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PRESTO-TANGO: an open-source resource for interrogation of the druggable human GPCR-ome

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Abstract

G protein-coupled receptors (GPCRs) are essential mediators of cellular signaling and important targets of drug action. Of the approximately 350 non-olfactory human GPCRs, more than 100 are still considered “orphans” as their endogenous ligand(s) remain unknown. Here, we describe a unique open-source resource that provides the capacity to interrogate the druggable human GPCR-ome via a G protein-independent β-arrestin recruitment assay. We validate this unique platform at more than 120 non-orphan human GPCR targets, demonstrate its utility for discovering new ligands for orphan human GPCRs, and describe a method (**PRESTO-TANGO;** Parallel Receptor-ome Expression and Screening via Transcriptional Output - TANGO) for the simultaneous and parallel interrogation of the entire human GPCR-ome.

G protein-coupled receptors (GPCRs) are proteins with seven transmembrane helices capable of transducing a wide variety of extracellular stimuli into intracellular signals, mediated by G proteins of four groups, G_s, G_i, $G_{12\ or\ 13}$ or G_q, as well as arrestins and other effectors[1]. The human genome encodes more than 350 different non-olfactory GPCRs, as well as a similar number of olfactory GPCRs[2–4]. In addition to their roles as signal transducers, GPCRs are the targets for more than one-third of currently prescribed medications[5, 6]. Approximately one-third of the non-olfactory GPCRs in the human genome are “orphan” GPCRs, *i.e.*, their endogenous or natural ligands are unknown[2–4], while many more are inadequately interrogated with respect to their ligands. Thus, much of the druggable GPCR-ome – like other drug target families such as the kinome[7], represents ‘dark

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matter' of the human genome. As many of these sparsely annotated GPCRs will likely represent fruitful future therapeutic targets, identifying drug-like chemical leads for the entire family of druggable GPCRs represents a major goal for chemical biology. Unfortunately, interrogating the druggable GPCR-ome *en masse* in a parallel and simultaneous fashion is currently technologically and economically unfeasible.

The difficulty in screening the entire druggable GPCR-ome in parallel is due mainly to the inherent diversity of signal transduction cascades, rendering attempts at parallel profiling challenging. Thus, for instance, functional assays for the identification of agonists at orphan and other sparsely annotated GPCRs have typically used readouts that depend on the native or forced[1] coupling of GPCRs with G proteins, *e.g.*, G_s, G_i, G_q or $G_{12\text{ or }13}$[8–14]. Unfortunately, these approaches are not well suited for the parallel and simultaneous genome-wide interrogation of the druggable GPCR-ome[1]. Alternatively, measurement of G-protein independent β-arrestin recruitment provides a feasible and universal assay platform, since nearly all tested GPCRs can induce arrestin translocation[15, 16] (Supplementary Table 1). A wide variety of approaches has been described to quantify GPCR-β-arrestin interactions, including high content screening (HCS)[17], bioluminescence resonance energy transfer (BRET)[18], enzyme complementation[19], and transcriptional activation following arrestin translocation (TANGO)[20], although none are routinely performed in a genome-wide, parallel manner. As we show here, the TANGO approach has a number of advantages for high throughput assays, including its independence from G protein coupling, generally high signal-to-background ratios, and its amplification of relatively small initial inputs into large readout signals. Independence from G protein coupling facilitates interrogation of orphan GPCRs, whose coupling partners are unknown. Some of the advantages of the TANGO assay might also be shared by other readout systems, including, for example, assays for changes in impedance or dynamic mass redistribution (reviewed in [21]) and indeed, arrestin recruitment may be part of the dynamic mass redistribution response measured in receptor-expressing cells responding to agonists, as suggested, but not directly shown, by the results of Hennen et al[22]. Our goal was to develop the TANGO assay into a platform that could encompass the entire druggable GPCR-ome. Although our assay does not differ significantly in terms of the general concept from that of Barnea et al.[20], several notable changes including the design of the plasmid constructs and assay execution have distinct advantages, as we describe below. We also demonstrate a method (**PRESTO-TANGO;** Parallel Receptor-ome Expression and Screening via Transcriptional Output-TANGO) that facilitates the rapid, efficacious, parallel, and simultaneous profiling of biologically active compounds across essentially the entire human druggable GPCR-ome. Additionally, we document how our approach leads to the facile identification of new synthetic and naturally-occurring agonists for orphan GPCRs. Finally, as our platform is open-source, our methods and reagents are made freely available to the scientific community.

RESULTS

Rationale and design

Our goal was to develop a platform suitable for the *parallel and simultaneous interrogation of every non-olfactory druggable GPCR in the human genome*. Accordingly, we devised a "modular" design strategy to produce a TANGO construct for each GPCR, extensively modifying the design of Barnea *et al.*[20]; the general scheme is shown in Figure 1a. The underlying principle was to make each GPCR construct in such a way that various "modules" could be conveniently included or excluded as desired (Fig. 1a). At the 5′-end, we included a cleavable signal sequence to promote membrane localization[23] and a FLAG epitope tag in order to be able to monitor cell surface expression by immunohistochemistry. At the 3′-end, the sequences for the Tobacco Etch Virus nuclear inclusion a endopeptidase (TEV) cleavage site and the tetracycline transactivator (*tTA*) protein were exactly as published by Barnea *et al.*[20]. GPCR sequences were then codon-optimized for expression in human cell lines, and each receptor sequence was followed by sequence from the C-terminus of the V_2 vasopressin receptor (V_2 tail) to promote arrestin recruitment[20, 24–26]. The receptor sequence and the V_2 tail were flanked by restriction sites, allowing the facile excision from or subcloning of other targets into those sites, and more efficient gene synthesis. The codon-optimized sequences were designed to exclude their flanking restriction sites. Additionally, it should be noted that by simple mutagenesis to insert a "stop" codon at the 3′-end of the receptor sequence, these TANGO constructs can also be used in other functional assays, *i.e.*, they can be "de-TANGO-ized".

The sequence of an entire prototypical TANGO construct is given in Supplementary Note 1 and the sequences of all of the receptor inserts produced can be found on-line at: (URL to be deposited upon manuscript acceptance). When multiple splicing isoforms existed for a given GPCR, the longest or the most prevalent form was used for the TANGO constructs. A few GPCRs with extremely long N-termini, and a few that could not be expressed in *E. coli*, were excluded from the project, resulting in a total of 315synthetic TANGO-ized GPCRs (Supplementary Table 2).

Validation of the PRESTO-TANGO platform

In preliminary experiments, each TANGO-ized construct was transfected and then examined by anti-FLAG immunofluorescence for both total and surface expression (Fig. 1b for examples). Of 315 constructs examined, 302 (96%) were surface-expressed (Supplementary Table 2). Of the remaining GPCRs that were not efficiently surface-expressed, receptor expression could be visualized in permeabilized cells (data not shown).

