Interaction of G-Protein βγ Complex with Chromatin Modulates GPCR-Dependent Gene Regulation

Anushree Bhatnagar¹, Hamiyet Unal¹, Rajaganapathi Jagannathan¹, Suma Kaveti², Zhong-Hui Duan¹,⁴, Sandro Yong¹, Amit Vasani³, Michael Kinter², Russell Desnoyer¹, Sadashiva S. Karnik¹*

¹Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, United States of America; ²Department of Cell Biology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, United States of America; ³Biomedical Imaging and Analysis Core, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, United States of America; ⁴Department of Computer Science, University of Akron, Akron, Ohio, United States of America

Abstract

HeterotrimERIC G-protein signal transduction initiated by G-protein-coupled receptors (GPCRs) in the plasma membrane is thought to propagate through protein-protein interactions of subunits, Gα and Gβγ in the cytosol. In this study, we show novel nuclear functions of Gβγ through demonstrating interaction of Gβγ with integral components of chromatin and effects of Gβ2 depletion on global gene expression. Agonist activation of several GPCRs including the angiotensin II type 1 receptor specifically augmented GβγGTP levels in the nucleus and Gβ2 interacted with specific nucleosome core histones and transcriptional modulators. Depletion of Gβ2 repressed the basal and angiotensin II-dependent transcriptional activities of myocyte enhancer factor 2. Gβ2 interacted with a sequence motif that was present in several transcription factors, whose genome-wide binding accounted for the Gβ2-dependent regulation of approximately 2% genes. These findings suggest a wide-ranging mechanism by which direct interaction of Gβγ with specific chromatin bound transcription factors regulates functional gene networks in response to GPCR activation in cells.

Introduction

The Gβ and Gγ subunits form a functionally inseparable Gβγ complex that generate the quiescent heterotrimeric G-proteins by associating with Gα-GDP. Current models show that G-protein activation by G-protein-coupled receptors (GPCRs) occur at the plasma membrane (PM). Second messengers or protein-protein interactions leading to spatio-temporal propagation of signals initiated by Gα and GβγGTP to the nucleus occurs in the cytoplasm, however translocation of G-protein subunits to nucleus is not frequently considered a possibility [1]. This view is changing due to the discovery of the shutting of Gα and Gβγ subunits from the PM to cell organelles, such as the Golgi, mitochondria, endosomes, and occasionally, the nucleus [2,3]. It is possible, therefore, that Gα or Gβγ complex translocates to nucleus and participate in gene regulation.

Gene regulation through G-protein signaling is crucial to human adaptation and survival which reflects the enormous success of therapeutics targeting GPCRs, the largest family of receptors encoded by the human genome. The finely tuned expression of an appropriate set of genes in a cell depends on multiple transcription factors (TFs) and transcriptional co-activators. GPCRs enhance gene transcription by facilitating the interaction of histone acetyl transferases (HATs), such as p300/ CBP, to TFs on chromatin [4]. Alternatively, recruitment of histone deacetylases (HDACs) to chromatin-bound TFs, such as myocyte enhancer factor 2A (MEF2A), represses transcription, and the repression is relieved by GPCR signals [5]. Nuclear localization of β-arræstins [6], GRK5 [7] and RGS proteins [8] is reported which suggests that these proteins recruited into the nucleus upon ligand activation of GPCRs may participate in the epigenetic processes that are essential for the functioning of cells. Whether Gα or Gβγ which are the primary transducers of GPCR signals, regularly enter the nucleus and directly participate in GPCR-coordinated transcriptional response remains unclear. Reports of Gβ1 or Gβ2 association with the glucocorticoid receptor [9], Gβ1γ2 association with HDAC5 [10,11], Gβ3 association with the nuclear shuttling of the R7 family of RGS proteins [8] and Gβγ association with the adipocyte enhancer binding protein [12] suggest a potential broad role of Gβγ in gene regulation. Therefore, we hypothesized that agonist activation of a typical GPCR such as the angiotensin II type 1 receptor (AT1R), changes the composition of chromatin-associated proteins which may include changes in the levels of specific G-protein subunits.

An unbiased high-throughput mass spectrometry analysis of the nuclear proteome upon activation of a GPCR led us to discover the interactions of Gβ2γ12 with chromatin. We found that the level of Gβ2 increased in the nucleus upon activation of diverse GPCRs and that Gβ2 was essential for agonist-induced MEF2A function. Gβ2 interacted with a sequence motif present in several TFs, and this interaction accounted for the coordinated gene regulatory function of Gβγ.
Materials and Methods

Reagents

The following reagents were used: HEK-293 cells (American Type Culture Collection) and NRVMs (Lonza); the pBudEA.1 plasmid, hygromycin and FuGENE 6 TM (Invitrogen); genetin (Gibco); Benzonase TM (Novagen); the agonists 5-hydroxytryptamine (5-HT), dobutamine (DOB), and isoproterenol (ISO); and anti-skeletal-actinin, anti-myc, and anti-FLAG antibodies, and anti-FLAG-M2 agarose beads (Sigma); antibodies against STAT1, STAT3, H2A, H2B, H4, MEF2A, TAF, Gzq, pan Gβ, Gβδ, NFAT, GATA4 and α-actinin-1 (Santa Cruz Laboratories); TBP (Abcam) and phospho- and total HDAC5 antibodies (Genscript); an anti-HA antibody (Zymed Laboratories); an α-actinin-4 specific antibody (Immunoglobulc); an amino-terminal FLAG-tagged human Gβδ plasmid and a myc-tagged human Gγ12 (UMR) plasmid; and an α-actinin-4 plasmid (Origene).

Nuclear and cytosolic fractionation

The nuclei and cytosol were isolated using the NUC101 nuclei isolation kit as detailed by the manufacturer (Sigma-Aldrich). The nuclear fractions were stained with DAPI, and subsequent visualization was performed using confocal microscopy to check for the integrity of nuclei. Nuclear protein was extracted using Benzonase (10 units/ml at 37°C for 60 min), which digests the nuclear acids without denaturing the proteins (chromatin proteins). The pellet was further extracted to isolate the tightly bound proteins using 0.45 N sulfuric acid (acid fractions). The purity of the fractions was determined by immunoblotting for specific cellular compartment markers, histones H1 and H2A (nuclear) and Gqα (cytosolic).

Site directed mutagenesis and plasmid construction

The amino terminus HA-tagged rat AT1R [13] under the control of the human cytomegalovirus (CMV) promoter was generated in the pcDNA3 plasmid. FLAG-Gβδ2 was subcloned into pBudEA.1 under the EF1 promoter. Nested primers were designed to delete each of the seven WD repeats in the FLAG-Gβδ2 construct. All of the subcloned plasmid constructs and the WD40 mutants were verified by DNA sequencing.

Transfection and generation of AT1R-expressing stable hek-293 cells

Routine transient transfection of HEK-293 cells was performed with FU Gene 6 TM per the manufacturer’s recommendations. The cell line stably expressing HA-AT1R was established by clonal isolation using genetin (600 μg/ml) selection.

