverse types of regulated exocytic events [**38**]. Rab5 localized in early endosomes, phagosomes, caveosomes and plasma membrane, mediates endocytosis and endosome fusion of clathrin-coated vesicles (CCVs). Rab20 regulates vacuolar-type H^+ ATPase (V-ATPase) trafficking. Rab33 mediates intra-Golgi trafficking. We showed that the localization and function of Rab proteins regulated the TRPC4β channel activity (**Fig. 6**). Second, we tested 14 Rho proteins. All Rho proteins did not induce mTRPC4β current in the absence of GTPγS. In the presence of GTPγS, Rho proteins reduced mTRPC4β currents (**Fig. 7 & 8 and Table 6 & 7**). Rho proteins are involved in different stages of tumor progression and regulate

lamellipodia and filopodia formation. RhoA and Rac1 are necessary for the formation of cell junctions. Activating RhoA leads to a loss of polarity, but antagonism of RhoA by RhoE overexpression induces the multilayering of epithelial cells. Activation of Rho GTPases also controls the degradation of filopodia and lamellipodia (**Fig. 9 & 10**). Third, 11 Arf/Sar proteins also did not affect mTRPC4β currents in the absence of GTPγS and almost all proteins decreased mTRPC4β current (**Fig. 11 and Table 8**). Arf/Sar proteins appeared to regulate TRPC4β channel activity by intracellular vesicle trafficking (**Fig. 12**). Arf proteins present a distinct localization and functions in the endoplasmic reticulum. Arf1 regulates the formation of lipid droplets and the replication of several viruses with COPII. In the trans-Golgi network (TGN), Arf4 and Arf5 recruit calcium-dependent activator protein for secretion (CAPS). Sar1 recruits COPII to allow vesicle transport to the Golgi from the ER (**Fig. 13**).

Lastly, whole-cell current experiments were performed to measure the effect of 32 Ras proteins expressions (**Fig. 14-16 and Table 9-11**). Ras, Rap, Rac biological effects was influenced to multiprotein complexes that localized in distinct cellular compartments (**Fig. 17**). Ras proteins Rasd1, Rasd2, and Rit increased mTRPC4β current in absence of GTPγS. Interestingly, these proteins present sequence homology (**Fig. 18)**. They have similar GTP binding pocket domains and an effector loop which participates in protein-protein interactions with other signaling molecules and is necessary for their full biological activity [**43**]. Rasd2 is reported to bind to both α- and β-subunits of heterotrimeric G proteins and to affect signaling by both $G\alpha_{i/o}$- and

$G\alpha_{s/olf}$- coupled receptors [**8**]. Rit is an interacting partner of $G\alpha_o$ protein [**18**]. Previously it was reported that not only, $G\alpha_i$ protein potently activated TRPC4β channel, but $G\alpha_o$ protein also activated it [**11**]. Rasd2 and Rit proteins may activate TRPC4β channel through $G\alpha_{i/o}$ proteins. Rasd1, rather than the other small G proteins, potently activated TRPC4β channel without GTPγS. Therefore, we studied the relationship between Rasd1 and TRPC4β channel.

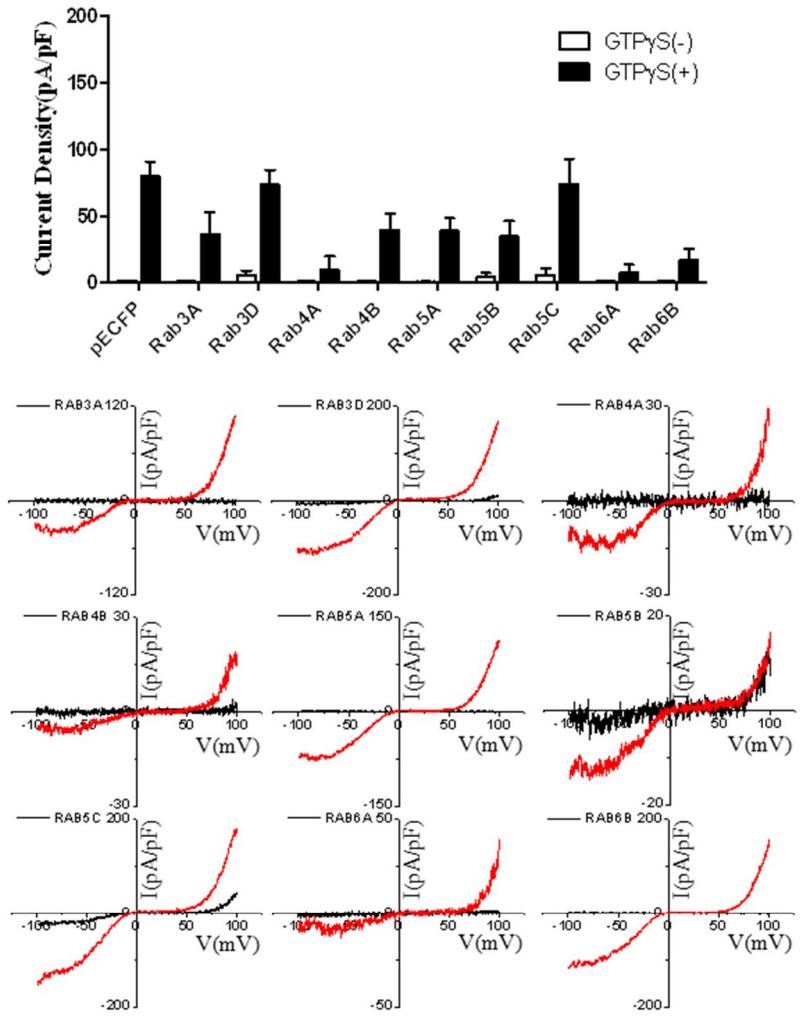


Figure 1. Specificity of Rasd1 to TRPC4β channels among different Rab proteins in Ras super family -1.

Various Rab proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rab proteins other than Rab3D, Rab5B and Rab5C such as Rab3A, Rab4A, Rab4B, Rab5A, Rab6A, and Rab6B could not activate TRPC4β channels.

Table 1. Current Densities of mTRPC4β Channel Activated by Rab Protein Families -1 (Total N = 1539).

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
Rab3A	10, 12	0.93 ± 0.23	37.1 ± 16.1
Rab3D	7, 6	5.53 ± 3.07	74.1 ± 10.9
Rab4A	5, 9	0.54 ± 0.45	9.66 ± 9.98
Rab4B	10, 10	0.92 ± 0.19	39.7 ± 12.6
Rab5A	3, 23	0.42 ± 0.18	39.4 ± 9.70
Rab5B	4, 3	4.10 ± 3.15	35.2 ± 11.4
Rab5C	4, 3	5.56 ± 4.54	74.5 ± 19.0
Rab6A	6, 6	0.69 ± 0.15	7.03 ± 6.07
Rab6B	3, 11	0.61 ± 0.34	16.5 ± 8.34

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

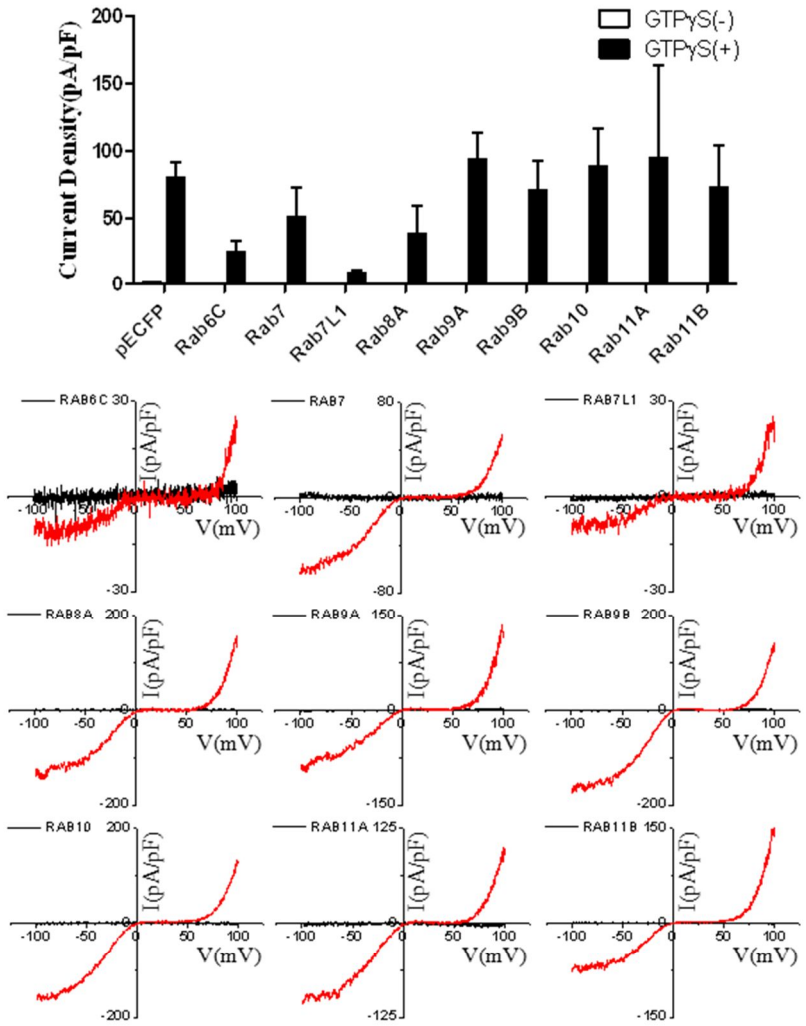


Figure 2. Specificity of Rasd1 to TRPC4β channels among different Rab proteins in Ras super family -2.

Various Rab proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rab proteins such as Rab6C, Rab7, Rab7L1, Rab8A, Rab9A, Rab9B, Rab10, Rab11A, and Rab11B could not activate TRPC4β channels.

Table 2. Current Densities of mTRPC4β Channel Activated by Rab Protein Families -2.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
Rab6C	3, 10	0.51 ± 0.10	24.4 ± 8.34
Rab7	3, 3	0.52 ± 0.13	50.3 ± 22.6
Rab7L1	6, 29	0.55 ± 0.22	9.03 ± 1.67
Rab8A	3, 5	0.58 ± 0.24	37.9 ± 21.5
Rab9A	3, 5	0.60 ± 0.10	94.0 ± 19.7
Rab9B	3, 3	0.86 ± 0.08	70.9 ± 22.0
Rab10	3, 6	0.67 ± 0.05	87.8 ± 29.3
Rab11A	3, 4	0.43 ± 0.08	94.5 ± 68.8
Rab11B	12, 10	0.72 ± 0.15	73.1 ± 30.4

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

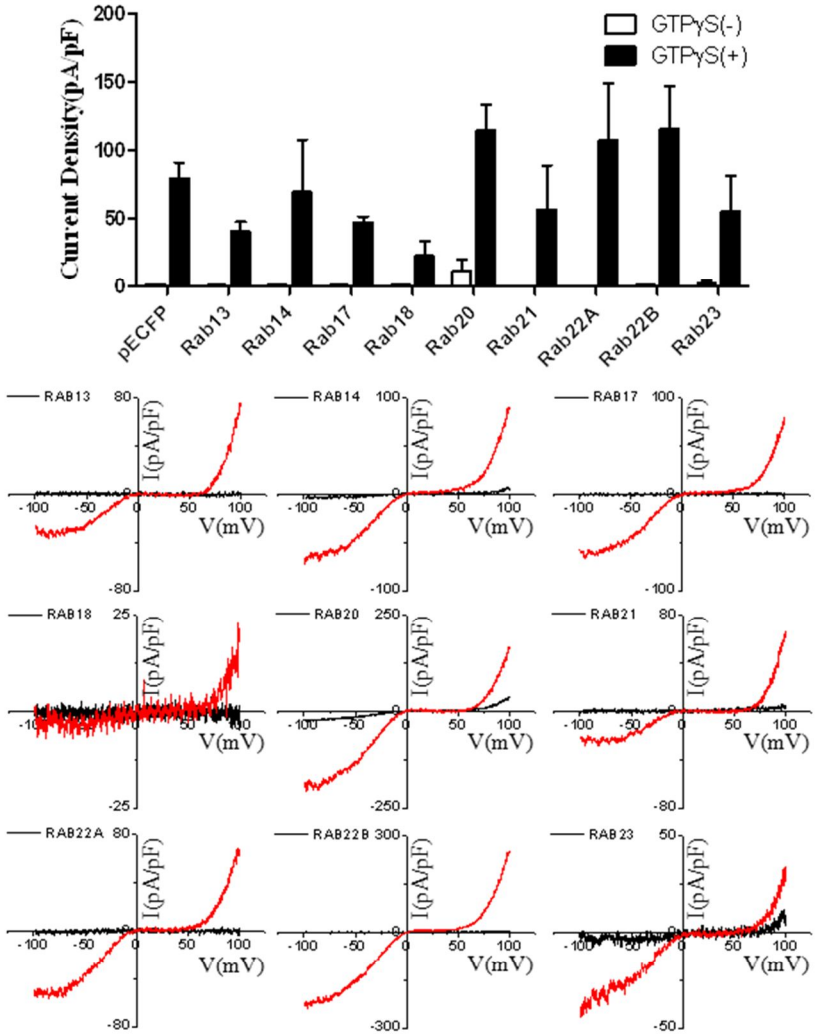


Figure 3. Specificity of Rasd1 to TRPC4β channels among different Rab proteins in Ras super family -3.

Various Rab proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rab proteins other than Rab20 such as Rab13, Rab14, Rab17, Rab18, Rab21, Rab22A, Rab22B, and Rab23 could not activate TRPC4β channels.

Table 3. Current Densities of mTRPC4β Channel Activated by Rab Protein Families -3.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
Rab13	4, 3	0.88 ± 0.14	40.7 ± 6.93
Rab14	4, 3	1.04 ± 0.57	69.5 ± 38.2
Rab17	3, 3	0.88 ± 0.21	47.6 ± 4.13
Rab18	6, 9	1.00 ± 0.32	22.7 ± 11.1
Rab20	7, 6	11.7 ± 8.25	115 ± 18.7
Rab21	3, 3	0.43 ± 0.19	57.0 ± 32.2
Rab22A	3, 4	0.49 ± 0.12	108 ± 41.9
Rab22B	3, 3	0.75 ± 0.20	116 ± 30.4
Rab23	3, 5	2.27 ± 1.61	55.1 ± 26.0

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

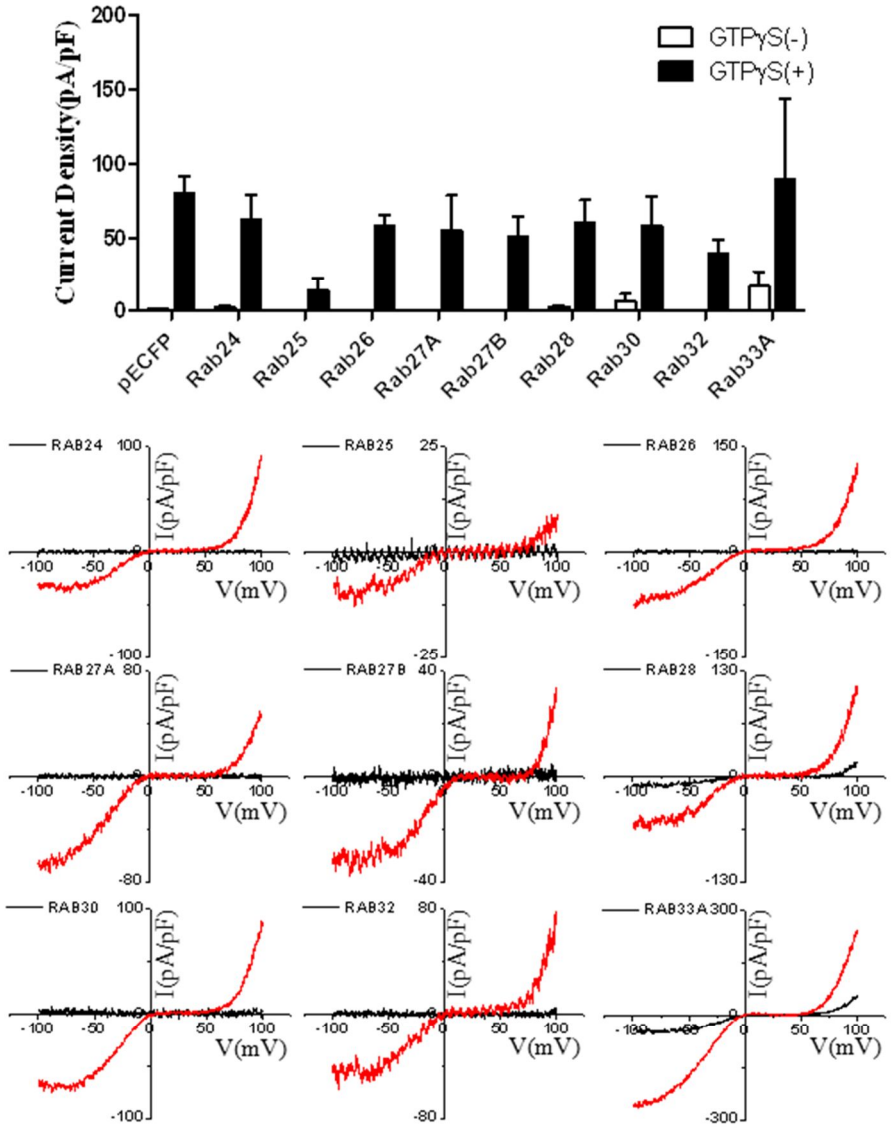


Figure 4. Specificity of Rasd1 to TRPC4β channels among different Rab proteins in Ras super family -4.

Various Rab proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rab proteins other than Rab30 and Rab33A such as Rab24, Rab25, Rab26, Rab27A, Rab27B, Rab28, and Rab32 could not activate TRPC4β channels.

