Arrestin domain-containing protein 3 recruits the NEDD4 E3 ligase to mediate ubiquitination of the β 2-adrenergic receptor

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Prolonged stimulation of the β 2-adrenergic receptor (β 2AR) leads to receptor ubiquitination and downregulation. Using a genome-wide RNA interference screen, we identified arrestin domain-containing 3 (*ARRDC3*) as a gene required for β 2AR regulation. The *ARRDC3* protein interacts with ubiquitin ligase neural precursor development downregulated protein 4 (NEDD4) through two conserved PPXY motifs and recruits NEDD4 to the activated receptor. The *ARRDC3* protein also interacts and co-localizes with activated β 2AR. Knockdown of *ARRDC3* expression abolishes the association between NEDD4 and β 2AR. Furthermore, functional inactivation of *ARRDC3*, either through small interfering RNA (siRNA)-mediated knockdown or overexpression of a mutant that does not interact with NEDD4, blocks receptor ubiquitination and degradation. Our results establish *ARRDC3* as an essential adaptor for β 2AR ubiquitination.

Keywords: *ARRDC3*; arrestin; β 2-adrenergic receptor; NEDD4; ubiquitination

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INTRODUCTION

The β 2-adrenergic receptor (β 2AR) is a prototypic member of the G protein-coupled receptor (GPCR) family (Pierce *et al*, 2002). The binding of agonists to β 2AR stimulates G proteins that alter the intracellular levels of cyclic AMP and the activity of protein kinase A, resulting in important physiological changes such as relaxation of airway smooth muscles and bronchodilation (Penn & Benovic, 2008). The attenuation of β 2AR signalling is equally crucial for the maintenance of normal physiology and homeostasis (Premont & Gainetdinov, 2007). This is achieved through receptor desensitization and is dependent on the duration of exposure to the agonist. Immediately after agonist activation, β 2AR becomes

phosphorylated and recruits cytosolic β -arrestins (DeWire *et al*, 2007). The interaction of β -arrestins with β 2AR uncouples the activated G protein complex from the receptor and terminates signalling. By contrast, prolonged stimulation is attenuated by receptor downregulation through endocytosis and protein degradation (Hanyaloglu & von Zastrow, 2008; Marchese *et al*, 2008).

A crucial event that precedes degradation of β 2AR is receptor ubiquitination (Shenoy *et al*, 2001). Protein ubiquitination is mediated by multiple enzymatic events that culminate with the covalent addition of one or more ubiquitin moieties to a substrate by an E3 ligase (Hershko & Ciechanover, 1998; Rotin & Kumar, 2009). Neural precursor development downregulated protein 4 (NEDD4), which is a member of the HECT (homologous to E6AP carboxyl terminus) family of E3 ubiquitin ligases, can ubiquitinate β 2AR (Shenoy *et al*, 2008). The NEDD4 E3 ligase contains WW domains, which can interact directly with proline-rich regions, such as the well-characterized PPXY motif, in target or adaptor proteins (Rotin & Kumar, 2009). However, neither β 2AR nor a putative adaptor protein β -arrestin 2 contains such a motif. Thus, the mechanism by which NEDD4 recruits to activated β 2AR remains unclear. In this study, we report a new arrestin domain-containing protein 3 (*ARRDC3*), as an essential adaptor that recruits the NEDD4 E3 ligase to mediate β 2AR ubiquitination.