LC-tandem mass spectrometry and protein identification

For Gel C analyses [14], the gels are run to attain 50% resolution of the electrophoresed proteins. The gels were cut into three regions, and each of these regions was further cut into five equal parts and digested with trypsin. The peptides were extracted and concentrated, and the digest was analyzed by LC-tandem MS [15]. The proteins contained in the nuclear fractions were identified using a shotgun sequencing approach [16]. Relative quantitation was determined using a spectrum counting approach. The MS results were also examined by plotting mass chromatograms for the respective peptides. Data were also searched using SEQUEST (ThermoFisher, San Jose, CA) with mass tolerances set at 3.0 Da for peptides and 2.0 for fragment ions using the standard variable oxidation of methionine (+16 Da) and carbamidomethylation on cysteine (+57 Da) as fixed modification. The MASCOT program (www.matrixscience.com) was used to compare all of the CID spectra with the NCBI non-redundant database and to identify the protein. Matching peptides were verified by manual interpretation.

Isolation of ventricular myocytes from adult C57BL6 mice

The isolation procedure for ventricular cardiac myocytes from adult C57BL6 mice has been reported in detail [17]. Handling of animals used for myocyte preparation is approved by IACUC using standard protocol recommendation. Cardiac myocytes were enzymatically dispersed via Langendorff perfusion of mouse hearts [18]. Following isolation, cells were treated with 1 μM AngII for 30 min and then fixed with 3% paraformaldehyde and subjected to immunocytochemical analysis.

Measurements of [Ca2+] fluct

Single ventricular myocytes were incubated with 1 mM fura-2 acetoxyethyl ester (Molecular Probes) for 10 min at room temperature in the dark. [Ca2+]i signaling was measured using a dual excitation spectrofluorometer (DeltaT RKF6002, Photon Technology International) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm as previously described [19]. Steady-state [Ca2+]i transient signals were recorded at a pacing frequency of 0.5 Hz in the absence of AngII. The stimulation was stopped and then followed by 1 μM AngII treatment. The resulting AngII-induced [Ca2+]i signal was recorded as a qualitative index of the initial sarcoplasmic reticulum (SR) [Ca2+]i, load in the cardiomyocytes.

Immunocytochemistry and confocal microscopy

Immunolocalization using confocal microscopy were performed essentially as described previously [20]. For 3D-visualization of confocal image stacks, the confocal image slices of myocytes labeled with DAPI and FITC (0.13 mm×0.13 mm×0.04 mm resolution in X, Y, and Z directions, respectively) were imported into Image-Pro 6.1 (Media Cybernetics, Silver Springs, MD) as a multi-plane sequence and subsequently split into blue and green channels. Customized sequence segmentation scripts were then applied to the blue (DAPI) channel to threshold and binarize each slice. The binarized image stack was then multiplied with the green channel stack (plane-by-plane) to extract green fluorescence localized to the nucleus. Both binarized nuclear slices and their corresponding green fluorescent slices were exported into Micro-View (GE Healthcare, Piscataway, NJ), reconstructed into 3D volumes (Z-dimension resolution was increased five times to improve definition of flattened nuclei), and rendered as iso-surfaces. Lastly, these iso-surfaces (DAPI and FITC) were merged together, and the opacity of the nucleus was adjusted to allow visualization of the underlying protein.

Reporter assays

The MEF2-luciferase assay (Promega) was performed as recommended by the manufacturer. Briefly, the MEF2-luciferase plasmid (1 μg) was transfected into AT1R-expressing cells in the presence or absence of Gβδ2 to evaluate the role of Gβδ2 in modulating MEF2A activity. The WD40 repeats of the N-terminal FLAG-tagged Gβδ2 (FLAG-Gβδ2) construct were sequentially deleted to create FLAG-Gβδ2 ΔWD(1–7) mutants. The HEK-AT1R cells were then transfected with FLAG-Gβδ2 ΔWD mutants, the MEF2-luciferase plasmid and 0.15 μg of the βGal plasmid (transfection control).
Immunoprecipitations with FLAG- Gβ2 and WD40 repeat mutants

The role of WD40 repeats in Gβ2 involved in the interaction with MEF2A was performed and quantified (Kodak Imager ID 3.6) as previously described [21].

Deacetylase assays

The HDAC activity (UPSTATE) was performed per the manufacturer’s guidelines. For deacetylase assays, the nuclear and cytosolic fractions were obtained from AT1R and AT1R-Gβ2i cells (+/- 30 min of 1 μM AngII). Actinin (1 μg anti-actinin-1 antibody)-associated deacetylase activity was measured in 100 μg each of the cytosolic and nuclear fractions. The samples were incubated with gentle mixing at 4°C overnight. Immunoprecipitates were collected via centrifugation and washed twice with 1 ml of ice-cold phosphate buffered saline (PBS). The resin was assayed for deacetylase activity.

RNAi-mediated knockdown of Gβ2

A DNA vector-based siRNA was designed to stably knockdown the expression of Gβ2 (gene name: GNB2) in HEK-293 cells [21]. The target sequence, ACTGCTACCTTGTTGTT [21], and the scrambled sequence, CGGTGTTCTACGTGGCTAT, were cloned into the pRNAU6.1/hygro plasmid under the control of the U6 promoter and a hygromycin selection marker. The cells were also co-transfected with the HA-AT1R-expressing plasmid that contained a neomycin selection marker. Selected clones were maintained in media containing hygromycin (100 μg/ml) and geneticin (600 μg/ml).

Microarray analysis

HEK-293 (AT1R; −/+AngII and AT1R-Gβ2i; −/+AngII) cells were harvested under RNA-free conditions, and RNA was isolated using the RNaseasy kit (Qiagen). RNA-based probe synthesis and hybridization were performed by the Gene Expression Array Core Facility at Case Western Reserve University (www.geacf.net) as described previously [21] using high-density oligonucleotide HG-U133 Plus 2 arrays (Affymetrix, Inc.; Santa Clara, CA). Gene expression changes predicted in the high-density oligonucleotide HG-U133 Plus 2 arrays (Affymetrix, University (www.geacf.net) as described previously [21] using Expression Array Core Facility at Case Western Reserve were harvested under RNase-free conditions, and RNA was

Statistical analysis

All experiments were performed three or more times. For image analysis, approximately 50–100 cells were analyzed in each set, and representative images are shown. All data are expressed as the mean ± SEM of at least three independent experiments. Each experiment was performed in triplicate unless otherwise indicated. Data were analyzed using an unpaired Student’s t-test (P<0.05) using GraphPad Prism 4 software.