Table 4. Current Densities of mTRPC4β Channel Activated by Rab Protein Families -4.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
Rab24	4, 3	2.40 ± 1.48	62.3 ± 16.8
Rab25	3, 6	0.03 ± 0.40	13.5 ± 9.13
Rab26	3, 3	0.36 ± 0.36	57.6 ± 7.47
Rab27A	3, 3	0.62 ± 0.19	54.8 ± 23.9
Rab27B	3, 3	0.61 ± 0.14	51.3 ± 12.7
Rab28	9, 5	3.00 ± 0.99	60.2 ± 15.3
Rab30	7, 4	6.84 ± 4.97	58.1 ± 20.1
Rab32	3, 6	0.33 ± 0.07	39.4 ± 9.09
Rab33A	5, 3	16.8 ± 10.0	89.4 ± 54.4

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

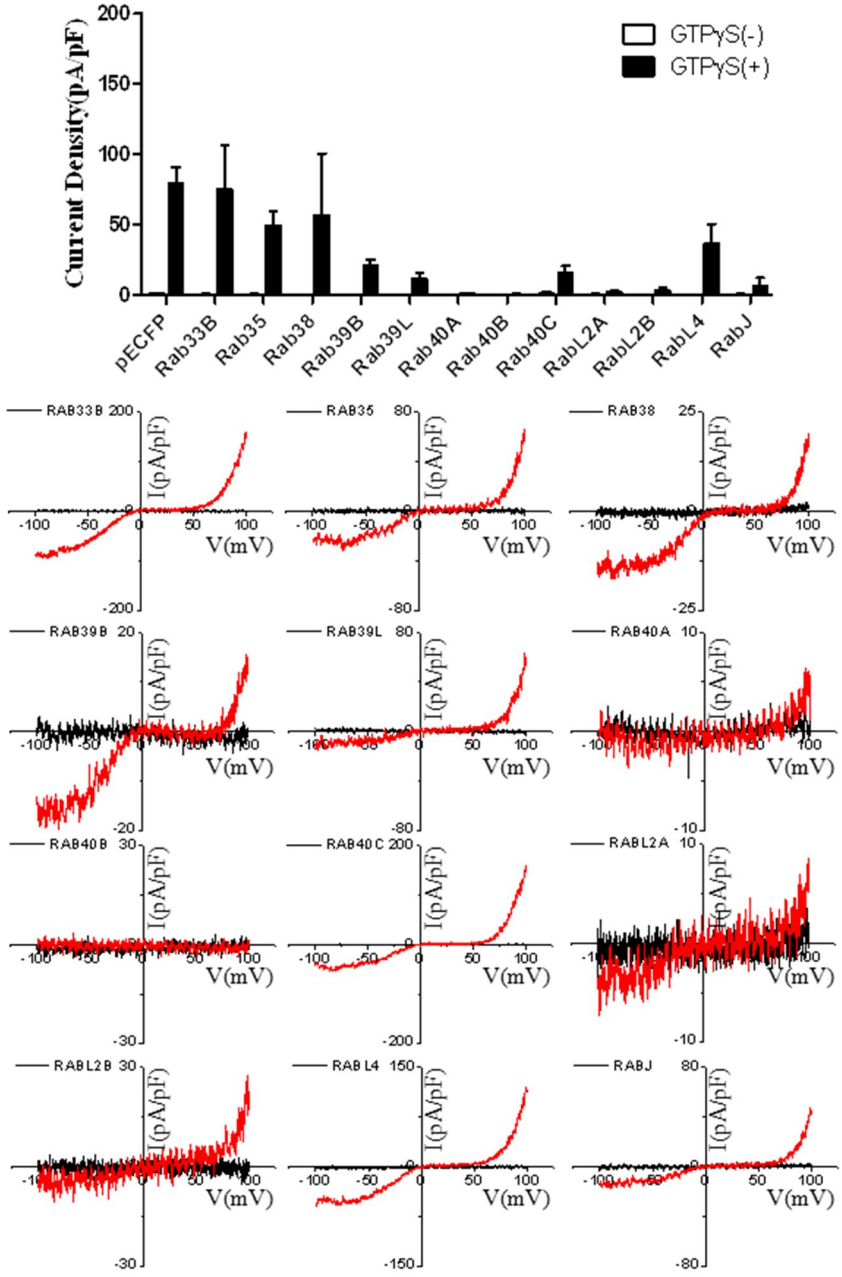

Current Density(pA/pF)
200
150
100
50
0
GTPγS(-)
GTPγS(+)
pECFP
Rab33B
Rab35
Rab38
Rab39B
Rab39L
Rab40A
Rab40B
Rab40C
RabL2A
RabL2B
RabL4
RabJ
RAB33B
RAB35
RAB38
RAB39B
RAB39L
RAB40A
RAB40B
RAB40C
RABL2A
RABL2B
RABL4
RABJ
I(pA/pF)
V(mV)

Figure 5. Specificity of Rasd1 to TRPC4β channels among different Rab proteins in Ras super family -5.

Various Rab proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rab proteins Rab33B, Rab35, Rab38, Rab39B, Rab39L, Rab40A, Rab40B, Rab40C, RabL2A, RabL2B, RabL4, and RabJ could not activate TRPC4β channels.

Table 5. Current Densities of mTRPC4β Channel Activated by Rab Protein Families -5.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
Rab33B	3, 8	0.62 ± 0.35	75.6 ± 30.8
Rab35	3, 7	0.56 ± 0.28	49.3 ± 10.7
Rab38	3, 3	0.38 ± 0.06	56.7 ± 44.1
Rab38B	7, 10	0.38 ± 0.14	20.7 ± 4.49
Rab39L	3, 5	0.60 ± 0.05	11.5 ± 4.27
Rab40A	3, 6	0.40 ± 0.21	1.17 ± 0.19
Rab40B	3, 4	0.59 ± 0.02	0.62 ± 0.46
Rab40C	5, 14	1.65 ± 1.05	15.4 ± 5.34
RabL2A	3, 7	0.54 ± 0.48	1.85 ± 1.53
RabL2B	3, 7	0.60 ± 0.54	3.31 ± 2.17
RabL4	3, 3	0.37 ± 0.15	36.6 ± 14.1
RabJ	3, 7	0.63 ± 0.35	6.68 ± 5.63

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

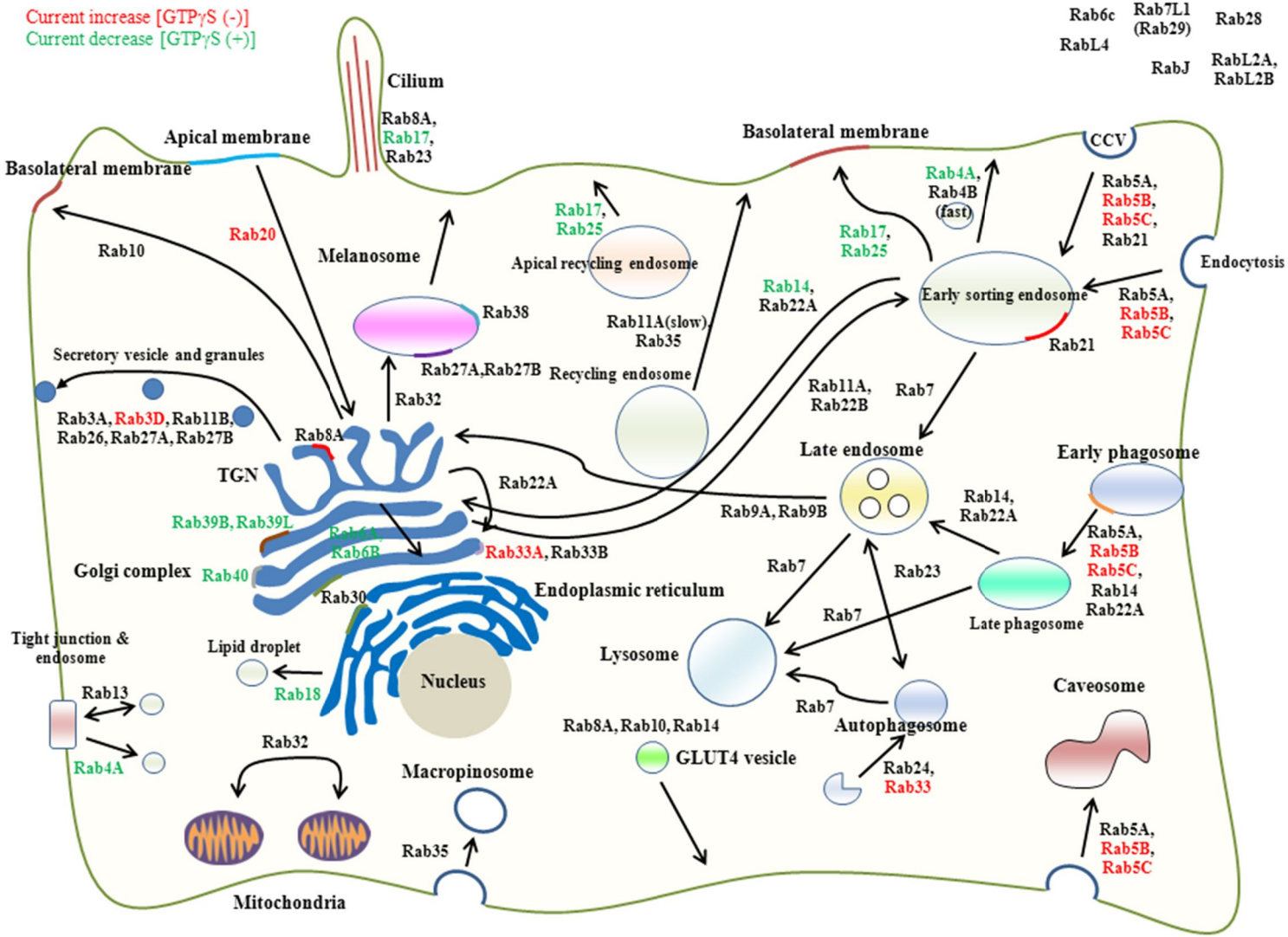

Current increase [GTPγS (-)]
Current decrease [GTPγS (+)]
Rab6c
Rab7L1 (Rab29)
Rab28
RabL4
RabJ
RabL2A, RabL2B
Cilium
Rab8A, Rab17, Rab23
Apical membrane
Basolateral membrane
Basolateral membrane
CCV
Rab4A, Rab4B (fast)
Rab5A, Rab5B, Rab5C, Rab21
Rab17, Rab25
Rab20
Rab10
Melanosome
Rab17, Rab25
Apical recycling endosome
Endocytosis
Rab14, Rab22A
Early sorting endosome
Rab5A, Rab5B, Rab5C
Rab38
Rab11A(slow), Rab35
Rab21
Secretory vesicle and granules
Rab27A,Rab27B
Recycling endosome
Rab32
Rab11A, Rab22B
Rab7
Rab3A, Rab3D, Rab11B, Rab26, Rab27A, Rab27B
Rab8A
Late endosome
Early phagosome
TGN
Rab22A
Rab9A, Rab9B
Rab14, Rab22A
Rab39B, Rab39L
Rab6A, Rab6B
Rab33A, Rab33B
Rab5A, Rab5B Rab5C, Rab14 Rab22A
Golgi complex
Rab40
Rab7
Rab23
Endoplasmic reticulum
Rab30
Late phagosome
Rab7
Tight junction & endosome
Lipid droplet
Lysosome
Nucleus
Rab18
Rab13
Caveosome
Rab7
Rab8A, Rab10, Rab14
Autophagosome
Rab32
Rab4A
GLUT4 vesicle
Macropinosome
Rab24, Rab33
Rab5A, Rab5B, Rab5C
Rab35
Mitochondria

Figure 6. Localization and function of Rab proteins regulated TRPC4β channel activity.

Rab proteins regulate the endocytic membrane trafficking, phagocytosis, ER to Golgi transport, and exocytosis. The red color represents an increase in TRPC4β currents in absence of GTPγS. The green color represents decreasing TRPC4β channel activity and the black color indicates no different compared to control with GTPγS

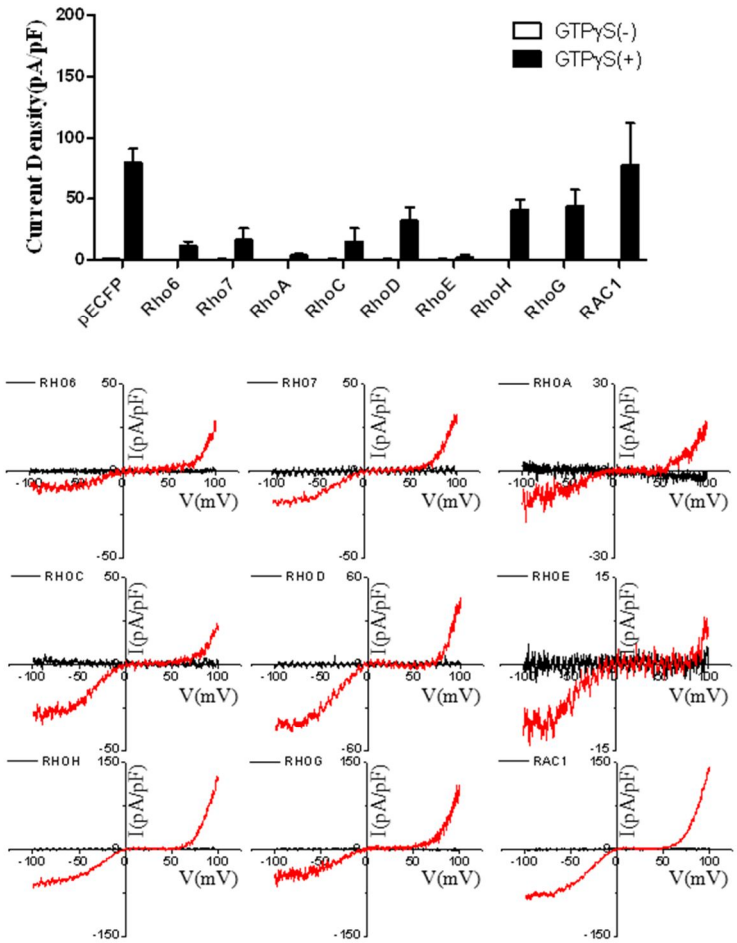


Figure 7. Specificity of Rasd1 to TRPC4β channels among different Rho proteins in Ras super family -1.

Various Rho proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rho proteins Rho6, Rho7, RhoA, RhoC, RhoD, RhoE, RhoH, RhoG, and Rac1 could not activate TRPC4β channels.

Table 6. Current Densities of mTRPC4β Channel Activated by Rho Protein Families -1.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
Rho6	7, 13	0.52 ± 0.51	11.5 ± 3.86
Rho7	3, 7	0.34 ± 0.58	16.6 ± 9.76
RhoA	15, 7	0.57 ± 0.22	4.02 ± 1.23
RhoC	3, 3	0.64 ± 0.36	15.0 ± 11.2
RhoD	3, 5	0.39 ± 0.53	32.6 ± 10.6
RhoE	3, 6	0.51 ± 0.35	2.33 ± 2.30
RhoH	3, 6	0.13 ± 0.44	41.5 ± 8.00
RhoG	3, 7	0.02 ± 0.71	44.3 ± 13.0
RAC1	3, 4	0.76 ± 0.06	77.7 ± 34.3

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

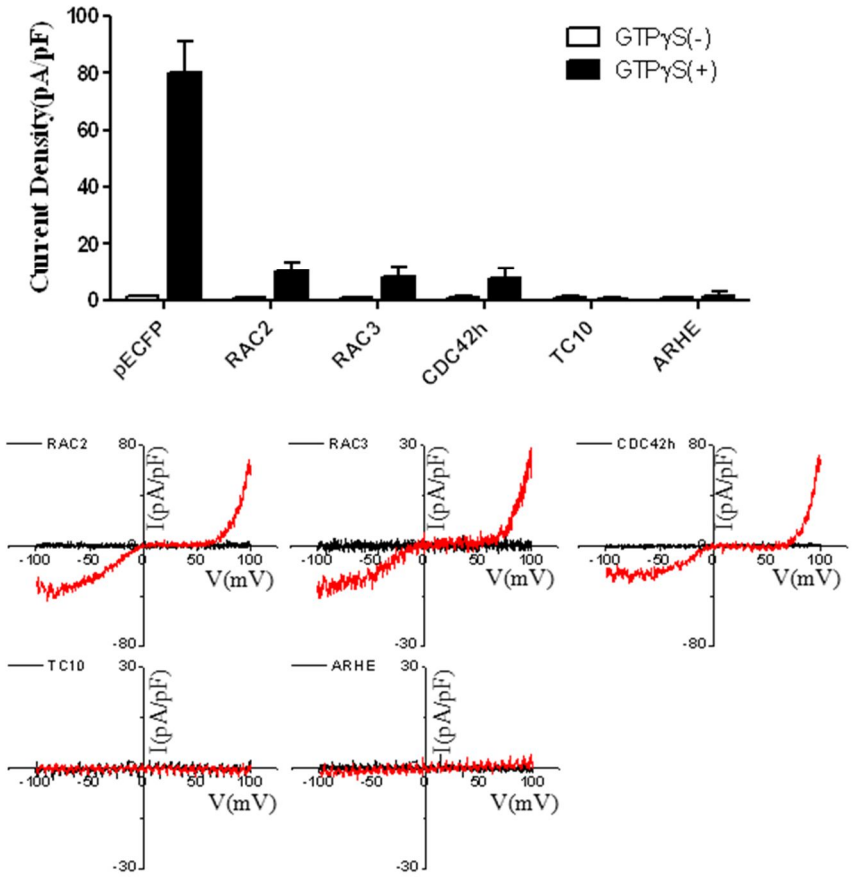


Figure 8. Specificity of Rasd1 to TRPC4β channels among different Rho proteins in Ras super family -2.

