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CHIP-MYTH: A novel interactive proteomics method for the assessment of agonist-dependent interactions of the human β_2-adrenergic receptor

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ABSTRACT

G-protein coupled receptors (GPCRs) are involved in a variety of disease processes and comprise major drug targets. However, the complexity of integral membrane proteins such as GPCRs makes the identification of their interacting partners and subsequent drug development challenging. A comprehensive understanding of GPCR protein interaction networks is needed to design effective therapeutic strategies to inhibit these drug targets. Here, we developed a novel split-ubiquitin membrane yeast two-hybrid (MYTH) technology called CHIP-MYTH, which allows the unbiased characterization of interaction partners of full-length GPCRs in a drug-dependent manner. This was achieved by coupling DNA microarray technology to the MYTH approach, which allows a quantitative evaluation of interacting partners of a given integral membrane protein in the presence or absence of drug. As a proof of principle, we applied the CHIP-MYTH approach to the human β_2-adrenergic receptor (β_2AR), a target of interest in the treatment of asthma, chronic obstructive pulmonary disease (COPD), neurological disease, cardiovascular disease, and obesity. A CHIP-MYTH screen was performed in the presence or absence of salmeterol, a long-acting β_2AR-agonist. Our results suggest that β_2AR activation with salmeterol can induce the dissociation of heterotrimeric G-proteins, G$\alpha\beta\gamma$, into Gα and G$\beta\gamma$ subunits, which in turn activates downstream signaling cascades. Using CHIP-MYTH, we confirmed previously known and identified novel β_2AR interactors involved in GPCR-mediated signaling cascades. Several of these interactions were confirmed in mammalian cells using LUminescence-based Mammalian IntERactome (LUMIER) and co-immunoprecipitation assays. In summary, the CHIP-MYTH approach is ideal for conducting comprehensive protein-protein interactions (PPI) screenings of full-length GPCRs in the presence or absence of drugs, thus providing a valuable tool to further our understanding of GPCR-mediated signaling.

1. Introduction

The G protein-coupled receptors (GPCRs) are a superfamily of transmembrane receptors that are broadly distributed in diverse tissues and cell types and that can collectively recognize a diverse array of ligands. Furthermore this large gene family encodes key components of cell signaling events. Activation or inhibition of GPCR signaling can affect numerous physiological processes, many of which are directly linked to human diseases. Accordingly, modulation of the activity of GPCRs is a subject of intense research with respect to the development of new drug therapies [1,2]. Currently,

Abbreviations: Ade, adenine; 3-AT, 3-amino-1,2,4-triazole; β_2AR, beta2-adrenergic receptor; Cub, carboxy-terminal part of ubiquitin; His, histidine; *lacZ*, gene for b-galactosidase; Leu, leucine; LUMIER, LUminescence-based Mammalian IntERactome; MYTH, membrane yeast two-hybrid; MCS, multiple cloning site; Minimal SD, minimal synthetic medium consisting of a dropout for the nutritional requirement of MYTH interaction; Nub, N-terminal part of ubiquitin; PPI, protein–protein interaction; SD, synthetic dropout; TF, transcription factor; Trp, tryptophan.

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~30% of all drugs target GPCRs [3], underscoring the need to understand these receptors in detail. Because the structure of these membrane proteins is highly hydrophobic and prone to aggregation, studying their potential interaction partners is difficult and thus, this class of proteins is underrepresented in genome-wide datasets.

To address this problem, we previously developed a yeast-based genetic technology for the *in vivo* detection of membrane protein interactions, the split-ubiquitin membrane yeast two-hybrid (MYTH) system [4,5]. MYTH allows for the use of full-length membrane proteins as baits, in their natural membrane context. The MYTH system is based on the principle of split-ubiquitin, whereby an N-terminal ubiquitin fragment (Nub) and a C-terminal ubiquitin fragment (Cub), are capable of spontaneous reconstitution into pseudo-ubiquitin [6]. In MYTH, a membrane bait protein is fused to Cub and an artificial transcription factor. Prey proteins, either cytosolic or membrane-bound, are fused to a mutant variant of Nub, NubG, which we engineered so that only bait-prey interaction allows for pseudo-ubiquitin formation. Subsequent recognition of pseudo-ubiquitin by deubiquitinating enzymes (DUBs) results in cleavage and release of the transcription factor, leading to reporter gene expression. To date, MYTH has been successfully applied to study interactions of membrane proteins such as ion channels, GPCRs and transporters from various organisms, and has only recently been adapted for the identification of interaction partners of mammalian receptor tyrosine kinases [7–10] .

Various yeast two-hybrid techniques have been previously used to systematically screen for PPIs of GPCRs [8,11]. Complementary techniques such as resonance energy transfer methods (BRET/FRET) have also been extensively used to identify interaction partners of GPCRs, however there have been significant limitations in our ability to study GPCRs under various conditions related to drug action (e.g. agonist activation/inactivation) in a high-throughput manner [12–14].

Here, we present a novel technique, CHIP-MYTH, capable of identifying novel partners of full-length integral GPCRs in a drug-dependent manner. In this proof-of-principle study, we identified known interactors, as well as new potential interactors, of ligand-occupied and -unoccupied β_2AR demonstrating the usefulness of this technology for studying GPCRs in a large-scale format under various physiological conditions. This approach can help define novel context-dependent interactomes of various GPCRs, thus helping to identify new interaction partners, which might be involved in the pathophysiology of human diseases.

2. Materials and methods

2.1. Reagents

Salmeterol was purchased from TOCRIS and dissolved in DMSO. A stock of 10 mM was generated and stored at −20 °C. To perform the experiments, salmeterol was thawed at room temperature and added to the yeast culture. Isoproterenol was purchased from Sigma–Aldrich and added at a final concentration of 10 μM.

2.2. Bait and prey constructs

The full-length transmembrane protein, human β_2-adrenergic receptor (Accession number NM_00024), was PCR-amplified and cloned into the pAMBV4 vector, where it was fused in frame to the carboxyl terminal half of ubiquitin (Cub) and a transcription factor (TF) consisting of the *Escherichia coli* DNA-binding protein LexA and the activation domain of VP16 from herpes simplex virus. The human fetal brain cDNA prey library was purchased from Dualsystems Biotech AG, Switzerland. These prey genes were cloned into the pNubG-HA-X vector, pPR3-N (X, any truncated cDNA derived from human fetal brain).

2.3. Yeast transformations

50 μl Of competent yeast cells expressing human β_2AR were treated with 50% (w/v) polyethylene glycol, 1 M LiOAc and 2.0 mg/ml single-stranded DNA. 100 ng Of prey plasmids were added into the transformation mixture and incubated at room temperature for 30 min. The competent yeast cells were heat-shocked at 42 °C for 40 min, pelleted by centrifugation, and resuspended in 100 μl of H_2O. Cells were plated on synthetic dropout (SD) agar plates devoid of Leu (to select for baits) or Trp (to select for preys) and transformants identified after incubation at 30 °C for 3 days.