Results

Gβ2 and Gγ12 traffic into the nucleus upon GPCR activation

To test the hypothesis that agonist activation of GPCRs changes the composition of chromatin-associated proteins, we examined angiotensin II type 1 receptor [AT1R], which is a peptide hormone GPCR. Mechanisms of AT1R signaling have been extensively studied in the attempt to improve AT1R-targeted therapies for hypertension, cardiac hypertrophy and end organ damage. Agonist (e.g., AngII)-mediated activation of AT1R has been reported to induce the nuclear mobilization of TFs, including GATA binding protein (GATA4), nuclear factor of activated T cells (NFAT), signal transducer and activator of transcription 3 (STAT3), nuclear factor-kappaB (NF-kB), extracellular signal-regulated kinases (ERK1/2), protein kinase C and HDAC5, during the progression of cardiovascular diseases [22–27]. We used a human embryonic kidney (HEK) 293 cell clone, HEK-AT1R, as a surrogate model system to identify the proteins that mobilize to the nucleus and associate with chromatin (Fig. 1). In HEK-AT1R cells, AngII induced Gq/11-PLC calcium signaling and pERK1/2 signaling (detailed in Fig. S1). Expression of early growth response genes was subsequently induced a result that was also found in neonatal cardiomyocytes stimulated with AngII [26,28]. The AngII effects were blocked by treatment with the AT1R-selective antagonist, losartan. To prepare the nuclear proteome, AT1R was activated for 30 min, which was determined as the time when AngII-induced pERK1/2 association with chromatin was maximal. The nuclear and cytosolic subcellular fractions isolated were well separated, as indicated by the absence of Gαz subunits in the chromatin preparation and the absence of the histone, H2A, in the cytosolic preparation (Fig. S2). When the nuclear proteome was queried for collision-induced dissociation (CID) spectra of peptides corresponding to abundant plasma membrane and cytosolic proteins, none corresponding to integrins, Gαz, GAPDH and cytochrome b5 were detected, which further confirmed the authenticity of our nuclear proteome preparation.
The largest groups of proteins found were RNA-binding proteins, heterogeneous nuclear ribonucleoproteins, splicing factors, nuclear proteins, ribosomal RNA-binding proteins and the proteins involved either directly in DNA binding or in cell cycle and gene regulation (Fig. 1b, Fig. S3). Most of these molecules are established nuclear proteins and/or shuttling proteins that contain a nuclear localization signal (NLS). Many signaling proteins (13.7%) without an obvious NLS present in nuclear proteome included Gβ2, Gγ12, and α-actinin-4 (Figs. 1b, S3, S4). The CID spectrum of the signature peptides, LLVSASQDGK for the Gβ2 isoform (Fig. 1c) and TASTNIAQAR for the Gγ12 isoform (Fig. 1d; see Table S1 for peptide coverage), were identified by SEQUEST (www.proteomicswiki/index.php/SEQUEST). The Gβ and Gγ subunits form an obligate functional monomer and translocate together. The nuclear partition coefficient estimated by WoLFPSORT (http://wolfpsort.org/) [29] for the Gβ2γ12 complex was –0.13 (equivalent to HDAC5, which is known to localize in both the nucleus and cytoplasm), indicating the potential for Gβ2γ12 to enter the nucleus upon GPCR activation. The nuclear partition coefficient of Gγ12 alone was –0.13. The nuclear partition coefficient of Gβ2 alone was identical to α-actinin-4 (–0.47), which is also known to localize both in the nucleus and cytoplasm [30], suggesting that Gβ2 most likely enters the nucleus upon GPCR activation.

Gβ2 content in the nuclear proteome

The label-free approach of spectrum counting [31] estimated a significant increase in Gβ2 translocation into the nucleus upon AT1R activation (Fig. 2a). The abundance of peptides from spiked-in trypsin was comparable in AngII treated HEK-293 and HEK-AT1R samples. The relative abundance of the Gβ2 peptide, LLVSASQDGK, in the chromatin of AngII activated HEK-AT1R sample (NL1.3E6) increased ≈3.1 fold when compared to HEK-293 sample. This fold increase was independently corroborated through additional analysis (Fig. S5). A variety of prohypertrophic agonists, including AngII, enhanced the nuclear translocation of Gβ2 (>1.7 fold) in human aortic smooth muscle (HASM) cells as validated by western blotting (Fig. 2b). The adrenergic receptor agonist (dobutamine) coupled to Gsε was as effective (Fig. 2b, see schematic) in the nuclear mobilization of Gβ2 as the Gsε-activating agonists (AngII and 5-HT). The activation of different GPCRs may release different Gβγ isoforms, which may participate in chromatin functions with different efficacies. We envision Gβ2 as a direct mediator of the nuclear effects of activated GPCRs.

Agonist-induced nuclear translocation of Gβ2

Indirect immunofluorescence staining demonstrated an increase in Gβ2 in the nucleus of HEK-AT1R and HASM cells and neonatal rat ventricular myocytes (NRVMs) upon treatment with AngII (Fig. 3a). Treatment with losartan prevented the increase in Gβ2 in the nucleus (Fig. S5a). To confirm that Gβ2 translocation was physiologically relevant in cells, we isolated adult mouse ventricular myocytes (AMVMs). Pacing and AngII treatment elicited calcium transients in AMVMs after isolation (Fig. 3b). AngII treatment stimulated the translocation of Gβ2 (Fig. 3c) from the cytoplasm into the nucleus of AMVMs (≈4.0-fold). Three-dimensional (3-D) image reconstruction of myocyte nuclei (Fig. 3d) showed the association of Gβ2 with chromatin. Thus, using different analytical methods, a 2.5- to 4.5-fold increase in the nuclear translocation of Gβ2 was observed in different types of cells upon AngII treatment (Fig. S5b).

Association of Gβ2 with components of chromatin

Our nuclear proteome preparation was enriched in protein complexes that were associated with an AngII-activated state of the genome. In this state, Gβ2 interacted with the AngII-responsive TF, MEF2A, the core histones, H2B and H4, the histone-modifying enzyme, HDAC5, and the calcium binding scaffold protein, α-actinin-4 (Fig. 4a). Gβ2 did not associate with histones H1, H2A and H3 or with pERK1/2.

Gβ2 association with MEF2A and histones H2B and H4 suggested that Gβ2 interacted with nucleosomes at the promoters of MEF2 regulated genes. The Gβ2 interactions with α-actinin-4 and HDAC5 suggested that Gβ2 played a role in AngII-mediated remodeling of chromatin by these two proteins. Actinin-1 and -4 are isoforms ubiquitously expressed in non-muscle tissues. They are calcium-sensitive proteins that engage class II HDACs in nucleo-cytoplasmic trafficking [32]. Class II HDACs, including HDAC5, regulate gene expression through association with TFs and alteration of the histone code at gene promoters [5]. We hypothesize that Gβγ is a component of the multiprotein complex at the promoters of MEF2 regulated genes that modulate transcription.