Various Rho proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Rho proteins Rac2, Rac3, CDC42h, TC10, and ARHE could not activate TRPC4β channels.

Table 7. Current Densities of mTRPC4β Channel Activated by Rho Protein Families -2.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
RAC2	3, 23	1.02 ± 0.14	10.5 ± 2.66
RAC3	7, 41	0.80 ± 0.15	8.40 ± 3.29
CDC42h	3, 7	0.63 ± 0.67	7.67 ± 3.50
TC10	3, 4	1.04 ± 0.18	0.41 ± 0.44
ARHE	3, 6	0.85 ± 0.11	1.22 ± 1.89

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

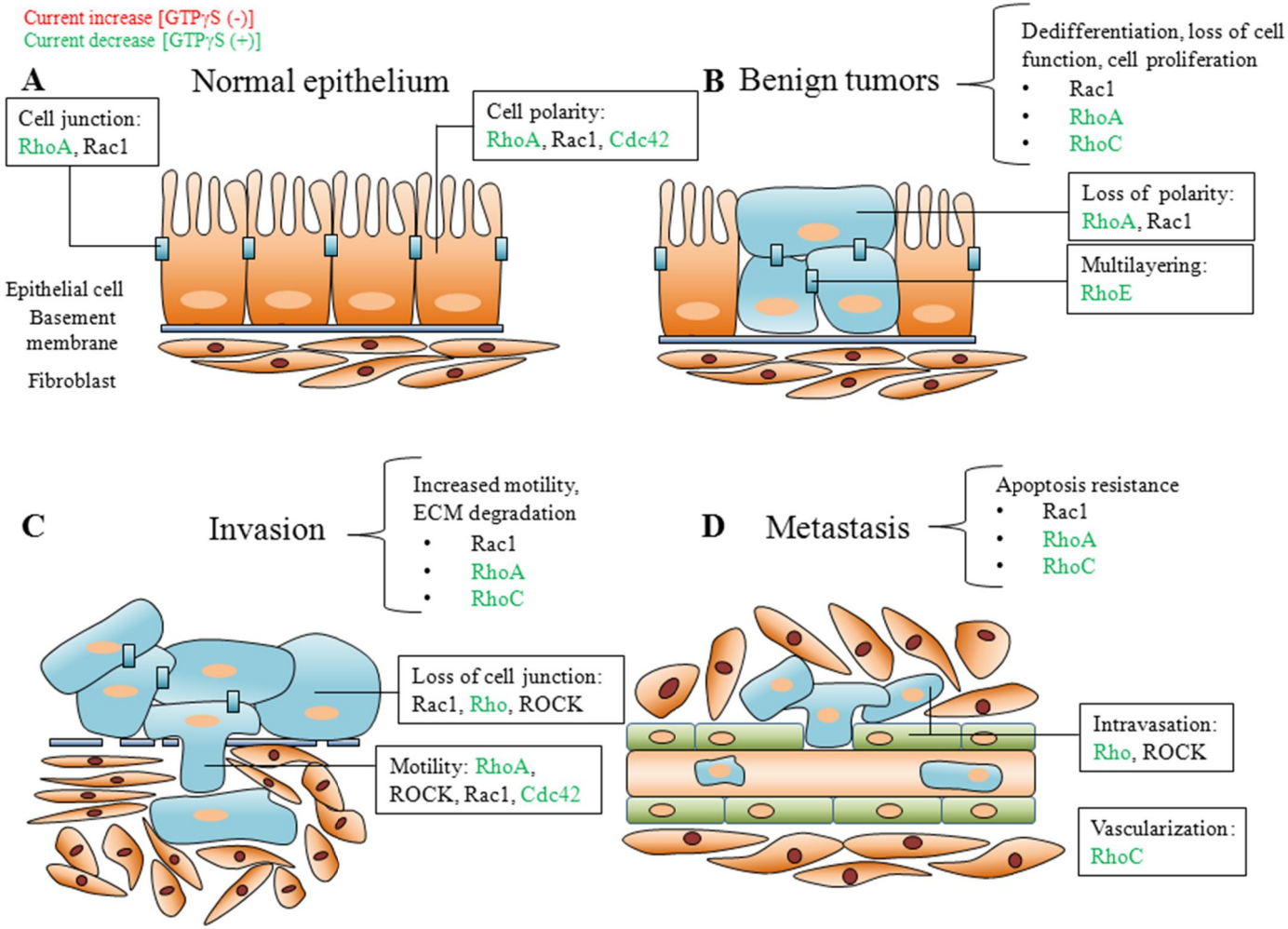

Current increase [GTPγS (-)]
Current decrease [GTPγS (+)]
A
Normal epithelium
Cell junction:
RhoA, Rac1
Cell polarity:
RhoA, Rac1, Cdc42
Epithelial cell
Basement membrane
Fibroblast
B Benign tumors
Dedifferentiation, loss of cell function, cell proliferation
• Rac1
• RhoA
• RhoC
Loss of polarity:
RhoA, Rac1
Multilayering:
RhoE
C
Invasion
Increased motility, ECM degradation
• Rac1
• RhoA
• RhoC
Loss of cell junction:
Rac1, Rho, ROCK
Motility: RhoA, ROCK, Rac1, Cdc42
D
Metastasis
Apoptosis resistance
• Rac1
• RhoA
• RhoC
Intravasation:
Rho, ROCK
Vascularization:
RhoC

Figure 9. Involvement of Rho proteins on TRPC4β channel activity

(A) Maintenance of normal epithelial polarity: Rac1 and RhoA are crucial for the formation of cell junction. Rac1, Cdc42 and RhoA are required for cell polarity. (B) Benign tumors: Loss of polarity and multilayering. Rho proteins induce tumor development by stimulating dedifferentiation and growth. Inhibition of Rac1 leads to a defect in depositing laminin asymmetrically and subsequent loss of polarity. Conversely, RhoA activation leads to the loss of polarity, but RhoA antagonism by RhoE overexpression induced the multilayering of epithelial cells. (C) Locally invasive tumors: Loss of tissue boundaries and increased motility. Rho proteins induce tumor development by altering cell-cell and cell-matrix adhesion. Modulation of Rho, ROCK, and Rac1 activation can lead to loss of cell junctions. Increased activity of Rac1, Cdc42, RhoA, and ROCK can cause elevated motility. Rac1 and RhoA regulate the expression of proteases that facilitate motility by degrading other extracellular matrix compounds and basement membrane. (D) Metastasis at a distant site: extravasation and intravasation. ROCK and Rho are required for tumor cells to cross endothelial cell layers. RhoC promotes the expression of angiogenic factors, leading to an increase in tumor vascularization. TRPC4β activity is indicated by the red color, showing an increase in TRPC4β currents in absence of GTPγS. The green color represents a decrease in the TRPC4β channel activity and the black color indicates no different with control with GTPγS.

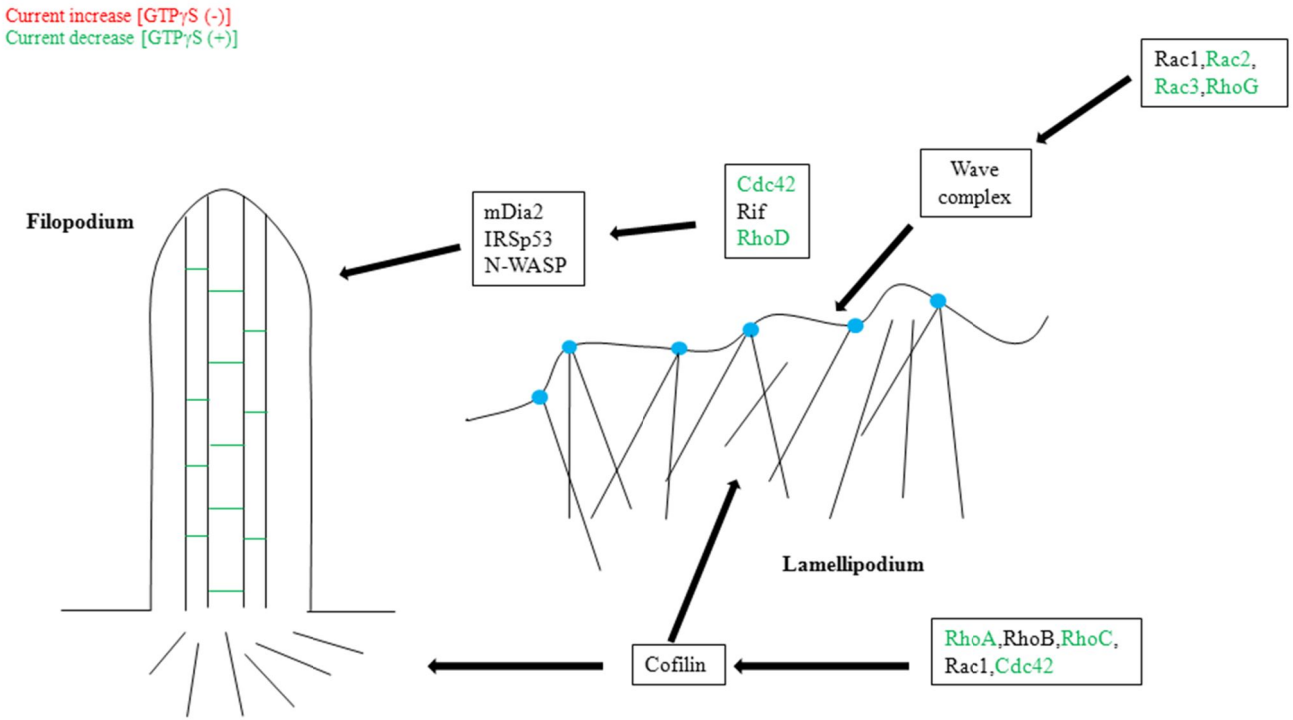

Current increase [GTPγS (-)]
Current decrease [GTPγS (+)]
Rac1,Rac2,
Rac3,RhoG
Wave
complex
Cdc42
Rif
RhoD
mDia2
IRSp53
N-WASP
Filopodium
Lamellipodium
RhoA,RhoB,RhoC,
Rac1,Cdc42
Cofilin

Figure 10. Rho GTPase regulation of lamellipodia and filopodia formation.

Membrane-bound, activated Rho GTPases initiate the formation of filopodia involving mDia2, IRSp53 and N-WASP and promote lamellipodia by interaction with the WAVE complex. The WAVE complex in turn binds to the Arp2/3 complex, which nucleates actin filaments. The WAVE complex can leave the filament and be replaced by VASP (following the hypothetical model of Urban et al. (2010)). Rho GTPases control the degradation of filopodia and lamellipodia by regulating the actin filament severing by cofilin.

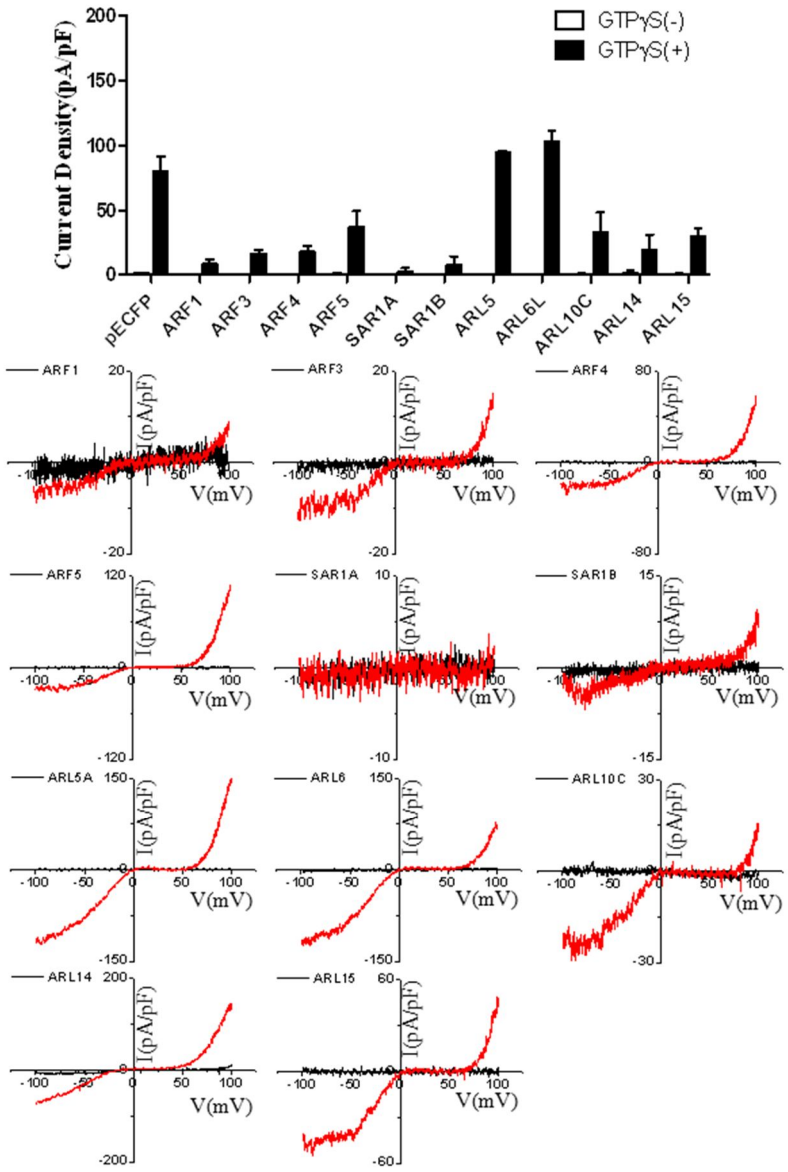


Figure 11. Specificity of Rasd1 to TRPC4β channels among different Arf/Sar proteins in Ras super family.

Various Arf proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Arf proteins Arf1, Arf3, Arf4, Arf5, Sar1A, Sar1B, Arl5, Arl6L, Arl10C, Arl14, and Arl15 could not activate TRPC4β channels.

Table 8. Current Densities of mTRPC4β Channel Activated by Arf/Sar Protein Families.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
ARF1	3, 13	0.79 ± 0.14	8.38 ± 4.02
ARF3	3, 7	0.76 ± 0.11	16.8 ± 2.96
ARF4	4, 10	0.20 ± 0.57	18.0 ± 4.06
ARF5	3, 5	0.97 ± 0.60	37.4 ± 11.9
SAR1A	6, 15	0.53 ± 0.27	3.09 ± 2.48
SAR1B	3, 3	0.72 ± 0.22	8.16 ± 6.48
ARL5	3, 3	0.32 ± 0.05	94.9 ± 1.38
ARL6L	5, 3	0.45 ± 0.25	103 ± 8.29
ARL10C	3, 3	0.17 ± 0.88	32.8 ± 15.7
ARL14	3, 4	1.90 ± 1.68	19.7 ± 11.5
ARL15	3, 5	0.79 ± 0.25	30.1 ± 6.12

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

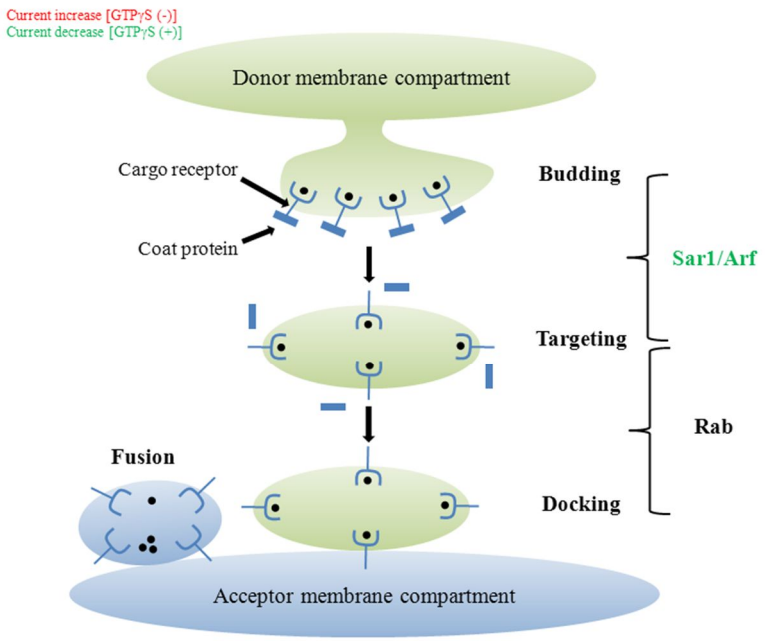


Figure 12. Intracellular vesicle trafficking. Rab and Arf proteins.

