Published in final edited form as:

Sci Signal. ; 11(549): . doi:10.1126/scisignal.aat7650.

Manifold roles of β -arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9**

Louis M. Luttrell^{1,2,3}, Jialu Wang⁴, Bianca Plouffe⁵, Jeffrey S. Smith^{4,6}, Lama Yamani⁷, Suneet Kaur⁴, Pierre-Yves Jean-Charles⁴, Christophe Gauthier⁸, Mi-Hye Lee¹, Biswaranjan Pani⁴, Jihee Kim⁴, Seungkirl Ahn⁴, Sudarshan Rajagopal^{4,6}, Eric Reiter⁸, Michel Bouvier⁵, Sudha K. Shenoy^{4,9}, Stéphane A. Laporte⁷, Howard A. Rockman^{4,9,10}, and Robert J. Lefkowitz^{4,6,11,*}

¹Department of Medicine, Medical University of South Carolina, Charleston, SC 29425, USA.

²Department of Biochemistry & Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA.

³Research Service of the Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, USA.

⁴Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA.

⁵Department of Biochemistry and Molecular Medicine, Institute for Research in Immunology and Cancer, University of Montreal, Montreal, QC H3C 1J4, Canada.

⁶Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, USA.

⁷Department of Medicine, Research Institute of the McGill University Health Center (RI-MUHC), McGill University, Montréal, QC, H4A 3J1, Canada.

⁸PRC, INRA, CNRS, Université duTours, 37380, Nouzilly, France.

⁹Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA.

¹⁰Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710, USA.

Publisher's Disclaimer: ***This manuscript has been accepted for publication in Science Signaling. This version has not undergone final editing. Please refer to the complete version of record at https://urldefense.proofpoint.com/v2/url?u=http-3A__www.sciencesignaling.org_&d=DwIFaQ&c=imBPVzF25OnBgGmVOlcsiEgHoG1i6YHLR0Sj_gZ4adc&r=2oD1LmRI8QCeZAY5GRb-HG5-36enPPHJUb8V1-ow6PI&m=f9Xq2hI-TIbHg9KBC_59ET9hovH5B7tphAiSx5_s7Tc&s=DwLvVPgh39IC3qb_mII5e_SH6IbLawMi8L6eLeO08vM&e=. The manuscript may not be reproduced or used in any manner that does not fall within the fair use provisions of the Copyright Act without the prior, written permission of AAAS."

*Corresponding author. lefko001@receptor-biol.duke.edu.

Author contributions: M.B., S.A.L., R.J.L., L.M.L., E.R., S.R., H.A.R., and S.K.S. conceived and designed all experiments, analyzed the data, wrote the manuscript, and contributed funding for experiments. S.A., C.G., P.-Y.J.-C., M.-H.L., B.P., J.K., S.K., E.R., J.W., and L.Y., performed MAPK activation assays, analyzed the data, and wrote the manuscript. B.P. performed BRET, FRET, and MAPK activation assays, analyzed the data, and wrote the manuscript. J.S.S. performed MAPK activation assays, collated data from all groups, prepared figures, and wrote the manuscript.

Competing interests: M.B. is a member of the Scientific Advisory Board of Domain Therapeutics, a company marketing platforms for GPCR based drug discovery. The BRET-based biosensors employed in this study have been licensed to Domain Therapeutics, but are available from the Bouvier laboratory free-of-charge for noncommercial academic use. R.J.L. and H.A.R. are founders of Trevena, Inc., a company that discovers and develops novel GPCR-targeted therapeutics.

Data and materials availability: All data needed to evaluate the conclusions of this study are available in the paper.

¹¹Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA.

Abstract

G protein–coupled receptors (GPCRs) use diverse mechanisms to regulate the mitogen-activated protein kinases ERK1/2. β -Arrestins (β Arr1/2) are ubiquitous inhibitors of G protein signaling, promoting GPCR desensitization and internalization and serving as scaffolds for ERK1/2 activation. Studies using CRISPR/Cas9 to delete β Arr1/2 and G proteins have cast doubt on the role of β -arrestins in activating specific pools of ERK1/2. We compared the effects of siRNA-mediated knockdown of β Arr1/2 and reconstitution with β Arr1/2 in three different parental and CRISPR-derived β Arr1/2 knockout HEK293 cell pairs to assess the effect of β Arr1/2 deletion on ERK1/2 activation by four G_s -coupled GPCRs. In all parental lines with all receptors, ERK1/2 stimulation was reduced by siRNAs specific for β -Arr2 or β -Arr1/2. In contrast, variable effects were observed with CRISPR-derived cell lines both between different lines and with activation of different receptors. For β_2 -adrenergic receptors (β_2 ARs) and β_1 ARs, β Arr1/2 deletion increased, decreased, or had no effect on isoproterenol-stimulated ERK1/2 activation in different CRISPR clones. ERK1/2 activation by the vasopressin V_2 and follicle-stimulating hormone receptors was reduced in these cells but was enhanced by reconstitution with β Arr1/2. Loss of desensitization and receptor internalization in CRISPR β Arr1/2 knockout cells caused β_2 AR-mediated stimulation of ERK1/2 to become more dependent on G proteins, which was reversed by reintroducing β Arr1/2. These data suggest that β Arr1/2 function as a regulatory hub, determining the balance between mechanistically different pathways that result in activation of ERK1/2, and caution against extrapolating results obtained from β Arr1/2- or G protein–deleted cells to GPCR behavior in native systems.

One-sentence summary:

β -arrestin proteins fine-tune different GPCR-stimulated pathways that converge on ERK1/2 activation.

Editor's summary: The balancing act of β -arrestins

G protein–coupled receptors (GPCRs) are thought to activate the kinases ERK1/2 through G protein– and β -arrestin–dependent pathways. The relative contribution of each is difficult to assess because β -arrestins prevent G protein–coupling by GPCRs (see the Focus by Gurevich and Gurevich). Studies based on CRISPR/Cas9-generated cell lines suggested that β -arrestins are dispensable for ERK1/2 activation. Luttrell *et al.* compared the effects of siRNA-mediated and CRISPR/Cas9-mediated knockdown of β -arrestins on ERK1/2 activation by several GPCRs in independent clones. Their data showed that signaling rewiring in the CRISPR clones rendered GPCR-dependent ERK1/2 activation more G protein–dependent, which was reversed by reconstitution with β -arrestins. Together, these findings suggest that β -arrestins balance signaling through the different pathways from GPCRs to ERK1/2 and suggest that experiments with deletion of β -arrestins or G proteins should be interpreted with caution.

