Genetically encoded intrabody sensors report the interaction and trafficking of β-arrestin 1 upon activation of G protein–coupled receptors

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#equal contribution

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Abstract

Agonist stimulation of G protein–coupled receptors (GPCRs) typically leads to phosphorylation of GPCRs and binding to multifunctional proteins called β-arrestins (βarrs). The GPCR–βarr interaction critically contributes to GPCR desensitization, endocytosis, and downstream signaling, and GPCR–βarr complex formation can be used as a generic readout of GPCR and βarr activation. Although several methods are currently available to monitor GPCR–βarr interactions, additional sensors to visualize them may expand the toolbox and complement existing methods. We have previously described antibody fragments (FABs) that recognize activated βarr1 upon its interaction with the vasopressin V2 receptor C-terminal phosphopeptide (V2Rpp). Here, we demonstrate that these FABs efficiently report the formation of a GPCR–βarr1 complex for a broad set of chimeric GPCRs harboring the V2R C terminus. We adapted these FABs to an intrabody format by converting them to single-chain variable fragments (ScFvs) and used them to monitor the localization and trafficking of βarr1 in live cells. We observed that upon agonist simulation of cells expressing chimeric GPCRs, these intrabodies first translocate to the cell surface, followed by trafficking into intracellular vesicles. The translocation pattern of intrabodies mirrored that of βarr1, and the intrabodies co-localized with βarr1 at the cell surface and in intracellular vesicles. Interestingly, we discovered that intrabody sensors can also report βarr1 recruitment and trafficking for several unmodified GPCRs. Our characterization of intrabody sensors for βarr1 recruitment and trafficking expands currently available approaches to visualize GPCR–βarr1 binding, which may help decipher additional aspects of GPCR signaling and regulation.

Introduction

G protein-coupled receptors (GPCRs) recognize a diverse set of ligands and initiate a broad spectrum of downstream signaling responses (1). Upon agonist-stimulation, GPCRs couple to three major sub-families of cellular proteins
namely, the heterotrimeric G-proteins, GRKs (GPCR kinases) and β-arrestins (βarrs) (1). Of these, βarrs are multifunctional adaptor proteins, which play a central role in regulatory and signaling paradigms of GPCRs (2,3). βarrs are evenly distributed in the cytoplasm under basal condition, and upon agonist-stimulation, they typically translocate to the plasma membrane to interact with activated and phosphorylated receptors (4).

Binding of βarrs to GPCRs at the plasma membrane results in termination of G-protein coupling and desensitization of receptors through a steric hindrance based mechanism (5). Subsequently, βarrs either dissociate from the receptors and re-localize back in the cytoplasm, or they traffic into endosomal vesicles in complex with the receptors (2,4). These two different patterns are referred to as “class A” and “class B”, respectively (4). βarrs also contribute in a number of downstream GPCR signaling pathways such as ERK1/2 MAP kinases activation although strict G-protein independence of such mechanisms are currently being discussed and debated (6-9).

Considering the multifaceted roles of βarrs, understanding the details of their interaction with GPCRs continues to be a frontier area in GPCR research (10). The interaction of βarrs with GPCRs involves two distinct components (11,12). One is receptor phosphorylation, primarily in the carboxyl-terminus but also in the intracellular loops, and the other is the intracellular side of receptor transmembrane bundle, referred to as receptor core (11,12). There are several assays that are currently used to measure GPCR-βarr interaction including those based on resonance energy transfer (13-15), enzyme complementation (16) and reporter responses (17,18). Still however, developing novel sensors is desirable to expand the currently available toolbox and complement the existing assays.

Previous studies have suggested that receptor phosphorylation is not only sufficient to promote βarr binding but it can also induce βarr conformations capable of mediating receptor endocytosis and signaling (19-21). These findings raise the possibility that biochemical reagents such as antibodies, which selectively recognize βarr conformation triggered by the interaction of phosphorylated receptor, may serve as sensors for βarr recruitment and trafficking. Here, we develop and characterize intrabody sensors derived from synthetic antibody fragments (FABs) against βarr1 that report the formation of GPCR-βarr1 complexes and allow us to monitor βarr1 trafficking in cellular context.

Results

**Synthetic antibody fragments report the formation of β2V2R-βarr1 complex**

Agonist-induced receptor phosphorylation is a key determinant for βarr recruitment (11). A phosphopeptide corresponding to the carboxyl-terminus of the human vasopressin V2 receptor, referred to as V2Rpp, has been used extensively as a surrogate to induce active βarr conformation in-vitro (22-25). We have previously generated and characterized a set of synthetic antibody fragments (FABs) that selectively recognize V2Rpp-bound βarr1 (26). We have also used one of these FABs, referred to as Fab30, to monitor the interaction of βarr1 with a chimeric β2 adrenergic receptor harboring V2R carboxyl-terminus (referred to as β2V2R) and V2R (25). As the first step towards developing these FABs as potential sensors of GPCR-βarr interaction and trafficking, we first confirmed their ability to report the formation of β2V2R-βarr1 complex in-vitro (Figure 1A-D). Here, we used lysates from cells expressing FLAG-β2V2R mixed with purified βarr1 and FABs, followed by co-immunoprecipitation (co-IP) and detection of the receptor as readout of complex formation. We observed that Fab30, and the additional FABs, selectively pull-down β2V2R upon agonist-stimulation through the formation of receptor-βarr1 complex (Figure 1A-D). A control FAB that does not interact with βarr1 failed to yield any detectable signal in the co-IP experiment (Figure 1A-B).
**Fab30 reports the formation of βarr1 complex for multiple chimeric GPCRs**

Before proceeding to generate potentially generic intrabody sensors from these FABs, we evaluated their ability to recognize βarr1 complex with other GPCRs. Considering that these FABs were selected against V2Rpp-bound βarr1, we reasoned that they should detect βarr1 complex for other chimeric GPCRs harboring the V2R carboxyl-terminus, similar to that in β2V3R. We generated six different chimeric GPCRs including the members from different sub-classes such as chemokine (CCR2-V3R), adrenergic (α2B-V3R), Complement (C5aR1-V3R), muscarinic (M5-V3R) and dopamine (D2-V3R and D5-V3R) receptors. Some of these receptors such as MSR, α2BR and D2R contain large 3rd intracellular loop (ICL3) while others have relatively shorter ICL3. We tested the ability of Fab30, which was most effective among all the FABs, to report the formation of receptor-βarr1 complex in co-IP assay for these receptors. As presented in Figure 2A-F, we observed that Fab30 efficiently recognized βarr1 for every chimeric GPCR tested here, similar to that of β2V3R. This finding allowed us to conceive that these FABs should work as generic intrabody sensors of βarr1 interaction and trafficking in cellular context for a broad set of chimeric GPCRs.