2.4. Immunofluorescence staining in yeast

Yeast cells containing human β_2AR were grown to midlog phase in SD-Leu media. Cells were then fixed in 10% formaldehyde at room temperature for 1 h with rotation, collected by centrifugation and resuspended in SK buffer (0.2 M K_2HPO_4 pH 7.5, 0.2 M KH_2PO_4, pH 7.5, 2 M Sorbitol). Cells were treated with 4 mg/ml Zymolyase-20T (Seikagaku Corp., Tokyo, Japan) dissolved in SK buffer plus β-mercaptoethanol (1 μl/ml buffer) and incubated at room temperature until cells became spheroplasts. Cells were washed three times in SK buffer, spotted on precoated poly-L-lysine slides (O. Kindler GmbH and Co., Freiburg, Germany) and permeabilized by acetone/methanol treatment. Washed cells were blocked with PBS+ 1% BSA and incubated with the polyclonal rabbit anti-VP16 (1:1000, Sigma–Aldrich, V4388) antibody followed by staining with a goat anti-rabbit FITC-conjugated antibody (1:500; Molecular Probes). The images were captured by confocal microscopy using a 63× oil-immersed objective. Cells were examined using a Leica DMI 6000 B microscope and images were analyzed using the Volocity software.

2.5. Growth assay

To determine the compound dose that inhibits yeast growth by approximately 15% (relative to DMSO), yeast strain THY.AP4 or the same strain over-expressing human β_2AR-Cub-TF was inoculated at an OD_{600} of 0.0625 in complete media or leucine dropout minimum media, respectively. Serial dilutions of salmeterol (agonist for β_2AR) were added to the final concentrations as listed in the figures. Yeast growth was monitored by measuring the OD_{600} every 15 min for 24 h with a TECAN Sunrise plate reader. Growth curves were performed in triplicate and curves shown are an average of three measurements per individual time point. The drug sensitivity is calculated by the growth inhibition in drug vs. DMSO.

2.6. Split-ubiquitin membrane yeast two-hybrid (MYTH) screen

Host *Saccharomyces cerevisiae* strain THY.AP4 (genotype *MATa*, *ura3*, *leu2*, *lexA*::*lacZ*::*trp1*, *lexA*::*HIS3*, *lexA*::*ADE2*) was grown in YPAD medium (1% yeast extract, 2% bacto peptone, 2% glucose, 0.01% adenine hemisulfate, 2% select agar). A MYTH screen was performed as previously described [15,16]. Human β_2AR over-expressed in THY.AP4 was transformed with 28 μg of fetal brain cDNA library. All transformants were plated on solid medium (SD-WL) selective for the presence of bait and prey, but not interaction, and grown for 2 days or approximately 20 generations. Transformants were then collected into liquid SD-WL and ~10×10^6 cells (1 OD_{600} unit) were re-cultured in either the presence or the absence of salmeterol in 2 mL of liquid SD-WL (SD-WL + D, and SD-WL − D, respectively). The transformants were collected by centrifugation and then grown for 6 generations in

2 ml of SD-WLAH (to select for interacting pairs) in either the presence or the absence of salmeterol (SD-WLAH + D and SD-WLAH − D, respectively) along with SD-WL ± D. To amplify the abundance of strong interactors, the selection was continued for an additional 6 generations of growth.

2.7. Isolation of hits

The prey plasmids of individual selected transformants were isolated using the Zymoprep™ Yeast Plasmid Miniprep kit (Zymo Research, USA). The genes encoding the specific interactors were then subjected to PCR amplification using common primers based on the NubG fusion and vector backbone (PCR-pPR3N-Fwd: GGA ATC CCT GGT GGT CCA TAC and PCR-pPR3N-Rev: GAC TCG AGG TCG ACG GTA TCG). The PCR reactions were carried out using 1× TaqPolymerase Buffer, 1.5 mM $MgCl_2$, 50 mM dNTPs, 50 ng of prey plasmids, 10 pmol of primers and 2.5 U of Taq Polymerase enzyme with the following cycling conditions; {95 °C for 2 min} × 1 cycle, {95 °C for 30 s, 60 °C for 45 s, 72 °C for 2 min} × 35 cycles, {72 °C for 8 min} × 1 cycle.

2.8. Probe labeling, purification and hybridization of DNA microarray

PCR products of selected interactors were purified using a PCR purification kit (Invitrogen). The purified products were biotinylated using the BioPrime Labeling Kit (Invitrogen). Briefly, 20 ml of PCR product were mixed with 20 ml of 2.5× Random Primers Solution and incubated on ice for 5 min. The probe was then denatured at 95 °C for 10 min and immediately chilled on ice. Five ml of 10× dNTPs, with dCTP-biotin and 1 U of Klenow Enzyme were added and incubated at 37 °C for 10 min before adding the stop buffer. To remove the free biotin, probe was passed through a CENTRI-SPIN^{-20} column. 50 ng Of probe was hybridized onto HuGene 1.0 ST array (Affymetrix) for 17 h at 45 °C, 60 rpm. Chips were washed and stained with 1 mg/ml streptavidin–phycoerythrin (SAPE), washed on an Affymetrix fluidics station and scanned. Signal intensity was extracted from HuGene 1.0 ST arrays using Affymetrix Power Tools v.1.12.0 (APT, http://www.affymetrix.com) using GC-background correction and PLIER for between chip normalization and probe summarization. Signal was expressed as log2 ratios relative to the reference array, to generate a ranked list of interactors in the absence or presence of drug.

2.9. Cell culture

HEK293T cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37 °C, 5% CO_2 incubator. Transfection experiments were performed using a modified calcium phosphate method. Briefly, for a 12-well plate, 65 µl ddH_2O and 75 µl 2 × BES (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) were added to the DNA and 7.5 µl 2.5 M $CaCl_2$ was then added. The mixture was vortexed, incubated at room temperature for 15 min and added dropwise to the cells. 8–16 h Later, the transfection mix was removed and replaced with indicated media.

2.10. Construction of mammalian bait and prey expression vectors

HA epitope-tagged human β_2AR was kindly provided by Dr. Michel Bouvier (University of Montreal) for immunoprecipitation purposes. The Renilla luciferase-tagged human β_2AR was generated by PCR and subcloning into pRluc-N2, and verified by sequencing before use in LUMIER screening. All the MYTH interactors used for LUMIER were generated by gateway cloning into pFLAG-V1899 (for N-terminal tagging) and pFLAG-V1900 (for C-terminal tagging), generous gifts from Dr. Jason Moffat (University of Toronto) and verified by DNA sequencing. The protein expression of these preys was confirmed by Western blotting.

2.11. LUMIER

The LUMIER assay was used to confirm the interactors identified by CHIP-MYTH. Additionally, this assay using β_2AR-Renilla luciferase fusion protein, was multiplexed by adding a CRE-Firefly luciferase reporter for a cAMP readout. LUMIER screens were modified from Barrios-Rodiles *et al* [17]. Briefly, HEK-293T cells were seeded onto poly-D-lysine coated 96-well plates (COSTAR, Corning) 24 h before transfection. 90 ng of β_2AR-Renilla luciferase (β_2AR-Rluc) and 50 ng of 3XFLAG-tagged cDNA preys, were transiently transfected using PolyFect (QIAGEN). The screens were carried out on an automated Dimension 4 Thermo Robotic platform. Transfected cells were maintained at 37 °C and 36 h later, salmeterol (10 µM) was added to the cells. 12 h after salmeterol activation, the cells were lysed in 200 µl of lysis buffer plus phosphatase and protease inhibitors. Measurements of luciferase activity in total protein cell lysates and immunoprecipitated samples were carried out using a Berthold Luminometer with the Renilla Luciferase Assay System (Promega). The selection of hits was done by calculating the NLIR (Normalized LUMIER Intensity Ratio) values as described in Ellis et al. [18], using a NLIR $\geqslant$ 3 as cut-off.

