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Increased ubiquitin-conjugation in cardiac myocytes and the involvement of crosstalk of G protein signaling

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Abstracts

Cardiac contractility is maintained mainly by β-adrenergic signaling which in response to ligand stimulation, then β-adrenergic receptor (β-AR) activates Gs causes production of cAMP. Chronic adaptive response of the heart to injury or abnormal hemodynamic load increases heart afterload and leads to hypertrophy. This condition often predisposed to heart failure with sudden death risk. Together with pathological cardiac hypertrophy, dampened response of β-adrenergic signaling to ligand stimulation is the hallmark of heart failure. On the other hand, it has been known that α-adrenergic signaling, which is associated with Gq is hyper-activated in cardiac hypertrophy. Thus, Gq signaling seems to be involved in the suppression of β-adrenergic signaling-regulated cardiac contractility in pathological cardiac condition. It has been suggested that the mechanism might involve reduced Gαs abundance via augmentation of Gαs ubiquitination.

To date, several non-receptor type regulators of G protein have been identified, including Ric-8 (resistance to inhibitors of cholinesterase 8). Different with invertebrates, mammals have two Ric-8 homologues i.e. Ric-8A and Ric-8B. Ric-8A interacts with and functions as GEF for Gαi/o, Gαq, and $G\alpha_{13}$ subunits whereas Ric-8B interacts with Gαs and Gαq. Recent study showed that Ric-8B stabilizes Gαs subunit by inhibiting its degradation mediated by ubiquitin proteasome pathway. The specific positive modulation of Ric-8B to Gαs requires interaction between the two proteins. Taken together, Ric-8B may play a critical role as mediator between Gs and Gq signaling.

In this study, I found the increase of a global ubiquitination in hypertrophied mice heart. Strikingly, the activation of Gq-signaling upon a stimulation of α_1-AR also resulted in the enhanced-ubiquitination of Gαs in the neonatal rat cardiomyocytes (NRCM). Ligand stimulation of Gαq-coupled receptor reduced Gαs expression and consequently, suppressed cAMP accumulation. This effect was canceled by pretreatment with the antagonist or Gq inhibitor. Furthermore, overexpression of Gαq resulted in the same attenuation of Gs signaling and expression level, which is due to the degradation of Gαs by a ubiquitin-proteasome pathway. In the previous study, our group reported that Ric-8B stabilizes the expression of Gαs and its signaling by suppressing the ubiquitination of Gαs. Remarkably, the enforced expression of Ric-8B effectively canceled the Gαq-caused ubiquitination of Gαs, and recovered the cAMP production by Gαs. I also demonstrated that the excess amount

of Gαq efficiently reduces the protein complex of Gαs and Ric-8B by sequestering Ric-8B in cells and *in vitro*.

Based on my current results I propose a molecular mechanism model of Gq-Gs signaling crosstalk in cardiac myocytes as follows. Hyperactivation of α_1-AR in myocytes, which activates Gq signaling, induces the dissociation of Gαq subunit from its Gβγ dimer and generates exaggerated free forms of Gαq which then capable to sequester Ric-8B from its preferable complexes with Gαs. In the absence of binding with Ric-8B, Gαs is rapidly ubiquitinated and degraded, resulting in reduced-β-AR response to ligand stimulation. My study may provide a new possible mechanism on how cardiac contractility is depressed in pathological hypertrophied heart and this maybe a part of mechanisms of chronic heart failure.

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Abbreviations

AC	adenylyl cyclase
ANF	atrial natriuretic factor
α-AR	α-adrenergic receptor
AV	arteriovenous
β-AR	β-adrenergic receptor
CAL	coronary artery ligation
cAMP	3',5'-cyclic adenosine monophosphate
CHF	chronic heart failure
CRE	cAMP response element
DAG	diacyl glycerol
DKO-MEFs	double knock out mouse embryonic fibroblasts
GDP/GTP	guanosine diphosphate/guanosine-5'-triphosphate
GEF	guanine nucleotide exchange factor
GPCR	G protein coupled receptor
GST	glutathione S-transferase
IBMX	3-isobutyl-1-methylxanthine
LPA	lysophosphatidic acid
β-MHC	β-myosin heavy chain
NRCM	neonatal rat cardiomyocytes
PE	phenylephrine
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
Ric-8	resistance to inhibitors of cholinesterase-8
α-SKA	skeletal α-actin
TAC	transverse aortic constriction

I. Introduction

I.1 G protein signaling

G protein-coupled receptors (GPCRs) transduce signals into the cells by activating G proteins, which are composed of α, β and γ subunits. When α subunit binds to GDP in inactive state, the α subunit forms a complex with β and γ subunits. Upon stimulation by ligands, GPCRs accelerate the GDP/GTP exchange reaction on the α subunit and induce the dissociation of G proteins into α (Gα) and βγ (Gβγ) subunits. Then both Gα and Gβγ independently or cooperatively modulate the activity of specific effectors (Gilman 1987; Kaziro et al. 1991). The Gα subunit has intrinsic GTP-hydrolysing activity, which reverses the heterotrimer to its inactive state. Fatty acylation of the α subunit and isoprenylation of the γ subunit facilitate G protein complex to localize in the cell membrane (Malbon 2005). The α subunits are divided into 4 subgroup proteins; Gαs, Gαi/o, Gαq, and $G\alpha_{12/13}$, on the basis of their amino acid sequences and their effectors. Specific coupling of the receptor to G proteins links to a different set of intracellular signaling networks. For example, Gαs is coupled with the β_1-adrenergic receptor (β_1-AR), activates adenylyl cyclase (AC), and subsequently induces cAMP production, whereas Gαq is linked to the α_1-adrenergic receptor (α_1-AR) and activates phospholipase Cβ (PLCβ), which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG), and subsequently induces intracellular Ca^{2+} increase and PKC activation (Malbon 2005).

Each component in G protein signaling system can be regulated independently by additional proteins or soluble modulators, which make it a complex system providing a variety of signaling pathways to serve particular functions in distinct cell types, including memory, sensory, development, and many biological systems in the organism (Wettschureck and Offermanns 2005).

I.2 Role of G protein signaling in cardiovascular system and pathological cardiac hypertrophy

Cardiac regulation is modulated by the sympathetic nervous system. The signal is transmitted by noradrenaline released from the sympathetic nerves and transduces into cardiomyocytes mediated mainly through β_1-AR. β_1-AR is a typical GPCR that is coupled to Gαs. Stimulation of β-AR by ligands such as catecholamines, results in Gαs-dependent activation of AC and subsequent cAMP accumulation, which activates the c-AMP-dependent protein kinase (PKA). Activated PKA then phosphorylates several proteins involved in excitation-contraction coupling within the cytoplasmic, sarcolemmal and myofibrillar cell components including L-type Ca^{2+} channels, phospholamban or troponin I (Wettschureck and Offermanns 2005). Thus, β-AR signaling is important to maintain cardiac contractility, rate and force.

Chronic adaptive response of the heart to injury or abnormal hemodynamic load increases heart pressure load or volume load, leading to increase in heart wall thickness (concentric hypertrophy) or cardiac dilation (eccentric hypertrophy), respectively (Opie et al. 2006; Hunter et al. 1999). This condition often predisposes to heart failure with sudden death risk, particularly when eventually heart fails to pump as strong as the healthy heart. Together with pathological cardiac hypertrophy, dampened response of β-adrenergic signaling to ligand stimulation is the hallmark of heart failure (Port and Bristow 2001). Pathological hypertrophy is different with physiological hypertrophy that usually occurs in highly trained athletes, which is reversible, associated with proportional increases in the length and width of cardiac myocytes, not associated with certain gene changes and does not cause cardiac contractility dysfunction. In contrast, pathological hypertrophy is associated with specific changes in genes and proteins that are expressed in newborn ventricle (fetal gene expression),

including ANF, β-MHC, *c-myc* and *c-fos* (Dorn and Brown 1999; Izumo et al. 1988; Nishida et al. 2008).

Early studies of hypertrophy in cultured neonatal rat cardiomyocytes revealed that ligand stimulation, including norepinephrine, phenylephrine, angiotensin II, endothelin, and prostaglandin $F_2\alpha$, increased cardiomyocyte cell size or other effects of hypertrophic parameter. Those results demonstrated an important feature, that the hypertrophic effects were induced by stimulation of Gq-coupled GPCRs expressed in cardiacmyocytes (Dorn and Brown 1999; Simpson 1983; Adams et al. 1996). On the contrary, inhibition of Gq signaling with neutralizing antibodies prevents cardiac hypertrophy (La Morte et al. 1994). The hypertrophic effects induced by activation of Gq-coupled receptor described above are associated with activation of phospholipase C as demonstrated by increased level of cardiomyocytes inositol triphosphate (IP3) and DAG (Adams et al. 1998; Sadoshima and Izumo 1993; Dorn and Brown 1999). PLC-mediated hypertrophy was also suggested by later studies using transgenic mice with cardiac targeted overexpression of α_{1B}-AR (Milano et al. 1994). Besides the PLC activation, protein kinase C (PKC) activation which is another downstream molecule of Gq signaling, might be involved in hypertrophy (D'Angelo et al. 1997). PKC is activated by diacylglycerol (DAG) that is a product of PLC-catalyzed phosphatidylinositol hydrolysis (Malbon, 2005). Moreover, no detectable ventricular hypertrophy induced by aortic constriction were observed in a study using a mouse line lacking both Gαq/Gα11 in cardiomyocytes (Wettschureck et al. 2001). Those findings prove that the Gq signaling is essential for cardiac hypertrophy.

Study using transgenic mice with cardiac targeted overexpression of Gαq (4-fold overexpression) exhibited a significant increase of the expression of hypertrophy markers (ANF, β-MHC and α-SKA) and an attenuation of β-AR-mediated stimulation of AC (D'Angelo et al. 1997). The α_1-AR or Gαq-induced suppression of β_1-AR-mediated Gs

signaling in the transgenic mouse lines has been reported in several other reports (Fig. 1) (Akhter et al. 1997; Lemire et al. 1998; Dorn et al. 2000). Those findings emphasize the possibility that excess Gq signaling induces the cardiac contractility dysfunction, since β-adrenergic signal transduction is a major pathway that maintains cardiac muscle contraction.

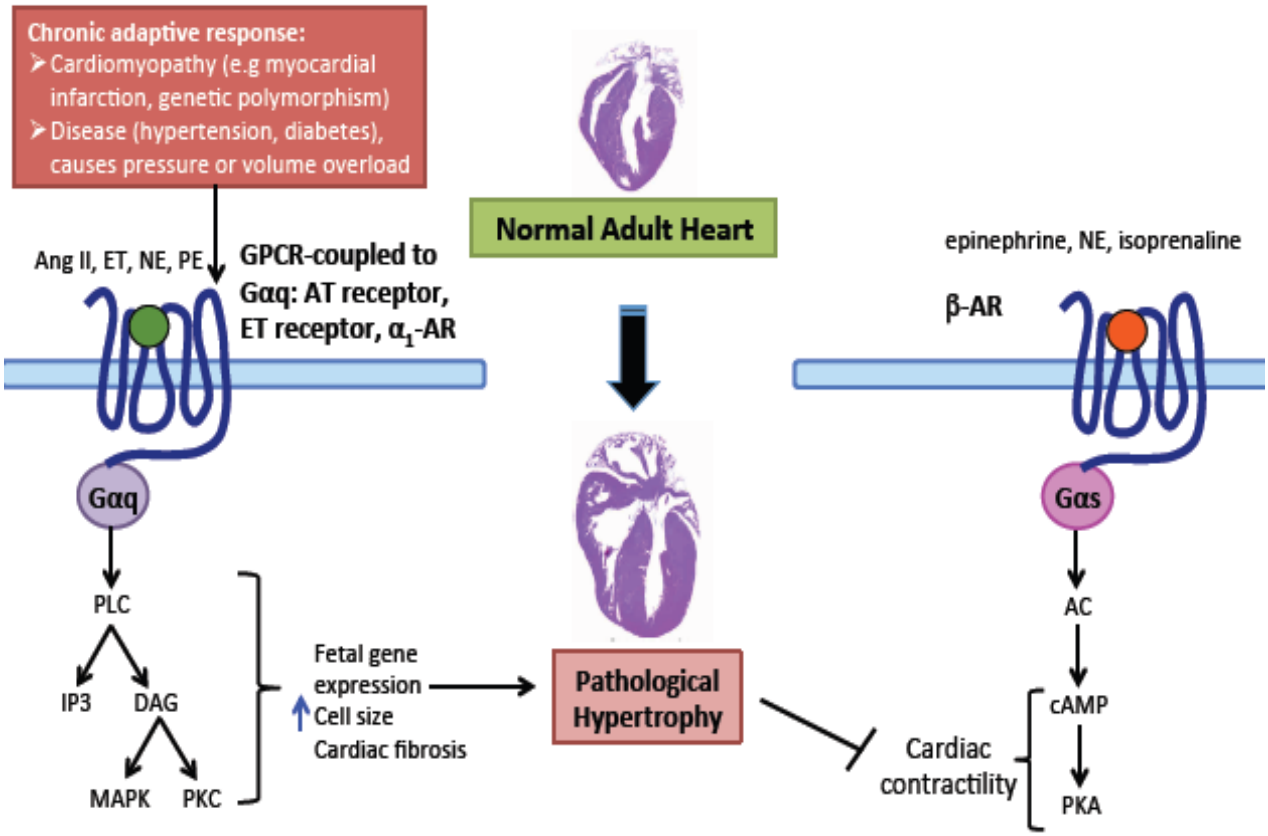


Figure 1. Crosstalk of Gq and Gs signaling in pathological cardiac condition. Cardiac contractility in normal adult heart is maintained by β-AR-coupled to Gs signaling. Chronic adaptive response causes hyper-activation of GPCR-coupled to Gq signaling, leading to various cellular responses, which induce ventricular remodeling (pathological hypertrophy). In this pathological condition, β-AR signaling was reported to be depressed. Those findings raise a possibility that Gq signaling may negatively modulate Gs signaling in pathological cardiac condition.