Rab11BP/Rabphilin-11, a downstream effector of Rab11, is mainly implicated in vesicle recycling, and directly interacts with mammalian Sec13. Substantial evidence has accumulated that most Rab proteins regulate targeting, docking, and fusion processes, but that some of them regulate the budding process, which is mainly regulated by Sar1/Arf proteins

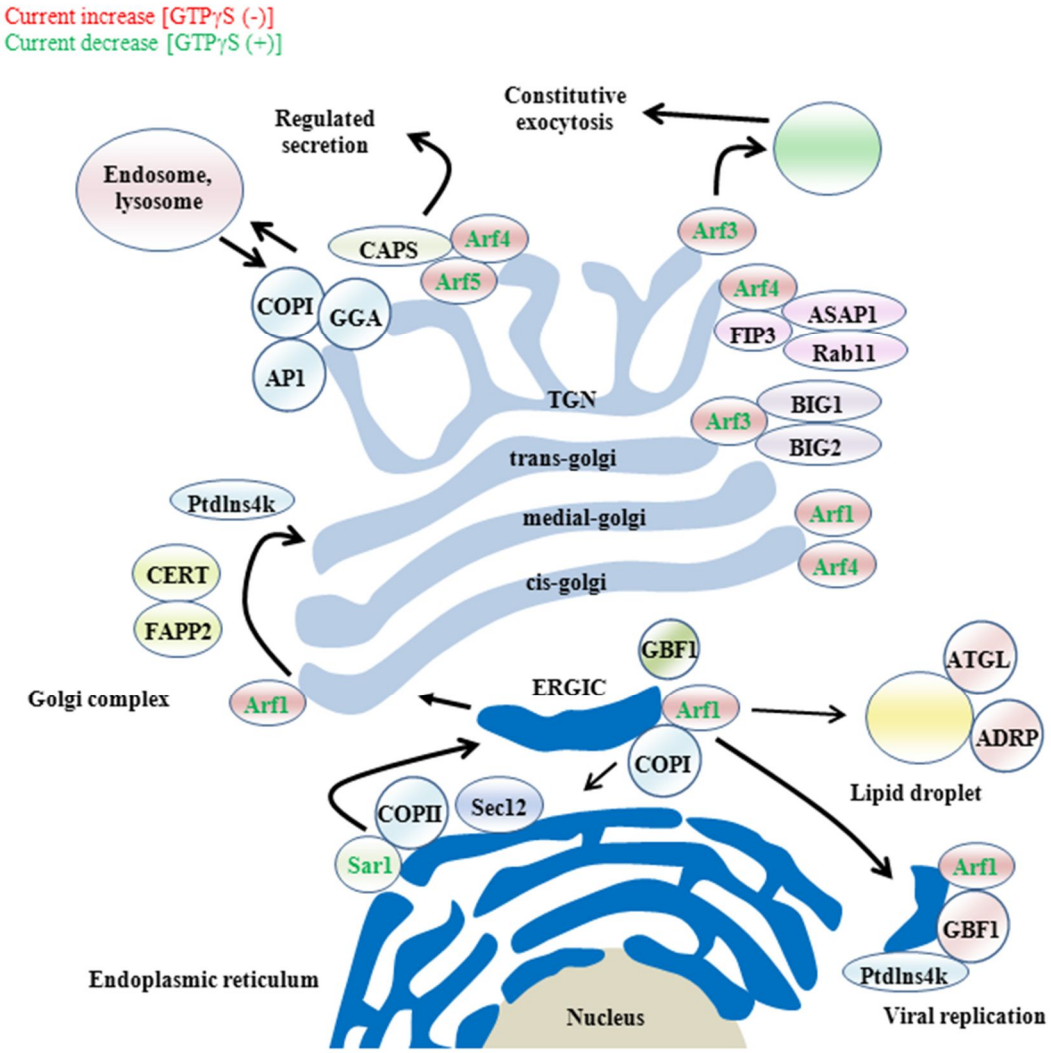

Current increase [GTPγS (-)]
Current decrease [GTPγS (+)]
Constitutive exocytosis
Regulated secretion
Endosome, lysosome
CAPS
Arf4
Arf5
Arf3
COPI
GGA
AP1
Arf4
ASAP1
FIP3
Rab11
TGN
Arf3
BIG1
BIG2
trans-golgi
Ptdlns4k
medial-golgi
Arf1
CERT
FAPP2
cis-golgi
Arf4
GBF1
ATGL
ERGIC
Golgi complex
Arf1
Arf1
ADRP
COPI
Lipid droplet
COPII
Sec12
Sar1
Arf1
GBF1
Endoplasmic reticulum
Ptdlns4k
Nucleus
Viral replication

Figure 13. Arf and Arl function in the secretory pathway and in specialized transport

Arf proteins present distinct localizations and functions in the endoplasmic reticulum (ER)–Golgi system. Arf1 and Arf4 localize to the early cis-Golgi and ARF3 specifically localizes to the trans-Golgi network (TGN). In addition to the recruitment of coat proteins (GGA (Golgi-localized, γ-ear-containing, ADP-ribosylation factor-binding protein), coat protein I (COPI), and adaptor protein 1 (AP1)) to the Golgi, Arf1 binds to ceramide transfer (CERT) and FAPP2 to mediate the transport of ceramide and glucosylceramide lipids from the cis-Golgi to the trans-Golgi. In the ER–Golgi intermediate compartment (ERGIC), Arf1 and its guanine nucleotide exchange factor (GEF) GBF1 act with COPII to regulate the formation of lipid droplets and for the replication of several viruses. CAPS (Calcium-dependent activator protein for secretion), which is involved in regulated secretion, is recruited to the TGN by Arf4 and Arf5. In the ER, Sar1, which is activated by SEC12, recruits COPII to allow vesicle transport to the Golgi

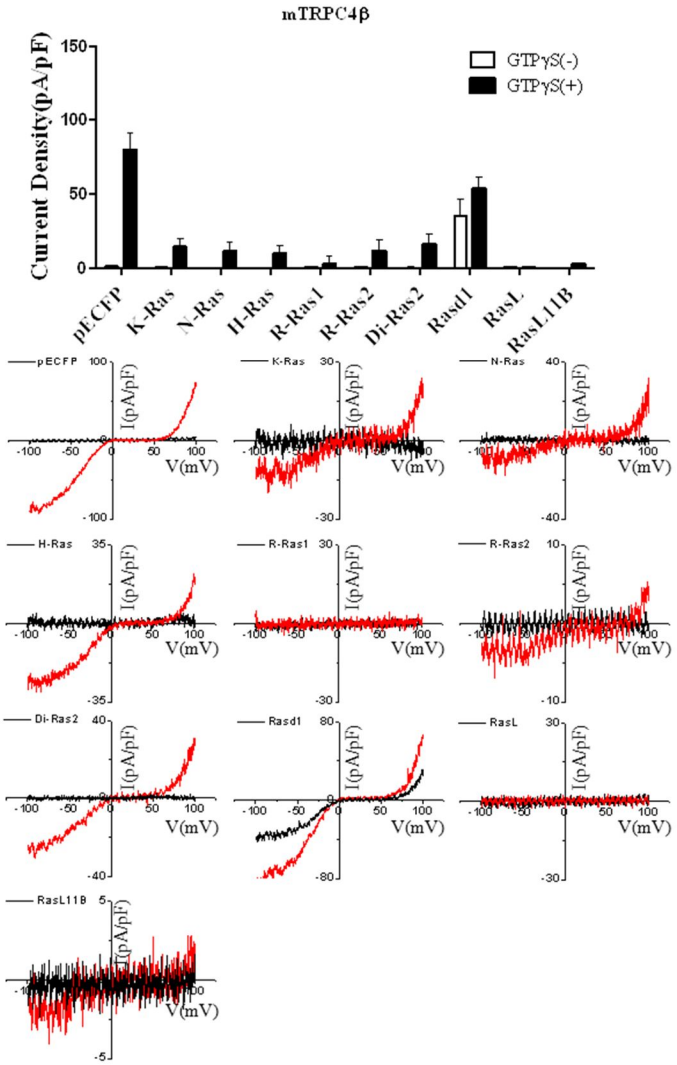


Figure 14. Specificity of Rasd1 to TRPC4β channels among different Ras proteins in Ras super family -1.

Various Ras proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Ras proteins other than Rasd1 such as K-Ras, N-Ras, H-Ras, R-Ras1, R-Ras2, Di-Ras2, RasL, and RasL11B could not activate TRPC4β channels.

Table 9. Current densities of mTRPC4β channel activated by Ras protein families -1.

Proteins	n*	I (pA/pF)† GTPγS (-)	I (pA/pF) GTPγS‡ (+)
ECFP§	215, 243	1.39 ± 0.24	79.9 ± 11.2
K-Ras	5, 15	0.57 ± 0.35	14.1 ± 6.00
N-Ras	5, 8	0.40 ± 0.10	11.2 ± 6.20
H-Ras	5, 5	0.23 ± 0.01	9.76 ± 5.73
R-Ras1	5, 5	0.72 ± 0.55	2.75 ± 5.22
R-Ras2	5, 5	0.74 ± 0.17	11.7 ± 7.35
Di-Ras2	5, 9	0.06 ± 1.08	16.2 ± 6.61
Rasd1	10, 10	35.7 ± 11.2	53.9 ± 7.91
RasL	5, 5	0.20 ± 0.23	0.60 ± 0.46
RasL11B	5, 5	0.43 ± 0.07	2.55 ± 0.24

*: Sample numbers of GTPγS(-) and GTPγS(+) groups.
†: Whole-cell current density
‡: Non-hydrolyzable Guanine Triphosphate
§: Enhanced cyanine fluorescent protein

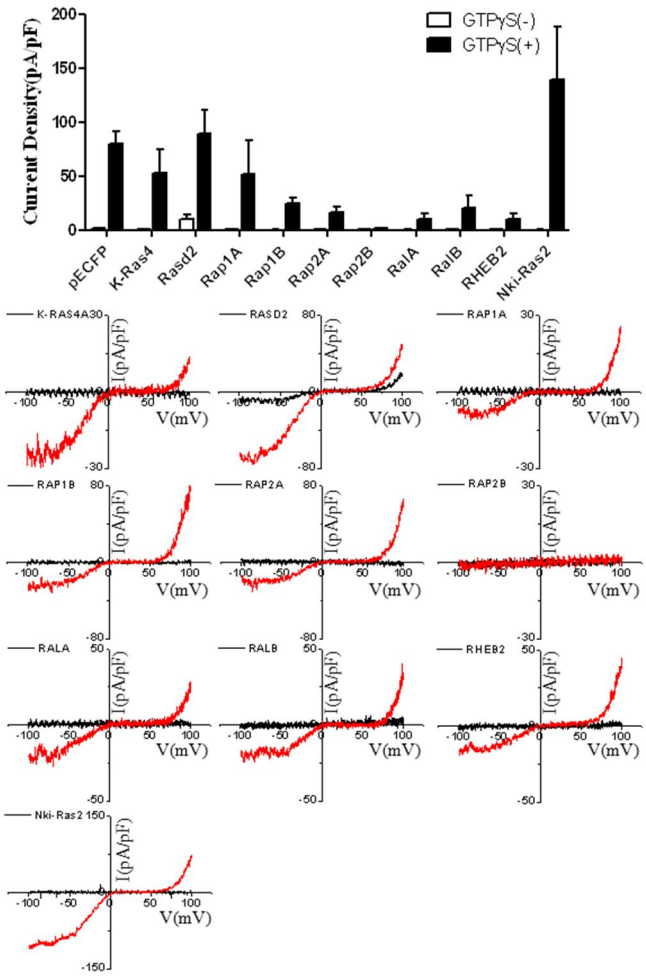


Figure 15. Specificity of Rasd1 to TRPC4β channels among different Ras proteins in Ras super family -2.

Various Ras proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Ras proteins other than Rasd2 such as K-Ras4, Rap1A, Rap1B, Rap2A, Rap2B, RalA, RalB, RHEB2, and Nki-Ras2 could not activate TRPC4β channels.

Table 10. Current Densities of mTRPC4β Channel Activated by Ras Protein Families -2.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
K-Ras4	3, 3	0.36 ± 0.29	11.3 ± 6.16
Rasd2	10, 12	9.86 ± 4.82	89.1 ± 21.9
Rap1A	3, 10	0.50 ± 0.19	51.5 ± 31.5
Rap1B	3, 3	0.33 ± 0.09	25.3 ± 4.50
Rap2A	5, 7	0.48 ± 0.22	16.9 ± 4.94
Rap2B	3, 10	0.33 ± 0.09	25.3 ± 4.50
RalA	3, 10	0.32 ± 0.05	9.88 ± 5.92
RalB	3, 8	0.60 ± 0.25	20.9 ± 11.2
RHEB2	3, 7	0.55 ± 0.15	10. 6 ± 4.79
Nki-Ras2	3, 3	0.25 ± 0.10	139.9 ±48.5

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

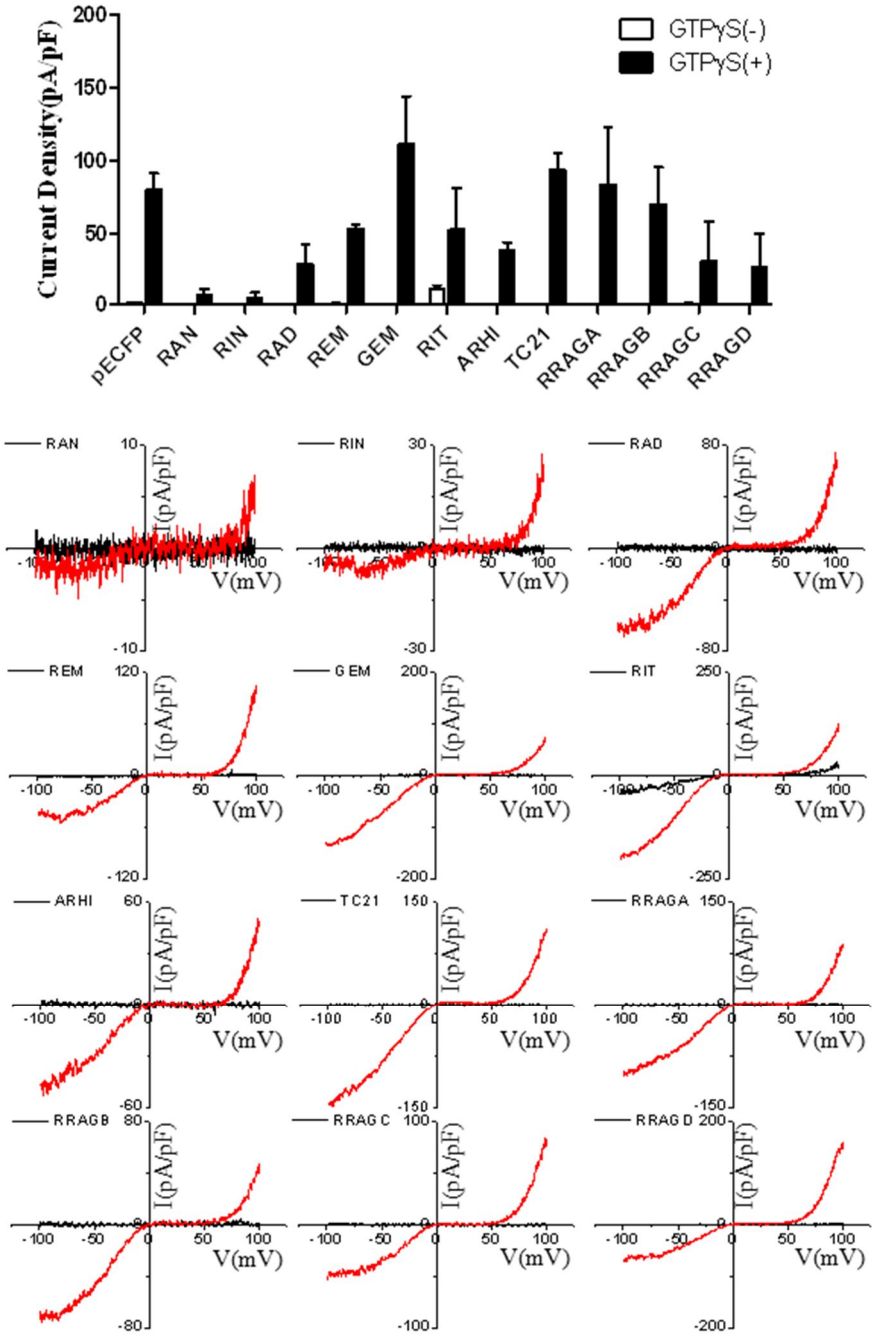

Current Density(pA/pF)
200
150
100
50
0
GTPγS(-)
GTPγS(+)
pECFP
RAN
RIN
RAD
REM
GEM
RIT
ARHI
TC21
RRAGA
RRAGB
RRAGC
RRAGD
RAN
RIN
RAD
REM
GEM
RIT
ARHI
TC21
RRAGA
RRAGB
RRAG C
RRAG D
I(pA/pF)
V(mV)

Figure 16. Specificity of Rasd1 to TRPC4β channels among different Ras proteins in Ras super family -3.

Various Ras proteins in Ras super family was screened if any protein could activate TRPC4β channels. GTPγS was used as a positive control for the activation. Ras proteins other than Rit such as RAN, RIN, RAD, REM, GEM, ARHI, TC21, RRAGA, RRAGB, RRAGC, and RRAGD could not activate TRPC4β channels.

Table 11. Current Densities of mTRPC4β Channel Activated by Ras and Ran Protein Families -3.