2.12. cAMP assay

HEK-293T cells were transiently transfected with mixtures composed of 90 ng of β_2AR-Renilla luciferase (β_2AR-Rluc), 50 ng of 3XFLAG-tagged cDNA preys and 70 ng of pCRE-Luc (Clontech). 24 h Posttransfection, the cells were serum starved (without drug) and 10 µM salmeterol was added. After 12 h of salmeterol activation, the cells were lysed in 200 µl of lysis buffer containing protease inhibitors. The Dual-Glo Luciferase Assay System (Promega) was applied to detect Firefly luciferase signal for cAMP levels and Renilla luciferase signal reflecting the expression level of β_2AR (used for normalization). The Firefly signals were normalized to Renilla signals using the Formula: (Firefly/Renilla) × 100 because the Renilla signals were 2 orders of magnitude larger than the Firefly signals. The normalized Firefly signals of the conditions where the FLAG-preys were co-transfected with pRluc (background) were subtracted from the Firefly normalized signals where the FLAG-preys were co-transfected with β_2AR-Rluc. Every condition in the presence or absence of salmeterol was tested 4 times ($n = 4$). To determine the basal cAMP levels we used conditions where CRE-Luc and β_2AR-Rluc or CRE-Luc and pRluc were co-transfected with empty vector (No Prey). All the 'No Prey' data points ($n = 16$) were averaged and used in the 2 graphs pV1899 and pV1900 in Fig. 5. Finally, the ratio of normalized Firefly signals in the presence of salmeterol over the normalized Firefly signals in the absence of salmeterol was calculated and plotted (Fig. 5).

2.13. Co-immunoprecipitation

5×10^5 HEK-293T cells were seeded in 6-well plate and were manually transfected using the calcium–phosphate precipitation method. After 48 h, cells were activated for 1.5 h with 10 µM isoproterenol and were lysed. The associated FLAG-epitope tagged proteins were then immunoprecipitated using anti-FLAG M2 monoclonal antibody (Sigma) at 4 °C for 2 h, followed by binding to protein G Sepharose. Total protein expression was confirmed by immunoblotting with rat anti-HA (Roche) antibody and mouse anti-FLAG M2 monoclonal antibody (Sigma–Aldrich) for human β_2AR and interacting partner, respectively.

3. Results

3.1. DNA microarray-membrane yeast two-hybrid (CHIP-MYTH) approach

To modify the conventional MYTH method, a DNA-microarray was introduced into the system to generate the final read-out. The screening approach begins by introducing an entire human fetal brain cDNA library into a *S. cerevisiae* host expressing a membrane protein of interest. An interaction between bait and preys allows yeast cells to survive under specific selective conditions. Specifically, transformants are initially grown on non-selective media and collected into liquid culture. Next, the selection of interactors is performed in a small culture volume to reduce the amount of drug used. Selection for interactions is performed in the absence or presence of drug for 6 generations. Finally, to select for stronger interactors, culturing is continued for another 6 generations in selective media (SD-WLAH) with and without drug (SD-WLAH – Drug and SD-WLAH + Drug, respectively). The plasmids harboring potential interactors are then extracted from yeast cells and inserts are amplified using PCR, biotinylated and hybridized onto a DNA microarray (Fig. 1).

3.2. The generation, expression and functionality of human β_2AR bait in yeast

As proof of principle for our CHIP-MYTH approach, we decided to use the human β_2-adrenergic receptor (β_2AR) as bait. This receptor is the main target of current market drugs such as salmeterol, used to treat asthma and chronic obstructive pulmonary disease

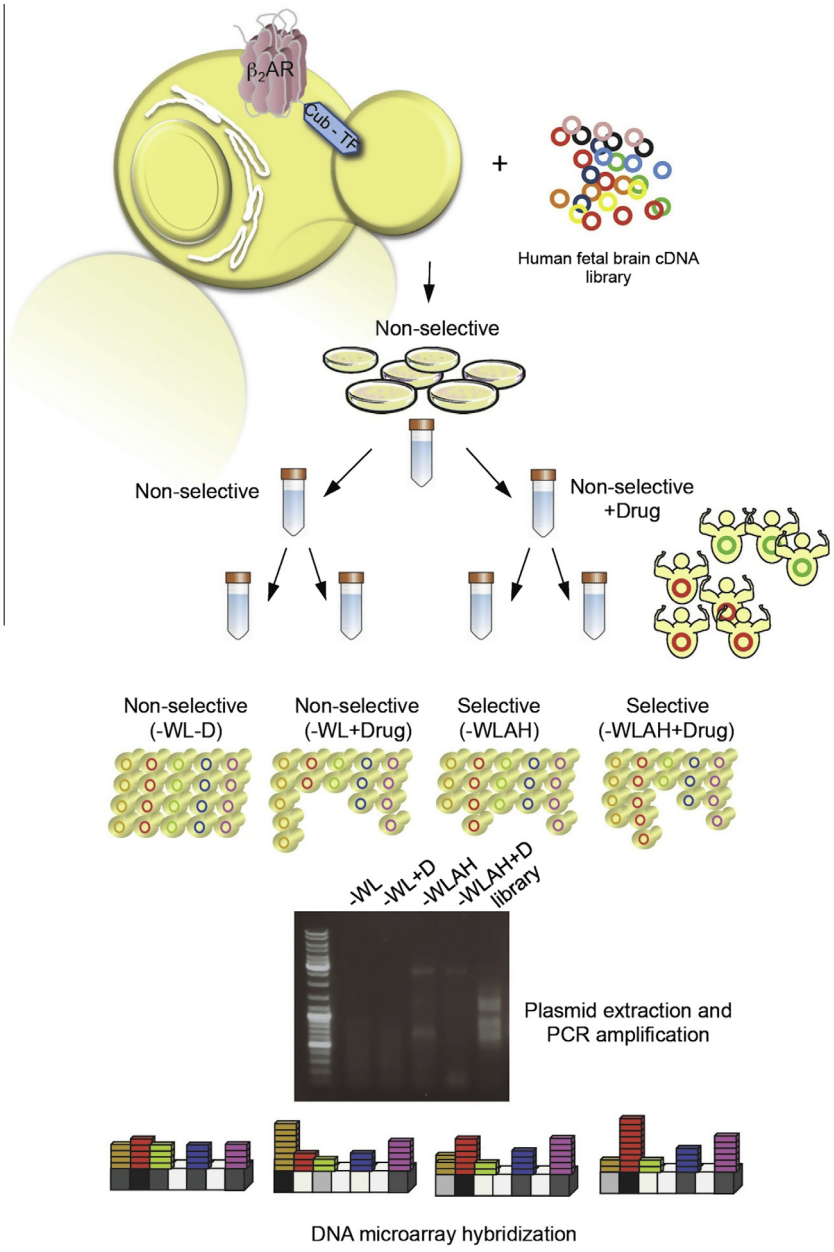

Fig. 1. CHIP-MYTH outline. A human fetal brain cDNA library is introduced into a *Saccharomyces cerevisiae* host expressing the membrane protein of interest. Transformants expressing interacting pairs are selected for, in the absence or presence of drug, by growth in selective media (SD-WLAH) for 6 generations. For selection of stronger interactors, growth in selective media can be performed for another 6 generations in selective media. The plasmids containing the potential interactors are then extracted from yeast cells, PCR-amplified and hybridized onto a DNA microarray.