However, the mechanism underlying the suppression of Gs signaling by Gq still remains to be elucidated, because previous reports indicated that the suppression is not mediated by a decreased number of β-AR (D'Angelo et al. 1997), reduced AC expression (Roth et al. 1999; Tang et al. 2004), or enhanced β-AR desensitization by β-adrenergic receptor kinase (βARK) (Dorn et al. 2000). A recent report suggested that the mechanism might involve reduced Gαs abundance via augmentation of Gαs ubiquitination (Tang et al. 2008). Several groups, including our group, reported that Gαs signaling is regulated by the ubiquitin-proteasome pathway in mammalian cells (Tang et al. 2008; Naviglio et al. 2004; Nagai et al. 2010).

I.3 Ubiquitin proteasome system and Gαs as novel substrate for ubiquitination

Ubiquitination is one of post-translational modifications of proteins, and involved in the regulation of numerous cellular functions, such as the DNA damage response, apoptosis, cell growth, and innate immune response (Portbury et al. 2012). Recently, several reports have described the mechanism by which ubiquitination regulates the signaling pathways in a number of inflammatory diseases, including cardiac hypertrophy, heart failure, ischemia reperfusion injury, and diabetes (Hofmann and Pickart 2001). Ubiquitination forms an isopeptide bond between the C-terminus of ubiquitin (G76) and a lysine residue on the substrate or in the case of polyubiquitin chains, on the ubiquitin itself (Hershko and Chiechanover 1998). Ubiquitination is catalyzed by three enzymes: E1 (ubiquitin-activating); E2 (ubiquitin-conjugating); and E3 (ubiquitin ligase). The specificity of ubiquitination process is determined by E3 ligases, which recognize and interact with, and accelerate the transfer of ubiquitin from E2 to substrates (Hershko and Chiechanover 1998). All known E3 ligases utilize one of two catalytic domains, which are HECT domain or RING finger, and crystal structures have provided detailed views of the active site of each type (Pickart 2001).

Since ubiquitin itself contains 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63), ubiquitin can also bind to other ubiquitin molecules, thereby causing the polyubiquitination on the substrate (Portbury et al. 2012). Monoubiquitination does not induce degradation, and in case of polyubiquitination, different linkages have distinct functions. Polyubiquitinations linked to K48 are the primary targeting signals for proteasomal degradation by 26 S proteasomes, whereas polyubiquitinations through K63 generally affect substrate activity via non-proteolytic mechanisms (Hofmann and Pickart 2001).

Ubiquitination of G proteins have been observed in several studies either in *S. cerevisiae* or in mammalian cells. Gpa1 protein, the α subunit of G protein in *S. cerevisiae*, which negatively regulates mating pathway in response to pheromones by sequestering Gβγ, was obviously be degraded by ubiquitin/proteasome pathway (Madura et al. 1994). In Chinese hamster ovary (CHO) cells, treatment of proteasome inhibitor MG132 prevented the degradation of four G protein subunits including Gαs, Gαi2, Gαq and Gβγ in the presence of protein synthesis inhibitor cycloheximide (Lee et al. 2003). In human osteosarcoma U2OS cells, MG132 enhanced the membrane AC activity accompanying with increased expression of Gαs, but not Gαi, and ubiquitinated-Gαs. (Naviglio et al. 2004). Recently, our group demonstrated that Ric-8B stabilizes Gαs by inhibiting Gαs ubiquitination (Nagai et al. 2010).

I.4. Ric-8B as non-receptor type regulator of G protein signaling

In addition to classic GPCR-dependent activation of G protein, recently, non-receptor type regulators are known to modulate G protein activity. Ric-8 (resistance to inhibitors of cholinesterase-8) is a cytoplasmic protein that was originally identified by a genetic screening of *Caenorhabditis elegans* and reported to function as a novel non-receptor type of the G protein regulator (Miller et al. 2000). *In vitro study* revealed that Ric-8 functions as a guanine nucleotide exchange factor (GEF) for Gα (Afshar et al. 2004). Invertebrates have one Ric-8,

whereas mammals have two homologues of Ric-8, named Ric-8A and Ric-8B (Tall et al. 2003). *In vitro* study using yeast two hybrid screening of a rat brain embryonic cDNA library with Gαo and Gαs as baits succeeded in isolating Ric-8-A and Ric-8B, respectively (Tall et al. 2003). *In vitro* biochemical studies have shown that Ric-8A and Ric-8B interacted with different class of G proteins. Ric-8A is a GEF for Gαq, Gαi, Gαo, and Gα13 *in vitro* (Tall et al. 2003) and potentiates Gq signaling (Nishimura et al. 2006). On the other hand, Ric-8B was shown to interact with Gαs and Gαq (Tall et al. 2003; Nagai et al. 2010).

In addition to their role in non-receptor G protein signaling, Ric-8 has been known to be involved in receptor-dependent signaling processes (Fig. 2). It was reported that Ric-8B is capable of enhancing cAMP accumulation in a ligand-dependent manner when β-AR and dopamine-1 receptor were co-expressed with Gαolf (von Dannecker et al. 2005). Ric-8A acts as a GEF for monomeric Gα subunits and not for heterotrimeric complex (Tall et al. 2003).Thus, Ric-8 interaction to Gα subunits is likely to depend on the dissociation of the α subunit from the heterotrimeric complex that can be induced by ligand binding to GPCR, generating Gα-GTP and Gβγ dimer. Following the hydrolysis of GTP by Gα intrinsic GTPaseactivity, Ric-8 could bind to Gα-GDP and potentiate GPCR signaling by catalyzing guanine nucleotide exchange and preventing the formation of a heterotrimeric complex with Gβγ (Hinrichs et al. 2012).

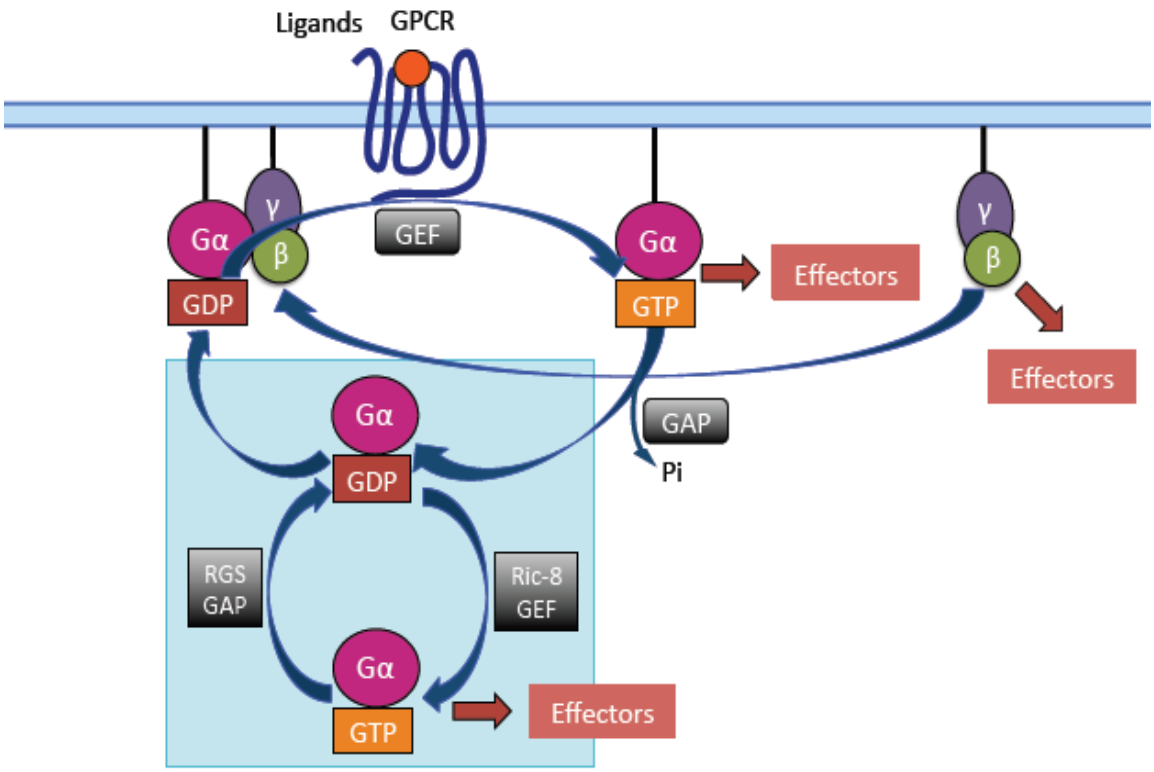


Figure 2. Conventional and unconventional G protein signaling regulation. In conventional signaling regulation, the GPCR functions as guanine nucleotide exchange factor (GEF) towards Gα subunit, resulting in GTP binding, heterotrimer dissociation, and subsequent Gα-GTP and Gβγ coupling to the effector protein to regulate signaling pathways. After GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the Gα subunit, the inactive Gα-GDP re-associates with Gβγ to regenerate heterotrimeric complex. In unconventional signaling regulation (blue square), Ric-8 interacts with Gα-GDP and exhibit GEF activity. The GTPase activity of RGS deactivates Gα-GTP by promoting GTP hydrolysis. This unconventional signaling potentiates the signal initiated by the ligand-GPCR complex.

As mentioned earlier, our group demonstrated that the ubiquitination of Gαs is inhibited by Ric-8B, thus Ric-8B stabilizes Gαs (Fig. 3) (Nagai et al. 2010). Furthermore, knockdown of Ric-8B attenuated β-AR response to ligand stimulation. The specific positive modulation of Ric-8B to Gαs requires interaction between the two proteins since Ric-8B splicing variants, which are defective for Gαs binding, failed to inhibit the ubiquitination (Nagai et al. 2010). Recently, the presence of GEF activity of Ric-8B to Gαs and putative role of Ric-8 as chaperone for nascent Gα subunits contributing to its proper membrane targeting were reported (Chan et al. 2011; Gabay et al. 2011), suggesting that Ric-8 is most likely to be a multifunctional G protein regulator.

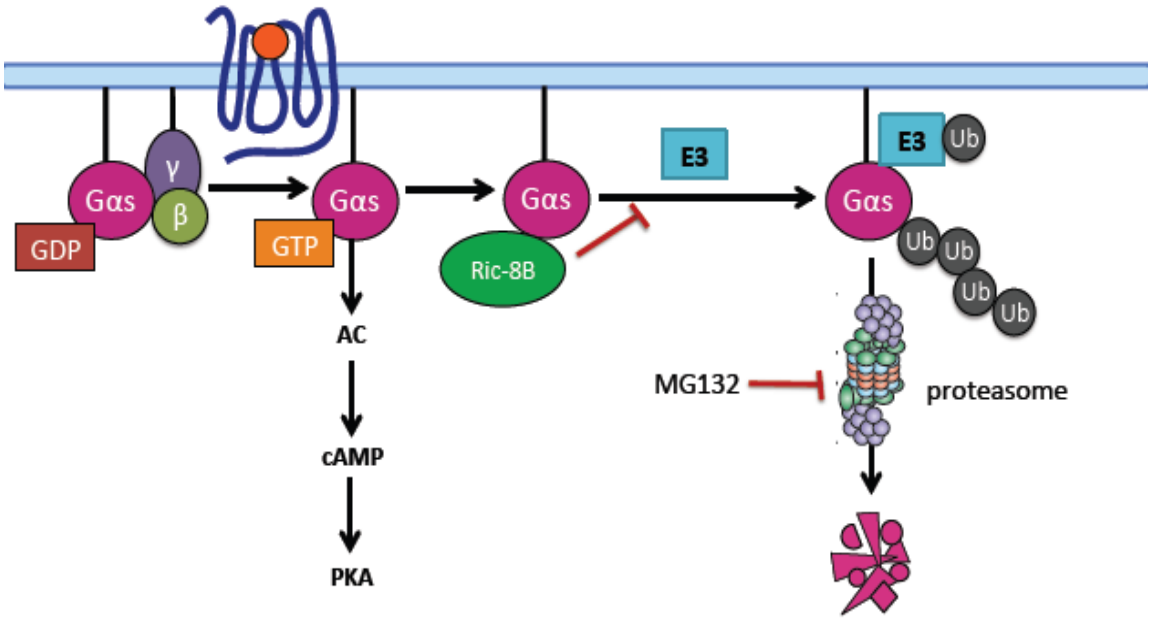


Figure 3. Ric-8B stabilizes Gαs. Ligand stimulation activates Gαs and induces dissociation of βγ subunit. Activated Gαs regulates its downstream effectors and also is known to be ubiquitinated by unknown E3 ligase, targeted for proteasomal degradation. Ric-8B inhibits Gαs ubiquitination by its direct interaction with Gαs. Thus, Ric-8B stabilizes Gαs and positively modulates Gs signaling (Nagai et al. 2010).

I.4 Purpose of this study

Hyperactivation of Gαq may interrupt Gs signaling and cause attenuation of β-adrenergic response to ligand stimulation as observed in pathological cardiac hypertrophy. The purpose of this study is to elucidate the molecular mechanism underlying the negative modulation of Gαq on Gs signaling. I hypothesize that the mechanism may take effect on Gs ubiquitination and Ric-8B may play an important role since it has an ability to stabilize Gαs by inhibiting ubiquitination and it binds both to Gαs and Gαq.

II. Materials and Methods

II.1 Reagents and antibodies

YM-254890 was kindly gifted by Jun Takasaki (Astellas Pharma, Inc.). Rabbit polyclonal antibodies against Gαs (C-18), Gαq (C-19), Gαi; mouse monoclonal antibodies against β-actin (C-4); and GST epitope (B-14) were purchased from Santa Cruz Biotechnology. A mouse monoclonal antibody against ubiquitin (FK2) was purchased from Enzo Life Sciences. The antibody against Ric-8B was prepared as described previously (Nagai et al. 2010). Mouse monoclonal anti-FLAG (M2) and anti-β tubulin antibodies were obtained from Sigma. The phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), was purchased from Nacalai Tesque. The protease inhibitor, MG132, was purchased from Calbiochem. Other reagents were obtained from Sigma, including isoproterenol, lysophosphatidic acid (LPA); α_1-AR agonist, phenylephrine (PE); α_1-AR antagonist, prazosin (Pz); PKC inhibitor, GF109203X; PI-3 kinase inhibitor, LY294002; ERK inhibitor, U0126; and JNK inhibitor, SP600125.

