Allosteric modulation of GPCR-induced β-arrestin trafficking and signaling by a synthetic intrabody

Mithu Baidya1,7, Madhu Chaturvedi1,7, Hemlata Dwivedi-Agnihotri1,7, Ashutosh Ranjan1, Dominic Devost2, Yoon Namkung3, Tomasz Maciej Stepniewski4,5, Shubhi Pandey1, Minakshi Baruah1, Bhanupriya Panigrahi1, Parishmita Sarma1, Manish K. Yadav1, Jagannath Maharana1, Ramanuj Banerjee1, Kouki Kawakami6, Asuka Inoue6, Jana Selent4, Stéphane A. Laporte2,3, Terence E. Hébert2 & Arun K. Shukla1

Agonist-induced phosphorylation of G protein-coupled receptors (GPCRs) is a primary determinant of β-arrestin (βarr) recruitment and trafficking. For several GPCRs such as the vasopressin receptor subtype 2 (V2R), agonist-stimulation first drives the translocation of βarrs to the plasma membrane, followed by endosomal trafficking, which is generally considered to be orchestrated by multiple phosphorylation sites. We have previously shown that mutation of a single phosphorylation site in the V2R (i.e., V2R360A) results in near-complete loss of βarr translocation to endosomes despite robust recruitment to the plasma membrane, and compromised ERK1/2 activation. Here, we discover that a synthetic intrabody (Ib30), which selectively recognizes activated βarr1, efficiently rescues the endosomal trafficking of βarr1 and ERK1/2 activation for V2R360A. Molecular dynamics simulations reveal that Ib30 enriches active-like βarr1 conformation with respect to the inter-domain rotation, and cellular assays demonstrate that it also enhances βarr1-β2-adaptin interaction. Our data provide an experimental framework to positively modulate the receptor-transducer-effector axis for GPCRs using intrabodies, which can be potentially integrated in the paradigm of GPCR-targeted drug discovery.

G protein-coupled receptors (GPCRs) recognize a broad spectrum of ligands and play critical roles in nearly every aspect of human physiology1,2, and these receptors continue to be a major class of targets for novel drug discovery1. The spatio-temporal aspects of GPCR signaling are tightly regulated by multifunctional proteins, β-arrestins (βarrs)3,4, and agonist-induced phosphorylation of GPCRs is a key determinant of βarr interaction and their ensuing functional outcomes5. While some GPCRs interact transiently with βarrs at the plasma membrane followed by rapid dissociation, others display a prolonged interaction resulting in endosomal trafficking of receptor-
βarr complexes. These two patterns of βarr interaction and trafficking have been used to categorize corresponding receptors as class A or class B GPCRs, respectively. Interestingly, distinct phosphorylation patterns on GPCRs have been linked to different βarr conformations, which in turn determine the resulting functional responses [10-11]. While cumulative phosphorylation on GPCRs is typically believed to determine the affinity of βarr interaction, emerging evidence now suggests that spatial positioning of even single phosphorylation sites may provide a decisive contribution to βarr recruitment and subsequent functional outcomes [12-13].

We previously reported that mutation of a single phosphorylation site in the vasopressin receptor subtype 2 (V2R) at Thr360 in the carboxyl-terminus (i.e., V2RT360A) dramatically altered βarr trafficking patterns [14]. V2R is a class B receptor in terms of βarr interaction and trafficking where agonist stimulation first results in membrane recruitment of βarrs, followed by endosomal co-localization. Interestingly, upon agonist-stimulation of V2RT360A, βarrs efficiently translocate to the plasma membrane, but do not traffic to endosomal compartments, unlike the wild-type receptor even after prolonged agonist-exposure (Fig.1a) [10]. V2RT360A also exhibits reduced levels of ERK1/2 activation compared to the wild-type receptor without any measurable effect on G protein-coupling as assessed by measuring cAMP production [15]. This mutation leads to the disruption of a salt-bridge with Lys294 in βarrs and consequently reduces the fraction of active βarr conformation as assessed using molecular dynamics simulation [10]. While V2RT360A exhibits a dramatic alteration in βarr trafficking pattern, the other phospho-site mutants behave either like wild-type (e.g., V2RS357A and V2RT359A) or exhibit a near-complete loss of activity (e.g., V2RppT360A) [12]. This prompted us to probe the conformation of βarr in the context of this receptor mutant (V2RT360A) and compare it with the wild-type V2R, using a previously described intrabody [30] (ib30) based sensor [13].

Here, we show that ib30 robustly recognizes βarr1 recruited to the plasma membrane upon agonist-stimulation of V2R ppT360A, and it rescues endosomal localization of βarr1 and ERK1/2 activation for V2RT360A to the levels of the wild-type receptor. We also discover that ib30 enriches an active-like conformational population of βarr1 and also enhances the interaction of βarr1 with β2arr-adaptin. These findings establish the capability of ib30 to allosterically modulate βarr1 trafficking and activation for V2R ppT360A, and potentially open a paradigm to modulate GPCR signaling using designer allosteric modulators.

**Results**

**βarr1 conformation induced by V2R ppT360A phospho-peptides**

Synthetic phospho-peptides corresponding to the carboxyl-terminus of V2R have previously been used to activate βarrs in-vitro and probe the activation-induced conformational changes [12-13]. Therefore, we first synthesized two phospho-peptides corresponding to V2R ppT360A and V2R ppT360A, which contain a non-phosphorylated threonine (T) or alanine (A) at position 360, respectively, while the rest of the sequence and phosphorylation patterns are identical to V2Rpp360 (referred to as V2R ppT360A) [11]. We used a previously described limited trypsin proteolysis assay [15] to compare βarr1 conformation induced by V2R ppT360A phospho-peptides with that of V2Rpp360. We observed that the activation of βarr1 by V2R ppT360A resulted in an accelerated cleavage of the 48 kDa band (Gly-Arg 2) [15], protection of 47 kDa and 45 kDa bands (Leu-Arg 2 and Leu-Arg, respectively) and appearance of a 21 kDa band (Leu-Arg 3) (Fig. 1c, d, Supplementary Fig. 1a, b) as reported previously [15]. Interestingly, V2R ppT360A phospho-peptides also induced a proteolysis pattern qualitatively similar to that observed for V2Rpp360, although there were noticeable differences such as relatively slower proteolysis of the 48 kDa band and a weaker intensity of the 21 kDa band (Fig. 1c, d and Supplementary Fig. 1a, b). The difference in the intensity of 48 kDa and 47 kDa bands is visualized better at 1: 50 ratio of trypsin:βarr1 (Fig. 1c, right half), while the difference in the 32 kDa and 21 kDa bands is visualized better at 1: 25 ratio (Fig. 1c, left half). This observation indicates that V2R ppT360A phospho-peptides are capable of binding βarr1; however, they do not induce active βarr1 conformation as stabilized by V2R ppT360A, but instead appear to promote an intermediate state between the basal and active-conformations.

**Fab30/ScFv30 sensors recognize V2R ppT360A-βarr1 complexes**

As an additional readout of βarr1 conformation induced by V2R ppWT vs. V2R ppT360A phospho-peptides, we measured the ability of antibody fragments referred to as Fab30/ScFv30 to recognize V2R-βarr1 complexes using co-immunoprecipitation (co-IP). Fab30 and ScFv30 are known to selectively recognize βarr1 conformation induced by V2R ppWT [15], and thus, they have been used previously as conformational biosensors to monitor βarr activation in vitro [15].