(COPD) [19]. Although the signaling cascade of β_2AR upon drug stimulation is not completely understood, we postulated that this receptor would share the common activation through G-proteins and subsequently turn on the signaling cascade. Human β_2AR was cloned into a MYTH bait expression vector, β_2AR-Cub-TF (Fig. 2A). The expression of this human protein in yeast, and its suitability for use in MYTH screening, was confirmed by genetic (Fig. 2B) and immunofluorescence (Fig. 2C) assays. As controls in our genetic assay, we used two non-interacting yeast integral membrane proteins; (1) Ost1p, an oligosaccharyltransferase complex expressed in the ER and (2) Fur4p, a uracil permease localized at the plasma membrane. These two proteins were fused to either NubI as positive controls (OstI-NubI and Fur4-NubI) or to NubG as negative controls (OstI-NubG and Fur4-NubG). Activation of the reporter system was measured as growth on media lacking histidine (-H) and adenine (-A) in the presence of the optimal concentration of 3-AT (SD-WLAH + 5 mM 3-AT; selective media). No growth was evident for cells expressing β_2AR-Cub-TF alongside the non-interacting Ost1-NubG and Fur4-NubG proteins on selective media, confirming that the bait did not activate the reporter system in the absence of an interacting protein partner (i.e. 'self-activate') and was therefore suitable for MYTH screening (Fig. 2B). Furthermore, the correct localization of human β_2AR to the plasma membrane was confirmed by immunofluorescence (Fig. 2C).

To test whether human β_2AR-Cub-TF expressed in yeast cells responds to any pharmacological β-adrenergic drugs, we performed growth inhibition assays (Fig. 2D). We originally examined the drug response by using the short-acting selective β-adrenergic agonist, isoproterenol (data not shown). Although yeast cells ectopically expressing human β_2AR exhibited a growth inhibition response, we could not use isoproterenol to perform the CHIP-MYTH screen since the short acting nature of isoproterenol was not compatible with the length of growth required in our screening pipeline. The long-acting drug salmeterol, a selective β-adrenergic agonist used in the control of moderate to severe asthma, was thus used for further experiments. We compared yeast growth between THY.AP4 yeast reporter strain and THY.AP4 overexpressing human β_2AR in the absence or presence of different doses of salmeterol. Yeast cells overexpressing human β_2AR-Cub-TF are sensitive to salmeterol treatment as observed by slow growth compared to non-treated yeast cells (Fig. 2D). These results demonstrate that the human β_2AR bait is correctly expressed at the plasma membrane and is functional, based on its ability to respond to the specific β-agonist, salmeterol.

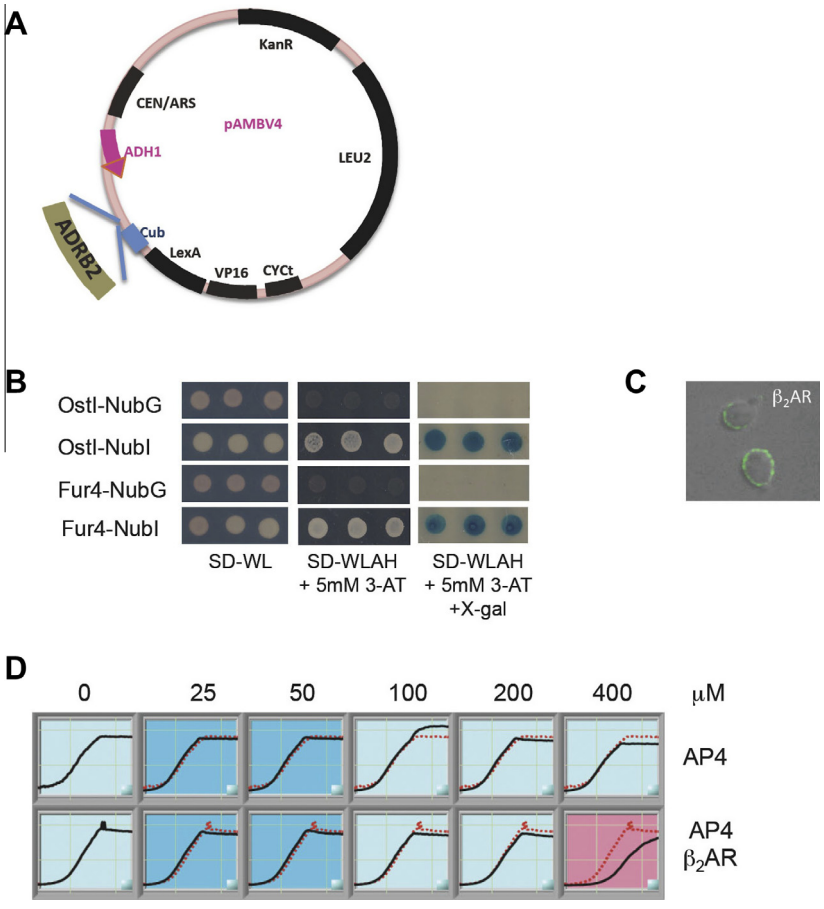

Fig. 2. Bait generation and verification. (A) The human β_2AR was cloned into the membrane yeast two-hybrid bait expression vector AMBV4, harboring an ADH1 promoter, the C-terminal ubiquitin half (Cub) and the chimeric transcription factor LexA-VP16. (B) To exclude self-activation, β_2AR-Cub-TF bait was tested for interaction with positive controls, OstI-NubI and Fur4-NubI or with the negative controls, OstI-NubG and Fur4-NubG. Activation of the reporter system was measured as growth on media lacking histidine (-H) and adenine (-A) in the presence of the optimal concentration of 3-AT (SD-WLAH + 5 mM 3-AT; selective media). Absence of interaction between β_2AR-Cub-TF and Ost1-NubG and Fur4-NubG indicated that β_2AR-Cub-TF bait is expressed properly, does not self-activate, and is therefore suitable for MYTH screening. (C) Immunofluorescence was performed to show that β_2AR is located at the membrane of yeast cells. Rabbit anti-VP16 antibody was used followed by anti-rabbit FITC treatment and imaging by confocal microscopy. (D) Yeast cells overexpressing human β_2AR-Cub-TF are sensitive to salmeterol treatment as observed by the slow growth compared to non-treated yeast cells. The *X*-axis exhibits time and *Y*-axis shows OD_{600} nm. Growth is compared between yeast THY.AP4 (top panel) and THY.AP4 overexpressing human β_2AR (bottom panel). The increasing amount of salmeterol is indicated as 0, 25, 50, 100, 200 and 400 μM, respectively. The dashed red line represents the reference yeast growth in the absence of drug, while the solid line shows yeast growth in the presence of salmeterol.