**Conversion of FABs into intrabodies and their expression analysis**

In order to develop these FABs into cellular sensors of βarr1 activation and trafficking, it is required to express them in functional form in the cytoplasm as intrabodies. We therefore converted the selected FABs into ScFvs (single chain variable fragments) by connecting the variable domains of their heavy and light chains through a previously optimized flexible linker (12), and then expressed them in HEK-293 cells as intrabodies, either with a carboxyl-terminal HA tag or as YFP fusion (Figure 3A-C). We observed robust expression of two of these intrabodies namely intrabody30 (Ib30) and intrabody4 (Ib4) in HEK-293 cells while others displayed relatively weaker expression (Figure 3B). For YFP-tagged intrabodies, we observed cytoplasmic as well as nuclear localization (Figure 3C). The underlying reason for nuclear localization of the intrabodies is not apparent to us although a previous study has also reported nuclear localization of an intrabody targeting β2 adrenergic receptor (27).

**Ib30 and Ib4 report the interaction of βarr1 with β2V2R and trafficking**

We next tested whether intrabodies can report the formation of receptor-βarr1 complex in cellular context. We first co-expressed β2V2R, βarr1 and HA-tagged intrabodies in HEK-293 cells, stimulated the cells with either an agonist (Isoproterenol) or inverse-agonist (carazolol) and immunoprecipitated the intrabodies using the HA tag. We observed that both intrabodies i.e. Ib30 and Ib4 recognized β2V2R-βarr1 complex upon agonist-stimulation although Ib30 was relatively more efficient (Figure 4A-B). We also tested the ability of Ib30 to recognize β2V2R-βarr1 complex formed upon stimulation of the receptor with a set of ligands with varying efficacies. Importantly, we observed that the level of recognition of the β2V2R-βarr1 complex by Ib30 mirrors the efficacy of the ligands (Figure 4C-D). This observation underscores the ability of Ib30 to report the formation of pharmacologically relevant receptor-βarr1 complex and corroborates its suitability as a reliable sensor of receptor-βarr1 interaction.

In order to probe the utility of intrabodies to monitor βarr1 trafficking upon receptor stimulation, we co-expressed β2V2R, βarr1-mCherry and YFP-tagged intrabodies in HEK-293 cells, and followed the localization of βarr1 and intrabodies using confocal microscopy after agonist treatment (Figure 4E-F). As expected, activation of β2V2R resulted in a typical “class B” pattern of βarr1 translocation, and interestingly, the intrabodies followed the localization of βarr1 and displayed robust colocalization (Figure 4E-F). We observed that Ib30 and Ib4 were first translocated to the cell
In order to probe whether \( V_2^R \) and \( \beta_{arr1} \), we employed a BRET assay. We also measured the ability of Ib30 to recognize endogenous \( \beta_{arr1} \) upon agonist-stimulation, and followed the localization pattern of \( \beta_{arr1} \) upon agonist-stimulation as reflected by translocation to the cell surface first followed by localization in intracellular vesicles. An additional band was observed on the Western blot in the co-IP experiment, which migrates below the \( V_2^R \) band, but its origin is currently not clear to us.

We also measured the ability of Ib30 to recognize endogenous \( \beta_{arr1} \) upon agonist-stimulation of \( V_2^R \), and observed a robust interaction in co-immunoprecipitation assay (Figure 6A-B). Furthermore, we evaluated the translocation pattern of Ib30-YFP upon agonist-stimulation for \( \beta_2 V_2^R \) and \( V_2^R \) in HEK-293 cells where \( \beta_{arr1} \) is overexpressed without any modification. As presented in Figure 6C, Ib30-YFP was robustly localized to intracellular vesicles after agonist-stimulation, which is reminiscent of typical translocation pattern of \( \beta_{arr1} \) for these receptors. These data further strengthen the utility of intrabody sensors described here in monitoring \( \beta_{arr1} \) recruitment and trafficking.

**Intrabodies do not alter \( \beta_{arr1} \) recruitment, receptor endocytosis, G-protein coupling and ERK1/2 phosphorylation**

In order for the intrabodies to be reliable sensors of \( \beta_{arr1} \) recruitment and trafficking, it is important that they do not significantly alter \( \beta_{arr1} \) recruitment, receptor endocytosis and G-protein coupling. Therefore, we first measured agonist-induced recruitment of \( \beta_{arr1} \) to \( V_2^R \) in presence of either a control intrabody (Ib-CTL) or Ib30/Ib4 using an intermolecular BRET assay. As presented in Figure 7A, we did not observe any significant difference in \( \beta_{arr1} \) recruitment. Next, in order to probe whether \( V_2^R \) is colocalized with Ib30 and \( \beta_{arr1} \) on intracellular vesicles, we performed three-color confocal imaging on HEK-293 cells expressing Flag-\( V_2^R \), \( \beta_{arr1} \)-YFP and Ib30-HA after agonist-stimulation (Figure 7B). Expectedly, we observed a robust co-localization of \( V_2^R \), \( \beta_{arr1} \) and Ib30 on intracellular vesicles suggesting that Ib30 does not alter the normal trafficking pattern of receptor-\( \beta_{arr1} \) complex in cellular context. This is further corroborated by the pattern of \( V_2^R \) colocalization with the early endosomal markers EEA1 and APPL1 which remains unaltered in presence of Ib-CTL vs. Ib30 (Figure 7C-D). Furthermore, we also measured \( \beta_{arr1} \) trafficking to endosomes upon \( V_2^R \) activation using an enhanced bystander BRET (ebbBRET) set-up (15) in presence of either Ib-CTL or Ib4/Ib30. Although we did not observe a significant difference in EC\(_{50} \) values (Figure 7E), Ib4/Ib30 appear to stabilize endosomal localization of \( \beta_{arr1} \) as reflected by \( \Delta BRET \) signal (Figure 7F). This observation is particularly relevant if the intrabody sensors are used in the context of receptor recycling where they might slow-down receptor recycling to the plasma membrane, and it would be interesting to probe this aspect further in future studies.