We observed that Fab30/ScFv30 robustly interacted with the V2R ppT360A-βarr1 complexes, albeit at lower levels than V2R ppWT (Fig. 2a–d). We carried out co-IP in the presence of either 10-fold or 50-fold molar excess of the phospho-peptides compared to βarr1, but the reactivity patterns of Fab30/ScFv30 did not change significantly (Fig. 2a–d). Similar to the limited proteolysis data presented in Fig. 1, these data also suggest that V2R ppT360A phospho-peptides induce a conformation in βarr1, which is qualitatively similar to that of V2R ppWT, but not identical. However, we cannot rule out the possibility that the binding affinities of Fab30 and ScFv30 for βarr1 V2R ppT360A-βarr1 complexes are relatively lower compared to βarr1 V2R ppWT complex, which requires additional experimentation.

Considering the patterns of limited proteolysis and Fab30/ScFv30 reactivity induced by these phospho-peptides, we next carried out limited proteolysis assays in the presence of ScFv30 (Fig. 2e, f, Supplementary Fig. 2). We observed that the 47 kDa band (Leu-Arg 2) was significantly protected in presence of ScFv30, and the bands at 32 kDa and 21 kDa (Leu-Arg 2 and Leu-Arg 3, respectively) did not appear (Fig. 2e, f, Supplementary Fig. 2). Interestingly, the proteolysis patterns observed in presence of ScFv30 were nearly-identical for V2R ppWT and V2R ppT360A phospho-peptides, although an additional band at ~30 kDa was observed only with V2R ppWT (Fig. 2e, f). The converging proteolysis patterns of βarr1 observed in the presence of ScFv30 for the wild-type and mutant peptides suggest that ScFv30 might be promoting the transition of V2R ppT360A-bound βarr1 conformation towards the active-like state.

**Structural insights into βarr1 conformation induced by V2R ppWT and V2R ppT360A**

Taking a lead from the limited proteolysis assays, we next analyzed the crystal structures of βarr1 in basal, V2R ppWT, and V2R ppT360A-bound states to gain further insights into βarr1 conformation. As the distal carboxyl-terminus of βarr1 is not resolved in these structures, we focused primarily on Arg358 and Arg458, which are the trypsin cleavage sites yielding the 32 kDa (Leu-Arg 2) and 21 kDa (Leu-Arg 3) bands, respectively. Both of these residues exhibit a reorientation of their side chains between basal and phospho-peptide-bound conformations (Fig. 3a). The network of interactions involving Arg358 and Arg458 are also mostly maintained between basal and peptide-bound conformations although there are some differences as well (Fig. 3a). Further analysis of the local interaction networks of Arg458 and Arg458 using CONTACT/ACT program within the CCP4 suite [16], which analyzes all possible contacts/interactions and distances between residues in protein structures, including water molecules within a specified distance, also converges to the same observation as evident from the crystal structures (Supplementary Fig. 3). We also carried out molecular dynamics (MD) simulation studies using the crystal structures of βarr1 as templates to probe the conformational ensemble sampled by Arg358 and Arg458, and observed that they explore similar conformational space in the wild-type and mutant phospho-peptide-bound structure.
states (Fig. 3b). Taken together, these structural insights provide a plausible explanation for the proteolysis patterns obtained for V2RppWT vs. V2RppT360, and support the hypothesis that V2RppT360 induces an intermediate conformation in βarr1 compared to apo- and V2RWT-bound states that may be further influenced by the binding of Fab30.

**Agonist-induced βarr1 recruitment to V2RWT and V2R<sup>T360A</sup>**

The experiments presented so far were carried out in-vitro using isolated phospho-peptides, and therefore, we next set out to measure the relative recruitment of βarr1 and the reactivity of Ib30, an intrabody derived from Fab30 that reports βarr1 activation and trafficking<sup>11,13</sup>, for the wild-type V2R (V2R<sup>WT</sup>) and the mutant receptor (V2R<sup>T360A</sup>). We first
used a NanoBiT assay that measures the direct binding of the receptor and βarr1 and, therefore, reports cumulative interaction resulting from both the cell surface and internalized pools (Fig. 4a). We observed that the total βarr1 recruitment to V2RppT360a mutant was significantly attenuated compared to the V2RppWT (Fig. 4b), and this is in excellent agreement with our previous study using the Tango assay. Next, we compared the surface recruitment of βarr1 to the wild-type and mutant receptor using a NanoBiT assay where the LgBiT component is tethered to the plasma membrane through CAAX sequence while βarr1 is tagged with SmBiT (Fig. 4c). Here, we observed a near-identical pattern of βarr1 recruitment for the V2RppWT and V2RppT360a suggesting that endosomal trafficking but not surface translocation is compromised by the Thr360Ala mutation in the V2R (Fig. 4d). These findings, therefore, set the stage for testing whether Ib30 can recognize the βarr1 conformation induced by V2RppT360a and influence its endosomal trafficking. In these experiments, surface expression of the V2RppWT and V2RppT360a were comparable as measured using whole-cell ELISA assay (Supplementary Fig. 4a, b).

Intrabody30 rescues endosomal trafficking of βarr1 for V2RppT360a

We first co-expressed SmBiT-βarr1 and LgBiT-Ib30 constructs with V2RppWT and V2RppT360a and measured agonist-induced changes in luminescence signal as a readout of βarr1-Ib30 interaction and conformational recognition of βarr1 by Ib30 (Fig. 5a). As Ib30 reactivity is expected to follow cumulative βarr1 recruitment upon receptor activation, we anticipated a relatively lower response for V2RppT360a compared to V2RppWT considering their total βarr1 recruitment patterns as presented in Fig. 4b. Surprisingly, however, we observed nearly identical response for Ib30 reactivity upon agonist-stimulation for both, V2RppWT and V2RppT360a (Fig. 5b). This finding not only suggests that the conformation of βarr1 induced by V2RppT360a in the cellular context is recognizable by the Ib30 sensor, but also that Ib30 might be rescuing endosomal trafficking of βarr1 and thereby, bringing the overall recruitment to the wild-type level. We tested this hypothesis by measuring the overall βarr1 recruitment for V2RppWT and V2RppT360a in a NanoBiT assay in presence of either a control intrabody (Ib-CTL) or Ib30. In fact, we observed that overall βarr1 recruitment for V2RppT360a becomes nearly-identical to that of V2RppWT upon co-expression of Ib30 (Supplementary Fig. 5). In these NanoBiT experiments, the V2RppWT and V2RppT360a were expressed at comparable levels as measured in terms of their surface expression (Supplementary Fig. 4c, d). We also measured agonist-induced G protein-coupling for the V2RppT360a in the presence of Ib-CTL and Ib30 but did not observe any significant difference, similar to the V2RppWT (Supplementary Fig. 6a, b), suggesting the specificity of Ib30 for receptor-βarr interaction without a measurable effect on G protein-coupling.

In order to directly visualize the ability of Ib30 to recognize βarr1 upon recruitment to V2RppT360a, we co-expressed Ib30-mYFP construct together with βarr1-mCherry in HEK-293 cells expressing V2RppT360a, and monitored localization of βarr1 and Ib30 by confocal microscopy. Ib30 translocated to the plasma membrane upon agonist-stimulation, similar to βarr1, and exhibited robust colocalization with βarr1 (Fig. 5c, d). Interestingly, we also observed that upon prolonged agonist exposure (>15 min), both βarr1 and Ib30 were translocated to endosomal vesicles and robustly co-localized (Fig. 5c, d). This observation further strengthens the hypothesis that Ib30 may potentially be rescuing endosomal trafficking of βarr1 for V2RppT360a as hinted in the NanoBiT-based Ib30 recognition assay (Fig. 5a, b) and overall βarr1 recruitment assay for V2RppT360a (Supplementary Fig. 5). To further corroborate these findings and directly establish the allosteric potentiation of endosomal trafficking of βarr1 by Ib30, we used three different approaches. First, we co-expressed a βarr1-mYFP construct in HEK-293 cells together with either V2RppWT or V2RppT360a in the presence or absence of HA-tagged Ib30. We monitored the localization of βarr1 in these cells upon agonist-stimulation using confocal microscopy and scored the localization pattern of βarr1 in terms of plasma membrane vs. internalized vesicles. We manually scored more than 500 cells for each condition and plotted the data as % normalized (i.e. % of total cells displaying membrane vs. punctate localization of βarr1). In line with data presented in Fig. 5c, d, we observed that the presence of Ib30 indeed promoted endosomal trafficking of βarr1 for V2RppT360a (Fig. 5e, f) while βarr1 remained localized primarily at the plasma membrane even after prolonged agonist-exposure in the absence of Ib30, as reported previously.