RESULTS

ARRDC3 is a novel regulator of β 2AR

We performed a genome-wide RNA interference-based genetic screen to identify genes required for β 2AR downregulation (Fig 1A). The screen was carried out in a cell line that expresses a distally truncated form (after residue 385) of β 2AR (β 2ARt), which undergoes fast and efficient receptor degradation on agonist stimulation (Cao *et al*, 1999; Gage *et al*, 2001). Briefly, β 2ARt cells were transduced by using a lentiviral genome-wide short hairpin RNA (shRNA) library and subjected to agonist (isoproterenol; ISO) stimulation for 16 h. We then performed several rounds of fluorescence-activated cell sorting to enrich for cells that, after ISO stimulation, maintained high levels of β 2AR, indicative of possible defects in β 2AR degradation. shRNAs that potentially interfere with β 2AR downregulation

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Fig 1 | Identification of *ARRDC3* as a new gene required for β 2AR degradation and ubiquitination. (A) Schematic diagram of the RNAi-based screen to identify regulators of β 2AR. (B) *ARRDC3* knockdown enhances β 2ARt membrane levels. FLAG- β 2ARt cells transduced with lentiviral particles carrying a non-targeting (NT) shRNA or an *ARRDC3*-specific shRNA and treated as indicated, before immunostaining and FACS analysis. The mean fluorescence intensity of NT or *ARRDC3* shRNA-expressing cells is indicated. (C,D) Effects of siRNA-mediated *ARRDC3* knockdown on (C) β 2AR and (D) β 2AR degradation. Normalized pixel densitometry values, shown as a bar graph, and s.e. values are averages of three independent experiments; ** $P < 0.01$ and *** $P < 0.001$. (E,F) Ubiquitination (Ub) of (E) β 2AR and (F) β 2AR in *ARRDC1* and *ARRDC3* knockdown cells. β 2AR, β 2-adrenergic receptor; β 2ARt, truncated β 2-adrenergic receptor; *ARRDC3*, arrestin domain-containing 3; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; IP, immunoprecipitation; ISO, isoproterenol; RNAi, RNA-mediated interference; shRNA, short hairpin RNA; WCE, whole cell extract.

were identified from the sorted cell population by genomic PCR and sequencing.

Two of the identified shRNAs corresponded to a new gene *ARRDC3*, also known as thioredoxin-binding protein-2-like inducible membrane protein (TLIMP; Oka *et al*, 2006). To confirm the effect of *ARRDC3* shRNA-mediated knockdown on β 2AR downregulation, we transduced β 2ARt cells with lentiviral particles carrying the identified *ARRDC3* shRNA sequence. Flow cytometry analysis showed that cells expressing the *ARRDC3*-specific shRNA exhibited a higher (>20%) level of cell-surface receptor after agonist stimulation, compared with the control non-targeting shRNA-expressing cells (Fig 1B). To ascertain that the stabilization of β 2AR was due to *ARRDC3* knockdown and was not caused by a potential off-target effect of the shRNA, we tested the effect of an *ARRDC3* small interfering RNA (siRNA) targeting a

sequence different from that of the identified shRNA. The *ARRDC3* protein expression was efficiently knocked down (>75%) by the siRNA and, as a result, ISO-induced degradation of β 2ARt was markedly inhibited: the level of undegraded receptor in *ARRDC3*-knockdown cells was approximately six-fold higher than that in the scrambled control siRNA-transfected cells (Fig 1C). This result demonstrates that *ARRDC3* is required for agonist-stimulated β 2AR degradation. The *ARRDC3*-knockdown cells also exhibited a slight increase (less than two-fold) in the basal level (before ISO stimulation) of the receptor, suggesting that *ARRDC3* might also have a role in the slow, yet constant, steady-state receptor degradation in the absence of an agonist (Morrison *et al*, 1996). To confirm that the effect of *ARRDC3* knockdown on β 2AR degradation is not limited to the modified β 2AR, we determined the effect of siRNA knockdown on the degradation of the

wild-type β 2AR. Consistent with a previous study (Cao *et al*, 1999), wild-type β 2AR exhibited slow degradation ($\sim 10\%$) on ISO stimulation (Fig 1D). Nevertheless, such slow degradation of β 2AR was blocked in ARRDC3 siRNA-transfected cells (Fig 1D). Together, our data identify *ARRDC3* as a new gene that is required for the efficient degradation of β 2AR.