Proteins	n*	I (pA/pF)† GTPγS‡ (-)	I (pA/pF) GTPγS (+)
RAN	3, 5	0.35 ± 0.12	6.23 ± 4.87
RIN	3, 7	0.20 ± 0.08	4.24 ± 4.35
RAD	3, 7	0.55 ± 0.44	28.4 ± 17.3
REM	3, 6	0.95 ± 0.30	53.1 ± 3.42
GEM	3, 6	0.08 ± 0.58	111 ± 33.3
RIT	6, 10	11.1 ± 2.33	52.2 ± 28.8
ARHI	3, 6	0.51 ± 0.15	37.8 ± 5.91
TC21	3, 3	0.24 ± 0.32	93.8 ± 11.4
RRAGA	3, 5	0.43 ± 0.46	83.4 ± 39.4
RRAGB	3, 4	0.43 ± 0.55	69.8 ± 25.3
RRAGC	3, 7	0.40 ± 0.66	30.6 ± 27.4
RRAGD	4, 10	0.07 ± 0.06	26.4 ± 23.2

*: Sample numbers of GTPγS(-) and GTPγS(+) groups
†: Whole-cell current density
‡: Non-hydrolysable guanine triphosphate
§: Enhanced cyanine fluorescent protein

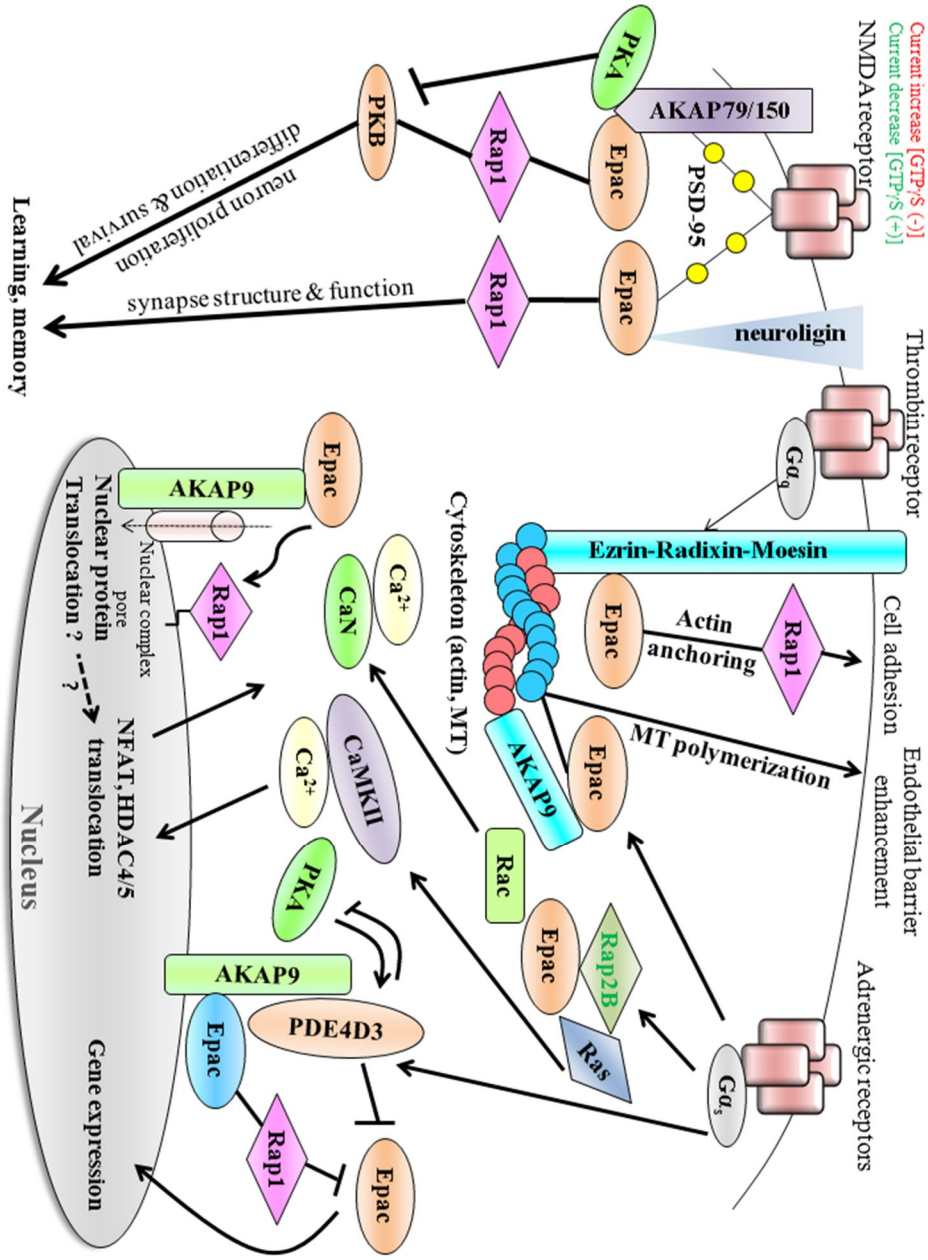

Current increase [GTPγS (-)]
Current decrease [GTPγS (+)]
NMDA receptor
PKA
AKAP79/150
Epac
PSD-95
Epac
neuroligin
Rap1
PKB
Rap1
neuron proliferation
differentiation & survival
synapse structure & function
Learning, memory
Thrombin receptor
Gαq
Ezrin-Radixin-Moesin
Epac
Actin
anchoring
Rap1
Cell adhesion
Epac
MT polymerization
Endothelial barrier
enhancement
AKAP9
Epac
Rac
Cytoskeleton (actin, MT)
Rap2B
Epac
Ras
Adrenergic receptors
Gαs
Epac
AKAP9
Nuclear complex
pore
Nuclear protein
Translocation ?
?
Rap1
Ca2+
CaN
CaMKII
Ca2+
PKA
AKAP9
PDE4D3
Epac
Rap1
Epac
NFAT, HDAC4/5
translocation
Gene expression
Nucleus

Figure 17. Multiprotein complexes localized in distinct cellular compartments and influence Rap, Ras, and Rac biological effects.

Epac2 activates PKB/Akt signaling which is involved in proliferation, differentiation and survival of neurons. By interacting with the anchoring proteins PSD-95 and/or neuroligins, Epac2 influences the structure and function of the synapses via Rap1. The small G proteins, Ran and Ran binding protein 2 (RanBP2), anchor Epac1 to the nuclear pore, allowing cAMP to activate a local pool of Rap1 at the nuclear envelope. This complex may participate in the nuclear/cytoplasmic shuttle of NFAT and HDAC proteins observed in Epac/Ras/Rac cardiac hypertrophic signaling.

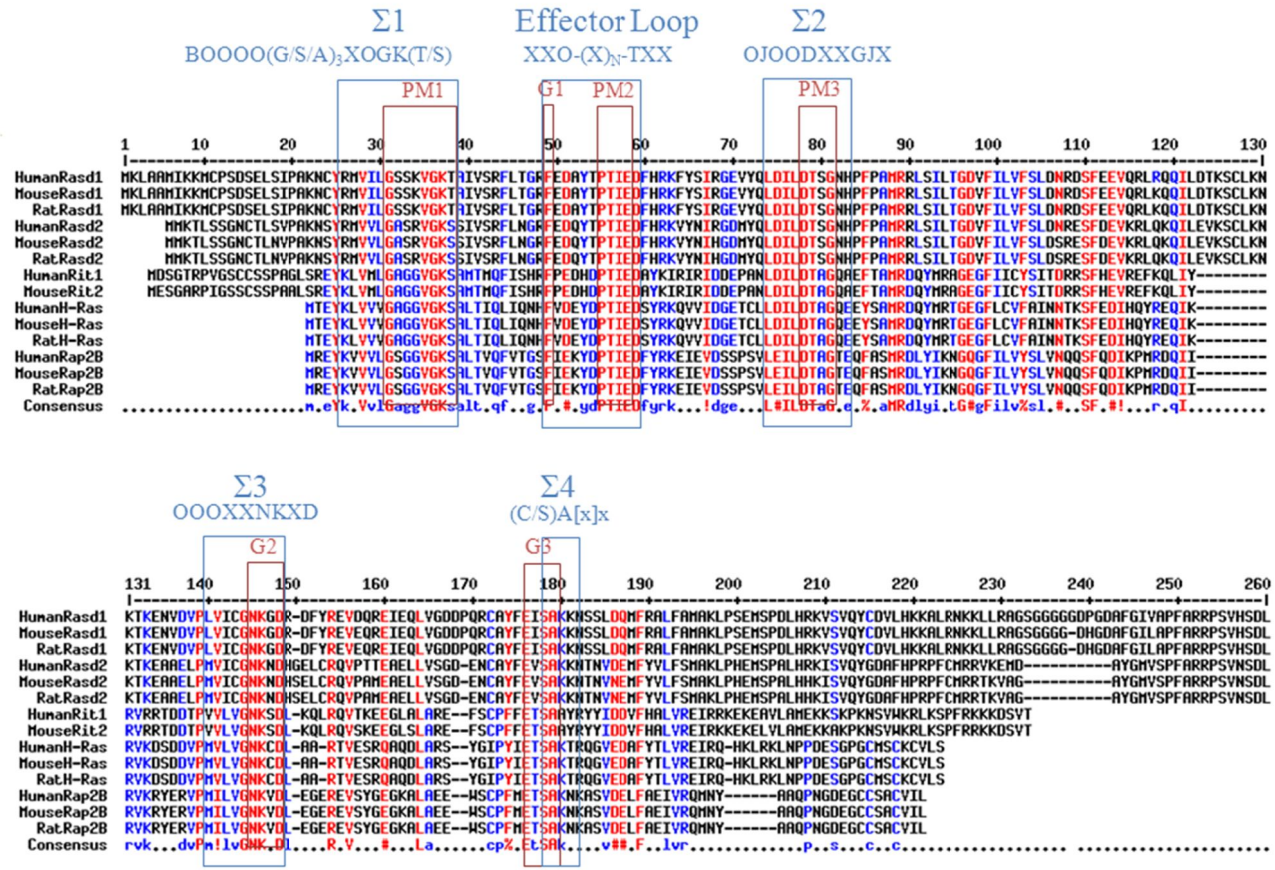

Σ1
BOOOO(G/S/A)3XOGK(T/S)
Effector Loop
XXO-(X)N-TXX
Σ2
OJOODXXGJX
PM1
G1
PM2
PM3
HumanRasd1
MouseRasd1
RatRasd1
HumanRasd2
MouseRasd2
RatRasd2
HumanRit1
MouseRit2
HumanH-Ras
MouseH-Ras
RatH-Ras
HumanRap2B
MouseRap2B
RatRap2B
Consensus
Σ3
OOOXXNKXD
Σ4
(C/S)A[x]x
G2
G3
HumanRasd1
MouseRasd1
RatRasd1
HumanRasd2
MouseRasd2
RatRasd2
HumanRit1
MouseRit2
HumanH-Ras
MouseH-Ras
RatH-Ras
HumanRap2B
MouseRap2B
RatRap2B
Consensus

Figure 18. Sequence alignment of several forms of Rasd1, Rasd2, and Rit proteins.

The following sequences were aligned by using multiple sequence alignment by Florence Corpet: human Rasd1, mouse Rasd1, rat Rasd1, human Rasd2, mouse Rasd2, rat Rasd2, human Rit, mouse Rit, human H-Ras, mouse H-Ras, rat H-Ras, human Rap2B, mouse Rap2B, and rat Rap2B. Regions defining the GTP-binding and hydrolysis domain (Σ1-Σ4) are boxed and annotated with their identifying consensus sequences. Consensus abbreviations: B, basic residue; J, polar residue; O, hydrophobic residue; X, any residue. The three conserved GDP/GTP binding motifs (G1-G3), and phosphate-magnesium binding motifs (PM1-PM3) are boxed. The red colors below the sequence indicate amino acid identity, whereas the blue color indicates similarity.

Rasd1 is a potent and selective activator of mTRPC4β channels

Rasd1 is functionally classified as a guanine nucleotide exchange factor (GEF) for $G\alpha_i$ proteins [**35**]. Rasd1 facilitates guanosine disphosphate (GDP) to GTP exchange of $G\alpha_i$ proteins and triggers it to be functionally active until intrinsic GTPase activity of $G\alpha_i$ protein turns off the signal by hydrolyzing GTP to GDP.

Previously, we reported that $G\alpha_i$ proteins are potent activators of mTRPC4β channels [**13, 16**]. Classically, stimulation of 7-TM G protein-coupled receptors (GPCR) such as histamine receptors, muscarinic acetylcholine receptors (mAChR), or purinergic receptors activates G proteins. Among the G proteins, $G\alpha_i$ proteins are shown to be activated by extracellular carbachol (100 μM) stimulating type 2 muscarinic acetylcholine receptor (M_2R or $mAChR_2$) by or the activation is mimicked by expressing constitutively active form of $G\alpha_i$ proteins. Since the function of Rasd1 as a GEF of $G\alpha_i$ may resemble the action of previously described activators of $G\alpha_i$ proteins, we expressed Rasd1 and mTRPC4β in HEK293 cells and detected the effect of Rasd1 on the activity of mTRPC4β.

Since Rasd1 proteins were tagged with cyan fluorescent protein (pECFP) proteins at the C-terminus, pECFP was transfected as a negative control for Rasd1 (**see "Materials and methods"**). As shown in **Figure 19A & D**, pECFP alone did not affect mTRPC4 current (0.83 ± 0.31 pA/pF, n = 30). $Rasd1^{S33V}$, a constitutively active form of Rasd1, induced large mTRPC4β current (**Figs. 19B & D**; 50.9 ± 9.9 pA/pF; n = 15). A mutation of serine at position 33 to valine (S33V) made Rasd1 unable to hydrolyse GTP to GDP,

hence holding it constitutively active. This constitutively active Rasd1^{S33V} induced strong morphological change [**12**]. In contrast, expressing dominant-negative form of Rasd1, Rasd1^{G31V}, did not activate mTRPC4β current (**Figs. 19C & D**, 0.54 ± 0.14 pA/pF, n = 6). Glycine at residue 31 is located at G_1 domain and crucial for GTP binding and hydrolysis [**26, 27**]. Substitution of glycine by valine (G31V) impairs GTP binding and hydrolysis to be the dominant-negative form of Rasd1.

Aside from Rasd1^{S33V}, other Rasd1 mutants, such as Rasd1^{G81A} and Rasd1^{A178V}, are also known to be constitutively active [**28, 37**]. Switching glycine at residue 81 to alanine (G81A) carries a point mutation in the G3 box guanine residue to interfere with its interaction with GTPase-activating proteins (GAPs), thus leading to a protein that is consistently bound to GTP [**37**]. In addition, switching alanine at residue 178 to valine (A178V) is expected to interrupt the guanyl nucleotide-binding pocket, resulting in an enhanced exchanged rate of guanine nucleotides. Therefore, we investigated the functions of the two mutants on mTRPC4 current. As a result, whole-cell currents of mTRPC4β were significantly increased in the presence of active Rasd1 mutants (**Figs. 20A & B**).

Although activation of mTRPC4β by Rasd1 was interesting, we could not exclude the possibility that Rasd1 activates other TRP channels such as mTRPC4α, hTRPC5, or mTRPC6 since amino acid sequence homology shows more than 40% similarity among those channels [**25**]. Thus, we tested whether Rasd1 activates these channels. As a result, Rasd1 could not activate mTRPC4α and mTRPC6 channels (**Fig. 21**). However, it was interesting that

Rasd1 reduced hTRPC5 current rather than increasing it (**Fig. 21**).

Collectively, these results suggest that mTRPC4β is the unique channel activated by Rasd1, compared to mTRPC4α, hTRPC5, or mTRPC6.

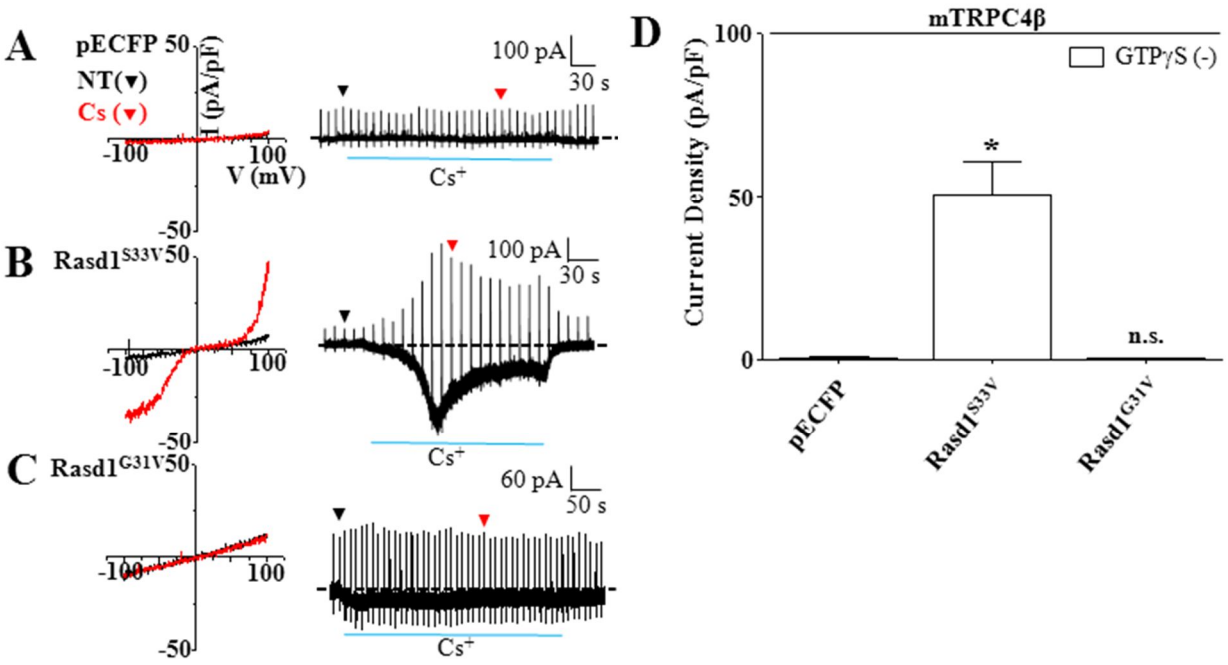


Figure 19. Rasd1 activates TRPC4 channel

HEK293 cells were transfected with mouse TRPC4β-EGFP and Rasd1 proteins (e.g., constitutively active Rasd1^{S33V} or dominant-negative Rasd1^{G31V}). Enhanced Cyan Fluorescent Protein (pECFP) was co-expressed as a negative control for Rasd1 proteins since they are tagged with pECFP at their C-terminus (see materials and methods). To increase the TRPC4 current, we measured TRPC4 channel activity with Cs^+-rich extracellular solution (Cs) since TRPC4 has greater permeability to Cs^+ than Na^+ (see materials and methods). Before and after Cs^+-rich solution, normal Tyrode (NT) solution was treated. (A) Empty vector pECFP could not induce TRPC4 current. (B) Constitutively active form of Rasd1 (Rasd1^{S33V}) induced TRPC4 current. (C) Dominant-negative form of Rasd1 (Rasd1^{G31V}) could not induce activation of TRPC4 channels. (D) Summarized current density measured above. Rasd1^{S33V} induced significant TRPC4-like current. The comparison between pECFP and Rasd1$^{S33V\text{ or }G31V}$ was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$. n.s., not significance. Black arrow heads (NT) and red arrow heads (Cs) indicate the time points where corresponding I-V curves were obtained.

