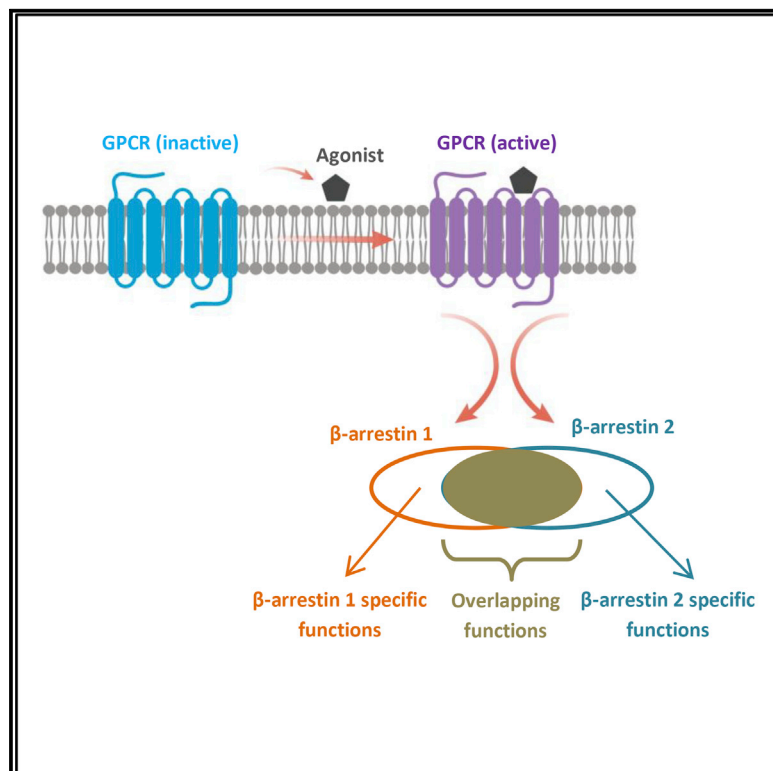


# Conformational Sensors and Domain Swapping Reveal Structural and Functional Differences between $\beta$ -Arrestin Isoforms

## Graphical Abstract



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## In Brief

Ghosh et al. discover structural differences between  $\beta$ -arrestin isoforms ( $\beta$ -arrestin 1 and 2), which are universal regulators of signaling and trafficking of G-protein-coupled receptors (GPCRs). These findings have direct implications for understanding the regulatory and signaling paradigms of GPCRs and designing novel therapeutics targeting this important class of receptors.

## Highlights

- $\beta$ -arrestin 1 and 2 differentially regulate signaling and trafficking of GPCRs
- There are conformational differences between receptor-bound  $\beta$ -arrestin 1 and 2
- Domain-swapped chimera of  $\beta$ -arrestin 1 and 2 exhibit functional complementation
- Functional differences of  $\beta$ -arrestin isoforms have implications for biased agonism



# Conformational Sensors and Domain Swapping Reveal Structural and Functional Differences between $\beta$ -Arrestin Isoforms

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## SUMMARY

Desensitization, signaling, and trafficking of G-protein-coupled receptors (GPCRs) are critically regulated by multifunctional adaptor proteins,  $\beta$ -arrestins ( $\beta$ arrs). The two isoforms of  $\beta$ arrs ( $\beta$ arr1 and 2) share a high degree of sequence and structural similarity; still, however, they often mediate distinct functional outcomes in the context of GPCR signaling and regulation. A mechanistic basis for such a functional divergence of  $\beta$ arr isoforms is still lacking. By using a set of complementary approaches, including antibody-fragment-based conformational sensors, we discover structural differences between  $\beta$ arr1 and 2 upon their interaction with activated and phosphorylated receptors. Interestingly, domain-swapped chimeras of  $\beta$ arrs display robust complementation in functional assays, thereby linking the structural differences between receptor-bound  $\beta$ arr1 and 2 with their divergent functional outcomes. Our findings reveal important insights into the ability of  $\beta$ arr isoforms to drive distinct functional outcomes and underscore the importance of integrating this aspect in the current framework of biased agonism.

## INTRODUCTION

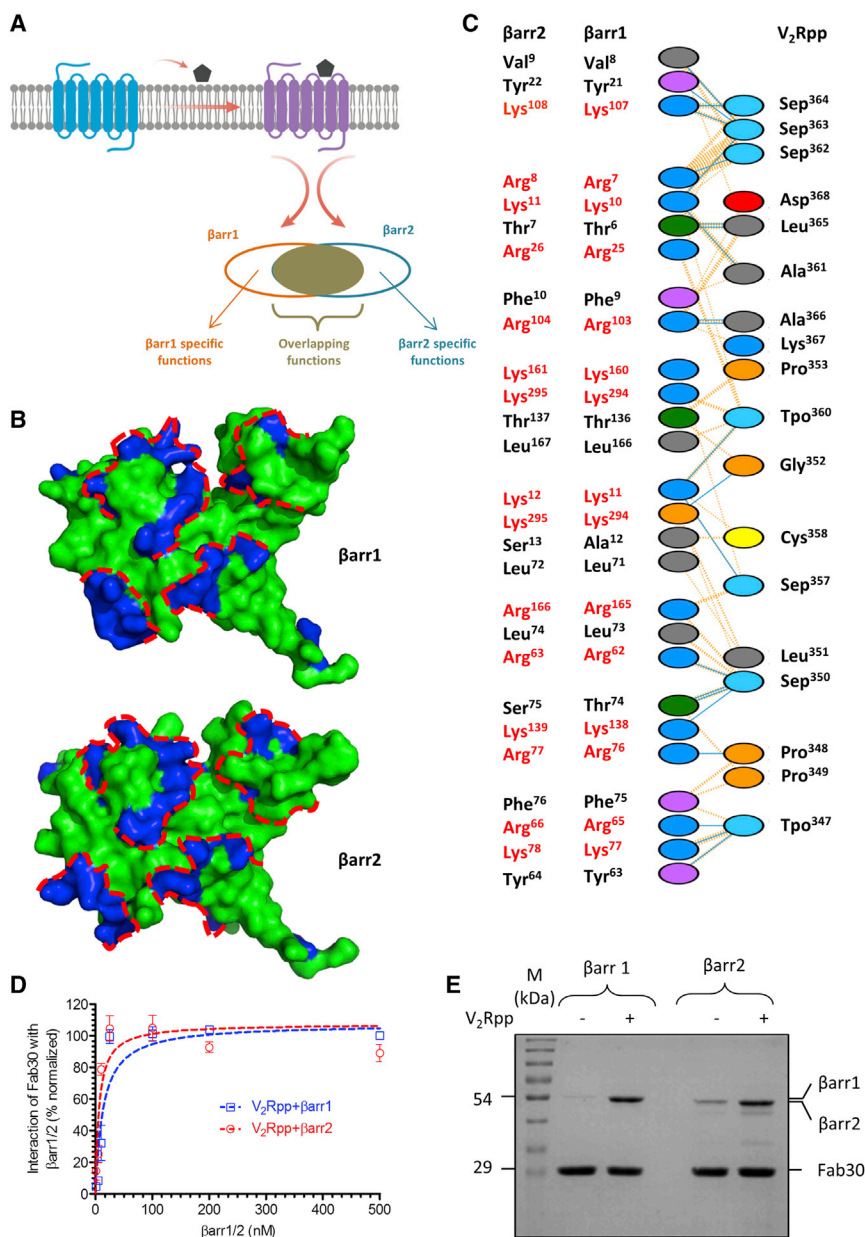
G-protein-coupled receptors (GPCRs) constitute a large family of integral membrane proteins in the human genome (Bjarnadóttir et al., 2006) and a major class of drug targets (Santos et al., 2017). Upon activation by agonists, GPCRs couple to heterotrimeric G proteins followed by the generation of second messen-

gers and downstream signaling. Subsequently, they are phosphorylated in their carboxyl-terminus and intracellular loops, which then results in coupling of  $\beta$ -arrestins ( $\beta$ arrs). Binding of  $\beta$ arrs interferes with further coupling of G proteins through steric hindrance, at least at the plasma membrane, and leads to receptor desensitization. Interestingly,  $\beta$ arrs also serve as adaptors for the components of clathrin machinery to mediate receptor endocytosis (Goodman et al., 1996; Laporte et al., 1999), and they can also scaffold various kinases to initiate several signaling pathways (DeWire et al., 2007; Luttrell et al., 1999, 2001; McDonald et al., 2000).

The two isoforms of  $\beta$ arrs, referred to as  $\beta$ arr1 and 2 (also known as arrestin 2 and 3, respectively) share a high level of sequence identity and both display an overall similar structure (Gurevich and Gurevich, 2015). For most GPCRs, both isoforms of  $\beta$ arrs are typically recruited upon agonist stimulation and participate in desensitization, endocytosis, and signaling. Emerging data suggest that a significant level of functional divergence between  $\beta$ arr1 and 2 exists for most GPCRs, and in some cases, they even display functional antagonism (Figure 1A) (Srivastava et al., 2015; Hara et al., 2011; Ahn et al., 2003). Interestingly, the functional differences of  $\beta$ arr isoforms is also manifested at the level of physiological outcomes downstream of several GPCRs (Srivastava et al., 2015; Walters et al., 2009; Trivedi et al., 2013).

The functional divergence of  $\beta$ arr isoforms has direct implications for the conceptual framework of  $\beta$ arr-dependent signaling, biased-agonism, and, in particular, for the development of  $\beta$ arr-biased ligands as novel GPCR therapeutics (Shukla et al., 2011). Still, however, the mechanistic basis of this phenomenon is currently lacking, and it represents a key knowledge gap in our understanding of the GPCR- $\beta$ arr interaction and signaling. The receptor- $\beta$ arr interaction is typically biphasic, involving the phosphorylated carboxyl-terminus





**Figure 1. Overall Structural and Conformational Similarity between  $V_2Rpp$ -Bound  $\beta$ arr1 and 2**

(A) Most GPCRs typically recruit both isoforms of  $\beta$ arrs, i.e.,  $\beta$ arr1 and 2, which mediate some overlapping functions, but they also display differential contributions in receptor desensitization, trafficking, and signaling outcomes.

(B) Crystal structure of  $V_2Rpp$ - $\beta$ arr1 reveals that Lys/Arg residues on the N-domain of  $\beta$ arr1 are aligned into a spatial groove (indicated by dotted red lines) and  $V_2Rpp$  docks into this groove. Corresponding Lys/Arg residues in  $\beta$ arr2, which are conserved at the primary sequence level, also exhibit a similar spatial arrangement as visualized using the crystal structure of  $\beta$ arr2 (PDB: 3P2D).

(C) Schematic representation of amino acids in  $\beta$ arr1 that are within the interacting distance of  $V_2Rpp$  based on previously determined crystal structure (PDB: 4JQI). The coordinates of the amino acid residues involved in binding with  $V_2Rpp$  were submitted into PDBSum, and the interactions were mapped as a simplified ladder. Corresponding residues in  $\beta$ arr2 are listed to highlight the conserved nature of  $V_2Rpp$ -interacting residues in  $\beta$ arr1 and 2. Lys/Arg residues, which form ionic interactions with the phosphate groups on  $V_2Rpp$ , are highlighted in red. Sep and Tpo represent phosphorylated serine and threonine residues in  $V_2Rpp$ , respectively. Dotted blue and orange lines denote hydrogen bonding and non-bonded contacts, respectively. The residues have been colored according to their types, viz., blue, positive; red, negative; green, neutral; purple, aromatic; gray, aliphatic; orange, Pro and Gly; and yellow, cysteine.