The ARRDC3 protein belongs to a protein family that includes five other ARRDC proteins (supplementary Fig S1A online; Alvarez, 2008; Aubry *et al*, 2009). Most of the ARRDC proteins have no annotated biological functions. However, two of the ARRDCs (ARRDC1 and ARRDC3), when expressed as green fluorescent protein (GFP) fusion proteins, localize to the plasma membrane (supplementary Fig S1B online), suggesting membrane-related functions. To determine whether ARRDC1, similarly to ARRDC3, has a role in ISO-induced β 2AR degradation, we examined the effect of *ARRDC1* knockdown. Despite efficient knockdown ($> 90\%$) of *ARRDC1* using siRNA, β 2AR degradation was not affected (supplementary Fig S1C online).

ARRDC3 is required for β 2AR ubiquitination

As ubiquitination is a prerequisite for efficient agonist-induced receptor degradation (Shenoy *et al*, 2001), we next tested whether ARRDC3 has a role in β 2AR ubiquitination. We determined the level of β 2AR ubiquitination by immunoprecipitation of β 2AR followed by anti-ubiquitin immunoblot analysis. As shown in Fig 1E, ISO stimulation led to a significant increase in the amount of ubiquitinated species of β 2AR in both scrambled control siRNA- and ARRDC1 siRNA-transfected cells, but the increase in ubiquitination was strongly inhibited in ARRDC3-knockdown cells ($\sim 70\%$ reduction in normalized amount of ubiquitinated β 2AR compared with the controls). Such an effect was specific for β 2AR ubiquitination as depletion of ARRDC3 did not grossly alter total ubiquitination levels (data not shown). Similarly, knockdown of ARRDC3 also diminished agonist-dependent ubiquitination of wild-type β 2AR ($\sim 30\%$ reduction in ubiquitinated β 2AR compared with ISO-treated control; Fig 1F). Consistent with the degradation data (supplementary Fig S1C online), knockdown of ARRDC1 did not inhibit β 2AR ubiquitination (Fig 1E,F). These observations demonstrate that ARRDC3 is required for agonist-dependent ubiquitination of β 2AR.

ARRDC3 recruits NEDD4 to ubiquitinate β 2AR

The human ARRDC3 protein contains two PPXY motifs at its carboxyl terminus, which are conserved among other orthologues (Fig 2A). As PPXY motifs are known to mediate protein–protein interactions through interaction with the WW domains (Rotin & Kumar, 2009), we hypothesized that ARRDC3 might interact with the WW domain-containing NEDD4, which was characterized recently as an ubiquitin ligase for β 2AR (Shenoy *et al*, 2008), to facilitate β 2AR ubiquitination. Indeed, we showed that ARRDC3 interacts with NEDD4 in a co-immunoprecipitation experiment (Fig 2B). To determine whether the PPXY motifs in ARRDC3 are required for the observed interaction with NEDD4, we generated PPXY mutants (for either single or double motifs) and showed that, although the first PPXY motif has a main role in the interaction, both PPXY motifs had to be mutated to fully abrogate the interaction of ARRDC3 with NEDD4 (Fig 2B). These results demonstrate that ARRDC3 interacts with NEDD4 through the conserved PPXY motifs.

To explore further the ARRDC3–NEDD4 interaction, we examined whether ARRDC3 and NEDD4 co-localize with each other. We expressed two fluorescent fusion proteins (ARRDC3–GFP and mCherry–NEDD4) in 293 β 2AR cells. The ARRDC3–GFP fusion protein, when expressed alone, is localized mostly at the plasma membrane (supplementary Fig S1B online). However, co-expression with mCherry–NEDD4 led to a cytosolic localization of ARRDC3 (Fig 2C, upper panel). This is probably owing to steady-state association between ARRDC3 and NEDD4, resulting in the redistribution of ARRDC3 to the cytosol where NEDD4 resides. On addition of agonist, both mCherry–NEDD4 and ARRDC3–GFP co-localized at the vicinity of the plasma membrane and in discrete bodies in the cytosol (Fig 2C). However, relocalization of NEDD4 was not very marked and most NEDD4 remained in the cytoplasm (Fig 2C). This probably reflects a transient recruitment of NEDD4 to the plasma membrane. Unlike the wild-type ARRDC3, the double PPXY motif mutant (ARRDC3 $\Delta\Delta$ PPXY) showed little co-localization with mCherry–NEDD4 either before or after agonist stimulation (Fig 2C, lower panels). These data reinforce the idea that ARRDC3 interacts with NEDD4 through the PPXY motifs.