Essential role of Gβ2 in MEF2A-regulated transcription

The mechanism of gene regulation by Gβ2 was determined using small interfering RNA (siRNA) based loss-of-function approach that was similar to that used by Krumins and Gilman [33]. The Gβ2 mRNA and protein levels were specifically reduced upon stable expression of Gβ2-targeted siRNA in cells, hereafter referred to as Gβ2i cells, whereas mRNA levels of other Gβ isoforms remained unchanged (Fig. S6, a–b). Normal Gq mediated signals upon activation of AT1R by AngII, such as the activation of ERK1/2 in the cytosol (Fig. S6, c–d), the accumulation of pERK1/2 in the nucleus (Fig. S6, e–f) and the mobilization of calcium from intracellular stores (Fig. S6g) remained unaltered in Gβ2i cells. In the Gβ2i and control HEK-AT1R cell lysates, MEF2A protein levels were similar (Fig. 4b; inset). But the basal MEF2-luciferase reporter gene expression driven by the MEF2 protein did not increase MEF2-luciferase. In the HEK-AT1R cells, AngII treatment increased MEF2-luciferase, whereas the AT1R blockers, losartan and candesartan, antagonized the AngII-mediated increase in MEF2-luciferase (Fig. S7a and S7b). The overexpression of Gβ2 in HEK-AT1R cells further increased AngII-mediated MEF2-luciferase (Fig. 4c). In the Gβ2i cells, α-actinin-4 and the α-actinin-associated HDACs were sequestered in the cytoplasm (Fig. S8a and S8b), suggesting that these shuttling proteins preferentially remained in the cytosol when Gβ2 was knocked down. The cytoplasmic sequestration of α-actinin-4-HDAC has been shown to act as a mechanism to increase MEF2A-dependent transcription [32]. However, the reduction of basal and AngII-induced MEF2-luciferase in Gβ2i cells when sufficient MEF2A was present.
indicates that Gβ2 plays a novel role in MEF2-luciferase gene transcription in normal cells.

We propose that the interaction between Gβ2 and MEF2A proteins is a GPCR-specific transcriptional cue that facilitates synergy between the MEF2A and TATA-binding protein (TBP) and transcription activating factor (TAF) complex in modulating transcription. As shown in Fig. 4d, in the presence of Gβ2, MEF2A interacted with the TBP/TAF complex (reverse co-immunoprecipitations (co-IPs) are shown in Fig. S9a). Knockdown of Gβ2 in the Gβi cells specifically disrupted the interaction of MEF2A with TBP; however, the interaction between TBP and TAF was not affected. These results suggest that the synergy between the MEF2A and TBP/TAF complex requires Gβ2. Hence, the knockdown of Gβ2 accounts for the decrease in basal as well as AngII-activated expression of MEF2-luciferase. In Fig. 4e, we independently assessed the nuclear localization of myc-tagged Gγ12 in HEK-AT1R cells upon AngII treatment. The myc-tagged Gγ12 associated with endogenous Gβ2, MEF2A and TBP. Thus, a novel Gβ2/Gγ12-dependent multiprotein complex is formed in the nucleus and is essential for the transcriptional activation of the MEF2 promoter.

Previous studies have shown that class II HDACs directly interact with MEF2A and repress transcription through histones deacetylation [5]. The MEF2A-HDAC interaction is dynamically regulated. Our results indicate that weak basal transcription may result from the involvement of Gβ2 in a complex with TBP/TAF and MEF2A-HDAC, as the nucleosomes were deacetylated locally in this state (basal in Fig. 4f). An agonist-activated increase in Gβ2 in the nucleus is expected to generate a nascent enhancer complex in which Gβ2 interacts with TBP/TAF and MEF2A without HDAC5. This complex may facilitate recruitment of HATs, leading to local acetylation of histones and promoting AngII-stimulated transcription. The knockdown of Gβ2 weakened the synergy between TAF/TRP and MEF2A, thereby attenuating transcription. The cytoplasmic localization of α-actinin-4 and HDAC in the Gβi cells suggests that HDAC shuttling regulates Gβ2-dependent transcription in the nucleus.
Figure 3. Agonist-activated nuclear translocation of Gβ2 in intact cells. (a) The HEK-AT1R, HASM and NRVM cells were treated with vehicle or 1 μM AngII for 30 min and fixed. Gβ2 is shown in green. The nucleus (blue, stained with DAPI) shows green staining that corresponds to Gβ2 in the nuclei. (b) Post-isolation viability and AngII response as assessed by calcium signals in AMVMs. The AMVMs that were paced at a frequency of 0.5 Hz displayed steady-state [Ca2+]i transient signals. When AMVM pacing was stopped, the [Ca2+]i signals ceased, and upon treatment with 1 μM AngII, the [Ca2+]i signal resumed. (c) Beating AMVMs were treated with vehicle or 1 μM AngII for 30 min and fixed. α-Actinin-1 was labeled red and Gβ was labeled green. The far right-hand inset shows a magnified image (1000×) of a single nucleus. The nucleus displays green staining that corresponds to Gβ. Note: α-actinin-1 is a sarcomeric marker and does not translocate to the nucleus. (d) 3-D reconstruction of a mouse cardiac myocyte nucleus (confocal microscopy image). Green fluorescence represents Gβ2, and blue represents DAPI staining. The top panel shows the localization of Gβ2 (in Z-plane) from the top to the bottom of the myocyte nucleus. The lower panel shows an intact AMVM nucleus and a slice through the nuclear image that depicts a significant accumulation of Gβ2 inside the nucleus of the AMVM cell upon AngII/AT1R activation. Note: all images were acquired using a 63× objective (1.4 N.A.) at 0.232 μM/pixel in the plane resolution and 0.041 μM/pixel in the Z-axis resolution. The confocal image is a representative
WD repeat structure in Gβ2 is essential for its interaction with MEF2A

To gain insight into the molecular interaction between Gβ2 and MEF2A, we used a mutagenesis approach. Each of the 7 WD repeats was sequentially deleted to create seven AWD mutants (Fig. S9b). All repeat deletion mutants interacted with MEF2A (Fig. S9c), and the AWD2, AWD3, and AWD5–AWD7 mutants stimulated MEF2A function, whereas the AWD1 mutant did not (Fig. S9d). Therefore, potentiating the MEF2A function appears to require a structure that includes the WD1 repeat of Gβ2. Rather surprisingly, the AWD1 mutant had a significant stimulatory effect, suggesting that WD1 in Gβ2 might be the site of its interaction with HDACs (which are MEF2A co-repressors). Thus, the WD1 repeat of Gβ2 is essential for promoting MEF2A function; however, all of the WD repeats contributed to interaction between Gβ2 and MEF2A. This led us to hypothesize that MEF2A makes contact with the central canal of the Gβ2 toroidal structure.