INTRODUCTION

The mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase 1 (ERK1) and ERK2 (ERK1/2) play critical roles in cell cycle progression, proliferation, survival, and apoptotic signaling by controlling phosphorylation of nuclear transcription factors, for example, Elk1, as well as diverse regulatory functions mediated through the phosphorylation of cytosolic substrates (1). Given their myriad roles, they are subject to multiple convergent forms of positive and negative control. As key regulators of these processes, G protein-coupled receptors (GPCRs) use multiple pathways to regulate ERK1/2 activity. Depending on the receptor and cell type, G_s -coupled GPCRs, for example, activate ERK1/2 through cAMP-dependent protein kinase (PKA) or direct cAMP-mediated effects on B-Raf and the Epac family of cAMP-regulated Rap1 guanine nucleotide exchange factors (GEFs) (2–4) or dampen mitogen-stimulated ERK1/2 activation through PKA-mediated inhibition of the upstream kinase cRaf1 (5). Adding to the diversity, Gq/11-coupled receptors often use protein kinase C (PKC)-dependent mechanisms to directly activate cRaf1 (6,7), whereas G_i/o -coupled receptors use $G\beta\gamma$ subunit-mediated mechanisms to trigger Ras-dependent ERK1/2 activation by stimulating the matrix metalloprotease-dependent shedding of tethered ligands that “transactivate” epidermal growth factor receptors (EGFRs) (8–10). Further complicating matters, in some cases multiple G protein species are involved downstream of the same receptor. For example, β_2 adrenergic receptor (β_2AR)-mediated ERK1/2 activation can involve “switching” of receptor coupling from G_s to G_i proteins, producing ERK1/2 signaling that is sensitive both to inhibitors of G_s -PKA and *Bordetella pertussis* toxin (PTX) (11,12).

The arrestins are a family of four cytosolic proteins that play a key role in the negative regulation of heterotrimeric G protein signaling pathways by binding to agonist-occupied GPCR kinase (GRK)-phosphorylated receptors and sterically inhibiting G protein coupling at the plasma membrane (13,14). The two non-visual arrestins, β -arrestin1 ($\beta Arr1$) and β -arrestin2 ($\beta Arr2$), also referred to as arrestin2 and arrestin3, respectively, further control G protein signaling by functioning as adaptor proteins that link activated GPCRs on the plasma membrane to the clathrin-dependent endocytic machinery (15–18). These combined actions keep downstream G protein signaling, including G protein-dependent activation of ERK1/2, in check by promoting desensitization and internalization of activated receptors. Arrestins also bind to numerous signaling proteins, notably the three component kinases of the ERK1/2 cascade: cRaf1, MEK1/2, and ERK1/2 (19–21). Importantly, this binding is sensitive to arrestin conformation, such that cRaf1 and ERK1/2 bind efficiently to arrestins in their GPCR-bound conformation yet have almost no affinity for the inactive cytosolic conformation (22,23). As a result, recruitment of $\beta Arr1/2$ to activated GPCRs promotes assembly of the cRaf1-MEK1/2-ERK1/2 complex, supporting activation of ERK1/2 and its retention in cytosolic GPCR-arrestin “signalsome” complexes (19,24). In their role as signaling scaffolds, $\beta Arr1/2$ affect the kinetics of GPCR-stimulated ERK1/2 activation, favoring prolonged activation, because $\beta Arr1/2$ -bound ERK1/2 is protected from rapid dephosphorylation by nuclear and cytosolic MAPK phosphatases (25–28), and determining its function, because the spatial constraints imposed on arrestin-bound ERK1/2 favor phosphorylation of cytosolic substrates but inhibit its nuclear functions. As a result, β -

arrestin-bound ERK1/2 is implicated in the regulation of GPCR internalization and trafficking (29,30), cytoskeletal rearrangement and chemotaxis (31,32), matrix metalloproteinase-dependent ectodomain shedding (33–35), and protein synthesis (36,37), but dampens Elk-1 dependent transcription (38,39).

Given their functional duality, the absence of β Arr1/2 would be expected to impair GPCR desensitization, potentially enhancing G protein-dependent ERK1/2 activation, while at the same time abrogating arrestin-supported signaling. Not surprisingly, germline deletion of *ARRB1/2* (the genes that encode β Arr1/2) produces complex effects on GPCR regulation of ERK1/2. In murine embryonic fibroblasts (MEFs) derived from β Arr1/2 knockout embryos, stimulation of predominantly $G_{i/o}$ -coupled lysophosphatidic acid receptors provokes robust and sustained ERK1/2 activation, which is mediated almost exclusively through transactivated EGFRs (40). When β -Arrestin is restored to the knockout cells, the duration of EGFR-dependent ERK1/2 activation is shortened and EGFR-dependent transcription is attenuated, consistent with the dampening of a G protein-dependent pathway by β Arr2-mediated desensitization. However, reintroducing β Arr2 restores the wild-type phenotype of sustained EGFR-independent ERK1/2 activation, consistent with a β Arr2-dependent signaling process that determines the kinetics of ERK1/2 activation and modifies lysophosphatidic acid receptor-driven transcription.

Consistent with previously published work in β Arr1/2 knockout MEFs, studies that used CRISPR/Cas9 and TALEN genome editing strategies to delete *ARRB1/2* or heterotrimeric $G\alpha$ subunit proteins in HEK293 cells demonstrated that β Arr1/2 are “dispensable” for activation of ERK1/2 by β_2 ARs, that is, that GPCR-dependent stimulation of ERK1/2 can be mediated by G proteins in the absence of β -arrestins. Grundmann *et al.* (41) using a HEK293 cell model system from which essentially all heterotrimeric G protein activity had been removed by CRISPR/Cas9 gene editing and PTX treatment, reported that several arrestin pathway-selective GPCR agonists fail to activate ERK1/2 in the absence of G proteins. O’Hayre *et al.* (42) reported that CRISPR/Cas9-mediated deletion of β Arr1/2 enhances, rather than diminishes, ERK1/2 activation by the G_s -coupled β_2 AR and vasopressin V_2 receptors (V_2 Rs), and that β_2 AR-dependent stimulation of ERK1/2 in a β Arr1/2 knockout background proceeds through a pathway involving heterotrimeric G proteins and the classical receptor tyrosine kinase mitogenic pathway components, c-Src, SHC, mSOS, and Ras (43). Based on the observations that β -arrestin-dependent signaling does not exist in β Arr1/2 null or “G-zero” HEK293 cells, the authors of these papers called into question more than 15 years of published work on the role of β Arr1/2 in ERK1/2 regulation (44–48), suggesting instead that GPCR regulation of ERK1/2 is a G protein-mediated process in which β Arr1/2 play a negligible role. The authors further suggest that, because the only ascertainable role of β Arr1/2 is to hinder G protein signaling through desensitization and internalization of GPCRs, that screening of “biased” GPCR agonists is most appropriately performed in genome-edited *ARRB1/2* knockout (KO) cells, in which the important G protein-mediated signaling is amplified (41,49).