To determine whether the ARRDC3–NEDD4 interaction is required for the degradation of β 2AR, we next tested the effect of overexpression of ARRDC3 $\Delta\Delta$ PPXY, which does not interact with NEDD4. As shown in Fig 2D, expression of the double PPXY ARRDC3 mutant inhibited ISO-dependent β 2AR degradation, whereas the control GFP or wild-type ARRDC3–GFP did not. Moreover, expression of ARRDC3 $\Delta\Delta$ PPXY blocked β 2AR ubiquitination, whereas vector or ARRDC3-transfected controls displayed robust receptor ubiquitination after ISO stimulation (Fig 2E). These data strongly suggest that the ARRDC3–NEDD4 interaction is required for β 2AR ubiquitination and subsequent degradation.

ARRDC3 interacts with activated β 2AR

The amino-terminal region of ARRDC3 is homologous to the arrestin proteins, which interact with activated (phosphorylated) GPCRs (Gurevich & Gurevich, 2006) and have important roles in attenuating GPCR signalling (DeWire *et al*, 2007). We envisioned that ARRDC3 might also interact with the receptor on agonist stimulation. We therefore investigated whether ARRDC3 associates with β 2AR. As shown in the co-immunoprecipitation experiments (Fig 3A), haemagglutinin (HA)-tagged ARRDC3 was pulled down with immunoprecipitated β 2AR. Although a weak association was observed before ISO addition, the ARRDC3– β 2AR association was enhanced greatly after agonist stimulation. This interaction does not require the PPXY motifs in ARRDC3, as the double PPXY mutant was also associated, though less robustly, with activated β 2AR (Fig 3A).

We investigated further the ARRDC3– β 2AR interaction by examining the co-localization of the two proteins. Cells expressing FLAG– β 2AR were transfected with control GFP or ARRDC3–GFP, and β 2AR was visualized by immunostaining with anti-FLAG. On ISO stimulation, β 2AR was internalized from the plasma membrane into cytosolic endosomal vesicles, as indicated by co-immunostaining with the early endosomal marker early endosomal antigen 1 (EEA1; Fig 3B). This was evident in both GFP and ARRDC3–GFP transfected cells. Prior to ISO stimulation, ARRDC3–GFP localized almost exclusively to the plasma membrane where β 2AR also resides. On agonist stimulation,