Gβ2 interacts with TFs that share an amino acid sequence motif

To localize a putative MEF2A binding site on Gβ2γ12, we evaluated chromatin-associated proteins that have been proven to interact with the central canal of the β-propeller proteins (see the methods). Combining molecular modeling, bioinformatics and evolutionary relationship (interactions of vertebrate β-propeller proteins in chromatin are not known) created a model for the interactions of MEF2A and histones with Gβ2. Several proteins that bind to the β-propeller central canal, including cyclin E binding to Cdc4, use a phosphopeptide (–LLTPPG–) docking site [34–36]. A similar sequence motif was conserved in MEF2A, and when aligned, an extended homology with the cyclin E region was found (Fig. 5a). Molecular modeling and docking experiments (detailed in the methods) indicated that MEF2A could dock at the central canal of Gβ2 (Fig. 5b) and that the proposed MEF2A binding site should not overlap with the interaction sites for cytoplasmic effectors of Gβ2 [37]. The histone H4 peptide binds WD7 in Drosophila β-propeller protein p55 [38]. In the Gβ2γ12 model (Fig. 5b), the binding site for histone H4 was conserved; thus, WD7 in Gβ2 may interact with the nucleosome.

Bioinformatic analysis revealed that one copy of the –LLTPPG– motif was conserved in several AngII-responsive TFs, including STAT1/3 and Nfat, but not in NFkB p65 or GATA4 (Fig. 5a). We tested this prediction by protein interaction analysis, and the data revealed that Gβ2 indeed associated specifically with NFAT and STAT1/3, but not with NFkB p65 or GATA4 (Fig. 5c). By interacting with multiple TFs via the conserved –LLTPPG– motif, Gβ2 can coordinate the expression of multiple target genes. The human genome harbors >105 sites for each of the Gβ2-interacting TFs, and transcription at some of these sites must be Gβ2-dependent in response to GPCR agonists. Gβ2 and other Gβγ isoforms may also coordinate GPCR-dependent gene transcription (Fig. 5d). The clinical success of GPCR-targeted drugs indicates that the therapeutic benefits of these drugs potentially include modulation of Gβγ-dependent chromatin remodeling. These insights led us to investigate the genome-wide transcription profile upon knockdown of Gβ2.

Gβ2-dependent global gene expression pattern

Expression profiling indicated that ~400 transcripts were differentially regulated by Gβ2-dependent signals (Fig. 6a, Table S5). The Ingenuity pathway analysis sorted the expression data to gene networks that reflected the capacity of the gene products (i.e., receptors, enzymes, scaffold proteins, and extracellular matrix components) to influence specific cellular functions. The most prominent cellular functions that were altered are shown in Figure 6b. Each of the significantly altered cellular functions consisted of a network of >60 molecules (Fig. 6c), indicating that Gβ2 knockdown substantially altered gene regulation (see ΔP-value). Gβ2 knockdown transformed the “cellular growth and function” network to the “cellular growth and function in disease” network (Fig. S10). The network score of 41 before Gβ2 knockdown indicated that there was a 10−41 chance that these genes were randomly present in the network. The network score after the knockdown was 40. Sixteen core molecules were unaffected by Gβ2 knockdown. The functional change in the Gβ2 cells appeared to be due to co-opted PM-resident transmembrane proteins (e.g., platelet-derived growth factor receptors and integrins) and secreted proteins (e.g., interleukin-1, serine protease inhibitors of the SERPIN gene family, insulin-like growth factor-1, and platelet-derived growth factor). Interestingly, the promoters of differentially expressed genes contained the binding sites for one or more of the TFs that associated with Gβ2 (Table S2). Analysis using Metacore™ revealed a set of genes (Fig. 6c) that were transcriptionally regulated by MEF2A, NFAT, STAT1, and STAT3. Gβ2 knockdown affected AngII-dependent transcription of these genes as confirmed by real time RTPCR, which may be the direct cause for the transformation of network function. In vivo, when the AT1R stimulus becomes chronic or when Gβ2 is not regulated properly, the dynamics of the signaling networks might tilt towards a disease state that can promote damage to the tissue as well as contribute to chronic disorders. We conclude that Gβ2 is a master regulator of gene expression programs in response to agonist activation of AT1R and likewise other the GPCRs.

Discussion

PM-to-nuclear translocation of Gβγ and its regulation of nuclear effectors is a novel paradigm in GPCR signaling. The most common role for Gβγ may be in mediating synergy between different transcriptional regulatory complexes at gene promoters. The specific and differential changes that are orchestrated by Gβγ could involve facilitating the interaction of the enhancer complex (MEF2A) with the TFIID complex (TBP/TAF), the stepwise dissociation of negative regulators (HDACs) from transcriptional regulatory complexes and the association of positive regulators (HATs, co-activators). Novel nuclear targets of Gβγ were identified in the present work, and the ability of the Gβγ complex to regulate nuclear translocation of glucocorticoid receptor [9] and HDAC5 [10,11] has been previously reported. The ability of Gβγ to facilitate interactions between multiple proteins that are involved in gene regulatory complexes can explain the signaling specificity and the high-level transcriptional output by G-proteins. Many proteins in the multiprotein complex can promote gene expression individually; however, none of these components, with the exception of Gβγ can function unequivocally as a GPCR-specific enhancer of gene transcription.

In specialized cells, such as cardiac and smooth muscle cells, the intracellular distribution of some GPCRs, including the AT1R, and G-proteins has been reported [39]. A consensus regarding how GPCRs signal in the subcellular compartments apart from
Figure 4. Interaction of Gβ2 in the nuclear proteome and mechanism of modulation of MEF2A transcriptional activity. (a) Gβ2 coimmunoprecipitates with α-actinin-4, HDAC5, MEF2A, and the histones H2B and H4. The nuclear fractions (100 μg) prepared from HEK-AT1R cells treated with AngII (1 μM for 30 min) were subjected to pull-down with only ProtG (−) or with a Gβ2 antibody and ProtG (+). The immunoblot on the right shows the abundance of the respective proteins in the immunoprecipitates (− and +) as well as input lysates for the − and + samples. Gel-C peptide index mining provided further supporting evidence for the provisional interactome of nuclear Gβ2 (Table S1). (b) A significant increase in MEF2-luciferase activity (*p = 0.039) when AT1R was exposed to AngII (bars 1 and 3 from right). The basal MEF2-luciferase activity was significantly (∼50%) attenuated in AT1R-Gβ2i cells when compared to wild-type AT1R (bars 1 and 2; **p = 0.002). The RLU is normalized to co-expressed β-gal activity in each sample. Data were further normalized to basal MEF2-luciferase activity in wild-type AT1R cells. Inset: No significant change was detected in MEF2A protein levels in the cell lysate. (c) A significant increase in MEF2-luciferase activity upon FLAG-Gβ2 overexpression. (d) In Gβ2-positive cells, immunoprecipitation with anti-TBP antibodies revealed the interaction of TBP with MEF2A and TAF. In the absence of Gβ2 (Gβ2i cells), TBP failed to co-immunoprecipitate MEF2A, but TAF was co-immunoprecipitated. The immunoblot on the right shows the abundance of the TAF, TBP, MEF2A and Gβ2 proteins in lysates (INPUT; Gβ2 and Gβ2i). (e) Upon AT1R activation with AngII, the transiently transfected myc-Gγ12 translocated to the nucleus with endogenous Gβ2 and associated with TBP and MEF2A. (f) Model depicting the modulation of MEF2A-dependent gene transcription by Gβ2-associated proteins (MEF2, HDAC5, α-actinin-4, TBP and TAF). Basal: In this state, Gβ2 forms a complex with MEF2A, HDAC5 and
PM resident GPCRs or how intracellular and cell surface GPCR signaling coordinated is still evolving. It is possible that extracellular agonists reach the intracellular compartments, such as the nucleus and promote local G-protein signaling in specialized cells. Local G-protein activation may also regulate the nuclear targets of Gbc as well as the generalized retrograde translocation of Gbc from the PM to the nucleus.

