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Distinct roles for β -arrestin2 and arrestin-domain-containing proteins in β_2 adrenergic receptor traffickingSang-Oh Han¹, Reddy P. Kommaddi¹ & Sudha K. Shenoy^{1,2+}¹Department of Medicine, and ²Department of Cell Biology, Duke University Medical Center, Durham, North Carolina, USA

β -arrestin 1 and 2 (also known as arrestin 2 and 3) are homologous adaptor proteins that regulate seven-transmembrane receptor trafficking and signalling. Other proteins with predicted 'arrestin-like' structural domains but lacking sequence homology have been indicated to function like β -arrestin in receptor regulation. We demonstrate that β -arrestin2 is the primary adaptor that rapidly binds agonist-activated β_2 adrenergic receptors (β_2 ARs) and promotes clathrin-dependent internalization, E3 ligase Nedd4 recruitment and ubiquitin-dependent lysosomal degradation of the receptor. The arrestin-domain-containing (ARRDC) proteins 2, 3 and 4 are secondary adaptors recruited to internalized β_2 AR–Nedd4 complexes on endosomes and do not affect the adaptor roles of β -arrestin2. Rather, the role of ARRDC proteins is to traffic Nedd4– β_2 AR complexes to a subpopulation of early endosomes.

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INTRODUCTION

The two mammalian non-visual arrestins (β -arrestin 1 and 2, also known as arrestin 2 and 3) function as essential adaptors for the seven-transmembrane receptors (7TMRs; also known as G protein-coupled receptors or GPCRs) and regulate receptor desensitization, trafficking and signalling [1,2]. The critical roles of β -arrestins are demonstrated by the perinatal lethality of β -arrestin1/ β -arrestin2 double knockout mice and defects observed in single knockout mice for 7TMR-stimulated responses [3]. Mammalian cells also express arrestin-domain-containing proteins (ARRDC 1–5 and the thioredoxin-interacting protein TXNIP), which have predicted structural similarities with arrestins but lack sequence homology [4–7].

All four arrestins (visual arrestin 1 and 4; non-visual arrestin 2 and 3) contain a characteristic α -helix in the N-terminal domain and a polar core that keeps the molecule constrained in a

basal conformation, which is disrupted on arrestin–receptor interaction [8,9]. Additionally, the β -arrestins have a well-defined clathrin-binding motif at the C-terminal domain [10]. Both β -arrestin1 and 2 bind HECT domain ligases and function as critical adaptors for ubiquitinating 7TMRs and ion channels/transporters [11–15].

TXNIP and ARRDC proteins 1–4 (but not ARRDC5) contain canonical PPXY motifs that bind WW domain-containing proteins, especially the HECT-domain-containing E3 ubiquitin ligases [4,5]. Some ARRDCs are predicted to have an alpha helix in the N-terminal domain [5]. In yeast, a small family of arrestin-domain-containing proteins with PPXY motifs function as arrestin-related trafficking adaptors for the yeast HECT domain E3 ligase Rsp5, which regulates ubiquitination and trafficking of plasma membrane amino-acid transporters [16]. Recently, ARRDC3 and not β -arrestin2 was reported to mediate Nedd4 recruitment to the β_2 adrenergic receptor (β_2 AR) to regulate receptor ubiquitination and degradation [17]. To explore what adaptors serve what functions in β_2 AR trafficking, we performed a series of biochemical and cellular assays to compare β -arrestin2 with ARRDC3.

RESULTS AND DISCUSSION

 β_2 AR endocytosis requires β -arrestin2, not ARRDC3

β -arrestins 1 and 2 mediate clathrin-dependent endocytosis of the β_2 AR [18–20]. Accordingly, exogenous expression of β -arrestin2, but not ARRDC3, led to an augmentation of Isoproterenol (Iso)-stimulated internalization of the β_2 AR in COS-7 cells (Fig 1A,B). Additionally, in HEK-293 cells β_2 AR internalization was dramatically decreased by gene silencing of β -arrestin2 but not ARRDC3 (Fig 1C,D). Concordant with previous findings on β -arrestin–clathrin interaction [19,21,22] both β -arrestin1 and 2 interacted with endogenous clathrin and Iso-stimulation increased β -arrestin2–clathrin binding by ~ 2 -fold (Fig 1E). In marked contrast, ARRDC3–clathrin binding was undetectable both basally and after Iso-stimulation (Fig 1E).