Next, we used an intermolecular bystander BRE assay to monitor the endosomal localization of βarr1 quantitatively by using βarr1-Luc and GFP-FYVE constructs, described previously (Fig. 6a). As shown in Fig. 6b, we observed a very low level of agonist-induced BRET for V2RppT360a in the presence of control intrabody (Ib-CTL), while V2RppWT exhibited a robust response as expected. Interestingly, however, co-expression of Ib30 rescued the BRET signal (i.e., endosomal trafficking of βarr1) to almost the same level as V2RppWT (Fig. 6b, c). We also observed an enhanced Emin in BRET assay for V2RppWT in the presence of Ib30, compared to Ib-CTL, although basal BRET was also higher and therefore, the change in BRET signal is significantly more pronounced for the V2RppT360a (Fig. 6c). Finally, we also carried out a similar experiment using the NanoBiT assay that measures endosomal trafficking of βarr1 based on similar principles as in BRE assay described above (Fig. 6d). We observed analogous potentiation of endosomal localization of βarr1 by Ib30 for V2RppT360a as in BRE assay while there was no significant change for V2RppWT (Fig. 6e, f). In these experiments, the surface expression of the V2RppWT and V2RppT360a were maintained at comparable levels to ensure that the observed differences did not arise from a difference in the expression level of the wild-type and mutant receptors (Supplementary Fig. 4e). Taken together with the confocal microscopy observations, these data establish that Ib30 recognizes V2RppT360a-bound βarr1 conformation and allosterically potentiates its trafficking to endosomal vesicles and thereby, rescues the trafficking pattern of βarr1 making it similar to the wild-type receptor.

Fig. 1 | V2RppWT and V2RppT360a impart different conformations on βarr1.

A Schematic representation of the inability of V2RppT360a mutant to promote endosomal trafficking of βarr1 as published previously. B Amino acid sequences of the V2RppWT and Thr360 mutant phospho-peptides (V2RpppT360a and V2RpppRT360a) were used in this study. Phosphorylated residues (serine, S; threonine, T) are highlighted in red, and the position 360 is indicated by an arrow. V2RpppT360a and V2RpppRT360a contain a non-phosphorylated threonine and an alanine at position 360, respectively. C Limited trypsin proteolysis of βarr1 (5 min) in the absence or presence of indicated phosphopeptides at two different trypsin: βarr1 ratio followed by visualization of the bands on SDS-PAGE. A representative gel from four independent experiments (left panel) and a schematic of the proteolysis pattern corresponding to 1:25 ratio of trypsin:βarr1 (right panel) is shown here. D Densitometry-based quantification (mean ± SEM) of indicated bands from four independent experiments, normalized with respect to V2RppWT condition (treated as 100%) (One-way ANOVA, Dunnnett’s multiple comparisons test). The exact p values are as follows: Gly4 to Arg284 (48 kDa) band (I: 25)- Apo (p < 0.0001), V2RpppT360a (p = 0.0159); Gly4 to Arg284 (48 kDa) band (I: 30)-Ap (p = 0.0039), V2RpppT360a (p = 0.0566), V2RpppT360a (p = 0.385); Leu1 to Arg418 (47 kDa) band (I: 25)-Ap (p = 0.0132), V2RpppT360a (p < 0.0001), V2RpppT360a (p = 0.0844); Leu1 to Arg418 (47 kDa) band (I: 30)-Ap (p < 0.0024), V2RpppT360a (p = 0.0001), V2RpppT360a (p = 0.059); Leu1 to Arg418 (32 kDa) band (I: 25)-Ap (p < 0.0001), V2RpppT360a (p = 0.0006), V2RpppT360a (p < 0.0001); Leu1 to Arg418 (32 kDa) band (I: 30)- Apo (p = 0.0005), V2RpppT360a (p = 0.0036); Leu1 to Arg418 (21 kDa) band (I: 25)-Ap (p < 0.0001), V2RpppT360a (p = 0.0028), V2RpppT360a (p = 0.0057); Leu1 to Arg418 (21 kDa) band (I: 30)- Apo (p = 0.0001), V2RpppT360a (p = 0.0035), V2RpppT360a (p = 0.0044). Source data are provided as a Source Data file (p < 0.05, "p<0.01, "p<0.001, ns=non-significant).
Intrabody30 rescues agonist-induced ERK1/2 activation for V2R\textsuperscript{T360A}

We have previously reported that agonist-induced ERK1/2 MAP kinase activation is significantly attenuated for V2R\textsuperscript{T360A} compared to the wild-type receptor\textsuperscript{10}. Considering the potentiating effect of Ib30 on β\textsuperscript{arr1} trafficking, and previous studies linking endosomal pool of β\textsuperscript{arrs} with ERK1/2 MAP kinase activation for GPCRs\textsuperscript{19}, we next measured the effect of Ib30 on agonist-induced ERK1/2 phosphorylation for the V2R\textsubscript{WT} and V2R\textsubscript{T360A}. Expectedly, Ib30 did not have a significant effect on ERK1/2 activation for the V2R\textsubscript{WT}, however, it robustly enhanced the level of phosphorylated ERK1/2 upon agonist-stimulation for V2R\textsubscript{T360A}, to the levels of the V2R\textsubscript{WT} (Fig. 7a, b). The surface expression of the wild-type V2R\textsubscript{WT} and V2R\textsubscript{T360A} was also measured, and the results showed a similar trend (Fig. 7c, d). The surface expression of the wild-type V2R\textsubscript{WT} and V2R\textsubscript{T360A} was also measured, and the results showed a similar trend (Fig. 7c, d).
and mutant receptors were at comparable levels in these experiments (Supplementary Fig. 4G). Taken together with the endocytosis data, these findings demonstrate an allosteric effect of Ib30 to positively modulate βarr-mediated functional responses for the V2R<sub>T360A</sub> mutant in the cellular context.

**Structural insights into the allosteric effect of Fab30 on βarr1 conformation**

The positive allosteric effect of Ib30 on endosomal trafficking of βarr1 and ERK1/2 activation for V2R<sub>T360A</sub> prompted us to probe the potential structural mechanism for this phenomenon at the level of phospho-peptide binding and βarr1 conformation. Therefore, we first analyzed the crystal structures of V2Rpp<sup>WT</sup>-βarr1 (PDB: 4Q0I) and V2Rpp<sup>T360A</sup>-βarr1 (PDB: 7DFA), determined previously. Interestingly, a segment of the V2Rpp<sup>T360A</sup> containing residues Pro<sup>533</sup> to Thr<sup>539</sup> showed a marked repositioning compared to the V2Rpp<sup>WT</sup>-βarr1 binding pose (Fig. 8a). In the V2Rpp<sup>WT</sup>-βarr1 crystal structure, pThr<sup>536</sup> engages Lys<sup>394</sup>, Lys<sup>395</sup> and Arg<sup>397</sup> in βarr1 through ionic interactions, which is expected absent in case of V2Rpp<sup>T360A</sup> mutation. Of these, Lys<sup>394</sup> in the lariat loop and Lys<sup>395</sup> in the β-strand I of βarr1 are particularly noteworthy as they constitute a key part of the polar core and phosphate sensor, respectively. These interactions are critical in the process of βarr1 activation upon binding of phosphorylated carboxyl-terminus of GPCRs. Interestingly, pThr<sup>539</sup> in V2Rpp<sup>T360A</sup> phospho-peptide engages with Lys<sup>394</sup> but not with Lys<sup>395</sup> or Arg<sup>397</sup>. This interesting structural rearrangement may, in part explain an intermediate active-like conformation induced by V2Rpp<sup>T360A</sup> phospho-peptides as observed in limited proteolysis and ScFv30 co-IP assay.