To test the utility of the platform, we assayed nearly all of the non-orphan receptors for activation by their canonical agonists. In total, we attempted to validate 167 non-orphan GPCRs and were successful with 125 (75%). Of the Family A GPCRs, 81% were validated, and these results are summarized in Table 1. Figures 1c and 1d show concentration-response curves for a prototypical non-orphan GPCR, the neuromedin B receptor (NMBR; also known as the BB1 bombesin receptor) for both β-arrestin recruitment activity (Fig. 1c) and G protein (calcium release) activity (Fig. 1d). Individual concentration-response curves for

every validated target are shown in Supplementary Data Set 1. Agonist-induced activation of the TANGO-ized GPCRs resulted in variable responses ranging from about 1.3-fold above baseline to 184-fold for the MLNR motilin receptor. Importantly, we discovered ligand-induced arrestin recruitment for the first time in 23 different GPCRs, including the NMBR bombesin receptor (also known as BB1; Fig. 1c), the CHRM5 muscarinic acetylcholine receptor (Fig. 2a), the CX3CR1 chemokine receptor (Fig. 2b), the DRD4 dopamine receptor (Fig. 2c; this, despite reports that this target does not interact with arrestin[27]), the GAL3 galanin receptor (Fig. 2d), the NMUR1 (Fig. 2e) and NMUR2 (Fig. 2f) neuromedin receptors, and others shown in Supplementary Data Set 1, including the AVPR1B vasopressin receptor, the CCKAR cholecystokinin receptor, the CMKOR1 orphan chemokine receptor, the GPBA bile acid receptor (but see also[28]), the HTR7 serotonin receptor, the LPAR5 lysophospholipid receptor (also known as GPR92), the MRGPRX4 orphan receptor, the NTSR2 neurotensin receptor, the P2RY13 and P2RY14 purinergic receptors, the PTGER1 and PTGFR (but see also[29]) prostanoid receptors, the RXFP4 relaxin receptor, the S1PR2 lysophospholipid receptor, the SSTR4 somatostatin receptor (but see also[30]), and the TACR2 tachykinin receptor.

Additionally, we tested whether the antagonist activity of test compounds could be quantified by simply pre-incubating cells with potential antagonists before agonist exposure. Thus, for instance we, like others[31, 32], found that neurotensin was inactive as an agonist at NTSR2 neurotensin receptors, although SR48692 and SR142948 are NTSR2 agonists (Supplementary Fig. 1a). Intriguingly, both neurotensin and the HRH1-histamine receptor antagonist levocabastine were antagonists of SR48692- or SR142948-induced arrestin recruitment responses in NTSR2-transfected cells (Supplementary Fig. 1b, c), as reported by others using orthogonal assays[31, 32]. Thus, these results validate the use of the TANGO β-arrestin recruitment assay for measurements of antagonist activity.

As noted previously, the constructs tested included the C-terminal tail of the V_2 vasopressin receptor (the "V_2 tail") in order to enhance arrestin interactions with the various receptors[20, 25]. Notably, the presence of the V_2 tail has been reported to have little to no effect on the TANGO assay for some receptors[24]. In our experience, removal of the V_2 tail had little effect on the ligand-induced responses of some receptors, *e.g.*, the LTBR4 leukotriene receptor (Supplementary Fig. 1d), increased the ligand-induced responses of others, *e.g.*, the CMKLR1 chemerin receptor (Supplementary Fig. 1e), and decreased the ligand-induced responses of others, *e.g.*, the FFAR2 free fatty acid receptor (GPR43; Supplementary Fig. 1f). A systematic and complete study of the effects of including or excluding the V_2 tail remains to be done, though the results shown in Supplementary Figs 1d–f provide a path forward for further optimization of the TANGO assay.

In additional experiments, we also validated the TANGO assay in "antagonist mode" by determining the effect of pre-exposure of GPCR-expressing cells to 1 μM clozapine on their responses to the agonist LSD. For some targets, clozapine had little or no effect on the responses of cells to LSD, *e.g.*, the HTR1A and HTR1D serotonin receptors (Supplementary Fig. 2a, b). For others, *e.g*, the HTR1B serotonin receptor and the ADRA2B adrenergic receptor (Supplementary Fig. 2c, d), the major effect of clozapine on LSD concentration-response curves was to shift them to the right. For still other GPCRs, *e.g.*, the HTR1E,

HTR1F, HTR2A and HTR5 serotonin receptors, and the DRD2 dopamine receptor, the effect of clozapine was to both shift the curves to the right and to decrease the Emax (Supplementary Fig. 2e–i). These results therefore further validate the use of the TANGO β-arrestin recruitment assay in "antagonist mode" and thus facilitate the discovery of novel modes of action of pharmacological agents.

Next, we tested each of the TANGO-ized constructs for constitutive activity, *i.e.*, recruitment of arrestin in the absence of known ligands, as detailed in **Methods,** and as seen in Supplementary Figure 3, Supplementary Table 3 and Figures 3a and 53b Constitutive activity varied over a range of more than about 500-fold among the various GPCRs; in one set of GPCRs tested, the ratio of the maximal to the minimal luminescence was 437, and in another, the ratio was 551 (Supplementary Table 3; Figures 3a, b. Detailed analyses revealed no readily apparent sequence-encoded pattern when comparing GPCRs with high and low constitutive activity, although we note that several members of the serotonin and purinergic receptor families had relatively high constitutive activity compared to the other tested GPCRs.

We also discovered that although our standard protocols specify overnight incubation with ligands (see **Methods**), very brief exposures to ligand (*i.e.*., 15 min) are sufficient to stimulate measurable responses in the TANGO assay, although overnight incubation is apparently required for maximum signal amplification (Supplementary Fig. 4). This feature facilitates testing compounds that, in long exposures, may be toxic to cells, but in short exposures may reveal activity at GPCR targets. Additional studies (not shown) suggest that the minimum incubation time for robust observation of responses to agonists in the TANGO assay depends on the target being tested, but that, for screening purposes, one or two hours is generally sufficient, provided that the signal is amplified overnight. Thus, optimization of agonist exposure time may improve assay performance depending on the individual targets or ligands to be studied.

"Many target-few compound" parallel GPCR-ome screening

Conventional small-molecule based screening often involves testing of hundreds of thousands of compounds at a single target and, as we have demonstrated previously (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=588463), TANGO assays are useful for such conventional 'one-target-at-a-time' GPCR screening. An alternative and potentially innovative approach, which we have named **"PRESTO-TANGO"** (Parallel Receptor-ome Expression and Screening via Transcriptional Output-TANGO) is to screen collections of perturbants (*e.g.*, small molecules, peptides, siRNAs, CRISPR-based editing constructs, and so on) against the druggable human GPCR-ome in a simultaneous fashion. Although simultaneous interrogation of the entire druggable GPCR-ome is clearly important, it has not been feasible for both technical and economic considerations. As we have developed a resource of most of the druggable GPCRs, we wondered if they could be screened in a 384-well format in a simultaneous and parallel fashion (Fig. 4a; see **Methods** and Supplementary Note 2 for a detailed description of the technology). As an initial validation, we screened the hallucinogen lysergic acid diethylamide (LSD) and and the serotonin-selective reuptake inhibitor fluoxetine against 133 non-orphan, non-olfactory GPCR targets (Supplementary

Note 3). LSD displayed activity at 15 of the tested targets, including several unexpected ones such as the α_{2B} adrenergic and the D_4 and D_5 dopamine receptors (Fig. 4b–d). Fluoxetine, which is reported to have few "off-targets",[33] showed modest agonist activity at the SSTR3 somatostatin receptor, which was not confirmed in follow-up assays (data not shown), thus supporting its selectivity for SERT and not for any GPCR targets.

