Supplementary section

Supplementary Figure legends

Figure S1. SUMOylation of β-arr2 using the UFDS system in DKO MEFs. Ubc9-β-arr2-FLAG or Ubc9C93S-β-arr2-FLAG were coexpressed with HA-SUMO2 in double β-arr1/2 knockout MEFs as indicated. The protein extracts were subject to immunoprecipitation using EZview Red ANTI-FLAG® M2 Affinity Gel, and fusions and SUMOylated fusions were detected by western blot using anti-FLAG antibodies. The arrows indicate Ubc9-β-arr2 or its SUMOylated form.

Figure S2. SUMOylation of a human β-arr2 peptide array identifies lysine 295 as a major SUMOylation site. (a) The peptide library was overlaid with SUMO conjugation assay mixture (Ubc9+) including recombinant His-SUMO1, His-SUMO2 and His-SUMO3. Dark spots represent a run of peptides that displayed successful conjugation of recombinant SUMO to immobilized peptides that are absent in the control array (Ubc9-). **(b)** Alanine substitution at K295 inhibits SUMO conjugation on the immobilized 276-300 peptide. Peptides were spotted four times.

Figure S3. β-arr2ΔSIM mutant displays defective nuclear accumulation. HeLa cells transfected with **(a)** YFP-tagged β-arr2 wild-type or β-arr2ΔSIM, **(b)** native β-arr2 or β-arr2ΔSIM, **(c)** FLAG-tagged β-arr2 wild-type or β-arr2ΔSIM, **(d)** Cherry-tagged β-arr2 wild-type or β-arr2ΔSIM were incubated with methanol control (CTL) or 20nM LMB for 60 min at 37°C, then fixed and processed for fluorescence microscopy using a wide-field microscope. Representative images are shown.

Figure S4. β-arr2ΔSIM mutant supports vasopressin V2 receptor endocytosis. (a) HEK293-KO-β-arr1/2 cells expressing exogeneous HA-V2R-venusYFP and Rluc-β-arr2-WT, or ΔSIM, at equivalent levels (Rluc counts graph) were treated with vehicle or 100nM arginine vasopressin (AVP) for 30 minutes. Data shown indicate cell surface expression of HA-V2R-venusYFP and represent the mean ± s.e.m. of five independent experiments (***$P<0.001$). (**b)** HEK cells were transfected with V2R fused to RlucII (V2R-RlucII), rGFP fused to the plasma membrane anchoring domain of KRas CAAX (rGFP-CAAX) and empty vector (mock) or β-arr2 constructs (WT and

ΔSIM). Transfected cells were stimulated with 100nM AVP for 30 minutes before BRET2 measurement, which was normalized against the signal measured for wild-type β-arr2. Data shown represent the mean ± s.e.m. of three independent experiments (*P<0.05; ns: non-significant). Schematic diagrams of the endocytosis assays were created using BioRender.com.

Figure S5. Effect of LMB on β-arr2 nuclear accumulation following mutation of potential *in silico* predicted SUMOylation sites. (a) Schematic displaying *in silico* predicted SUMOylation sites. **(b and c)** HeLa cells transfected with FLAG-tagged β-arr2ΔSIM, β-arr2-K295R, β-arr2-K11R, β-arr2-K25R, β-arr2-K53R, β-arr2-K158R, β-arr2-K293R, β-arr2-K400R, β-arr2-K11R-K295R, β-arr2-K25R-K295R, β-arr2-K53R-K295R, β-arr2-K158R-K295R, β-arr2-K293R-K295R and β-arr2-K295R-K400R were incubated with methanol control (CTL) or 20 nM LMB for 60 min at 37°C, then fixed, processed and imaged using either wide-field **(b)** or confocal **(c)** fluorescence microscopy. Representative images are shown.