Next, we used molecular dynamics (MD) simulation on V2Rpp<sup>WT</sup>, and V2Rpp<sup>T360A</sup>-bound βarr1. We have previously reported that Thr<sup>536</sup>Ala mutation resulted in a significant shift in the population of βarr1 towards inactive-like conformation compared to the V2Rpp<sup>WT</sup> as measured in terms of the inter-domain rotation angle<sup>15</sup>. We also found that the Thr<sup>536</sup>Ala mutation leads to the disruption of a salt-bridge with Lys<sup>394</sup> in the lariat loop of βarr1, which links the N- to the C-domain via the phospho-peptide, and removing this inter-domain connector may reverse the inter-domain rotation leading to the transition of βarr1 towards inactive conformation<sup>11</sup>. Now, in this study, we first reproduced this behavior for the V2Rpp<sup>T360A</sup> mutant, demonstrating that introducing the Thr<sup>536</sup>Ala mutation in the V2Rpp-βarr1 complex leads to a dramatic shift towards inactive-like conformation with an inter-domain rotation angles <15° (i.e. V2Rpp<sup>WT</sup>: 24° vs. V2Rpp<sup>T360A</sup>: 63%) (Fig. 8b–d). Strikingly, simulations of the V2Rpp<sup>T360A</sup>-βarr1 complex in presence of Fab30 revealed that Fab30 binding significantly stabilizes the population of active-like βarr1 conformations (V2Rpp<sup>T360A</sup> + Fab30: 70% vs. V2Rpp<sup>T360A</sup>: 37%) (Fig. 8b–d). This interesting observation can be rationalized by the fact that Fab30 simultaneously binds to the N- and the C-domain of βarr1, which blocks the reversal of the inter-domain rotation towards inactive-like βarr1 conformations. This stabilizing contribution of Fab30 towards active-like βarr1 conformations may offer a plausible mechanism for the positive allosteric effect of Ib30 observed on βarr1 trafficking to endosomes and agonist-induced ERK1/2 activation for the V2R<sub>T360A</sub>. It is worth noting here that despite an accumulated simulation time of 100s per system (i.e. 5 runs of 2 µs), our simulation set up does not allow for a converged sampling of βarr1 ensemble. However, the running averages of the inter-domain rotation angle show that βarr1 can transition between active- and inactive-like states in all three conditions (i.e. V2Rpp<sup>WT</sup>, V2Rpp<sup>T360A</sup> and V2Rpp<sup>T360A</sup> + Fab30). This indicates that βarr1 explores a wide range of conformational landscape and that observed tendencies are not an artifact but rather describe an actual property of each system.

**Intrabody30 enhances βarr1-β2-adaptin interaction**

Next, we set out to identify a potential functional correlate of Ib30-induced enrichment of active-like βarr1 conformation and to reveal the mechanism of Ib30-mediated endosomal targeting of βarr1. As the interaction of βarrs with β2-adaptin is a prominent mechanism that drives GPCR endocytosis<sup>12</sup>, we measured the effect of Ib30 on βarr1-β2-adaptin interaction. We used the ear-domain of β2-adaptin (592–951) tagged with GST at the N-terminus and assessed its interaction with βarr1 in the presence of lysate prepared from 5P9 cells expressing V2R<sub>T360A</sub>. The ear domain represents the C-terminal appadage of the β2-adaptin subunit of the clathrin adaptador A2 complex, and this region has been shown previously to interact with βarr2. As presented in Fig. 9a, b, we observed a low but statistically significant interaction between βarr1 and β2-adaptin in the presence of Ib-CTL. More interestingly, the presence of Ib30 enhanced this interaction several fold suggesting the ability of Ib30 to promote βarr1-β2-adaptin interaction (Fig. 9a, b).

To further corroborate this interesting finding in cellular context, we next used a previously described BRET-based assay<sup>22</sup> to monitor agonist-induced interaction of βarr1 with β2-adaptin in the presence of either Ib-CTL or Ib30 for V2R<sub>WT</sub> and V2R<sub>T360A</sub> (Fig. 9c). There was a robust interaction between βarr1 and β2-adaptin for the V2R<sub>WT</sub> upon agonist-stimulation in the presence of Ib-CTL, while the response was significantly lower for the V2R<sub>T360A</sub>. This is in line with significantly attenuated endosomal trafficking of βarr1 for the V2R<sub>T360A</sub> compared to V2R<sub>WT</sub>. Interestingly, co-expression of Ib30 significantly enhanced βarr1-β2-adaptin interaction for V2R<sub>T360A</sub>, bringing it to almost the same level as V2R<sub>WT</sub>. However, the basal BRET signal was also higher under Ib30 expression conditions compared to Ib-CTL, and it may reflect the propensity of Ib30 to enhance βarr1-β2-adaptin interaction, even under the basal condi-tion i.e. without receptor activation (Fig. 9d). To test this hypothesis, we carried out a titration experiment, where we expressed Ib30 at increasing levels and assessed βarr1-β2-adaptin interaction in the BRET assay. As presented in Fig. 9e, increasing expression of Ib30 indeed enhanced BRET in a saturable manner suggesting the ability of Ib30 to promote basal interaction between βarr1 and β2-adaptin. Taken together, these observations provide a mechanistic basis of Ib30-induced allosteric modulation of βarr1 trafficking pattern observed for V2R<sub>T360A</sub>. 
Discussion

In this study, we demonstrate that a synthetic intrabody (Ib30) can allosterically modulate agonist-induced trafficking patterns of βarr1 for a vasopressin receptor subtype 2 mutant (V2RRT360A) lacking a key phosphorylation site in its carboxyl-terminus. Ib30 induces the transition of βarr1 trafficking pattern for V2RRT360A from class A to class B by enriching the fraction of active-like conformational population of βarr1, and allosterically enhancing βarr1-β2-adaptin interaction. Moreover, Ib30 also rescues the attenuated ERK1/2 activation for V2RRT360A to levels induced by the wild-type receptor. A previous study has demonstrated a critical role of βarr-β2-adaptin interaction in βarr-mediated ERK1/2 activation for V2R using a small molecule inhibitor of this interaction\(^2\). Therefore, an increase in βarr1-β2-adaptin interaction in presence of Ib30 may provide a plausible mechanism for its ability to rescue agonist-induced ERK1/2 activation for V2RRT360A. However, additional mechanisms may also contribute to this intriguing observation, and it would be interesting to probe this further in subsequent studies. Although Ib30 appears to slightly enhance the endosomal trafficking of βarr1 for V2R\(^{WT}\) (Fig. 6b, c), an observation that is consistent with our

Fig. 3 | Structural insights into binding of phospho-peptides to βarr1.

\(a\) Structural snapshots comparing the relative orientation and local interaction networks of trypsin cleavage sites Arg\(^{188}\) and Arg\(^{285}\) in the crystal structures of βarr1 in basal (PDB: 1G4M, grey), V2Rpp\(^{WT}\)-bound (PDB: 4JQI, orange) and V2RppT360-1-bound (PDB: 7DFA, violet) conformations. The dotted lines represent hydrogen bonds and polar interactions. 

\(b\) Molecular dynamics simulations based on the crystal structures confirm an overall similar conformational space sampled by Arg\(^{188}\) and Arg\(^{285}\), the two trypsin proteolysis sites which are protected by ScFv30.
future studies may also help clarify the underlying mechanism for the lack of cAMP potentiation in case of V2RppT360A despite enhanced endosomal trafficking of βarr1 in the presence of Ib30.