(D) Fab30 recognizes  $V_2Rpp$ -bound  $\beta$ arr1 and 2 to a comparable level as measured using an ELISA-based approach described in the STAR Methods section. Data represent mean  $\pm$  SEM of three independent experiments, with each carried out in duplicate and normalized with respect to the maximum signal obtained for  $V_2Rpp$ - $\beta$ arr1 condition (treated as 100%).

(E) Similar reactivity of Fab30 toward  $V_2Rpp$ -bound  $\beta$ arr1 and 2 is further confirmed by a coIP experiment.

A representative image of three independent experiments is shown here, and densitometry-based quantification of all three experiments is presented in Figure S3A. See also Figure S1.

(i.e., receptor tail) and the transmembrane bundle (i.e., receptor core) (Gurevich and Gurevich, 2004; Shukla et al., 2014). These two sets of interactions result in the formation of partially engaged (i.e., tail engaged) and fully engaged (i.e., core engaged) receptor- $\beta$ arr complexes, respectively. Recent studies have suggested that distinct functional outcomes are associated with these two conformations of receptor- $\beta$ arr complexes (Kumari et al., 2016; Cahill et al., 2017; Kumari et al., 2017; Sente et al., 2018). Thus, we envisioned that key determinants of the functional divergence of  $\beta$ arr isoforms may be encoded at the level of structural and conformational differences between receptor-bound  $\beta$ arrs.

Accordingly, we set out to probe the conformations of receptor-bound  $\beta$ arrs by using a battery of complementary approaches, including biochemical and functional assays, synthetic antibody-based conformational biosensors, single-particle electron microscopy, bimane fluorescence spectroscopy, and molecular dynamics simulation. We discover potential structural differences between receptor-bound  $\beta$ arr1 and 2 and identify key regions in  $\beta$ arrs that are critical for imparting these differences. By using a domain-swapped chimera of  $\beta$ arrs, we also observe that the structural differences between  $\beta$ arr1 and 2 manifest in their distinct functional contributions downstream of GPCRs. Our findings provide important insights into the

GPCR- $\beta$ arr interaction, and they have direct implications for refining the framework of biased agonism at GPCRs.

## RESULTS

### Sequence and Structural Analysis of $\beta$ arrs

The crystal structure of  $\beta$ arr1 in complex with a phosphopeptide corresponding to the carboxyl-terminus of the human vasopressin receptor ( $V_2R$ ; referred to as  $V_2Rpp$ ) has revealed the interaction interface between receptor-attached phosphate groups and positively charged residues in the N-domain of  $\beta$ arr1 (Shukla et al., 2013). Here,  $V_2Rpp$  serves as a surrogate for the phosphorylated receptor tail, and therefore, the  $V_2Rpp$ - $\beta$ arr1 complex represents a close proxy of the partially engaged receptor- $\beta$ arr1 complex. Our analysis of the phosphate-interacting residues on  $\beta$ arr1 in this crystal structure, and their spatial surface mapping, revealed a groove along the N-domain of  $\beta$ arr1 that constitutes the docking interface for  $V_2Rpp$  through a number of charge-charge interactions (Figure 1B, top panel). As  $V_2Rpp$  binds both  $\beta$ arr1 and 2 with comparable affinities (Nobles et al., 2007; Xiao et al., 2004), we analyzed the sequence and the three-dimensional structure of  $\beta$ arr2 to assess whether the spatial arrangement of phosphate-interacting residues may be conserved in both isoforms. Indeed, we observed not only that phosphate-interacting residues are highly conserved in  $\beta$ arr2 but also that their spatial distribution on the N-domain of  $\beta$ arr2 forms a groove identical to  $\beta$ arr1 (Figures 1B, bottom panel, 1C, and S1). This observation suggests a potentially similar binding mechanism of receptor tail with  $\beta$ arr isoforms and provides a rationale to probe it experimentally.

### Fab30 as a Sensor of $\beta$ arr Activation Reveals Overall Similarity between $V_2Rpp$ -Bound $\beta$ arr1 and 2

Based on sequence and structural analyses, we conceived that the overall conformation of  $V_2Rpp$ -bound  $\beta$ arr1 and 2 may be similar, and to probe this, we used a synthetic antibody fragment (referred to as Fab30) as a sensor of  $\beta$ arr conformation that preferentially recognizes  $V_2Rpp$ -bound  $\beta$ arr1 (Shukla et al., 2013, 2014; Kumari et al., 2016, 2017). As the ability of Fab30 to recognize  $\beta$ arr2 has not been evaluated previously, we first identified the paratope residues for Fab30 binding on  $\beta$ arr1 based on the crystal structure of the  $V_2Rpp$ - $\beta$ arr1-Fab30 complex (Shukla et al., 2013) and confirmed that they are mostly conserved in  $\beta$ arr2 (Figures S2A–S2C). Thus, Fab30 should recognize  $V_2Rpp$ -bound  $\beta$ arr2 as well, and in fact, we observed a robust interaction of Fab30 with  $V_2Rpp$ -bound  $\beta$ arr2 at comparable levels to  $\beta$ arr1 in two parallel assays based on ELISA and co-immunoprecipitation (coIP) (Figures 1D and 1E; Figure S3A). In addition, a single-chain variable fragment version of Fab30, referred to as ScFv30, also exhibited a similar pattern of reactivity toward  $V_2Rpp$ -bound  $\beta$ arr1 and 2 (Figures S3B and S3C). Under similar experimental conditions, a control Fab (Fab-CTL) that does not recognize  $\beta$ arr1 failed to exhibit any significant specific binding in coIP experiments (Figures S3D and S3E). These data suggest that the overall conformations of  $\beta$ arr1 and 2 in complex with  $V_2Rpp$ , as detected by Fab30 reactivity, are similar.

### Fab30 Reactivity Suggests Potential Conformational Differences between Receptor-Bound $\beta$ arr1 and 2

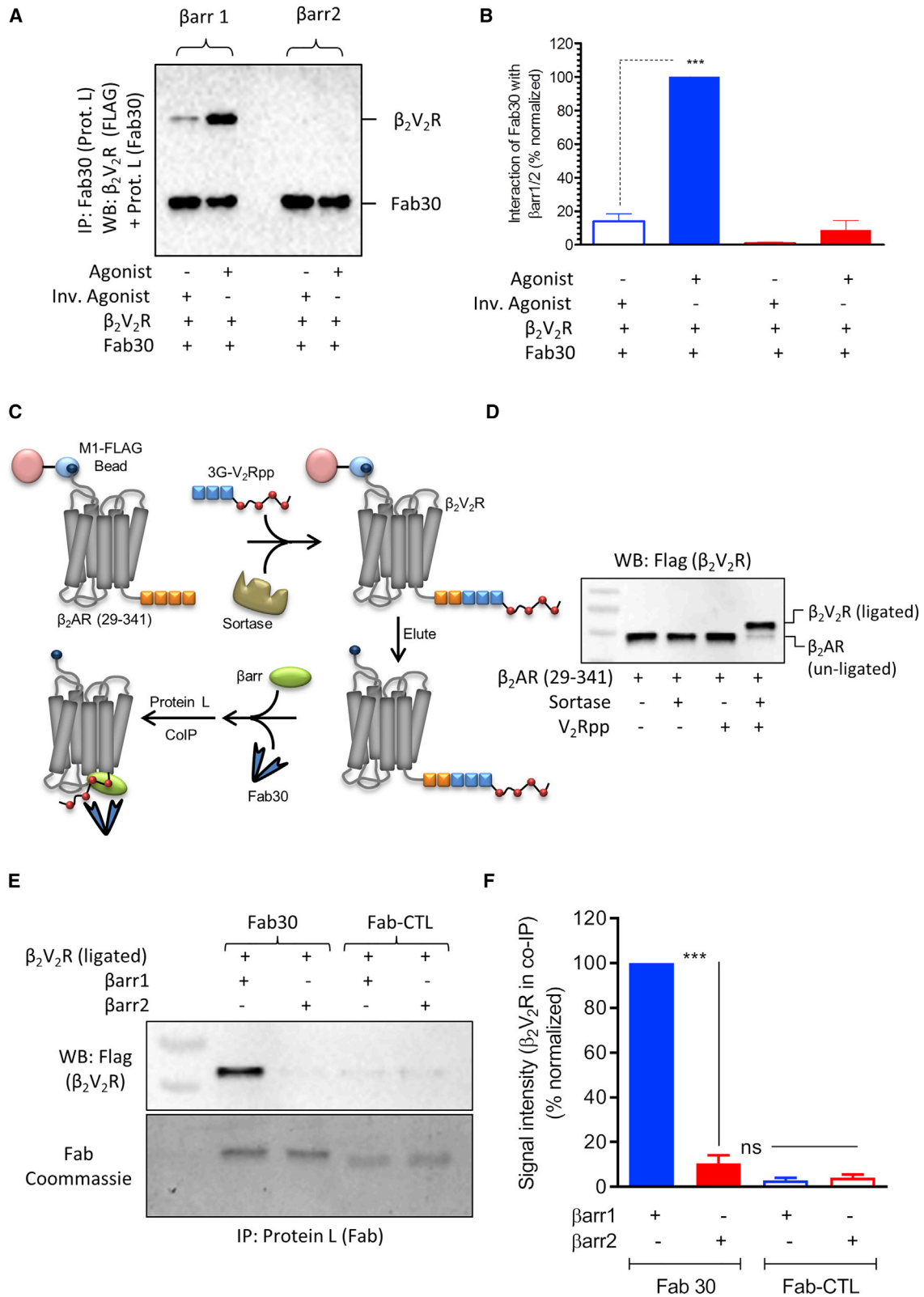
We next set out to measure the reactivity of Fab30 toward activated and phosphorylated receptor-bound  $\beta$ arr1 and 2. Here, we used two different GPCRs, the  $V_2R$  and a chimeric  $\beta_2$ -adrenergic receptor, referred to as  $\beta_2V_2R$ , where the carboxyl-terminus of the  $\beta_2AR$  is replaced with that of the  $V_2R$  (Oakley et al., 2000; Thomsen et al., 2016). Although we have previously reported that Fab30 robustly recognizes receptor- $\beta$ arr1 complexes (Shukla et al., 2014; Kumari et al., 2016), in order to further establish Fab30 as a reliable sensor of receptor-bound  $\beta$ arr conformation, we measured its reactivity toward  $\beta_2V_2R$ - $\beta$ arr1 complexes formed in response to a set of ligands having different efficacies ranging from inverse agonists, partial agonists, to full agonists. We observed an excellent correlation between Fab30 reactivity (measured using coIP assay) and the relative efficacy of ligands, as measured by their cAMP response (Figures S4A–S4E). This observation underlines the ability of Fab30 to report a pharmacologically relevant receptor-bound  $\beta$ arr1 conformation and, therefore, allows us to compare the conformation of receptor-bound  $\beta$ arr1 and 2.