Figure S6. Expression of p53 in doxycycline-induced H1299-p53-TETON cells and endogenous p53 in MCF-7 cells. (a) H1299-p53-TETON cells were incubated overnight with increasing levels of doxycycline (10, 50, 100 and 200ng/ml). Western blot shows p53 expression relative to MCF-7 cells. Loading control western blot using Hsp70 antibodies is shown in the lower inset. **(b)** Western blots of MCF-7 cell lysates demonstrating endogenous expression of RanBP2, SUMO-RanGAP, Mdm2, p53, β-arr2, Ubc9, SUMO1 and SUMO2. **(c)** Western blot of MCF-7 cell lysates demonstrating comparable expression of endogenous β-arr2 with exogenously expressed Rluc-β-arr2.

Supplementary Materials and Methods

Reagents and antibodies

Cell culture media and fetal bovine serum were from Invitrogen. Leptomycin B was purchased from Sigma. The following antibodies were from Cell Signaling Technologies: anti-Flag-DYKDDDDK-Tag D6W5B (#14793), anti-HA-Tag C29F4 (#3724), anti-Myc-Tag 71D10 (#2278), anti-βarr2 C16D9 (#3857), anti-βarr1/2 D24H9 (#4674), anti-GFP D5.1 (#2956), anti-RanGAP (#14675), anti-Ubc9 D26F2 (#4786), anti-SUMO-1 C9H1 (#4940) or -2/3 18H8 (#4971) and anti-GAPDH (#5174). Anti-RanBP2 was from Abcam (ab 64276). Anti-Hsp70 was from Santa Cruz (sc-32239). Anti-LEDGF (p52/p75) was from BD Bioscience (#611715). Antibodies were used at 1:1000 dilution in western blot, and at 1:200 dilution in immunofluorescence assay. Anti-IgG secondary antibodies coupled with Alexa Fluor 488 (LifeTechnologies #A11034) or Rhodamine (SantaCruz sc-2091) were used at a 1:500 dilution. For FACS experiments, Anti-HA antibody (HA-11) was purchased from Covance. Anti-mouse Alexa Fluor 647 secondary antibody was purchased from Invitrogen. Immunoprecipitation experiments used EZview Red ANTI-FLAG® M2 Affinity Gel (Sigma #F2426). All standard laboratory chemicals were purchased from Sigma. Horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch #111-035-003), was used at a 1:10,000 dilution. GeneJuice transfection reagent was from Novagen, Lipofectamine 3000 from Invitrogen, Ni-NTA agarose beads (nickel-charged resin) were purchased from Qiagen, and SUMO agarose beads were from ENZO (SUMO1 BML-UW0095; SUMO2 BML-UW0100). SMARTpool siRNAs were from HorizonDiscovery.

Plasmids

All β-arr2-expressing plasmids contained human β-arr2 cDNA, which was inserted into relevant constructs using standard PCR subcloning. pβ-arr2 containing native β-arr2 was obtained from the cDNA Resource Center. pβ-arr2-FLAG, pCherry-β-arr2, pYFP-β-arr2, GFP-Mdm2, V2R-RlucII, rGFP-CAAX and pIRESP-HA-V2R-vYFP plasmids have been described previously (1-4). All SUMOylation and SIM mutants were generated using the QuikChange (Stratagene) site-directed mutagenesis methodology according to manufacturer's instructions. The β-arr2ΔSIM mutant plasmids were transfected with a higher amount of plasmid, as with

equivalent amounts, this mutant was expressed less well compared to wild-type β-arr2. UFDS fusions were constructed by amplifying the Ubc9 or Ubc9C93S cDNAs by PCR and subcloning upstream and in frame with a β-arr2-FLAG cDNA. pHis-SUMO1, pHis-SUMO2 and pUbc9 were kind gifts of Dr. George S. Baillie. pHA-SUMO1 and pHA-SUMO2 were kind gifts of Dr. Claudine Pique. All constructs were verified by nucleotide sequencing (Institut Cochin GENOM'IC sequencing facility). Sequences of all primers used are available on request.