An elegant study has recently reported crystal structures of βarr1 in complex with several different phospho-peptides derived from the carboxyl-terminus of V2R, including the V2RppT360A. While the binding affinities of βarr1 to V2RppWT and V2RppT360A are comparable, V2RppT360A exhibits a slightly altered binding mode compared to V2RppWT in these crystal structures. Therefore, it is unlikely that distinct trafficking patterns of βarr1 for V2RppWT vs. V2RppT360A originate from an affinity difference, and it points towards a conformational mechanism underlying this phenomenon. This is indeed supported by our MD simulation studies, where Fab30 binding enriches active-like βarr complexes and deciphered functional outcomes associated with these distinct conformations. A recent study using NMR spectroscopy demonstrated that Fab30 binding to a partially-engaged GPCR-βarr1 complex facilitates additional conformational changes in βarr1 leading to a transition towards fully-activated conformation. Our study now draws an interesting parallel with this recent NMR study by demonstrating that Ib30 allosterically facilitates the transition of a

![Diagram](https://example.com/diagram.png)

**Fig. 4 | Agonist-induced βarr1 recruitment to V2RWT and V2RppT360A mutant.**

a | Schematic representation of NanoBiT-based βarr1 recruitment assay. b | HEK-293 cells expressing the indicated receptor and βarr constructs were stimulated with varying doses of AVP for 30 min followed by the measurement of luminescence (mean ± SEM; n = 4 independent experiments; normalized with luminescence signal for V2RWT at maximal ligand dose as 100%). Two-way ANOVA, Sidak’s multiple comparisons test; ****p < 0.0001). c | Schematic representation of NanoBiT-based assay for measuring βarr1 translocation to the cell surface. d | HEK-293 cells expressing the indicated receptor and βarr constructs together with LgBiT-CAAX were stimulated with varying doses of AVP for 30 min followed by the measurement of luminescence (mean ± SEM; n = 4 independent experiments; normalized with luminescence signal for V2RWT at maximal ligand dose as 100%). Two-way ANOVA, Sidak’s multiple comparisons test; ns = non-significant). Source data are provided as a Source Data file.
functionally-compromised βarr1 conformation to a fully-competent conformation for V₂R<sup>T360A</sup>, and rescues downstream functional responses. It would be interesting to explore in future studies whether the effect of Ib30 observed here for V₂R<sup>T360A</sup> is somehow linked to the transition between the partially- and fully-engaged βarr conformations in complex with the receptor.

The paradigm of βarr-AP2 interaction through β<sub>2</sub>-adaptin in driving GPCR endocytosis via a clathrin-mediated pathway is mostly conserved across GPCRs<sup>5</sup>. Therefore, our study raises the possibility of Ib30 being a potentially generic positive modulator of βarr trafficking for other GPCRs as well, especially those exhibiting Class A pattern of βarr recruitment. Interestingly, we have already demonstrated the
ability of Ib30 to recognize βarr1 in complex with several native GPCRs, although it was selected from a phage display library using V2Rpp-βarr1 as the target. Therefore, it would be worth probing the effect of Ib30 in the context of endocytosis and ERK1/2 phosphorylation for other receptors in future studies. Another interesting avenue where Ib30 may serve as a useful tool is the emerging paradigm of catalytic activation of βarrs, where they may continue to generate functional outputs even after dissociation from activated receptors. It is plausible that Ib30 may recognize and stabilize such conformational “memory” in βarrs and thereby, facilitate its visualization in the cellular context as well as at high resolution using direct structural approaches. In summary, we demonstrate that agonist-induced trafficking of βarrs and downstream responses can be allosterically modulated using conformation-specific intrabodies targeting protein-protein interactions. These findings open a paradigm for positively modulating GPCR signaling in cellular context and may catalyze the discovery of previously unknown aspects of GPCR-βarr interaction and functional outcomes.

Methods

General reagents

Most chemicals and molecular biology reagents were purchased from Sigma-Aldrich unless mentioned otherwise. HEK-293 cells (ATCC; cat. no. CRL-3216) were maintained at 37°C under 5% CO₂ in Dulbecco’s Modified Eagle Medium ( Gibco; cat. no. 12800-085) supplemented with 10% FBS (Gibco; cat. no. 10270-106) and 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Gibco; cat. no. 15140-122). Cells were cultured in 10 cm dishes ( Corning; cat. no. 430167) at 37°C under 5% CO₂ and passedaged at 70 to 80% confluence using 0.05% trypsin-EDTA for detachment. S/9 cells (Expression Systems; cat. no. 94-001 F) were maintained as suspension cultures in ES 921 media (Expression Systems; cat. no. 96-001-O1). Lauryl Maltose Neopentyl Glycol (LMNG) was purchased from Anatrace (cat. no. NG310).

Construct design and expression plasmids

The expression constructs for the wild-type human V2R and V2RΔ360A mutants have been described previously. Briefly, the cDNA coding for V2RWT with an N-terminal HA signal sequence and FLAG tag was PCR amplified and cloned in a customized pcDNA3.1 (+) vector. This construct was also cloned in pVL1393 vector for expression in S/9 cells. The ThrΔ360 mutation was generated on the V2RWT backbone using Q5 Site-Directed Mutagenesis Kit (NEB). The βarr1-mYFP plasmid used for confocal imaging experiments was obtained from Addgene (cat. no. 36916). βarr1-mCherry plasmid was a gift from Dr. Mark Scott, Institut Cochin, France. The plasmids encoding ScFv-CTL, ScFv30, Ib-CTL-HA, Ib30-HA and Ib30-YFP have been described previously. The V2RWT and V2RΔ360A constructs were also fused with a 15 amino-acid flexible linker to the small subunit of NanoLuc i.e., SmBiT at its C-terminus. Similarly, Ib30 were N-terminally fused with LgBiT fragment in pCAGGs vector for NanoLuc complementation-based NanoBit assay. For in-vitro assays, i.e., trypsin proteolysis and ScFv30/Fab30 co-IP experiments, βarr1 was purified from BL21 cells by Glutathione Sepharose (GS) affinity chromatography. All the constructs were sequence verified (Macrogen). V2R agonist AVP (arginine-vasopressin) was synthesized by GenScript, and phospho-peptides V2RppWT, V2RppΔ360A and V2RppΔ360A when were synthesized by the peptide synthesis facility at Tufts University. The construct for GST-tagged β2-adaptin (residues 592–951, Rat, isoform 2) in pGEX4T1 vector was received as a kind gift from Dr. Thomas Pucadyil (Pune, India).

Limited trypsin proteolysis assay

To qualitatively assess the effect of different V2R phospho-peptides i.e., V2RppWT, V2RppΔ360A and V2RppΔ360A on βarr1 conformation, limited trypsin proteolysis of βarr1 in the presence or absence of these phospho-peptides was performed. The protocol for trypsin proteolysis of βarr1 has been described previously. Briefly, βarr1 (5–10 μM) was incubated in the absence or presence of (50:1 molar ratio, phospho-peptide: βarr1) the phospho-peptides for 30 min at 4°C. Thereafter, L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated Trypsin (Sigma-Aldrich; cat. no. T1426) was added to the βarr1-phospho-peptide mixture at a ratio of 1: 25 and 1: 50 (w/w) and the samples were incubated at 37°C for 5 min. In addition to the indicated ratio of trypsin: βarr1, other ratios like 1: 10, 1: 100 and 1: 250 were also tried. At 1: 10 ratio, βarr1 was completely digested while at lower trypsin concentrations, the resolution of the digested fragments was poor. At each time point, 20 μl of the reaction mix (5 μg of βarr1) was withdrawn and transferred to a fresh microcentrifuge tube containing 5 μl of 5x SDS loading buffer to quench the proteolysis reaction. The digested samples were separated on 12% SDS-polyacrylamide gels by electrophoresis to determine the effect of phospho-peptides on the digestion pattern of βarr1. In addition, to study how ScFv30 affects the digestion pattern of βarr1 when activated with different phospho-peptides, a 50-fold molar excess of ScFv30 was added to the βarr1 samples prior to proteolysis. Samples without ScFv30 were used as references for comparison. After proteolysis with a 1: 50 ratio of trypsin: βarr1, the samples were quenched at 30 min and resolved by SDS-PAGE as described earlier.