Fig 2 | ARRDC3 interacts with and recruits NEDD4 E3 ligase to the activated β 2AR to mediate receptor ubiquitination. (A) Alignment of the PPXY-containing domains of ARRDC3 orthologues. PPXY motifs are highlighted in red in the alignment and were mutated as indicated to generate individual PPXY motif mutants ARRDC3-AASA and ARRDC3-AALA, or the double PPXY motif mutant ARRDC3-ΔAPPXY. (B) ARRDC3 interaction with NEDD4 requires the PPXY motifs. FLAG-tagged NEDD4 was co-transfected into 293T cells with HA-tagged ARRDC3 or its mutants. FLAG immunoprecipitated complexes were immunoblotted with the indicated antibodies. (C) Co-localization of ARRDC3 with NEDD4. GFP-ARRDC3- and mCherry-NEDD4-transfected β 2AR-expressing cells were treated as indicated and fixed with paraformaldehyde before visualization by confocal microscopy. White arrowheads indicate NEDD4 and ARRDC3 co-localization. (D) Effects of ARRDC3 or ARRDC3-ΔAPPXY expression on β 2AR degradation. GFP or GFP-tagged ARRDC3 (or the double PPXY mutant) was co-transfected with FLAG- β 2AR expression vector into 293T cells. Cells were treated with vehicle (water) or ISO. β 2AR degradation was assayed by anti-FLAG immunoblotting. Anti-GFP was used to assess the expression of the GFP constructs. The asterisk indicates non-specific bands. (E) Effect of ARRDC3 or ARRDC3 PPXY mutant overexpression on β 2AR ubiquitination. HA-tagged ARRDC3 (or the double PPXY mutant) was transfected into β 2ARt-expressing cells. β 2AR ubiquitination was determined by FLAG IP followed by anti-ubiquitin (Ub) immunoblotting. All results are representative of duplicate independent experiments. β 2AR, β -adrenergic receptor; β 2ARt, truncated β -adrenergic receptor; ARRDC3, arrestin domain-containing 3; GFP, green fluorescent protein; HA, haemagglutinin; IP, immunoprecipitation; ISO, isoproterenol; NEDD4, neural precursor development downregulated protein 4; WCE, whole cell extract; wt, wild type.

some of the ARRDC3 signal internalized into cytosolic vesicles (supplementary Fig S2A online) that co-localize with both β 2AR and EEA1 (Fig 3B, right panels). This is consistent with the ARRDC3- β 2AR interaction observed in the co-immunoprecipitation experiment, and suggests that ARRDC3 might continue to associate with β 2AR after agonist stimulation and during receptor endocytosis. Similarly, co-localization of ARRDC3 with the fast-degrading receptor β 2ARt was also observed (supplementary Fig S2B online). The co-localization of ARRDC3 with the receptors was also observed in the PPXY mutant (supplementary Fig S2A,B online), although less clearly as it occurred at the plasma membrane. Together, these data show that ARRDC3 associates with β 2AR and that such an association is enhanced on agonist stimulation.

ARRDC3 mediates the NEDD4 and β 2AR association

The NEDD4 protein was found previously to associate with β 2AR on agonist stimulation and it was suggested that β -arrestin 2 might mediate the NEDD4- β 2AR association (Shenoy *et al*, 2008). We asked whether ARRDC3 is required for the association between NEDD4 and activated β 2AR. We transfected β 2ARt-expressing cells with siRNAs targeting ARRDC3, β -arrestin 2 or a non-targeting sequence, and then assessed the NEDD4- β 2AR interaction using a co-immunoprecipitation assay. As shown in Fig 4A, NEDD4 associated with β 2AR in scrambled control siRNA-transfected cells, and such association was augmented moderately on ISO stimulation. The knockdown of ARRDC3 by siRNA completely abrogated the association of NEDD4 with the receptor in the presence or absence of ISO stimulation. By contrast, a 60% reduction in the level of

Fig 3 | ARRDC3 interacts with activated β 2AR. (A) Association of ARRDC3 with activated β 2AR. Cells stably expressing FLAG- β 2ARt were transfected with the indicated plasmids then subjected to a treatment with vehicle or ISO. Anti-FLAG immunoprecipitates from the corresponding lysates and WCEs were analysed with the indicated antibodies. (B) ARRDC3 co-localizes with β 2AR and EEA1 after agonist stimulation. β 2AR-expressing cells transfected with GFP (control) or ARRDC3-GFP were incubated with a rabbit-raised FLAG antibody and subjected to treatment with ISO (or vehicle) as indicated. Cells were fixed and permeabilized as detailed in the 'Methods' section then incubated with a mouse-raised EEA1 antibody. Cells were later stained with a rabbit IgG TRITC-conjugated antibody and a mouse IgG Alexa 647-conjugated antibody before mounting on slides and visualization. White arrowheads indicate regions of co-localization. β 2AR, β 2-adrenergic receptor; β 2ARt, truncated β 2-adrenergic receptor; ARRDC3, arrestin domain-containing 3; EEA1, early endosomal antigen 1; GFP, green fluorescent protein; ISO, isoproterenol; TRITC, tetramethylrhodamine isothiocyanate; WCE, whole cell extract.