Cell culture and transfection

HeLa human cervical cancer and Human Embryonic Kidney 293T (HEK293T) cell lines were obtained from the American Type Culture Collection (ATCC). MEFs were a kind gift from Prof. Robert J. Lefkowitz (Duke University). H1299-Tet-ON-p53 cells were a kind gift from Prof. Steven B. McMahon (Thomas Jefferson University). HEK293-KO-β-arr1/2 cells were a kind gift from Prof. Asuka Inoue (Tohoku University). Cells were cultured at 37°C, 5%CO_2, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. All cells were regularly tested to ensure they were free from mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza). Gene Juice was used at a ratio of 3:1 Gene Juice:DNA, and Hela/HEK cells transfected according to the manufacturer's instructions. Lipo3000 was used to transfect H1299 and MCF-7 cells according to the manufacturer's instructions. For BRET experiments, HEK 293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% newborn calf serum, 100 units of penicillin and 100 µg/ml streptomycin at 37˚C. Cells were transiently transfected at a density of 400 000 cells/mL using a PEI/DNA ratio of 4:1. For siRNA transfections in HeLa cells, control and RanBP2 SMARTpool siRNA targeting duplexes (50nM) were reverse transfected using Lipo3000. 24 hours later cells were transfected with Cherry-β-arr2ΔNES using GeneJuice.

His-tagged protein purification using Ni-NTA beads

48h hours after transfection, cells were lysed under highly denaturing conditions in buffer A containing 6M guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM imidazole, pH 7.6. The samples were boiled at 95°C for 10 min then sonicated with 15 sec pulses until the solution was no longer viscous. Cell lysates were cleared

by centrifugation at 13,000 rpm for 25 min, and the supernatants were incubated with Nickel-NitriloTriAcetic (Ni-NTA) agarose beads (Qiagen) for 3 hours on a wheel at room temperature. The beads were washed six times with decreasing amounts of guanidium-HCl, by diluting buffer A with buffer TI containing 25 mM Tris/HCl and 20 mM imidazole (pH 6.8). Finally, bound proteins were eluted by boiling 5 min in Laemmli buffer with 200 mM imidazole.

Coimmunoprecipitation

HEK cells were transiently transfected with plasmids as indicated in the figure legends. 48h hours posttransfection, cells were lysed in 1 mL of 50 mM Hepes, 250 mM NaCl, 2 mM EDTA, 0.5% NP40, 10% glycerol, 100 µM Na3VO4, 1 mM NaF, 10mM NEM supplemented with protease inhibitors (Roche), and clarified by centrifugation at 13,000 rpm for 20 min at 4°C. Immunoprecipitations were performed with constant agitation, overnight at 4°C, on 1 mg of the supernatants with EZview Red ANTI-FLAG® M2 Affinity Gel. The day after, the beads were washed 4 times with lysis buffer, and finally the immunoprecipitated proteins were eluted in Laemmli buffer, and detected by western blot.

Live cell imaging and Immunofluorescence

Live cell imaging and immunofluorescence experiments were conducted as previously described (5). Briefly, HeLa, HEK, H1299-p53-TETON or MCF-7 cells were seeded into a µ-Dish (IBIDI; 35mm or 4 well µ-Slide). One day later, subconfluent cells were transiently transfected with GeneJuice (Hela, HEK) or Lipo3000 (H1299, MCF-7). Cells were maintained in culture for the next 24 h prior to fixation and immunofluorescence experiments, or direct live cell imaging. For nuclear translocation experiments, cells were treated with methanol vehicle control or with 20 nM of LMB and incubated at 37 °C for 60 min in complete DMEM. For Mdm2 displacement experiments, cells were co-transfected with 0.2 µg of GFP-Mdm2 and 1 µg of a Cherry-β-arr2 (wild-type or mutated) constructs. Cells were fixed in 4% PFA and processed for fluorescence microscopy. Confocal images of cells were acquired using a Leica spinning-disk microscope (40X or 63X oil immersion lens) equipped with a CoolSnap HQ2 CCD camera. Subsequently confocal microscopy images were processed, merged and cropped, and the intensity values were determined using ImageJ software (NIH). All images were acquired using identical parameters and

cells expressing comparable Cherry fluorescence intensities were quantified for Mdm2 displacement. For quantification of the nuclear:cytoplasmic ratio of YFP-β-arr2 fluorescence in live cells a previously described homemade ImageJ routine (5) was used.