3.3. Identification of interactors of human β_2AR overexpressed in yeast by DNA microarray analysis

A CHIP-MYTH screen was performed under 2 conditions (see Section 2 for details): in the absence of salmeterol (to detect general interactions) and in the presence of salmeterol (to detect changes in interactions in response to drug-addition). We collected the cells after growth in different conditions, extracted genomic DNA and PCR-amplified prey-ORFs. A DNA microarray was used as the readout of the screen. Following hybridization of biotinylated samples to independent arrays, the signal intensity for each probeset (corresponding to a unique ORF) was used as a measure of the 'strength' of an interaction. We selected potential partners based on their signal intensity and then compared the signal for these hits in selective versus non-selective media. These comparisons are expressed as the log2 intensity ratio of samples obtained from selective media in the presence of salmeterol (*Y*-axis) and its absence (*X*-axis) (Fig. 3). A log2 ratio of signal intensity (drug/background) and (no drug/background) were then listed and ranked. The primary hits selected for validation were those top 10% of the enriched ratio. The potential hits were narrowed down by clone availability and sequence-verification. In total, we selected 55 unique FLAG-tagged preys for validation via an orthogonal LUMIER protein interaction assay [17].

In general, upon activation of a GPCR, the receptor acts as a guanine nucleotide exchange factor (GEF) for its cognate heterotrimeric G-protein (Gα, Gβ and G), resulting in G-protein dissociation and subsequent regulation of the activity of several second messengers such as cAMP and intracellular calcium and several protein kinases [20,21]. Moreover, the activation of GPCR leads to changes in gene expression by turning on a GEF for the GTPase Rho, resulting in a variety of effects such as protein phosphorylation and receptor internalization [21]. Several regulators of G-protein signaling (RGS) proteins are also reported as direct interactors of not only the G-protein, but also of the GPCR [22]. The multiple interaction modes underscores the complexity of GPCR activation and regulation [21].

Our CHIP-MYTH screen revealed a number of interesting interacting partners of β_2AR and was able to detect β_2AR (ADRB2)-homodimerization [23] in both the absence and presence of salmeterol. Also, several Regulator of G-protein Signalling (RGS) proteins [24], RGS2, RGS10, RGS16, RGS18 and RGS19, showed high signal intensity on the DNA microarray compared to background in both conditions (Fig. 3). These data indicate that CHIP-MYTH successfully detected both known and novel potential interacting partners. Interestingly, a majority of salmeterol-induced interactors belonged to the GPCR family, demonstrating heterodimerization of β_2AR to other GPCRs.

3.4. Confirmation of novel interactors using LUMIER in mammalian cells

To independently confirm the physical interactions between β_2AR receptor and partners identified by CHIP-MYTH, we used LUMIER, a high-throughput assay for measuring protein–protein interactions in mammalian cells [17]. We cloned the putative interactors into mammalian expression vectors harboring an N- or C-terminal FLAG-tag. The human β_2AR receptor was fused to Renilla luciferase (β_2AR-Rluc). The β_2AR-Rluc and the FLAG-tagged potential partners were co-expressed in mammalian HEK293T cells and potential interactions were assessed after immunoprecipitation and Renilla-luciferase readout using an automated platform.

We calculated the Normalized luminescence intensity ratio (NLIR) as previously described [18,25] for both the N-terminal FLAG-tagged preys (pV1899) and C-terminal FLAG-tagged preys,

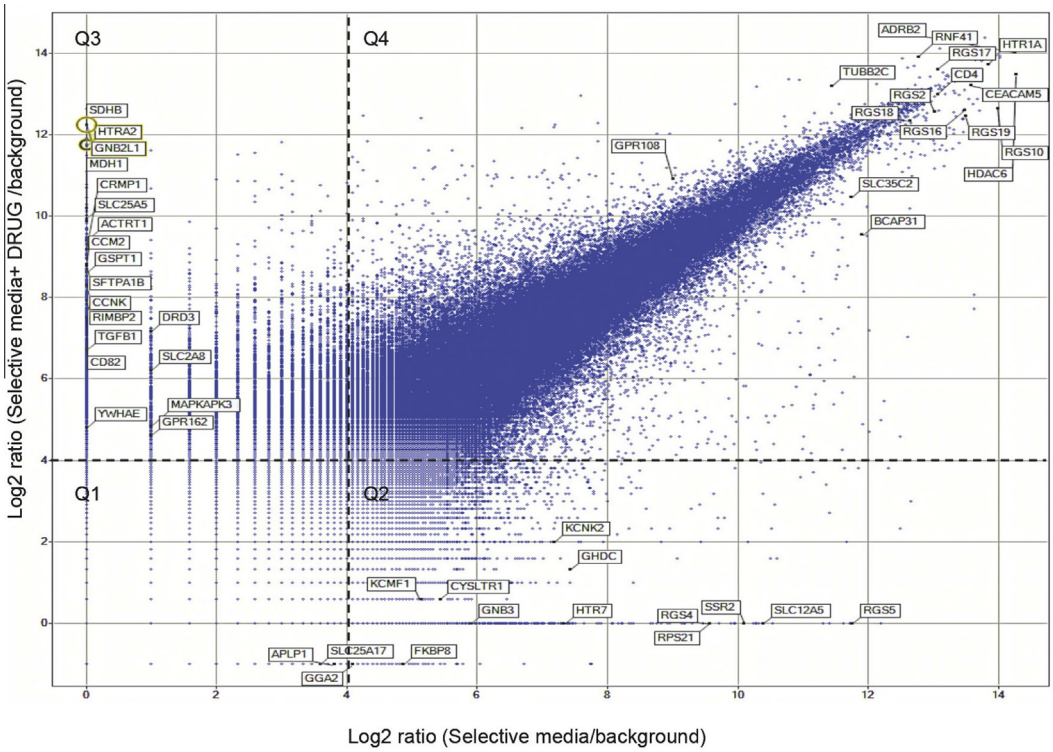

Fig. 3. β_2AR-interactors identified by CHIP-MYTH. Screen results were visualized by plotting the log2 of the signal intensity for each prey in the presence (*y*-axis) and absence (*x*-axis) of salmeterol. Genes with a log2 (drug/background) > 4 were considered as potential strong interactors (drug-specific, Q3) and a log2 (no drug/background) > 4-enrichment were potential interactors in the absence of drug, Q2. The majority of genes were found in both absence and presence of drug (Q4).

(p1V900) (Fig. 4). We originally subcloned 80 preys into the Flag-tagging vectors. However, due to PCR amplification or sequence-verification attrition, we present the data for 55 unique Flag-tagged preys that were tested by LUMIER. Among these 55 preys, 28 proteins showed an interaction to the human β_2AR-Rluc in HEK293T cells with an NLIR above 3, which we considered our cut-off for positive interactions, resulting in an overall confirmation rate of 50.9%. Out of the 28 LUMIER-positive hits, 8 interacted with β_2AR in the absence of salmeterol, whereas 20 proteins interacted with β_2AR in the absence and presence of salmeterol (Fig. 4).