Surface expression of receptor mutants

The surface expression of V2RWT and V2RΔ360A used in different cellular assays was measured by whole-cell surface ELISA. For this, HEK-293 cells transfected with either V2RWT or V2RΔ360A were seeded at a density of 0.2 million per well in a 24-well plate precoated with 0.01% poly-D-Lysine (Sigma-Aldrich; cat. no. P0899). After 24 h, cells were fixed with 4% (w/v) paraformaldehyde (pH 6.9) on ice for 20 min and washed three times with 1× tris-buffered saline (TBS) buffer (150 mM NaCl and 50 mM Tris-HCl (pH 7.4)). Subsequently, nonspecific sites were blocked with 1% bovine serum albumin (BSA; prepared in 1× TBS) for 90 min, followed by the incubation of cells with horseradish peroxidase (HRP)-coupled anti-FLAG M2 antibody (dilution-1: 5000; Sigma-Aldrich; cat. no. A8592), prepared in 1% BSA for 90 min. Cells were then washed three times with 1% BSA in TBS, and 200 μl of...
tetramethylbenzidine (TMB) ELISA substrate (Thermo Fisher Scientific; cat. no. 34028) was added to each well. Once blue color appeared in the wells, the reaction was stopped by transferring 100 μl of the solution to a different 96-well plate already containing 100 μl of 1 M H₂SO₄. Absorbance was measured at 450 nm in a multimode plate reader (Victor X4-Perkin-Elmer). For normalization of signal across different wells, cell density was estimated using Janus Green (Sigma-Aldrich; cat. no. 201677) staining. TMB solution was removed from the wells; cells were washed with 1× TBS followed by incubation with 0.2% (w/v) Janus Green for 20 min. Thereafter, cells were washed three times with distilled water and 800 μl of 0.5 N HCl was added to each well. 200 μl of this solution was used for measuring the absorbance at
Fig. 6 | Ib30 potentiates endosomal trafficking of βarr1 for the V2R<sup>T360A</sup> mutant. a Co-expression of Ib30 promotes endosomal trafficking of βarr1 for V2R<sup>T360A</sup> as assessed by BRET assay. HEK-293 cells expressing the indicated constructs were stimulated with varying doses of AVP followed by BRET measurement. Data (mean ± SEM) from three independent experiments are presented here (Two-way ANOVA, Tukey’s multiple comparisons test; **p < 0.0001). Comparison of ΔBRET (difference in the BRET signal at the lowest and highest dose of AVP) in the BRET assay based on the data presented in panel B from three independent experiments (mean ± SEM, One-way ANOVA, Tukey’s multiple comparisons test; **p < 0.001, ***p < 0.0001). b Schematic representation of NanoBiT-based endosomal localization assay for βarr1. Co-expression of Ib30 robustly promotes endosomal trafficking of βarr1 for V2R<sup>T360A</sup> as assessed by NanoBiT assay. HEK-293 cells expressing the V2R<sup>WT</sup> or V2R<sup>T360A</sup> together with SmBiT-tagged βarr1 and Ib-CTL/Ib30 were stimulated with indicated doses of AVP followed by luminescence measurement. Data (mean ± SEM) from five independent experiments, normalized with maximal response under V2R<sup>WT</sup> + Ib-CTL condition (treated as 100%) (Two-way ANOVA, Tukey’s multiple comparisons test; ***p < 0.0001) are presented here. Comparison of maximal response (at 1 µM AVP) in the NanoBiT-based endosomal trafficking assay presented in panel E from five independent experiments (mean ± SEM, One-way ANOVA, Tukey’s multiple comparisons test; *p < 0.01, **p < 0.001, ns = non-significant). Source data are provided as a Source Data file.

**NanoBiT assay**

NanoBiT assays were carried out following a previously published protocol<sup>12</sup>. Briefly, HEK-293 cells were transfected with the plasmids as indicated in the corresponding figures using PEI (Polyethylenimine; 1 mg ml<sup>-1</sup>) as transfection agent at a DNA: PEI ratio of 1:3. For total βarr1 recruitment (Fig. 4b), 4 µg of receptor-SmBiT and 3 µg of LgBiT-βarr1 were used, while for measuring surface recruitment (Fig. 4d), 3 µg of receptor, 2 µg of SmBiT-βarr1 and 5 ug of LgBiT-CAAX were transfected. For the Ib30 reactivity assay (Fig. 3b), 5 µg of receptor, 5 µg of LgBiT-Ib30, and 2 µg of SmBiT-βarr1 were transfected. For endosomal trafficking experiment (Fig. 6e), 3 µg of receptor, 2 µg of SmBiT-βarr1, and 5 µg of FVYE-LgBiT were transfected. To measure the effect of Ib30 on total βarr1 recruitment (Supplementary Fig. 3b), 4 µg of receptor-SmBiT, 3 µg of LgBiT-βarr1 and either 6 µg of Ib-CTL or 1 µg of Ib30 were used. After 16–18 h of transfection, cells were harvested in PBS solution containing 0.5 mM EDTA and centrifuged. Cells were resuspended in 3 ml assay buffer (HBSS buffer with 0.01% BSA and 5 mM HEPES, pH 7.4) containing 10 µM coelenterazine (GoldBio; cat. no. CZ05) at final concentration. The cells were then seeded in a white, clear-bottom, 96-well plate at a density of 0.7–0.9 × 10<sup>3</sup> cells per 100 µl per well. The plate was kept at 37 °C for 90 min in the CO<sub>2</sub> incubator followed by incubation at room temperature for 30 min. Basal readings were taken in luminescence mode of a multi-plate reader (Victor X4-Perkin-Elmer). The cells were then stimulated with varying doses of ligand AVP ranging from 1 pM to 1 µM (6x stock, 20 µl per well) prepared in drug buffer (HBSS buffer with 5 mM HEPES, pH 7.4). Luminescence was recorded for 60 min immediately after addition of ligand. The initial counts of 4–10 cycles were averaged and fold increase was calculated with respect to vehicle control (unstimulated values) and analyzed using nonlinear regression four-parameter sigmoidal concentration–response curve in GraphPad Prism software (v9.3).

