Review

Current applications of mini G proteins to study the structure and function of G protein-coupled receptors

Byron Carpenter*

Warwick Integrative Synthetic Biology (WISB) centre, School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

* **Correspondence:** Email: b.carpenter@warwick.ac.uk; Tel: +442476574242.

Abstract: G protein-coupled receptors (GPCRs) regulate intracellular signalling pathways that contribute to virtually all aspects of cell function. Characterising GPCRs in each of their conformational states is key to understanding their mechanism of action, but structure determination of receptors in their active state, bound to a heterotrimeric G protein or β-arrestin, has proved challenging. A number of G protein surrogates have been developed to simplify this process, including G protein-derived peptides, nanobodies and, most recently, mini G proteins. The aim of these surrogates is to bind the receptor and stabilise its active conformation, whilst eliminating the problems inherent to native signalling proteins, namely their large size, instability and conformational dynamics. Mini G proteins are composed of a single domain from the G protein α-subunit that has been engineered to form a stable complex with GPCRs. They induce comparable pharmacological and structural changes in the receptor to those elicited by heterotrimeric G proteins, and retain their native receptor-coupling specificity. At least one member of each G protein family has been converted into a mini G protein, which means that they can be used to characterise a wide variety of GPCRs. Since their initial publication two years ago, mini G proteins have facilitated the structure determination of three different receptors in their active state and enabled the development of a methodology to thermostabilise GPCRs in their fully active conformation. They have also been used to develop a range of assays that can measure mini G protein coupling to receptors *in vitro*, and a sensitive cell-based assay that is capable of accurately reporting ligand efficacy and quantifying G protein coupling *in vivo*. This review presents an overview of the current applications of mini G proteins to study the structure and function of GPCRs.

Abbreviations: 5-HT_{1B}R: serotonin 5-HT_{1B} receptor; 5-HT_{2A}R: serotonin 5-HT_{2A} receptor; α_{2A}-AR: α_{2A}-adrenergic receptor; A_1R: adenosine A_1 receptor; A_{2A}R: adenosine A_{2A} receptor; AT_1R: angiotensin II receptor; β_1AR: β_1-adrenergic receptor; β_2AR: β_2-adrenergic receptor; BRET: bioluminescence resonance energy transfer; CB_1R: cannabinoid receptor type 1; CRF_1R: corticotrophin-releasing factor receptor 1; cryo-EM: cryo-electron microscopy; CGRP: calcitonin gene-related peptide; CTR: calcitonin receptor; D1R: D1 dopamine receptor; D2R: D2 dopamine receptor; D5R: D5 dopamine receptor; ETAR: endothelin A receptor; GLP-1R: glucagon-like peptide 1 receptor; GPCR: G protein-coupled receptor; kDa: kilodalton; M3R: M3 acetylcholine receptor; M4R: M4 acetylcholine receptor; μOR: μ-opioid receptor; NTSR1: neurotensin receptor; PtdIns(4,5)P_2: phosphatidylinositol-4,5-bisphosphate; V2R: vasopressin receptor 2

1. Introduction

G protein-coupled receptors (GPCRs) mediate cellular communication by regulating signal transduction through heterotrimeric G proteins and β-arrestins. This family of receptors represent one of the most important therapeutic targets for the pharmaceutical industry [1], and, as such, its members are key targets for structure determination. Crystallisation of GPCRs has proved to be one of the biggest challenges in structural biology in recent years, but one that has ultimately been overcome by technical innovation. The development and application of novel methodologies, including T4 lysozyme fusion proteins [2], conformational thermostabilisation [3] and lipidic cubic phase crystallisation [4,5], has resulted in 277 structures of 54 different receptors being determined to date [6]. These have provided unparalleled insight into the mechanism of ligand binding and receptor activation, and are currently being used by the pharmaceutical industry to facilitate the development of novel therapeutic compounds [7]. However, it has become evident that to understand the activation mechanism of individual receptors, and to fully exploit the potential of structure-based drug design, each receptor should ideally be characterised in a minimum of four distinct conformational states, bound to either: an inverse agonist; an agonist; an agonist and G protein; or an agonist and arrestin [8].

Structural and functional characterisation of GPCRs in their active conformation [8], *i.e.* bound to a heterotrimeric G protein [9] or β-arrestin [10], has proved to be particularly challenging, due to the large size, instability and conformational dynamics of these complexes [11,12]. Whilst it is true that structure determination of receptors bound to native signalling proteins is most desirable, the success rate of crystallising these complexes has been particularly low. Therefore, driven by the need to expedite the structure determination of receptors in their active conformation, a number of G protein mimetics have been developed, including G protein-derived peptides [13,14], nanobodies [15] and mini G proteins [16]. The aim of these surrogates is to bind the receptor and stabilise its active conformation, whilst eliminating the problems inherent to native signalling proteins. However, these mimetics have both advantages and disadvantages compared to heterotrimeric G proteins. The C-terminal peptide (GαCT) from the G protein α-subunit (Gα) is the smallest and simplest of the G protein mimetics, and it has facilitated the determination of numerous

active-state rhodopsin structures [13,17–22]. However, such peptides do not appear to efficiently stabilise the active conformation of other receptors, and are therefore of limited use for studying the wider GPCR family. Nanobodies have proved to be effective G protein surrogates that are able to induce comparable pharmacological and structural changes in the receptor to those elicited by heterotrimeric G proteins [23–27]. Their small size and rigid structure makes them particularly well suited to X-ray crystallography applications [28], but their inability to recapitulate the native GPCR-G protein interface means that these structures do not provide insight in to the molecular determinants of G protein coupling specificity. They also require laborious immunisation and selection procedures that must be performed for each receptor being studied. Mini G proteins (see Section 2) induce comparable pharmacological and structural changes in the receptor to those elicited by heterotrimeric G proteins, retain their natural coupling specificity [16,29] and recapitulate the native GPCR-G protein interface [30]. Mini G protein versions of at least one member of the four G protein families have been developed, they can therefore be used off-the-shelf to study a wide variety of receptors. However, the G_q class proved difficult to develop, and required the construction of chimeric proteins that are less biologically relevant [29].