It is concerning that the conclusions of these first published studies applying CRISPR/Cas9 KO cells to the study of GPCR-mediated ERK1/2 activation contrast so starkly with a large and diverse previous literature using various other validated technologies and

physiologically relevant models. Accordingly, we determined the contributions of β Arr1/2 to ERK1/2 activation by different G_s -coupled GPCRs in three CRISPR/Cas9 genome-edited HEK293 cell lines derived independently in different laboratories. Each receptor was probed using complementary loss-of-function and gain-of-function approaches wherein endogenous β Arr1/2 in parental HEK293 lines was abruptly knocked down using siRNAs, or in which β Arr1/2 was reintroduced into CRISPR β Arr1/2 KO lines by transient transfection. Together, our data provide insight into the complex roles of arrestins in regulating signal strength in two pathways, one G protein-dependent and the other β Arr1/2-dependent, that converge downstream on a common effector.

RESULTS

β Arr1/2 gene editing has variable effects on β_2 AR-stimulated activation of ERK1/2 in three independently derived β Arr1/2 CRISPR KO HEK 293 cell lines

The ERK1/2 cascade is subject to control by both G protein-dependent and β -arrestin-dependent mechanisms that contribute to pathway activation (47,48). Because β -arrestins both negatively regulate G protein-dependent signaling to ERK1/2 by promoting GPCR desensitization and support pathway activation by functioning as ligand-regulated scaffolds, deletion of both β Arr1/2 isoforms would be expected to augment G protein-dependent ERK1/2 activation while simultaneously abrogating the β -arrestin-dependent pathway. The net effect, either augmentation or reduction of GPCR-mediated ERK1/2 activation, would likely be dependent on the relative contribution of each pathway in a given cell background. For example, data from a study showing enhanced β_2 AR-stimulated ERK1/2 activation in HEK293 cell lines in which β Arr1/2 were deleted using CRISPR/Cas9 or combined β Arr1 TALEN knockout and β Arr2 siRNA approaches suggest that in those cells, the augmentation of G_s signaling due to loss of β Arr1/2-dependent desensitization is greater than the loss of signal arising from β -arrestin-dependent ERK1/2 activation (42).

To test whether deletion of β Arr1/2 by genome-editing strategies consistently produced a net enhancement of GPCR-stimulated ERK1/2 activation, we initially compared β_2 AR-mediated ERK1/2 responses in three independently derived HEK293 cell lines in which both the *ARRB1* and *ARRB2* genes were targeted using a CRISPR/Cas9 system. The three parental lines differed in the relative ratios of endogenous β Arr1 and β Arr2 abundance, with β Arr1 being more abundant than β Arr2 in with two lines (AI-HEK293 and SL-HEK293) whereas β Arr2 was more abundant than β Arr1 in the third (HAR-HEK293) (Fig. 1). All three derived CRISPR β Arr1/2 KO lines were devoid of detectable β Arr1/2. We then compared the time course of ERK1/2 activation in each parental-CRISPR HEK293 cell pair upon stimulation of endogenously expressed β_2 ARs with a saturating concentration of isoproterenol (Fig. 1, A to C). The maximal isoproterenol-stimulated ratio of phosphorylated ERK1/2 (pERK1/2) to total ERK1/2 in each parental cell line was normalized to 100% and the response in the corresponding CRISPR cell line was expressed as a percentage of the response in the parental cells. Consistent with the earlier report (42), deletion of β Arr1/2 in the AI-CRISPR β Arr1/2 KO clone led to a two-fold increase in pERK1/2 abundance relative to that in its parental line. However, this increase was not observed in either the SL-CRISPR β Arr1/2 KO or HAR-CRISPR β Arr1/2 KO cell lines, in which β Arr1/2 deletion had no

substantial effect on β_2 AR mediated ERK1/2 activation. The augmented response seen in the AI-CRISPR β Arr1/2 KO line was preserved when the β_2 AR was overexpressed (Fig. 1D), indicating that at both high and low β_2 AR abundance, deletion of β Arr1/2 resulted in a net increase in ERK1/2 activation in those cells.

Knockdown of β Arr1/2 using siRNA in parental HEK293 cells and reconstitution of β Arr1/2 in CRISPR β Arr1/2 KO cells have reciprocal effects on β_2 AR-stimulated activation of ERK1/2

A limitation of gene-editing strategies such as CRISPR/Cas9 is that the derived clonal cell lines have, of necessity, been selected for their ability to survive in the absence of the deleted protein(s). In the case of β Arr1/2, which are key regulators of virtually all GPCR signaling, surviving CRISPR clones must have undergone some degree of “rewiring” of GPCR signaling pathways to compensate for the absence of these physiologically relevant GPCR regulatory processes. The divergent results we obtained using independently derived CRISPR β Arr1/2 KO lines (Fig. 1) suggest that comparison of CRISPR clones with their parental lines is not an optimal strategy for delineating the functions of the missing proteins due to undefined adaptations during clonal selection. To further characterize the complex roles of β Arr1/2 in β_2 AR-mediated ERK1/2 activation, we adopted reciprocal loss-of-function and gain-of-function strategies in which we compared the effects of β Arr1/2 knockdown by siRNA in the parental HEK293 lines with those of β Arr1/2 reconstitution by transient transfection of the CRISPR β Arr1/2 KO lines.

We first determined the effects of knocking down β -arrestins using siRNAs (Fig. 2). Because previous studies of β -arrestin-dependent ERK1/2 activation have suggested that for some GPCRs, for example the β_2 AR and the angiotensin AT1A receptor (AT1AR), β Arr1 and β Arr2 play specialized desensitizing and signaling roles, respectively (50–54), these experiments used well-validated siRNA sequences targeting either β Arr2 alone or both β Arr1/2 (53,54). We found that siRNA-mediated knockdown of β Arr2 or β Arr1/2 produced about an 80% reduction in the abundance of the targeted isoform(s) 48 hours after transient transfection. Knockdown of β Arr2 alone led to a statistically significant reduction in β_2 AR-stimulated ERK1/2 activation compared to that in all three HEK293 parental lines and with both endogenously expressed and transiently overexpressed β_2 AR (Fig. 2, A to D). Similar to previous studies (54), these data indicate that in the continued presence of β Arr1, selective knockdown of β Arr2 produces a net decrease in β_2 AR-stimulated ERK1/2 activation that is consistent with β Arr2-dependent augmentation of ERK1/2 signaling. To mimic the effects of β Arr1/2 deletion in the CRISPR lines, we also used siRNA targeting both β -arrestin isoforms (Fig. 2, E to H). As in the CRISPR cell lines, this would be expected to reduce both β Arr2-dependent signaling and any contribution of β Arr1 or β Arr2 to receptor desensitization; that is, to shift β_2 AR-stimulated ERK1/2 activation more strongly toward G protein-dependent, and away from β -arrestin-dependent, mechanisms. Knockdown of β Arr1/2 in the SL-parental and HAR-parental HEK293 lines reversed the attenuation of ERK1/2 activation that was observed when β Arr2 only was targeted, mimicking our findings in the SL-CRISPR and HAR-CRISPR β Arr1/2 KO lines (Fig. 2, F and G). The absence of either a net increase or decrease in ERK1/2 activation upon β Arr1/2 knockdown suggests that β_2 AR-mediated ERK1/2 activation in the absence of β Arr1/2