Novel activities in the GPCR-ome

Given our initial success with two highly annotated small molecules and well-known GPCRs, we next determined if we could expand this approach by screening a collection of approved drugs (NIH Clinical Collection of compounds, NCC-1 library; http://www.nihclinicalcollection.com) against 91 orphan and poorly annotated GPCRs. A heatmap of the results obtained is shown in Figure 5a, and the entire dataset is shown in Supplementary Table 4. As can be seen in Figure 5a and in Supplementary Table 4, some of the tested drugs displayed promiscuous inhibitory activity. Thus, for example, resveratrol produced a greater than 2-fold reduction at 70 of 91 (77%) and homoharringtonine at 58 of 91 (64%) targets tested; twelve additional compounds produced a greater than 2-fold reduction in luminescence at 20 or more of the 91 targets tested. This sort of inhibition could be due to cytotoxicity, compound aggregation, or inhibition of the luciferase reporter[34]. In contrast, a few others -- most notably the aminopeptidase inhibitor bestatin (> 2-fold increase at 59 of 91 (65%) targets tested) -- induced increased activity. Although we did not investigate the mechanism for this promiscuous 'agonist' effect, such promiscuous activity has been previously ascribed to enhanced luciferase stability[35, 36]. These results illustrate the value of screening at multiple targets simultaneously and in a parallel fashion, enabling the separation of false positive "frequent hitters" from screening hits that can be productively pursued.

Among the most intriguing activities revealed by our screen of 446 compounds at 91 targets (*i.e.*, 40,586 compound-target tests) was the activity of the K_{ATP}-channel blocker nateglinide at the MRGPRX4 orphan GPCR (Fig. 5b and Supplementary Table 4). Nateglinide induced a 45-fold increase in luminescence over basal levels in the initial screen and, of the 91 targets tested, nateglinide was apparently selective for MRGPRX4 (Fig. 5b and Supplementary Table 4). The concentration-dependent activity of nateglinide at MRGPRX4 was subsequently confirmed by TANGO (Fig. 5c) and PI hydrolysis assays (Fig. 5d). Nateglinide had exceedingly modest activity at high concentrations when cAMP was measured at MRGPRX4, and was inactive at MRGPRX1, MRGPRX2 or MRGPRX3 (Supplementary Fig. 5a). The magnitude of the cAMP response was much lower than the response to isoproterenol in MRGPRX4-expressing cells, which is due to the responses of the constitutively expressed β_2 adrenergic receptor in HEK cells (Supplementary Fig. 5b). These data, combined with the inability of nateglinide to inhibit a cAMP response to isoproterenol in these cells (Supplementary Fig. 5c), and the activity of nateglinide in the PI hydrolysis assay (Fig. 5d), indicate for the first time that MRGPRX4 is primarily a G_q-coupled receptor. Next, we prepared stably expressing lines for MRGPRX1, MRGPRX2 and MRGPRX4 receptors; calcium mobilization assays done with these cell lines showed concentration-dependent responses of MRGPRX1-expressing cells to the cognate ligand BAM8-22 (Supplementary Fig. 6a, b), of MRGPRX2-expressing cells to SB 205,607

(Supplementary Fig. 6c, d), and of MRGPRX4-expressing cells to nateglinide (Supplementary Fig. 6e, f), further indicating that this group of receptors is primarily G_q-coupled in HEK cells.

In preliminary screening studies, we also identified the HIV protease inhibitor saquinavir as a potential agonist at an orphan GPCR, the so-called BB3 bombesin receptor. To further investigate saquinavir's BB3 activity, and validate the specificity of our platform, we confirmed its concentration-dependence at BB3 (Fig. 5e), and showed that the related bombesin receptors BB1 (Supplementary Fig. 7a) and BB2 (Supplementary Fig. 7b) were insensitive to saquinavir in the TANGO assay. In orthogonal calcium mobilization assays, saquinavir did not stimulate a response in BB1- or BB2-expressing cells (Supplementary Fig. 7c, 7d), but did stimulate a response in BB3-expressing cells (Supplementary Fig. 7e), and this activity was also confirmed by an assay of PI hydrolysis (Fig. 5f). Thus, these data confirm that saquinavir has significant off-target activity at the BB3 orphan GPCR, and serve to validate that the apparent target specificity of the TANGO assay can be recapitulated in orthogonal assays.

Our initial screen of the NCC-1 library also included the related target MRGPRX2 (Supplementary Table 4), at which the most active compounds included the delta opioid receptor agonist SB 205,607 (also known as TAN-67), confirming a prior report[37]. Interestingly, two other opioids, levorphan and dextromethorphan, as well as the antihistaminergic and antiserotonergic compound cyproheptadine, the antihistaminergic compound ketotifen, and the antiserotonergic compound pizotyline, also showed activity in the TANGO assay at MRGPRX2 (Supplementary Table 4)—all of which were confirmed in concentration-response studies (Supplementary Fig. 8a). The concentration-dependent activities of SB 205,607 and dextromethorphan at MRGPRX2 were also confirmed by PI hydrolysis (Supplementary Fig. 8b). An intriguing discovery was the finding that the kappa opioid receptor selective antagonist JDTic and the selective salvinorin A analogue RB64 displayed significant activity at MRGPRX2 in the TANGO assay (Supplementary Fig. 8c).

DISCUSSION

Here we provide the first open-source resource, which we have named **PRESTO-TANGO**, for the parallel and simultaneous interrogation of the druggable GPCR-ome, as summarized in Figure 6 The development of this unique platform was facilitated by modifying and expanding an arrestin recruitment (TANGO) assay[20] for GPCR activation that is sensitive, easily executed, and amenable to both HTS and simultaneous parallel screening at many GPCRs. Since knowledge of the G protein partners of each GPCR is not required, this assay is particularly suitable for "first-pass" screening of compound libraries and for identifying ligands of orphan receptors, as has been previously suggested[20, 37]. Importantly, we have demonstrated that: (i) activation of the majority of GPCRs can be measured using the TANGO arrestin recruitment assay; (ii) the TANGO assay can also be used for measurement of antagonist activity when canonical or newly discovered agonists are available; and (iii) the simultaneous parallel screening of a few compounds using the PRESTO-TANGO approach, or the parallel screening of compound libraries using the TANGO method, reveals new activities for known drugs and compounds, and identifies new ligands for both sparsely

annotated and orphan GPCRs. Although the TANGO β-arrestin recruitment assay has previously been used by many others, including us for the DRD2 dopamine receptor[38], the DOR[39] and KOR[40, 41] opioid receptors, and several serotonin receptors[42], the present study is the first to adapt it to almost the entire GPCR-ome, and to make the entire resource publicly available to the scientific community. This resource is thus likely to find widespread use by structural biologists who focus on GPCRs, chemical biologists intent on deconstructing the actions of drug-like compounds, molecular biologists searching for GPCR perturbants, molecular pharmacologists and systems biologists.