 β -arrestin and ARRDC3 bind a key endosomal protein

We compared β -arrestin 1 and 2 and ARRDC3 for their binding to hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), a protein that constitutively associates with early endosomes and binds ubiquitinated cargo during sorting of

¹Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, USA

²Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, USA

⁺Corresponding author. Tel: +919 681 5061; Fax: +919 681 7851; E-mail: sudha@receptor-biol.duke.edu

Fig 1 | Agonist-stimulated β_2 AR endocytosis requires β -arrestin2, but not ARRDC3. (A) COS-7 cells were transfected with vector, β -arrestin2 (β arr2) or ARRDC3 and cell-surface β_2 ARs assessed with and without 1 μ M Isoproterenol (Iso) stimulation for 30 min. Mean $\pm$ s.e.m. from three to seven independent experiments are shown ($n = 3$ for ARRDC3-5 μ g; rest $n = 7$). *** $P < 0.0001$ versus vector and ** $P < 0.001$ versus ARRDC3 conditions. (B) Western blots showing endogenous and overexpressed ARRDC3 (2 μ g), β arr2 and β -actin levels. (C) Internalization of cell-surface receptors were assessed as in (A) in cells transfected with a control non-targeting siRNA (CTL, $n = 8$), siRNA-targeting β arr2 (β arr2-1 $n = 5$, β arr2-2 $n = 3$) or ARRDC3 (DC3-1 $n = 6$, DC3-2 $n = 5$). *** $P < 0.0001$ versus CTL, DC3-1 and DC3-2. (D) ARRDC3, β arr1, β arr2 and actin in each sample were detected by serial immunoblotting of whole-cell lysates. (E) COS-7 cells transfected with vector, HA- β arr1, β arr2 or ARRDC3 plasmids were serum deprived for 1 h, and stimulated for indicated times with 10 μ M Iso. Isolated anti-HA immunoprecipitates (IPs) were probed for clathrin heavy chain. Shown are representative blots for clathrin, and HA- β arr2 and ARRDC3 from one of three independent experiments. (F) COS-7 cells were transfected with pCDNA3-HA, β -arrestin1-HA, β -arrestin2-HA or ARRDC3-HA and HA IPs were analysed for endogenous HRS protein. (G) Quantification of HRS signals in each IP is summarized from three independent experiments where maximal binding in each experiment = 100%. ARRDC, arrestin-domain-containing; HA, haemagglutinin; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; IP, immunoprecipitation; Iso, Isoproterenol; siRNA, short interfering RNA; β_2 AR, β_2 adrenergic receptor; β arr2, β -arrestin2.

internalized receptors. HRS has been shown to regulate post-endocytic sorting of 7TMRs and growth factor receptors [23,24]. β -arrestins and ARRDC3 interacted with endogenous HRS, consistent with their roles in sorting internalized receptors into endosomes (Fig 1F,G). Together with our internalization data, these data suggest that during β_2 AR trafficking, β -arrestin2 facilitates both early endocytic and post-endocytic steps, whereas ARRDC3 might facilitate just post-endocytic steps.