II.2 Recombinant adenovirus

Adenovirus expressing green fluorescent protein (GFP) and Gαq was prepared as described previously (Maruyama et al. 2002) and kindly provided by Dr. Kurose (Kyushu University). Infection of cells by adenovirus was monitored by GFP fluorescence.

II.3 Hypertrophied heart

Mouse hypertrophied heart samples were prepared by pressure overload with transverse aortic constriction (TAC) surgery as described previously (Nishida et al. 2008) and kindly provided by Dr. Kurose (Kyushu University). Briefly, TAC surgery and sham surgery were performed on 8- to 10-week-old male C57BL/6J mice. After surgery, mice were kept for 6

weeks. Heart functions were measured using echocardiography and catheter before sacrificed.

II.4 Cell culture and transfection

Human embryonic kidney 293T (HEK293T) and mouse embryo-derived fibroblast (NIH3T3) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (JRH Biosciences), 100 μg/ml streptomycin, and 100 units/ml penicillin at 37°C, with 5% CO_2. Plasmid DNAs were transfected into HEK293T cells using the calcium phosphate method or Lipofectamine 2000 (Invitrogen). Cultures of neonatal rat cardiac myocytes (NRCMs) isolated from the ventricles of 1- to 2-day-old Wistar rat pups were performed as described previously (Yamashita et al. 1997). Cardiac myocytes were plated on 2% gelatin-coated plates and cultured in DMEM with 10% FBS. After 24 h, cells were then starved in serum free low-glucose DMEM containing 10 nM insulin and 1 mM taurine and infected with recombinant adenoviruses at a multiplicity of infection (MOI) of 30 as indicated in figures. When Ric-8B was overexpressed in NIH3T3 cells or cardiac myocytes, plasmid DNAs were transfected using Fugene 6 (Roche).

II.5 Retroviral production and infection

HEK293T cells were transfected with Psi2 helper retroviral plasmid together with pMSCV-puro vectors encoding Gαq or other type of Gα proteins (Gαi) and/or FLAG-Ric-8B. Viruses were harvested 48 h post-transfection, and pooled. NIH3T3 cells ($1x10^5$ cells/60-mm dish) were infected once with 1.5 ml of retrovirus-containing supernatant supplemented with 8μg/ml polybrene. If cells needed to be infected with another virus, the second infection was done 2 h after the first infection. Twenty-four hours after infection, cells were selected in 7.5 μg/ml puromycin for 72 h.

II.6 Measurement of intracellular cAMP accumulation

NIH3T3 cells infected with retroviruses to overexpress Gαq and Ric-8B were pretreated with 0.5 mM IBMX for 30 min and subsequently stimulated with 10 μM isoproterenol for 15 min. Cyclic AMP was measured using the AlphaScreen cAMP assay kit (PerkinElmer Life Sciences) according to the manufacturer's protocol. When cardiac myocytes expressing Gαq were used, cells were infected with adenoviruses indicated in the figures; 48 h later, cAMP accumulation was measured. In other experiments, uninfected NIH3T3 cells or NRCMs were stimulated with ligands described in the figures, and measurements were performed.

II.7 *In vivo* ubiquitination assay

An *in vivo* ubiquitination assay was performed as described previously (Kuo et al. 2004). HEK293T cells were transfected with pMT107-6xHis-ubiquitin and other indicated expression plasmids including pCMV5-Gαs, pCMV5-Gαq, or pCMV5-FLAG-Ric-8B. Cells were harvested by centrifugation 48 h post-transfection. Cells were lysed with urea lysis buffer (10 mM Tris-HCl, pH8.0, 10 mM NaH_2PO_4, 8 M urea, 10% glycerol, 0.1% Triton X-100, 0.5 M NaCl, 10 mM imidazole, and 10 mM β-mercaptoethanol) and disrupted by sonication. Lysates were centrifuged at 14,000 rpm for 5 min at room temperature, and supernatants were collected. For purification of His_6-tagged ubiquitinated proteins, Ni-NTA agarose (Qiagen) was added to the supernatant and gently agitated for 4 h at room temperature. The resins were washed 5 times with 20 mM imidazole in urea lysis buffer and treated with 150 mM Tris-HCl, pH 6.8, containing 200 mM imidazole, 5% SDS, 30% glycerol, and 0.72 M β-mercaptoethanol. Ubiquitinated Gαs protein was detected by immunoblotting using anti-Gαs antibody.

II.8 Immunoprecipitation and immunoblotting

Cardiac myocytes were starved in serum-free DMEM for 24 h before being stimulated with 10 μM PE for 24 h. Ten micromolar of MG132 was added 12 h before PE stimulation was ended. Cells were then lysed in a lysis buffer (10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 20 mM β-

glycerophosphate, 2 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethane sulfonylfluoride (PMSF), 1 μg/ml leupeptin) per 60-mm dish. The extracts were centrifuged at 14,000 rpm for 10 min. The supernatants were incubated with 1 μg anti-Gαs antibody and protein G-Sepharose (GE Healthcare), for 1.5 h at 4 °C. HEK293T cells were transfected with the indicated combinations of plasmids, including pCMV5-Gαq or pCMV5-FLAG-Ric-8B. Cells were lysed and immunoprecipitated with 1 μg anti-FLAG antibody 48 h after transfection. The immunoprecipitates were washed with a lysis buffer 4 times, denatured by adding an SDS-PAGE sample buffer, and subjected to SDS-PAGE. The proteins were transferred to PVDF membranes (Millipore), blocked with 5% non-fat skim milk, and probed with antibodies as indicated in the figures. To examine polyubiquitinated proteins of ventricles from TAC mouse hearts by immunoblot, the ventricles from frozen heart samples were excised in small cubes and homogenized in ice-cold lysis buffer with the same composition as described above using a Potter homogenizer at a setting of 1,000 rpm for 20 strokes. The homogenate was centrifuged at 14,000 rpm for 10 min to obtain the supernatant. Equal amounts of protein were analyzed by immunoblot assays with the indicated antibodies.

II.9 *In vitro* binding assay

In vitro binding of GST- Ric-8B to Gαs and Gαq was performed as described previously (Tall et al. 2003). Recombinant GST-Ric-8B, Gαq, and Gαs proteins were prepared as previously described (Nagai et al. 2010). Briefly, 30 nM of GST-Ric-8B was incubated with 1 μM Gαq in binding buffer (20 mM HEPES-NaOH, pH 8.0, 100 mM NaCl, 10 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, and 0.05% Lubrol PX) for 30 min at 25 °C. One hundred nanomolar of Gαs was added to the mixture for further incubation in the binding buffer for 1 h at 25 °C. Glutathione-Sepharose 4B (GE Healthcare) was added to the reaction mixture and gently agitated for 30 min at 4 °C. The resins were washed 3 times with binding buffer and treated with 20 mM free glutathione in binding buffer (pH 7.5). The eluted proteins were resolved by SDS-PAGE and immunoblotted with anti GST, anti-Gαs or anti-Gαq antibodies.

II.10 Reporter gene assay

NIH3T3 cells were seeded on 24-well plate at about 70% confluence. Cells were transfected with 90 ng/well firefly CRE-luciferase reporter gene plasmids (pCRE-Luc), 10 ng/well *Renilla*-Luciferase (pEF-*Renilla*-Luc) plasmid, and different amounts of FLAG-Ric-8B plasmid (0, 1, 10, and 100 ng/well). Twenty-four hours after transfection, cells were stimulated with 10 μM isoproterenol for 16 h, and luciferase activity in cell lysates was measured with a luminometer (ARVO MX, PerkinElmer Life Sciences) using the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to the internal control activity of *Renilla*-Luc.

III. Results

III.1 Global ubiquitination is increased in cardiac hypertrophic model

In the previous study, our group reported that α subunit of Gs protein is regulated by ubiquitin-proteasome pathway in a manner dependent on Ric-8B (Nagai et al. 2010), and suggested that this maybe involved in heart failure, since Gs signaling is essential for the heart's function. First, I analyzed the total ubiquitination in the cardiac hypertrophic model. Transverse aortic constriction (TAC) in the mouse is an experimental model for the cardiac hypertrophy induced by pressure overload. Six weeks after TAC operation, mouse hearts showed 70% increase of weight and the elevated expression of pathological hypertrophy markers including ANP, β-MHC, c-myc and c-fos (Nishida et al. 2008). Furthermore, in TAC mouse heart, Gq signaling was activated, and β-adrenergic response was reduced (Esposito *et al*. 2001; Pandya *et al*. 2009). I prepared the homogenates of the hypertrophic hearts and performed immunoblot analysis with anti-ubiquitin antibody. As shown in Fig. 4A, the total ubiquitination was obviously potentiated in cardiac hypertrophy. Next, to investigate that cardiac Gαs is regulated by ubiquitin-proteasome pathway, the effect of proteasome inhibitor MG132 on cardiomyocytes was examined (Fig. 4B). MG132 effectively enhanced the expression level of Gαs protein in cardiomyocyte, suggesting that the ubiquitination of Gαs contributes to maintenance of its expression level. Previous studies indicated that the Gαq molecule predominantly regulates the development of hypertrophy (Adams et al. 1998; Wettschureck et al. 2001). To test the possibility that prolonged stimulation of Gq signaling may cause the ubiquitination of Gαs, I utilized neonatal rat cardiomyocytes (NRCMs) that express endogenous α- and β-adrenergic receptors and have similar gene expression observed in *in vivo* pathological hypertrophy (Izumo et al. 1988). NRCMs were stimulated with α_1-AR-specific agonist, phenylephrine (PE), then analyzed by immunoblotting. Strikingly, PE

effectively enhanced the total ubiquitination and reduced Gαs expression level (Fig. 4C). This effect was canceled by treatment with the α_1-AR antagonist, prazosin, and MG132. Moreover, the ubiquitination in immunoprecipitates with the anti-Gαs antibody was specifically augmented by the PE stimulation in the presence of MG132 (Fig. 4C). These data suggest that cardiac hypertrophy induces global ubiquitination, and continuous activation of Gq signaling enhances the proteasomal degradation of the Gαs protein.

III. 2 Continuous stimulation of Gq signaling decreases cAMP accumulation

To test whether the reduction of Gαs induced by continuous Gq activation affects Gs signaling, cardiomyocytes were pretreated with PE prior to stimulation with the β-AR-specific agonist, isoproterenol. As shown in Fig. 5A, stimulation with isoproterenol strongly enhanced cAMP accumulation in the cardiomyocytes; this response was reduced by pretreatment with PE. The addition of α_1-AR antagonist, prazosin, attenuated the suppression. This result correlated well with reduced Gαs expression, as shown in Fig. 4C. In addition to cardiomyocytes, NIH3T3 cells were utilized to confirm whether another endogenous Gq signal diminishes Gs-mediated cAMP accumulation. Since NIH3T3 cells do not express α_1-AR, I used lysophosphatidic acid (LPA), which is known to stimulate Gq signaling. Similarly, the isoproterenol-induced cAMP production was effectively suppressed by long stimulation with LPA (Fig. 5B). The inhibitory effect was canceled by treatment with a Gq-specific inhibitor, YM-254890 (Nishimura et al. 2010), indicating that the inhibitory effect of LPA should be mediated through Gq.

III.3 Overexpression of Gαq reduces Gαs expression

Hyperactivation of Gq signaling should increase the free form of Gαq. Transgenic mice with cardiac-targeted overexpression of α_1-AR or Gαq showed hypertrophy and reduced β-

adrenergic response in the heart (D'Angelo *et al*. 1997; Akhter *et al*. 1997). To investigate the effect of excess amounts of Gαq on the Gαs expression level and Gs signaling, I forcibly expressed Gαq in NRCMs by using adenovirus. When Gαq was overexpressed, the protein amount of Gαs was drastically reduced (Fig. 6A). Similarly, the overexpression of Gαq strongly inhibited the cAMP accumulation induced by isoproterenol (Fig. 6B). In NIH3T3 cells, overexpression of Gαq induced the reduction of Gαs and cAMP accumulation (Figs. 6C,D). On the other hand, overexpression of Gαi2 in NIH3T3 did not induce any reduction in the Gαs protein level or cAMP accumulation (Figs. 7A,B). These observations suggest that excess Gαq interrupted Gs signaling by reducing Gαs expression. It was reported that transgenic overexpression of Gαq in mice induces hypertrophy-associated expression of fetal cardiac genes and activates PKCε, but does not activate MAP kinase (D'Angelo et al. 1997). To test the involvement of the downstream signaling molecules of Gq in the crosstalk between Gq and Gs, I treated NIH3T3 cells overexpressing Gαq with several inhibitors prior to the stimulation with isoproterenol. All inhibitors used in this experiment, including the PKC inhibitor (GF109203X) and MAPK pathway inhibitors (U0126 and SP600125) failed to have any effect on either Gαs expression or cAMP accumulation (Figs. 8A,B). These results suggest that the negative regulation of Gαq on Gαs protein and its signaling pathway is mediated by a novel mechanism rather than by the downstream signaling of Gq such as PLC-PKC and MAPK pathways.

III.4 Ric-8B attenuates Gq-induced suppression of Gs signaling

Ric-8B is known as specific positive modulator for Gs by inhibiting Gαs ubiquitination (Nagai et al. 2010). To test whether Ric-8B could protect Gαs expression and its signaling from a suppressive effect by Gαq, I co-expressed Gαq and Ric-8B in NIH3T3 cells. As expected, Gq-induced reduction of the Gαs protein was canceled by co-expression

of Ric-8B (Fig. 9A). Furthermore, expression of Ric-8B partially abrogated the Gαq-induced suppression of cAMP accumulation in response to isoproterenol (Fig. 9B). These results suggest that Ric-8B plays an important role in the crosstalk of Gq on Gs signaling and regulates the degradation machinery of Gαs for fine-tuning Gs signaling. To clarify the dose dependency of Ric-8B expression, NIH3T3 cells were transfected with various amounts of expression vectors of Ric-8B. As shown in Fig. 10A, ectopic expression of Ric-8B enhanced the isoproterenol-induced transcriptional activation through cAMP-response element (CRE) with the bell-shaped dose dependency. A higher amount of Ric-8B expression showed a weaker effect on isoproterenol-induced CRE activation. I also used NRCMs to test the effect of Ric-8B on cAMP production (Fig. 10B). Enforced expression of Ric-8B enhanced the isoproterenol-induced cAMP production. The data utilizing cardiomyocytes emphasize the critical roles of Ric-8B in Gs signaling.