Clearly, none of these surrogates represent a silver bullet for characterising GPCRs in their active conformation, and the stability of individual receptors remains a key factor in the success of structural studies. Nonetheless, together they comprise a toolkit of binding proteins that have greatly simplified the characterisation of active-state GPCRs, and have provided valuable insight into the mechanism of receptor activation. Mini G proteins are the latest addition to this toolkit, and, in this review, I present an overview of their current applications to study the structure and function of GPCRs.

2. Development of mini G proteins

The observation that led to the development of mini G proteins came from structure of the β_2-adrenergic receptor (β_2AR) in complex with heterotrimeric G_s [9]. Despite the G protein having a molecular weight of over 90 kDa, the structure revealed that virtually all of the molecular contacts that it formed with the receptor were mediated by the ~30 kDa GTPase domain from $G\alpha_s$ ($G\alpha_s$-GTPase). The original mini G protein (mini-G_s) was constructed by isolating the $G\alpha_s$-GTPase domain (Figure 1), and engineering it to increase its thermostability and reduce conformational dynamics. This was achieved using rational design mutagenesis, based primarily on the structure of Arl2 [31], the small G protein that is most structural similarity to $G\alpha_s$ [32]. The optimal construct contained deletions in three key regions, namely the α-helical domain (Gα-AH), switch III and the N-terminus. The Gα-AH domain was the most conformationally dynamic region in the β_2AR–G_s complex [9,11]; its deletion not only reduced conformational dynamics, but also enabled the Gα-GTPase domain to couple receptors in the absence of the G protein βγ subunits (Gβγ) [16]. Switch III was disordered in the receptor-bound conformation [9], and is completely absent from small G proteins. Its deletion was designed to replace this flexible region with the defined secondary structure elements found in Arl2 [16,30,31]; this resulted in an increase in both the thermostability and expression level of the mini G protein [16]. The N-terminal helix normally interacts with Gβγ, and is therefore not explicitly required in the mini G protein. Deletion of the complete N-terminus had a detrimental effect on both the thermostability and expression level of the mini G protein, consequently, only residues 1–25 were deleted. Nevertheless, this removed a substantial region of

conformationally dynamic protein, and also eliminated the lipidation sites that tether Gα to the cell membrane [33]. The optimal construct also contained seven point mutations [16], none of which were located within the receptor-binding site. The G49D, E50N, A249D and S252D mutations were designed to stabilise the nucleotide-binding pocket and phosphate-binding loop; they resulted in increased thermostability of the mini G protein, as well as increased expression levels. The L272D mutation was incorporated to conformationally constrain the flexible switch II region. The I372A and V375I mutations, which are located within the α5 helix, were designed to stabilise the receptor-bound conformation of the mini G protein, particularly in the presence of detergent, by improving packing of the α5 helix against the core of the protein. The I372A mutation was also shown to render the complex largely resistant to dissociation by cellular concentrations of guanine nucleotides [16]. Residue I372 appears to act as a relay, transmitting the conformational rearrangements induced by receptor binding from the α5 helix to the nucleotide-binding pocket; the I372A mutation appears to prevent the propagation of these conformational changes, and thus uncouples receptor binding from nucleotide exchange [16]. The final mini-G_s construct was shown to induce a shift in the agonist-binding affinity of both the β_1-adrenergic receptor (β_1AR) and the adenosine A_{2A} receptor (A_{2A}R) comparable to that elicited by G_s, demonstrating that it functioned as an effective mimetic of the heterotrimeric G protein [16,30]. To prove that mini-G_s could facilitate the structure determination of a receptor in its active state, it was crystallised in complex with A_{2A}R, and the structure solved at 3.4 Å resolution [30,34] (see Section 3.2). Comparison of this structure with that of β_2AR–G_s revealed striking similarities in the organisation of the complex. Critically, the α5 helix and C-terminus of the mini G protein undergo comparable conformational changes to those observed in the heterotrimeric G protein, demonstrating that the mini G protein does indeed recapitulate the native GPCR-G protein interface [30].
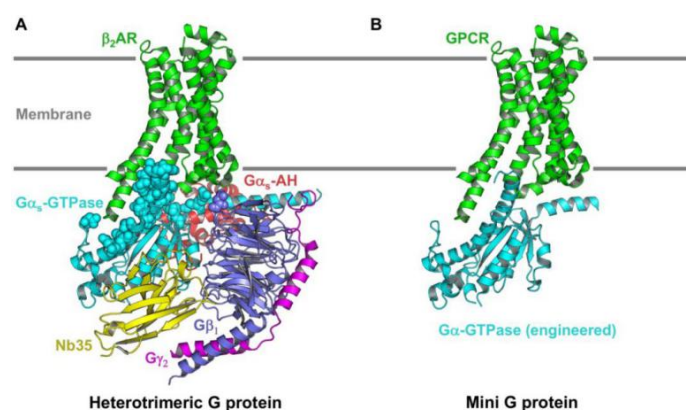

Figure 1. Development of the original mini G protein. A: Structure of β_2AR in complex with heterotrimeric G_s and a nanobody (Nb35) that stabilised the complex [9]. Despite the G protein having a molecular weight of over 90 kDa, virtually all of the residues that interact with the receptor (shown as spheres) were localised to the $G\alpha_s$-GTPase domain (coloured cyan). B: Model of a mini G protein in complex with a receptor. A truncated, engineered version of the Gα-GTPase domain, with a molecular weight of ~27 kDa, was developed to act as a functional mimetic of the heterotrimeric G protein [16]. Figures prepared using PyMOL (The PyMOL Molecular Graphics System, Version 1.8.4 Schrödinger, LLC).

Following the successful development of mini-G_s, the concept was transferred to the other classes of heterotrimeric G proteins; the goal being to convert at least one member of each family into a mini G protein [29]. To date, this has been directly achieved for members of the G_s, G_i and G_{12} families (see Table 1 for details), but only indirectly for G_q family members (discussed below). Mini-G_q could not be expressed in a functional form, so instead a chimera was constructed, in which residues from mini-G_s that interact with either $A_{2A}R$ or β_2AR in the respective crystal structures [9,30] were mutated to match those found in G_q. This approach is not ideal since it cannot account for structural differences between G_s and G_q, or variations in the molecular contacts that they form with G_s- or G_q-coupled receptors, respectively. Nonetheless, the mini-$G_{s/q}$ chimera *did* interact with G_q-coupled receptors such as the neurotensin receptor (NTSR1) and the angiotensin II receptor (AT$_1$R), and *did not* bind G_s-coupled receptors such as $A_{2A}R$ [29]. Therefore, it is a useful tool for the functional characterisation of G_q-coupled receptors, but, since it is unlikely to fully stabilise the receptor in its active state or perfectly recapitulate the native GPCR-G protein interface, it may not be the best option for structural studies. Development of mini-G_i also proved difficult, and so a chimera with mini-G_s (mini-$G_{s/i}$) was constructed. This has been used successfully in cell-based assays (see Section 4.2), although mini-G_o remains the best G_i family member for structural studies (see Table 1).