reflects the balance between augmented G protein-dependent signaling, which would increase the ERK1/2 signal, and reduced β -arrestin-dependent signaling, which would decrease it. More complex effects were seen using the AI-parental HEK293 line, where siRNA-mediated knockdown of β Arr1/2 reduced peak ERK1/2 activation upon stimulation of endogenous, but not overexpressed, β_2 AR (Fig. 2, E and H). These results are directly opposite those seen in the comparison of the AI-parental and AI-CRISPR β Arr1/2 KO lines (Fig. 1), where the gene-edited clone exhibited a G protein–dominant phenotype wherein β_2 AR-stimulated ERK1/2 activation was consistently enhanced by removal of β Arr1/2.

We then performed the complementary experiment, in which β Arr2 or β Arr1/2 were reintroduced into each CRISPR β Arr1/2 KO background by transient transfection. Because these cells were devoid of both β Arr1 and β Arr2, the expectation would be that reintroduction of either or both isoforms would restore β_2 AR desensitization and dampen G protein–dependent downstream signaling, while at the same time restoring the capacity for β -arrestin-dependent ERK1/2 activation. Because β Arr1/2 were re-introduced by transient transfection, these experiments were performed in CRISPR β Arr1/2 KO lines co-transfected to express the β_2 AR. The abundance of β Arr1/2 in the transfected CRISPR β Arr1/2 KO lines was comparable with that of endogenous β Arr1/2 in the respective parental HEK293 lines (Fig. 3 A to C). Not surprisingly given its complex functions, restoration of β Arr2 alone had cell line-specific effects. In the AI-CRISPR β Arr1/2 KO line (Fig. 3A), which showed enhanced maximal β_2 AR-stimulated ERK1/2 activation compared to that of its parental line (Fig. 1), there was no net reduction in ERK1/2 activation, suggesting that any decrease in G protein–dependent signaling that resulted from restored β_2 AR desensitization was offset by some increase in ERK1/2 activation in the presence of β Arr2. Similar results were observed in the HAR-CRISPR β Arr1/2 KO line, where reintroduction of β Arr2 led to a statistically insignificant reduction in maximal β_2 AR–stimulated ERK1/2 activation (Fig. 3C). The opposite effect was seen in the SL-CRISPR β Arr1/2 KO line, where reintroduction of β Arr2 statistically significantly increased the maximal β_2 AR-stimulated ERK1/2 signal (Fig. 3B). Identical results were obtained when both β Arr1/2 were reintroduced into the β Arr1/2 KO background to mimic the phenotype of the parental HEK293 cell lines (Fig. 3, D to F). No statistically significant net effect on β_2 AR-stimulated ERK1/2 activation was seen in the AI-CRISPR β Arr1/2 KO line (Fig. 3D), whereas the SL-CRISPR and HAR-CRISPR β Arr1/2 KO lines (FIG 3, E and F) exhibited the enhanced ERK1/2 signal seen when only β Arr2 was reintroduced (Fig. 3B), although at different times.

If the only role of β -arrestins was to inhibit G protein signaling by promoting homologous desensitization and sequestration of GPCRs, then the consequences of β Arr1/2 knockdown in parental HEK293 cells by siRNA (Fig. 2) and reconstitution in CRISPR β Arr1/2 KO cells by transient transfection (Fig. 3) would be a consistent augmentation and reduction, respectively, of β_2 AR-stimulated ERK1/2 activation. The most parsimonious interpretation of the seemingly discordant results obtained using different parental-CRISPR pairs is that β Arr1/2 perform dual roles, serving as both negative regulators of G protein signaling and as signaling scaffolds that enhance ERK1/2 activation (44–48). As such, their abundance determines the signal strength in two pathways that converge downstream on a common effector, a G protein–dependent pathway that is attenuated by β -arrestin, and a β -arrestin–dependent pathway that is conferred by their presence. In such a case, a net increase,

decrease, or no effect on ERK1/2 activation might all be observed depending on whether the cell background is poised for G protein–dominant or β -arrestin-dominant activation of ERK1/2. The marked variability in the responses we observed in the three different CRISPR β Arr1/2 KO lines supports this interpretation and also highlights a major drawback in using such genetically altered cells for complex signaling experiments. Marked differences in the expression of myriad signaling elements in the different lines due to the rewiring of cellular pathways required to keep these genetically edited cells viable is likely responsible for the different response patterns observed.

β Arr1/2 determine both the efficiency of β_2 AR-Gs coupling and the dominant mechanism of β_2 AR-mediated ERK1/2 activation

The results of our complementary loss-of-function and gain-of-function analyses of the effect of β Arr1/2 on ERK1/2 pathway activation by β_2 ARs lead to several testable hypotheses. First, deletion of β -arrestins should increase the efficiency of β_2 AR-G_s coupling by removing the restraint imposed by homologous desensitization. Second, deletion of β -arrestins should reduce or eliminate β_2 AR internalization, leaving more agonist-responsive receptors on the plasma membrane. Third, deletion of β -arrestins should cause downstream signaling to ERK1/2 to become more dependent on G protein-regulated effectors, such as PKA.

To test the first hypothesis, we measured the efficiency of β_2 AR-mediated G_s activation using a bioluminescence resonance energy transfer (BRET) approach (55). In this assay, changes in BRET efficiency between a *Renilla* luciferase-II (*Rluc*-II)-tagged G_s subunit and green fluorescent protein 10 (GFP10)-tagged G γ 2 subunit upon isoproterenol stimulation of co-expressed β_2 ARs report on conformational rearrangements within the G_s heterotrimer that occur upon activation. Isoproterenol stimulation produced a concentration-dependent decrease in BRET (BRET) that was greater in the CRISPR β Arr1/2 KO lines than in their respective parental controls for all the clones, indicating a gain in G_s activation efficiency when β Arr1/2-mediated desensitization was removed (Fig. 4A). The effects of restoring β Arr1 or β Arr2 to the β Arr1/2 KO background were also examined. Reconstitution with even low amounts of either arrestin isoform was sufficient to fully revert G_s activation to the levels observed in the parental HEK293 lines. The finding that either β -arrestin isoform alone could restore β_2 AR desensitization is consistent with our ERK1/2 activation data (Fig. 2), where knockdown of β Arr2 in the continued presence of endogenous β Arr1 consistently led to a net reduction in β_2 AR-mediated ERK1/2 activation, whereas knockdown of β Arr1/2, which would impair desensitization and amplify G protein signaling, produced no net change in the ERK1/2 response.