Although this unique resource and the overall approach we used are both quite powerful, the resource is currently limited by our inability to validate assays for some GPCRs. Why assays for these particular GPCRs (see Fig. 6) could not be validated remains largely unknown, although it is likely that upon further optimization, useful assays for many of these can be perfected. Alternatively, it is conceivable that some receptors do not interact with arrestins in an agonist-dependent fashion, as has been claimed for the α_{1A} adrenergic receptor (ADRA1A)[43], the AT_2 angiotensin II receptor (AGTR2)[44], and the D_4 dopamine receptor (DRD4)(see [27] for example). Indeed, for the β3 adrenergic (ADRB3) receptor, which lacks the consensus sequences for GRK phosphorylation and thus for arrestin binding, downstream signaling appears to proceed by direct interaction with kinases in the ERK pathway.[45] For other GPCRs, for which receptor-arrestin interactions have been reported in the literature (Supplementary Table 1) but for which we were not able to initially validate assays, it may be that the TANGO assay requires further optimization, perhaps by removal of the V_2 tail, or that the other arrestin recruitment assays used in the literature are more tractable than the TANGO assay for these particular GPCRs. Interestingly, there were also a few targets for which the TANGO assay proved suitable, although there had been reports in the literature that these targets did not interact with arrestin – these included the GPBA bile acid receptor[28], the prostaglandin F2α (PTGFR) receptor[29], the SSTR4 somatostatin receptor[30], and the aforementioned DRD4-dopamine receptor. It is also possible that some of the targets that could not be validated in our study interact with other members of the arrestin family, but not the β-arrestin2 as used here. In order to adapt our realization of the TANGO assay to interactions with other members of the arrestin family, all that would be required would be the creation of a cell line expressing alternative β-arrestins. Importantly, our validation studies revealed several agonist-induced receptor-arrestin interactions for the first time; these included at least 23 different receptor targets (see Table 1 and Supplementary Data Set 1). Finally, and importantly, for some GPCR targets, these assays are the first reliable functional assays for receptor activation to be reported, *e.g.*, the HTR5 serotonin receptor. All of the validated TANGO assays, and all of those that could not be validated, are summarized in Supplementary Table 5.

In addition to providing a resource for testing the function of nearly the entire druggable human GPCR-ome, we have also devised a method by which one or a few compounds can be tested at all 315 GPCR targets simultaneously and in parallel. Our preliminary results, in which we tested two compounds against 133 targets, showed novel activities for a well-known compound, LSD, and verified the relative selectivity of the SSRI fluoxetine. Such

simultaneous and parallel screening also facilitates the identification of promiscuous compounds (*i.e.*, "frequent hitters"), thereby minimizing futile follow-up studies.

We also screened a small library of US FDA-approved drugs at 91 different GPCR targets using the TANGO platform. Our intention was: (i) to discover ligands at poorly annotated or "orphan" receptors: (ii) to discover new targets for known drugs; (iii) to test whether the activity of compounds in the TANGO assay could be confirmed in orthogonal assays; and (iv) to demonstrate the value of massively parallel screening to separate promiscuous compounds from novel ligand-receptor pairs. Among the most striking results from this screen (Fig. 5b–d, Supplementary Table 4) was the remarkable activity shown by the diabetes drug nateglinide, a K_{ATP} channel blocker, at MRGPRX4, a member of the MAS-related GPCR family. There are four MRGPRX receptors in the human genome and they have been reported to be expressed only in primates, with expression largely limited to the dorsal horn of the spinal cord (reviewed by [46]). For the most part, the MRGPRX family is thought to be peptidergic, although a few small molecule ligands are also reported[46]. Based on analogy with the larger family of MAS-related GPCRs that has been extensively studied in rodents, MRGPRX receptors are thought to have a role in pain and itch[46]. Thus, it is interesting that the itch-inducing compound chloroquine activates MAS-related GPCRs in mice[47], and that there are reports of "rash, itching and urticaria" as occasional side-effects of nateglinide treatment[48]. Our initial screen of the NCC-1 library also revealed a large number of compounds belonging to a variety of pharmacological classes that were active at MRGPRX2, including the delta opioid receptor agonist SB 205,607 (also known as TAN-67), as previously reported[37]. Some other opioid receptor ligands were also active at MRGPRX2, which may reflect the role of similar receptors in pain[46]. Many other potential ligand-orphan GPCR pairings were identified in the initial screen and will be important to pursue in subsequent studies.

Importantly, the results of our validation studies demonstrate that our approach facilitates the ***simultaneous profiling of hundreds of GPCRs*** in a cost-effective and robust manner. Additionally, as many novel GPCR-ligand interactions are revealed, we have begun to illuminate a previously unknown and subterranean pharmacology for known drugs and new ligands at orphan and non-orphan GPCRs. As this is an open-source resource, (ADDGENE link to be inserted here upon publication) this platform will be of immense value to the scientific community.

ONLINE METHODS

Transfections

All transfections were done using an optimized calcium phosphate method.[49]

"Standard" arrestin recruitment assay

HTLA cells, (an HEK293 cell line stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV fusion gene) were a gift from the lab of Richard Axel, and were maintained in DMEM supplemented with 10% FBS, 2 μg/ml puromycin and 100 μg/ml hygromycin B in a humidified atmosphere at 37°C in 5% CO_2. For transfection, cells were plated at 9 to 10 × 10^6 cells per 150 mm cell culture dish (day 1). The following day (day 2),

cells were transfected using the calcium phosphate method. On day 3, transfected cells were transferred at 15,000 to 20,000 cells per well in 40 µl of medium into poly-L-lysine coated and rinsed 384-well white clear-bottom cell culture plates (Greiner Bio-one). On day 4, 3.5x drug stimulation solutions were prepared in filter-sterilized assay buffer, which consisted of 20 mM HEPES and 1x HBSS at pH 7.4, and 20 µl added to each well. On day 5, medium and drug solutions were removed from the wells (by aspiration or shaking), and 20 µl per well of Bright-Glo solution (Promega) diluted 20-fold in assay buffer were added to each well. After incubation for 15 to 20 minutes at room temperature, luminescence was counted in a Trilux luminescence counter. Results in the form of RLU (relative luminescence units) were exported into Excel spreadsheets, and Graphpad Prism was used for analysis of data. To measure constitutive activity, no ligand was added on day 4.

PRESTO-TANGO GPCR-ome screening β-arrestin recruitment assay

see Supplementary Text File 2.

Immunofluorescence

On day 1, cells (15,000/well in 384-well clear-bottom black plates) were prefixed with 4% paraformaldehyde (PFA) for 30 min at RT, incubated with anti-FLAG antibody (1:500, polyclonal rabbit anti-Flag, Sigma #F1804), and incubated for 1 hour at room temperature and then overnight at 4°C. On day 2, cells were incubated with Alexa Fluor 594-conjugated goat anti-rabbit antibody (1:200, Invitrogen) and nuclear dye (Hoechst 33342, 1:2000, Invitrogen) for 1 hour at RT in the dark. After thorough washing with PBS (1X PBS, 0.5 mM $CaCl_2$, pH 7.4), cells were post-fixed with 4% PFA for 30 min on ice and stored at 4 °C in the dark. Images were obtained using the BD Pathway Bioimaging System (BD).