Since mammals have another homologue of Ric-8, named Ric-8A, I also tested the effect of Ric-8A on Gαq-induced suppression of Gs signaling. Unexpectedly, co-expression of Ric-8A also rescued the reduction of Gαs expression and isoproterenol-induced cAMP accumulation (Figs. 11A,B). These results indicate that the attenuation of Gαq-induced suppression on Gs signaling is not Ric-8B specific. Ric-8A binds to Gαq, but does not bind to Gαs (Tall et al. 2003). It suggests that excess amount of free Gαq in cells is regulated by Ric-8A and Ric-8B, and the regulation is important in maintaining Gs signaling.

III.5 Ric-8B cancels Gαs ubiquitination induced by Gαq

The ubiquitination of Gαs was enhanced when Gαq was ectopically expressed in HEK293 cells (Tang et al. 2008). A similar regulation mechanism for controlling Gs signaling seems to work in cardiac myocytes, because treatment of MG132 enhanced the endogenous Gαs expression when Gαq was overexpressed in cardiac myocytes (Fig. 12A).

The previous reports and my current result suggest that Gαs protein is most likely to be degraded by ubiquitin-proteasome pathway in cardiomyocytes. Next, to investigate the involvement of Ric-8B in Gαs ubiquitination induced by Gαq, I performed *in vivo* ubiquitination assay by using HEK293T cells. Cells were transfected with His-ubiquitin and the indicated combination of plasmids including Gαq, Gαs, FLAG-Ric-8B. After cells were lysed in denaturing conditions, ubiquitinated Gαs was precipitated by Ni-agarose resin and detected by anti-Gαs antibody. I found a more intense ladder of ubiquitinated Gαs proteins in the presence of ectopic Gαq. Interestingly, co-expression with Ric-8B completely blocked the Gαq-induced ubiquitination (Fig. 12B). These findings strongly support my hypothesis that negative modulation by Gq on Gs signaling may have an effect on Gαs stability that is maintained by Ric-8B. Ric-8A also attenuated Gαs ubiquitination induced by Gαq (Fig. 13). This result suggests again that excess free Gαq induces the promotion of Gαs ubiquitination by sequestering Ric-8B, because Ric-8A has an ability to inhibit the Gαq-induced Gαs ubiquitination by making a complex with excess Gαq.

III.6 Excess Gαq sequesters Ric-8B from its binding to Gαs

The positive regulation of Ric-8B to Gαs requires their direct interaction; however, Ric-8B binds to both Gαs and Gαq (Nagai et al. 2010). Therefore, I hypothesized that the competitive binding of Gαs and Gαq to Ric-8B may cause the Gαq-induced reduction of Gαs. To test this hypothesis, I performed a co-immunoprecipitation assay using lysate from HEK293T cells, which express Gαq with FLAG-Ric-8B. I found that endogenous Gαs was co-immumoprecipitated with FLAG-tagged Ric-8B (Fig. 14A). Strikingly, exogenous expression of Gαq effectively inhibited the physical interaction between Gαs and Ric-8B. To confirm the competitive binding, I performed *in vitro* pull down assay using recombinant proteins of GST-Ric-8B, Gαq and Gαs, which were purified from bacterial lysate. Both Gαs

and Gαq directly bind to Ric-8B (Nagai et al. 2010). I set the molar ratio of GST-Ric-8B, Gαs and Gαq as 1:3:30, respectively. GST-Ric-8B was incubated with Gαq for 30 min, and then Gαs was added. After further 1 h incubation, GST-Ric-8B was precipitated with glutathione-Sepharose. Similar to the result from the co-immunoprecipitation assay, the addition of Gαq inhibited the binding of Ric-8B to Gαs (Fig. 14B). These findings indicate that excess free form of Gαq released from Gαqβγ upon Gq-coupled receptor activation competes with Gαs for binding to Ric-8B, resulting in the promotion of Gαs ubiquitination and the reduction of Gαs protein and Gs signaling. This may provide new possible mechanism on how β-adrenergic signaling is depressed in pathological hypertrophic heart.

III.7 Involvement of PKC in suppression of Gs signaling

Though in previous experiment I have demonstrated that PKC inhibitor didn't cancel Gαq-induced suppression of Gs signaling in overexpression system (Figs. 8A,B), one report suggested that PKC phosphorylates type VI adenylyl cyclase (AC), thereby inhibits its activity (Lin et al. 2002). Since AC type VI is a dominant AC isoform in mammalian cardiac myocytes (Sunahara et al. 1996), therefore, I investigated effect of PKC specific inhibitor, GF109203X, on cAMP accumulation in hyper-activation of endogenous Gq signaling condition. Similar to experiment in Figure 5A, I stimulated cardiomyocytes with PE in the absence or presence of the PKC inhibitor, and then measured cAMP accumulation in response to isoproterenol stimulation (Fig. 15). In contrast to ectopic Gαq-induced suppression of Gs signaling, the α_1-AR-mediated suppression was partially recovered by GF109203X. This result suggests the involvement of PKC activation in downregulation of Gs signaling after prolonged activation of Gq-coupled receptor.

III.8 Mdm2 may not be the E3 ligase for Gαs

Previously, Tang et al. reported the possible involvement of Mdm2 in the ubiquitination of Gαs (Tang et al. 2008). To verify the possibility, I utilized the MEFs lacking p53 and Mdm2 (DKO MEFs, double knockout mouse embryonic fibroblasts) (Weber et al. 2000), and tested whether the Gαq-caused suppression of Gαs protein and signaling requires Mdm2. In DKO MEFs, enforced expression of Gαq still suppressed the reduction of Gαs protein and isoproterenol-induced cAMP accumulation. Overexpression of Mdm2 in DKO MEFs failed to affect neither isoproterenol-stimulated cAMP production nor the protein expression of Gαs (Fig. 16). This result suggests that Mdm2 may not be involved in Gαq-induced suppression of Gs signaling.

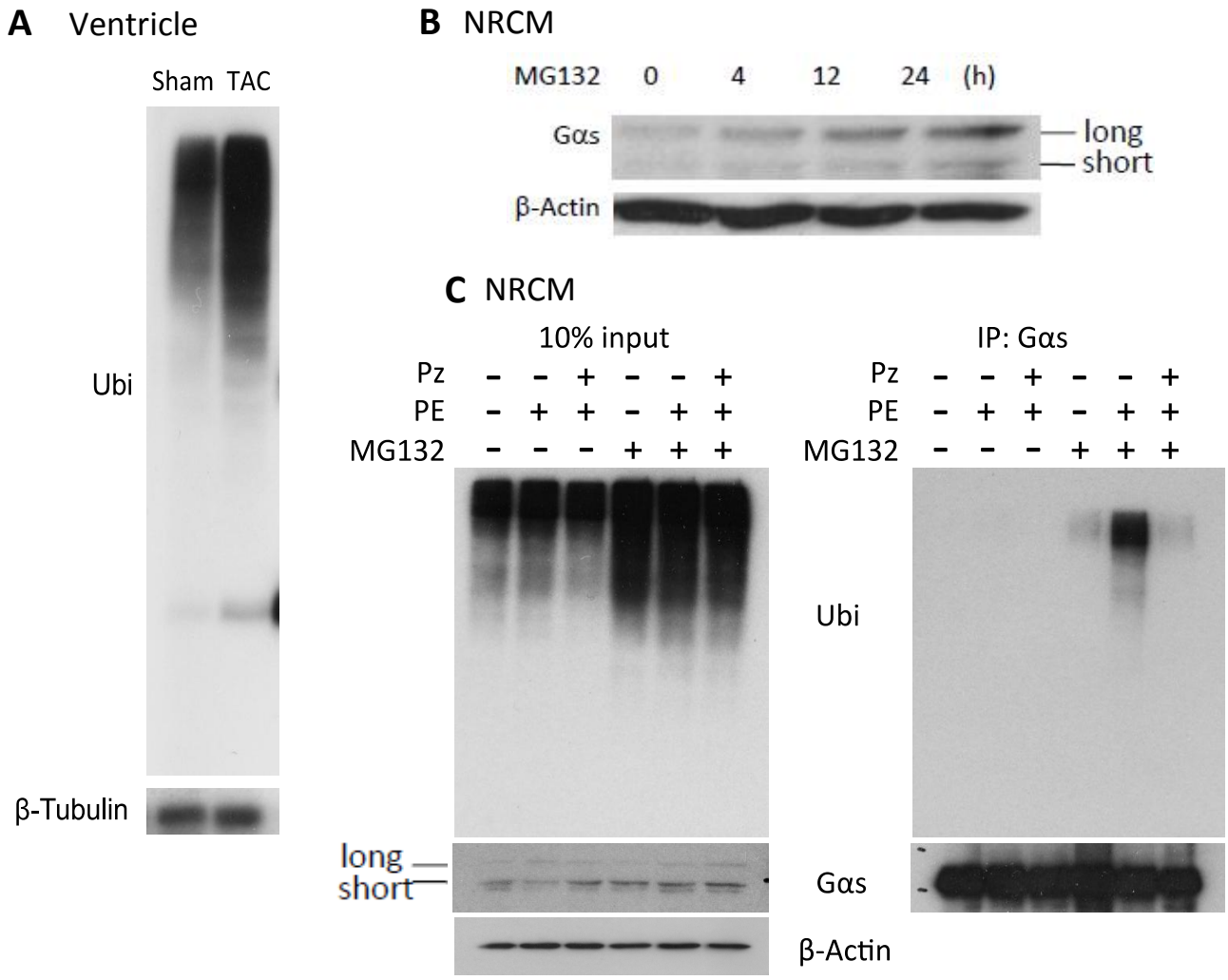


Figure 4. Increased ubiquitination in cardiac hypertrophic model and Gq signal induced the suppression of Gαs protein and the ubiquitination. A, Immunoblot analysis of polyubiquitinated proteins from the ventricle of sham and transverse aortic constriction (TAC) mice. **B,** Neonatal rat cardiomyocytes (NRCMs) were treated with 10 μM proteasome inhibitor, MG132, for the indicated periods. Expression of Gαs was analyzed by immunoblot. There are four spliced forms of Gαs (Kozasa et al. 1988), and major long and short forms are indicated by lines. The ratio of the spliced forms depends on the cell types. **C,** NRCMs were treated with 10 μM prazosin (Pz) and 10 μM phenylephrine (PE) for 24 h in the presence or absence of 10 μM MG132 for 12 h before stimulation was ended. Cells were lysed, and Gαs was immunoprecipitated. The immunoprecipitates and lysates (10% input) were analyzed by immunoblot with the indicated antibodies.

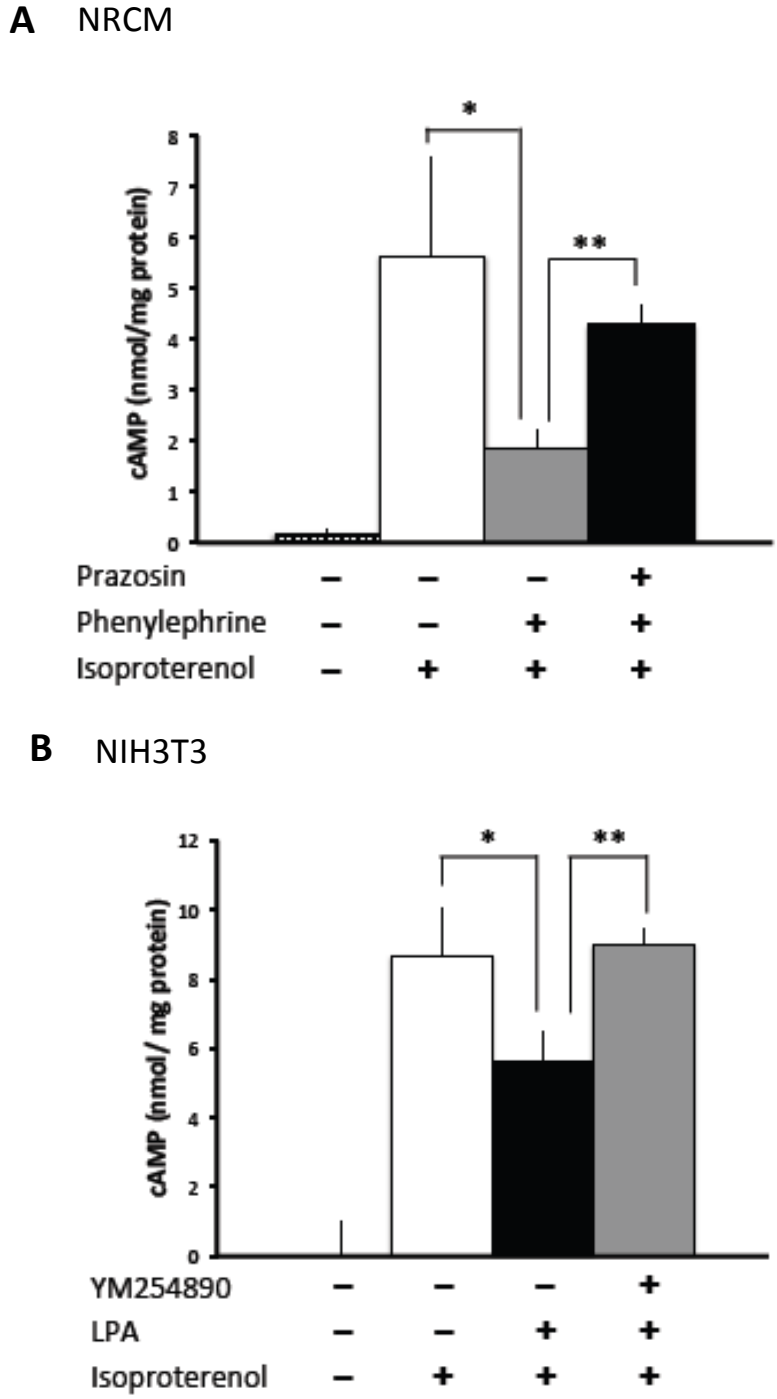


Figure 5. Stimulation of Gq signaling suppresses the β-AR-induced cAMP accumulation. A, NRCMs were stimulated with 10 μM PE for 24 h with or without 10 μM Pz. Then cells were stimulated with 1 μM isoproterenol for 5 min, and cAMP accumulation was measured. **B,** NIH3T3 cells were treated with LPA for 6 h with or without 1 μM of YM-254890. Cells were stimulated with 10 μM isoproterenol for 15 min, and cAMP accumulation was measured. Error bars mean s.d. (n=3, *P<0.05; **, p < 0.005, Student's t test).