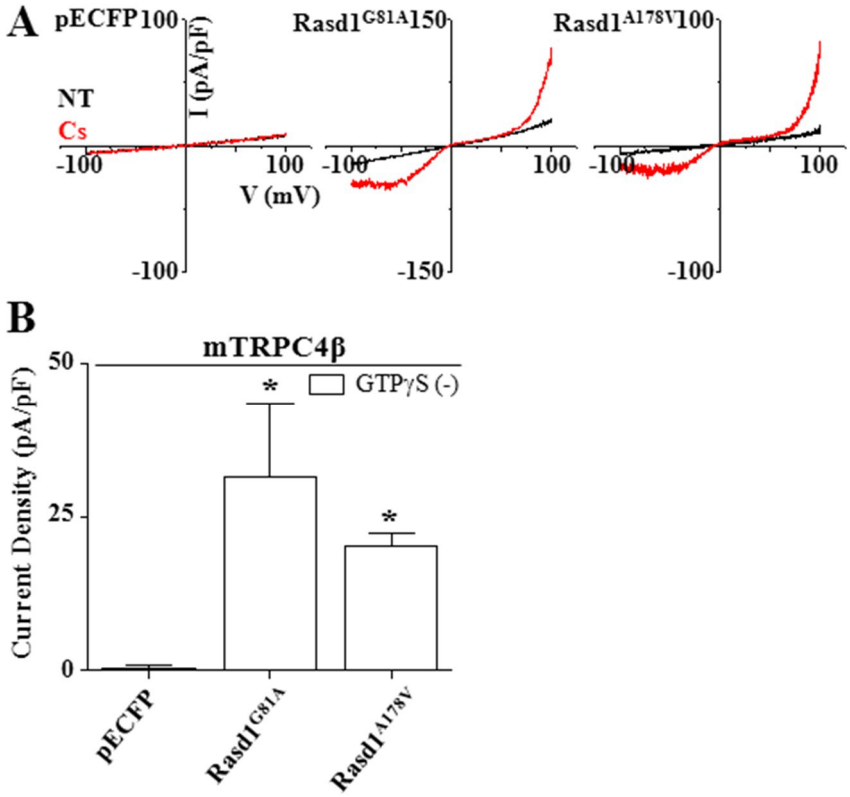


Figure 20. Specificity of mouse TRPC4β to Rasd1

(A) All panels indicate I-V relationship of currents measured from HEK293 cells expressing mouse TRPC4β-ECFP channel and constitutively active Rasd1 proteins, Rasd1^{G81A} and Rasd1^{A178V}. Enhanced Cyan Fluorescent Protein (pECFP) was expressed as a negative control for Rasd1 proteins since they are tagged with pECFP at their C-terminus (see materials and methods). All Rasd1 proteins were able to activate TRPC4 channels. (B) Summarized current density measured above. Rasd1^{G81V} and Rasd1^{A178V} induced significant TRPC4-like current increase. The comparison between pECFP and Rasd1^{G81A} or Rasd1^{A178V} was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$.

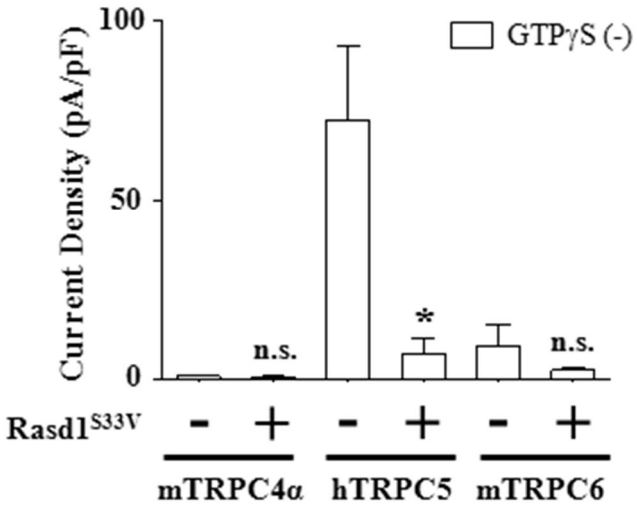


Figure 21. Specificity of Rasd1 to TRPC4β channels among other TRP channels.

The effect of Rasd1^{S33V} on other TRPC channels, TRPC4α, TRPC5 or TRPC6. Rasd1^{S33V} could not activate mouse TRPC4α or mouse TRPC6. It rather decreased the activity of human TRPC5 channels. The comparison between TRPC channels and TRPC channels + Rasd1^{S33V} was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$. n.s., not significant.

$G\alpha_i$ proteins are crucial for activation of mTRPC4β by Rasd1.

Although Rasd1 is known to act as a GEF of $G\alpha_i$ proteins, the underlying mechanism between Rasd1 and mTRPC4β channel needs to be verified. Therefore, we used pertussis toxin (PTX) to characterize possible activation mechanism. Pertussis toxin has been widely used as a reagent to characterize the involvement of heterotrimeric G proteins in signaling. This toxin catalyzes the ADP ribosylation of α subunits of $G\alpha_i$ family, and this modification prevents the occurrence of the receptor-G protein interaction [**45**]. Because the population of functional $G\alpha_i$ is strongly reduced by PTX, we examined whether PTX antagonizes the action of Rasd1.

The whole-cell current was measured in HEK293 cells expressing Rasd1 and mTRPC4β with or without pretreatment of PTX, respectively. Rasd1-activated mTRPC4β currents at -60 mV were 50.82 ± 9.93 pA/pF (**Figs. 22A & C**; n = 17) but was significantly reduced when PTX was pretreated (**Figs. 22A & C**, 0.01 ± 0.82 pA/pF, n = 10). Whole-cell mTRPC4β current without Rasd1 was 0.83 ± 0.31 pA/pF (n = 10) and whole-cell current with dominant-negative Rasd1 ($Rasd1^{G31V}$) was 0.12 ± 0.27 pA/pF (n = 7) (**Figs. 22A & C**). Pretreated PTX showed similar but almost zero current density in those groups (**Figs. 22A & C**, 0.88 ± 0.13 pA/pF, n = 15; 0.06 ± 0.35 pA/pF, n = 15).

When GTPγS was added to internal solution as a positive control for the activation, mTRPC4β current was measurable in all three groups of expression conditions, i.e., pECFP (65.99 ± 3.20 pA/pF, n = 10), $Rasd1^{S33V}$ (61.18 ± 17.28 pA/pF, n = 15), and $Rasd1^{G31V}$ (74.41 ± 22.41 pA/pF, n = 8) (**Figs. 22A & C**). PTX reduced GTPγS-activated mTRPC4β currents from

pECFP-expressing cells (0.17 ± 0.27 pA/pF, n = 9) and Rasd1^{G31V}-expressing cells (0.67 ± 0.37 pA/pF, n = 8) (**Figs. 22A & C**). However, PTX could not reduce GTPγS-activated mTRPC4β currents from Rasd1^{S33V}-expressing cells (**Figs. 22A & C**, 74.77 ± 11.71 pA/pF, n = 11).

At this point, we reasoned that Rasd1 requires certain population of functional $G\alpha_i$ proteins to activate mTRPC4β channels. However, there are some upstream activators, usually G-protein coupled- receptors, which require the same condition. Muscarinic acetylcholine receptor type 2 (M_2R), for example, is strong mTRPC4β channel activator, and functional $G\alpha_i$ proteins are quintessential for its action onto mTRPC4β channels. In a sense, if Rasd1 and M_2R both exist, the competitive action for functional $G\alpha_i$ protein would be expected. Therefore, we expressed both Rasd1 and M_2R in HEK293 cells and tested how the mTRPC4β channel current results.

Without Rasd1, M_2R strongly activated mTRPC4β channels (**Figs. 22B & D and Fig. 23A**; 69.93 ± 1.18 pA/pF, n = 14) in response to extracellular carbachol (100 μM). When Rasd1 was co-expressed, however, M_2R-activated TRPC4 current was significantly reduced (**Figs. 22B & D and Fig. 23B**, 30.15 ± 5.06 pA/pF, n = 10) which clearly demonstrates competitive action between Rasd1 and M_2R. Dominant-negative form of Rasd1 (Rasd1^{G31V}) showed no competition, and M_2R-activated mTRPC4β current was fully recovered (**Figs. 22B & D and Fig. 23C**; 56.08 ± 21.53 pA/pF, n = 10).

These results indicate that activation signaling of Rasd1 to mTRPC4β involves $G\alpha_i$ protein, and certain population of functional $G\alpha_i$ protein is essential for activation of mTRPC4β by Rasd1.

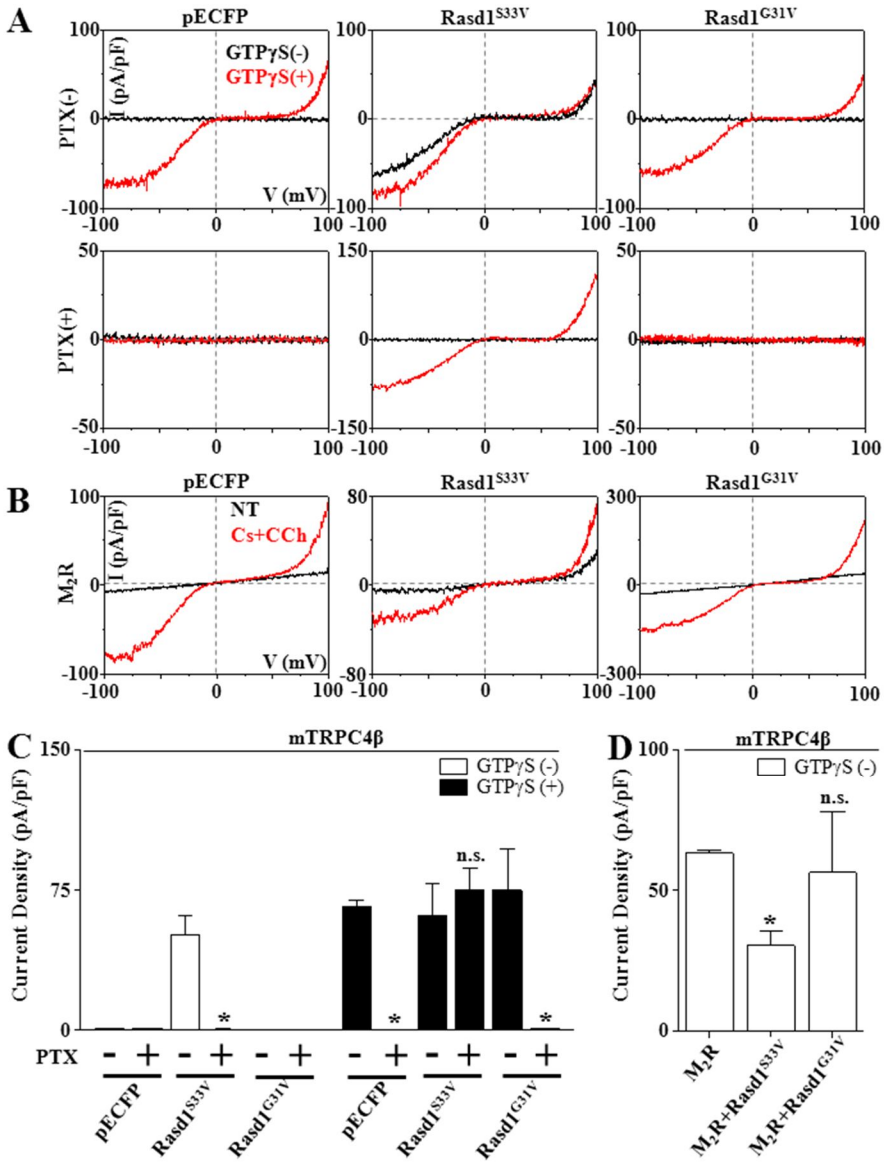

A
pECFP
Rasd1S33V
Rasd1G31V
GTPγS(-)
GTPγS(+)
I (pA/pF)
V (mV)
PTX(-)
PTX(+)
B
NT
Cs+CCh
M2R
C
mTRPC4β
GTPγS (-)
GTPγS (+)
Current Density (pA/pF)
n.s.
PTX
pECFP
Rasd1S33V
Rasd1G31V
D
M2R
M2R+Rasd1S33V
M2R+Rasd1G31V

Figure 22. Functional population of $G\alpha_i$ proteins is necessary for the activation of TRPC4β channels by Rasd1

(A) The effect of pertussis toxin (PTX) on Rasd1-activated TRPC4β. PTX strongly inhibited Rasd1-activated current. When intracellular GTPγS was applied, $Rasd1^{S33V}$-activated TRPC4β was insensitive to PTX. (B) The effect of Rasd1 on M_2 (muscarinic acetylcholine receptor type 2)-activated TRPC4β. *Left panel*, without $Rasd1^{S33V}$, M_2 activated TRPC4β channels. *Middle panel*, $Rasd1^{S33V}$ inhibited M_2-activated TRPC4β current. *Right panel*, $Rasd1^{G31V}$, a dominant negative form of Rasd1, showed no effect on M_2-activated TRPC4β current. (C) Summarized current density measured above. PTX strongly inhibited $Rasd1^{S33V}$-activated TRPC4β current. The comparison between pre-treatment without PTX and with PTX was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$. n.s., not significance. (D) Summarized current density measured above. $Rasd1^{S33V}$ inhibited M_2-activated TRPC4β current whereas $Rasd1^{G31V}$ showed no effect on M_2-activated TRPC4β current. The comparison between M_2R and M_2R + Rasd1 mutant ($Rasd1^{S33V \text{ or } G31V}$) was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$. n.s., not significance.

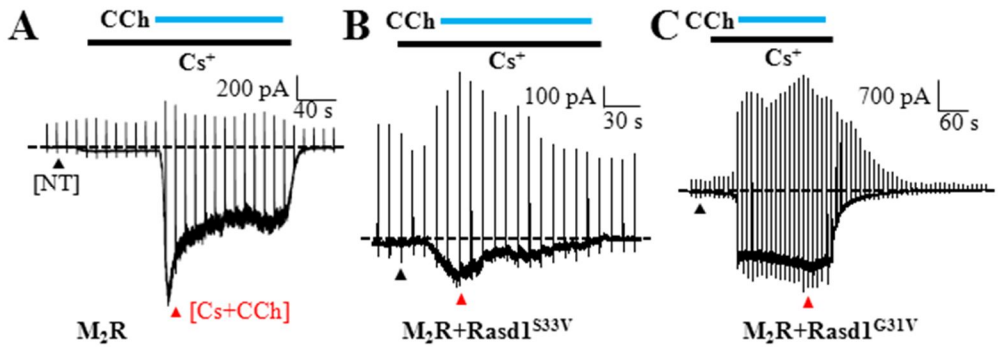


Figure 23. Time course of M2R-activated TRPC4β channels co-expressed with Rasd1 proteins

All panels indicate time course of currents measured from HEK293 cells expressing mouse TRPC4β channels, human muscarinic acetylcholine receptor type 2 (M_2R) and indicated Ras family proteins, $Rasd1^{S33V}$ or $Rasd1^{G31V}$. 100 μM of extracellular carbachol was applied in order to stimulate muscarinic receptors. (A) Under carbachol stimulation, TRPC4β channel was strongly activated with fast activation. (B) Under carbachol stimulation, TRPC4β channel showed minute activation, hence small inward current when $Rasd1^{S33V}$ was co-expressed. (C) The carbachol-M_2R-mediated TRPC4β channel activation was fully recovered when $Rasd1^{G31V}$, a dominant negative form of Rasd1, was co-expressed instead of $Rasd1^{S33V}$. Black arrow heads (NT) and red arrow heads (Cs + CCh) indicate the time points where corresponding I-V curves were obtained.

$G\alpha_{i1}$ is crucial for the activation of mTRPC4β by Rasd1.