β -arrestin 2 by siRNA transfection did not affect the interaction between NEDD4 and β 2AR (Fig 4A). These data establish ARRDC3 as the adaptor that mediates the association between NEDD4 and β 2AR.

DISCUSSION

In this study, we report the discovery of a new ARRDC protein, ARRDC3, as a critical component of the β 2AR downregulation process. Our data demonstrate that ARRDC3 functions as an adaptor for β 2AR ubiquitination by recruiting the NEDD4 E3 ligase to activated receptor (Fig 4B). The ARRDC3 protein interacts with NEDD4 through its conserved PPXY motifs and with β 2AR, particularly after β -agonist stimulation. Through the interaction with ARRDC3, NEDD4 is recruited to the proximity of the receptor, allowing the E3 ligase to recognize and ubiquitinate the receptor. Although our study characterizes how ARRDC3 interacts with NEDD4, it remains to be determined whether the arrestin-like region of ARRDC3 mediates the interaction with activated (phosphorylated) β 2AR in a manner similar to arrestins (Mittal & McMahon, 2009). Nevertheless, our study represents the first demonstration of the interaction of an ARRDC protein with a prototypical GPCR and its crucial role in receptor downregulation, indicating that structural similarities between ARRDCs and arrestins yield interesting functional parallels.

An arrestin protein, β -arrestin 2, has been shown previously to interact with β 2AR and to be required for receptor downregulation (Shenoy *et al*, 2001, 2008). Although both ARRDC3 and β -arrestin 2 are required for efficient β 2AR downregulation, our data show that only ARRDC3 is required for the recruitment of NEDD4 to the activated receptor. β -Arrestin 2 has been shown to have an important role in receptor internalization by recruiting components of the internalization machinery, such as clathrin and assembly protein AP-2 (Goodman *et al*, 1996; Santini *et al*, 2002). Our data do not suggest a direct role for ARRDC3 in receptor internalization. Thus, ARRDC3 and β -arrestin 2 seem to have distinct roles in β 2AR downregulation. Whether and how the two proteins coordinate during β 2AR downregulation remains to be determined.

The role of ARRDC3 as an ubiquitination adaptor is remarkably similar to that of arrestin-related trafficking (ART) adaptor proteins in yeast. ART proteins have been shown to recruit the yeast HECT domain E3 ligase Rsp5 to ubiquitinate target plasma membrane proteins (Lin *et al*, 2008; Nikko *et al*, 2008; Nikko & Pelham, 2009). Although the sequence similarity is low between ARRDC3 and the yeast ART proteins (supplementary Fig S3 online; Alvarez, 2008), they share a similar functional domain organization: an arrestin-like domain at the N-terminus and PPXY motifs at the C-terminus. The conservation of structural domains and the analogous functions of the ARTs and ARRDC3 indicate that

Fig 4 | ARRDC3 is an essential adaptor for β 2AR ubiquitination. (A) ARRDC3 is required for the NEDD4- β 2AR association. Cells stably expressing FLAG- β 2ARt were transfected with siRNAs targeting nothing (scrambled), ARRDC3 or β -arrestin 2 and then subjected to a treatment with vehicle or ISO. Anti-FLAG immunoprecipitates from the corresponding lysates and WCE were analysed by immunoblotting with the indicated antibodies. (B) A schematic model of ARRDC3 functioning as an adaptor for β 2AR ubiquitination (Ub). β 2AR, β 2-adrenergic receptor; β 2ARt, truncated β 2-adrenergic receptor; ARRDC3, arrestin domain-containing 3; IP, immunoprecipitation; ISO, isoproterenol; NEDD4, neural precursor development downregulated protein 4; siRNA, small interfering RNA; WCE, whole cell extract.