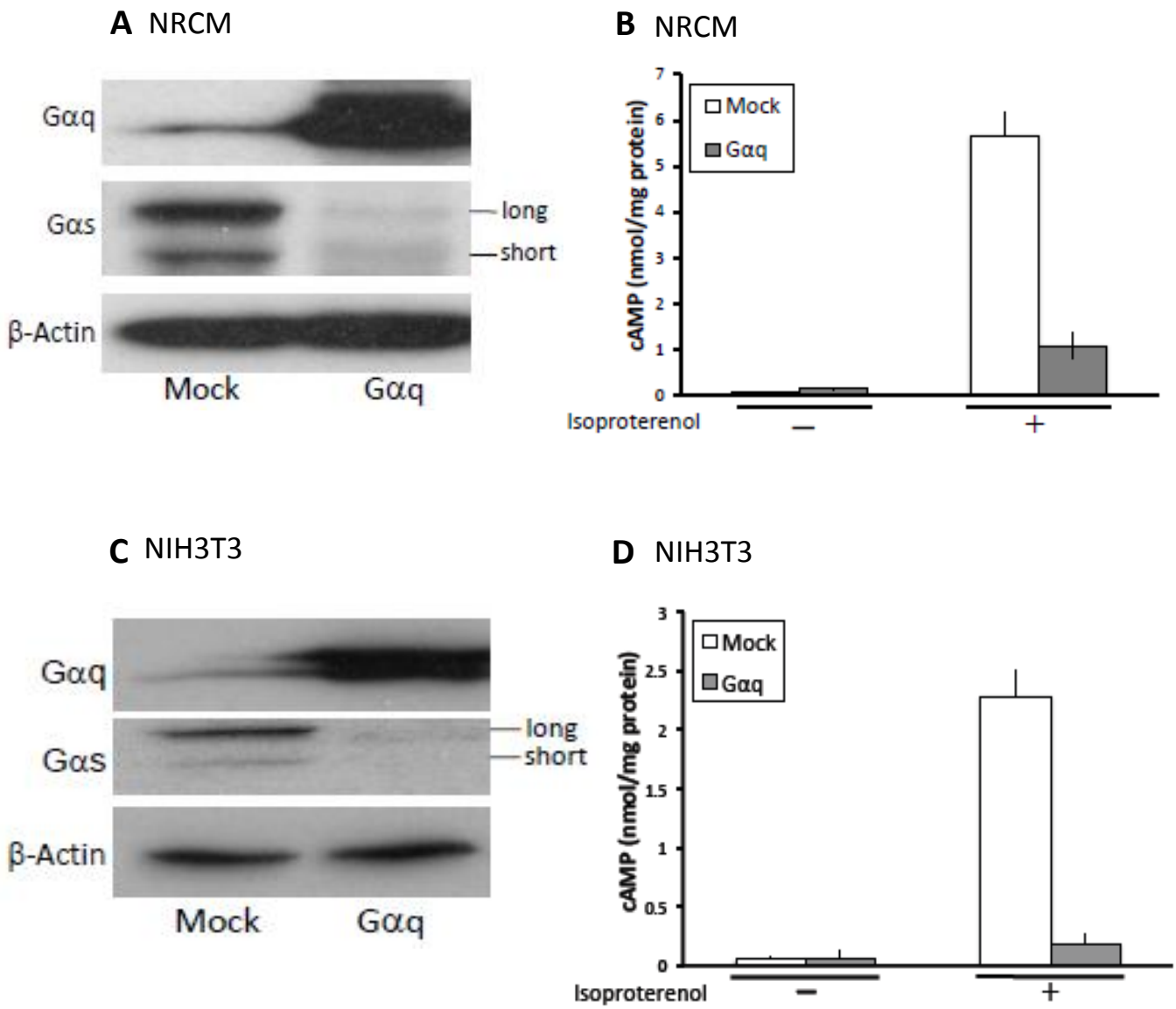


Figure 6. Overexpression of Gαq reduces Gαs protein and suppresses Gs signaling. A, NRCMs were infected with adenoviruses harboring GFP and Gαq at MOI 30. Cell lysates were analyzed by immunoblot using the indicated antibodies after 48 h of infection. **B,** NRCMs infected with adenoviruses were stimulated with 1 μM isoproterenol for 5 min, then cAMP accumulation was measured. **C,** Cell lysates from NIH3T3 cells infected with either an empty retrovirus or a retrovirus harboring Gαq were subjected to immunoblot analysis using the indicated antibodies. **D,** NIH3T3 cells infected with retrovirus were stimulated with 10 μM isoproterenol for 15 min, then cAMP accumulation was measured. Error bars mean s.d. (*n*=3).

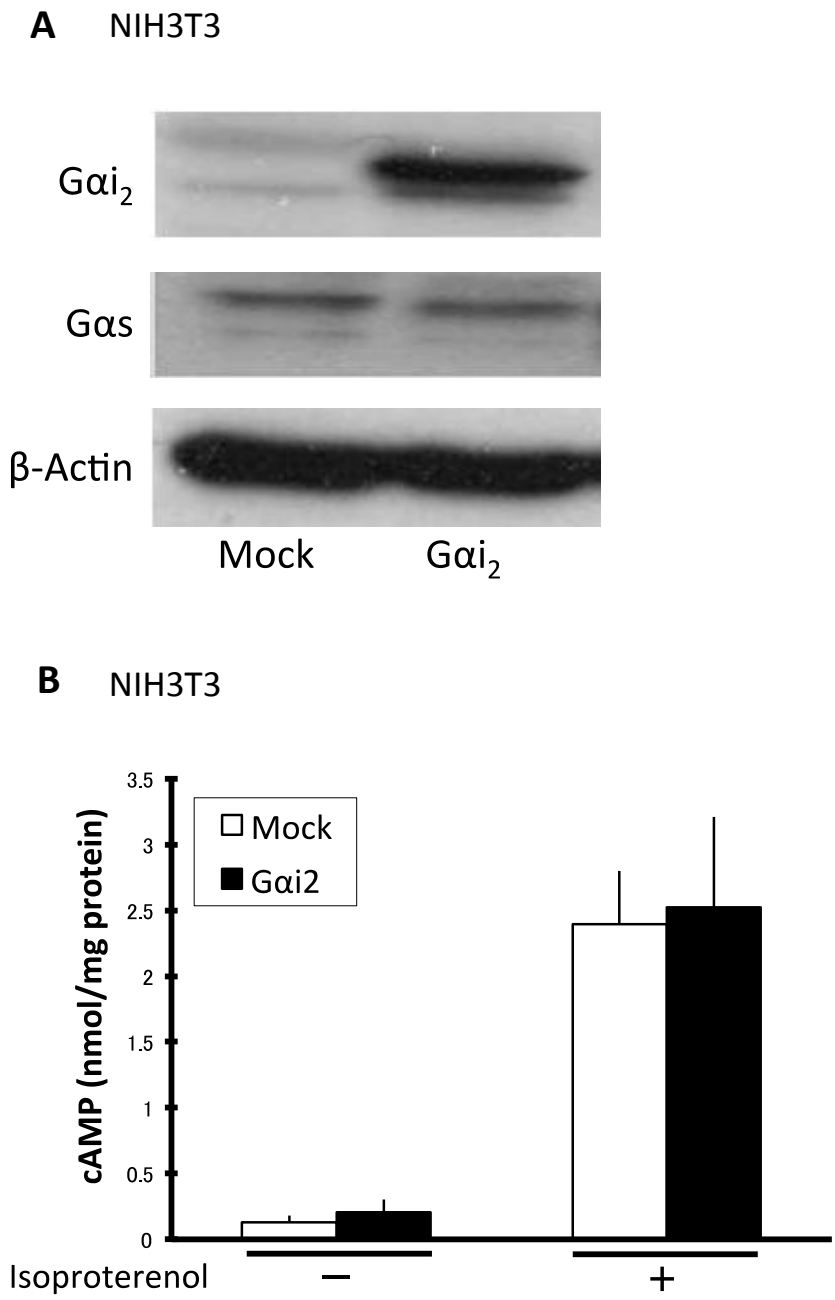


Figure 7. Overexpression of Gαi does not suppress Gs signaling. A, Cell lysates from NIH3T3 cells infected with the indicated retroviruses were subjected to immunoblot analysis using the indicated antibodies. **B,** The infected NIH3T3 cells were stimulated with 10 μM isoproterenol for 15 min, then cAMP accumulation was measured. Error bars mean s.d. (*n*=3).

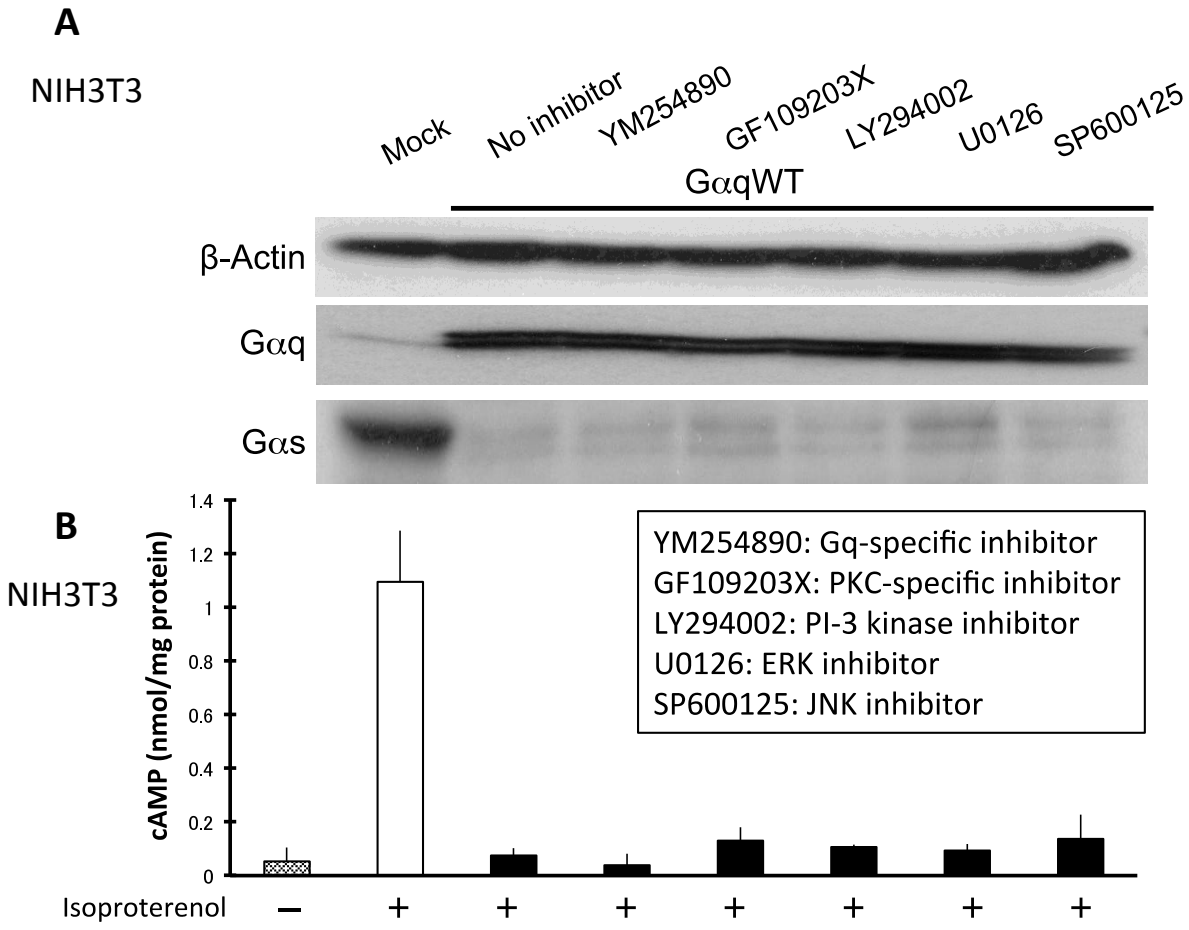


Figure 8. Gq downstream effectors do not mediate Gαq-induced Gs signaling suppression. A, NIH3T3 cells were infected with either an empty retrovirus or a retrovirus harboring Gαq. Cell lysates were analyzed by immunoblot using the indicated antibodies after 3 h treatment with the indicated Gq downstream signaling inhibitors. **B**, The infected NIH3T3 cells were treated with the indicated inhibitors prior to stimulation with 10 μM isoproterenol for 15 min, then cAMP accumulation was measured. Error bars mean s.d. (*n*=3).