PTX experiment demonstrated that a functional $G\alpha_i$ protein is essential for the action of Rasd1 onto mTRPC4β channels. However, HEK293 cells present several types of $G\alpha_i$ proteins [**1**], and verifying the subtype of $G\alpha_i$ proteins would be important as well. Therefore, we tested the effect of the subtype of wild-type (WT) $G\alpha_i$ proteins on mTRPC4β currents. WT $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ proteins were co-transfected with the mTRPC4β channel in HEK293 cells. **Figure. 24** showed that mTRPC4β currents increased from 0.44 ± 0.52 pA/pF (n = 4) to 29.5 ± 8.79 pA/pF (n = 16) with $G\alpha_{i2}$ or 27.9 ± 9.12 pA/pF (n = 19) with $G\alpha_{i3}$. However, $G\alpha_{i1}$ did not affect mTRPC4β currents (2.89 ± 2.36 pA/pF, n = 8). mTRPC4β currents were increased by activating endogenous $G\alpha_i$ proteins *via* Rasd1. We expressed mTRPC4β channel, $Rasd1^{S33V}$ and $G\alpha_i$ protein subtypes. mTRPC4β currents by $Rasd1^{S33V}$ and $G\alpha_{i1}$ protein (34.6 ± 8.62 pA/pF, n = 28), $Rasd1^{S33V}$ and $G\alpha_{i2}$ protein (44.1 ± 24.4 pA/pF, n = 16), or $Rasd1^{S33V}$ and $G\alpha_{i3}$ protein (37.4 ± 10.5 pA/pF, n = 24) showed no difference compared with those by $Rasd1^{S33V}$ protein (25.3 ± 5.82 pA/pF, n = 10) (**Fig. 24**). Because Rasd1 fully activated endogenous $G\alpha_i$ proteins, mTRPCβ currents may not be further increased by co-expressing $G\alpha_i$ proteins with $Rasd1^{S33V}$. However, $Rasd1^{S33V}$-activated mTRPC4β currents were increased dramatically by co-expressing $G\alpha_{i1}$ protein with $Rasd1^{S33V}$. We tested dominant-negative mutant of $G\alpha_{i1}^{G202T}$, $G\alpha_{i2}^{G203T}$ and $G\alpha_{i3}^{G202T}$ proteins in HEK293 cells and measured Rasd1-activated mTRPC4β currents. Glycine at position 202 and 203 was suggested as a member of guanine nucleotide-binding motif (G3) of $G\alpha_i$ proteins and switching it to threonine facilitates

GTP to GDP exchange [**2**]. As shown in **Figure. 24**, Rasd1-activated mTRPC4β currents were 22.00 ± 11.50 pA/pF in the presence of an empty vector (n = 25). Dominant-negative $G\alpha_{i1}$ and $G\alpha_{i3}$ significantly reduced Rasd1-activated mTRPC4β currents (**Fig. 24**, 0.88 ± 0.35 pA/pF, n = 33; 0.97 ± 0.32 pA/pF, n = 21). However, co-expression with dominant-negative $G\alpha_{i2}$ did not affect Rasd1-activated mTRPC4β currents (**Fig. 24**, 15.81 ± 5.64 pA/pF, n = 29). While dominant-negative $G\alpha_{i3}$ decreased mTRPC4β currents, WT $G\alpha_{i3}$ did not affect mTRPC4β currents. These results indicate that Rasd1 activated TRPC4β channels. This raised the question of whether the channel activation requires direct interaction with Rasd1. To address this question, we transfected TRPC4β, TRPC5, $G\alpha_i$, Gβγ, and Rasd1, and their association was analyzed by co-immunoprecipitation and FRET (**Fig. 25**). We observed an interaction between Rasd1 and TRPC4β-GFP by co-immunoprecipitation. Rasd1 interacted with $G\alpha_{i2}$, but not with TRPC4, TRPC5 and Gβγ by FRET. FRET analysis is important where florescent located proteins. Thus, this result needs further investigation. These results suggest that $G\alpha_{i1}$ protein is crucial for mTRPC4β activation by Rasd1. However, because of the co-immunoprecipitation results, we could not exclude the possibility that Rasd1 directly activates TRPC4β channels.

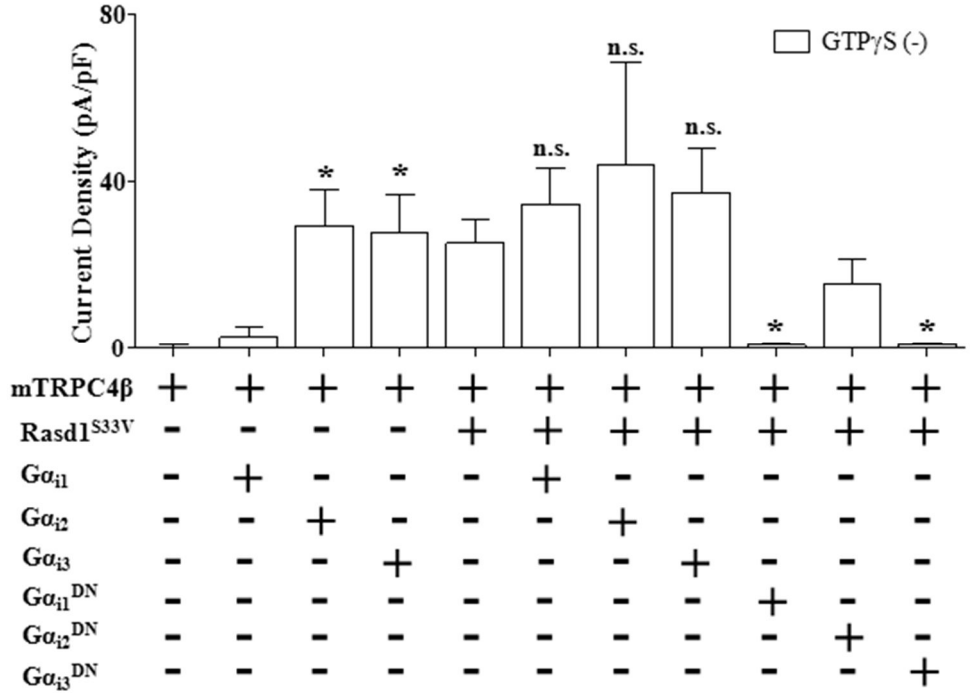
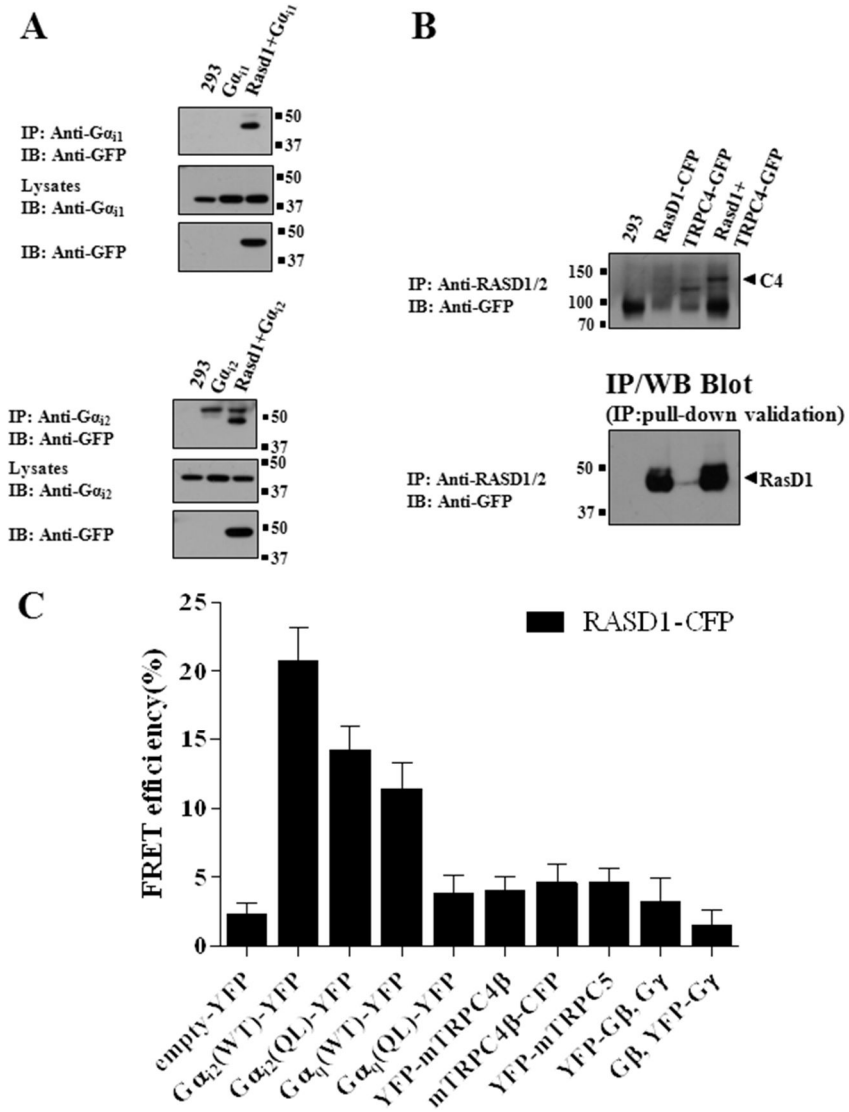


Figure 24. $G\alpha_{i1}$ is essential for the activation of TRPC4β channels by Rasd1

$G\alpha_{i2}$ and $G\alpha_{i3}$ activated TRPC4β currents but $G\alpha_{i1}$ did not affect TRPC4β channels. The comparison between PCDNA3.1 and $G\alpha_i$ proteins was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$. mTRPC4β currents activated by $G\alpha_i$ proteins + $Rasd1^{S33V}$ showed no difference from those induced by $Rasd1^{S33V}$. The comparison between $Rasd1^{S33V}$ and $Rasd1^{S33V}$ + $G\alpha_i$ proteins was carried out with Student's t-test. $G\alpha_{i1}^{G202T}$, $G\alpha_{i2}^{G203T}$ and $G\alpha_{i3}^{G202T}$ were used as dominant-negative (DN) forms of the corresponding $G\alpha_i$ proteins. $G\alpha_{i1}^{DN}$ and $G\alpha_{i3}^{DN}$ strongly inhibited Rasd1-activated TRPC4β currents whereas Ga_{i2}^{DN} showed little effect on Rasd1-activated TRPC4β currents. $G\alpha_{i1}$ significantly increased Rasd1-activated TRPC4β current. Statistical significance was denoted by an asterisk (*) at $P < 0.05$.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IP: Anti-Gαi1
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Lysates
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IB: Anti-GFP
50
37
293
Gαi2
Rasd1+Gαi2
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IB: Anti-GFP
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IB: Anti-Gαi2
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B
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IB: Anti-GFP
150
100
70
C4
IP/WB Blot
(IP:pull-down validation)
IP: Anti-RASD1/2
IB: Anti-GFP
50
37
RasD1
C
RASD1-CFP
FRET efficiency(%)
25
20
15
10
5
0
empty-YFP
Gαi2(WT)-YFP
Gαi2(QL)-YFP
Gαq(WT)-YFP
Gαq(QL)-YFP
YFP-mTRPC4β
mTRPC4β-CFP
YFP-mTRPC5
YFP-Gβ, Gγ
Gβ, YFP-Gγ

Figure 25. $G\alpha_i$ and TRPC4β interact with Rasd1

(A) Rasd1-CFP, $G\alpha_{i1}$, and $G\alpha_{i2}$ were co-transfected in HEK293 cells. Immunoprecipitation was performed with $G\alpha_{i1}$, $G\alpha_{i2}$ antibody followed by immunoblotting with GFP antibody. Total proteins of $G\alpha_{i1}$ and $G\alpha_{i2}$ were detected using $G\alpha_{i1}$ and $G\alpha_{i2}$ antibody. (B) Rasd1-CFP and TRPC4β-GFP were transfected in HEK293 cells. Immunoprecipitation was conducted with Rasd1 antibody and visualized by western blotting with GFP antibody. Total protein of Rasd1 was detected using GFP antibody. (C) Fluorescence was measured in HEK293 cells Rasd1-CFP, $G\alpha_{i2}$ WT-YFP, $G\alpha_{i2}$ QL-YFP, Gq WT-YFP, Gq QL-YFP, YFP-mTRPC4β, mTRPC4β-CFP, YFP-mTRPC5, YFP-GβGγ, and GβYFP-Gγ. Among these proteins, Rasd1 bound $G\alpha_{i2}$ WT, $G\alpha_{i2}$ QL, and Gq WT.

Gβγ subunits are not obligatory for the action of Rasd1 to mTRPC4β channels.

Since Rasd1 is GEF of $G\alpha_i$ proteins, it removes GDP from alpha subunit and change GDP to GTP. This exchange dislocates alpha subunit from betagamma subunits (Gβγ). Although classical pathway of heterotrimeric G-protein describes alpha subunit mostly, the release of Gβγ subunit is equally important. Therefore, we tested the involvement of Gβγ subunits in Rasd1-activation of mTRPC4β.

First, $G\beta_1$ and $G\gamma_2$ proteins or $G\beta_1$ and $G\gamma_7$ proteins were co-expressed with mTRPC4β channels. Neither of them was able to activate mTRPC4β channels (**Fig. 26A**). On the other hand, co-expression of β-adrenergic receptor kinase (βARK) that scavenges free Gβγ proteins, did not reduce Rasd1-activated mTRPC4β currents (**Fig. 26B**). Furthermore, we tested $G\beta^{W99A}$, which holds G-proteins as a heterotrimer [**12**] making G proteins to be insensitive to activating signaling. $G\beta^{W99A}$ mutant did not induce significant difference in Rasd1 activation of mTRPC4β **Fig. 26C)**. Lastly we tested $G\beta^{I80A}$ that keeps Gβγ dimers in free form [**12**]. As shown in **Fig. 26D**, $G\beta^{I80A}$ mutant did not induce significant difference in Rasd1 activation of mTRPC4β. Together, these results establish that Gβγ signaling can be excluded from possible signaling candidates of Rasd1.

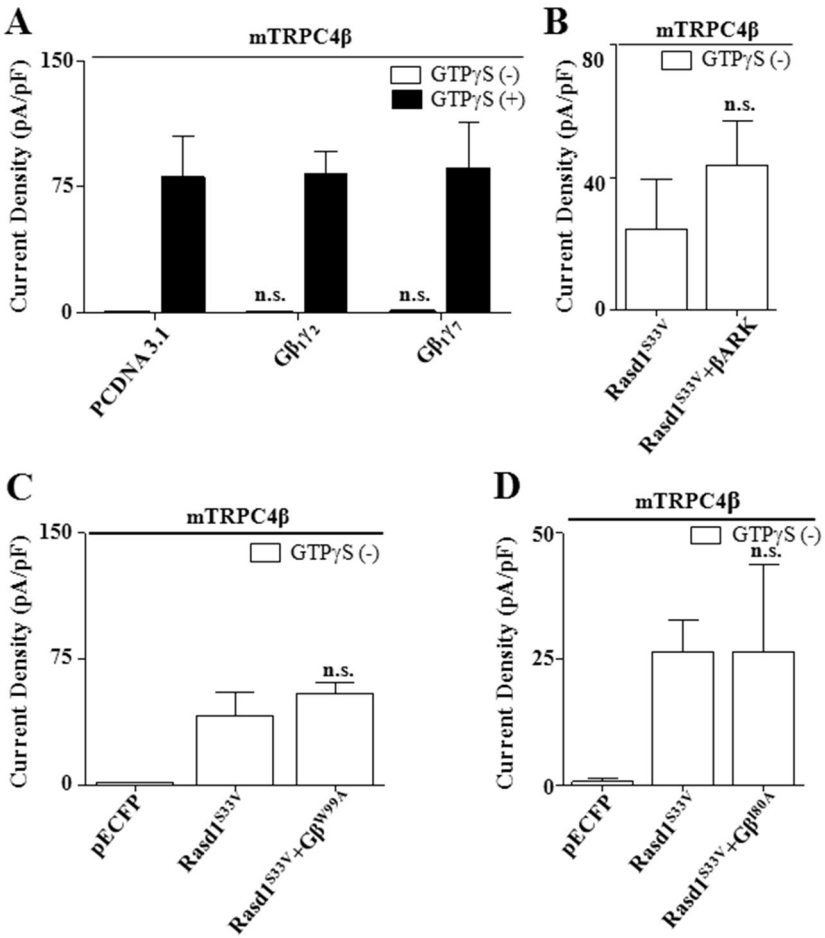
A
mTRPC4β
150
75
0
Current Density (pA/pF)
GTPγS (-)
GTPγS (+)
n.s.
n.s.
PCDNA3.1
Gβ1γ2
Gβ1γ7
B
mTRPC4β
80
40
0
Current Density (pA/pF)
GTPγS (-)
n.s.
Rasd1S33V
Rasd1S33V+βARK
C
mTRPC4β
150
75
0
Current Density (pA/pF)
GTPγS (-)
n.s.
pECFP
Rasd1S33V
Rasd1S33V+GβW99A
D
mTRPC4β
50
25
0
Current Density (pA/pF)
GTPγS (-)
n.s.
pECFP
Rasd1S33V
Rasd1S33V+GβI80A

Figure 26. Gβγ is not obligatory for the Rasd1-mediated activation of TRPC4β channels

(A) $G\beta_1\gamma_2$ and $G\beta_1\gamma_7$ could not activate TRPC4 channels. GTPγS was used as a positive control for the activation. The comparison between PCDNA 3.1 and Gβγ proteins was carried out with Student's t-test. (B) β-adrenergic receptor kinase (βARK) was used as a free Gβγ scavenger. βARK could not reduce Rasd1-activated TRPC4β currents. The comparison between $Rasd1^{S33V}$ and $Rasd1^{S33V}$ + βARK proteins was carried out with Student's t-test. n.s., not significant. (C) $G\beta^{W99A}$ that is known to hold heterotrimeric G-proteins as a heterotrimer and makes it insensitive to activating signal, could not reduce Rasd1-activated TRPC4β current. The comparison between $Rasd1^{S33V}$ and $Rasd1^{S33V}$ + $G\beta^{W99A}$ proteins was carried out with Student's t-test. n.s., not significant. (D) $G\beta^{I80A}$, which is known to keep Gβγ dimers in free form, could not affect Rasd1-activated TRPC4β current. The comparison between $Rasd1^{S33V}$ and $Rasd1^{S33V}$ + $G\beta^{I80A}$ proteins was carried out with Student's t-test. n.s., not significant.

Dexamethasone, a synthetic glucocorticoid and immunosuppressant, increases TRPC4 current via Rasd1.

Dexamethasone is a synthetic glucocorticoid, a widely used immunosuppressant and insulin secretion regulator. Treatment of AtT-20 cells with dexamethasone was shown to increase mRNA expression of Rasd1 [**13**]. Based on this study, we tested whether dexamethasone increases mTRPC4β current by activating Rasd1. It should be noted that we used wild-type (WT) Rasd1 instead of constitutively active form for these experiments to pinpoint the susceptibility of dexamethasone–dependent activation signaling.

HEK293 cells were incubated with 100 μM dexamethasone for 48 h. **Figs. 27A & B** showed that mTRPC4 current was significantly increased from 22.92 ± 12.71 pA/pF (n = 19) in control to 91.40 ± 53.60 pA/pF with dexamethasone (**Figs. 27A & B**, n = 8).

These results suggest that dexamethasone activates mTRPC4β channels *via* Rasd1-dependent signaling.