We next measured the effect of intrabodies on G\(_{\alpha}\)-coupling to the \( V_2^R \) using cAMP response as readout. Once again, we did
not observe any significant difference in cAMP dose response or time-kinetics for Ib-CTL vs Ib30/Ib4 conditions (Figure 8A-B). Finally, we also evaluated the effect of intrabodies on agonist-induced ERK1/2 MAP kinase activation, a prototypical readout of V₂R signaling, and did not detect a significant alteration by the intrabodies (Figure 8C-D). Taken together, these data establish that intrabodies do not have a major effect on transducer coupling and receptor endocytosis making them suitable sensors to record βarr1 interaction and trafficking for GPCRs.

**Ib30 as a generic sensor of agonist-induced βarr1 trafficking for multiple chimeric GPCRs**

Taking lead from the ability of Fab30 to recognize βarr1 complex with several chimeric GPCRs as presented in Figure 2, we next evaluated Ib30 as a sensor to report βarr1 trafficking for these chimeric GPCRs in cellular context. Similar to previous experiments, we co-expressed the chimeric receptors with βarr1-mCherry and Ib30-YFP in HEK-293 cells, and followed the localization of βarr1 and intrabodies using confocal microscopy after agonist treatment (Figure 9A-F). We observed that similar to β₂V₂R, Ib30 followed βarr1 translocation pattern by first localizing to the cell surface followed by trafficking into intracellular vesicles for all of these chimeric receptors (Figure 9A-F). It is worth noting here that the receptors used in Figure 9A-C contain most of the phosphorylation sites in their carboxyl-terminus while their 3rd intracellular loops are relatively small. On the other hand, receptors included in Figure 9D-F, harbor a larger 3rd intracellular loop, which also contains most of the potential phosphorylation sites, and their carboxyl-terminus is relatively smaller. Therefore, the data presented in Figure 9 not only demonstrate the generality of Ib30 as a sensor to monitor agonist-induced βarr1 recruitment and trafficking for chimeric GPCRs but also its versatility for receptors differing in terms of their carboxyl-terminus and intracellular loops.

**Ib30 sensor suggests conformational diversity in GPCR-βarr1 complexes**

Finally, we evaluated the ability of Ib30 sensor to report the trafficking of βarr1 for a set of GPCRs without the fusion of V₂R-tail. We observed that Ib30-YFP followed agonist-induced translocation pattern of βarr1 for several different receptors including the complement C5a receptor 1 (C5aR1), the neurotensin receptor 1 (NTSR1), the muscarinic acetylcholine receptor subtype 2 (M2R), and the atypical chemokine receptor subtype 2 (ACKR2) (Figure 10A-D). We also validated the ability of Ib30 to recognize receptor-bound βarr1 for C5aR1 and ACKR2 by co-immunoprecipitation experiment (Figure 10E-F). These findings suggest that Ib30 can act as a sensor for monitoring agonist-induced βarr1 translocation for at least some GPCRs with their native carboxyl-terminus as well. Interestingly however, we observed that Ib30 did not robustly follow βarr1 translocation for the bradykinin subtype 2 receptor (B2R) upon agonist-agonist-stimulation (Figure 10G) although there was clear translocation of βarr1, first to the plasma membrane and then in intracellular vesicles. Taken together, these data potentially hint at conformational differences in GPCR-βarr1 complexes, even if the overall recruitment patterns are apparently similar. Future studies focused on measuring conformational differences in different GPCR-βarr complexes may provide additional insights and possibly link the conformational diversity to functional outcomes.

**DISCUSSION**

Monitoring βarr interaction and subsequent trafficking has been used extensively to study the activation and regulatory framework of GPCRs. A number of approaches are commonly utilized for this including direct fusion of fluorescent proteins to βarrs (4), resonance energy transfer (FRET/BRET) based assays (14,28), enzyme complementation methods (16) and reporter assays (17,18). Each of these methods necessitates a significant engineering
and modification of the receptor, the βarr, or both. Intrabody sensors described here recognize receptor-bound βarr1 and report its trafficking in cellular context without the need for any modification of βarr1.

Although we observe that the intrabody sensors are capable of recognizing βarr1 for several GPCRs without the modification of their carboxyl-terminus, a potential drawback is that they are not likely to be universal for every GPCR as reflected for B3R in Figure 10G. On the other hand, these intrabody sensors are able to recognize βarr1 more generally in the context of chimeric GPCRs harboring V2R carboxyl-terminus. It is conceivable that a similar strategy can be employed for other GPCRs as well by using, for example, phosphopeptides derived from the corresponding receptors. It is also worth noting here that many of the βarr1 assays such as PRESTO-TANGO also utilize chimeric GPCRs with V2R carboxyl-terminus (V2R tail) (18). Engineering V2R tail typically imparts “class B” pattern on GPCRs and thereby, makes the detection of βarr1 interaction more robust compared to the unmodified receptors (29). It is also important to note that out of five different FABs tested here, only two expressed efficiently as intrabodies in the cytoplasm. Therefore, starting with a larger number of FABs may be desirable to obtain more functional intrabodies in future endeavors.

Considering that YFP fusion does not alter the ability of intrabodies to interact with βarr1 and follow their translocation, it is also conceivable that they can be adapted in resonance energy transfer assays, or even in NanoBit format, for quantitative measurements of receptor-βarr1 interaction. Such strategies may yield even more sensitive versions of these intrabody sensors compared to approaches utilized here. In addition, while the intrabody sensors developed here are specific to βarr1 (25), it is plausible to design and develop similar intrabodies for Barr2 as well. Such an effort may help uncover novel insights into the functional divergence of the two βarr isoforms (30).

Another interesting aspect of GPCR-βarr1 interaction is the ability of differential receptor phosphorylation patterns to induce distinct functional conformations in βarrs (31,32). For several GPCRs, different phosphorylation patterns arising in ligand-specific, cell-type specific and kinase-specific manners have been mapped and correlated with βarr mediated functional outcomes (33-35). Thus, it is tantalizing to hypothesize that intrabodies designed against different phosphopeptides derived from a given receptor may illuminate interesting attributes of receptor signaling and regulation in future.

In conclusion, our study expands the currently available toolbox to monitor GPCR-βarr interaction and trafficking, and the intrabody sensors described here should facilitate drawing novel insights into GPCR signaling and regulatory paradigms.