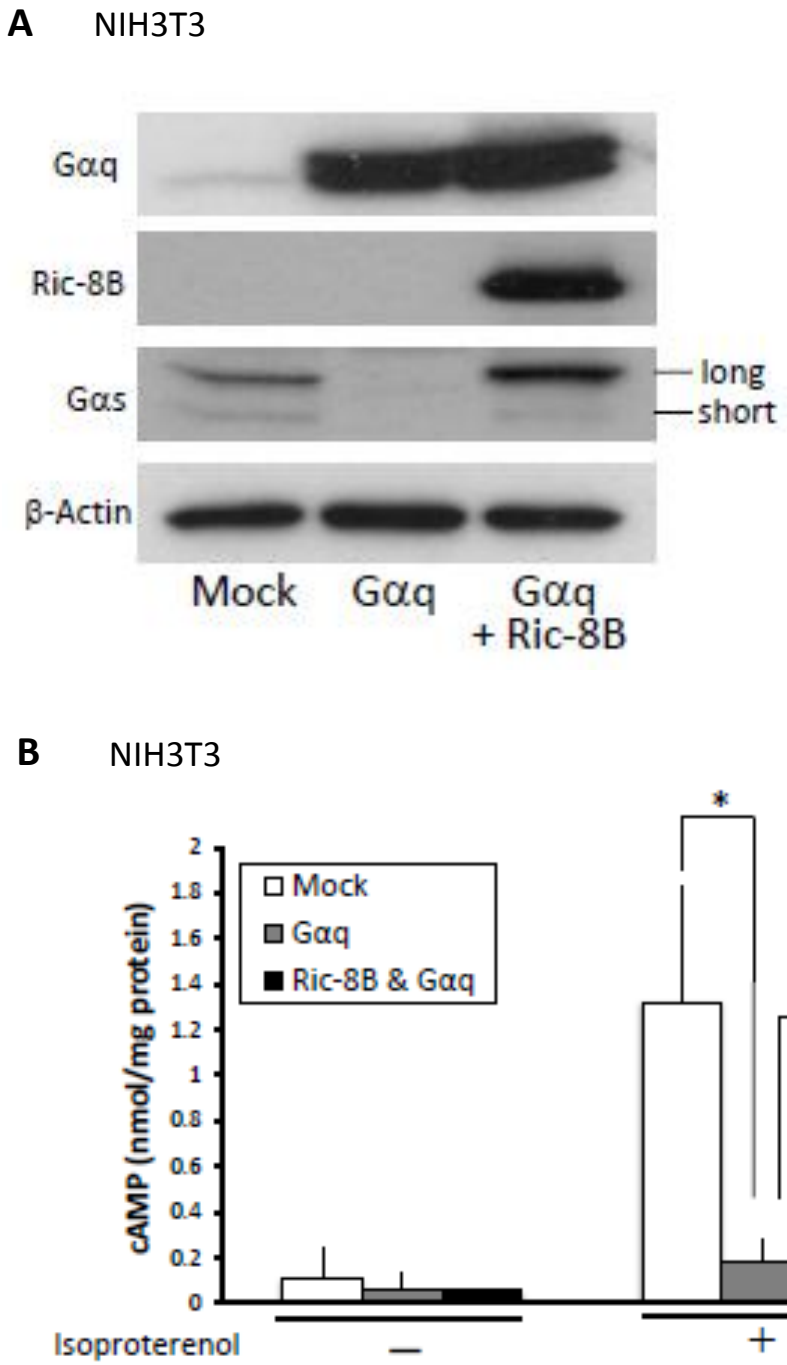
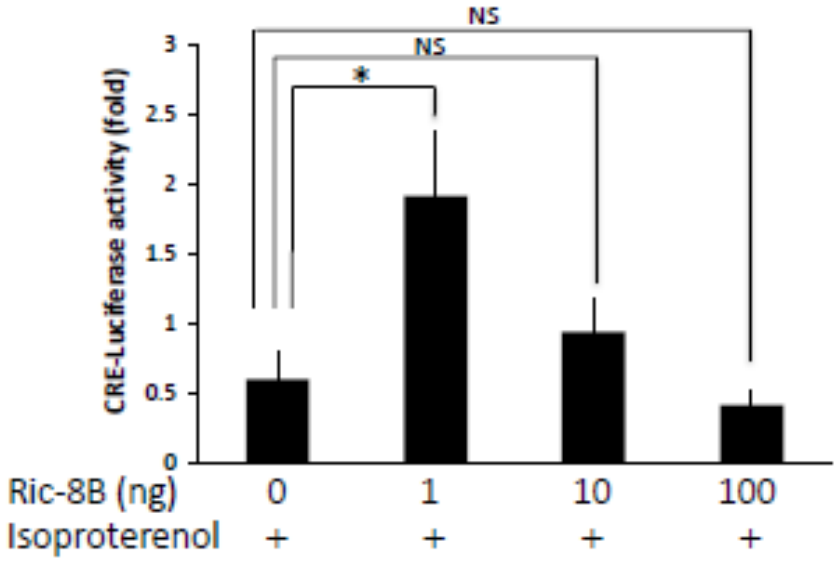


Figure 9. Ric-8B attenuates Gαq-induced suppression of Gs signaling. A, Cell lysates from NIH3T3 cells infected with an empty retrovirus or a retroviruses harboring Gαq and FLAG-Ric-8B were analyzed by immunoblotting using the indicated antibodies. **B**, NIH3T3 cells infected with retroviruses were stimulated with 10 μM isoproterenol for 15 min, then cAMP accumulation was measured. Error bars mean s.d. (n=3, *P<0.05, Student's t test).

A NIH3T3



B NRCM

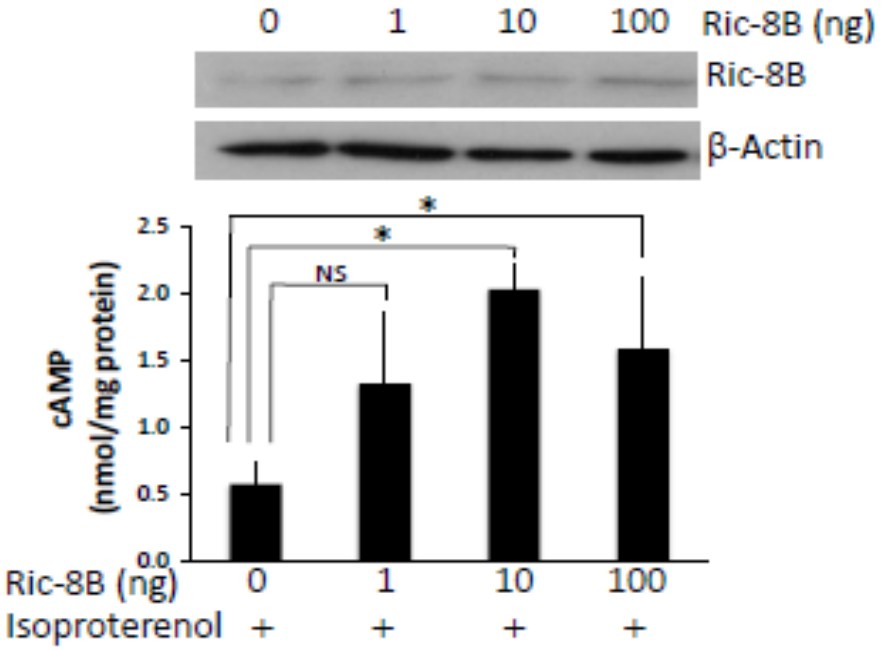
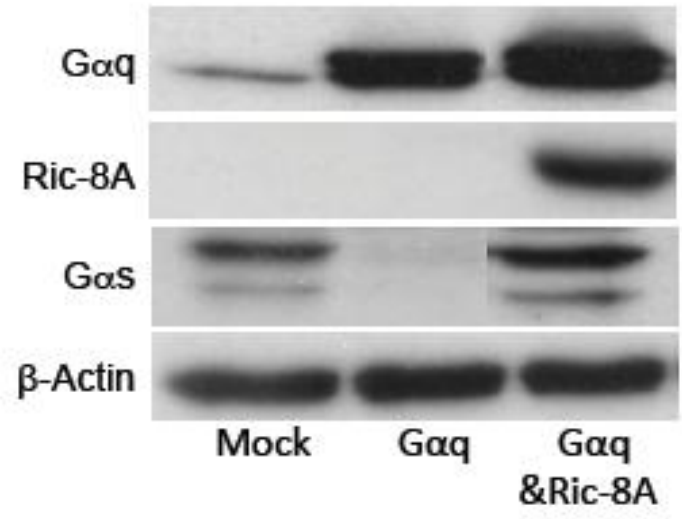


Figure 10. Ric-8B needs optimum expression level to enhance Gs signaling. A, NIH3T3 cells were co-transfected with pCRE-luciferase, a *Renilla*-luciferase plasmid, and the indicated amount of plasmid harboring FLAG-Ric-8B. After 24 h, cells were stimulated with 10 μM isoproterenol for 16 h, and luciferase activity in cell lysates was measured as described in Experimental Procedures. **B,** NRCMs were transfected with the indicated amount of plasmid harboring FLAG-Ric-8B. After 48 h, cells were stimulated with 1 μM isoproterenol for 5 min, and cAMP accumulation was measured. Error bars mean s.d. (n=3, NS=non-significant; *P<0.05, Student's t test).

A NIH3T3



B NIH3T3

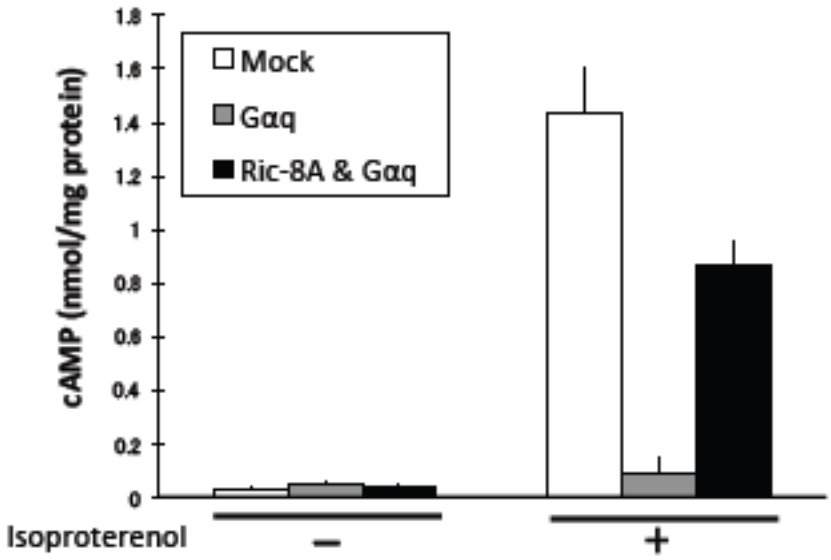


Figure 11. Ric-8A rescues Gαq-induced suppression of Gs signaling. A, Cell lysates from NIH3T3 cells infected with an empty retrovirus or a retroviruses harboring Gαq and FLAG-Ric-8A were analyzed by immunoblotting using the indicated antibodies. **B**, NIH3T3 cells infected with retroviruses were stimulated with 10 μM isoproterenol for 15 min, then cAMP accumulation was measured. Error bars mean s.d. (*n*=3).

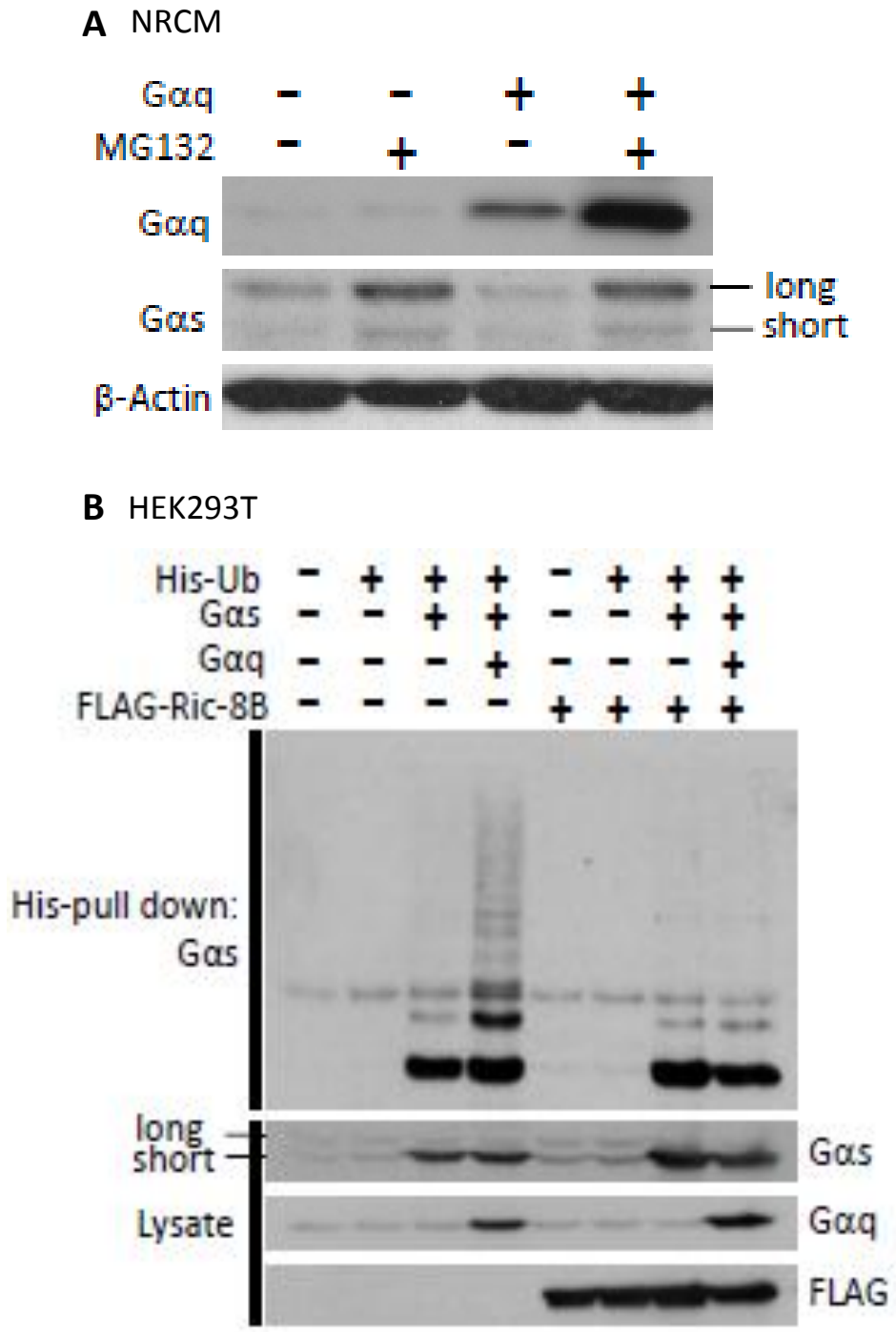


Figure 12. Gαq enhances ubiquitination of Gαs, but Ric-8B attenuates the effect of Gαq. A, NRCMs were infected with adenoviruses harboring GFP and Gαq at MOI 30 and sequentially treated with 10 μM MG132. After 24 h of infection, cell lysates were analyzed by immunoblot analysis using the indicated antibodies. **B,** HEK293T cells expressing $[His]_6$-ubiquitin, Gαs, and Gαq with or without FLAG-tagged Ric-8B were lysed, and the ubiquitinated proteins were precipitated by Ni-NTA agarose resin. The ubiquitinated Gαs was detected by immunoblot.