A direct role for G-protein subunits in orchestrating gene responses to GPCRs is thought to be limited because the repertoire of conventional signaling targets of heterotrimeric G-proteins are localized in the PM and cytosol. The discovery of Gb2 translocation to the nucleus and its role in the regulation of gene networks define Gbc as a key missing link through which GPCRs modulate gene expression. A variety of GPCR agonists promote the nuclear translocation of Gβγ in physiologically relevant cells, indicating its universal significance.

WD repeat β-propeller proteins are integral components of chromatin-modifying complexes in lower eukaryotes [40]. Members of this family (>165 proteins) exhibit similar structures and remarkably, perform similar types of nuclear functions [40–45]. However, the nuclear functions of heterotrimeric Gβγ proteins, which are the founding members of the β-propeller protein family, have remained elusive. Taken together with previous data [8–12], our findings suggest that Gβγ proteins mediate chromatin remodeling, which may be an evolutionarily ancient and essential function in vertebrates. Histone gene clusters and histone-modifying enzymes were indeed modulated in Gb2i cells (Table S3), similar to the regulation of histone genes by β-propeller.

Figure 5. 3-D model of the proposed Gβ2 interaction with MEF2A and histone H4. (a) Multiple sequence alignment using the CLUSTAL W program revealed that the phosphopeptide motif (–LLpTPPG–) was conserved in the TFs that associated with Gβ2 (MEF2A, STAT1, STAT3 and NFAT), but not in NFkB and GATA4. (b) Surface model of Gβ2γ12 based on Gβ1γ2 crystal coordinates. Gβ2γ12 is shown in gray, and the common site of interaction with cytoplasmic effectors (Gα and PLCβ) is shown in teal. The –LLpTPPG– peptide (shown as red ball and stick) anchors to the central core of the β-propeller structure and makes contact with the amino acid residues shown in green and purple. The purple side chains contacting the peptide are conserved charge interactions. The histone H4 tail peptide, shown in brown, may interact on the surface of the WD7 repeat. (c) Co-immunoprecipitation of Gβ2 with the AngII-responsive TFs, NFAT, STAT1, and STAT3, but not with GATA4 and p65 NFkB. The nuclear fractions (100 mg) prepared from HEK-AT1R cells treated with AngII (1 μM for 30 min) were subjected to pull-down with only ProtG (−) or with a Gβ2 antibody and ProtG (+). The immunoblot on the right shows the abundance of the respective proteins in the immunoprecipitates (− and +) and input lysates for the − and + samples. (d) Gβ2 interaction with selective AngII-responsive TFs, suggesting a role for Gβ2 in genome wide transcription that eventually leads to changes in cellular functions.

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Figure 6. Gβ2-dependent global gene expression patterns. Altered gene expression patterns and gene networks that engage common biological processes are shown. Of the >47,000 transcripts monitored, 705 unique and annotated transcripts (2% of the transcriptome) were differentially affected by AngII stimulation in the Gβ2i cells (see Table S5). Out of these, 299 transcripts were identical to the transcripts in the Gβ2Sc control, indicating that these transcripts were regulated by Gβ2-independent signals from AT1R, and the remaining ~400 transcripts were specifically regulated by Gβ2. The false discovery rate was <3%. (a) Venn diagram: a total of 800 genes were modulated in Gβ2Sc cells, and 705 genes were modulated upon Gβ2 knockdown (Gβ2i) in AT1R-expressing cells treated with AngII (1 μM for 30 min). (b) The altered cell functions upon Gβ2 knockdown. (c) The hierarchy of gene functions, Δp-value, number of molecules involved and genes regulated by the Gβ2-interacting TFs, MEF2A, NFAT, and STAT1/STAT3 (derived from the ‘Build Networks – Expand by one group interaction’ algorithm in MetaCore™). Shown in green are down regulated genes, in red are up regulated genes in an independent experiment. Additional promoter information is shown in Table S2.

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proteins in drosophila [42] and histone deacetylases in chicken [43]. Thus, controlling the expression of chromatin-regulating complexes may be a critical function of vertebrate Gβγ.

Global gene regulation dependent on Gβγ provides a mechanism for direct gene regulatory function of an activated GPCR in a variety of biological contexts. Nearly 2% of the modulated genes in Gβ2i cells are members of the GPCR superfamily or are involved in signal transduction activated by GPCRs (Table S4), and they also include ~30% of cardiac hypertrophic marker genes (Table S4) [24]. Increased G-protein signaling is a trigger for the reactivation of the fetal gene program, which is a hallmark feature of cardiac hypertrophy and heart failure [24–25]. The extent to which deregulation of gene expression in zebrafish is due to extensive reconfiguration of the epigenome and/or involves Gβ2 is a critical question that remains to be elucidated. Conceivably, the Gβγ pathway could be targeted pharmacologically to control physiological and pathological chromatin responses and may be particularly useful in the setting of chronic disorders [46], in which dysregulated GPCR signaling is known to play an important role. Therefore, it is essential to gain a better understanding of the role of different Gβγ isoforms in epigenetic regulation.

Supporting Information

Methods S1 Details of experimental protocol for some methods described. (DOC)

Figure S1 Pharmacological and biochemical analysis of HEK-293 cells stably expressing HA-tagged AT1R. (a) Scatchard analysis; the kinetics of binding of 125I-[Sar1,Leu8]AngII (measured Kd (812 pM) and Bmax (5.3 pmol/mg) to AT1R. (b) AngII ligation with AT1R mobilizes calcium from intracellular stores. (c) Immunocytochemical analysis of HEK-293 cells stably expressing HA-tagged AT1R (labeled green with FITC) and visualized by confocal microscopy. Under quiescent conditions, the receptors are localized at the plasma membrane. Receptor activation with 1 μM AngII caused PM ruffles (white arrows) followed by a significant increase in the immunoreactivity of pERK1/2 (labeled red) in the nucleus (blue) for up to 60 min. Note that the confocal image shown here is after 10 min of AngII stimulation. In all subsequent experiments, 30 min of stimulation was used. (TIF)