**Experimental procedures**

**General reagents, plasmids and cell culture**

HEK-293 cells (ATCC) were maintained in DMEM containing 10% FBS and penicillin/streptomycin (100U/mL) at 37°C in 5% CO2. Transient transfection of plasmids was performed using PEI and cells were typically assayed 48h post-transfection. The plasmids encoding FLAG-β2V2R, FLAG-V2R, Ib-CTL-HA, Ib4-HA, Ib30-HA, βarr1-mCherry have been described previously (25). YFP-tagged intrabodies were generated by sub-cloning their coding region in pCMV6-AC-YFP vector. The chimeric GPCRs were generated by grafting the V2R-tail sequence at residue 324 in CCR2, 443 in α2BR, 443 in D2R, 379 in D5R, 514 in M5R and 326 in C5aR1. All constructs were verified by DNA sequencing. The antibodies were purchased from Sigma (HRP-coupled mouse anti-FLAG M2), Cell Signaling Technology (βarrs), Santa Cruz Biotechnology (rabbit anti-HA) and Thermo Fisher (goat anti-rabbit Alexa-Fluor647 and goat anti-mouse Alexa-Fluor555). Other general chemicals were purchased from Sigma, APEXBIO and local
suppliers. Recombinant human CCL7 was purified following a previously published protocol (36).

**Co-immunoprecipitation assay**

In order to probe the reactivity of FABs towards β₂VαR (Figure 1), SF9 cells expressing FLAG-tagged receptor were lysed and incubated with purified βarr1 and FABs. For the co-IP data presented in Figure 2, the plasmids encoding FLAG-tagged receptor and βarr1 were transfected in HEK-293 cells. 48h post-transfection, cells were serum-starved for 4-6h, stimulated with agonist, lysed by douncing and incubated with FAB30 for 1h at room temperature. Subsequently, the receptor-βarr1-FAB complex were solubilized with 1% MNG for 1h, centrifuged to collect the clarified solubilized complex, and 20μl of pre-equilibrated (in 20mM HEPES, 150mM NaCl, pH 7.4 buffer) Protein L beads (GE Healthcare) were added. After additional 1h incubation, beads were washed three times with wash buffer (20mM HEPES, 150mM NaCl, pH 7.4, 0.01% MNG) and eluted with 2XSDS loading buffer. Eluted samples were run on 12% SDS-PAGE, and the receptors were detected using HRP-coupled anti-FLAG M2 antibody while the FABs were visualized using Coomassie staining.

In order to assess the ability of intrabodies to report the formation of receptor-βarr1 complex (Figure 4 and Figure 6A-B), HEK-293 cells expressing the FLAG-tagged receptor, βarr1 and HA-tagged intrabodies were stimulated with saturating concentration of indicated ligands for 30min at 37°C. Afterwards, cells were lysed in NP-40 lysis buffer (Tris 50 mM; NaCl 150mM; PhosStop 1X; Protease inhibitor 1X; NP-40 1%) followed by incubation with 20μl of pre-equilibrated HA beads (Sigma, Cat. No. 100350) for 2h at 4°C. Beads were washed three times with wash buffer (20mM HEPES, 150mM NaCl, pH7.4), eluted with 2XSDS loading buffer and proteins were visualized by Western blotting (HRP-coupled anti-FLAG M2 antibody at 1:2000 dilution and anti-HA antibody, sc-805 from Santa Cruz Biotechnology at 1:5000 dilution).

**Confocal microscopy**

In order to monitor the translocation of βarr1 and intrabodies by confocal microscopy (Figure 3C-D, Figure 4E-F, Figure 5C-E, Figure 6C, Figure 9A-F, Figure 10A-D and Figure 10G), HEK-293 cells were transfected with plasmids encoding the indicated receptor, βarr1-mCherry and YFP-tagged intrabodies. 24h post-infection, cells were seeded onto confocal dishes (GenetiX; Cat. No. 100350) pretreated with 0.01% poly-D-lysine (Sigma). After another 24h, cells were serum-starved for 4-6h prior to stimulation with saturating concentration of indicated agonists. For live cell confocal imaging, we used Zeiss LSM 710 NLO confocal microscope and samples were housed on a motorized XY stage with a CO₂ enclosure and a temperature-controlled platform equipped with 32x array GaAsP descanned detector (Zeiss). YFP was excited with a diode laser at 488 nm laser line while m-cherry was excited at 561 nm. Laser intensity and pinhole settings were kept in the same range for parallel set of experiments and spectral overlap for any two channels was avoided by adjusting proper filter excitation regions and bandwidths. Images were scanned using the line scan mode and images were finally processed in ZEN lite (ZEN-blue/ZEN-black) software suite from ZEISS. Colocalization was analyzed by calculating Pearson’s correlation coefficient (PCC) between the indicated channels using JACoP plugin in ImageJ software (37). At least three regions of interest (ROIs) per cell were analyzed and the mean±SEM of PCC are presented in the respective figure legends together with the number of cells and independent experiments.

For three color imaging (Figure 7B) and colocalization with early endosomal markers (Figure 7C), receptor imaging of live or fixed cells was monitored by “feeding” cells with anti-FLAG antibody (15 min, 37°C) in phenol-red-free DMEM prior to agonist treatment. Fixed cells were washed three times in
PBS/0.04% EDTA to remove FLAG antibody bound to the remaining surface receptors, fixed using 4% PFA (20 min at RT), permeabilized and stained using HA primary antibody followed by Alexa-Fluor 555 or 647 secondary antibodies. For co-localization of FLAG-V2R with endosomal markers, cells were treated as above except incubated with either of the following primary antibodies post-permeabilization; EEA1 (rabbit anti-EEA1 antibody from Cell Signaling Technology) or APPL1 (rabbit anti-APPL1 antibody from Cell Signaling Technology). Cells were imaged using a TCS-SP5 confocal microscope (Leica) with a 63x 1.4 numerical aperture (NA) objective and solid-state lasers of 488 nm, 561 nm, and/or 642 nm as light sources. Leica LAS AF image acquisition software was utilized. All subsequent raw-image tiff files were analyzed using ImageJ or LAS AF Lite (Leica), and colocalization was measured by calculating the Pearson’s correlation coefficient (PCC) using JACoP plugin in ImageJ software as mentioned above.