SUMO beads pulldown assay

We assayed the SIM/SUMO interaction with SUMO-beads (ENZO) using 1 mg of cell lysate (lysis buffer: 50 mM Hepes, 250 mM NaCl, 2 mM EDTA, 0.5% NP40, 10% glycerol, 100 µM Na3VO4, 1 mM NaF, supplemented with protease inhibitors (Roche)) from cells transfected with either YFP-β-arr2 or YFP-β-arr2ΔSIM and 40µl of beads. Beads were collected after 3 hours incubation by centrifugation, washed three times and resuspended in Laemmli buffer. β-arr2 bound to the beads was then assayed by western blotting.

Flow Cytometry

48 h after transfection, HEK293-KO-β-arr1/2 cells expressing HA-V2R-vYFP and Rluc-β-arr2-WT or ΔSIM were treated with vehicle or 100nM AVP for 30 minutes. Cell surface localized receptors were labelled on ice using a monoclonal anti-HA antibody followed by an anti-mouse Alexa Fluor 647 secondary antibody. Cells were analyzed through an LSR II flow cytometer (BD Biosciences) set to detect YFP and Alexa Fluor 647. Data analysis was performed using BD FACSDiva software.

Enhanced bystander bioluminescence resonance energy transfer (ebBRET)

For the endocytosis assay, cells were transfected with V2R fused to RlucII (V2R-RlucII), rGFP fused to the plasma membrane anchoring domain of KRas CAAX (rGFP-CAAX) and β-arr2 constructs (WT and ΔSIM). 100µL of transfected cells were seeded in white 96-well microplates (Greiner). 48 hours later, cells are washed with DPBS (Dulbecco’s Phosphate Buffered Saline) to remove cell media and HBSS (Hank's Balanced Salt Solution) was added to the wells followed by 30min treatment with arginine vasopressin (AVP). Coelenterazine 400a (2,5µM) was added to the cells 5 minutes before BRET2 measurement on a Mithras microplate reader equipped with donor filter (410 ± 80 nm) and acceptor filter (515 ± 30 nm). The BRET2 signal is calculated as the ratio of the light emitted by energy acceptor and the light emitted by energy donor and is normalized against the signal measured for

the β-arr2 WT condition. For the nuclear localization assay, cells were transfected with β-arrestin2 constructs (WT, ΔNES and ΔSIMΔNES) fused at their C-terminus to the BRET donor Renilla luciferase II (β-arrestin2-RlucII) and the BRET acceptor Renilla green fluorescent protein (rGFP) fused to a nuclear localization signal (NLS-rGFP). Light measurements and BRET2 signals were calculated as for the endocytosis assay.

References

1. Paradis JS, Ly S, Blondel-Tepaz E, Galan JA, Beautrait A, Scott MG, et al. Receptor sequestration in response to beta-arrestin-2 phosphorylation by ERK1/2 governs steady-state levels of GPCR cell-surface expression. Proc Natl Acad Sci U S A. 2015;112(37):E5160-8.
2. Boularan C, Scott MG, Bourougaa K, Bellal M, Esteve E, Thuret A, et al. beta-arrestin 2 oligomerization controls the Mdm2-dependent inhibition of p53. Proc Natl Acad Sci U S A. 2007;104(46):18061-6.
3. Namkung Y, Le Gouill C, Lukashova V, Kobayashi H, Hogue M, Khoury E, et al. Monitoring G protein-coupled receptor and beta-arrestin trafficking in live cells using enhanced bystander BRET. Nat Commun. 2016;7:12178.
4. Storez H, Scott MG, Issafras H, Burtey A, Benmerah A, Muntaner O, et al. Homo- and hetero-oligomerization of beta-arrestins in living cells. J Biol Chem. 2005;280(48):40210-5.
5. Blondel-Tepaz E, Guilbert T, Scott MGH. Methods to Investigate the Nucleocytoplasmic Shuttling Properties of beta-Arrestins. Methods Mol Biol. 2019;1957:251-69.