CRISPR KO of β Arr1/2 also abolished the isoproterenol-induced loss of cell surface receptor assessed by enhanced bystander BRET (ebBRET), which monitors BRET between an *Rluc*-tagged β_2 AR and *Renilla reniformis* GFP anchored at the plasma membrane by a prenylated CAAX motif (rGFP-CAAX) (Fig. 4B) (56). Consistent with the well-established role of β -arrestins in β_2 AR endocytosis, isoproterenol stimulation produced a concentration-dependent reduction in ebBRET in parental HEK cells with EC₅₀ values ranging between 208 and 395 nM for the three clones, about 10-fold higher than the EC₅₀ for G_s activation.

Reconstitution of either β Arr1 or β Arr2 in the CRISPR β Arr1/2 KO cells was sufficient to restore isoproterenol-stimulated β_2 AR internalization; however, unlike for the desensitization experiments, the effect of β Arr1 was only partial and required greater protein abundance than that of β Arr2. This is consistent with published data showing that activated β_2 ARs have a higher affinity for β Arr2 than for β Arr1 (53,57,58), and suggests that whereas both β -arrestin isoforms readily support β_2 AR desensitization, β Arr2 plays a greater role in β_2 AR endocytosis.

Finally, to test the hypothesis that ERK1/2 activation in a β Arr1/2 KO background would be more dependent upon G protein signaling, we determined the extent to which β_2 AR-mediated ERK1/2 activation in parental HEK293 and CRISPR β Arr1/2 KO cells was sensitive to inhibition of G protein signaling. Previous studies, both in intact HEK293 cells and an in vitro reconstituted system, demonstrated that β_2 ARs can use multiple G protein-mediated pathways to activate ERK1/2 (2–4), and that in some cases, PKA-dependent phosphorylation of the receptor confers the capacity to activate ERK1/2 through PTX-sensitive $G_{i/o}$ -mediated pathways (11,12). Depending on the cell background, β_2 AR-dependent stimulation of ERK1/2 can be sensitive to inhibition of PKA, treatment with PTX, or both. Therefore, we compared the effects of pretreatment with the PKA inhibitor 6–22, PTX, or both, on isoproterenol-stimulated ERK1/2 activation assayed using the Alphascreen Surefire system, a fluorescence resonance energy transfer (FRET)-based assay in which detection of activated ERK1/2 is by immuno-sandwich capture of endogenous pERK1/2 in cell lysates (Fig. 4C) (59). Isoproterenol stimulation of parental HEK293 cells produced a two-fold increase in FRET that was unaffected by 6–22 or PTX alone but was reduced by pretreatment with both inhibitors. In contrast, ERK1/2 activation in the corresponding CRISPR β Arr1/2 KO cells was inhibited in the presence of either the PKA inhibitor or PTX alone, confirming a shift toward G protein-dependent ERK activation in the β Arr1/2 null cells.

Together, these data indicate that compared to β Arr1/2-replete parental cells, the CRISPR β Arr1/2 KO cells have been rewired to shift β_2 AR-mediated ERK1/2 activation from dual β -arrestin- and G protein-mediated pathways to a predominately G_s -cAMP-PKA pathway, most likely in compensation for the loss of β -arrestin-dependent signaling in the β Arr1/2 KO CRISPR clones. The change results from amplified G_s signaling in the absence of β Arr1/2-mediated desensitization and abrogated β Arr2-dependent β_2 AR internalization.

β -Arrestin-dependent ERK1/2 activation is conserved amongst GPCRs that differ in their GPCR-arrestin complex stability

Another facet of GPCR regulation that may affect β -arrestin regulation of ERK1/2 is the lifetime of the GPCR-arrestin complex. Most GPCRs fall into two groups based on their relative avidity for the two β -arrestin isoforms and the stability of the GPCR-arrestin complex (58, 60). Members of the first group, termed ‘class A’, such as the β_2 AR, exhibit higher avidity for β Arr2 than for β Arr1 and form transient GPCR-arrestin complexes that dissociate upon receptor internalization, enabling the receptors to recycle to the plasma membrane. Members of the second group, ‘class B’, such as the V_2 R, exhibit equivalent avidity for β Arr1 and β Arr2 and form stable receptor-arrestin complexes that persist as the

receptor undergoes endosomal sorting, which slows recycling and promotes lysosomal degradation. The first reports describing β -arrestin-dependent ERK1/2 activation focused on the class B receptor PAR2 (protease-activated receptor 2) and AT1AR, where pERK1/2 is retained in GPCR- β -arrestin “signalsome” complexes, leading to sustained ERK1/2 activation that is targeted to endosomal vesicle membranes (19,24,51). Subsequent studies found evidence for β -arrestin-dependent ERK1/2 activation by class A GPCRs, which also use β -arrestin scaffolds to augment ERK1/2 activation (33,35,54).

To investigate the effects of GPCR-arrestin complex stability on ERK1/2 activation using the CRISPR/Cas9 genome-editing approach, we selected an additional panel of receptors that all couple primarily to the G_s -cAMP-PKA pathway but that represent different modes of β Arr1/2 interaction: the β_1 adrenergic receptor (β_1 AR), V_2 R, and the follicle-stimulating hormone receptor (FSHR). The β_1 AR, like the closely related β_2 AR, uses the class A mode of β -arrestin interaction (34). Previous studies showed that β_1 AR-mediated ERK1/2 activation is sensitive to knockdown of either β -arrestin isoform individually or together (33,34) and that it requires concomitant activation of $G_{i/o}$ proteins (61). We characterized β_1 AR-mediated ERK1/2 activation both in parental HEK293 lines in which β Arr1/2 were knocked down using siRNAs and in CRISPR β Arr1/2 KO lines in which β Arr1/2 were reconstituted by transient transfection (Fig. 5). The efficiency of siRNA-mediated knockdown of β Arr1/2 in the HEK293 parental cell lines co-transfected with plasmid encoding the murine β_1 AR was comparable to that achieved in experiments using the β_2 AR (Fig. 5, A to C). Consistent with previous reports (33,34), siRNA-mediated knockdown of β Arr1/2 caused a statistically significant reduction in β_1 AR-stimulated ERK1/2 activation in all three HEK293 parental lines (Fig. 5, A to C). These results contrast with those from experiments with cells expressing the β_2 AR, where knockdown of β Arr2, but not β Arr1/2, consistently impaired ERK1/2 activation across cell lines (Fig. 2) and suggest that in the case of the β_1 AR, any amplification of G protein-mediated signaling that might result from knockdown of β Arr1/2 is insufficient to compensate for the loss of β -arrestin-mediated signaling. We then compared β_1 AR-stimulated ERK1/2 activation in CRISPR β Arr1/2 KO lines with CRISPR cells in which β Arr1/2 was reconstituted by transient transfection (Fig. 5, D to F). We found that restoring β Arr1/2 had no net effect on β_1 AR-stimulated ERK1/2 activation in either the AI-CRISPR or HARCISPR β Arr1/2 KO lines and only modestly enhanced it in the SL-CRISPR β Arr1/2 KO cells. This finding mirrors our results from experiments with the β_2 AR (Fig. 3) and supports the interpretation that any attenuation of G protein signaling by β Arr1/2-dependent desensitization and internalization of β_1 ARs obtained by restoring β Arr1/2 was offset by the recovery of β Arr1/2-dependent ERK1/2 activation.