**GloSensor assay and ERK1/2 phosphorylation**

In order to measure the effect of intrabodies on Gαs-coupling, if any, we measured agonist-induced cAMP response in GloSensor assay following a previously described protocol (25). Briefly, HEK-293 cells were transfected with plasmids encoding the V2R, the luciferase-based cAMP biosensor (pGloSensorTM-22F plasmid) and the intrabodies. 16h post-transfection, media was aspirated, cells were flushed and pooled together in assay buffer containing 1X Hanks balanced salt solution, pH 7.4 and 20 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]. Cell density was measured and adjusted such as to yield approximately 125,000 cells in 100µl. Cells were pelleted at 2000rpm for 3min to remove the assay buffer and then the pellet was re-suspended in the desired volume of sodium luciferin solution prepared in the same assay buffer. After seeding the cells in a 96 well plate, the plate was incubated at 37°C for 90min followed by an additional incubation of 30 min at room temperature. Subsequently, various doses of the indicated ligand were added to the cells, and the luminescence reading was recorded using a micro-plate reader (Victor X4; Perkin Elmer). Agonist-induced phosphorylation of ERK1/2 MAP kinase was measured by Western blotting following a previously described protocol (38).

**BRET assay**

For measuring βarr1 recruitment and endosomal localization by BRET (Figure 8A and 8D), transfections were performed on HEK-293 cells seeded (40,000 cells/100 µl/well) in 96-well white micro-plates (Greiner) using PEI at a ratio of 4:1 (PEI: DNA). In order to monitor V2R-βarr1 interaction, we used βarr1-RlucII and V2R-YFP plasmids described previously (39). To monitor endosomal translocation of βarr1, we used enhanced bystander BRET (ebBRET) where the BRET acceptor (Renilla green fluorescent protein; rGFP), is fused to the FYVE domain from endofin protein targeted to early endosomes (rGFP-FYVE) and βarr1 fusion with the BRET donor Renilla luciferase II (RlucII) (40). 48h post-transfection, culture media was removed, cells were washed with DPBS (Dulbecco’s Phosphate Buffered Saline) and replaced by HBSS (Hank’s Balanced Salt Solution). Afterwards, cells were stimulated with increasing concentrations of AVP for 10min and 2.5µM coelenterazine H (BRET1) or coelenterazine 400a (BRET2) was added 5min before BRET measurement. BRET signals were recorded on a Mithras (Berthold scientific) micro-plate reader equipped with the following filters: 480/20 nm (donor) and 530/20 nm (acceptor) for BRET1 or 400/70 nm (donor) and 515/20 nm (acceptor) for BRET2. The BRET signal was determined as the ratio of the light emitted by the energy acceptor over the light emitted by energy donor. Raw BRET values are presented in Figure 7A and 7E while agonist-induced change in BRET signal (ΔBRET) obtained by calculating the difference in BRET values for the highest and lowest concentrations of AVP is presented in Figure 7F.
Statistical analysis and data presentation

Quantified data were plotted and analyzed using GraphPad Prism software, and the details of experimental replicates and statistical analysis are mentioned in the corresponding figure legends.

Data availability

All data are available in the manuscript.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contribution

M.Ba. and P.K. carried out the co-immunoprecipitation assay, confocal microscopy and ERK1/2 phosphorylation with help from H.D.-A., S.P., M.C., D.R. and A.S.; B.S. performed the BRET experiments under the supervision of M.Bo.; S.S. carried out three color imaging and colocalization with endosomal markers using confocal microscopy under the supervision of A.H.; AKS supervised and coordinated the overall project.

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**Figure legends**

**Figure 1. Synthetic antibody fragments (FABs) that recognize β2V2R-βarr1 complex.** A. Fab30 selectively recognizes agonist-induced β2V2R-βarr1 complex as assessed by co-immunoprecipitation. SF9 cells expressing FLAG-tagged β2V2R and GRK2CAAX were stimulated with either carazolol (1μM) or BI-167107 (100nM), lysed and mixed with purified βarr1 and Fab30 (or a control Fab). Subsequently, Fab was immunoprecipitated using protein L agarose beads and co-purification of the receptor was visualized by Western blotting using HRP-coupled anti-FLAG M2 antibody. Fabs were detected by coomassie staining. B. Densitometry-based quantification of Western blot signal in panel A presented as mean±SEM of four independent experiments normalized with respect to maximal response (treated as 100%). C. The ability of additional Fabs to recognize agonist-induced β2V2R-βarr1 complex assessed by co-immunoprecipitation following the protocol mentioned above. D. Densitometry-based quantification of Western blot signal in panel C presented as mean±SEM of four independent experiments normalized with respect to carazolol condition (treated as 1). Data in panel B and D are analyzed using One-Way-ANOVA (****p<0.0001; ***p <0.001; **p <0.01; *p <0.05).

**Figure 2. Fab30 reports agonist-induced interaction of βarr1 with chimeric GPCRs.** A-F. HEK-293 cells expressing N-terminally FLAG-tagged chimeric GPCRs harboring the V2R carboxyl-terminus and βarr1 were stimulated with corresponding agonists (100nM CCL7, 20μM Dopamine, 100nM C5α, 20μM Epinephrine, 20μM Dopamine and 20μM carbachol, respectively), lysed and mixed with purified Fab30. Subsequently, Fab30 was immunoprecipitated using protein L agarose beads and co-purification of the receptor was visualized by Western blotting using HRP-coupled anti-FLAG M2 antibody. Fabs were detected by Coomassie staining. Graphs in every panel show densitometry-based quantification of Western blot signal presented as mean±SEM of four independent experiments (three for D5V2R and D2V2R) normalized with respect to maximal response (treated as 100%) and analyzed using One-Way-ANOVA (***p <0.001).

**Figure 3. Conversion of FABs into intrabodies and their expression analysis.** A. Schematic representation of conversion of FABs into ScFv (Single Chain Variable Fragment) format for intracellular expression as intrabodies. B. Expression profile of intrabodies in HEK-293 cells visualized by Western blotting. Lysate prepared from HEK-293 cells expressing the indicated intrabodies with carboxyl-terminus HA tag were separated on SDS-PAGE followed by visualization using anti-HA antibody. C-D. Intracellular expression of Ib30-YFP/Ib4-YFP and βarr1-mCherry as visualized by confocal microscopy.
HEK-293 cells expressing the corresponding plasmids were subjected to live cell imaging and it revealed localization of Ib30-YFP and Ib4-YFP in both, cytoplasm and nucleus. Scale bar is 10μm.