Table 1. Mini G proteins.

Family	Mini G proteins	GPCRs shown to bind *in vitro*[a]	GPCRs shown to bind *in vivo*[b]	Structures determined
G_s	mini-G_s	$A_{2A}R$	$A_{2A}R$	$A_{2A}R$–mini-G_s
				$A_{2A}R$–mini-G_s–$\beta_1\gamma_2$[c]
		β_1AR		
			β_2AR	
			D1R	
			D5R	
			V2R	
	mini-G_{olf}	$A_{2A}R$		
G_i	mini-G_{i1}	5-HT$_{1B}$R		
	mini-$G_{s/i1}$[d]	5-HT$_{1B}$R		
			α_{2A}-AR	
			A$_1$R	
			CB$_1$R	
			D2R	
			M4R	
	mini-G_{o1}	5-HT$_{1B}$R		5-HT$_{1B}$R–mini-G_{o1}–$\beta_1\gamma_2$[c]
		Rhodopsin		Rhodopsin–mini-G_{o1}
G_q	mini-$G_{s/q}$[d]	NTSR1		
		AT$_1$R	AT$_1$R	
			ETAR	
			M3R	
			5-HT$_{2A}$R	

Continued on next page

Family	Mini G proteins	GPCRs shown to bind in vitro[a]	GPCRs shown to bind in vivo[b]	Structures determined
G_{12}	mini-G_{12}	None	None[e]	

[a] Detergent-solubilised or membrane embedded GPCRs.

[b] Primary coupling interactions.

[c] Heterotrimeric versions of the mini G proteins.

[d] Chimeric mini G proteins.

[e] Only secondary coupling interactions have been observed for mini-G12 *in vivo* [35].

Mini G proteins have a number of advantages in structural biology applications, compared to the heterotrimeric G proteins from which they were derived, including their smaller size (~27 kDa), reduced conformational dynamics, increased solubility and improved thermostability, particularly in short-chain detergents. They also exhibit additional characteristics that have broadened their range of applications, including: (i) their high-level expression (up to 100 mg/l) in *E. coli* [36], which makes them both simple and inexpensive to produce in large quantities, and thus ideal for high-throughput screening applications (see Section 3.1); (ii) their potential modification to restore binding to Gβγ, which is particularly useful in cryo-electron microscopy (cryo-EM) applications (see Section 3.2); and (iii) their resistance to dissociation from the receptor by physiological concentrations of GTP [16], which allows them to be used in cell-based assays (see Section 4.2).

3. Structural biology applications of mini G proteins

Mini G proteins have been used to both improve the properties of receptors for structural studies (see Section 3.1), and to facilitate structure determination of GPCRs in their active state (see Section 3.2). To date, structures of three different receptors have been solved in complex with mini G proteins, using either X-ray crystallography or cryo-EM, namely $A_{2A}R$ [30,37], the serotonin 5-HT_{1B} receptor (5-$HT_{1B}R$) [38] and rhodopsin [39]. Interestingly, structures of the $A_{2A}R$ complex have been solved using both approaches, which has enabled direct comparison of these techniques [37].

3.1. Thermostabilisation of GPCRs for structural studies

GPCRs are extremely labile when extracted from the membrane by detergent solubilisation, and the relative stability of individual receptors is a key factor in whether or not their structure can be determined. At present, structures have been reported for only ~15% of non-olfactory GPCRs encoded in the human genome. Considering that these receptors have often been selected for characterisation based on their above-average stability, it is telling that the vast majority have only had their structures determined bound to an antagonist, which is typically the most stable conformation. One solution to this problem has been the development of conformational thermostabilisation, which uses systematic mutagenesis coupled with a radioligand binding assay to stabilise detergent-solubilised receptors in a defined conformational state [3]. This approach has previously been used to increase the thermostability of either antagonist- or agonist-bound receptors sufficiently to facilitate their crystallisation [3,40–42]. Notably, conformational thermostabilisation

of $A_{2A}R$ in the presence of an agonist alone did not result in stabilisation of the fully active state, but rather a biologically relevant intermediate-active conformation [40,41,43–46]. This suggests that in order to stabilise the fully active state of the receptor, both the agonist and G protein must be present during the selection process.

Using heterotrimeric G proteins in this type of conformational thermostabilisation procedure poses several problems. First, G proteins are composed of multiple subunits, which may be susceptible to dissociation when heated in the presence of detergent. Second, to ensure saturation of the receptor, the G protein must be added at a relatively high concentration (ideally in the micromolar range), which would potentially require hundreds of milligrams of G protein to conduct a full screen. Third, the heterotrimer must be expressed in insect cells using the baculovirus expression system, which means that producing large quantities of pure protein is both time consuming and expensive. In contrast, mini G protein are small, single domain proteins that are highly expressed in *E. coli*, and stable even in harsh detergent [16]. The advent of mini G proteins has therefore enabled conformational thermostabilisation to be applied to an agonist–GPCR–G protein complex, namely the corticotrophin-releasing factor receptor 1 (CRF_1R) in complex with the peptide agonist sauvagine and mini-G_s [47]. The initial screen identified 17 individual point mutations that increased the apparent melting temperature (T_m) of the sauvagine–CRF_1R–mini-G_s complex by up to 5.1 °C. These results are comparable to those obtained during the initial thermostability screen of agonist-bound $A_{2A}R$ [40], which ultimately resulted in its successful crystallisation [41], suggesting that conformational thermostabilisation is indeed a viable approach for stabilising agonist–GPCR–G protein complexes.