We used two parallel approaches based on ELISA and coIP assays by using activated and phosphorylated  $\beta_2V_2R$  and  $V_2R$  (Kumari et al., 2016, 2017). As expected, Fab30 robustly recognized receptor-bound  $\beta$ arr1; surprisingly however, it failed to recognize receptor-bound  $\beta$ arr2 for both the  $\beta_2V_2R$  (Figures 2A, 2B, and S5A) and the  $V_2R$  (Figures S5B and S5C). ScFv30 also exhibits a pattern identical to Fab30, i.e., it recognizes receptor-bound  $\beta$ arr1 but not  $\beta$ arr2 (Figures S5D and S5E). As the key residues in  $\beta$ arr1 responsible for binding Fab30 are mostly conserved in  $\beta$ arr2, and Fab30 can robustly bind  $V_2Rpp$ - $\beta$ arr2 complex, the lack of Fab30 reactivity toward receptor-bound  $\beta$ arr2 is unlikely to result from differences in its interaction interface between  $\beta$ arr1 and 2. In agreement with previous studies (Oakley et al., 2000), we also observed that  $\beta_2V_2R$  and  $V_2R$  robustly interact with  $\beta$ arr2 (Figures S6A–S6C), and therefore, the lack of Fab30 reactivity is also not because of the inability of  $\beta_2V_2R$  to bind  $\beta$ arr2.

In order to rule out the affinity difference of Fab30 for  $\beta$ arr1 versus  $\beta$ arr2, we carried out titration coIP experiments, first with increasing concentrations of  $V_2Rpp$ , and second, with increasing concentrations of  $\beta_2V_2R$  while keeping the concentrations of  $\beta$ arr1 and  $\beta$ arr2 constant. Fab30 recognizes  $V_2Rpp$ -bound  $\beta$ arr2 as efficiently as  $V_2Rpp$ -bound  $\beta$ arr1, even at the partial occupancy of  $V_2Rpp$  (Figures S7A and S7B). Moreover, even at 9-fold higher concentration of  $\beta_2V_2R$ , we still did not observe any detectable reactivity of Fab30 toward receptor-bound  $\beta$ arr2 (Figure S7C). These data suggest that the lack of Fab30 reactivity toward receptor-bound  $\beta$ arr2 is not due to an affinity difference of Fab30 or available stoichiometry of phosphorylated tail between  $V_2Rpp$  versus  $\beta_2V_2R$  experiments.

In order to validate our data in a cellular context, we next expressed hemagglutinin (HA)-tagged intrabody version of Fab30 (referred to as Ib30) together with  $\beta_2V_2R$  and either  $\beta$ arr1 or  $\beta$ arr2 in HEK293 cells, followed by a coIP experiment. Similar to *in vitro* experiments performed with purified proteins, we found that even in the cellular context, Ib30 recognizes receptor-bound  $\beta$ arr1 but not  $\beta$ arr2 (Figure S7D). Taken together,





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these findings suggest that there are potential conformational differences between  $\beta$ arr1 and 2 in complex with activated and phosphorylated receptors, which, in turn, results in the lack of Fab30 reactivity toward the receptor- $\beta$ arr2 complex.

### Fab30 Reactivity toward $\beta$ arr1 and 2 in the Presence of Homogeneously Phosphorylated Receptors

For the experiments mentioned in Figures 2A, 2B, and S5, we have utilized *in-cellulo*-phosphorylated receptors. A potential concern with respect to Fab30 reactivity may be heterogeneous phosphorylation of the receptor carboxyl-terminus when compared to synthetic  $V_2$ Rpp with a well-defined phosphorylation pattern. To rule out this possibility, we used two parallel approaches. First, we used Sortase enzyme-based chemical ligation of  $V_2$ Rpp to truncated  $\beta_2$ AR (29–341) in order to generate a chimeric  $\beta_2V_2R$  with a well-defined and homogeneous phosphorylation pattern identical to that present in  $V_2$ Rpp, following a slightly modified version of a previously published protocol (Staus et al., 2018) (Figures 2C and 2D). Interestingly, we observed that similar to the *in-cellulo*-phosphorylated receptor, this chemically ligated version of the receptor also induces a conformation in  $\beta$ arr2 that is not recognized by Fab30 (Figures 2E and 2F). Second, we generated a series of  $V_2$ R phosphorylation mutants lacking either the individual phosphorylation sites or cluster of phosphorylation sites and compared the ability of Fab30 to recognize receptor-bound  $\beta$ arr1 and 2 for these mutants. However, we did not observe any significant gain of Fab30 reactivity in any of these receptor mutants (Figure S7E). Taken together, these data suggest that the lack of Fab30 recognition for receptor-bound  $\beta$ arr2 does not arise from heterogeneous or site-specific phosphorylation of the receptor.

### Corroborating Evidence for Potential Conformational Differences between $\beta$ arr1 and 2

Leading up to this point, our data based on Fab30 recognition suggest potential conformational difference between receptor-bound  $\beta$ arr1 and 2. In order to corroborate these findings further, we tested a series of additional Fabs that we have recently generated and characterized (Ghosh et al., 2017). Similar to Fab30, these additional Fabs also interacted comparably with  $V_2$ Rpp-bound  $\beta$ arr1 and 2 (Figures S8A and S8B) and efficiently recognized the complex of  $\beta$ arr1 with an activated and phosphorylated receptor (Figures S8C and S8D). Interestingly however, these additional Fabs also displayed no detectable

recognition toward receptor-bound  $\beta$ arr2 (Figures S8C and S8D). Although the binding epitope of these additional Fabs on  $\beta$ arr1 has not been precisely determined yet, their reactivity pattern supports the notion of conformational differences between receptor-bound  $\beta$ arr isoforms.

As an additional line of evidence for the conformational differences between receptor-bound  $\beta$ arr1 and 2, independent of Fab30 reactivity as readout, we used bimane fluorescence spectroscopy. Here, we used purified  $\beta$ arr1 and 2 that are bimane labeled in their C-loop ( $\beta$ arr1<sup>245C</sup> and  $\beta$ arr2<sup>246C</sup>), which forms a key interface for receptor interaction and exhibits conformational rearrangement during receptor interaction (Latorraca et al., 2018) (Figure 3A). We observed a decrease in bimane fluorescence for  $\beta$ arr1 upon its interaction with the receptor, whereas there was significant increase for  $\beta$ arr2 (Figure 3B). These directionally opposite changes in bimane fluorescence intensities for  $\beta$ arr1 and 2 upon their interaction with the receptor suggest that their C-loops are positioned in different environments and provide additional corroborating evidence for their conformational difference in receptor-bound states. We did not observe a significant change in bimane fluorescence intensity upon  $V_2$ Rpp binding, which can be interpreted to reflect conformational similarity between  $V_2$ Rpp-bound  $\beta$ arr1 and 2 with respect to C-loop.

### Structural Insights into Conformational Differences between Receptor-Bound $\beta$ arrs

In order to better understand the Fab30 reactivity pattern and conformational differences between receptor-bound  $\beta$ arr1 and 2, we used molecular dynamics (MD) simulation to gain structural and mechanistic insight. The crystal structure of  $\beta$ arr1 in complex with  $V_2$ Rpp and Fab30 has revealed a major rotation of the C-domain relative to the N-domain by approximately 20° (Shukla et al., 2013). We postulated that this inter-domain rotation in  $\beta$ arrs may indeed be the primary determinant for effective recognition by Fab30, and in order to test this, we performed MD simulations monitoring the stability of Fab30 binding to  $\beta$ arr1 conformers with different inter-domain rotation angles in solution (Figures 3C, 3D, and S8E). Here, the Fab30-binding stability is assessed as root-mean-square deviation (RMSD) of the backbone atoms along the simulation time. We found that Fab30 remains stably bound to  $\beta$ arr1 conformers with a rotation angle > 15° (i.e., stable RMSD progression) (Figures 3C and 3D). In contrast, a significant instability of Fab30 interaction is observed for  $\beta$ arr1 conformers with a rotation angle < 15° (i.e., drastic

#### Figure 2. Fab30 Reactivity Pattern Reveals Potential Conformational Differences between Receptor-Bound $\beta$ arr1 and 2

(A) Fab30 robustly recognizes  $\beta_2V_2R$ -bound  $\beta$ arr1 but not  $\beta$ arr2, as assessed by colP. Here, carazolol (1  $\mu$ M) and BI-167107 (1  $\mu$ M) are used as an inverse agonist and agonist, respectively. A representative image from three independent experiments is shown.

(B) Densitometry-based quantification of data presented in (A). Values represent mean  $\pm$  SEM of three independent experiments analyzed using one-way ANOVA with Bonferroni post-test (\*\*\*)  $p < 0.001$ . Data are normalized with respect to the agonist- $\beta_2V_2R$ - $\beta$ arr1 condition (treated as 100%).

(C) Schematic flow-chart of sortase-based chemical ligation of  $V_2$ Rpp with truncated  $\beta_2$ AR (29–341) and subsequent colP to measure Fab30 reactivity.

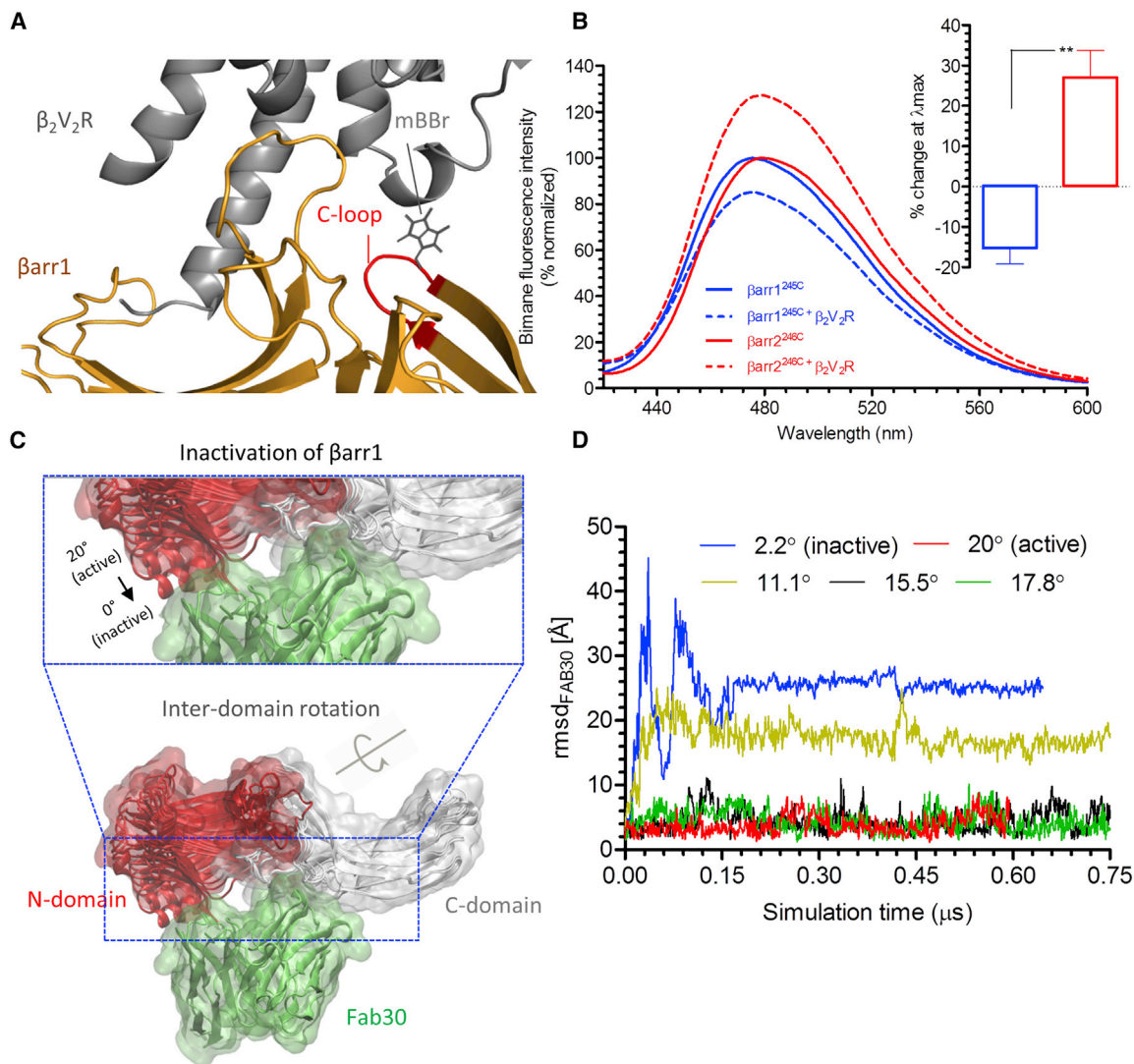
(D) Efficiency of sortase-based ligation of  $V_2$ Rpp to  $\beta_2$ AR (29–341), as measured by western blotting. A representative blot from two independent experiments is shown.