PI hydrolysis

HEK-293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were transfected with 10 µg of receptor DNA per 15 cm cell culture dish and incubated overnight at 37°C in a humidified 5% CO_2; the next day, cells were seeded into poly-L-lysine coated 96-well plates in 200 µl per well of DMEM supplemented with 1% dialyzed FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin. After attaching to the plate, cells were incubated for 16 hours as above in inositol-free DMEM (United States Biological, Swampscott, MA) containing 1% dialyzed FBS, and 1 µCi/well of ^{3}H-inositol. Next, cells were washed with 100 µl drug buffer (1X HBSS, 24 mM $NaHCO_3$, 11 mM glucose, 15 mM LiCI, pH 7.4) and treated with 100 µl of drug buffer containing 10 µM drug in quadruplicate for 1 hour at 37°C in a 5% CO_2 incubator. Alternatively, for concentration-response curves, cells were treated with a range of concentrations in quadruplicate in 100 µl of drug buffer and incubated for 1 hour at 37°C in a 5% CO_2 incubator. Following treatment, drug solution was removed and 40 µl of 50 mM formic acid was added to lyse cells for 30 minutes at 4°C. After cell lysis, 40 µl of acid extracts were transferred to polyethylene terephthalate 96-well sample plate (PE#1450-401) and mixed with 75 µl of Perkin Elmer RNA Binding YSi SPA Beads (#RPNQ0013) at a concentration of 0.2 mg beads/well and incubated for 30 minutes at 4°C. Bead/lysate mixtures were then counted using a Perkin Elmer 2450 MicroPlate Counter.

Ca^{2+} mobilization assay

Cells were plated (15,000 cells/well) into poly-L-lysine coated 384-well clear-bottom black-walled microplates (Greiner Bio-one) with 40 μl of media (DMEM media supplemented with 500 ug/ml geneticin sulfate (G-418), 1% dialyzed fetal bovine serum, and 50 U of penicillin/50 μg of streptomycin) and incubated overnight (37 °C, 5% CO_2). The following day, media was replaced with 20 μl of calcium dye (FLIPR Calcium 4 Assay Kit; Molecular Devices) diluted in assay buffer (1x HBSS, 2.5 mM probenecid, 20 mM HEPES, pH 7.4–7.8) and incubated for 45 min at 37 °C and 15 min at room temperature. Compounds were initially dissolved in DMSO at 10 mM. The 16-point curves were prepared as 3x serial dilutions for each compound with final concentrations ranging from 10 μM to 0.003 nM. Basal fluorescence was measured for 10 s, then 10 μl of test or control compounds were added, followed by continued fluorescence measurement for an additional 120 s. Raw data was plotted as a function of molar concentration of test compound using Prism 5.0 (GraphPad Software).

Generation of stable cell lines

Inducible cell lines expressing MRGPRX1, MRGPRX2 or MRGPRX4 were generated using the Flp-In T-Rex Core Kit (Invitrogen) according to the manufacturer's instructions. In brief, genes were subcloned into the pcDNA5/FRT/TO vector and cotransfected with the POG44 expression plasmid into the Flp-In T-Rex HEK-T cell line using FuGENE HD transfection reagent (Promega). Receptor-expressing cells were selected and maintained in DMEM containing 10% FBS, 15 μg/ml blasticidin, 100 μg/ml hygromycin B, and 100 IU Penicillin and 100 μg/ml Streptomycin. Receptor expression following 24 hours of 1ug/ml Tetracycline treatment was confirmed via immunofluorescence with an anti-FLAG antibody (Sigma #F1804) as above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Allen JA, Roth BL. Strategies to discover unexpected targets for drugs active at G protein-coupled receptors. Annu Rev Pharmacol Toxicol. 2011; 51:117–144. [PubMed: 20868273]
2. Bjarnadottir TK, et al. Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse. Genomics. 2006; 88:263–273. [PubMed: 16753280]
3. Fredriksson R, Schioth HB. The repertoire of G-protein-coupled receptors in fully sequenced genomes. Mol Pharmacol. 2005; 67:1414–1425. [PubMed: 15687224]
4. Vassilatis DK, et al. The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A. 2003; 100:4903–4908. [PubMed: 12679517]

5. Hopkins AL, Groom CR. The druggable genome. Nat Rev Drug Discov. 2002; 1:727–730. [PubMed: 12209152]
6. Rask-Andersen M, Masuram S, Schioth HB. The druggable genome: Evaluation of drug targets in clinical trials suggests major shifts in molecular class and indication. Annu Rev Pharmacol Toxicol. 2014; 54:9–26. [PubMed: 24016212]
7. Edwards AM, et al. Too many roads not taken. Nature. 2011; 470:163–165. [PubMed: 21307913]
8. Coward P, Chan SD, Wada HG, Humphries GM, Conklin BR. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. Anal Biochem. 1999; 270:242–248. [PubMed: 10334841]
9. Eglen RM, Bosse R, Reisine T. Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening. Assay Drug Dev Technol. 2007; 5:425–451. [PubMed: 17638542]
10. Emkey R, Rankl NB. Screening G protein-coupled receptors: measurement of intracellular calcium using the fluorometric imaging plate reader. Methods Mol Biol. 2009; 565:145–158. [PubMed: 19551361]
11. Hill SJ, Williams C, May LT. Insights into GPCR pharmacology from the measurement of changes in intracellular cyclic AMP; advantages and pitfalls of differing methodologies. Br J Pharmacol. 2010; 161:1266–1275. [PubMed: 21049583]
12. Liu B, Wu D. Analysis of the coupling of G12/13 to G protein-coupled receptors using a luciferase reporter assay. Methods Mol Biol. 2004; 237:145–149. [PubMed: 14501047]
13. Rodrigues DJ, McLoughlin D. Using reporter gene technologies to detect changes in cAMP as a result of GPCR activation. Methods Mol Biol. 2009; 552:319–328. [PubMed: 19513660]
14. Siehler S, Guerini D. Novel GPCR screening approach: indirect identification of S1P receptor agonists in antagonist screening using a calcium assay. J Recept Signal Transduct Res. 2006; 26:549–575. [PubMed: 17118798]
15. Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. Science. 2005; 308:512–517. [PubMed: 15845844]
16. Roth BL, Marshall FH. NOBEL 2012 Chemistry: Studies of a ubiquitous receptor family. Nature. 2012; 492:57. [PubMed: 23222609]
17. Barak LS, Ferguson SS, Zhang J, Caron MG. A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. J Biol Chem. 1997; 272:27497–27500. [PubMed: 9346876]
18. Angers S, et al. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci U S A. 2000; 97:3684–3689. [PubMed: 10725388]
19. Yan YX, et al. Cell-based high-throughput screening assay system for monitoring G protein-coupled receptor activation using beta-galactosidase enzyme complementation technology. J Biomol Screen. 2002; 7:451–459. [PubMed: 14599361]
20. Barnea G, et al. The genetic design of signaling cascades to record receptor activation. Proc Natl Acad Sci U S A. 2008; 105:64–69. [PubMed: 18165312]
21. Fang Y, Li G, Ferrie AM. Non-invasive optical biosensor for assaying endogenous G protein-coupled receptors in adherent cells. J Pharmacol Toxicol Methods. 2007; 55:314–322. [PubMed: 17207642]
22. Hennen S, et al. Decoding signaling and function of the orphan G protein-coupled receptor GPR17 with a small-molecule agonist. Sci Signal. 6:ra93. [PubMed: 24150254]
23. Guan XM, Kobilka TS, Kobilka BK. Enhancement of membrane insertion and function in a type IIIb membrane protein following introduction of a cleavable signal peptide. J Biol Chem. 1992; 267:21995–21998. [PubMed: 1331042]
24. Hanson BJ, et al. A homogeneous fluorescent live-cell assay for measuring 7-transmembrane receptor activity and agonist functional selectivity through beta-arrestin recruitment. J Biomol Screen. 2009; 14:798–810. [PubMed: 19531663]
25. Kim KM, Caron MG. Complementary roles of the DRY motif and C-terminus tail of GPCRS for G protein coupling and beta-arrestin interaction. Biochem Biophys Res Commun. 2008; 366:42–47. [PubMed: 18036556]