**Figure 4. Intrabodies report agonist-induced formation of β₂V₂R-βarr1 complex and trafficking of βarr1 upon β₂V₂R stimulation.** A. The ability of intrabodies (Ib30 and Ib4) to recognize receptor-bound βarr1 upon agonist-stimulation. HEK-293 cells expressing β₂V₂R, βarr1 and Ib30/Ib4/Ib-CTL were stimulated with either inverse agonist (carazolol; 1μM) or agonist (Isoproterenol; 10μM) followed by co-immunoprecipitation (co-IP) using anti-HA antibody agarose. Subsequently, the proteins were visualized by Western blotting using anti-FLAG M2 antibody and anti-HA antibody. B. Densitometry-based quantification of the data in panel A presented as mean±SEM from four independent experiments normalized with maximal response (treated as 100%) and analyzed using One-Way-ANOVA (****p <0.0001). C. The ability of Ib30 to report the formation of receptor-βarr1 complex mirrors ligand efficacy. HEK-293 cells expressing β₂V₂R, βarr1 and Ib30 (or Ib-CTL) were stimulated with saturating concentrations of indicated ligands followed by co-immunoprecipitation and Western blotting as mentioned above. For isoproterenol condition, which yielded maximal signal, only 10% of the total elution from the co-IP is loaded on the gel to avoid signal saturation. D. Densitometry-based quantification of the data in panel C presented as mean±SEM from three independent experiments normalized with respect to maximal response (treated as 100%). E-F. HEK-293 cells expressing β₂V₂R, βarr1-mCherry and YFP-tagged Ib30/Ib4 were stimulated with isoproterenol (10μM), and the localization of βarr1 and intrabodies were visualized using confocal microscopy at indicated time-points. Pearson's correlation coefficients (PCC) were measured to assess the colocalization of βarr1 and Ib30 using JACoP plugin in ImageJ. Ib30 - 0.28±0.03 from 13 cells, 0.74±0.05 from 9 cells, 0.76±0.02 from 29 cells for the upper, middle and lower panels, respectively; 4 independent experiments. Ib4 - 0.24±0.03 from 10 cells, 0.84±0.03 from 9 cells, 0.94±0.01 from 20 cells for the upper, middle and lower panels, respectively, 3 independent experiments.

**Figure 5. Intrabodies report the formation of V₂R-βarr1 complex and trafficking of βarr1 upon V₂R stimulation.** A. The ability of intrabodies (Ib30 and Ib4) to recognize V₂R-bound βarr1 upon agonist-stimulation. HEK-293 cells expressing V₂R, βarr1 and Ib30/Ib4/Ib-CTL were stimulated with either inverse agonist (Tolvaptan; 100nM) or agonist (AVP; 100nm) followed by co-immunoprecipitation (co-IP) using anti-HA antibody agarose. Subsequently, the proteins were visualized by Western blotting using anti-FLAG M2 antibody and anti-HA antibody. B. Densitometry-based quantification of the data in panel A presented as mean±SEM from four independent experiments normalized with maximal response (treated as 100%) and analyzed using One-way-ANOVA with Bonferroni post-test (****p <0.0001). C-D. HEK-293 cells expressing V₂R, βarr1-mCherry and YFP-tagged Ib30/Ib4 were stimulated with AVP (100nM), and the localization of βarr1 and intrabodies were visualized using confocal microscopy at indicated time-points. Pearson's correlation coefficients (PCC) were measured to assess the colocalization of βarr1 and Ib30 using JACoP plugin in ImageJ. Ib30 - 0.31±0.02 from 16 cells, 0.81±0.03 from 16 cells, 0.80±0.02 from 20 cells for the upper, middle and lower panels, respectively; 6 independent experiments. Ib4 - 0.27±0.02 from 20 cells, 0.74±0.02 from 21 cells, 0.75±0.01 from 47 cells for the upper, middle and lower panels, respectively, 3 independent experiments. E. Time-lapse confocal imaging of HEK-293 cells expressing V₂R, βarr1-mCherry and Ib30-YFP to demonstrate agonist-induced translocation of βarr1 and Ib30 in the same cells over time. A representative image panel from three independent experiments is shown here. Scale bar is 10 μm.
Figure 6. Intrabody30 recognizes receptor-bound endogenous βarr1 and reports the trafficking of native βarr1. A. The ability of intrabodylb30 to recognize V2R-bound endogenous βarr1 upon agonist-stimulation. HEK-293 cells expressing V2R and HA-tagged lb30/lb-CTL were stimulated with either inverse agonist (Tolvaptan; 100nM) or agonist (AVP; 100nM) followed by co-immunoprecipitation (co-IP) using anti-HA antibody agarose. Subsequently, the proteins were visualized by Western blotting using anti-βarr and anti-HA antibodies. B. Densitometry-based quantification of the data in panel A presented as mean±SEM from three independent experiments normalized with maximal response (treated as 100%) and analyzed using One-Way-ANOVA (****p<0.0001). C. HEK-293 cells expressing β2,V2R/V2R and lb30-YFP were stimulated with isoproterenol (10μM) and AVP (100nM), respectively, and the localization of lb30-YFP was visualized using confocal microscopy. Representative images from three independent experiments are shown here. Scale bar is 10 μm.