(E) Fab30 fails to recognize  $\beta$ arr2 in complex with homogeneously phosphorylated  $\beta_2V_2R$ . After sortase-based chemical ligation of  $V_2$ Rpp, the resulting  $\beta_2V_2R$  was incubated with equal concentrations of  $\beta$ arr1/2 and Fab30/Fab-CTL followed by colP. The reactivity of Fab30 with receptor-bound  $\beta$ arr1/2 was evaluated by western blot.

(F) Densitometry-based quantification of Fab30 reactivity toward receptor-bound  $\beta$ arr1 and 2, as measured in (E).

Data represent mean  $\pm$  SEM of three independent experiments, normalized with respect to  $\beta$ arr1 (treated as 100%).

See also Figures S2, S3, S4, S5, S6, and S7.



**Figure 3. Fluorescence Spectroscopy and MD Simulation Provide Insights into Conformational Differences between barr Isoforms**

(A) Schematic representation of the bimane fluorescence spectroscopy experiment where monobromobimane (mBBR) is chemically attached to a cysteine, engineered in the C-loop of barrs.

(B) The fluorescence intensity of  $\beta\text{arr1}^{\text{mBBR}}$  decreases upon its interaction with  $\beta_2\text{V}_2\text{R}$ , whereas that of  $\beta\text{arr2}^{\text{mBBR}}$  increases significantly. Here, purified barrs<sup>mBBR</sup> were incubated with purified  $\beta_2\text{V}_2\text{R}$  (agonist-bound and phosphorylated) at a molar ratio of  $\beta\text{arr1}:\beta_2\text{V}_2\text{R}$  (1:3 in a concentration range of 1–5  $\mu\text{M}$ ). As a reference, the fluorescence intensity of barrs<sup>mBBR</sup> alone was measured first and used for normalization (treated as 100%). The inset shows the differences in bimane fluorescence at  $\lambda_{\text{max}}$  for receptor-bound barr1 and 2.

(C) Fab30-binding stability depends on the inter-domain rotation angle of barr1.

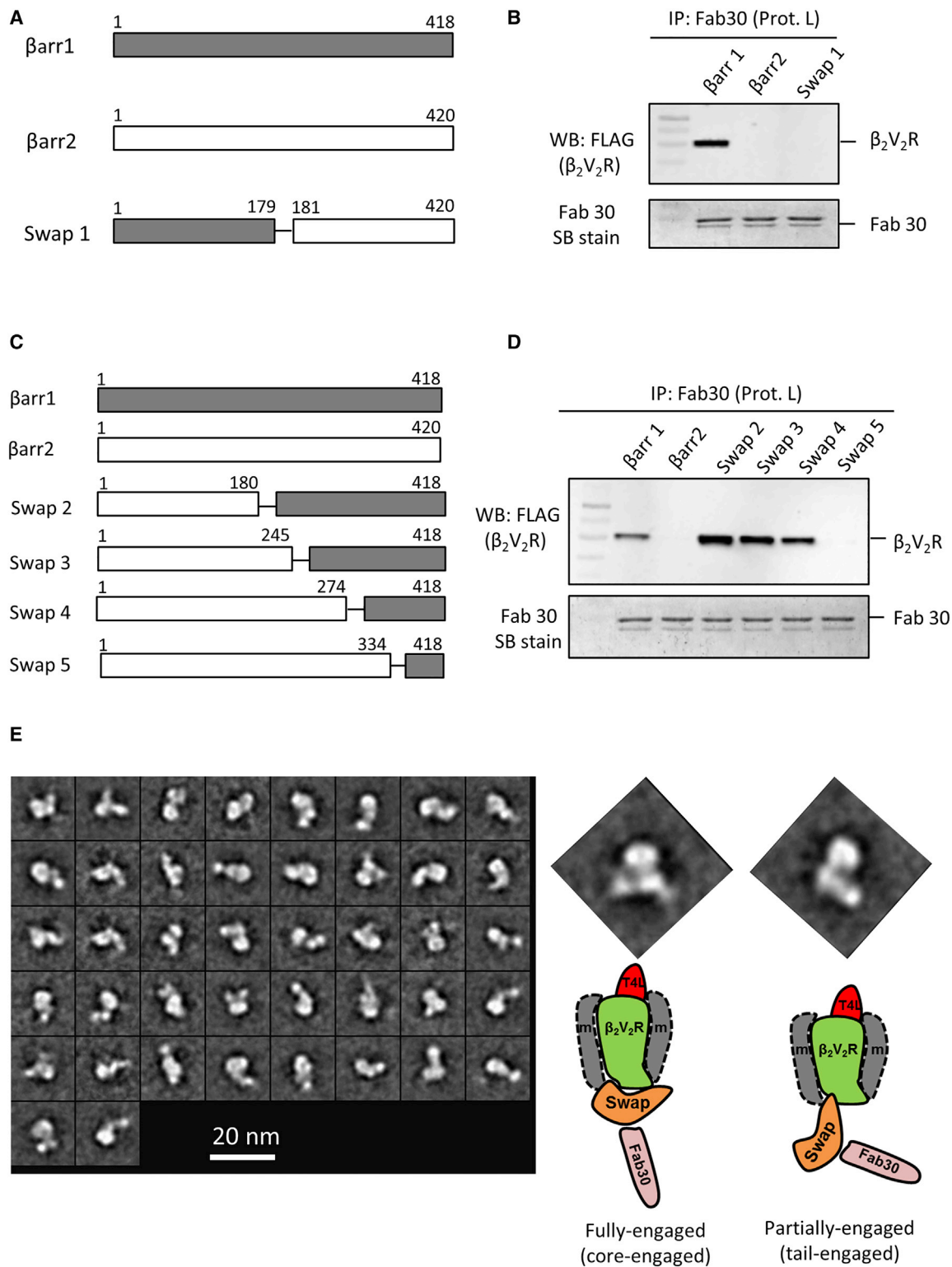
(D) The binding stability of the ScFv version of Fab30 (green surface) in complex with barr1 (N-domain, red surface; C-domain, white surface) is measured as RMSD of Fab30 backbone atoms ( $\text{RMSD}_{\text{Fab30}}$ ) for different activation states of barr1 (inter-domain rotation angles 2.2°, 11.1°, 15.5°, 17.8°, and 20°). Inter-domain rotation angles 15.5° and 20° in barr1 result in a stable  $\text{RMSD}_{\text{Fab30}}$  progression. Rotation angles of 2.2° and 11.1° provoke a rapid increase of the  $\text{RMSD}_{\text{Fab30}}$  (i.e., binding instability) during the first 50 ns of simulation time due to the clash of the N-domain with Fab30.

See also [Figure S8E](#).

increase in RMSD) (Figures 3C and 3D). Such instability is not surprising because the N-domain of barr1 approaches Fab30 when the inter-domain rotation relaxes toward the inactive (or basal) state (i.e., rotation angle decreasing from 20° to 0°), which, in turn, results in unfavorable contacts and steric clashes (Figures 3C and 3D). In other words, Fab30 reactivity can be considered as readout of the degree of inter-domain rotation in barrs

upon activation, and therefore, it is plausible that the inter-domain rotation in receptor-bound barr2 is significantly different than barr1, resulting in the lack of recognition by Fab30.

MD simulation data presented above raise the possibility that the core engagement with the receptor may contribute toward potential conformational differences between receptor-bound barr1 and 2. In line with this possibility, we observed a relatively



**Figure 4. Identification of Structural Regions That Impart Conformational Differences between Receptor-Bound  $\beta$ arr1 and 2**

(A) Schematic representation of the swap1 construct that harbors the N-domain of  $\beta$ arr1 and the C-domain of  $\beta$ arr2.

(B) Fab30 fails to detect receptor-bound conformation of swap1 as evaluated by colP, carried out in a similar fashion as in Figure 2A. A representative image of three independent experiments is shown here.

(legend continued on next page)



greater interaction of  $\beta$ arr2 with  $V_2R$  in the absence of “tail-engagement” by using a carboxyl-terminus-truncated receptor construct (referred to as  $V_2R^{\Delta C-term}$ ) (Figure S9A). This finding may be interpreted to suggest that the core interaction for  $\beta$ arr2 is stronger compared to  $\beta$ arr1, and therefore, receptor- $\beta$ arr2 complexes may predominantly exist in a fully engaged conformation that is different from that of  $\beta$ arr1. This provides a plausible explanation for the near-complete lack of Fab30 reactivity toward receptor-bound  $\beta$ arr2. However, in the absence of high-resolution structures of fully engaged receptor- $\beta$ arr complexes, it is not feasible to precisely determine the contribution of core engagement toward imparting distinct  $\beta$ arr conformations, and future structural studies are necessary to illuminate the mechanism of distinct  $\beta$ arr conformations upon their interaction with the receptors.

### Distal C-Domain in $\beta$ arrs Is Important for the Structural Differences between the Two Isoforms

To identify the key regions in  $\beta$ arrs that are potentially responsible for imparting distinct conformations on  $\beta$ arr1 and 2, we generated a series of chimeric  $\beta$ arr constructs and tested the reactivity of Fab30 toward their complexes with the receptor. Unlike visual arrestins and  $\beta$ arr1,  $\beta$ arr2 lacks the c-edge loops (these are different from the C-loop described earlier) that are proposed to anchor the C-domain of visual arrestin to the plasma membrane upon its recruitment to rhodopsin (Lally et al., 2017). Therefore, we first generated a  $\beta$ arr2 construct where we grafted the c-edge loop1 of  $\beta$ arr1 in the corresponding position of  $\beta$ arr2 and tested the reactivity to Fab30 (Figures S9B and S9C). However, similar to  $\beta$ arr2, this loop-grafted construct also failed to exhibit detectable recognition by Fab30 (Figures S9B and S9C), suggesting that the lack of c-edge loop1 in  $\beta$ arr2, and thereby, the potential lack of membrane anchoring, is not responsible for its conformational difference with  $\beta$ arr1.