3.2. Structure determination of GPCRs in their active conformation

Mini G proteins were originally developed to facilitate structure determination of receptors in their active conformation using X-ray crystallography. Only one structure of a GPCR bound to a heterotrimeric G protein has been solved using this approach, namely the β_2AR–G_s complex, and even this required a nanobody (Nb35) to stabilise the complex sufficiently to enable its crystallisation [9] (Figure 1A). Mini G proteins have facilitated the determination of two additional structures by X-ray crystallography. The structure of $A_{2A}R$ bound to mini-G_s and the agonist NECA was solved at 3.4 Å resolution by vapour diffusion crystallisation in the detergent octylthioglucoside (OTG) [30] (Figure 2A). This was the first structure of $A_{2A}R$ to be solved in its active conformation, and it completed a series of structures comprising the inactive, intermediate-active and active states that provided unparalleled insight in the activation mechanism of this receptor [48]. The structure of rhodopsin bound to mini-G_o and all-*trans* retinal was solved at 3.1 Å resolution by vapour diffusion crystallisation in the detergent octyl glucose neopentyl glycol (OGNG) [39] (Figure 2A). In the visual system, rhodopsin naturally couples to transducin (G_t), which is a member of the G_i family; attempts to construct a mini G protein version of G_t were unsuccessful, so crystallisation was performed using another G_i family member, mini-G_o (see Table 1). This structure revealed that a common active conformation of rhodopsin is responsible for coupling both the G protein and arrestin [10,39]. It is noteworthy that both of these mini G protein complexes were crystallised by vapour diffusion, since high-quality crystals could not be obtained using LCP. The β_2AR–G_s complex however was crystallised using LCP [9], so this method should also be compatible with mini G proteins. It is likely that further screening will be required to identify the optimal combination of

detergent, lipids and additives to successfully crystallise mini G protein complexes using this approach.
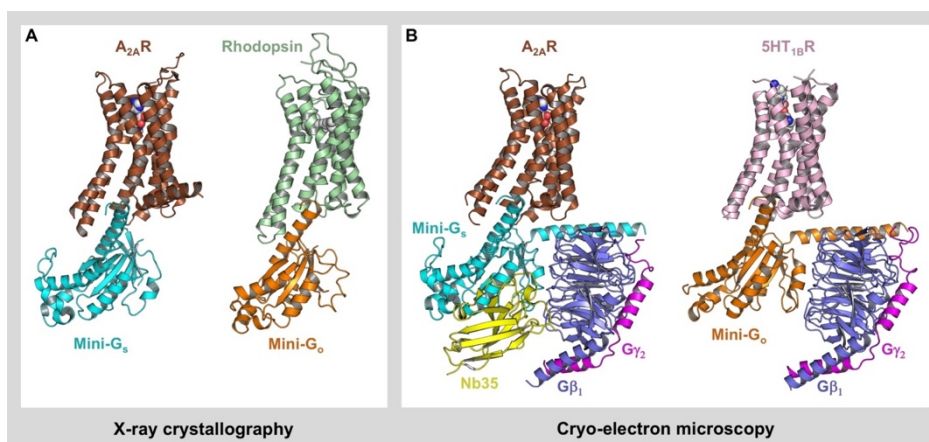

Figure 2. Structures of GPCRs in complex with mini G proteins. A: Structures of two mini G protein complexes have been solved using X-ray crystallography: $A_{2A}R$ bound to mini-G_s and the agonist NECA (PDB: 5G53) [30]; and rhodopsin bound to mini-G_o and all-*trans* retinal (PDB: 6FUF) [39]. B: Structures of two heterotrimeric mini G protein complexes have been solved using cryo-EM: $A_{2A}R$ bound to mini-G_s–$\beta_1\gamma_2$, NECA and Nb35 (PDB: 6GDG) [37], and $5HT_{1B}R$ bound to mini-G_o–$\beta_1\gamma_2$ and the agonist donitriptan (PDB: 6G79) [38].

Recent advances in cryo-EM have brought this technique to the forefront of efforts to determine structures of GPCR–G protein complexes. During the past two years it has accounted for structures of three receptors in complex with G_s, namely the calcitonin receptor (CTR) [49], the calcitonin gene-related peptide (CGRP) receptor [50] and the glucagon-like peptide 1 receptor (GLP-1R) [51], as well as three receptors in complex with G_i, namely the μ-opioid receptor (μOR) [52], the adenosine A_1 receptor (A_1R) [53] and rhodopsin [54]. Despite the fact that cryo-EM is currently limited to proteins larger than ~65 kDa [55], which is similar to that of a receptor in complex with a mini G protein, it has not yet proved possible to resolve such complexes using this approach. However, mini G proteins can easily be modified to restore binding to Gβγ [16], and these heterotrimeric versions have facilitated the determination of two structures using cryo-EM. The structure of $A_{2A}R$ bound to mini-G_s–$\beta_1\gamma_2$ and the agonist NECA was solved at 4.1 Å resolution in the detergent lauryl maltose neopentyl glycol (LMNG) [37] (Figure 2B). The organisation of the interface in this structure was virtually identical to that in the $A_{2A}R$–mini-G_s complex solved using X-ray crystallography, which further validates the use of mini G proteins as surrogates for heterotrimeric G proteins. The structure of 5-$HT_{1B}R$ bound to mini-G_o–$\beta_1\gamma_2$ and the agonist donitriptan was solved at 3.8 Å resolution in the detergent decylmaltoside (DM) [38] (Figure 2B). This was the first structure of a receptor to be solved in complex with G_o, and it provided unique insight into the molecular determinants of G protein coupling specificity.

At present, it is unclear whether the heterotrimeric versions of mini G proteins have a significant advantage over native heterotrimeric G proteins in cryo-EM applications. While mini G proteins do lack the conformationally dynamic Gα-AH domain [16], this is not necessarily an

advantage in cryo-EM, since flexible regions can easily be masked and omitted from data processing. Furthermore, cryo-EM is compatible with milder long-chain detergents, in which the stability of complexes composed of either a mini G protein or heterotrimeric G protein appear to be similar [16], although this has not been extensively tested. The use of mini G protein heterotrimers does enable shorter chain detergents such as DM to be screened during sample preparation for cryo-EM; an approach that significantly improved the homogeneity of the 5-HT$_{1B}$R complex (R. Nehmé, personal communication). Furthermore, if technical developments continue to reduce the protein size limit for cryo-EM [56], then, in the future, it may be possible to determine structures of GPCRs in complex with the mini G protein alone. This would significantly increase the throughput of complex preparation and screening, which should translate into an increased rate of successful structure determination.