Figure 7. Effect of intrabodies on βarr1 recruitment, V2R endocytosis and endosomal localization of βarr1. A. Intrabodies do not significantly alter agonist-induced βarr1 recruitment to V2R as assessed in intermolecular BRET assay. HEK-293 cells expressing V2R-venus, βarr1-RlucII and indicated intrabodies were stimulated with varying doses of AVP and the levels of BRET signal were recorded using a plate reader. Data represent mean±SEM from three independent experiments, each performed in duplicate. B. lb30 colocalizes with internalized V2R and βarr1 upon agonist-stimulation as visualized using confocal microscopy of HEK-293 cells expressing FLAG-V2R, βarr1-YFP and lb30-HA. The merged image shows colocalization of all three protein upon receptor internalization. Cells were ‘fed’ anti-FLAG M2 antibody prior to agonist stimulation (AVP 100 nM, 12 min), and subsequently, fixed, permeabilized, treated with HA antibody and imaged (Pearson’s correlation coefficient of V2R and βarr1 in unstimulated cells = 0.38±0.03 and in stimulated cells = 0.88±0.03, lb30 and V2R in unstimulated cells = 0.29±0.04 and in stimulated cells = 0.83±0.01, and βarr1 with lb30 in unstimulated cells = 0.43±0.08 and in stimulated cells = 0.63±0.04, no. of cells = 3). A representative image of n=3 cells/condition is shown here and the scale bar is 5 μm. C. lb30 does not significantly alter agonist-induced internalization of V2R as assessed by confocal microscopy. Comparative analysis of V2R colocalization with two early endosomal markers, EEA1 and APPL1, upon agonist-stimulation in presence of either lb-CTL or lb30. Cells expressing FLAG-V2R and lb30-HA were treated with anti-FLAG antibody prior to agonist stimulation (AVP, 100nM, 3-12 min) followed by fixation, permeabilization and staining for endosomal markers APPL1 or EEA1. Pearson’s coefficient of V2R and EEA1 in lb-CTL cells = 0.70±0.01 and in lb30 cells = 0.42±0.01, no. of cells = 4, and Pearson’s coefficient of V2R and APPL1 in lb-CTL cells = 0.69±0.07 and in lb30 cells = 0.30±0.07, no. of cells = 4. D. Colocalization was also measured by manual counting of punctae in confocal images and quantified data representing mean+SEM from four different cells per condition are presented. E. An intermolecular BRET assay to measure the effect of intrabodies on the endosomal localization of βarr1 upon agonist-stimulation. HEK-293 cells expressing V2R, βarr1-RlucII, rGFP-FYVE and indicated intrabodies were stimulated with varying doses of AVP and the levels of BRET signal were recorded using a plate reader. Data represent mean±SEM from four independent experiments, each performed in duplicate. F. Agonist-induced change in BRET signal (i.e. the difference in BRET signal between the highest and the lowest AVP doses) as measured in panel D is presented as ΔBRET and analyzed using One-Way ANOVA (**p<0.01; ***p<0.001).

Figure 8. Effect of intrabodies on G-protein coupling and ERK1/2 phosphorylation. A. lb30 does not significantly alter Gαs-coupling of V2R as reflected by cAMP response. HEK-293 cells expressing V2R,
indicated intrabodies, and a luciferase-based cAMP biosensor (F22) were stimulated with varying doses of AVP and levels of cAMP were measured in terms of bioluminescence using a micro-plate reader. Data are normalized with respect to the maximal response obtained in presence of Ib-CTL (treated as 100%) and the graph represents mean±SEM of three independent experiments, each performed in duplicate.

**B.** Time-course of agonist-induced cAMP response in HEK-293 cells expressing V2R and indicated intrabodies. Data are derived from the experiments described in panel A at AVP concentration of 100nM. **C-D.** Intrabodies do not significantly alter agonist-induced ERK1/2 MAP kinase phosphorylation. HEK-293 cells expressing V2R and indicated intrabodies were stimulated with AVP (100 nM) for indicated time-points followed by detection of ERK1/2 phosphorylation using Western blotting. Representative images from four independent experiments are shown here, and densitometry-based quantification of data, normalized with Ib-CTL, 30 min condition treated as 100%, are presented in the lower panels.

**Figure 9.** Ib30 reports agonist-induced trafficking of βarr1 for chimeric GPCRs. **A-F.** HEK-293 cells expressing the indicated chimeric GPCRs with V2R carboxyl-terminus, βarr1-mCherry and Ib30-YFP were stimulated with saturating concentration of respective agonists (100nM CCL7, 20μM Dopamine and 100nM C5a, 20μM Epinephrine, 20μM Dopamine and 20μM carbachol, respectively), and the localization of βarr1 and Ib30 was visualized using confocal microscopy at indicated time-points. Scale bar is 10 μm. Pearson's correlation coefficients (PCC) were measured to assess the colocalization of βarr1 and Ib30 using JACoP plugin in ImageJ, and the values for the upper, middle and lower panels, respectively, are presented here. CCR2V2R - 0.21±0.02 from 17 cells, 0.84±0.06 from 5 cells, 0.83±0.02 from 26 cells; 4 independent experiments. D5V2R - 0.36±0.04 from 9 cells, 0.87±0.04 from 6 cells, 0.82±0.03 from 30 cells; 3 independent experiments. C5aR1V2R - 0.31±0.03 from 34 cells, 0.87±0.01 from 40 cells, 0.85±0.01 from 53 cells; 4 independent experiments. α2BV2R - 0.30±0.04 from 7 cells, 0.90±0.02 from 8 cells, 0.91±0.02 from 11 cells; 4 independent experiments. D2V2R - 0.27±0.04 from 18 cells, 0.88±0.02 from 11 cells, 0.83±0.03 from 13 cells; 3 independent experiments. M5V2R - 0.27±0.02 from 15 cells, 0.79±0.04 from 22 cells, 0.82±0.05 from 9 cells; 4 independent experiments. Scale bar is 10 μm.

**Figure 10.** Ib30 reports agonist-induced trafficking of βarr1 for several unmodified GPCRs. **A-D.** HEK-293 cells expressing the indicated receptor, Barr1-mCherry and Ib30-YFP were stimulated with saturating concentration of respective agonists (100nM C5a, 20μM carbachol, 100nM NTS1 and 100nM CCL7, respectively), and the localization of βarr1 and Ib30 was visualized using confocal microscopy at indicated time-points. Pearson's correlation coefficients (PCC) were measured to assess the colocalization of βarr1 and Ib30 using JACoP plugin in ImageJ, and the values for the unstimulated and stimulated conditions, respectively, are presented here. C5aR1 - 0.27±0.03 from 20 cells, 0.75±0.03 from 25 cells; 5 independent experiments. M2R - 0.30±0.04 from 8 cells, 0.85±0.02 from 25 cells; 4 independent experiments. NTSR1 - 0.24±0.04 from 15 cells, 0.87±0.01 from 16 cells; 3 independent experiments. ACKR2 - 0.88±0.02 from 9 cells, 0.81±0.01 from 29 cells; 3 independent experiments. Scale bar is 10 μm. For ACKR2, we observed significant membrane localization of βarr1 and Ib30, even before agonist-treatment, which results into higher PCC values for unstimulated condition. **E-F.** HEK-293 cells expressing the C5aR1 and ACKR2, respectively, together with Barr1 and Ib30 were stimulated with either respective agonists (100nM) for indicated time-points followed by co-immunoprecipitation (co-IP) using protein L agarose beads. Subsequently, the proteins were visualized by Western blotting using anti-FLAG M2 antibody and anti-HA antibody. The right panels show densitometry-based quantification of four independent experiments normalized with signal at 30min (treated as 100%) and analyzed using One-
Way ANOVA (**p<0.01; ***p<0.001). G. Ib30 does not follow agonist-induced translocation of βarr1 for the bradykinin subtype 2 receptor (B₂R) as assessed by confocal microscopy on HEK-293 cells expressing B₂R, βarr1-mCherry and Ib30-YFP, and stimulated with 100nM bradykinin. The Pearson's correlation coefficients in the upper, middle and lower panels were 0.33±0.03 from 15 cells, 0.34±0.03 from 20 cells, and 0.34±0.04 from 16 cells, respectively, based on 5 independent experiments. Scale bar is 10 μm.
Figure 1