Next, we generated a construct, referred to as swap1, harboring the N-domain of  $\beta$ arr1 and the C-domain of  $\beta$ arr2. Fab30 did not recognize receptor-bound swap1, suggesting that it adopts a conformation similar to  $\beta$ arr2 and that the primary determinants of distinct conformations of receptor-bound  $\beta$ arr1 and 2 are likely encoded in the C-domain (Figures 4A, 4B, and S9D). This is further confirmed by a reverse chimera, referred to as swap2, harboring the N-domain of  $\beta$ arr2 and the C-domain of  $\beta$ arr1 which is effectively recognized by Fab30 (Figures 4C, 4D, and S9E). We also generated additional chimeric constructs, referred to as swap3-5, harboring the N-domain of  $\beta$ arr2 and different segments of  $\beta$ arr1 C-domain. CoIP experiments revealed that the structural determinants of conformational differences between  $\beta$ arr isoforms, as measured by Fab30 reactivity, primarily reside in the distal C-domain at the primary sequence level (Figures 4C and 4D). The pattern of Fab30 reactivity also indicates that the

conformation of receptor-bound swap1 is similar to  $\beta$ arr2, whereas that of swap2 is similar to receptor-bound  $\beta$ arr1. To provide additional support for the interaction of swap2 with the receptor in a manner similar to  $\beta$ arr1, we carried out negative-staining-based single-particle electron microscopy (EM) analysis of a Fab30-stabilized  $\beta_2V_2R$ - $\beta$ arr<sup>swap2</sup> complex (Figure 4E). This complex also exhibits a biphasic interaction with conformational distribution between the partially engaged and fully engaged complexes, similar to what was previously observed for the  $\beta_2V_2R$ - $\beta$ arr1-Fab30 complex (Shukla et al., 2014). We also tested the ability of Ib30 to recognize receptor-bound swap2 in a cellular context, and in agreement with the data presented in Figures 4C and 4D, Ib30 robustly recognizes receptor-bound swap2, at a level similar to that of  $\beta$ arr1 (Figures 5A and 5B). This observation further suggests an overall similar conformation adopted by receptor-bound  $\beta$ arr1 and swap2 and provides supporting evidence for the cellular relevance of the data obtained with chimeric  $\beta$ arr constructs *in vitro*.

### A Potential Link between $\beta$ arr Conformations and Their Distinct Functional Contributions

To probe whether conformational differences between receptor-bound  $\beta$ arr isoforms may be directly linked to their functional divergence, we first measured the contribution of  $\beta$ arr1 and 2 in agonist-induced endocytosis, extracellular signal regulated kinase 1/2 (ERK1/2) microtubule-associated protein mitogen-activated protein (MAP) kinase phosphorylation, and cyclic AMP (cAMP) response for the  $V_2R$  under  $\beta$ arr1 or 2 knockdown conditions (Figures 5C–5F and S10A–S10C). We found that the presence of either  $\beta$ arr1 or 2 is capable of supporting agonist-induced endocytosis of the  $V_2R$  (Figure 5C), suggesting that the two isoforms are functionally redundant in mediating receptor endocytosis. Interestingly, agonist-induced ERK1/2 MAP kinase phosphorylation downstream of  $V_2R$  is sensitive to the depletion of either isoform (Figures 5D and S10C), suggesting that both are involved. Strikingly however, agonist-induced cAMP response is significantly enhanced upon  $\beta$ arr2 depletion (Figure 5E), potentially indicating a predominant role of  $\beta$ arr2 in receptor desensitization compared to  $\beta$ arr1. The surface expression of  $V_2R$  in  $\beta$ arr1- or 2-depleted cells are comparable to each other (Figure S10B). As expected, the exogenous expression of  $\beta$ arr2 in  $\beta$ arr2 knockdown cells lowers the level of cAMP (Figure 5F). Most interestingly, the exogenous expression of swap1, which is conformationally similar to  $\beta$ arr2, also effectively lowers the enhanced level of cAMP in  $\beta$ arr2 knockdown cells (Figure 5F). Taken together, these data suggest that receptor-bound  $\beta$ arr2 is potentially more effective in driving receptor desensitization compared to  $\beta$ arr1 and, thus, provide a possible link between the conformational differences of receptor-bound  $\beta$ arrs and their functional divergence.

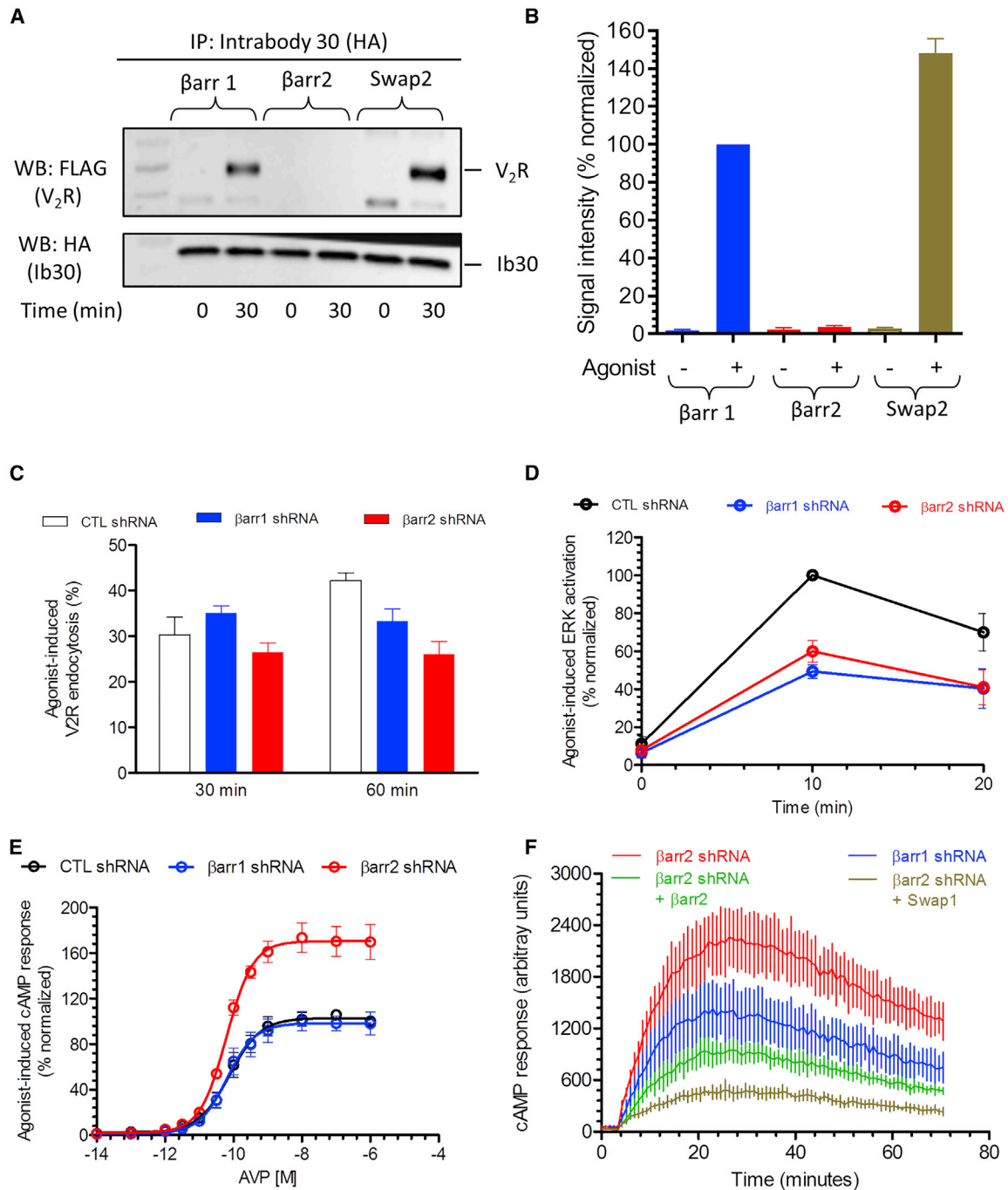
(C) Schematic representation of swap2-5 constructs that harbor N-domain of  $\beta$ arr2 and different stretches of the C-domain of  $\beta$ arr1.

(D) Fab30 reactivity in coIP reveals that the distal C-domain imparts conformational differences between  $\beta$ arr1 and 2.

(E) 2D class averages derived from single-particle negative-staining EM analysis of the agonist- $\beta_2V_2R$ -swap2-Fab30 complex carried out as described in the STAR Methods section. The right panel shows two representative 2D class averages depicting the partially engaged and fully engaged complexes.

A schematic representation of these two conformations is presented for the ease of visualization.

See also Figure S9.



**Figure 5. A Domain-Swapped Chimera of βarrs Gains Fab30 Reactivity in Cellular Context and Exhibits Functional Complementation**

(A) Intrabody 30 (Ib30) efficiently recognizes receptor-bound swap 2 upon agonist stimulation in a cellular context, as assessed by a coIP experiment using the lysate of HEK293 cells expressing FLAG-V<sub>2</sub>R, βarr1, and 2 or swap 2 and Ib30. Cells were stimulated with arginine vasopressin (AVP; 100 nM) for indicated time points, followed by coIP, and a representative image from three independent experiments is shown.

(B) Densitometry-based quantification of data presented in (A). Values represent mean signal intensity ± SEM normalized with respect to agonist-V<sub>2</sub>R-βarr1 condition (treated as 100%).

(C) Either isoform of βarrs is sufficient to mediate agonist-induced endocytosis of V<sub>2</sub>R measured by whole-cell ELISA in HEK293 cells. Data represent mean ± SEM of five independent experiments, with each carried out in duplicate. Percent endocytosis as measured by the surface level of V<sub>2</sub>R before and after agonist-stimulation is presented in the graph.

(legend continued on next page)

## DISCUSSION

Most GPCRs recruit both  $\beta$ arr1 and 2 upon agonist stimulation, which, in turn, mediate and regulate receptor desensitization, endocytosis, and signaling. Although both isoforms are individually capable of mediating the above-mentioned functions, interestingly, they often display a differential contribution toward these functions and subsequent physiological outcomes for different receptor systems. This paradigm, now observed across multiple GPCRs (Srivastava et al., 2015), suggests potential differences at structural and conformational levels in receptor-bound states of  $\beta$ arr1 and 2. We observed that although the docking interface for the phosphorylated receptor tail and the resulting conformations in  $\beta$ arr1 and 2 are similar to each other, they appear to adopt different conformations upon their engagement with activated and phosphorylated receptors.

These findings suggest that a differential conformational rearrangement may happen in  $\beta$ arr1 versus 2 when they transition from the partially engaged to fully engaged complex involving the receptor core. In other words, the core engagement between the receptor and  $\beta$ arrs may impart distinct structural changes in the two isoforms of  $\beta$ arrs. In fact, a comparison of the crystal structures of pre-activated visual-arrestin (i.e., splice variant p44) with the rhodopsin-visual-arrestin complex reveals significant structural changes in visual arrestin (Kang et al., 2015; Zhou et al., 2017; Kim et al., 2013). Moreover, a recent study on the rhodopsin-visual-arrestin system has also suggested that both the receptor tail and the receptor core are capable of inducing activating conformational changes in visual arrestin, independent of each other (Latorraca et al., 2018). Additional studies in a cellular context have also reported that the core engagement can drive an active conformation in  $\beta$ arr2, which allows it to enrich in clathrin-coated structures, even in the absence of a stable complex with the receptor (Eichel et al., 2016, 2018). Although these previous studies align with our findings raising the possibility of core engagement driving the conformational differences between receptor-bound  $\beta$ arr1 and 2, future structural studies should illuminate the structural mechanism underlying this interesting phenomenon. As different GPCRs have diverse signatures of phosphorylatable residues in their carboxyl-terminus or in intracellular loops and may have different levels of core engagement, it may not be surprising to discover additional levels of conformational diversity in  $\beta$ arrs, which makes this system precisely tunable in a context-dependent manner (Ranjan et al., 2017).