β_2 AR ubiquitination/degradation requires β -arrestin2

Next, we compared β -arrestin2 and ARRDC3 for mediating agonist-stimulated Nedd4 recruitment to activated β_2 ARs. As previously described [12], β -arrestin2 knockdown eliminated Iso-induced Nedd4- β_2 AR association (Fig 2A-C). However, despite equivalent reduction of protein expression, ARRDC3 knockdown did not inhibit Iso-induced Nedd4- β_2 AR association (Fig 2A-C). Concordantly, ARRDC3 knockdown did not affect Iso-stimulated

Fig 2 | β_2 AR ubiquitination/degradation requires β -arrestin2, but not ARDC3. (A) HEK-293 cells stably expressing Flag- β_2 AR (293- β_2 AR) were transfected with control, β -arrestin2 (β arr2) or ARDC3-specific siRNA. Serum-starved cells were stimulated with 1 μ M Iso for 10 min, Flag IPs isolated and lysates immunoblotted for Nedd4 and then β_2 AR. (B) Whole-cell extracts were serially immunoblotted for β -arrestin2 with either anti- β arr2 (A2CT), anti- β arr1 (A1CT) antibodies, ARDC3 and actin. (C) Quantification of Nedd4 in receptor IPs from four independent experiments where maximal binding = 100%. ** P < 0.01 Iso versus NS, *** P < 0.001, β -arr2-siRNA-Iso versus all other conditions. (D) 293- β_2 AR cells were transfected with siRNA that target nothing (control), β arr2 or ARDC3, stimulated $\pm$ Iso for 15 min. Flag- β_2 AR IPs were serially probed for ubiquitin (FK1) and β_2 AR (H-20). (E) Ubiquitin signal in each sample was normalized to the respective receptor amount and plotted as mean $\pm$ s.e.m. from four independent experiments. ** P < 0.01, *** P < 0.001 Iso versus NS; *** P < 0.001, β -arr2-siRNA-Iso versus all other conditions. (F) Flag- β_2 AR-mYFP stable HEK-293 cells transfected with control, β -arr2, or ARDC3 siRNA were stimulated or not with 1 μ M Iso for 6 h along with LysoTracker dye. Scale bar, 10 μ m. (G) Pearson's correlation coefficients calculated for β_2 AR and LysoTracker Red colocalization (mean $\pm$ s.e.m.) in the respective cells for NS- and Iso-stimulated conditions: *** P < 0.001 versus NS; n > 50 cells for all conditions. (H) 293- β_2 AR cells under control or depleted conditions for β arr2 (β -arr2-1, β -arr2-2) or ARDC3 (DC3-1, DC3-2) were stimulated with vehicle or 10 μ M Iso for 24 h and receptor expression was determined by radioligand binding. The bar graphs depict degraded receptors as a percentage of total receptors in each sample; ** P < 0.001 versus CTL. ARDC, arrestin-domain-containing; IB, immunoblotting; IP, immunoprecipitation; Iso, Isoproterenol; siRNA, short interfering RNA; β_2 AR, β_2 adrenergic receptor; β arr2, β -arrestin2.

Fig 3 | Comparison of β -arrestin2, ARDC3 and ARDC3^{AXXA} for subcellular distribution and protein interactions. (A) 293- β_2 AR cells were transfected with vector, HA- β arr2, ARDC3 or ARDC3^{AXXA} and HA IPs were isolated from cells stimulated with vehicle, or Iso for indicated times. Endogenous Nedd4 in the IPs were detected and shown in the upper panels. The lower panels display the amounts of β arr2, ARDC3 and ARDC3^{AXXA} in each IP. (B) Data are shown as means $\pm$ s.e.m., n = 4; * P < 0.05, *** P < 0.0001 against respective NS samples. Confocal images show subcellular distribution $\pm$ Iso stimulation (1 μ M, 2 min) for β arr2-GFP (C), ARDC3-GFP (D) and ARDC3^{AXXA}-GFP (E). (F,G). Binding of endogenous HRS with β arr and ARDC3 determined as in Fig 1; * P < 0.05, versus rest. (H) 293- β_2 AR cells were transfected with ARDC3 or ARDC3^{AXXA} and the subcellular distribution of ARDC3 and endogenous HRS was determined by immunostaining and confocal microscopy. ARDC, arrestin-domain-containing; GFP, green fluorescent protein; HA, haemagglutinin; HRS, hepatocyte growth factor-regulated tyrosine kinase substrate; IB, immunoblotting; IP, immunoprecipitation; Iso, Isoproterenol; β arr2, β -arrestin2; NS, nonstimulated.