4. Applications of mini G proteins for the functional characterisation of GPCRs

Mini G proteins have been used in a number of applications to functionally characterise GPCRs. *In vitro*, they have been used to develop a suite of easy-to-use assays that are capable of reporting mini G protein binding to either membrane-embedded or detergent-solubilised receptors. They have also been used in a cutting-edge native mass spectrometry study to identify endogenous lipids that modulate GPCR-G protein interactions. *In vivo*, they have been used to develop a sensitive assay that is capable of accurately quantifying mini G protein coupling to variety of receptors.

4.1. Characterising G protein coupling to GPCRs in vitro

In many cases, simply detecting G protein binding to a receptor *in vitro* is extremely challenging, particularly when working with detergent-solubilised samples. This means that there is no single assay that is suitable for characterising G protein coupling to the entire GPCR family. Therefore, five different assays have been developed to monitor mini G protein coupling to a range of different GPCRs. First, a competition binding assay was used to demonstrate mini G protein coupling to membrane-embedded A$_{2A}$R [30,37], β$_1$AR [16] and 5-HT$_{1B}$R [29]. This assay measures the inhibitory constant (K_i) of an agonist binding to a receptor in either the presence or absence of a mini G protein (Figure 3A). For many receptors, G protein binding elicits a significant increase (10 to 100-fold) in their agonist-binding affinity [57], and, in these cases, the competition binding assay can be used to reliably measure mini G protein coupling. The advantages of this assay are that it is very sensitive and can be performed in high-throughput format. The disadvantages are that a radiolabelled antagonist is required for each receptor being investigated and some receptors exhibit only a small shift in agonist-binding affinity upon G protein coupling, in which case this assay is not suitable. Second, a thermostability assay [3] was used to report binding of mini G proteins to detergent-solubilised A$_{2A}$R [29,30], AT$_1$R [29], β$_1$AR [16], CRF$_1$R [47], NTSR1 [29] and 5-HT$_{1B}$R [29]. This assay measures the apparent T_m of a non-purified detergent-solubilised receptor in the presence of either an agonist, or both an agonist and a mini G protein (Figure 3B). All receptors that have been tested in this assay display higher thermostability when bound to both the agonist and mini G protein, compared to the agonist alone; this response can therefore be used as a reliable indicator of mini G protein coupling. The advantages of this assay are that it can be used to evaluate the stability of the complex in different detergents and can be performed in high-throughput. The main disadvantage is that, in this format, a radiolabelled agonist is required for each receptor

being investigated. However, it may be possible to use a different type of thermostability assay that does not require a radioligand, such as differential scanning fluorimetry (DSF) [58], although this has not been tested. Third, fluorescence size exclusion chromatography (FSEC) was used to evaluate mini G protein coupling to non-purified detergent-solubilised $A_{2A}R$, β_1AR and 5-HT$_{1B}R$ [29]. In this approach, samples of a GFP-tagged mini G protein, either alone or mixed with a non-purified detergent-solubilised receptor bound to either an agonist or antagonist, are loaded onto a gel filtration column and GFP signal in the eluate is monitored using a fluorescence detector. A shift in the retention volume of the GFP-tagged mini G protein peak in the presence of the agonist-bound receptor is indicative of complex formation (Figure 3C). The advantages of this assay are that it uses non-purified receptor samples and it allows rapid screening of detergent or buffer conditions for optimal complex formation. The disadvantages are that only a low concentration of GFP-tagged mini G protein can be used in the assay and high detergent concentrations are often required for efficient membrane solubilisation, which can affect the stability of the complex. Fourth, a fluorescence saturation binding assay (FSBA) was used to estimate the affinity of mini G protein binding to detergent-solubilised $A_{2A}R$, β_1AR and 5-HT$_{1B}R$ [29]. This assay measures the apparent equilibrium dissociation constant (K_D) of a GFP-tagged mini G protein binding to a non-purified detergent-solubilised receptor, immobilised on a microtiter plate *via* its histidine tag, in the presence of either an agonist or antagonist (Figure 3D). The main advantage of this assay is that it can be used to estimate G protein binding affinity in different detergents or buffers. The main disadvantage is that the affinity of the interaction is likely to be affected by the presence of detergent, and thus is not representative of native interactions within the cell. Fifth, analytical gel filtration chromatography was used to demonstrate mini G protein binding to $A_{2A}R$ [29], β_1AR [16] and 5-HT$_{1B}R$ [29]. In this approach, samples of purified receptor and mini G protein, either individually or mixed, are resolved by analytical gel filtration chromatography. A decrease in the retention volume of the mixed sample, compared to the receptor alone, indicates complex formation (Figure 3E). Also, co-elution of the receptor and mini G protein, which is assessed by SDS-PAGE analysis of the gel filtration eluate, gives a reliable indication of coupling (Figure 3F). The advantages of this method are that it can be used to screen detergents or buffer conditions for optimal complex formation and it enables the stability of a complex to be assessed over a period of days. The main disadvantage is that it requires a significant quantity of purified receptor, which can be challenging to obtain.