We performed an analogous set of experiments using the class B V_2 R (Fig. 6). Unlike class A receptors, V_2 Rs form long-lived GPCR-arrestin complexes that are retained in endosomes after receptor internalization. Because active ERK1/2 has high affinity for β Arr1/2 only when the arrestin is in its “active,” GPCR-bound conformation (22), the capacity for β Arr1/2 to remain bound to class B GPCRs enables them to regulate both the duration and spatial compartmentalization of ERK1/2 activation (19,25,27). Thus, ERK1/2 activation by the V_2 R is strongly dependent upon β Arr1/2 scaffolding (62–66). We used siRNA to knockdown β Arr1/2 in the HEK293 parental cell lines co-transfected with plasmid encoding

the human V₂R (Fig. 6, A to C). Consistent with previous reports, knockdown of β Arr1/2 caused a substantial reduction in V₂R-stimulated ERK1/2 activation in all three HEK293 parental lines, indicating that β Arr1/2 signaling in arrestin-replete cells cannot be compensated for by the loss of desensitization that occurs when their abundance is abruptly reduced by siRNA-mediated knockdown. We then compared V₂R-stimulated ERK1/2 responses in the CRISPR β Arr1/2 KO lines with those in which β Arr1 or β Arr2 was restored by transient transfection (Fig. 6, D to F). Perhaps reflecting the larger contribution of β Arr1/2 scaffolds to ERK1/2 activation by class B GPCRs, reintroducing either β Arr1 or β Arr2 into the β Arr1/2-null background was sufficient to rescue ERK1/2 activation, resulting in a substantial increase in V₂R-stimulated ERK1/2 activation in all three CRISPR lines.

We also examined the FSHR, another G_s-coupled GPCR for which β -arrestin-dependent ERK1/2 activation has been well characterized (67,68). The FSHR exhibits a β Arr1/2 binding profile that is distinct from that of either the β_1 AR or V₂R. Whereas it possesses a class B-like cluster of serine and threonine residues in its C terminus that is lacking in class A GPCRs and recruits both β -arrestin isoforms equivalently, the FSHR releases β Arr1/2 soon after its internalization and is efficiently recycled (69,70). We used siRNAs to target β Arr1/2 in the HEK293 parental cell lines co-transfected with plasmid encoding the human FSHR (Fig. 7, A to C). Similar to experiments with the β_1 AR and V₂R, siRNA-mediated knockdown of β Arr1/2 caused a statistically significant reduction in FSHR-stimulated ERK1/2 activation in all three HEK293 parental lines. For the complementary reconstitution experiments, we overexpressed β Arr1/2 in the CRISPR β Arr1/2 KO lines to a similar extent (Fig 7, D to F). Despite the fact that the FSHR has an atypical mode of arrestin binding, reintroducing β Arr1/2 into the β Arr1/2 KO background resulted in a marked enhancement in receptor-mediated ERK1/2 activation in all three CRISPR lines, mimicking the results obtained from experiments with the V₂R.

β Arr1/2 are required for β_1 AR- and β_2 AR-mediated ERK1/2 activation by the biased agonist carvedilol

The complex web of G protein- and β Arr1/2-dependent mechanisms regulating ERK1/2 activity downstream of different GPCRs lends itself to divergent outcomes when β Arr1/2 are deleted by CRISPR/Cas9 gene editing or knocked down using siRNAs, mostly due to an increase in G protein pathway activity that may or may not offset the loss of β -arrestin-mediated signaling. A more direct way to test the role of β -arrestins in GPCR-stimulated ERK1/2 activation is to use a biased agonist that lacks intrinsic efficacy for heterotrimeric G protein activation but retains the capacity to promote β Arr1/2 recruitment to the receptor. With such ligands, the confounding role of β Arr1/2 as negative regulators of G protein signaling is eliminated, because the G protein pathways remain inactive. Carvedilol is a clinically useful, non-subtype selective β -AR antagonist (71,72). In HEK293 cells, carvedilol behaves as an inverse agonist for β_2 AR stimulation of G_s, while retaining the capacity to promote receptor phosphorylation by GRKs and activate ERK1/2 in a β -arrestin-dependent manner (73). In the case of the β_1 AR, carvedilol behaves as a very low potency partial agonist for both G_s- and G_{i/o}-coupling, while promoting ERK1/2 activation through a β Arr1/2-dependent mechanism (34). As well as recruiting β -arrestins, carvedilol recruits G_i

to the β_1 AR, but not the β_2 AR. β -Arr-dependent ERK1/2 activation by carvedilol acting on the β_1 AR is also PTX-sensitive, indicating a requirement for some Gi protein-mediated co-stimulus (61). Prolonged exposure (24 hours) to high concentrations ($>10 \mu\text{M}$) of carvedilol also has been reported to directly inhibit ERK1/2 activation in vascular smooth muscle cells (74).

We performed the complementary loss-of-function and gain-of-function experiments using carvedilol to preferentially activate β Arr-dependent signaling pathways in cells coexpressing either β_2 AR or β_1 AR (Fig. 8). We used siRNAs to knock down β Arr1/2 in HEK293 parental cells and monitored the effects of knockdown on the ERK1/2 response to carvedilol in cells expressing the β_2 AR (Fig. 8A). Knockdown of β Arr1/2 caused the nearly complete loss of carvedilol-stimulated ERK1/2 activation in the parental cells. We then tested the β_2 AR response to carvedilol in the corresponding CRISPR β Arr1/2 KO line with and without β Arr1/2 reconstitution (Fig. 8B). Carvedilol had no statistically significant effect on ERK1/2 activity in the CRISPR β Arr1/2 KO background; however, reintroducing β Arr1/2 restored the response. We performed analogous experiments with the β_1 AR (Fig. 8, C and D). Similar to the experiments with β_2 AR, carvedilol provoked a modest increase in ERK1/2 activation that was eliminated by β Arr1/2 knockdown (Fig. 8C). Conversely, carvedilol had no effect on ERK1/2 activity in the CRISPR β Arr1/2 KO background, but stimulated a response in cells reconstituted with β Arr1/2 (Fig. 8D). Together, these data support the conclusion that the responses of ERK1/2 to carvedilol, which occurred in the absence of measurable G_s signaling, were dependent upon the presence of β Arr1/2.