Figure S2 Preparation and validation of the chromatin proteome. Nuclear fraction extraction for mass spectrometry analysis. Cytoplasmic and nuclear fractions were prepared from untransfected (UT) and AT1R-expressing HEK-293 cells treated with different ligands (AngII, losartan and candesartan). Fifty micrograms of protein was loaded onto 10% Nu-PAGE gels and subjected to western blot analysis. The G-protein α-subunit, Gαq was only found in the cytoplasmic fraction, whereas histone H2A was found in the nucleus, and T-ERK1/2 was present in both fractions. Note: the chromatin proteome was queried for CID spectra of peptides corresponding to plasma membrane and cytosolic marker proteins (e.g., integrins, Gαq, GAPDH, Baxin, and cytosome b3). None of the peptides corresponding to the above abundant proteins were detected in the nucleus, which confirms the fractionation procedure. (TIF)

Figure S3 Classification of the chromatin proteome of AT1R-activated cells. All peaks with at least 15 product ions in the MS/MS spectra were extracted. The peak lists from three replicate experiments were searched against mouse and rat reference sequences using search parameters for human protein trypic fragments and allowing for standard modifications and cleavage variation (1 missed cleavage/peptide). Quantitative analysis was performed by label-free spectrum counting after applying a threshold peptide ion score of 30 for MS/MS interpretation. All peptides were manually validated. The minimum criterion for positive identification of any protein was the presence of one signature peptide with a manually validated CID spectra. A total of 173 proteins were present on the peak list, of which 137 proteins met the selection criteria applied. (TIF)

Figure S4 The CID spectra of α-actinin-4 peptides. The chromatin proteome of AT1R-activated cells consisted of peptides (CISQEQMQOXQEFR, TINEVENQILTR, FAIQDISVEET-SAK) assigned (MASCOT/NCBI non-redundant database) to α-actinin-4. (TIF)

Figure S5 Gβ2 accumulates in the nucleus upon AT1R activation by AngII and is blocked by treatment with the AT1R antagonist, losartan. (a) The HEK-AT1R cells untreated or treated with 1 μM losartan and HA-AT1R were labeled red, and Gβ2 was labeled green. The inset in the right top corner of the middle Gβ2 panel shows a magnified image (1000×) of a single cell (arrow in overlay). The nucleus of the cell shows green staining that corresponds to Gβ2 in the nuclei. (b) Different analytical methods, including mass spectrometry (MS), immuno blot (IB) analysis and immunocytochemistry (ICC), showed equivalent fold changes in Gβ2 accumulation in the nucleus upon AT1R activation. A pixel counting approach estimated (50 cells, n = 3) that ~30% of the Gβ2 pool was localized in the nucleus when AT1R was activated with 1 μM AngII for 30 min in HASM and HEK-AT1R cells. This distribution accounts for ~2.5–4.5-fold increases in Gβ2 levels in the nucleus which is similar to that estimated by other methods. (TIF)

Figure S6 AT1R-mediated cytoplasmic signaling events are unaffected upon RNAi-mediated silencing of Gβ2. (a) Total lysates were prepared from untransfected HEK293 cells, dual plasmid-transfected clones expressing AT1R with scrambled Gβ2-scrambled (Gβ2Sc) and AT1R with a Gβ2RNAi plasmid. Lysates were subjected to immunoblot analysis to detect AT1R expression (anti-HA), Gβ (pan antibody) and β actin (loading control). Both of the cell lines exhibited equivalent levels of AT1R. The Bmax (maximal specific binding) obtained for AT1R-Gβ2Sc was 8.7±/−0.9 pmol/mg and 9.7±/−0.9 pmol/mg for AT1R-Gβ2i with a Kd value of 1732.5±/−170 pM. Taken together, both cell lines expressed comparable levels of AT1R. (b) Table showing the Affymetrix array gene expression data from Gβ2i stable cell lines compared to Gβ2+ cells revealed a knockdown specifically for GNB2. (c–d) Both cell types were serum starved for a minimum of 18 hr and then exposed to vehicle (−) or 1 μM AngII (+) for 5, 10, 15, 20, 30 and 60 min. Lysates were immunoblotted for pERK1/2 and total ERK1/2 in Gβ2Sc and Gβ2i cells. The phosphorylation of ERK1/2 upon AngII activation of AT1R was preserved in the absence of Gβ2. (c–f) Immunocytochemical analysis followed by confocal imaging of pERK1/2 (labeled green) localized in the nuclei (labeled blue with DAPI) in Gβ2Sc and Gβ2i cells upon AT1R activation with AngII. (g) Calcium mobilization upon AngII activation of AT1R was preserved in Gβ2Sc and Gβ2i cells (fluorescence-based assay using FLEX Station 3). (TIF)
Figure S7 The AT1R blockers, losartan and candesartan, prevented the increase in AngII-mediated MEF2 reporter activity. (a) AngII treatment increased MEF2-luciferase expression, and this increase was blocked by treatment with the AT1R antagonist, losartan (−54%), and (b) candesartan (−96%). Note: losartan is a less potent AT1R antagonist compared to candesartan. Data are expressed as % RLU normalized to the AngII response (100%) with losartan/candesartan alone as 0%. Error bars indicate standard error of the mean (n = 3) of experiments performed in duplicate. P values were * = 0.03 and ** = 0.02 using an unpaired t-test (two-tailed with Welch’s correction in GraphPad Prism software).

Figure S8 Gβ2 modulates the export of the α-actinin-4-HDAC complex from the nucleus to the cytosol. (a) Immunocytochemical analysis of AT1R and AT1R-Gβ2RNAi cells revealed increased cytoplasmic localization of α-actinin-4 (green) compared with control. (b) An actinin-associated HDAC activity assay on the cytosolic fraction of AT1R in AT1R-Gβ2 RNAi cells (no agonist and AngII 1 μM for 30 min). There was a significant increase in actinin-associated HDAC activity upon AngII treatment of AT1R cells. There was a significant increase under quiescent conditions in Gβ2i cells (no agonist). P value: * = 0.049 and † = 0.034. No significant change was observed upon agonist exposure under conditions of Gβ2 knockdown.

Figure S9 The WD repeats in Gβ2 form a platform to allow for the formation of a multimeric protein complex. Reverse Co-IPs were performed with Prot G (−) and Prot G (+) antibodies. (a) Gβ2, MEF2, TBP and TAF antibodies (+) were used for immunoprecipitation (IP), and the samples were immunoblotted (IB) for interacting proteins as shown here. (b) Schematic representation of sequential WD repeat deletions in Gβ2. (c) Co-immunoprecipitation with anti-M2 FLAG beads in FLAG-Gβ2/mutants and MEF2-expressing cells showed no significant change in MEF2 association with Gβ2 (n = 3). (d) MEF2 functional activity stimulated by Gβ2/WD repeat deletion mutants. Error bars indicate standard error of the mean (n = 3), and P values were †, ‡ < 0.003 and * = 0.011 as calculated using an unpaired t-test (two-tailed) with Welch’s correction in GraphPad Prism software.