It is also intriguing that the differences between  $\beta$ arr1 and 2 appear to arise primarily from the distal C-domain, a region

that is not only most diverse between the two isoforms at the primary sequence level but also harbors the interface for several interaction partners, such as clathrin, adaptin, and TRAF6. Therefore, it is tempting to speculate that receptor-bound  $\beta$ arr1 and 2 may also differ in their ability to scaffold different partners, owing to their conformational differences. In fact, such a scenario is supported by a global interactomics analysis where a significant difference between the interactome of  $\beta$ arr1 and 2 is reported (Xiao et al., 2007). Future studies may shed light on this interesting possibility, including a precise mapping of  $\beta$ arr residues that may be responsible for the differential reactivity of Fab30 toward  $\beta$ arr isoforms.

A previously published hydrogen/deuterium exchange (HDX) study of  $\beta$ arrs using pre-activated mutants ( $\beta$ arr1<sup>R169E</sup> and  $\beta$ arr2<sup>R170E</sup>) provides additional corroborating evidence for our findings (Yun et al., 2015). A re-analysis of this previous study reveals a significant difference between the HDX pattern of lariat loop peptides when we compared  $\beta$ arr1<sup>WT</sup>- $\beta$ arr1<sup>R169E</sup> and  $\beta$ arr2<sup>WT</sup>- $\beta$ arr2<sup>R170E</sup> with each other. For example, we observed a significantly higher rate of deuterium uptake in the 279–289 segment of  $\beta$ arr1 between the wild type (WT) and R<sup>169E</sup> mutant, but the corresponding region in  $\beta$ arr2 does not exhibit a significant difference between the WT and R<sup>170E</sup> mutant (Figure S10D). Although pre-activated mutants may not be a perfect surrogate of fully active  $\beta$ arr conformations, the HDX pattern does suggest that the lariat loop region may adopt different conformations for activated  $\beta$ arr1 versus  $\beta$ arr2, and it further complements our domain-swapping data described above.

Our functional data link the conformational differences between receptor-bound  $\beta$ arr1 and 2, as reported by the Fab30-based sensor, with their different contributions in V<sub>2</sub>R desensitization. Previous studies have discovered that agonist-induced V<sub>2</sub>R endocytosis and ERK1/2 MAP kinase phosphorylation can be efficiently supported by partially engaged receptor- $\beta$ arr complexes, whereas receptor desensitization is driven primarily by the core-engaged complex (Kumari et al., 2017). This aligns with the possibility of differential core engagement for  $\beta$ arr1 and 2 leading to their distinct contribution in receptor desensitization but comparable contribution in V<sub>2</sub>R endocytosis and ERK MAP kinase activation. It is also conceivable, however, that an additional level of conformational differences in  $\beta$ arr isoforms may exist and drive their functional divergence with respect to endocytosis, signaling, and ubiquitination for other GPCRs.

Our findings also raise some interesting questions and open new avenues for future investigations in this therapeutically important research area. For example, some GPCRs, such as

(D) Both isoforms of  $\beta$ arrs contribute to agonist-induced ERK MAP kinase activation downstream of V<sub>2</sub>R, as measured by western blotting in HEK293 cells. Data represent mean  $\pm$  SEM of five independent experiments and are normalized with maximal ERK activation in control condition (treated as 100%). A representative image of these experiments is shown in Figure S10C.

(E) Depletion of  $\beta$ arr2 enhances agonist-induced cAMP response in HEK293 cells expressing V<sub>2</sub>R, as measured using the GloSensor assay. Data represent mean  $\pm$  SEM of three independent experiments, with each carried out in duplicate and normalized with the maximal cAMP response in CTL condition (treated as 100%).

(F) Exogenous expression of  $\beta$ arr2 or swap1 lower the enhanced levels of cAMP in  $\beta$ arr2 knockdown cells, suggesting a potential link between receptor-bound  $\beta$ arr2 conformation and receptor desensitization.

A representative profile from three independent experiments, each performed in duplicate, is shown here. See also Figure S10.

muscarinic receptors, contain a very short carboxyl-terminus but harbor phosphorylation sites in their 3<sup>rd</sup> intracellular loops. Do such receptors also use a biphasic mechanism of interaction with  $\beta$ arrs, and do the two  $\beta$ arr isoforms adopt distinct conformations for such receptors? Might there exist different conformations of receptor-bound  $\beta$ arr1 and 2 in response to stimulation by  $\beta$ arr-biased ligands, and how do such conformational signatures govern the ensuing bias at the functional level? In addition, high-resolution structures of GPCR- $\beta$ arr complexes, preferably of different  $\beta$ arr isoforms and different receptors, are still required to better understand the commonalities and differences in these signaling complexes. Future investigations to address some of these aspects should clearly offer novel insights into GPCR- $\beta$ arr interaction and reveal how conformational differences in receptor-bound  $\beta$ arrs fine-tune their functional outcomes.

In conclusion, we discover structural and conformational differences between receptor-bound  $\beta$ arr isoforms that are potentially associated with their functional divergence in the context of GPCR regulatory and signaling paradigms. Our findings underline the importance of carefully considering both isoforms of  $\beta$ arrs when designing and characterizing  $\beta$ arr-biased GPCR ligands and, thus, have direct implications for an ever-growing area of biased agonism aimed at designing novel GPCR therapeutics.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.08.053>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, E.G., A.K.S., and J.S.; Methodology, E.G., A.K.S., and J.S.; Investigation, E.G., H.D., M.B., A.S., P.K., T.S., H.R.K., M.-H.L., J.v.G., M.C., D.R., S.P., J.M., R.G.-G., and S.D.; Writing – Original Draft, A.K.S.; Writing – Review & Editing, E.G., A.K.S., and J.S.; Funding Acquisition, A.K.S., L.M.L., K.Y.C., S.D., and J.S.; Resources, L.M.L.; Supervision, A.K.S., L.M.L., K.Y.C., S.D., and J.S.; Project Administration, A.K.S.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Streptavidin-HRP	Genscript	Cat# M00091
Protein L-HRP	Genscript	Cat# M00098
Monoclonal ANTI-FLAG M2-HRP antibody	Sigma-Aldrich	Cat# A8592;RRID:AB_439702
Phospho-p44/42 MAPK (Erk1/2)	Cell Signaling Technology	Cat# 9101; RRID:AB_331646
p44/42 MAPK (Erk1/2) Antibody	Cell Signaling Technology	Cat# 9102; RRID:AB_330744
β-Arrestin 1/2 (D24H9) Rabbit mAb	Cell Signaling Technology	Cat# 4674; RRID: AB_10547883
HA-probe (Y-11) antibody	Santacruz Biotechnology	Cat# sc-805, RRID:AB_631618
Anti-rabbit IgG secondary antibody	GenScript	Cat# A00098
<b>Bacterial and Virus Strains</b>		
<i>E. coli</i> DH5α	NEB	Cat# C29871
<i>E. coli</i> BL21 (DE3)	NEB	Cat# C25271
<i>E. coli</i> 55244	ATCC	ATCC,55244
Baculovirus carrying β2V2R, V2R and GRK2	Cloned in pVL1393	N/A
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Luria Bertani Broth, Miller	Sisco Research Laboratories (SRL)	Cat# 29817 (LM019)
2 XYT Growth Medium	Himedia	Cat# G034-500G
DSP (Dithiobis succinimidyl-propionate)	Sigma Aldrich	Cat# D3669
Paraformaldehyde (PFA)	Sigma Aldrich	Cat# P6148,CAS no. 30525-89-4
Isopropyl-B-D-Thiogalactopyranoside (IPTG)	SRL	Cat# 67208 (094866)
Lysozyme (3x cryst) ex. Egg white	SRL	Cat# 45822 (124013)
Phenylmethane Sulphonyl Fluoride (PMSF)	SRL	Cat# 84375 (84375)
Benzamidine Hydrochloride	SRL	Cat# 93014 (0248255)
Phosphatase inhibitor cocktail (Phosstop)	Roche	4906837001
Monobromobimane	Sigma Aldrich	Cat# B4380,CAS no. 71418-44-5
Thrombin, Bovine	Merck	Cat# 605157,CAS no. 9002-04-4
L-Glutathione reduced	Sigma Aldrich	G4251, CAS no. 70-18-8
Lauryl Maltose Neopentyl Glycol (MNG)	Anatrace	NG310 CAS no.1257852-96-2
FLAG peptide	Genscript	N/A
TMB (Tetramethylbenzidine)	Genscript	M00078
Luciferin sodium salt	Gold Biotech	Cat# LUCNA,CAS no. 103404-75-7
Puromycin dihydrochloride	Gold Biotech	Cat# P-600-100
PEI (Polyethylenimine)	Polysciences	Cat# 23966
Bovine Serum Albumin, BSA	SRL	83803 (0140105)
HBSS - Hank's Balanced Salt Solution	Thermo Fisher Scientific	Cat# 14065
GIBCO Fetal Bovine Serum	Thermo Fisher Scientific	Cat# 10270-106
DMEM	Cellclone	Cat# CC3004
GIBCO Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# 15140122
ESF 921 Insect Cell Culture Medium	Expression Systems	Cat# 96-001-01
FLAG Peptide	Genscript	N/A
Isoproterenol Bitartrate	Sigma Aldrich	Cat# I2760 CAS No.54750-10-6
Carazolol	ApexBio	Cat# C5802.
BI 167107	Synthesized	N/A
Tolvaptan	Sigma Aldrich	Cat# T7455 CAS No.150683-30-0

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Arginine Vasopressin Peptide (AVP)	Genscript	N/A
V2Rpp and 3G-V2Rpp	Tuft's Peptide Synthesis Facility	N/A
Critical Commercial Assays		
Glosensor	Promega	Cat# E2301
Experimental Models: Cell Lines		
Human: HEK293T	ATCC	Cat# CRL-3216
Insect: Sf9	Expression systems	N/A
Oligonucleotides		
N/A	N/A	N/A
Recombinant DNA		
$\beta$ arr1 N245C expression plasmid	Genscript	N/A
$\beta$ arr2 S246C expression plasmid	Genscript	N/A
$\beta$ arr1/2-mcherry	Dr Mark G.H. Scott	N/A
$\beta$ arr1/2 shRNA	Dr Hyder Ali	N/A
cDNA cassette for $\beta$ 2V2R	cDNA resource center	N/A
cDNA cassette for V2R	cDNA resource center	N/A
cDNA cassette for GRK2	cDNA resource center	N/A
Sortase A pentamutant (eSrtA) in pET29	<a href="#">Chen et al., 2011</a>	Addgene Plasmid #75144
Software and Algorithms		
Image Lab	Bio-Rad	N/A
Graphpad Prism	Graphpad	N/A
Zen lite, Zeiss	Zeiss	<a href="https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html">https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html</a>
Pymol	Schrodinger LLC	N/A
ProteinLynx Global Server 2.4	Waters	N/A
DynamX program	Waters	N/A
ACEMD simulation package	<a href="#">Harvey et al., 2009</a>	N/A
CHARMM36m forcefield	<a href="#">Huang et al., 2017</a>	N/A
CHARMM36 forcefield	<a href="#">Klauda et al., 2010</a>	N/A
MOE software package	Chemical Computing Group (CCG)	<a href="https://www.chemcomp.com/">https://www.chemcomp.com/</a>
Other (Resins)		
M1-FLAG resin	In-house	N/A
Captol (Protein L)	GE Lifesciences	17547802
Glutathione resin	Genscript	Cat# L0026
Mouse anti-HA (Hemagglutinin) resin	Sigma	Cat# A2095
His60 Ni Superflow resin	Clontech	Cat# 635660

## LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Arun K. Shukla ([arshukla@iitk.ac.in](mailto:arshukla@iitk.ac.in)). This study did not generate new unique reagents.