the ubiquitin ligase adaptor functions have been conserved throughout evolution. Similar to yeast ARTs, there are several ARRDC proteins in humans (supplementary Fig S3 online). The fact that the other plasma membrane-localized ARRDC, ARRDC1, has no obvious role in β 2AR ubiquitination indicates little functional redundancy among the ARRDC family members. Unlike yeast, which has only one HECT domain ligase (Rsp5), mammalian cells have at least nine such ubiquitin ligases (Rotin & Kumar, 2009), all of which (including NEDD4) contain WW domains that might interact with PPXY motifs. Indeed, ARRDC3 interacts with at least three other HECT domain E3 ligases (supplementary Fig S4 online). It is thus possible that ARRDC3, through its interactions with other members of the NEDD4 E3 ligase family, might function as an ubiquitination adaptor for multiple receptor protein substrates.

Our study identifies ARRDC3 as an essential mediator of β 2AR downregulation. Meticulous control of receptor levels through downregulation ensures appropriate levels of signalling in tissues such as the lung airways, whereas excessive downregulation can lead to the reduction or loss of functional β 2AR, thus severely limiting therapies (such as β -agonists for asthma treatment) that rely on efficient β 2AR activation and signalling (Shore & Moore, 2003). Future studies on the role of ARRDC3 in physiological sites of β 2AR activity might provide new insights into *in vivo* β 2AR regulation and lead to new approaches that improve β 2AR-based therapies.

METHODS

β 2AR ubiquitination and degradation assays. Cells expressing β 2AR or β 2ARt were subjected to 10 μ M ISO (Sigma) stimulation, lysed in 0.5% Nonidet-40 lysis buffer and immunoblotted with anti-FLAG-horseradish peroxidase (Sigma). For β 2AR ubiquitination, β 2ARt- or wild-type β 2AR-expressing cells were first incubated with 10 μ M alprenolol antagonist (Sigma) for 15 min (to reduce constitutive background ubiquitination), stimulated with 10 μ M ISO and lysed in buffer supplemented with 10 mM N-ethylmaleimide (Sigma) to inhibit deubiquitinase activity. Anti-FLAG EZview beads (Sigma) used for the immunoprecipitation were subjected to three 10 min washes with lysis buffer and resuspended in 50 μ l 2 $\times$ lithium dodecyl sulphate sample buffer (Invitrogen) containing 10% β -mercaptoethanol (Sigma) and analysed by immunoblotting.

Co-immunoprecipitations. 293T cells were transfected using Eugene 6 (Roche) or Turbofect (Fermentas). After 48 h, cells were lysed, pre-cleared with protein A agarose, and HA- or FLAG-tagged proteins were immunoprecipitated using anti-HA EZview or anti-FLAG EZview beads. Washed beads were resuspended in sample buffer and analysed by immunoblotting. Where noted, crosslinking was achieved by incubation for 40 min with 2 mM crosslinker dithio-bismaleimidoethane (Pierce) in phosphate-buffered saline containing 10 mM HEPES (pH 7.5), as previously described (Shenoy et al, 2008) with minor modifications. The immunoprecipitation complexes were washed three times consecutively in lysis, high-salt and low-salt buffers before being resuspended in sample buffer for immunoblotting.

Immunofluorescence assays and confocal microscopy. The β 2AR-expressing cells seeded on glass cover slips (VWR) were transfected and then incubated with 10 μ g per ml anti-FLAG M2 polyclonal or monoclonal antibodies (Sigma) before stimulation

with ISO. Cells were washed and fixed with 3.8% paraformaldehyde and permeabilized with 0.1% Triton X-100 buffer in Tris-buffered saline supplemented with 3% bovine serum albumin. Primary and secondary antibody incubations were carried out as indicated, before visualization. Image acquisition was carried out using a Leica TCS-NT laser scanning confocal microscope (Leica) fitted with air-cooled argon and krypton lasers.

Details of other experimental methods can be found in the supplementary information online.

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

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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