DISCUSSION

Our results underscore the multiple roles played by β Arr1/2 in the regulation of ERK1/2, a nodal kinase with essential roles in cell cycle progression and survival that is controlled by convergent signals downstream of multiple GPCRs and growth factor receptors (1–10). In the physiologic setting (Fig. 9), β Arr1/2 play both positive and negative regulatory roles. By promoting the desensitization of activated GPCRs, they maintain a brake on the G protein-mediated signals that activate ERK1/2, while at the same time serving as ligand-regulated scaffolds for the cRaf1-MEK1/2-ERK1/2 cascade (Fig. 9A). Deletion of β Arr1/2, by definition, removes both functions. As a result, G protein signaling is amplified while β Arr1/2 signaling is eliminated (Fig. 9B). The net effect on GPCR-stimulated ERK1/2 activation may be either an increase or a decrease depending on whether the enhanced signal strength in the unrestrained G protein pathway exceeds the contribution of β -arrestin-mediated signaling that exists in the wild-type environment. Our data, obtained using three independently derived CRISPR/parental HEK293 cell pairs and with four different G_s -coupled GPCRs, illustrate this point. Deletion of β Arr1/2 may augment ERK1/2 activation, diminish it, or produce no net effect, as the gain in signal strength in one pathway attempts to offset the loss of another. A more complete understanding of the role of β Arr1/2 scaffolds in GPCR control of ERK1/2 requires one to dissociate its two core functions, not to eliminate both. One approach that has been informative is the use of so-called β -arrestin-biased agonists, ligands that have little effect on, or even inhibit, receptor-G protein coupling, but that act as agonists or partial agonists for β Arr1/2 recruitment (Fig. 9C). Using these probes mitigates the confounding effects of amplified G protein signaling, enabling the

effects of β Arr1/2 engagement to be observed in isolation. Other tools that either have been, or may potentially be, useful in the future, are GPCR mutants deficient in G protein coupling or β -arrestin mutants that retain the ability to activate signaling but lose the ability to desensitize G protein activation or vice versa (51,54,75,76). Pharmacological inhibitors that selectively disrupt only some β -arrestin actions can also be useful tools. As an example, Barbadin, an inhibitor of the interaction between β -arrestins and the adaptor protein AP2 that blocks endocytosis, but not the recruitment of β -arrestin to many GPCRs, inhibits ERK1/2 activation by the V_2 R (77).

In general, β -arrestins exhibit three properties that enable them to function as central regulators of GPCR signaling (14). First, like their distant cousins the “arrestin-fold proteins” (78), they have the conformational flexibility to bind to multiple cargos, including, in the case of β Arr1/2, several catalytically active proteins, such as ERK1/2, c-Src, and Mdm2 (47,48,79). Second, they exist in equilibrium between different intracellular pools, for example, the cytosol, nucleus, microtubules, and the plasma membrane, wherein they adopt different conformations and perform different functions (23,80–82). Finally, they possess the unique capacity to recognize the active conformations of hundreds of different GPCRs. Thus, β -arrestins can constrain signaling cargos to one subcellular location, compartmentalizing or dampening pathway activity (83), until an external GPCR-mediated stimulus prompts them to undergo a conformational change that releases some cargos and engages others. Within such a complex network of interactions, differences in the relative abundance of GPCRs and their signaling effectors, as might exist between tissues or in disease states, are likely to cause substantial shifts in signal strength between G protein– and β -arrestin–mediated pathways. Our data from CRISPR β Arr1/2 KO cells highlight this. Despite being derived from common HEK293 cell progenitors, independent CRISPR KO clones react differently to the deletion of β Arr1/2. In some, for example, AI-CRISPR β Arr1/2 KO cells, deletion of β Arr1/2 led to an unambiguous amplification of β_2 AR-dependent stimulation of the ERK1/2 pathway compared to the parental clone, because G protein signaling was unhindered by β Arr1/2-dependent desensitization (Fig. 1A) (42). Such findings suggest that the selective pressure applied by CRISPR/Cas9-mediated knockout of β Arr1/2 can favor the isolation of clones that compensate for the loss of β -arrestin dependent signaling by rewiring to a predominately G_s -cAMP-PKA pathway. But this degree of change is not apparent in other clones, for example, SL-CRISPR β Arr1/2 KO and HAR-CRISPR β Arr1/2 KO cells, likely due to subtle background differences between them (Fig. 1, B and C). With GPCRs that are more tightly β Arr1/2-coupled, for example, the V_2 R, deletion of β Arr1/2 produced a consistent reduction in ERK1/2 signaling that was rescued by restoring β -arrestin expression (Fig. 6). Curiously, O’Hayre *et al.* (42) did not observe a reduction in V_2 R-stimulated ERK1/2 in the same AI-CRISPR β Arr1/2 KO cells that we used in our study, a discrepancy that we currently do not understand. Nonetheless, a range of experimental approaches, including CRISPR β Arr1/2 KO cells (this study), siRNA-mediated knockdown, pharmacological inhibition of the β -arrestin–AP2 interaction, and dominant-negative β -arrestin mutants (62–66,77), all converge to indicate that β -arrestins play an important role in the early and late phases of ERK1/2 activation by the V_2 R.

It should also be stressed that the phrases “arrestin-dependent” and “G protein–independent” are not synonymous. An arrestin-dependent signal is one that is enhanced or

conferred by the presence of arrestins and diminished or abolished by their deletion. In considering arrestin-dependent regulation of the ERK1/2 cascade, it is important to recognize that the upstream element, c-Raf1, is not a constitutively active kinase. β Arr1/2 scaffolding of the c-Jun N-terminal kinase 3 (JNK3) cascade, where simple co-expression of β Arr1/2 together with the component kinases ASK1, MKK4 or MKK7, and JNK3 leads to a marked activation of JNK3, is not only G protein-independent, but also GPCR-independent (21,84–86). In contrast, the affinity of cRaf1 and ERK1/2 for arrestins is sensitive to β Arr1/2 conformation such that only GPCR-bound β Arr1/2 supports the efficient assembly of the ERK1/2 activation complex (22). As a result, in the absence of GPCR activation, β Arr1/2 act as “silent scaffolds,” sequestering MEK1/2 in a microtubulebound pool and dampening basal ERK1/2 pathway activity until the ligand-induced formation of high-affinity GPCR docking sites on the plasma membrane recruits β Arr1/2, promotes complex assembly, and brings cRaf1 into the proximity of an activating stimulus. In cells in which basal Ras signaling is high, simple β Arr1/2-dependent assembly of the cRaf1-MEK1/2-ERK1/2 complex on a membrane surface may be sufficient to exceed the pathway activation threshold. Indeed, GPCR-independent recruitment of cyclophilin FRB domain-tagged β Arr2 to a myristoylated-cyclophilin FKBP domain expressed on the plasma membrane is sufficient to activate ERK1/2, as is expression of a constitutively internalized neurokinin NK1 receptor- β Arr1 chimera (28,87).