3.5. cAMP assay reveals the activation of G-protein signaling cascade

The β2AR receptor is a G protein-coupled transmembrane receptor that activates the enzyme adenylyl cyclase. Activation of adenylyl cyclase leads to increased cyclic adenosine monophosphate (cAMP) levels. To determine whether any of the interactors identified by CHIP-MYTH can increase intracellular cAMP level upon binding to β_2AR, we used a pCRE-Firefly luciferase reporter system (pCRE-Luc-pASV40, where Luc is firefly luciferase). The pCRE- promoter contains the cAMP-response element (CRE) activated by CREB1. Therefore, the Firefly luciferase signal was

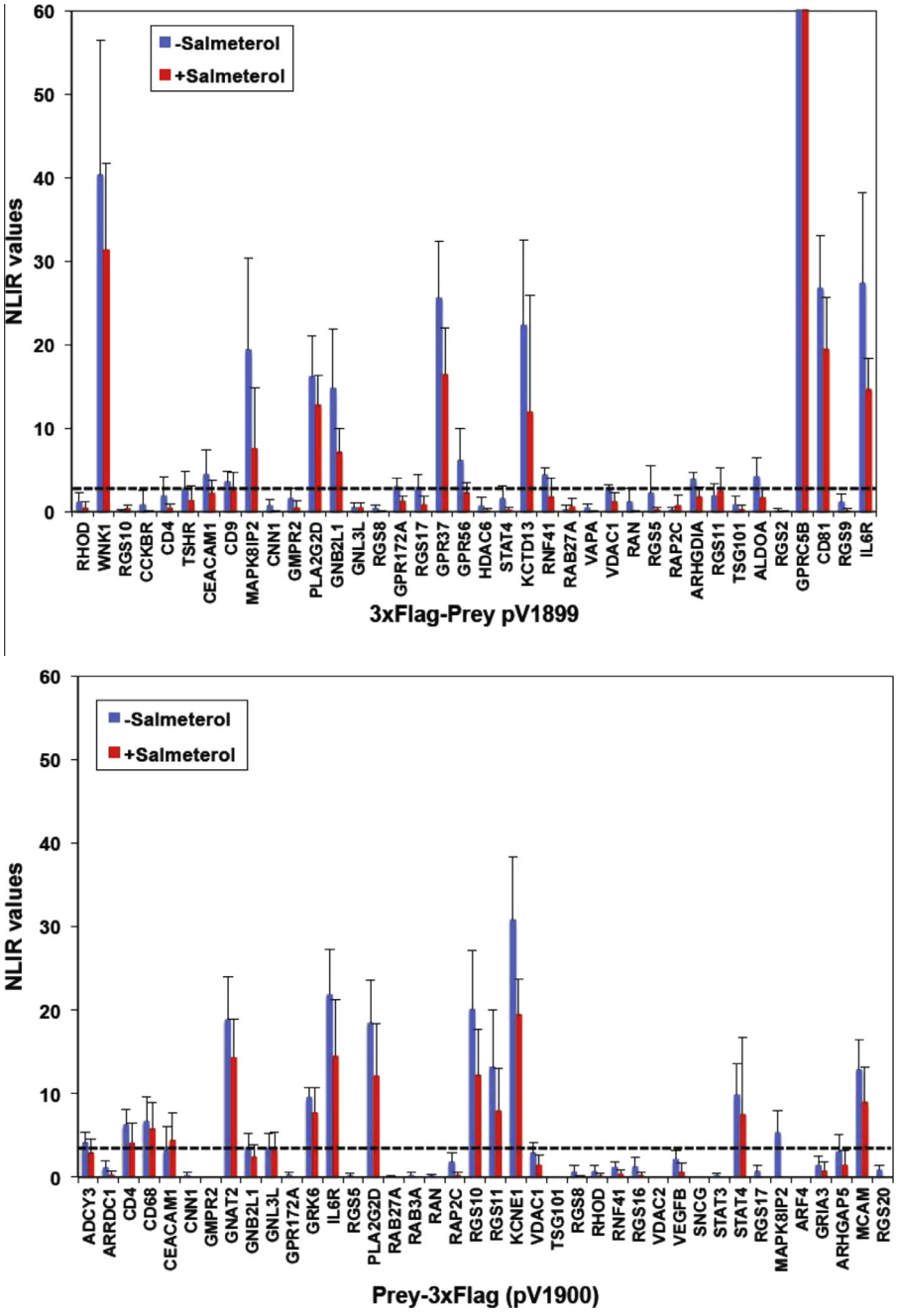

Fig. 4. Confirmation of novel β_2AR interactors. LUMIER assay for protein–protein interactions in HEK293T cells. 55 Selected hits from CHIP-MYTH were tagged with 3XFLAG and tested against β_2AR-Rluc used as bait. *Y*-axis represents the normalized luminescence intensity ratio (NLIR) calculated as previously described [18], plus standard deviation ($n = 4$). *X*-axis shows the tested FLAG-tagged preys. The dashed line at NLIR value of 3 indicates the cut-off for positive interactions. The blue bars indicate interactions tested in the absence of salmeterol and the red bars show interactions tested in the presence of salmeterol. N-terminal, FLAG-tagged preys, pV1899, are shown in the top panel. C-terminal, FLAG-tagged preys, pV1900, are shown in the bottom panel. Out of the 55 preys, 23 of them were tested as both N- and C-terminal, FLAG-tagged preys. The NLIR values for GPRC5B are not shown to better compare the 2 graphs with differentially tagged preys. The NLIR values for GPRC5B were 147 (+/−28) in the absence of salmeterol) and 116 (+/−35) in the presence of salmeterol.

considered as reflection of the cAMP levels. For these experiments, 3 plasmids, human β_2AR-Rluc (or pRluc as control), FLAG-tagged prey and pCRE-Luc, were transiently transfected into HEK293T cells. 24 h post-transfection, 10 μM salmeterol was added or not, for 12 more hours to ensure that the pCRE-Luc system is fully translated. We normalized the Firefly luciferase values from the pCRE-Luc vector to the Renilla luciferase levels as described in Section 2. We considered as basal cAMP levels the conditions where no FLAG-tagged prey was present (no Prey, Fig. 5A). The ratio of normalized cAMP between the conditions where salmeterol was present over the ones where salmeterol was absent was calculated. A ratio above 1 was considered having an effect on intracellular cAMP levels by a given prey. As shown in Fig. 5A, CCKBR, a GPCR and GRK6, a GPCR kinase, induced a slight increase in cAMP-level when coexpressed with β_2AR. Additionally, the regulatory G proteins RGS10 and RGS11 induced a very modest increase in cAMP levels upon coexpression with β_2AR. These data, along with the LUMIER results in the presence of drug, suggested that the activation of β_2AR-Rluc with salmeterol was perhaps not optimal under our experimental conditions. Nonetheless, taken together, our results show that the CHIP-MYTH approach can be successfully used to discover novel protein–protein interactions for GPCRs in response