A

IP : Fab (Protein L)

|       | Fab CTL | Fab 30 |
|-------|---------|--------|
| 54 kDa|         |        |
| WB: β₂V₂R|        |        |

Coomassie (Fab)

β₂V₂R  +  +  +  +
Carazolol  +  -  +  -
BL-167107  -  +  -  +

B

Signal intensity (% normalized)

C

IP : Fab (Protein L)

|       | Fab 4  | Fab 7  | Fab 9  | Fab 12 |
|-------|--------|--------|--------|--------|
| 54 kDa|         |        |        |        |
| WB: β₂V₂R|        |        |        |        |

Coomassie (Fab)

Carazolol  +  -  +  -  +  -  -  +
BL-167107  -  +  -  +  -  +  +  +
Figure 2

A) CCR2-V_{2,R}  
Agonist - +  
54kDa  
WB: FLAG  
33kDa  
Fab30  

B) D5-V_{2,R}  
Agonist - +  
WB: FLAG  
71kDa  
54kDa  
33kDa  
Fab30  

C) C5aR1-V_{2,R}  
Agonist - +  
WB: FLAG  
54kDa  
43kDa  
33kDa  
Fab30  

D) α2B-V_{2,R}  
Agonist - +  
WB: FLAG  
71kDa  
54kDa  
33kDa  
Fab30  

E) D2-V_{2,R}  
Agonist - +  
WB: FLAG  
71kDa  
54kDa  
33kDa  
Fab30  

F) M5-V_{2,R}  
Agonist - +  
WB: FLAG  
71kDa  
54kDa  
33kDa  
Fab30
Figure 3

(A) Diagram showing the process of creating an Intrabody (Ib) plasmid with ScFv-ORF. The plasmid results in cytoplasmic expression.

(B) Western Blot (WB) analysis showing bands at 33 kDa and 43 kDa. The bands are labeled as Vector control, Intrabody 30, Intrabody 4, Intrabody 7, Intrabody 9, and Intrabody 12. The WB is for actin or HA (Intrabody).

(C) Merged images showing βarr1-mCherry, Ib30-YFP, and merged images.

(D) Merged images showing βarr1-mCherry, Ib4-YFP, and merged images.
Figure 4

A

[Image showing WB and IP results for Ib-CTL, Ib30, and Ib4]

B

[Graph showing signal intensity (% normalized) for Isoproterenol and Carazolol]

C

[Image showing WB and IP results for Isoproterenol, Carazolol, Salmeterol, Salbutamol, Clenbuterol, and Norepinephrine]

D

[Graph showing signal intensity (% normalized) for Isoproterenol, Carazolol, Salmeterol, Salbutamol, Clenbuterol, and Norepinephrine]

E

[Images showing βarr1-mCherry and Ib30-YFP with Merged images at 0 min, 10 min, and 25 min]

F

[Images showing βarr1-mCherry and Ib4-YFP with Merged images at 0 min, 5 min, and 35 min]
Figure 5

A

WB: V₂R
54 kDa
33 kDa

IP: Intrabody (HA)

V₂R + + + + + +
Tolvaptan + - + - + -
AVP - + - + - +

B

Signal intensity (% normalized)

Tolvaptan AVP

C

βarr1-mCherry Ib30-YFP Merged

D

βarr1-mCherry Ib4-YFP Merged

E

βarr1-mCherry Ib30-YFP Merged

βarr1-mCherry Ib4-YFP Merged
Figure 6

A

IP: Protein L agarose

| Treatments | Ib-CTL | Ib30 |
|------------|--------|------|
| Tol (100nM) + | - | + |
| AVP (100nM) - | + | - |

- 71kDa
- 54kDa
- 33kDa
- 29kDa

B

Reactivity of intrabodies to βarr1 (% normalized)

- Tolvaptan
- AVP

C

0 min | ~45 min

β2V1R | Ib30-YFP
Figure 7

A

βarr1 recruitment
βarr1-RlucII + V2R-venus

BRET (raw values)

Log(AVP) [M]

BRET (raw values)

Ib4
Ib30
Ib-CTL

V2R (red)
βarr1(green)
Ib30 (magenta)
Merge

Basal
AVP (12 min)

V2R (red)
βarr1(green)
Ib30 (magenta)
Merge

3min 12min 3min 12min
V2R + EEA1
V2R + APPL1

C

+Ib-CTL
+Ib30

FLAG-V2R
EEA1
Merge

D

V2R colocalization with EEA1 and APPL1

E

Endosomal localization of βarr1
βarr1-RlucII + rGFP-FYVE

F

Agonist-induced ΔBRET

0
20
40
60
80

20
40
60
80
90
100

0.0
0.2
0.4
0.6
0.8

-12 -11 -10 -9 -8 -7 -6

0.0
0.2
0.4
0.6
0.8

** ***
**Figure 10**

(A) Basal

(B) Stimulated

(C) Basal

(D) Stimulated

(E) IP: Ib30 (Protein L)
   C5a (min) 0 5 30
   54kDa 43kDa 33kDa 29kDa
   WB: FLAG
   WB: HA

(F) IP: Ib30 (Protein L)
   CCL7 (min) 0 5 30
   54kDa 33kDa
   WB: FLAG
   WB: HA

(G) B2R

0 min

~2 min

~15 min