Mini G proteins have also been used to study the effect of endogenous lipids on the G protein coupling selectivity of $A_{2A}R$, β_1AR and NTSR1 using native mass spectrometry [59]. Hotspots were identified on the cytoplasmic surface of the receptors that specifically bound phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$). This lipid appears to function as an allosteric modulator of G protein binding, by both stabilising the active state of the receptor and mediating bridging interactions with the G protein. Mini G proteins proved ideal for this application, since they are more stable than heterotrimeric G proteins, and, whilst nanobodies could also be used, they did not bind PtdIns(4,5)P$_2$ and thus did not recapitulate the native bridging interactions.
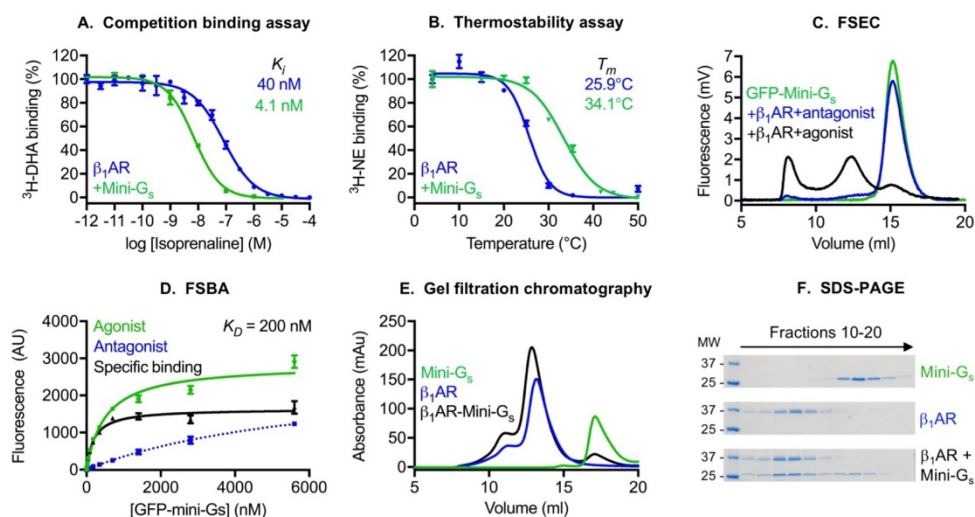
Figure 3. Five different assays have been developed to characterise mini G protein coupling to a range of GPCRs *in vitro*; the example of mini-G_s binding to β_1AR is used to illustrate each assay. A: A competition binding assay that measures the K_i of agonist binding to membrane-embedded receptors in either the presence or absence of a mini G protein. Coupling to mini-G_s resulted in an increase in the isoprenaline-binding affinity of β_1AR by ~10-fold [16]. B: A thermostability assay that measures the apparent T_m of non-purified detergent-solubilised receptors in either the presence or absence of a mini G protein. Coupling to mini-G_s resulted in an increase in the thermostability of DDM-solubilised β_1AR by ~8 °C [16]. C: Fluorescence size exclusion chromatography (FSEC) that measures binding of a GFP-tagged mini G protein to non-purified detergent-solubilised receptors. In the presence of DDM-solubilised β_1AR and the inverse agonist ICI118551 no shift was observed in the retention volume of GFP-mini-G_s (15.1 ml), but in the presence of β_1AR and the agonist isoprenaline the retention volume shifted to 12.1 ml, demonstrating complex formation; a peak of aggregated protein was also observed in the void volume of the column (8 ml) [29]. D: A fluorescence saturation binding assay (FSBA) that measures the apparent K_D of a GFP-tagged mini G protein binding to non-purified detergent-solubilised receptors. Non-specific binding of GFP-mini-G_s to DDM-solubilised β_1AR was measured in the presence of the inverse agonist ICI118551, this was subtracted from the total binding measured in the presence of the agonist isoprenaline to give specific binding; the apparent K_D was 200 nM [29]. E: Analytical gel filtration chromatography that measures mini G protein coupling to purified detergent-solubilised receptors. Mini-G_s had a retention volume of 17.1 ml and DDM-solubilised β_1AR had a retention volume of 13.2 ml, compared to a retention volume of 12.9 ml for the β_1AR–mini-G_s complex [16]. F: SDS-PAGE analysis of the gel filtration eluate confirmed that the receptor and mini G protein co-eluted [16].

4.2. Characterising G protein coupling to GPCRs in vivo

Mini G proteins have been used to measure G protein coupling to GPCRs in live mammalian cells using confocal microscopy, bioluminescence resonance energy transfer (BRET) spectroscopy

and luciferase complementation [35]. Initially, confocal microscopy was used to demonstrate agonist-dependent recruitment of mini-G_s (fused to the fluorescent protein venus) to membrane-localised β_2AR. This confirmed that the resistance of mini G protein complexes to dissociation by cytosolic concentrations of guanine nucleotides makes them ideally suited to cell-based applications. Next, a BRET spectroscopy assay was developed using mini G proteins from each of the four families. Mini-G_s and mini-G_{12} were both well expressed in mammalian cells, but neither mini-G_q or mini-G_i were expressed in a soluble form, therefore the chimeric versions of these proteins, mini-$G_{s/i}$ and mini-$G_{s/q}$, were used (see Table 1). BRET was measured between venus-tagged versions of these mini G proteins and a panel of GPCRs (fused to the *Renilla* luciferase Rluc8). The cytosolic localisation of mini G proteins means that they displayed a significantly higher BRET signal-to-noise ratio than heterotrimeric G proteins [60,61], which are tethered to the membrane through lipid anchors [33]. The assay was able to accurately profile coupling of the mini G proteins to a panel of receptors including: The α_{2A}-adrenergic receptor (α_{2A}-AR); A_1R; $A_{2A}R$; AT_1R; β_2AR; the cannabinoid receptor type 1 (CB_1R); the D1, D2 and D5 dopamine receptors (D1R, D2R and D5R, respectively), the endothelin A receptor (ETAR); the M3 and M4 acetylcholine receptors (M3R and M4R, respectively); the serotonin 5-HT_{2A} receptor (5-$HT_{2A}R$); and the vasopressin receptor 2 (V2R; see Table 1). The mini G proteins, including the two chimeras, were shown to maintain appropriate coupling specificity *in vivo* (see Table 1). Furthermore, their secondary coupling interactions were also accurately reported, and in the case of D1R and D5R, previously unknown secondary interactions were also identified. The assay was also able to precisely report the efficacy of agonists, partial agonists, antagonists and inverse agonists at β_2AR by measuring mini-G_s coupling [35]. Similar results were obtained when the assay was modified to use luciferase complementation [62], demonstrating the versatility of this assay format. Confocal microscopy and BRET spectroscopy were also used to measure mini G protein recruitment to receptors localised to intracellular compartments, including A_1R in the Golgi apparatus and β_2AR in early endosomes [35]. Thus, mini G proteins can be used as biosensors for monitoring the subcellular localisation and internalisation of receptors in a similar manner to nanobodies [63,64].