## EXPERIMENT MODEL AND SUBJECT DETAILS

### Bacterial cell culture

Three bacterial strains *viz.*, *E. coli* DH5 $\alpha$ , *E. coli* BL21(DE3) pLysS and *E. coli* 55244 were used in the current study and were cultured according to standard protocols.



### Mammalian cell culture

HEK293 cells (Female) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and further supplemented with penicillin and streptomycin. Cells were regularly checked for mycoplasma contamination.

### Insect cell culture

Sf9 cells (Female) were grown in serum-free media and maintained at 27°C. Cells were infected with baculoviruses as per standard protocols.

## METHOD DETAILS

### Construct design and protein expression

*E. coli* expression constructs for  $\beta$ arr1 and 2, Fab30 and ScFv30 are described earlier, and these proteins were purified using previously described protocols (Kumari et al., 2017).  $\beta$ arr1<sup>N245C</sup>/ $\beta$ arr2<sup>S246C</sup> were generated on a minimal cysteine background (i.e., harboring C59V, C68L, C125S, C140L, C150V, C242V, C251V, C269S mutations) by site-directed mutagenesis, and purified using a similar protocol as for wild-type. Expression constructs for  $\beta_2$ V<sub>2</sub>R, GRK2, V<sub>2</sub>R, and their purification details have also been published previously (Kumari et al., 2017). Briefly, FLAG- $\beta_2$ V<sub>2</sub>R and FLAG-V<sub>2</sub>R were co-expressed with GRK2 in Sf9 cells (cultured in ESF921 media from Expression Systems), and 60–66h post-infection; cells were stimulated with indicated ligands and harvested by centrifugation.

### Sequence and structural analysis of $\beta$ arrs

Phosphate interacting residues in V<sub>2</sub>Rpp-bound  $\beta$ arr1 were identified based on the previously determined crystal structure (PDB ID: 4JQI). They were compared to  $\beta$ arr2 by sequence alignment, structural visualization in PyMol and subsequent analysis in PDBsum as indicated in respective figure legends.

### ELISA assay

In order to assess the interaction of Fab30 with V<sub>2</sub>Rpp-bound  $\beta$ arrs (presented in Figures 1D and S3E), we first immobilized purified protein L (Genscript) onto MaxiSorp ELISA plates (Nunc). Subsequently, we incubated the wells with 1% BSA (Bovine Serum Albumin) to block non-specific binding. Afterward, we added purified Fabs (in 20 mM HEPES, pH 7.4, 150mM NaCl; 1–3  $\mu$ g per well in 100  $\mu$ L volume) followed by gentle washing to remove unbound Fabs, and then added purified biotinylated  $\beta$ arrs (in 20 mM HEPES, pH 7.4, 150mM NaCl, 0.01% MNG; 1–3  $\mu$ g per well in 100  $\mu$ L volume) (with or without pre-incubation with V<sub>2</sub>Rpp). After an incubation of 15–30 min, wells were washed extensively (using 20mM HEPES, pH 7.4, 100mM NaCl, 0.01% MNG), and incubated with HRP-coupled streptavidin (Genscript; 1:2000 dilution of 1  $\mu$ g/ml). After another round of extensive washing, the reactivity of Fab30 with  $\beta$ arrs was visualized by adding TMB ELISA (Genscript). Colorimetric reaction was stopped by adding 2M H<sub>2</sub>SO<sub>4</sub> and absorbance was measured at 450nm using a Victor X4 plate reader (Perkin-Elmer, USA).

In order to assess the recognition of receptor-bound  $\beta$ arrs by Fab30 (presented in Figure S5A), we followed a recently described protocol (Kumari et al., 2016). Here, we first immobilized Fab30 on the ELISA plate followed by the addition of activated and phosphorylated receptor (in the form of cell lysate) mixed with purified  $\beta$ arrs. Their interaction was detected using HRP-coupled anti-FLAG M2 antibody (Sigma, 1:2,000 dilution) as the receptor contains an N-terminal FLAG tag.

### Co-immunoprecipitation assay

For coimmunoprecipitation based detection of Fab30 reactivity toward V<sub>2</sub>Rpp-bound  $\beta$ arrs (presented in Figures 1E, S3B–S3D, S8A, and S8B), purified proteins were mixed together (in buffer containing 20mM HEPES, pH 7.4, 150mM NaCl, 0.01% MNG; 1:3 fold molar ratio of  $\beta$ arr:Fab; final concentrations in the range of 1–10  $\mu$ M) and incubated at room-temperature for 1h. Subsequently, protein L agarose beads were added to the reaction mix and incubated for an additional 1h at room-temperature. Beads were washed three times by centrifugation (using 20mM HEPES, pH 7.4, 150mM NaCl, 0.01% MNG), bound proteins were eluted using SDS sample buffer and separated by SDS-PAGE.

In order to evaluate the interaction of receptor-bound  $\beta$ arrs with Fabs by coIP, we used cell lysate prepared from Sf9 cells co-expressing recombinant FLAG-tagged receptor and GRK2. Cells were first stimulated with an inverse agonist (to generate inactive and non-phosphorylated receptor) or an agonist (to generate activated and phosphorylated receptor). Cell lysate was pre-incubated with purified  $\beta$ arrs and Fab30 at room temperature for 1h. Subsequently, protein L beads (equilibrated in 20mM HEPES, pH 7.4, 100mM NaCl and 0.01% MNG) were added to the reaction mix and tumbled for an additional 1h. Beads were washed three times with washing buffer (same as equilibration buffer), bound proteins were eluted with SDS sample loading buffer and analyzed by western blotting (HRP-coupled anti-FLAG M2 HRP from Sigma at 1:2000 dilution; HRP-coupled Protein L from Genscript at 1:2000 dilution).

In an alternative coIP set-up (presented in Figure S7C), HEK293 cells expressing  $\beta_2$ V<sub>2</sub>R,  $\beta$ arr-mCherry and HA tagged ScFv30 as an intrabody, were stimulated with agonist or inverse agonist followed by coIP using anti-HA antibody coupled agarose beads (Sigma). Interaction of ScFv30 intrabody with  $\beta$ arr1 and 2 were visualized by western blotting.

### Sortase ligation protocol

For the preparation of receptor with homogeneous phosphorylation, we followed a slightly modified version of a previously published protocol (Staus et al., 2018). Briefly, Sf9 cells expressing  $\beta_2$ AR (29-341) was first stimulated with 10nM BI-167107 for 30min and then resuspended in lysis buffer (50mM HEPES pH 7.4, 150mM NaCl, 10nM BI-167107, 1mM PMSF and 2mM benzamidine). Cells were lysed by dounce homogenization and lysate was solubilized in 1%(v/v) MNG for 2h at room temperature and cleared by centrifugation at 15000 rpm for 30min. Supernatant was incubated with pre-equilibrated M1-FLAG beads supplemented with 2mM  $\text{CaCl}_2$  for 2h at 4°C. Beads were washed alternately with low salt buffer(50mM HEPES pH 7.4, 150mM NaCl, 0.01% MNG, 10nM BI-167107 and 2mM  $\text{CaCl}_2$ ) and high salt buffer(50mM HEPES pH7.4, 350mM NaCl, 0.01% MNG, 10nM BI-167107 and 2mM  $\text{CaCl}_2$ ) respectively. For ligation reaction, beads were resuspended in buffer containing 50mM HEPES, pH 7.4, 100mM NaCl, 0.01% (v/v) MNG, 10nM BI-167107, 5mM  $\text{CaCl}_2$ , 50uM GGG-V<sub>2</sub>Rpp (GGGARGRpTPPpSLGPQDEpSCpTpTApSpSpSLAKDTSS) and 2  $\mu$ M sortaseA. Slurry was incubated overnight at 4°C and next day beads were alternately washed with low salt buffer and high salt buffer respectively. Ligated receptor was eluted with FLAG peptide and protein-L coIP assay was performed to assess the interaction of  $\beta$ arrs with receptor.

### Confocal microscopy

HEK293 cells (ATCC) were cultured in DMEM (Sigma) supplemented with 5% Fetal Bovine Serum (Thermo Scientific) and 1% Penicillin-Streptomycin at 37°C under 5% CO<sub>2</sub>. Cells were transfected with indicated plasmids using PEI (Poly Ethylene Imine) at a DNA to PEI ratio of 1:3. 24h post-transfection, cells were split and seeded onto poly-L-lysine coated coverslips. After an additional 24h, cells were serum starved for 2h and then used for live cell imaging (LSM780NLO confocal microscope from Carl Zeiss) (Figures 2E and S7B). Cells were stimulated with agonists for indicated time-points as mentioned in the respective figure legends.

### Bimane fluorescence assay

Experimental details of bimane labeling and fluorescence measurements have been described in detail previously (Kumari et al., 2016). Briefly, purified  $\beta$ arr1<sup>N245C</sup> and  $\beta$ arr1<sup>S246C</sup> were buffer exchanged in labeling buffer (20mM HEPES, 150mM NaCl, pH 7.5), and then incubated (approximately at a concentration of 1mg/ml) with freshly prepared monobromobimane (mBBr, Sigma-Aldrich) at a 10-fold molar excess. After 1h incubation on ice, unreacted mBBr was separated on a PD10 desalting column (GE Healthcare). Labeled  $\beta$ arrs were either used right away in the fluoresce measurements or they were flash frozen with 10% glycerol and stored at -80°C for later usage. For measuring the conformational change in the finger loop, mBBr labeled  $\beta$ arrs were mixed at 1:3 molar ratios with purified  $\beta_2$ V<sub>2</sub>R. Purified receptors were also buffer exchanged in 20mM HEPES, pH 7.5, 150 mM NaCl, 0.01% MNG (Maltose Neopentyl Glycol), and final reactions were prepared such to maintain a consistent buffer (and detergent conditions). After incubating the mixture for 1h at room temperature, bimane fluorescence intensity was measured using a Fluorometer (Perkin Elmer, USA model LS-55) in photon counting mode as described previously (Kumari et al., 2016).

### Functional assays

HEK293 cells were infected with previously described and validated lentiviral shRNAs targeting either  $\beta$ arr1 or 2 followed by generation of stable cells lines with puromycin selection using standard protocol (Vibhuti et al., 2011). Agonist-induced cAMP, receptor endocytosis and ERK MAP kinase phosphorylation was measured using previously described protocols (Kumari et al., 2017).