HEK293T

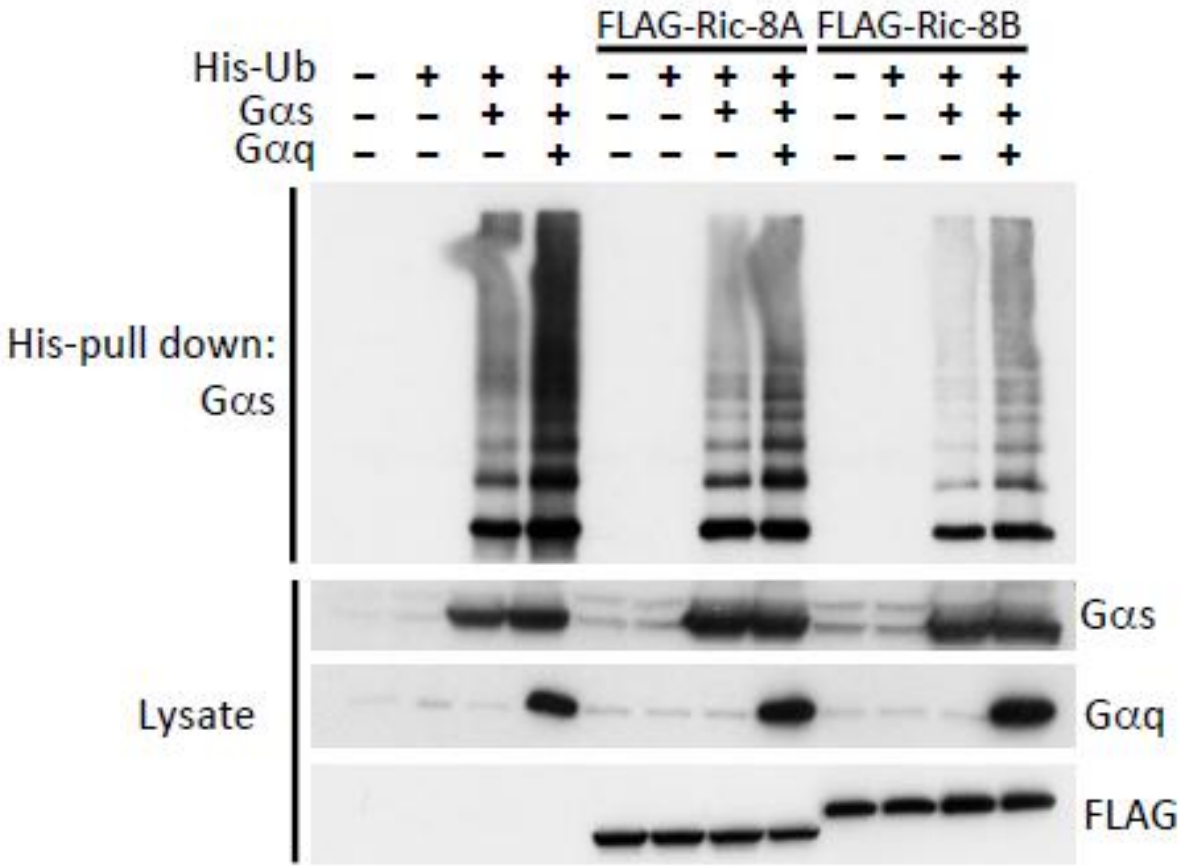


Figure 13. Ric-8A attenuates Gαq-enhanced Gs ubiquitination. HEK293T cells expressing $[His]_6$-ubiquitin, Gαs, and Gαq with or without FLAG-tagged Ric-8A or FLAG-tagged Ric-8B were lysed, and the ubiquitinated proteins were precipitated by Ni-NTA agarose resin. The ubiquitinated Gαs was detected by immunoblot.

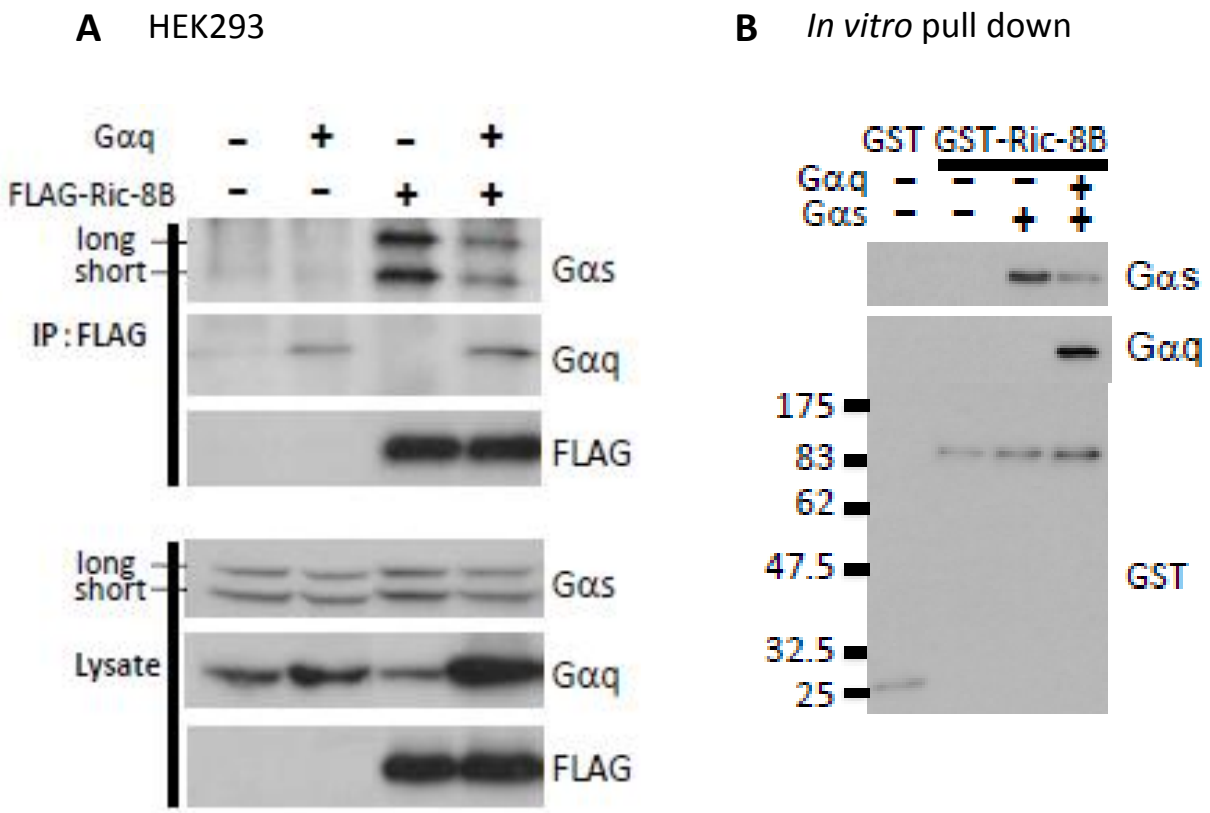


Figure 14. Excess amount of Gαq interrupts the interaction of Gαs and Ric-8B. A. HEK293T cells were transfected with the indicated combinations of plasmids. The transfected cells were lysed, and the immunoprecipitation of Ric-8B was performed with M2 antibody. The immunoprecipitates and lysates were subjected to immunoblot analysis with the indicated antibodies. **B.** GST-Ric-8B (30 nM) was incubated with 1 μM Gαq for 30 min at 25 °C. Then Gαs (100 nM) was added to the mixture, and continuously incubated for 1 h at 25 °C. The protein complexes were precipitated with Glutathione-Sepharose resin and analyzed by immunoblot with anti-Gαs or anti-Gαq antibody.

NRCM

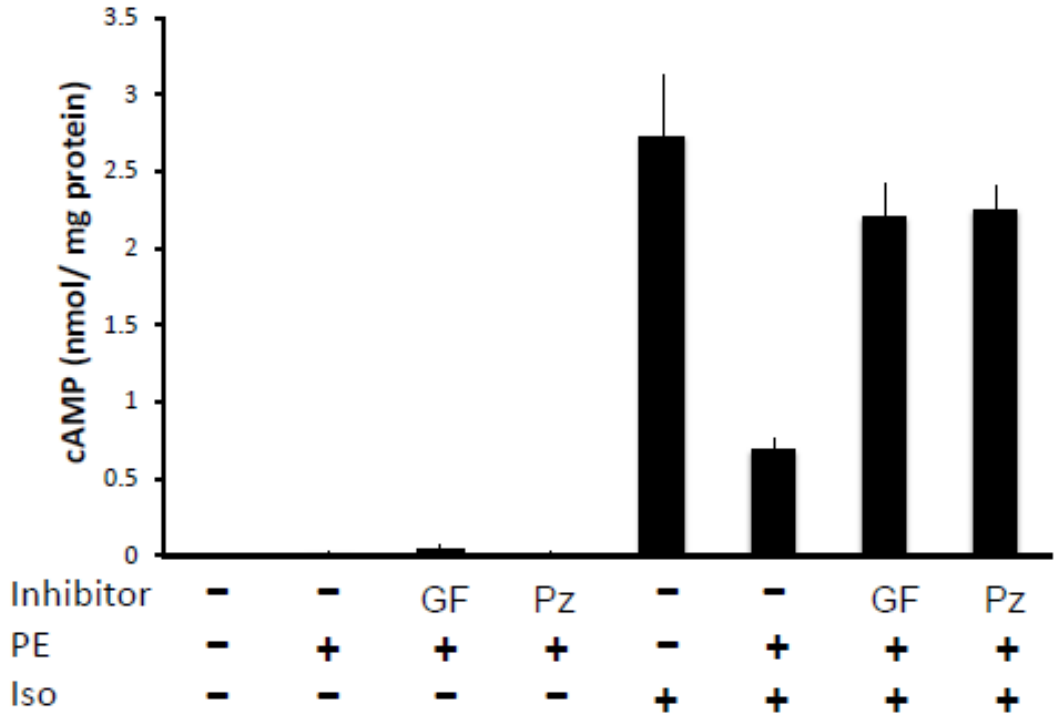
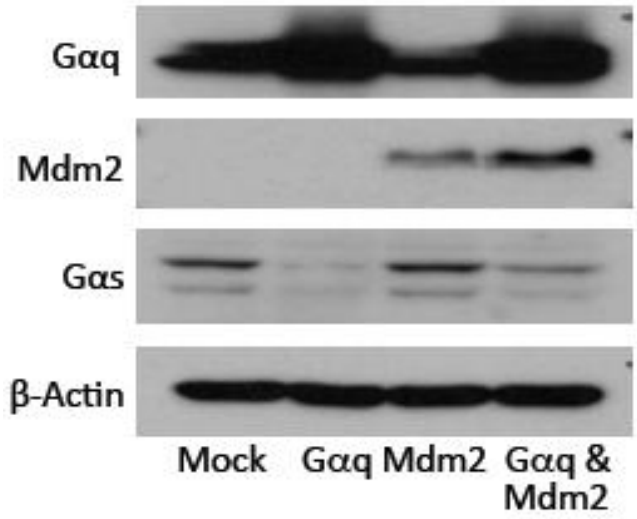


Figure 15. PKC is involved in α_1-AR-induced suppression of Gs signaling. NRCMs were treated with 10 μM PE for 24 h with or without 10 μM PKC inhibitor, GF109203X (GF) or 10 μM Pz. Then cells were stimulated with 1 μM isoproterenol (Iso) for 5 min, and cAMP accumulation was measured. Error bars mean s.d. (*n*=3).

A DKO MEFs



B DKO MEFs

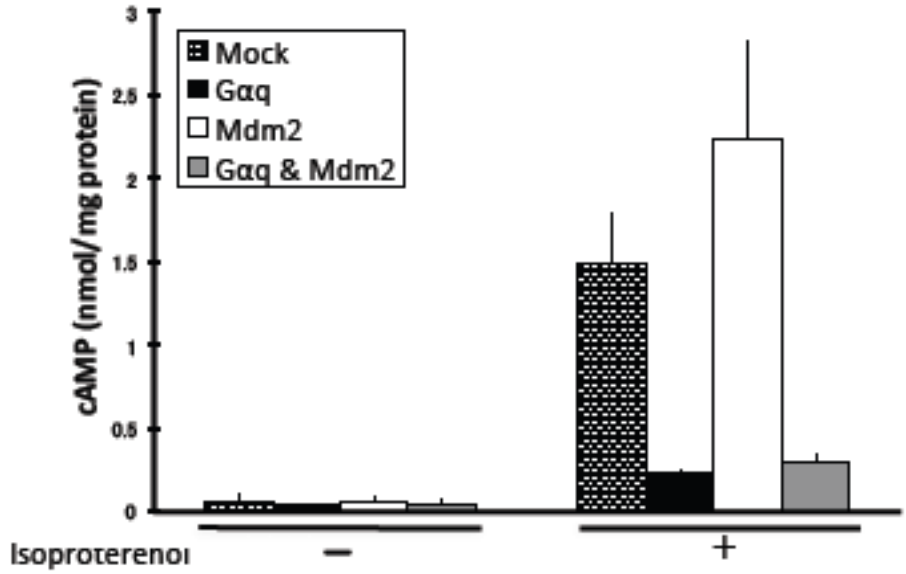


Figure 16. Mdm-2 did not suppress Gs expression and signaling. A, MEFs lacking Mdm2 and p53 (DKO MEFs) were infected with an empty retrovirus or retroviruses harboring Gαq and Mdm-2. The cell lysates from DKO MEFs were analyzed by immunoblotting using the indicated antibodies. **B,** Infected DKO MEFs were stimulated with 10 μM isoproterenol for 15 min, then cAMP accumulation was measured. Error bars mean s.d. (*n*=3).