5. Conclusions and future perspectives

Mini G proteins have facilitated the structure determination of three different receptors in their active state, and have enabled the development of a novel methodology to thermostabilise agonist–GPCR–G protein complexes. These structures have provided valuable insight into the mode of agonist binding, the mechanism of receptor activation, and the molecular determinants of G protein coupling specificity. Their potential applications in structure-based drug design means there is high demand for structures of all GPCRs to be solved in their active state; both cryo-EM and X-ray crystallography will be key techniques in determining these structures, and mini G proteins have proven to be useful tools in both approaches. Beyond structural biology, mini G proteins have been used to develop a range of assays that have facilitated the characterisation of G protein coupling both *in vitro* and *in vivo*. The cell-based BERT spectroscopy assay will have immediate applications in GPCR drug discovery, enabling the efficacy and functional selectivity [65] of novel drug candidates to be measure at potentially any receptor. It will also facilitate the characterisation of allosteric modulators that can be used to enhance the receptor subtype specificity of orthosteric ligands [66]. The construction of some mini G protein subtypes has proved difficult, particularly G_q family

members, thus further work is required to complete development of the full mini G protein panel. The relatively high degree of sequence homology shared by the different mini G proteins means that it should be possible to use gene shuffling approaches to pinpoint areas responsible for the poor stability of certain subtypes. This would enable the development of more biologically relevant chimeras in which only minimal regions of the mini G protein need to be substituted to achieve optimal expression and stability [67]. Although a number of different tools are available for characterising receptors in their active state, the fact that mini G proteins can be used off-the-shelf without the need for laborious receptor-specific development makes them an ideal first choice in many applications. Further development and modification of mini G proteins should result in many more novel applications being identified, particularly in relation to GPCR functional studies. Therefore, in the future, mini G proteins will continue to contribute to our understanding of these important receptors, and aid in the development of drugs to treat a wide variety of diseases.

Acknowledgements

Conflict of interest

The author is an inventor on a patent application covering mini G proteins, but has no other conflict of interest.

References

1. Wise A, Gearing K, Rees S (2002) Target validation of G-protein coupled receptors. *Drug Discov Today* 7: 235–246.
2. Cherezov V, Rosenbaum DM, Hanson MA, et al. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318: 1258–1265.
3. Serrano-Vega MJ, Magnani F, Shibata Y, et al. (2008) Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. *Proc Natl Acad Sci USA* 105: 877–882.
4. Caffrey M (2015) A comprehensive review of the lipid cubic phase or in meso method for crystallizing membrane and soluble proteins and complexes. *Acta Crystallogr F Struct Biol Commun* 71: 3–18.
5. Landau EM, Rosenbusch JP (1996) Lipidic cubic phases: A novel concept for the crystallization of membrane proteins. *Proc Natl Acad Sci USA* 93: 14532–14535.
6. Chan W, Said M, Zhang C, et al. (2018) GPCR-EXP: A semi-manually curated database for experimentally-solved and predicted GPCR structures. Available from: https://zhanglab.ccmb.med.umich.edu/GPCR-EXP/.
7. Jazayeri A, Dias JM, Marshall FH (2015) From G Protein-coupled receptor structure resolution to rational drug design. *J Biol Chem* 290: 19489–19495.
8. Carpenter B, Tate CG (2017) Active state structures of G protein-coupled receptors highlight the similarities and differences in the G protein and arrestin coupling interfaces. *Curr Opin Struct Biol* 45: 124–132.

9. Rasmussen SG, Devree BT, Zou Y, et al. (2011) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* 477: 549–555.

10. Kang Y, Zhou XE, Gao X, et al. (2015) Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* 523: 561–567.

11. Westfield GH, Rasmussen SG, Su M, et al. (2011) Structural flexibility of the G alpha s alpha-helical domain in the beta2-adrenoceptor Gs complex. *Proc Natl Acad Sci USA* 108: 16086–16091.

12. Van EN, Preininger AM, Alexander N, et al. (2011) Interaction of a G protein with an activated receptor opens the interdomain interface in the alpha subunit. *Proc Natl Acad Sci USA* 108: 9420–9424.

13. Scheerer P, Park JH, Hildebrand PW, et al. (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 455: 497–502.

14. Hamm HE, Deretic D, Arendt A, et al. (1988) Site of G protein binding to rhodopsin mapped with synthetic peptides from the alpha subunit. *Science* 241: 832–835.

15. Pardon E, Laeremans T, Triest S, et al. (2014) A general protocol for the generation of Nanobodies for structural biology. *Nat Protoc* 9: 674–693.

16. Carpenter B, Tate CG (2016) Engineering a minimal G protein to facilitate crystallisation of G protein-coupled receptors in their active conformation. *Protein Eng Des Sel* 29: 583–594.

17. Blankenship E, Vahedi-Faridi A, Lodowski DT (2015) The high-resolution structure of activated opsin reveals a conserved solvent network in the transmembrane region essential for activation. *Structure* 23: 2358–2364.

18. Choe HW, Kim YJ, Park JH, et al. (2011) Crystal structure of metarhodopsin II. *Nature* 471: 651–655.

19. Deupi X, Edwards P, Singhal A, et al. (2012) Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II. *Proc Natl Acad Sci USA* 109: 119–124.

20. Park JH, Morizumi T, Li Y, et al. (2013) Opsin, a structural model for olfactory receptors? *Angew Chem Int Ed Engl* 52: 11021–11024.

21. Singhal A, Ostermaier MK, Vishnivetskiy SA, et al. (2013) Insights into congenital stationary night blindness based on the structure of G90D rhodopsin. *EMBO Rep* 14: 520–526.

22. Standfuss J, Edwards PC, D'Antona A, et al. (2011) The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature* 471: 656–660.

23. Huang W, Manglik A, Venkatakrishnan AJ, et al. (2015) Structural insights into micro-opioid receptor activation. *Nature* 524: 315–321.

24. Kruse AC, Ring AM, Manglik A, et al. (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* 504: 101–106.

25. Rasmussen SG, Choi HJ, Fung JJ, et al. (2011) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* 469: 175–180.

26. Ring AM, Manglik A, Kruse AC, et al. (2013) Adrenaline-activated structure of beta2-adrenoceptor stabilized by an engineered nanobody. *Nature* 502: 575–579.

27. Weichert D, Kruse AC, Manglik A, et al. (2014) Covalent agonists for studying G protein-coupled receptor activation. *Proc Natl Acad Sci USA* 111: 10744–10748.

28. Manglik A, Kobilka BK, Steyaert J (2017) Nanobodies to study G protein-coupled receptor structure and function. *Annu Rev Pharmacol Toxicol* 57: 19–37.

29. Nehme R, Carpenter B, Singhal A, et al. (2017) Mini-G proteins: Novel tools for studying GPCRs in their active conformation. *PLoS One* 12: e0175642.

30. Carpenter B, Nehme R, Warne T, et al. (2016) Structure of the adenosine A(2A) receptor bound to an engineered G protein. *Nature* 536: 104–107.