### Negative staining single particle analysis of $\beta_2$ V<sub>2</sub>R- $\beta$ arr<sup>swap2</sup>-Fab30 complex

Samples for negative stain EM was prepared by conventional negative staining method (Ohi et al., 2004). Around 3.5  $\mu$ l of the purified complex of  $\beta_2$ V<sub>2</sub>- $\beta$ arr<sup>swap2</sup>-Fab30 was adsorbed on glow discharged carbon coated copper grid for around one minute. This was followed by washing with three drops of water and staining with 0.5% uranyl formate for 30 s. Negatively stained  $\beta_2$ V<sub>2</sub>- $\beta$ arr<sup>swap2</sup>-Fab30 complex sample was imaged by using Tecnai T12 transmission electron microscope furnished with LaB6 filament and operating at 120kV accelerating voltage. Images were collected at magnification of 80k through side mounted Olympus VELITA (2K2K) CCD camera. All the images were collected at a defocus range of  $\sim$ -1.5 to -1.8 $\mu$ m yielding a final pixel size of  $\sim$ 2 Å at specimen level. Total about 10000 particles from 150 micrographs were selected manually using e2boxer.py of EMAN 2.12 suite and used for 2D classification. The particle stack was classified into 50 classes using simple\_prime2D script of SIMPLE software package.

### MD simulation set-up and analysis

#### Fab30 binding stability in solution

From the crystallized active  $\beta$ arr1 (PDB code: 4JQI), we removed the co-crystallized phosphopeptide and part of the Fab30 maintaining only residues 5 to 108 of the light chain and residues 1 to 123 of the heavy chain. The missing loop segment (309 to 310) of  $\beta$ arr1 was modeled using the loop modeler tool in the MOE software package (<https://www.chemcomp.com/>). Afterward, we generated different inter-domain rotation states using linear interpolation between the active  $\beta$ arr1 (PDB code: 4JQI) and the inactive  $\beta$ arr1 (PDB code: 1G4R). Complexes were subjected to a geometrical optimization using the MOE package (CHARMM27 force field and born solvation). During this optimization, we applied constraints to the backbone atoms of the  $\beta$  sheets and helices of  $\beta$ arr1. To verify that obtained  $\beta$ arr1 conformations reflect a low-energetic conformation along the inactivation pathway, we compared interpolated structures to conformations observed in unbiased simulations of  $\beta$ arr1 inactivation (see Figure S8E). Interpolated structures were populated with an RMSD less than 1 Å in unbiased inactivation simulations proving low-energetic conformations.

Fab30 complex was obtained by the following procedure. To ensure correct placement of Fab30 with respect to the C-domain (i.e., the main Fab30 binding interface) of the  $\beta$ arr1, all interpolation states were aligned to the C-domain of the active  $\beta$ arr1-Fab30 complex (PDB code: 4JQI). The Fab30 binding interface is optimized during the equilibration phase with only backbone atoms constrained (see section simulation setup below). Note that this procedure does not remove clashes of the Fab30 with the N-domain produced by low inter-domain rotation angles domain in the inactive  $\beta$ arr1 compared to the active state. In fact, these clashes are likely the reason for experimentally observed downregulation of Fab30 binding and are the focus of our simulation experiments.

The obtained  $\beta$ arr1-Fab30 complexes were solvated and ionized to 150 mM NaCl using VMD (Humphrey et al., 1996) yielding a system of approximately 86000 atoms. Systems were equilibrated for 10 ns in the NPT ensemble applying harmonic positional restraints to the protein backbone atoms and allowing side chains, water molecules and ions to relax (see detailed information below). Then, NVT production runs (see section simulation setup below) were used to assess Fab30 binding stability during simulation by monitoring the RMSD of the backbone atoms of the Fab30  $\beta$  sheet. In order to ensure a correct detection of Fab30 movement, we aligned the simulated complex to the C-domain of  $\beta$ arr1 prior to RMSD measurements.

### Dynamics of active and inactive $\beta$ arr1

To sample the conformational flexibility of inactive and active  $\beta$ arr1, we started simulations from the active (PDB code: 4JQI) and inactive (PDB code: 1G4R) crystal structure. In the active structure, we removed the co-crystallized Fab30, maintaining the co-crystallized phosphopeptide to stabilize the active conformation. In both of the structures missing loops were modeled using the loop modeler tool in the MOE software package. Structures were solvated and ionized according to the protocol described above yielding systems above 56000 atoms. Afterward, solvated complexes were subjected to equilibration and simulation (see section simulation setup below).

Simulation times ( $\mu$ s) for  $\beta$ arrs in receptor-bound complexes

Simulation system	Simulation time
$\beta$ arr1 in complex with Fab30 with different inter-domain rotation angles (10 systems)	accumulated 7.6
Peptide stabilized active $\beta$ arr1 (3 replicates)	accumulated 3.1
Inactive $\beta$ arr1 (3 replicates)	accumulated 2.0
Total	12.7

### Simulation set-ups

All simulations were carried out using the ACEMD simulation package (Harvey et al., 2009) and the CHARMM36m forcefield (Huang et al., 2017) and CHARMM36 forcefield (Kluda et al., 2010) force fields for proteins and lipids, respectively. NPT simulations were carried out at 310 K and 1 bar using the Berendsen barostat (Berendsen, 1984) with a relaxation time of 400 fs and 2 fs integration time step and harmonic constraints applied to all backbone atoms. NVT simulations were run at 310 K, using the Langevin thermostat (Grest and Kremer, 1986) with a damping coefficient of  $5 \text{ ps}^{-1}$  and 4 fs integration time step. No harmonic constraints are applied in this phase. In all simulations, we used a van der Waals and short-range electrostatic interactions with a cut-off of 9 Å and the particle mesh Ewald method (Darden, 1993) for long-range electrostatic interactions.

### Analysis of inter-domain rotation angle

The inter-domain rotation angle is used as metric to assess the conformational landscape of  $\beta$ arrs in the receptor-bound state. For this purpose, we computed the displacement of the C-domain relative to the N-domain between the inactive (PDB code: 1G4R) and active  $\beta$ arr1 crystal structures (PDB code: 4JQI) as previously described in Latorraca et al. (2018). The corresponding script was kindly provided by Naomi Latorraca.

### Evaluation of inactivation pathway generated by interpolation

To verify that obtained  $\beta$ arr1 conformations reflect a low-energetic conformation along the inactivation pathway, we compared interpolated structures to conformations observed in unbiased simulations of  $\beta$ arr1 inactivation. The active structure of  $\beta$ arr1 (PDB code: 4JQI) was solvated and simulated for 800ns in NVT conditions in three separate runs (see section simulation set-ups), allowing it to spontaneously inactivate.

Interpolation states were aligned to the frames of the  $\beta$ arr1 inactivation using the backbone atoms of  $\beta$  sheets). Then, we quantified the presence of interpolated states along the inactivation pathway (Figure S8E). We find that all ten interpolated structures with an inter-domain rotation angle between  $0^\circ$  and  $20^\circ$  are sampled by unbiased simulation with an RMSD of  $\beta$  sheets lower than 1. This indicates that generated states of  $\beta$ arr1 in Fab30 complexes adopt a low-energetic conformation along the inactivation pathway.

### Hydrogen/deuterium exchange analysis

Protein expression and purification conditions are as previously described (Yun et al., 2015). HDX-MS and data processing methods are also as previously described (Yun et al., 2015). Briefly, Purified protein was prepared in 60–100  $\mu$ M in H<sub>2</sub>O buffer (20 mM HEPES, pH 7.4 and 150 mM NaCl). Hydrogen/deuterium exchange was initiated by mixing 2  $\mu$ L of protein with 28  $\mu$ L of D<sub>2</sub>O buffer (20 mM HEPES, pD 7.4, 150 mM NaCl in D<sub>2</sub>O), and the mixture was incubated for various time intervals (10, 100, 1000 and 10,000 s) on ice. At the indicated time points, the mixture was quenched by adding 30  $\mu$ L of ice-cold quench buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 2.01). For non-deuterated (ND) samples, 2  $\mu$ L of purified protein was mixed with 28  $\mu$ L of H<sub>2</sub>O buffer to which, 30  $\mu$ L of ice-cold quench buffer was added. Quenched samples were digested online by passing them through an immobilized pepsin column (2.1 X 30 mm) (Life Technologies, Carlsbad, CA, USA) at a flow rate of 100  $\mu$ L/min with 0.05% formic acid in H<sub>2</sub>O at 11°C. Peptide fragments were subsequently collected on a C18 VanGuard trap column (1.7  $\mu$ m X 30 mm) (Waters, Milford, MA, USA) for desalting with 0.05% formic acid in H<sub>2</sub>O and were then separated by ultra-pressure liquid chromatography using an Acquity UPLC C18 column (1.7  $\mu$ m, 1.0 X 100 mm) (Waters) at a flow rate of 35  $\mu$ L/min with an acetonitrile gradient starting with 8% B and increasing to 85% B over 8.5 min. To minimize the back-exchange of deuterium to hydrogen, the system from trapping column to UPLC column was maintained at 0.5°C and the buffers were adjusted to pH 2.5. Mass spectral analyses were performed with a Xevo G2 Qtof equipped with a standard ESI source (Waters). Mass spectra were acquired in the range of m/z 100–2000 for 12 min in positive ion mode. Peptide identification and HDX-MS data processing Peptic peptides were identified in non-deuterated samples with ProteinLynx Global Server 2.4 (Waters). Searches were run with the variable methionine oxidation modification. To process HDXMS data, the amount of deuterium in each peptide was determined by measuring the centroid of the isotopic distribution using the DynamX program (Waters). Back-exchange was not corrected because the data consisted of comparisons between  $\beta$ -arrestin1 and  $\beta$ -arrestin2 or between wild-type and R169E mutants. All of the data was derived from at least three independent experiments.

### Receptor- $\beta$ arr chemical cross-linking

For assessing receptor  $\beta$ arr interactions, Sf9 cells expressing  $\beta_2$ V<sub>2</sub>R and GRK2 were first stimulated with an inverse agonist and agonist respectively for 30 minutes. Cells were lysed with lysis buffer (20mM HEPES pH 7.4, 100mM NaCl, 1X phosstop, 1mM PMSF and 2mM benzamidine) and incubated with purified  $\beta$ arr 1 or 2 at room temperature for 30 min. This was followed by the addition of 1mM dithiobis (succinimidyl-propionate) from a freshly prepared 100mM stock solution in DMSO. The lysate was incubated at room temperature for 40 min, and the reaction was quenched by adding 1M Tris pH 8.5. The lysate was solubilised in 1%(v/v) MNG for 1 h at room temperature and cleared by centrifugation at 15000 rpm for 30min. The supernatant was incubated with pre-equilibrated M1-FLAG beads supplemented with 2mM CaCl<sub>2</sub> for 2h at 4°C. Beads were washed alternately with low salt buffer(20mM HEPES pH 7.4, 150mM NaCl, 0.01% MNG, 2mM CaCl<sub>2</sub>) and high salt buffer(20mM HEPES pH7.4, 350mM NaCl, 0.01% MNG, 2mM CaCl<sub>2</sub>) respectively. Cross-linked proteins were eluted in FLAG-elution buffer (20mM HEPES pH 7.4, 150mM NaCl, 2mM EDTA, 0.01% MNG and 250ug/mL FLAG peptide). Samples were resolved on SDS-PAGE and visualized by western blotting.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Gel and blot densitometry analysis was done on ImageJ and Image lab (Bio-Rad) software respectively. Experimental data from ELISA assays, bimane fluorescence assay and densitometry based quantification of western blots were plotted using GraphPad Prism software. Data represents means  $\pm$  SEM and details of statistical analysis and number of biological replicates are indicated in the respective figure legends.

### DATA AND CODE AVAILABILITY

This study did not generate any datasets and code for analysis.