A

B

C βarr2-GFP

D ARRDC3-GFP

E ARRDC3^{AAXA}-GFP

F

G

H

Fig 4 | Interaction of ARRDC proteins with the β_2 AR. (A) 293- β_2 AR cells were transfected with β -arrestin2-GFP and its subcellular distribution along with that of Flag- β_2 AR was determined by immunostaining and confocal microscopy after Iso stimulation for indicated times. Similar experiment as in (A) was performed with ARRDC3-GFP (B) or ARRDC3^{AXXA}-GFP (C). Scale bar, 10 μ m. 293- β_2 AR cells were transfected with β -arrestin2-GFP (D), ARRDC3 or ARRDC3^{AXXA} (E) and Flag- β_2 AR IPs were immunoblotted for β arr2 or ARRDC3. (F) Quantification (mean $\pm$ s.e.m.) of ARRDC3 signals normalized to receptor levels in IPs from four independent experiments. **** P < 0.0001 versus others. (G) 293- β_2 AR cells were transfected with ARRDC1-GFP, ARRDC2-GFP or ARRDC4-GFP and subcellular distribution $\pm$ Iso (30 min) was determined as in (A). (H) ARRDC2^{AXXA} and ARRDC4^{AXXA} were transfected into 293- β_2 AR cells and their subcellular distribution after Iso stimulation (1 μ M, 30 min) was determined as in (A). (I) Schematic showing the patterns of β -arrestin and ARRDC protein binding with the β_2 AR. On agonist stimulation and G protein-coupled receptor kinase (GRK) phosphorylation, β -arrestin2 is rapidly recruited to the activated β_2 AR at the plasma membrane. β -arrestin initiates Nedd4 recruitment as well as clathrin-AP2 dependent receptor internalization. ARRDC proteins constitutively associated with HRS-containing vesicles recognize and bind Nedd4- β_2 AR complexes leading to post-endocytic sorting of internalized, ubiquitinated β_2 ARs. ARRDC, arrestin-domain-containing; GFP, green fluorescent protein; GRK, G protein-coupled receptor kinase; IB, immunoblotting; IP, immunoprecipitation; Iso, Isoproterenol; β_2 AR, β_2 adrenergic receptor; β arr2, β -arrestin2; NS, nonstimulated.

β_2 AR ubiquitination (Fig 2D,E), lysosomal trafficking (Fig 2F,G) or degradation as measured by radioligand binding (Fig 2H, supplementary Table S1 online). In marked contrast, β -arrestin2 knockdown resulted in a significant inhibition of Iso-stimulated ubiquitination (Fig 2D,E); caused a substantial retention of β_2 ARs at the plasma membrane even after 6 h of Iso stimulation; significantly reduced colocalization of β_2 AR vesicles with the LysoTracker dye (Fig 2F,G); and markedly inhibited receptor degradation after 24 h of Iso stimulation (Fig 2H, supplementary Table S1 online). These data confirm our earlier findings that β -arrestin2 acts as an essential adaptor for Nedd4 recruitment, receptor ubiquitination and lysosomal degradation [11,12,25] and demonstrate that ARRDC3 is not essential for promoting these effects.

Comparison of β -arrestin2, ARRDC3 and ARRDC3^{AXXA}

Iso-stimulation increased the interaction between Nedd4 and β -arrestin2, as we reported before (Fig 3A,B) [12]. In contrast, although ARRDC3–Nedd4 binding was much more robust than β -arrestin–Nedd4 binding, there was little agonist-dependent increase (Fig 3A,B). When the proline and tyrosine residues in the two PPXY motifs (supplementary Fig S1 online) were replaced with alanines, the resulting mutant ARRDC3^{AXXA} showed no Nedd4 binding because of the loss of WW domain interaction (Fig 3A,B). In contrast, Nedd4 interaction with β -arrestin2 and another adaptor Grb10 does not involve WW domains [12,26].