26. Vrecl M, et al. Beta-arrestin-based Bret2 screening assay for the “non”-beta-arrestin binding CB1 receptor. J Biomol Screen. 2009; 14:371–380. [PubMed: 19403920]
27. Cho DI, Beom S, Van Tol HH, Caron MG, Kim KM. Characterization of the desensitization properties of five dopamine receptor subtypes and alternatively spliced variants of dopamine D2 and D4 receptors. Biochem Biophys Res Commun. 2006; 350:634–640. [PubMed: 17022946]
28. Jensen DD, et al. The bile acid receptor TGR5 does not interact with beta-arrestins or traffic to endosomes but transmits sustained signals from plasma membrane rafts. J Biol Chem. 2013; 288:22942–22960. [PubMed: 23818521]
29. Goupil E, et al. Biasing the prostaglandin F2alpha receptor responses toward EGFR-dependent transactivation of MAPK. Mol Endocrinol. 2012; 26:1189–1202. [PubMed: 22638073]
30. Tulipano G, et al. Differential beta-arrestin trafficking and endosomal sorting of somatostatin receptor subtypes. J Biol Chem. 2004; 279:21374–21382. [PubMed: 15001578]
31. Richard F, Barroso S, Martinez J, Labbe-Jullie C, Kitabgi P. Agonism, inverse agonism, and neutral antagonism at the constitutively active human neurotensin receptor 2. Mol Pharmacol. 2001; 60:1392–1398. [PubMed: 11723247]
32. Vita N, et al. Neurotensin is an antagonist of the human neurotensin NT2 receptor expressed in Chinese hamster ovary cells. Eur J Pharmacol. 1998; 360:265–272. [PubMed: 9851594]
33. Keiser MJ, et al. Predicting new molecular targets for known drugs. Nature. 2009; 462:175–181. [PubMed: 19881490]
34. Auld DS, et al. Characterization of chemical libraries for luciferase inhibitory activity. J Med Chem. 2008; 51:2372–2386. [PubMed: 18363348]
35. Auld DS, Thorne N, Maguire WF, Inglese J. Mechanism of PTC124 activity in cell-based luciferase assays of nonsense codon suppression. Proc Natl Acad Sci U S A. 2009; 106:3585–3590. [PubMed: 19208811]
36. Auld DS, Thorne N, Nguyen DT, Inglese J. A specific mechanism for nonspecific activation in reporter-gene assays. ACS Chem Biol. 2008; 3:463–470. [PubMed: 18590332]
37. Southern C, et al. Screening beta-Arrestin Recruitment for the Identification of Natural Ligands for Orphan G-Protein-Coupled Receptors. J Biomol Screen. 2013; 18:599–609. [PubMed: 23396314]
38. Allen JA, et al. Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy. Proc Natl Acad Sci U S A. 2011; 108:18488–18493. [PubMed: 22025698]
39. Fenalti G, et al. Molecular control of delta-opioid receptor signalling. Nature. 2014; 506:191–196. [PubMed: 24413399]
40. White KL, et al. Identification of novel functionally selective kappa-opioid receptor scaffolds. Mol Pharmacol. 2014; 85:83–90. [PubMed: 24113749]
41. Wu H, et al. Structure of the human kappa-opioid receptor in complex with JDTic. Nature. 2012; 485:327–332. [PubMed: 22437504]
42. Wacker D, et al. Structural features for functional selectivity at serotonin receptors. Science. 2013; 340:615–619. [PubMed: 23519215]
43. Stanasila L, Abuin L, Dey J, Cotecchia S. Different internalization properties of the alpha1a- and alpha1b-adrenergic receptor subtypes: the potential role of receptor interaction with beta-arrestins and AP50. Mol Pharmacol. 2008; 74:562–573. [PubMed: 18523139]
44. Turu G, et al. Differential beta-arrestin binding of AT1 and AT2 angiotensin receptors. FEBS Lett. 2006; 580:41–45. [PubMed: 16359671]
45. Cao W, et al. Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. J Biol Chem. 2000; 275:38131–38134. [PubMed: 11013230]
46. Solinski HJ, Gudermann T, Breit A. Pharmacology and signaling of MAS-related G protein-coupled receptors. Pharmacol Rev. 2014; 66:570–597. [PubMed: 24867890]
47. Liu Q, et al. Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. Cell. 2009; 139:1353–1365. [PubMed: 20004959]
48. Twaites B, Wilton LV, Layton D, Shakir SA. Safety of nateglinide as used in general practice in England: results of a prescription-event monitoring study. Acta Diabetol. 2007; 44:233–239. [PubMed: 17874223]

49. Jordan M, Schallhorn A, Wurm FM. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res. 1996; 24:596–601. [PubMed: 8604299]

50. Katrich V, Cherezov V, Stevens RC. Diversity and modularity of G protein-coupled receptor structures. Trends Pharmacol Sci. 2012; 33:17–27. [PubMed: 22032986]

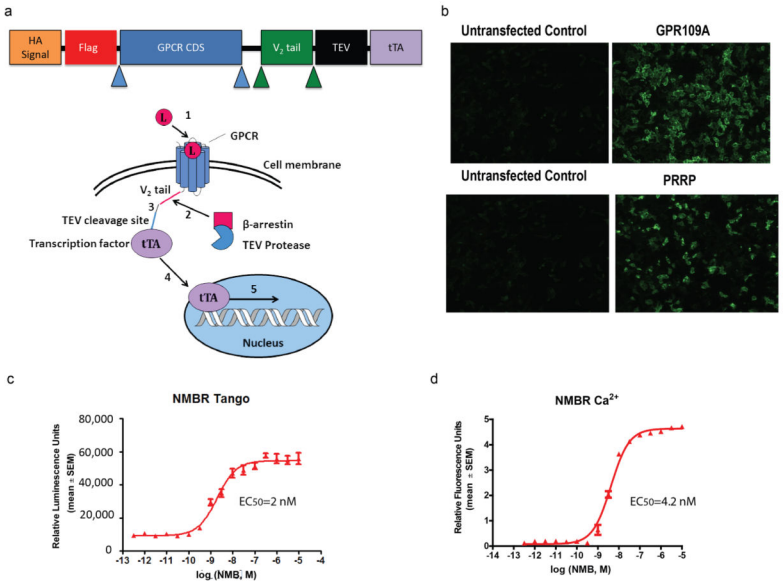

Figure 1. Design, principle, and validation of selected TANGO assays

(a) Modular design of TANGO constructs (top); blue arrowheads indicate Cla I sites, and green arrowheads indicate Age I sites. General scheme for the β-arrestin (TANGO) recruitment assay (bottom). Upon activation of the GPCR by an agonist (1), β-arrestin is recruited to the C-terminus of the receptor (2). This is followed by cleavage of the GPCR fusion protein at the TEV protease site (3). Cleavage results in the release of the tTA transcription factor (4), which, after transport to the nucleus, activates transcription of the luciferase reporter gene (5). **(b)** Surface expression of two selected TANGO constructs as shown by immunofluorescence using an anti-FLAG antibody. Concentration-response curves of a prototypical non-orphan GPCR, the neuromedin B receptor stimulated by neuromedin B (NMB) in the TANGO assay **(c)** and in a calcium-release assay **(d);** data are shown as the mean ± SEM of typical experiments done in quadruplicate. Curves were fitted using Graphpad Prism 5.0.