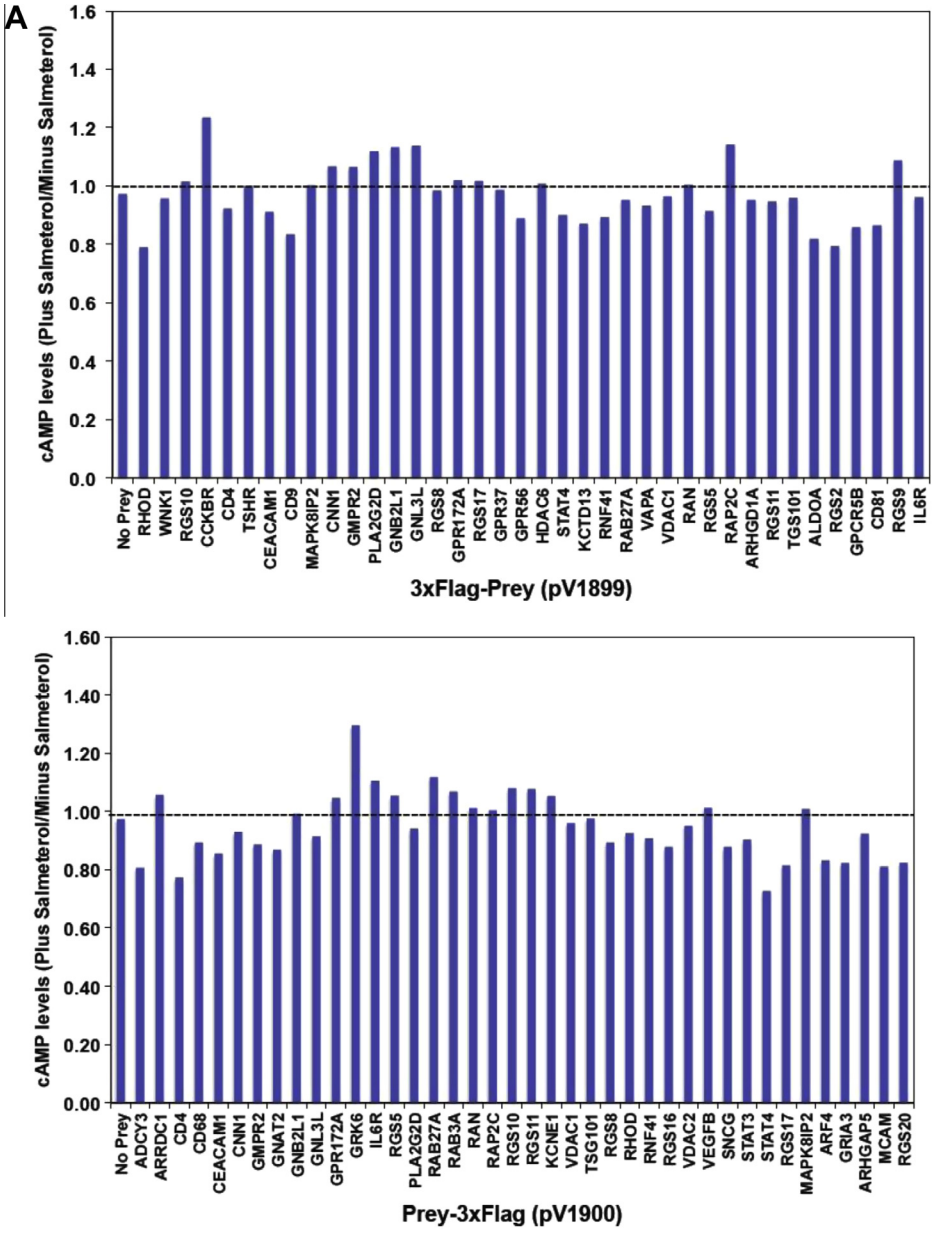

Fig. 5. Downstream β_2AR signaling activation. (A) cAMP levels upon salmeterol addition. Three plasmids (human β_2AR-Rluc, FLAG-tagged prey and pCRE-Luc) were transiently transfected into HEK293T cells. 24 h post-transfection, cells were treated +/− salmeterol for 12 h. *Y*-axis exhibits the ratio of the normalized Firefly luciferase (reflecting cAMP levels, calculated as described in Section 2) in the presence of salmeterol over the conditions in the absence of drug. The dashed line indicates the basal cAMP levels at 1 where no FLAG-tagged prey (no prey) was transfected. Prey names are indicated on the X-axis. N-terminal, FLAG-tagged preys, pV1899 are shown in the top panel and C-terminal, FLAG-tagged preys, pV1900, are shown in the bottom panel. (B) Protein interaction network for β_2AR. Shown are 55 β_2AR interactors detected by CHIP-MYTH that were further tested by LUMIER, with and without salmeterol. LUMIER detected 28 interactions: 8 in the absence of salmeterol (yellow edges) and 20 in the absence and presence of salmeterol (red edges). 27 were detected by CHIP-MYTH, but not confirmed by LUMIER (green edges). Previously known interactions, obtained from the Interologous Interaction Database version 2.3 (http://ophid.utoronto.ca/i2d) [30], are shown as solid edges. Proteins that raised cAMP levels in the presence of salmeterol are indicated by nodes with red outlines. To reduce network complexity, remaining nodes and edges are made partially transparent. Network visualization in NAViGaTOR 2.3.1 (http://ophid.utoronto.ca/navigator) [31].

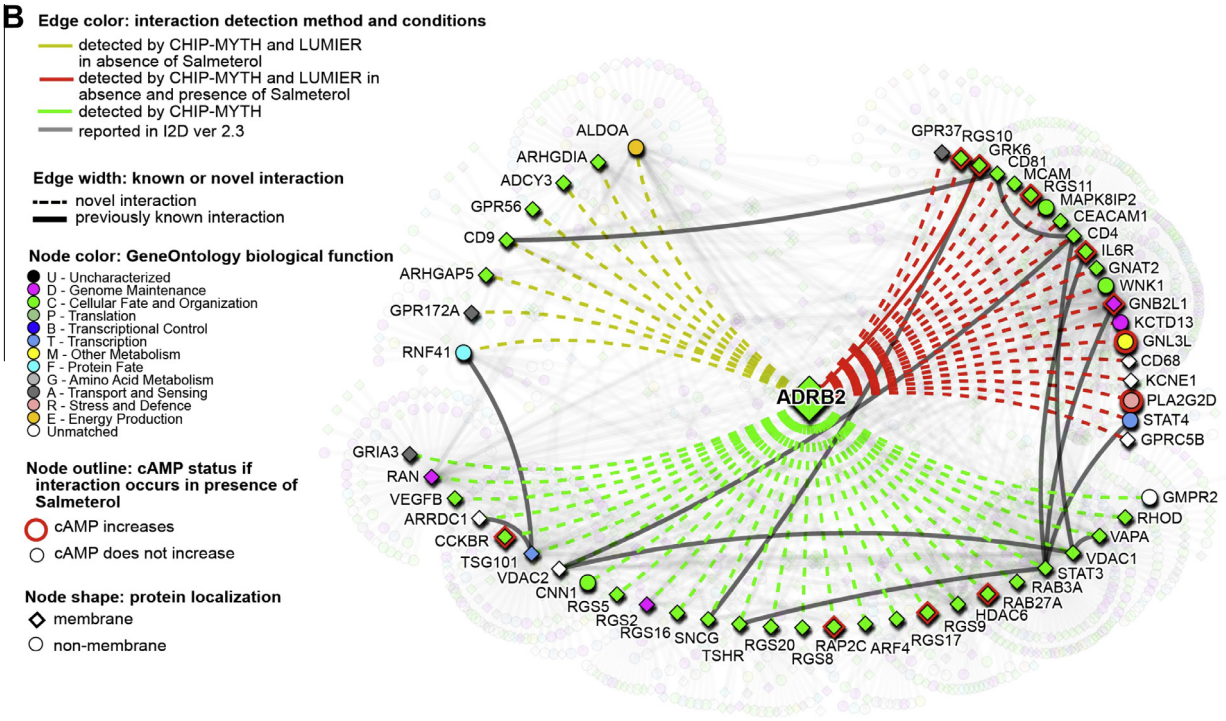

Fig. 5 (*continued*)

to drug activation and furthermore show that our strategy can uncover important regulatory partners for the activation of downstream GPCR signaling. Fig. 5B shows a summary of our data as a network graph with 55 novel partners for β_2AR found through CHIP-MYTH and confirmed by LUMIER in the absence or presence of salmeterol.