The rapid agonist-dependent recruitment of cytoplasmic β -arrestin2-GFP to the plasma membrane [18] is displayed in Fig 3C. In contrast, agonist stimulation did not markedly affect the subcellular distribution of ARRDC3-GFP in the cytoplasm and at the plasma membrane (Fig 3D). Its cytoplasmic distribution is not homogeneous, but concentrated in vesicles, which have partial overlap with EEA1, an early endosome marker and LAMP2, a late endosomal/lysosomal marker (supplementary Fig S2 online). The subcellular distribution of ARRDC3^{AXXA} differs from the WT: the mutant protein is localized at the plasma membrane but not concentrated in vesicles (Fig 3E). Although WT ARRDC3 binds endogenous HRS, ARRDC3^{AXXA} is totally impaired (Fig 3F,G). Additionally, although vesicles containing overexpressed WT-ARRDC3-GFP colocalized with HRS-positive endosomes, no endosomal localization is detected for ARRDC3^{AXXA} (Fig 3H).

Internalized β_2 ARs colocalize with ARRDC3

Next we determined if β_2 AR and ARRDC3 can colocalize in HEK-293 cells and compared it with the known pattern of β -arrestin2–

β_2 AR interaction. Before agonist stimulation, no colocalization is detected between the cytoplasmic β -arrestin2-GFP and the plasma-membrane-localized β_2 ARs (nonstimulated (NS) images, Fig 4A). On agonist stimulation, a rapid and robust recruitment of β -arrestin2 to the activated β_2 ARs at the plasma membrane results (Fig 4A). After 30 min of agonist activation, β -arrestin2 begins to redistribute to the cytoplasm and internalized β_2 ARs are seen in endosomes (Fig 4A). This dissociation of internalized β_2 AR and β -arrestin2 is attributed to the deubiquitination of the latter by ubiquitin-specific protease 33 [27].

In unstimulated cells, regions of plasma membrane showed some overlap of β_2 AR and ARRDC3-GFP (Fig 4B). At 5 min after Iso-stimulation, few vesicles are detected with internalized β_2 AR and ARRDC3. However, after 30 and 60 min of Iso, most vesicles containing internalized β_2 ARs colocalized with ARRDC3-containing vesicles (Fig 4B), but the membrane-distributed ARRDC3 remained unchanged. This pattern suggests that rather than a co-trafficking event wherein ARRDC3 from the plasma membrane co-internalizes with the β_2 AR, actually, the internalized β_2 ARs are targeted to endosomes containing ARRDC3. ARRDC3^{AXXA} that is impaired in endosomal distribution but unimpaired in plasma membrane localization and incapable of interaction with Nedd4 and HRS (as shown in Fig 3) did not colocalize with the internalized β_2 AR (Fig 4C). Additionally, ARRDC3^{AXXA} did not affect β_2 AR internalization (see distribution of β_2 AR mostly in endosomes in Iso-treated cells, Fig 4C, lower panels). Collectively, these data do not support the notion that ARRDC3 recruits Nedd4 to the agonist-stimulated β_2 AR. Rather, they suggest the possibility that Nedd4, which is bound to the β_2 AR, might recruit the β_2 AR to ARRDC3 and HRS-containing endosomes.

Concordant with the confocal data, co-IP assays showed a marked increase in β -arrestin2-GFP– β_2 AR binding after 5 min of Iso stimulation, and a subsequent time-dependent decrease in the amount of β_2 AR-bound β -arrestin2 (Fig 4D). In contrast, co-IP assays showed increased association of the β_2 AR with ARRDC3 only after 30 min of Iso treatment (Fig 4E,F). Some basal binding of overexpressed ARRDC3^{AXXA} with the β_2 AR is detected in these chemically cross-linked samples. However, unlike the WT ARRDC3, there is no agonist-promoted increase in binding between the β_2 AR and ARRDC3^{AXXA} (Fig 4E,F). The exact significance of the basal interaction between ARRDC3 and the β_2 AR at the plasma membrane remains to be determined; ARRDC3 could have a role in recycling of receptors or their targeting to membrane micro-domains. Nonetheless, these data suggest that

the observed β_2 AR–ARRDC3 interaction on endosomes relies on the poly-proline–WW domain interaction and is dependent on prior Nedd4 binding to the β_2 AR. Furthermore, ARRDC3– β_2 AR interaction appears to be an event secondary to a previous recruitment of Nedd4 by β -arrestin2 to the internalizing receptor.