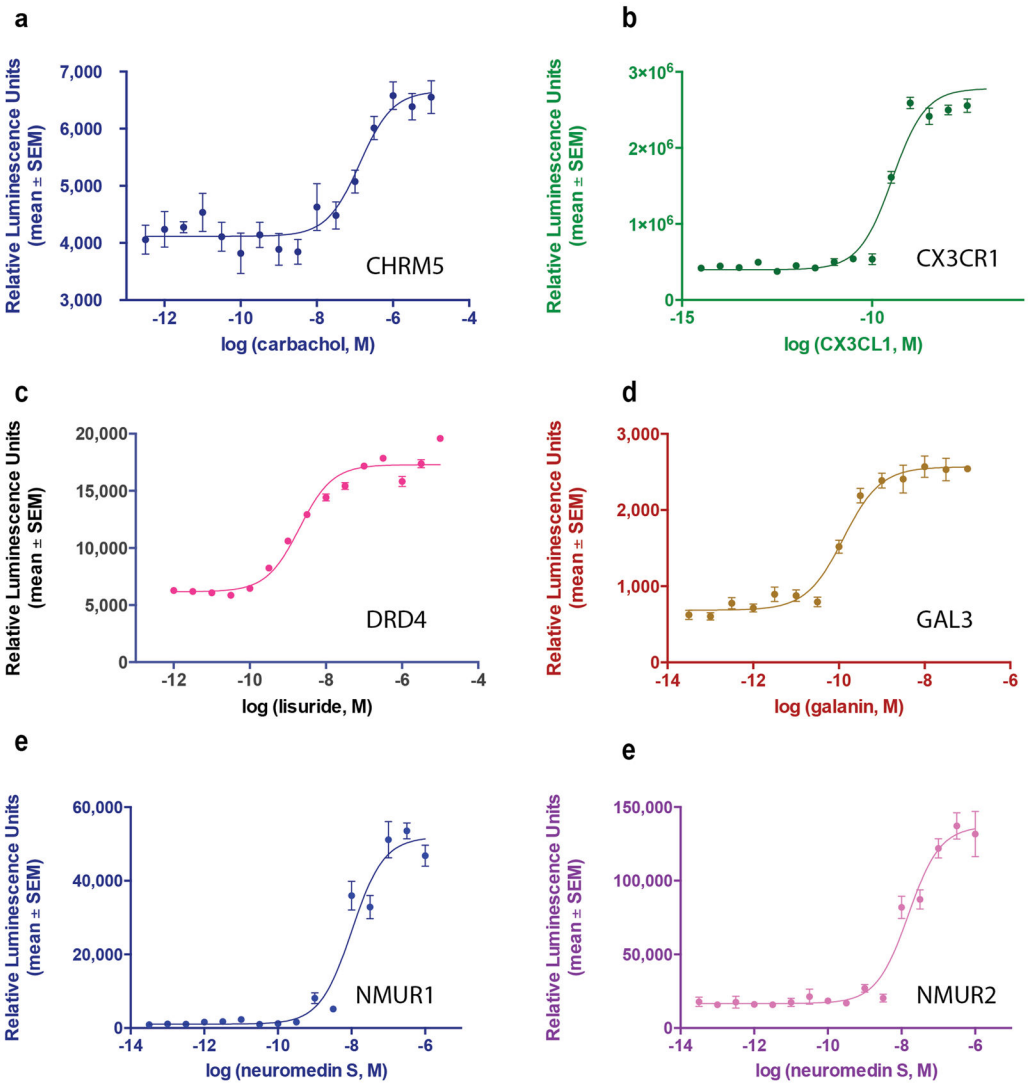

Figure 2. First-time demonstration of arrestin mobilization using the TANGO assay

Shown are concentration-response curves for the response of **(a)** the CMRM5 muscarinic acetylcholine receptor to carbachol (EC50 = 133.8 nM, n = 4), **(b)** the CX3CR1 chemokine receptor to its ligand CX3CL1 (EC50 = 0.34 nM, n = 4), **(c)** the DRD4 dopamine receptor to lisuride (EC50 = 2.0 nM, n = 3), (d) the GAL3 galanin receptor to galanin (EC50 = 0.13 nM, n = 4), **(e)** the NMUR1 neuromedin receptor to neuromedin S (EC50 = 10.2 nM, n = 4), and **(f)** the NMUR2 neuromedin receptor to neuromedin S (EC50 = 15.4 nM, n = 4).

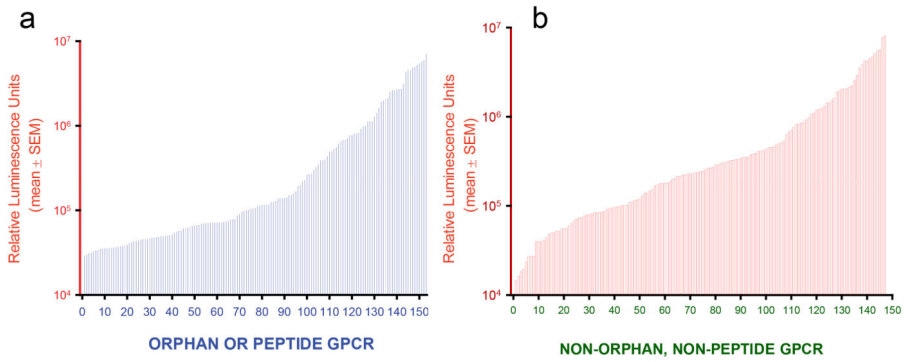

Figure 3. Constitutive (or basal) activity for TANGO-ized constructs

Data are expressed in RLU (relative luminescence units) for the orphan and peptide GPCRs **(a)** and the non-orphan **(b)** GPCRs (n = 4 for each target.