Figure S10 Influence of Gβ2 knockdown on the cellular growth and proliferation network. The knockdown of Gβ2 affected function of this network. (a) The Cellular Growth and Proliferation Network was derived from the IPA analysis of differentially regulated genes in the wild-type cells (in AngII vs. untreated cells). (b) The Cellular Growth and Proliferation Network derived from differentially regulated genes in the Gβ2i cells (in AngII vs. untreated cells). The assigned function for the Cellular Growth and Proliferation Network in Gβ2i cells (i.e., cellular growth and proliferation in connective tissue disorders and in nervous system development and function). (c) The Cellular Growth and Proliferation Network derived from Gβ2i cells is superimposed onto the wild-type network and shows the presence of molecules that now participate in the network and thus assigns it new specialized function.

Table S1 The Peptide Index.

Table S2 Location of Binding Sites for Gβ2 Interacting Transcription Factors within Promoters of Genes Associated with Cellular Growth and Proliferation Network.

Table S3 Gβ2 Modulates the Expression of Histone and Histone Modifier Genes.

Table S4 Differentially Expressed Genes (p<0.01) Upon Gβ2 Knockdown.

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Author Contributions

Prepared illustrations: AB SK HU RJ SSK. Read and approved the manuscript: AB HU RJ SK ZHD SY AV MK RD SSK. Conceived and designed the experiments: AB SSK. Performed the experiments: AB HU SK SY RD. Analyzed the data: AB SK AV HU MK RJ ZHD SSK. Contributed reagents/materials/analysis tools: AB SK AV HU MK RD SSK. Wrote the paper: AB HU SSK.

References

1. Clapham DE, Neer EJ (1997) G protein beta gamma subunits. Annu Rev Pharmacol Toxicol 37: 167–203.
2. Lambert NA (2008) Dissociation of heterotrimeric G proteins in cells. Sci Signal 1(25):re5.
3. Saini DK, Chiarti M, Gautam N (2009) Shuttling and translocation of α-actinin-4 into the nucleus of cardiomyocytes. Proc Natl Acad Sci 105: 12457–12462.
4. Martini JS, Raake P, Vinge LE, DeGeorge BR Jr, Chaprun JK, et al. (2008) Uncovering G protein-coupled receptor kinase-5 as histone deacetylase kinase in the nucleus of cardiomyocytes. Proc Natl Acad Sci 105: 12457–12462.
5. Hepler JR (2005) RhoB, a surprising new link between G proteins, RGS proteins, and nuclear signaling. Science STKE. doi: 10.1126/stke.2942005pe30.
21. Bhatnagar A, Sheffler DJ, Kroeze WK, Compton-Toth B, Roth BL (2004) Large-scale characterization of HeLa cells nuclear phosphoproteins. Proc Natl Acad Sci 101: 12130–12135.

20. Bhatnagar A, Willins DL, Gray JA, Woods J, Benovic JL, et al. (2001) The dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT2A) serotonin receptors reveals differential sorting of arrestins and 5-HT2A receptors during endocytosis. J Biol Chem 276: 8269–8277.

19. Kanaya N, Murray PA, Damron DS (1998) Propofol and ketamine only inhibit intracellular Ca2+ transients and contractions in rat ventricular myocytes at supraclimical concentrations. Anesthesiology 89: 397–403.

18. Do¨ring HJ (1990) The isolated perfused heart according to Langendorff technique function application. Physiol Bohemoslov 39: 481–504.

17. Yong SL, Wang QK (2006) Animal models for cardiac arrhythmias. Methods Mol Med 129: 127–140.

16. Kinter M, Sherman NE (2000) Protein Sequencing and Identification using Tandem Mass Spectrometry, D. M. Desiderio and N. M. Nibbering, Eds. (Wiley, New York).

15. Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Ville ´n J, et al. (2004) Caveolin-1 interacts with 5-HT2A serotonin receptors and profoundly modulates dynamin-dependent, arrestin-independent internalization of 5-hydroxytryptamine 2A (5-HT2A) serotonin receptors during endocytosis. J Biol Chem 276: 8269–8277.

14. Han Z, Guo L, Wang H, Shen Y, Deng XW, et al. (2006) Structural basis for the specific recognition of methylated histone H3 lysine 4 by the WD-40 protein WDR5. Mol Cell 22: 137–144.

13. Yaffe MB, Smerdon SJ (2004) The use of in vitro peptide-library screens in the analysis of phosphoserine/threonine-binding domain structure and function. Annu Rev Biophys Biomol Struct 33: 225–244.

12. Orlicky S, Tang X, Willems A, Tyers M, Sicheri F (2003) Structural basis for phoshodependent substrate selection and orientation by the SCF(Cdc4 ubiquitin ligase. Cell 112: 243–256.

11. Ford CE, Skiba NP, Bae H, Daaka Y, Reaveny E, et al. (1998) Molecular basis for interactions of G-protein beta-gamma subunits with effectors. Science 280: 1271–1274.

10. Song JJ, Garlick JD, Kingston RE (2008) Structural basis of histone H4 recognition by p55. Genes Dev 22, 1313–1318.

9. Boivin B, Vaniotis G, Allen BG, He ´bert TE (2008) G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm? J Recept Signal Transduct Res 20: 15–20.

8. DeSilva H, Lee K, Osley MA (1998) Functional dissection of yeast Hir1p, a WD repeat-containing transcriptional coresspressor. Genetics 148: 667–677.

7. Dynlacht BD, Weinzierl RO, Admon A, Tjian R (1993) The dTAFII80 subunit of Drosophila TFIID contains beta-transducin repeats. Nature 363, 176–179.

6. Martinez-Halban MA, Tsukiyama T, Gdula D, Wu C (1998) Drosophila NURF-55, a WD repeat protein involved in histone metabolism. Proc Natl Acad Sci 95: 132–137.

5. Suka N, Nakashima E, Shinmyozu K, Hieda M, Jinguji H (2006) The WD40-repeat protein Pwp1p associates in vivo with 25S ribosomal chromatin in a histone H4 tail-dependent manner. Nucleic Acids Res 34: 3555–3567.

4. Zhang Q, Vo N, Goodman RH (2000) Histone binding protein RbAp48 interacts with a complex of CREB binding protein and phosphorylated CREB. Mol Cell Biol 20: 4970–4978.

3. Ahmad A, Takami Y, Nakayama T (1999) WD repeat protein Pot1p in vivo with 25S ribosomal chromatin in a histone H4 tail-dependent manner. Nucleic Acids Res 34: 3555–3567.

2. Yokoyama H, Yamada T, Endo R, Ino Y, Gotoki M, et al. (1998) Actinin-4, a novel actin-bundling protein associated with cell motility and cancer invasion. J Cell Biol 140, 1383–1393.

1. Liu H, Sachyev RG, Yates JR (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76: 4191–4201.

0. Chakraborthy S, Reineke EL, Lnn M, Li X, Liu Y, et al. (2006) Alpha-actinin 4 potentiates myocyte enhancer factor-2 transcription activity by antagonizing histone deacetylase 2. J Biol Chem 281: 35070–35080.

9. Krumins AM, Gilman AG (2006) Targeted knockdown of G protein subunits selectively prevents receptor-mediated modulation of effectors and reveals complex changes in non-targeted signaling proteins. J Biol Chem 281: 10250–10262.