**Confocal microscopy**

For visualizing the effect of intrabody on βarr-mediated receptor trafficking, HEK-293 cells were co-transfected with 3 µg of either V2R<sup>WT</sup> or V2R<sup>T360A</sup> along with 2 µg of βarr1-mYFP in the presence or absence of 2 µg of Ib30 with help of polyethylenimine (Polysciences; cat. no. 23966) reagent (21 µl) in 10 cm plates. Transfection was performed in FBS-deficient DMEM (Gibco; cat. no. 12800-017) after which cells were replaced with DMEM supplemented with FBS (Gibco; cat. no. 10270-106). Post 24 h, cells were seeded onto poly-D-lysine (Sigma-Aldrich; cat. no. P0899) precoated glass bottom confocal dishes (SPL Life Sciences; cat. no. 100350) at a density of 1 million per dish. Cells were allowed to adhere to confocal dishes for 24 h. The next day, cells were starved in FBS-deficient DMEM for 4 h and then stimulated with 100 nM AVP, and live cells were visualized under the confocal microscope (Zeiss LSM 710 NLO). The confocal microscope was equipped with a motorized XY stage along with a temperature and CO<sub>2</sub> controlled platform. For visualizing Ib30 and βarr1 together, cells were transfected with βarr1-mCherry (2 µg) and Ib30-mYFP (2 µg) along with V2R<sup>T360A</sup> (3 µg). To excite mYFP, a multi-line argon laser source was used and for the mCherry, a diode pump solid-state laser source was used.
The emitted signal was detected with a 32× array GaAsP descanned detector (Zeiss). For related experiments, all microscopic settings including laser intensity and pinhole slit were kept in the same range and for avoiding any spectral overlap between two channels filter excitation regions and bandwidths were adjusted accordingly. Images were acquired in line scan mode and were subsequently processed post imaging in ZEN lite (ZEISS) software suite. For quantifying βarr trafficking to either membrane or endosomes, confocal images were categorized into early (1 to 8 min) and late time points (9 to 30 min) post agonist stimulation. The cells with βarr1-mYFP fluorescence in the plasma membrane were scored as surface localized, and the cells with punctate structures in the cytoplasm were scored as internalized. In
Fig. 8 | Intrabody30 (Ib30) stabilizes the active conformation of βarr1. A Structural snapshots of βarr1 crystal structures in complex with V2RppWT (PDB: 4JQ) and V2Rpp360A (PDB: 7DFA). The superimposed structures display repositioning of the V2Rpp360A N-terminal segment harboring Thr360 residue (cyan) relative to the V2RppWT (green). Also, changes in ionic interactions of Thr360 with neighboring residues are shown. For the V2RppWT bound βarr1, Thr360 engages with Lys419, Lys434, and Arg51. In the V2Rpp360A bound state, the Thr360 is non-phosphorylated, and the side-chain of Thr359 is repositioned to interact with Lys42.

Agonist-induced cAMP responses measured by GloSensor assay
To measure cAMP accumulation (as a readout for G protein activation), 50–60% confluent HEK-293 cells were co-transfected with either V2RWT or V2Rpp360A DNA (2 μg), luciferase expressing F/CAM biosensor construct (3.5 μg) and Ib-CTL (2 μg) or Ib30 (1 μg) DNA. After 18–20 h of transfection, cells were washed with PBS and treated with trypsin-EDTA (0.05%). Detached cells were harvested and centrifuged at 1500 g for 10 min, and the cell pellet was resuspended in 0.5 mg/ml luciferin (GoldBio; cat. no. LUCNA) solution prepared in 1X HBSS buffer (Gibco; cat. no. 14065) at 200,000 cells per well. The next day, cells were serum-starved in DMEM for 6 h and were then stimulated with 100 nM AVP (agonist for V2R) for indicated time points. After stimulation for selected time points, the media was aspirated and the cells were lysed in 100 μl of 2× SDS protein loading buffer. Cellular lysates were heated at 95 °C for 15 min. Following centrifugation at 21130 X g for 15 min, 10 μl of samples were loaded per well and separated by 12% SDS-polyacrylamide gel electrophoresis. Phosphorylated ERK1/2 signal was detected by Western blotting using anti-phospho-ERK1/2 antibody (dilution-1: 5000; CST; cat. no. 9102) followed by horseradish peroxidase-conjugated secondary antibody. Densitometry-based quantification was carried out using the ACEMD3 engine37. Both systems underwent a 20 ns equilibration in conditions of constant pressure (1 atm) and the side-chain of Thr359 is repositioned to interact with Lys42. b–d MD

BRET assay for βarr1 trafficking
HEK-293T cells (ATCC) were grown in complete culture media (DMEM high glucose (Wistet; cat. no. 319-015-CL) supplemented with 10% FBS (Wisent; cat. no. 098150) and penicillin/streptomycin (Wisent; cat. no. 450-201-EL) in a tissue culture incubator set at 37 °C providing 5% CO2. The day before transfection, cells were plated into well of a 6-well plate (Thermo scientific; cat. no. 140675) at 400,000 cells per well. The next day, media was changed for DMEM high glucose supplemented with only 2.5% FBS and cells were transfected using PEI (Polysciences; cat. no. V9879) at different concentrations prepared in Kreb's/HEPES. To assess BRET, 10 μl of a 20 μM coelenterazine 400 A (GoldBio; cat. no. C320) solution diluted in Krebs/HEPES was added 5 min before the end of the stimulation period. BRET was then monitored by measuring 3 consecutive luminescence readings at both 410 nm and 515 nm using a Tristar2 plate reader (Berthold Technologies GmbH & Co. KG). BRET was calculated as the emission at 515 nm/emission at 410 nm and the 3 values were averaged. BRET data were plotted as dose-response curves using GraphPad Prism (v6).

Effect of Ib30 on agonist induced ERK1/2 phosphorylation
To assess the effect of Ib30 on βarr1 mediated signaling downstream to V2RWT and V2Rpp360A mutant, agonist-induced ERK1/2 phosphorylation was measured. For this, 50–60% confluent HEK-293 cells were co-transfected with 150 μg of indicated V2R constructs and 1 μg of HA-tagged Ib30. A control intrabody (Ib-CTL) that does not recognize receptor-bound βarr1 was also transfected in parallel at levels comparable to Ib30 (3 μg) to achieve normalized expression levels of both the intrabodies. 24 h after transfection, cells were seeded into six-well plates at a density of 1 million cells per well. The next day, cells were serum-starved in DMEM for 6 h and were then stimulated with 100 nM AVP (agonist for V2R) for indicated time points. After stimulation for selected time points, the media was aspirated and the cells were lysed in 100 μl of 2× SDS protein loading buffer. Cellular lysates were heated at 95 °C for 15 min, followed by centrifugation at 21130 X g for 15 min. 10 μl of samples were loaded per well and separated by 12% SDS-polyacrylamide gel electrophoresis. Phosphorylated ERK1/2 signal was detected by Western blotting using anti-phospho-ERK1/2 antibody (dilution-1: 5000; CST; cat. no. 9101) followed by reprobing of the blots with anti-total-ERK1/2 antibody (dilution-1: 5000; CST; cat. no. 9102). The sequence of βarr1, V2Rpp360A, and V2RppWT was analyzed using the ChemDoc imaging system (Bio-Rad), and densitometry-based quantification was carried out using ImageJ software suite.

Molecular dynamics simulations
Data without Fab30 was adapted from a previous study30. To generate V2RppWTβarr1, V2Rpp360Aβarr1, and V2Rpp360Aβarr1-Fab30 complexes, we used previously determined crystal structure36. The sequence of βarr1 was reverted to match the isoform used in the in-vitro experiments [Uniprot AC: P29066]. The phosphorylation state of the V2Rpp was retained from the used crystal structures. Missing fragments in the βarr1 and V2Rpp structures were modeled using the loop modeller module available in the MOE package (www.chemcomp.com). In Fab30 we maintained residues 5 to 108 of the light chain and residues 1 to 123 of the heavy chain. The complexes were solvated (TIP3P water) and neutralized using a 0.15 concentration of NaCl ions. System parameters were obtained from the Charmm36M force field36. Simulations were carried out using the ACEMD3 engine37. Both systems underwent a 20 ns equilibration in conditions of constant pressure (NPT ensemble, pressure maintained with Berendsen barostat, 1.01325 bar pressure), using a timestep of 2 fs. During this stage
Restraints were applied to the backbone. This was followed with 5 × 2 µs of simulation for each system in conditions of constant volume (NVT ensemble) using a timestep of 4 fs. This allowed us to amass a total of 10 µs simulation time per system. Simulations of inactive βarr1, as well as the V2RWTβarr1 complex were carried out in a 3 x 500 ns setup. For each of the simulations we used a temperature of 310 K, which was maintained using the Langevin thermostat, hydrogen bonds were restrained using the RATTLE algorithm. Non-bonded interactions were cut-off at a distance of 9 Å, with a smooth switching function applied at 7.5 Å. The inter-domain rotation angle of βarr1 was analysed using a script kindly provided by Naomi Latoracca.