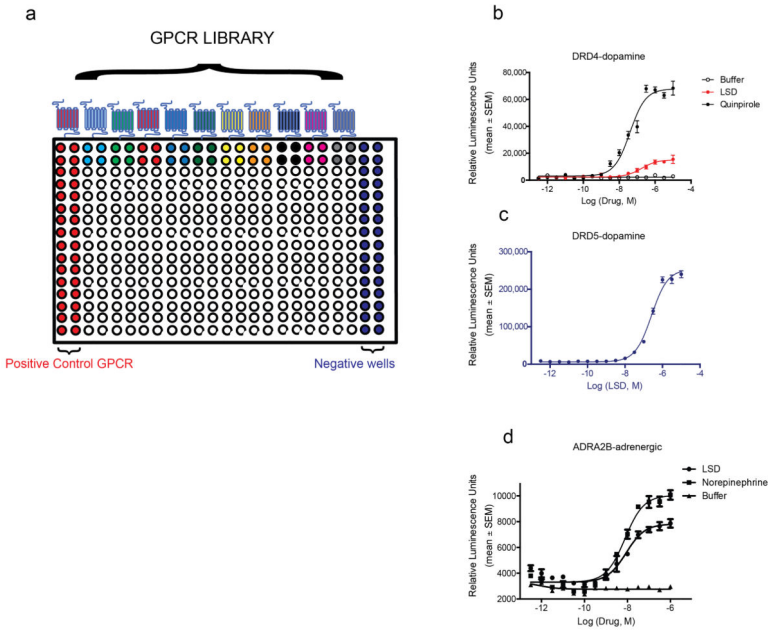

Figure 4. Compound profiling using the PRESTO-TANGO method at the human GPCR-ome

(a) General format in which compounds are screened against a large number of GPCRs in quadruplicate in 384-well plates; for clarity only the first row of individual GPCRs is shown. **(b–d)** Concentration-response curves of LSD at various targets; data are shown as mean ± SEM, and curves were fitted with Graphpad Prism. **(b)** DRD4-dopamine, **(c)** DRD5-dopamine, **(d)** ADRA2B-adrenergic.

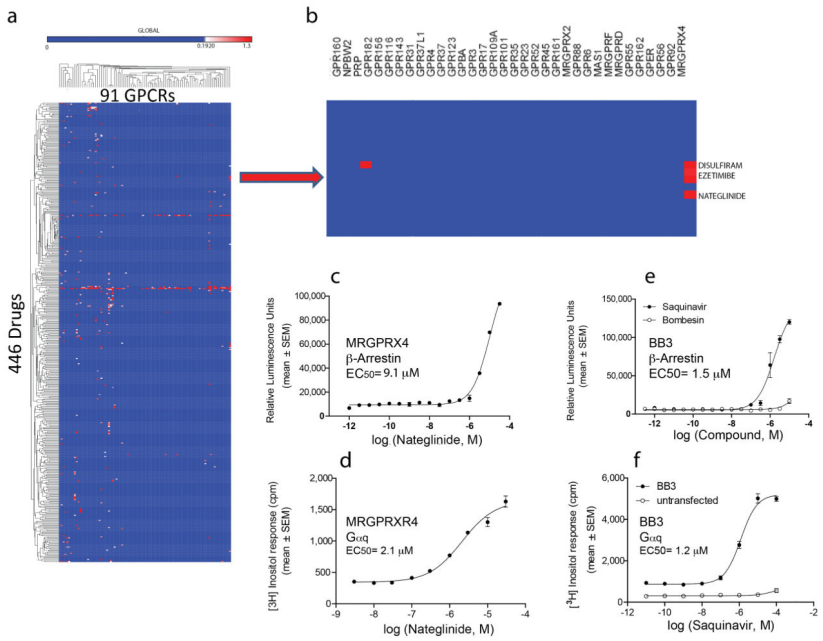

Figure 5. Novel ligand-target interactions detected by parallel GPCR-ome screening

Results of screening of the 446-compound NCC-1 library at 91 GPCR targets in the TANGO assay, and follow-up studies are shown. **(a)** Heatmap of the entire matrix (red = stimulation of luminescence over background). **(b)** Closeup view of a section of the heatmap, showing the activity of nateglinide at MRGPRX4. **(c)** Concentration-response curve of nateglinide at MRGPRX4 in the TANGO assay (n = 4). **(d)** Concentration-response curve of nateglinide at MRGPRX4 by PI hydrolysis (n = 3). **(e)** Concentration-response curve of saquinavir at the BB3 bombesin receptor in the TANGO assay (n = 4). **(f)** Concentration-response curve of saquinavir at the BB3 bombesin receptor by PI hydrolysis (n = 3).

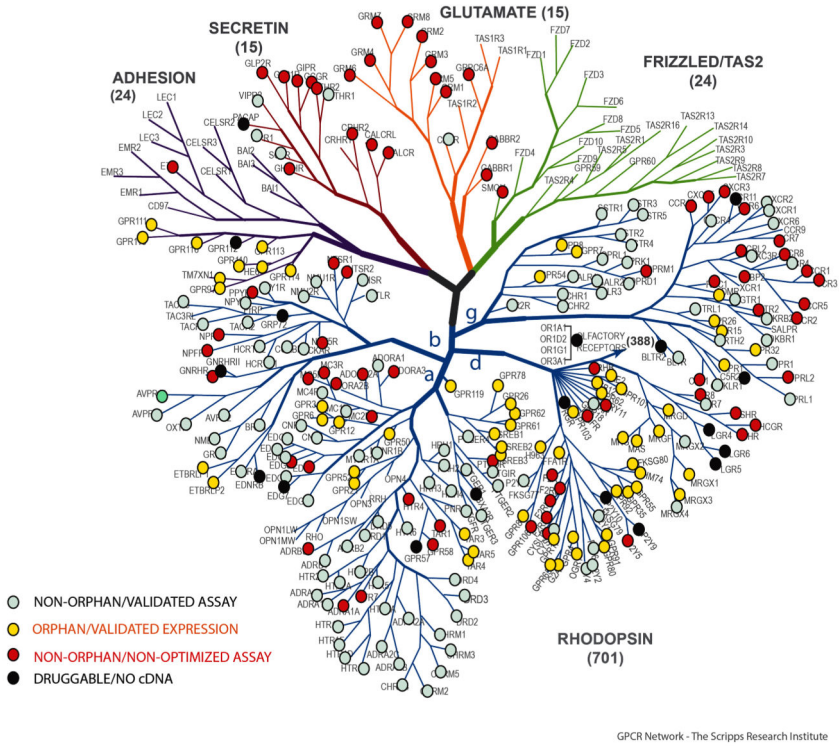

Fig. 6. TANGO-izing the druggable GPCR-ome

Tree-based phylogram of the non-olfactory GPCR-ome showing the status of the TANGO assay for each GPCR. Cyan circles represent those non-orphan GPCRs for which assays were validated; yellow circles represent those assays for which expression of orphan GPCRs were validated; blue circles represent those non-orphan GPCRs for which optimized assays are not yet available, and black circles represent those GPCRs for which no TANGO-ized construct is available.

Table 1

Assay validation statistics.

GROUP	VALIDATED IN THIS STUDY	COULD NOT BE VALIDATED	VALIDATION ATTEMPTED	NOT TRIED OR ORPHAN	TOTAL
A	127 (82%)	28 (18%)	155	110	265
B	8 (57%)	6 (43%)	14	2	16
C	0	8 (100%)	8	7	15
ADHESION	0	0	0	17	17
OTHER	0	0	0	2	2
TOTAL	135 (76%)	42 (24%)	177	139	315

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