IV. Discussion

Hypertrophy in chronic heart failure is often accompanied by dampened β-AR response, resulting in impairment of heart output and performance that leads to fatal effect. In this study, I provide a new possible molecular mechanism of the reduced β-AR response in cardiac hypertrophy, involving the crosstalk of G protein signaling mediated by the Ric-8B-dependent ubiquitination and degradation of Gαs.

I observed that the total ubiquitination was increased in the cardiac hypertrophy (Fig. 4A), although the target proteins of ubiquitination have not been identified. Previously our group demonstrated that the protein stability of Gαs and its signaling are regulated by ubiquitin-proteasome machinery, and Ric-8B has the ability to protect Gαs from proteasomal degradation by interacting with Gαs (Nagai et al. 2010). Several groups reported that the signaling pathway mediated by Gαs is weakened in the cardiac hypertrophy model (D'Angelo et al. 1997; Akhter et al. 1997; Lemire et al. 1998; Dorn et al. 2000; Harris et al. 2009). Furthermore, Gαs expression level and function were decreased in hearts from volume overload model rat with an arteriovenous fistula (AV shunt) (DiFusco et al. 2000). This volume overload model caused hypertrophy with no hypertension. A reduced level of Gαs and response to β-AR stimulation were also observed in cardiomyocytes derived from a chronic heart failure (CHF) rat with coronary artery ligation (CAL) (Yoshida et al. 2000). However, the molecular mechanism of the reduced expression of Gαs and impaired β-AR response remains to be clarified. Importantly, I observed similar impairment in neonatal rat cardiomyocytes. Prolonged stimulation of α_1-AR coupling with Gq caused a reduction of Gαs expression and suppression of Gs signaling (Figs. 4C,5A). Studies on transgenic mice expressing α_1-AR or Gαq in the heart suggested that Gq signal activation is essential for the onset of hypertrophy (D'Angelo et al. 1997; Akhter et al. 1997). These findings suggest that

Gαs could be a target protein of ubiquitination in cardiac hypertrophy. My observation in cardiac myocytes indicated that ubiquitination of Gαs is increased when Gαq is continuously activated with α_1-AR stimulation (Fig. 4C). The pattern of fetal gene expression and the increase of cardiomyocyte size are known to be the parameter of hypertrophy. Hypertrophic hearts derived from Gαq-expressing transgenic mice and pressure overload mice after aortic banding indicated the similar change of these parameters (Dorn and Brown 1999; Sakata et al. 1998). Indeed, I observed the enforced expression of Gαq in both rat cardiac myocytes and HEK293T induced the increase of total ubiquitination, and Gαs was drastically degraded by ubiquitin-proteasome pathway.

The possibility of the involvement of Ric-8B in the crosstalk of Gq and Gs signalings was raised by previous findings that Ric-8B binds to Gαq and Gαs (Tall et al. 2003) and stabilizes Gαs by inhibiting ubiquitin-dependent degradation (Nagai et al. 2010). Although Ric-8B was reported to preferably bind to Gαs (Chan et al. 2011), I hypothesize that an excessive amount of Gαq can inhibit the binding of Gαs to Ric-8B. In this study, I demonstrated the competitive interaction of Ric-8B with Gαs and Gαq in cells and *in vitro* (Fig. 14). Ric-8B attenuated the effect of Gαq on Gαs expression and Gαs ubiquitination (Figs. 9,12). Co-expression of Ric-8A with Gαq also exhibited a similar effect to that shown by Ric-8B (Figs. 11,13). This result suggested that the interaction of Gαq with Ric-A is also important for maintaining the Gs signaling. The expression level and intracellular localization of Ric-8A and Ric-8B should be involved in the regulation of Gq and Gs signaling in physiological and pathological conditions.

Previously, Tang *et al*. also reported that the overexpression of Gαq induced the ubiquitination of Gαs and that the Akt activation was involved in this ubiquitination (Tang et al. 2008). However, I observed that the inhibitor of PI-3 kinase that behaves as an upstream molecule of Akt failed to affect the Gαq-induced reduction of the Gαs protein and Gαs

signaling (Fig. 8). Furthermore, Tang's paper described the possible involvement of Mdm2 in the ubiquitination of Gαs. Mdm2 was originally identified as a ubiquitin E3 ligase for p53 tumor suppressor (Honda et al. 1997), and reported to regulate the function of β-arrestin which is mediated by its ubiquitin ligase activity (Wang et al. 2003), suggesting that Mdm2 could be involved not only in the ubiquitin-mediated regulation of tumor suppressors but also the regulation of cytosolic signaling. However, my observations using double-knockout mouse embryonic fibroblasts (DKO MEFs), lacking Mdm2 and p53, did not support the involvement of Mdm2 in the regulation of Gs signaling (Fig. 15).

Previous studies reported that activated Gq signaling attenuated the β-AR-mediated signaling, and oppositely, the inhibition of Gq signaling improves β-AR-mediated responses in both models of high blood pressure and cardiac hypertrophy (Harris et al. 2009; Akhter et al. 1998). Similarly, when I stimulated endogenous Gq signaling in neonatal rat cardiomyocytes with phenylephrine, cAMP accumulation was reduced (Fig. 5A). Importantly, a similar result was observed in NIH3T3 cells when Gq activation was inhibited by treatment of a specific Gq inhibitor, YM-254890 (Fig. 5B). This agent specifically inhibits the GDP/GTP exchange reaction of the Gαq subunit by inhibiting the GDP release from Gαq (Nishimura et al. 2010). Klattenhoff *et al*. reported that in response to isoproterenol and carbachol, ligands for Gs- and Gq-coupled receptors, respectively, Ric-8B was translocated from cytosol to the plasma membrane (Klattenhoff et al. 2003). In addition, our group also observed that polyubiquitinated Gαs proteins were localized in the plasma membrane (Nagai et al. 2010). These findings support my model showing that the release of Gαq from βγ subunit seems to be required to sequester Ric-8B from Gαs, and this facilitates the Gαs destabilization and impairment of Gs signaling.

Ubiquitination mediated through K48 is known to be a trigger for the proteasomal protein degradation. However, other polyubiquitin chains, including the K63-linked

ubiquitination, are generally involved in the regulation of substrate activity mediated by non-proteolytic mechanisms (Hofmann and Pickart 2001). Depending on cell type, a different type of ubiquitination may work for the regulation of Gs signaling, and if so, a different enzymatic system (probably including different E2 and E3 enzymes) would catalyze the ubiquitination of Gαs. Although I have no evidence to describe the detailed mechanism and types of Gαs ubiquitination, I would like to emphasize that the Gs signaling seems to be regulated by ubiquitination in cardiac cells like other types of cells such as HEK293T and NIH3T3 cells, and the dysregulation of Gαs ubiquitination could be involved in the onset of heart failure.

The Gs-mediated signaling pathway is regulated by the ubiquitin-proteasome pathway in several signaling steps. Lignitto *et al*. reported that the RING-type ubiquitin E3 ligase praja2 regulates the protein stability of regulatory subunits of PKA and tunes the strength and continuity of PKA signaling in response to cAMP (Lignitto et al. 2011). The Mahogunin RING finger-1 (MGRN1) E3 ubiquitin ligase inhibits the melanocortin receptor-mediated cAMP production by competition with Gαs, although the target protein of MGRN1-mediated ubiquitination was not identified (Pérez-Oliva, 2009). To date, the ubiquitin E3 ligase for Gαs is not identified; however, the ubiquitin E3 ligase involved in the ubiquitin-mediated regulation of Gαs should be a critical molecule for tuning the intensity of Gαs signaling. In our laboratory, a project for identifying the Gαs E3 ligases using an *in vitro* ubiquitination assay and high-through put screening with cell-free RING finger protein synthesis is in progress.

Based on my findings, I propose a molecular mechanism model of increased Gαs ubiquitination and suppressed Gs signaling in cardiac myocytes, as shown in Figure 17. In pathological cardiac condition, Gq signaling induced by GPCRs, including α_1-AR, an angiotensin II receptor, and an endothelin receptor, is hyperactivated and induces the

dissociation of Gαq subunit from the Gβγ dimer. This chronic condition generates excess free forms of Gαq, which are able to sequester Ric-8B from its preferable complexes with Gαs (Fig. 14). In the absence of binding with Ric-8B, Gαs is rapidly ubiquitinated and degraded by proteasome, and β-AR-mediated signaling is reduced. This mechanism may provide a new possible mechanism for the development of cardiac contractility dysfunction in heart failure, particularly in the step that trigger the heart to go through compensation state which leads to increased sympathetic nervous system.

However, my proposed model cannot rule out other possible mechanism underlying dampened response of β-adrenergic signaling in pathological cardiac condition. I have shown in this study that PKC should also be involved in the Gq signaling-evoked suppression of cAMP accumulation when α_1-AR was activated by phenylephrine (Fig. 15). This is not surprising since it has been reported that the activity of the dominant type AC VI expressed in cardiomyocytes is inhibited by PKC phosphorylation *in vitro* (Lin et al. 2002). However, in intact cells PKC activation does not inhibit the activity of AC VI. Rather, PKC potentiates AC VI activation in cells (Beazely et al. 2004).

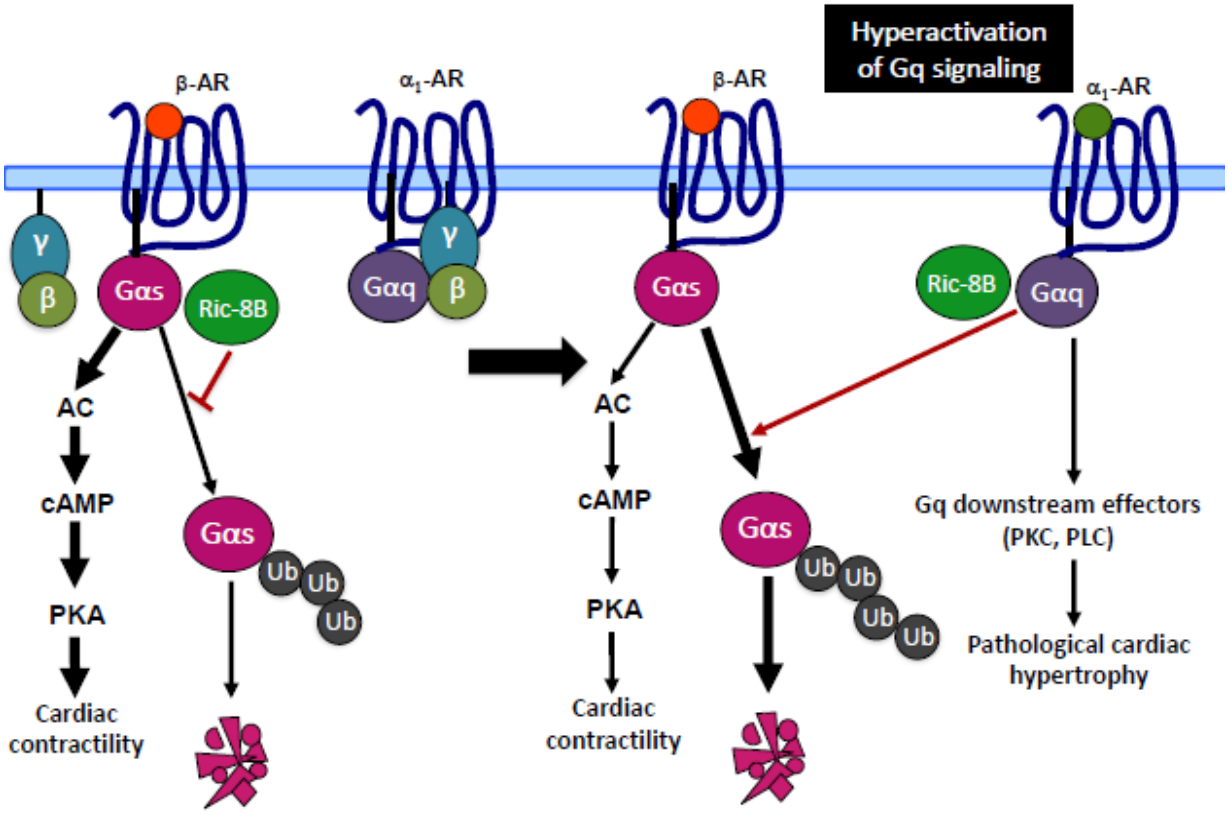


Figure 17. Proposed molecular mechanism model of dampened response of β-adrenergic signaling in cardiac pathological condition. The β-adrenergic signaling is the main signaling that regulates cardiac contractility. This signaling is mediated through Gαs, which is known as a substrate for ubiquitination. Ric-8B positively modulated Gs signaling by inhibiting Gαs ubiquitination. Ric-8B can also bind to Gαq. In a cardiac pathological condition where Gq signaling is hyperactivated and leads to hypertrophy, the amount of free-form Gαq released from Gαqβγ may be increased. In this condition, an excess amount of Gαq sequesters Ric-8B from Gαs. Gαs is then rapidly ubiquitinated and degraded by the ubiquitin-proteasome pathway, resulting in the impairment of Gs signaling. Thus, hyperactivation of Gq signaling negatively modulates Gs signaling.

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Nara, August 2013,

Riris Istighfari Jenie

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