3.6. Drug-dependent interactions confirmed by co-immunoprecipitation assay

Among the CHIP-MYTH interacting hits, we tested 3 interactors that were confirmed by either LUMIER or cAMP assays (KCNE1, GNB2L1 and RNF41) by co-immunoprecipitation in the absence and presence of the β_2AR-agonist isoproterenol. We decided to use isoproterenol in order to test whether this short-acting agonist has an influence on β_2AR-interactions, because salmeterol did not exhibit a pronounced effect on these confirmed interactions by LUMIER (see Fig. 4). HA-β_2AR and FLAG-tagged preys were co-transfected into HEK293T cells and 18 h later, treated with either DMEM media or DMEM + 10 μM isoproterenol. Fig. 6 shows that after 1.5 h, a marked increase of interaction between β_2AR and its known partner β-arrestin, is observed. Remarkably, a strong drug effect was observed with GNB2L1, which only interacted with HA-β_2AR upon isoproterenol treatment. RNF41 did not interact with β_2AR in the co-IP assay, whereas KCNE1 showed interaction with HA-β_2AR both in the absence and presence of isoproterenol. Therefore, although the protein–protein interactions confirmed by LUMIER were not significantly different with or without salmeterol under our experimental conditions, the interaction between β_2AR and GNB2L1, was drug-dependent as originally found by CHIP-MYTH.

4. Discussion

Impairment of GPCR function has serious consequences for proper cell function and has been proposed to underlie various pathologies such as pulmonary and cardiovascular diseases, schizophrenia, Parkinson's disease, hypertension and several cancers [1,26,27]. As a consequence of their fundamental importance, combined with the ability to modulate their activity using extracellular means, they are primary targets for current drugs and drug development programs. However, the molecular cascades triggered upon drug activation and the induced protein interactions are poorly described. Here, we introduced a novel functional proteomics approach, called CHIP-MYTH, which is a robust and comprehensive method for identifying novel partners of full-length GPCRs in ligand-occupied/unoccupied states. The assay can be easily performed in a high-throughput manner in yeast, and it is therefore cost-effective, non-resource intensive, and sparing in terms of drug-expenditure.

In our proof of principle study we identified various previously known β_2AR interactors, such as β_2AR itself and GRK6 [28]. We also discovered novel interactors, which were confirmed in human cells by two independent orthogonal assays such as the high-throughput LUMIER assay and co-immunoprecipitation assays. Furthermore, we also presented a modified version of LUMIER where, in addition to detecting PPIs, we introduced a functional readout with the transcriptional activation of a *cis*-reporter gene to evaluate the changes of cAMP levels upon β_2AR activation by salmeterol. Various novel β_2AR interactors may play a role in regulating cAMP levels and one of them, GNB2L1, showed isoproterenol-induced interaction with β_2AR, comparable to β-arrestin, suggesting an activation-dependent interaction between β_2AR and GNB2L1. Interestingly, GNB2L1 is a G-protein recently reported to regulate the internalization of the muscarinic receptor CHRM2, another GPCR receptor [29].

Our novel method allows for detection of ligand-occupied and unoccupied β_2AR interactors, which in turn can give invaluable insight into β_2AR signaling. Therefore, CHIP-MYTH can be widely applied to identify novel PPIs of any transmembrane receptor in response to a variety of compounds. As with any microarray-based assay, a natural extension would be to adapt the read-out step to

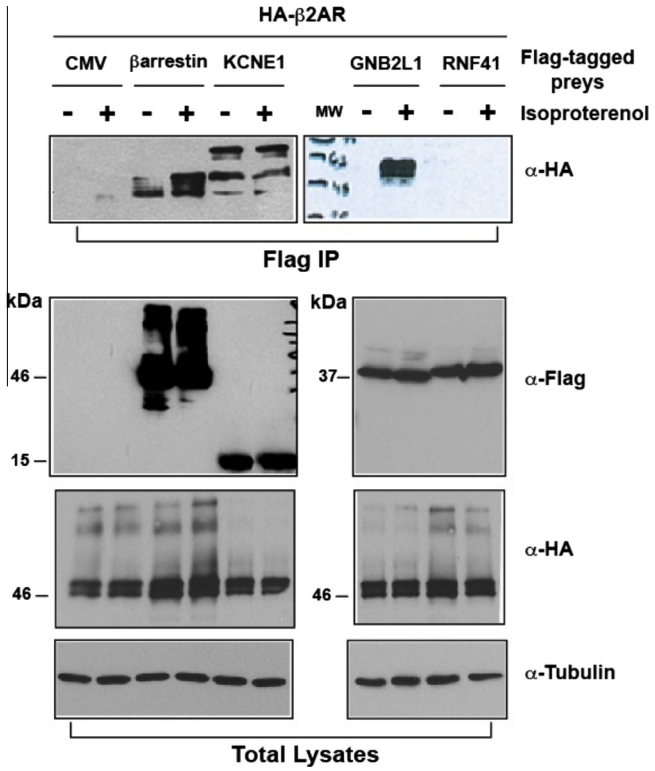

Fig. 6. Drug-dependent interactions confirmed by co-immunoprecipitation. HA-β_2AR and FLAG-tagged preys were transiently co-transfected into HEK293T cells. 18 h after transfection, cells were treated with either DMEM or DMEM + 10 μM isoproterenol. After 1.5 h, cells were lysed and coimmunoprecipitated with anti-FLAG antibodies. Western blots of the immunoprecipitation fraction (probed with anti-HA antibody to detect β_2AR), total bait expression (anti-HA), total prey expression (anti-FLAG), and loading control (anti-tubulin) in the absence (−) and presence (+) of 10 μM isoproterenol are shown. CMV indicates empty vector (no prey) and MW molecular weight markers.

next-generation sequencing (NGS). Nonetheless, there are advantages and disadvantages for either platform. For arrays, costs are low and turn-around time is rapid, but cross-hybridization is a liability, which can complicate discriminating between closely related sequences. NGS has the advantage of being independent of library clone content, and can distinguish between nearly identical sequences. An often overlooked liability of NGS is the sensitivity of this readout for highly abundant species, as in the case for ribosomal RNA in RNA-seq. By analogy, a selected population of clones could have low diversity, leading to significant oversampling by NGS. Ultimately, the particular high-throughput readout will be dictated by the nature of the interaction and the required sensitivity.

In the future, CHIP-MYTH (and potential variants making use of NGS) can serve as a powerful proteomics platform to identify novel interactors of any full-length integral transmembrane protein in the presence of a variety of compounds, which will give invaluable insight into signaling cascades conferred by disease-related membrane proteins.

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