Our data and conclusions differ from the recently reported effects of ARRDC3 on β_2 AR degradation [17]. These authors relied mostly on the characterization of a truncated β_2 AR, which would be impaired in normal GRK phosphorylation, β -arrestin recruitment and perhaps in ubiquitination in the C-terminal domain [25,28]. They did observe a decrease in receptor binding with the polypoline mutant in immunoprecipitation assays, but concluded this to be a small decrease in receptor interaction; however, the expression levels of WT and mutant ARRDC3 were not carefully controlled in their experiments. These authors also used a single short interfering RNA (siRNA) oligo to knock down ARRDC3, which in our hands decreases both β -arrestin1 and 2 levels (supplementary Fig S3 online).

To address the differences between our studies and that by Nabhan *et al* [17], we further tested the specific siRNA used by the authors to knockdown ARRDC3 (oligo DC3-3). After Iso stimulation, both ubiquitination and internalization of the β_2 AR were impaired on knockdown with DC3-3 (supplementary Fig S4 online). Moreover, only β -arrestin2 transfection rescued these defects, whereas a siRNA-resistant ARRDC3 ‘ARRDC3(W)’ did not rescue either internalization or ubiquitination of the β_2 AR (supplementary Fig S4 online). ARRDC3(W) showed identical subcellular distribution, as the WT ARRDC3 and colocalized with internalized β_2 ARs just as the WT ARRDC3 does (supplementary Fig S4 online) and its PPXY motifs are intact; therefore, its inability to rescue the siRNA effects was not because of any alteration in its expression or binding functions. Accordingly, the effects observed by Nabhan *et al* [17] on β_2 AR trafficking on siRNA knockdown were most probably because of a decrease in β -arrestin2 rather than a depletion of ARRDC3 levels.

ARRDC3 homologs also bind internalized β_2 ARs

Bioinformatics predictions [4,5] suggest that ARRDC3 is closely related to ARRDC2 and ARRDC4 but only distantly related to ARRDC1, TXNIP and ARRDC5. As shown in Fig 4G, ARRDC1-GFP is mainly localized at the plasma membrane and does not form a complex with internalized β_2 ARs on endosomes. TXNIP is mainly localized in the nucleus with little cytoplasmic distribution, and does not colocalize with internalized β_2 AR (supplementary Fig S5 online). On the other hand, ARRDC2-GFP is expressed more on endosomes with some plasma membrane distribution, whereas ARRDC4-GFP showed identical subcellular distribution as ARRDC3-GFP (present both at the plasma membrane and in vesicles) and both proteins displayed a robust colocalization with internalized β_2 ARs on endosomes (Fig 4G). Interestingly, when ARRDC3-cherry and either ARRDC2-GFP or ARRDC4-GFP were co-expressed, there was a significant overlap of subcellular distribution, especially on endosomes between these homologous proteins (supplementary Fig S6 online). ARRDC2 and ARRDC4 also immunoprecipitated with the β_2 AR after 30 min of agonist stimulation (supplementary Fig S7 online). ARRDC2 and ARRDC4 polypoline mutants were also impaired in endosomal distribution and did not colocalize with internalized β_2 AR similar to the ARRDC3^{AAXA} mutant (Fig 4H). These data suggest that, like ARRDC3, ARRDC2

and ARRDC4 might regulate post-endocytic trafficking of the β_2 AR. Our attempts to knock down all three adaptors simultaneously were unsuccessful; future studies with new approaches would be required to delineate the redundant roles of ARRDCs. Indeed, the failure of ARRDC3 silencing to affect β_2 AR trafficking might be attributable to functional overlap among ARRDCs 2, 3 and 4.