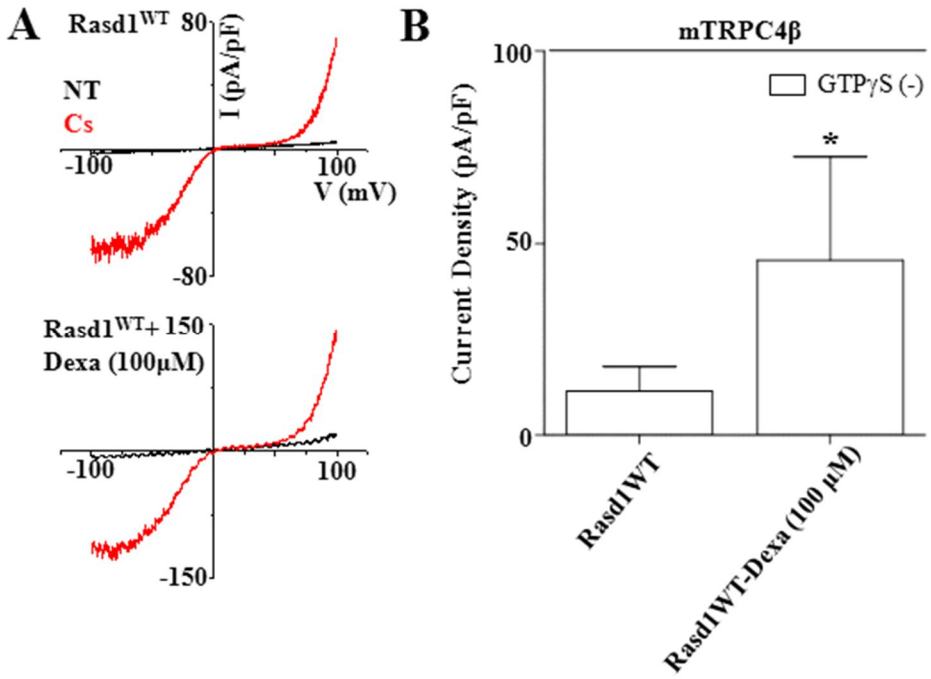

Figure 27. Dexamethasone enhances Rasd-1 activated TRPC4 currents.

Dexamethasone, a synthetic glucocorticoid, is known to increase mRNA levels of Rasd1 [**27**]. (A) 100 μM of dexamethasone pre-treated 48 hours before patch-clamp increased Rasd-1 activated TRPC4β current. (B) Summarized current density measured above. 100 μM of dexamethasone significantly increased Rasd1-activated TRPC4β current. The comparison between Rasd1 WT and Rasd1 WT + Dexa was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$.

Rasd1-TRPC4 interaction is responsible for cationic current in pancreatic β-cells (INS-1) in response to dexamethasone.

Recently, TRPC4 channel activity was reported important in leptin-mediated insulin secretion in pancreatic β-cells [**41**]. Since both Rasd1 and TRPC4 are detectable in pancreatic β-cells, we tested whether Rasd1 activation by dexamethasone is sufficient to generate TRPC4-like current in INS-1 cells.

We found that 100 nM dexamethasone was sufficient to generate cationic current in INS-1 cells and the I-V curve resembled that of TRPC4 (**Figs. 28A & B**, 59.59 ± 8.48 pA/pF, n = 13), suggesting that dexamethasone activates TRPC4 in INS-1 cells. Inhibition of Rasd1 with siRNA blunted dexamethasone-induced TRPC4 current in INS-1 cells (**Figs. 28A & B**, 18.45 ± 4.45, n = 8), indicating that the effect of dexamethasone on TRPC4 is mediated by Rasd1.

To investigate whether dexamethasone activates TRPC4 *via* increasing Rasd1 protein expression, we detected protein level of Rasd1 in INS-1 cells before and after dexamethasone treatment. As shown in **Fig. 28C**, dexamethasone increased protein level of Rasd1 in INS-1 cells in basal but not with Rasd1-siRNA.

These results suggest that dexamethasone activates TRPC4 *via* Rasd1 in INS-1 cells.

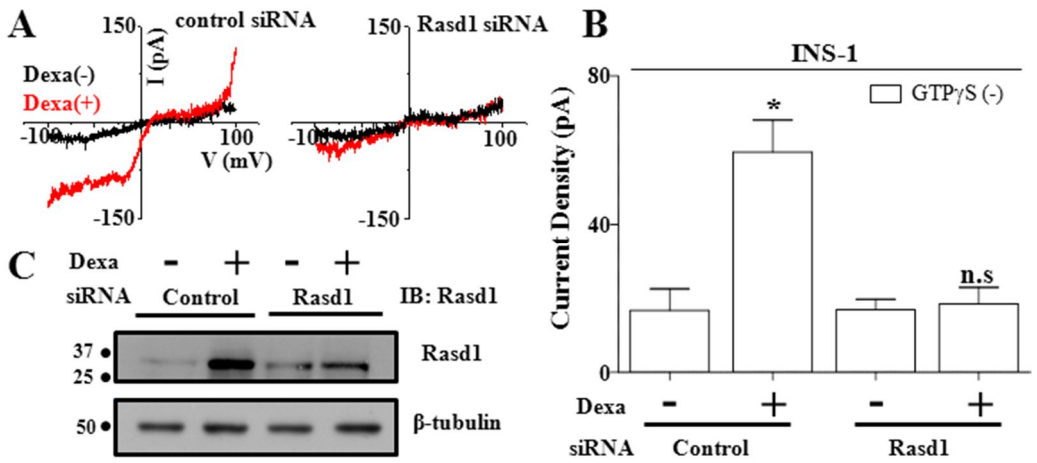

Figure 28. Dexamethasone induced TRPC4-like current in INS-1 cells by increasing expression level of Rasd1

(A) 100 nM of dexamethasone induced TRPC4-like current in INS-1 cells measured by perforated patch clamp configuration. This dexamethasone-induced TRPC4-like current was abolished when siRNA for Rasd1 was treated. (B) Summarized TRPC4-like current density measured above. Dexamethasone induced significantly large TRPC4-like current in INS-1 cells. The comparison between control siRNA and control siRNA + Dexa, or Rasd1 siRNA and Rasd1 siRNA + Dexa was carried out with Student's t-test. Statistical significance was denoted by an asterisk (*) at $P < 0.05$. n.s., not significant. siRNA for Rasd1 made the cells insensitive to extracellularly applied dexamethasone. (C) Protein expression level of Rasd1 measured by western blotting. Dexamethasone increased expression level of Rasd1.

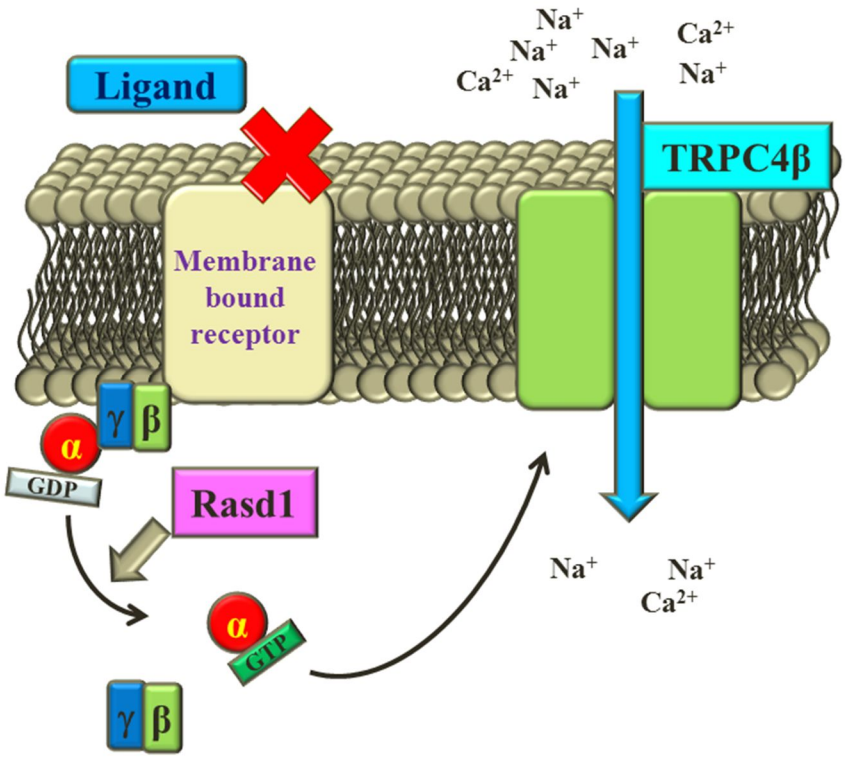

Figure 29. Schematic representation of the interaction between mTRPC4β channel, heterotrimeric G proteins, and Rasd1.

Rasd1 is classified as a guanine nucleotide exchange factor (GEF) for $G\alpha_i$ proteins. Rasd1 promotes GDP to GTP exchange in $G\alpha_i$ proteins and triggers it to be functionally active until the intrinsic GTPase activity of $G\alpha_i$ proteins turns off the signal by hydrolyzing GTP to GDP. $G\alpha_i$-GTP protein activates TRPC4β channel.

Discussion

In this study, small G proteins could closely affect TRPC4β channels. Rab protein has been implicated in the regulation of intracellular vesicular trafficking with specialized transport functions of the different Rab proteins in the secretory and endocytic pathways. Rab5 protein is present on early endosomes and regulates initial steps of the endocytic process [**22**]. Rab3 is involved in Ca^{2+} dependent exocytosis [**39**]. Rab11A is associated with vesicles in the apical portion of epithelial cells near the centrosome and beneath the apical plasma membrane. Additionally, Rab11A participates in the regulation of recycling endosomal trafficking [**38**] (**Fig. 6**). Early studies showed that Rho proteins regulate cell morphology and the actin cytoskeleton [**36**]. However, it is now clear that they also affect gene expression, survival, and cell proliferation. These cellular functions are important in tumorigenesis, and subsequent studies have shed light on how Rho proteins contribute to the deregulation of these processes. Inhibitory mutants of RhoA, RhoH, Rac1, Cdc42 and TC10 prevent Ras transformation of fibroblasts, and activated mutants of these proteins are transforming, albeit weakly compared with the Ras oncogenes. RhoA expression levels correlated positively with the tumor progression. RhoC is overexpressed in pancreatic ductal adenocarcinoma and inflammatory breast cancer. RhoH is genetically altered in tumors of myeloid origin. The correlation between Rho protein overexpression and clinical outcome raises the possibility that these protein expression levels could be useful prognostic indicators (**Figs. 8 & 9**) [**36**].

Arf/Sar proteins regulate membrane trafficking and organelle structure by recruiting cargo-sorting coat proteins, interacting with regulators of other G proteins, and modulating membrane lipid composition [**6**]. Arf1 and Arf3 appear to be released from membranes during GTP hydrolysis in cells. Arf4 and Arf5 remain bound to the ER-Golgi intermediate compartment (ERGIC) membranes in their GDP-bound form. This raises the possibility that Arf proteins that are bound to membranes in their GDP-bound form might interact with membrane-localized partners and mediate signaling. Sar1, which is present in all eukaryotes examined to date, presents an amino-terminal amphipathic helix and functional similarities with Arf1 in recruiting a coat complex during vesicle budding (**Fig. 12**) [**6**]. We were interested in fact that TRPC4 currents were increased by small G proteins in absence of GTPγS. Some proteins decreased TRPC4β currents in the presence of GTPγS, but some Rab proteins, Rasd1, Rasd2, and Rit protein increased TRPC4 currents without GTPγS (**Fig. 1, 3, 4, 14, 15, and 16**). Rasd1 strongly increased TRPC4β current compared to other small G proteins. The relationship between Rasd1 and TRPC4β channels was investigated. However, further studies are warranted to examine the role of the other small G proteins that affected TRPC4β currents. Rasd1, rather than other small G proteins, potently increased TRPC4β channel without GTPγS. mTRPC4β channel is the only channel susceptible to Rasd1 (hTRPC5 and mTRPC6 are not activated by Rasd1). The action of Rasd1 on mTRPC4β requires the activation of $G\alpha_i$ proteins, specifically, $G\alpha_{i1}$ (**Fig. 24 & 29**).

Traditionally, the activation mechanism of TRPC channel was confined to

classical heterotrimeric G-protein and G-protein coupled receptor pathway [**31**]. This is plausible since the discovery of the channel was from *Drosophila Melanogaster* retinogram where $G_{q/11}$-PLC pathway is the predominant governing signaling [**25**]. Recently, we have reported that $G\alpha_i$ proteins are potent activators of TRPC4 and TRPC5 channels [**11**]. This though, however, still entails heterotrimeric G-protein pathway. In the current study, we clearly demonstrate that small G-proteins, aside from heterotrimeric G-proteins, are capable of activating TRPC channels. We expect this study could expand the current understandings of the activation mechanisms of TRPC channels.

Although we describe, for the first time, the link between small G-protein and TRPC4 channel activation, the relationship between small G-protein and TRPC5 channel has been reported earlier. Bezzerides *et al.*, [**1**] reported that rapid translocation of TRPC5 channels to plasma membrane is induced by epidermal growth factor (EGF) *via* Rac1 small G-protein-dependent signaling pathway. Similarly, the functional relationships between rasd1 and G protein-gated inwardly rectifying K^+ channels (GIRK) or rasd1 and voltage-gated Ca^{2+} channels have been reported [**26, 35**].

In this regard, it should be avoided to restrict Rasd1 as an only small GTPase that can activate TRPC4 channels, although it is among Ras small GTPase family. In this study, we found that GTPγS-activated mTRPC4β current in $Rasd1^{S33V}$ expressing cell was insensitive to pertussis toxin (**Figs 15A & C**). This may due to chemical nature of GTPγS; it can activate any kinds of heterotrimeric or small GTPases. EGFR signaling including Rac1 [**2, 32**] or leptin pathway [**37**] might be involved. Second, it may due to increased

GTPγS affinity of $G_{i/o}$ proteins which is triggered by GEFs such as Rasd1 [**42**]. The inhibitory action of pertussis toxin to $G\alpha_i$ proteins *via* ADP ribosylation and enhancing action of Rasd1 to $G\alpha_i$ proteins *via* increasing GTPγS may compete each other. Lastly, an 'unexpected regulatory domain' of $G\alpha_{i/o}$ may be obligatory [**41**]. The interaction of GEFs (e.g., GAP-43, NG-GPA, presenilin 1 and Rasd1) between $G\alpha_i$ proteins through 'unexpected regulatory domain' could rescue $G\alpha_i$ proteins once inhibited by pertussis toxin. The mechanism needs further investigation.

The finding of TRPC-like current in pancreatic β-cell in response to dexamethasone was interesting since similar study regarding leptin and glucose regulation of TRPC4 was recently published [**37**]. In this study, leptin is shown to activate TRPC4 channel and Ca^{2+} influx through TRPC4 activates AMPK which then triggers translocation of ATP-dependent K^+ channel (K_{ATP}) to plasma membrane to hyperpolarize the resting membrane potential. Another study reported that dexamethasone increased the protein expression of Rasd1 in pancreatic β cells and diminished insulin secretion [**24**]. These studies indicated that Rasd1 and TRPC4 could be common molecular candidates in controlling insulin secretion in β-cells. However, whether Rasd1-activated TRPC4 delivers its signal to AMPK-K_{ATP} pathway is not known. Further studies are warranted to investigate the significance and downstream mechanisms of Rasd1-TRPC4. In human, dexamethasone stimulates leptin release from adipocytes and decreases insulin secretion [**5, 21**].

Clinically, the effect of glucocorticoid is important in care of hospitalized

diabetes mellitus (DM) patients [**40**]. It is known that long-term prescription of dexamethasone could lead to insulin resistance and decrease in insulin secretion [**40**]. If the patient has been treated with dexamethasone as an anti-inflammatory drug or immunosuppressant, diabetes mellitus as his/her underlying disease could occur as a paradoxical effect of the drug. The relationship among Rasd1, TRPC4 and insulin secretion might suggest new therapeutic agent for this particular clinical situation.

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국문 초록

Canonical transient receptor potential (TRPC4) 이온통로는 칼슘 투과성을 가진 비선택성 이온투과 이온통로로 포유류에 널리 분포하고 있다. 일반적으로 TRPC4 이온통로는 $G_{q/11}$-PLC 통로 또는 $G_{i/o}$ 단백질을 통하여 직접적으로 활성화 시킨다고 추측되고 있다. TRPC4 에 관한 많은 메커니즘 연구는 heterotrimeric G-protein 를 통해 이루어 졌으나, 우리는 TRPC4 와 small GTPase 사이의 기능적 관계에 대해 보고하고자 한다. 우리는 patch clamp, western blotting, FRET 의 방법을 통하여서 이들을 확인해보았다. Rab 단백질들과 Rasd1, Rasd2, Rit 단백질이 GTPγS 없이 TRPC4 채널을 활성화시키는 것을 확인하였다.

Rasd1 은 선택적으로 TRPC4 이온통로를 활성화 시키며 Small G protein family 중에서 TRPC4 이온통로를 가장 강력하게 활성 시켰다. 또한 Rasd1 을 통한 TRPC4 이온통로 활성을 위해서는 $G\alpha_{i1}$ 의 기능적인 분포가 필요하다는 것도 알 수 있었다. 한편 당질코르티코이드 및 소염제로 알려져있는 dexamethasone 은 췌장베타세포 (INS-1)에서 Rasd1 의 mRNA level 을 증가시킨다고 알려져 있다. 우리는 INS-1 세포에서 dexamethasone 처리시 Rasd1 의 단백질 증가를 통한 TRPC4 와 유사한 전류를 발견하였다. dexamethasone 으로 인한 Rasd1 과 TRPC4 의 관계는 dexamethasone 처방을 장기적으로 받아야 하는

hospitalized diabetes mellitus 환자의 새로운 치료제로 제시될 수 있을 것이다.

주요어 : TRPC4, dexamethasone, Small G protein, Rasd1, GPCR, insulin
학 번 : 2010-21910