31. Hanzal-Bayer M, Renault L, Roversi P, et al. (2002) The complex of Arl2-GTP and PDE delta: From structure to function. *EMBO J* 21: 2095–2106.

32. Sunahara RK, Tesmer JJ, Gilman AG, et al. (1997) Crystal structure of the adenylyl cyclase activator Gsalpha. *Science* 278: 1943–1947.

33. Spiegel AM, Backlund PS, Butrynski JE, et al. (1991) The G protein connection: Molecular basis of membrane association. *Trends Biochem Sci* 16: 338–341.

34. Carpenter B, Tate CG (2017) Expression, purification and crystallisation of the adenosine A2A receptor bound to an engineered Mini G protein. *Bio Protoc,* 7.

35. Wan Q, Okashah N, Inoue A, et al. (2018) Mini G protein probes for active G protein-coupled receptors (GPCRs) in live cells. *J Biol Chem* 293: 7466–7473.

36. Carpenter B, Tate CG (2017) Expression and purification of mini G proteins from escherichia coli. *Bio Protoc* 7: e2235.

37. Garcia-Nafria J, Lee Y, Bai X, et al. (2018) Cryo-EM structure of the adenosine A2A receptor coupled to an engineered heterotrimeric G protein. *Elife* 7: e35946.

38. Garcia-Nafria J, Nehme R, Edwards PC, et al. (2018) Cryo-EM structure of the serotonin 5-HT1B receptor coupled to heterotrimeric Go. *Nature* 558: 620–623.

39. Tsai CJ, Pamula F, Nehmé R, et al. (2018) Crystal structure of rhodopsin in complex with a mini-Go sheds light on the principles of G protein selectivity. *Sci Adv* 4: eaat7052.

40. Lebon G, Bennett K, Jazayeri A, et al. (2011) Thermostabilisation of an agonist-bound conformation of the human adenosine A(2A) receptor. *J Mol Biol* 409: 298–310.

41. Lebon G, Warne T, Edwards PC, et al. (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature* 474: 521–525.

42. Warne T, Serrano-Vega MJ, Baker JG, et al. (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* 454: 486–491.

43. Lebon G, Edwards PC, Leslie AG, et al. (2015) Molecular determinants of CGS21680 binding to the human adenosine A2A receptor. *Mol Pharmacol* 87: 907–915.

44. Lebon G, Warne T, Tate CG (2012) Agonist-bound structures of G protein-coupled receptors. *Curr Opin Struct Biol* 22: 482–490.

45. Xu F, Wu H, Katritch V, et al. (2011) Structure of an agonist-bound human A2A adenosine receptor. *Science* 332: 322–327.

46. Ye L, Van EN, Zimmer M, et al. (2016) Activation of the A2A adenosine G-protein-coupled receptor by conformational selection. *Nature* 533: 265–268.

47. Strege A, Carpenter B, Edwards PC, et al. (2017) Strategy for the thermostabilization of an agonist-bound GPCR coupled to a G protein. *Method Enzymol* 594: 243–264.

48. Carpenter B, Lebon G (2017) Human adenosine A2A receptor: Molecular mechanism of ligand binding and activation. *Front Pharmacol* 8: 898.

49. Liang YL, Khoshouei M, Radjainia M, et al. (2017) Phase-plate cryo-EM structure of a class B GPCR-G-protein complex. *Nature* 546: 118–123.

50. Liang YL, Khoshouei M, Deganutti G, et al. (2018) Cryo-EM structure of the active, Gs-protein complexed, human CGRP receptor. *Nature* 561: 492–497.

51. Zhang Y, Sun B, Feng D, et al. (2017) Cryo-EM structure of the activated GLP-1 receptor in complex with a G protein. *Nature* 546: 248–253.

52. Koehl A, Hu H, Maeda S, et al. (2018) Structure of the micro-opioid receptor-Gi protein complex. *Nature* 558: 547–552.

53. Draper-Joyce CJ, Khoshouei M, Thal DM, et al. (2018) Structure of the adenosine-bound human adenosine A1 receptor-Gi complex. *Nature* 558: 559–563.

54. Kang Y, Kuybeda O, de Waal PW, et al. (2018) Cryo-EM structure of human rhodopsin bound to an inhibitory G protein. *Nature* 558: 553–558.

55. Khoshouei M, Radjainia M, Baumeister W, et al. (2017) Cryo-EM structure of haemoglobin at 3.2 A determined with the Volta phase plate. *Nat Commun* 8: 16099.

56. Renaud JP, Chari A, Ciferri C, et al. (2018) Cryo-EM in drug discovery: Achievements, limitations and prospects. *Nat Rev Drug Discov* 17: 471–492.

57. Green SA, Holt BD, Liggett SB (1992) Beta 1- and beta 2-adrenergic receptors display subtype-selective coupling to Gs. *Mol Pharmacol* 41: 889–893.

58. Niesen FH, Berglund H, Vedadi M (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protoc* 2: 2212–2221.

59. Yen HY, Hoi KK, Liko I, et al. (2018) PtdIns(4,5)P2 stabilizes active states of GPCRs and enhances selectivity of G-protein coupling. *Nature* 559: 423–427.

60. Gales C, Rebois RV, Hogue M, et al. (2005) Real-time monitoring of receptor and G-protein interactions in living cells. *Nat Methods* 2: 177–184.

61. Hein P, Frank M, Hoffmann C, et al. (2005) Dynamics of receptor/G protein coupling in living cells. *EMBO J* 24: 4106–4114.

62. Dixon AS, Schwinn MK, Hall MP, et al. (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem Biol* 11: 400–408.

63. Irannejad R, Pessino V, Mika D, et al. (2017) Functional selectivity of GPCR-directed drug action through location bias. *Nat Chem Biol* 13: 799–806.

64. Irannejad R, Tomshine JC, Tomshine JR, et al. (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* 495: 534–538.

65. Kenakin T, Christopoulos A (2013) Signalling bias in new drug discovery: Detection, quantification and therapeutic impact. *Nat Rev Drug Discov* 12: 205–216.

66. Gentry PR, Sexton PM, Christopoulos A (2015) Novel allosteric modulators of G protein-coupled receptors. *J Biol Chem* 290: 19478–19488.

67. Skiba NP, Bae H, Hamm HE (1996) Mapping of effector binding sites of transducin alpha-subunit using G alpha t/G alpha i1 chimeras. *J Biol Chem* 271: 413–424.