β -arrestin and ARRDCs function sequentially

As shown in the schematic in Fig 4I, β -arrestin2 recruitment to activated β_2 ARs primarily initiates receptor endocytosis, Nedd4 recruitment and receptor ubiquitination. β -arrestin deubiquitination by USP33 causes dissociation of β -arrestin2 from the β_2 AR [27,29]. Nedd4 bound to the β_2 AR connects the cargo to ARRDC containing endosomes promoting the sorting of Nedd4– β_2 AR complexes to HRS-positive endosomes. The ARRDC–Nedd4 interaction also regulates the binding of ARRDC and HRS on endosomes. Overall, β -arrestin2, Nedd4 and ARRDC function in a hierarchical manner to traffic the agonist-stimulated β_2 AR to sorting endosomes; herein, the role of ARRDC is fulfilled by ARRDC 2, 3 or 4 all of which are equipotent in binding the cargo (β_2 AR), the regulatory ligase (Nedd4) and the endosomal-sorting molecule HRS.

The binding of internalized β_2 AR and ARRDC proteins on endosomes reveals an as yet unappreciated regulatory sorting mechanism for this 7TMR. Although the β_2 AR has been the most studied receptor since its cloning decades ago [30], its intracellular itinerary is not fully understood. Several molecular mechanisms, including receptor phosphorylation/dephosphorylation, ubiquitination/deubiquitination, hydroxylation and so on, govern the residence time of the receptor at the cell surface and are in interplay in determining whether the agonist-stimulated receptor has to be deactivated and degraded or resensitized and recycled to the plasma membrane. Whether the interaction with ARRDC proteins is a ‘by-stander’ effect on cargo traffic through endosomes or a decision-rendering event during post-endocytic sorting demands further careful scrutiny.

METHODS

Immunoprecipitation assays. For all protein–protein interaction or ubiquitination assays, sub-confluent monolayers of cells were used. After agonist or vehicle treatments, cells were scraped into ice-cold lysis buffer supplemented with protease inhibitors as reported before [25]. Detection of Nedd4– β_2 AR complexes and β_2 AR–ARRDC3 interaction involved chemical cross-linking methods that have been described before [12]. Protein concentrations of clarified extracts were determined by a modified Bradford method. Roughly 600–800 μ g of soluble protein solution was used for immunoprecipitation with anti-Flag affinity (Sigma) or anti-haemagglutinin (HA) affinity agarose (Thermo Fisher) as required. The IP mixture was rotated at 4 °C and bound proteins were separated from unbound and non-specific interactions by repeated centrifugation and wash steps. Immunoprecipitated complexes were eluted by addition of SDS–polyacrylamide gel electrophoresis sample buffers and separated by 4–20% gradient gels or 10% gels (Invitrogen). Signals were detected and acquired with a CCD camera system (Bio-Rad Chemidoc-XRS) and analysed with Image-Lab software (Bio-Rad).

Confocal microscopy. HEK-293 β_2 AR stable cells were transiently transfected with GFP-tagged β -arrestin or ARRDC constructs.

At 24–48 h post-transfection, cells were plated on collagen-coated 35-mm glass bottom plates. Next day, cells were starved for 1 h in serum-free medium, and then stimulated with Iso for the desired times, fixed with 5% formaldehyde, diluted in PBS containing calcium and magnesium, permeabilized with 0.1% Triton X-100 in PBS containing 2% bovine serum albumin for 30 min and incubated with appropriate primary antibody overnight (either EEA1, HRS, LAMP2 or β_2 AR) at 4 °C, followed by the respective secondary antibody conjugated with Alexa-594. Confocal images were obtained on a Zeiss LSM510 laser-scanning microscope using multitrack sequential excitation (488 and 568 nm) and emission (515–540 nm, GFP; 585–615 nm, Texas Red) filter sets. Other relevant methods are included in the supplementary information online.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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Author contributions: S.-O.H. designed research, conducted experiments, produced analytic tools, performed data analyses and contributed to writing; R.P.K. conducted experiments and performed data analyses; S.K.S. designed research, conducted experiments, performed data analyses and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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