The angle was measured by comparing the displacement of the C-domain relative to the N-domain between the inactive (PDB code: 1G4R) and active βarr1 crystal structures (PDB code: 4JQI). Each simulation frame was aligned to a representative snapshot from the inactive structure. A total of 10 µs simulation time per system was collected for analysis. Simulations of inactive βarr1, as well as the V2RWTβarr1 complex were carried out in a 3 x 500 ns setup. For each of the simulations we used a temperature of 310 K, which was maintained using the Langevin thermostat, hydrogen bonds were restrained using the RATTLE algorithm. Non-bonded interactions were cut-off at a distance of 9 Å, with a smooth switching function applied at 7.5 Å. The inter-domain rotation angle of βarr1 was analysed using a script kindly provided by Naomi Latoracca. The angle was measured by comparing the displacement of the C-domain relative to the N-domain between the inactive (PDB code: 1G4R) and active βarr1 crystal structures (PDB code: 4JQI). Each simulation frame was aligned to a representative snapshot from the inactive structure.
to the reference structures using the Ca atoms of the β-strands present within the N-domain, while the same atoms present in the C-domain were used to calculate the rotation angle. We have deposited all the simulation data presented in the current manuscript in the GPCRmd portal.

**Co-immunoprecipitation (co-IP) assay**

Co-IP was performed to evaluate the interaction between V_{R\beta\text{pp}}^{360\alpha} and V_{R\beta\text{pp}}^{360\alpha} with βarr1 in presence of Fab30 and ScFv30. 5 μg of purified βarr1 was activated with 10-fold and 50-fold molar excess of phospho-peptides for 1 h at room temperature (25 °C) in binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl). Thereafter, the activated βarr1 was incubated with 2.5 μg of purified Fab30 or ScFv30. Subsequently, 20 μl of pre-equilibrated Protein G beads (GE Life-sciences; cat. no. 17547802) were added to the reaction mixture and incubated for an additional 1 h at room temperature, which was followed by extensive washing (3–5 times) with binding buffer + 0.01% LMNG. Elution was taken with 2X SDS loading buffer. Interaction of Fab30 and ScFv30 with βarr1 in presence of phospho-peptides was visualized using Coomassie staining of the gels. Band intensity was analyzed by ImageJ gel analysis software.

To assess the effect of ScFv30 on V_{R\beta\text{pp}}^{360\alpha} induced βarr1-β2-adaptin interaction, we performed co-immunoprecipitation assay (co-IP). The V_{R\beta\text{pp}}^{360\alpha} receptor was expressed in Sf9 cells, stimulated with 100 mM AVP and centrifuged to obtain receptor pellet. The receptor pellet was resuspended in appropriate volume of lysis buffer containing 20 mM HEPES, 150 mM NaCl, 1X PhosSTOP (Roche; cat. no. 04906837001), and 1X protease inhibitor (Roche; cat. no. 04693116001), subjected to Dounce homogenization and incubated with 1 μg of purified βarr1 for 30 min at room temperature. The receptor-βarr complex was again incubated with 5 μg of purified ScFv30 or ScFv-CTL for another 30 min and solubilized with 1% LMNG for 1 h. Meanwhile, GST or GST-β2-adaptin protein (2.5 μg) was immobilized on 20 μl buffer (20 mM HEPES, 150 mM NaCl) equilibrated GS beads (1 h at room temperature) and washed once to remove any unbound protein. Subsequently, the supernatant from solubilized complex was allowed to bind with protein bound GS beads (1 h at room temperature) followed by three washes with wash buffer (20 mM HEPES, 150 mM NaCl, 0.01% LMNG). The bead-bound complex was eluted in 2X SDS loading buffer. Eluted samples were separated by 12% SDS–polyacrylamide gel electrophoresis and probed using βarr antibody and HRP-coupled protein G antibody (dilution-1: 10000; CST; cat. no. sc-47778) was used for loading control. After solubilization, 20 μl of lysate was set aside for confirming equal loading of βarr1 and ScFv. The lysate was run on separate 12% SDS–polyacrylamide gel and probed using βarr antibody and HRP-coupled protein G antibody (dilution-1: 10000; CST; cat. no. 4674). BRET signals were measured using a plate reader (Victor X4-Perkin-Elmer). Coelenterazine h (Nanolight™, final concentration of 5 μM) was added 25 min prior to BRET measurement. The filter set used was 460/80 nm and 535/30 nm for detecting the Renilla, Renilla luciferase (donor) and YFP (acceptor) light emissions, respectively. The BRET ratio was determined by calculating the ratio of light emitted by YFP over light emitted by Renilla. For BRET analysis, HEK-293 cells were transfected with 120 ng of βarr1-RlucII and 1 μg of β2-adaptin-YFP along with various amounts (0 to 3 μg) of Ibb30 in 100 mM dishes or scaled down to 1/6 in a well in 6well plates. BRET signals were measured in absence of ligand stimulation. Expression levels of Ibb30 were accessed by western blotting with anti-HA-peroxidase conjugate (dilution-1: 1000, Sigma-Aldrich; cat. no. 12013819001). Anti-β-actin antibody (dilution-1: 2000, Santa Cruz Biotechnology; cat. no. sc-47778) was used for loading control.

**Data quantification and statistical analysis**

The experiments were conducted at least three times and data (mean ± SEM) were plotted and analyzed using GraphPad Prism software (v9.3). The data were normalized with respect to proper experimental controls and appropriate statistical analyses were performed as indicated in the corresponding figure legends.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The original raw data for gels, immunoblots and confocal micrographs have been deposited in Mendeley Data (https://doi.org/10.17632/8wmkcw8ht7.1). This paper does not report any original code. The coordinates for V_{R\beta\text{pp}}^{360\alpha}-βarr1 and V_{R\beta\text{pp}}^{360\alpha}-βarr1 crystal structures used in this study are available in PDB with ID 4QJL and 7DFA, respectively. Any additional information required to reanalyze the data reported in this paper is available from the corresponding author upon reasonable request. Source data are provided with this paper. Original data pertaining to MD simulation are deposited in GPCRmd (https://submission.gpcrmd.org/dynadb/publications/14868). Source data are provided with this paper.

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Author contributions

M.B. (MBA) carried out confocal microscopy, assisted in the NanoBiT assay, β2-adaptin interaction experiments using co-immunoprecipitation and ERK1/2 MAP kinase activation experiments; M.C. generated the receptor constructs with the help from P.S., carried out the limited proteolysis experiment with A.R., participated in β2-adaptin interaction experiments using co-IP, and performed ERK1/2 MAP kinase activation experiments with S.P.; HD-A carried out GloSensor assay with the help from M.C. and NanoBiT assay with M.B. and PS, participated in β2-adaptin interaction experiments using co-IP; M.B. (MB), B.P., and M.K.Y. performed Fab30/SceFv30 co-IP assay; R.B. and J.M. carried out structural analysis of crystal structures; D.D. performed BRET experiments to monitor endosomal trafficking of βar1 under the
supervision of TEH; T.M.S. performed MD simulation studies under the supervision of J.S.; Y.N. performed βarr1-β2-adaptin BRET experiments under the supervision of S.A.L.; K.K. and A.I. provided new reagents; all authors contributed in data interpretation and manuscript writing; T.E.H. and S.A.L. edited the manuscript; A.K.S. coordinated and supervised the overall project.

**Competing interests**
The authors declare no competing interests.

**Additional information**

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**Correspondence** and requests for materials should be addressed to Arun K. Shukla.

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