On the other hand, formal demonstration that an “arrestin-dependent” signal is “G protein-independent” would require either in vitro reconstitution of the complex with pure proteins or examination of GPCR signaling in cells devoid of heterotrimeric G proteins. In more native systems, it is inevitable that β -arrestins and G proteins would act cooperatively to regulate ERK1/2, because activation of G protein effectors can provide a cRaf1-activating stimulus and β -arrestins can recruit and orient the ERK1/2 pathway components on the plasma membrane in proximity to that stimulus. For example, it is known that the β -arrestin-dependent activation of ERK1/2 by many $G_{i/o}$ coupled receptors is also PTX-sensitive (61,88). Similarly, whereas the G protein-coupling efficacy of β -arrestin-biased agonists is disproportionally reduced relative to their ability to stimulate β -arrestin recruitment, most are not devoid of the ability to activate G proteins. For example, Sar¹-Ile⁴-Ile⁸ acting on the AT1AR (89) and carvedilol acting on the β_1 AR (61) behave as weak partial agonists for G protein signaling. Thus, it is unsurprising that these ligands can activate ERK1/2 in a β Arr1/2 KO background. β -Arr-biased agonists that exhibit true reversal of efficacy, that is, that act as inverse agonists for G protein coupling while still promoting β -arrestin recruitment, for example [D-Trp¹², Tyr³⁴]-bovine PTH (7–34) acting on the human type 1 parathyroid hormone receptor (PTH1R) (90) and carvedilol acting on the β_2 AR (61,73), are rare. But their capacity to cause β -arrestin-dependent ERK1/2 activation while suppressing basal G protein signaling is difficult to reconcile with the proposed universal requirement for heterotrimeric G proteins in the action of β -arrestin-biased agonists (41). Observations from CRISPR/Cas9 genome-edited cell lines virtually devoid of heterotrimeric G protein activity reinforce the notion that G proteins and β -arrestins often act cooperatively, while at the same time demonstrating that ERK1/2 activation by many GPCRs is markedly diminished by CRISPR/Cas9-mediated deletion of β Arr1/2 (41). Although our data are not inconsistent with previous studies using CRISPR/Cas9-edited

HEK293 cells (41,42), the focus of those studies on the role of heterotrimeric G proteins fails to recognize that β -arrestin scaffolds act not only as signal amplifiers, but also redirect ERK1/2 into functionally discrete pools, targeting ERK1/2 to nonnuclear substrates involved in the regulation of GPCR trafficking, protein translation, and cytoskeletal rearrangement (29–39). Assaying total cellular ERK1/2 activity captures only the effects on signal amplitude and duration and can miss β -arrestin functions related to the compartmentalization of functionally distinct ERK1/2 pools (38,39). Developing a more complete understanding of the roles of β -arrestins would at minimum require the use of reporters that discriminate between the activity of nuclear and cytosolic ERK1/2.

Our data highlight another point, that β Arr1/2 do not perform the same functions downstream of all GPCRs, even in a common cellular background. Consistent with previous reports (58,62), we found that β Arr2 played a larger role than did β Arr1 in supporting β_2 AR internalization and ERK1/2 activation, whereas both isoforms efficiently support β_2 AR desensitization (Figs. 2 and 4). Similar evidence of β Arr1/2 isoform specialization has been published for the Gq/11-coupled AT1AR, where again β Arr2 appears to play the major signaling role, and for the PAR2 receptor, for which β Arr1 is the dominant isoform (25,31,38,91). In contrast, our data, together with data from published studies, indicate that for other receptors, for example, the V₂R, either β Arr1 or β Arr2 is sufficient to support ERK1/2 activation (Fig. 6) (62–66), whereas for still others, for example, the FSHR and PTH1R, both β Arr1 and β Arr2 are required (Fig. 7) (27,67,68). β -Arrestins also exert GPCR-specific effects on the duration of ERK1/2 activation. In the case of the predominantly G_s-coupled β_2 AR, β_1 AR, V₂R, and FSHR studied here, depletion of β -arrestins affects the early (2 to 15 min) phase of ERK1/2 activation (33,54,66,68), whereas for receptors with slow rates of ligand dissociation, such as the G_q/11-coupled angiotensin receptor AT_{1A}R, the G_s/G_q/11-coupled PTH₁R, and the G_i/G_q-coupled PAR2 receptor, the effects of β -arrestin depletion are most evident at later times (26,27,31).

What might be the physical basis for these apparently contradictory results? Certainly, the intracellular domain architecture of different activated GPCRs affects their avidity for β Arr1 and β Arr2, just as it determines G protein coupling selectivity (58). Biophysical data obtained using intramolecular FRET or BRET probes suggest even greater nuance, in that different GPCRs appear to induce distinct “active” β -arrestin conformations, enabling them to determine not only which β -arrestin isoforms are activated, but also what functions they perform (92,93). Comparison of the population average “conformational signatures” of β Arr2 induced by different GPCRs in a common cellular background demonstrates that receptors that use β -arrestins to activate ERK1/2 cause characteristic shifts in β Arr2 conformation that are not seen with GPCRs that use β -arrestins in a predominantly desensitizing role. Beyond the influences of cell background and GPCR structure, it is likely that the functions of β Arr1/2 are determined by multiple factors, including ligand structure (94,95), GPCR C-tail phosphorylation “bar codes” produced by different GRKs (96–98), and posttranslational modifications that stabilize or destabilize the receptor-arrestin complex (99), which collectively influence β Arr1/2 conformation.

Based on work performed using CRISPR/Cas9 and TALEN knockout approaches to demonstrate that β Arr1/2 are dispensable for the β_2 AR-dependent stimulation of ERK1/2

and that β -arrestin-biased agonists are capable of activating ERK1/2 in the absence of β -arrestins, it has been suggested that genome-edited cells are superior to alternatives, such as cells subjected to short-term, siRNA-mediated knockdown, for interrogating the mechanisms underlying biased drug responses (41,42). Although there are clear advantages to genome-edited cell lines for many applications, CRISPR cell clones are selected based on their capacity to exist in the absence of the deleted protein(s). Hence, whereas such cells are outstanding tools for studying how cells can survive without a missing protein, they are less useful for understanding how cells survive with it or how biased ligands affect cells with intact signaling networks. For example, although germline deletion of either β -arrestin isoform individually is tolerated, simultaneous deletion of β Arr1/2 results in embryonic or very early postnatal lethality, which is characterized by severe developmental defects (100). However, it is possible to derive immortalized MEF clones from *ARRB1/2* knockout embryos (50) and to delete *ARRB1/2* using CRISPR/Cas9-dependent gene-editing approaches (101). Thus, although they are apparently indispensable for mammalian development and organismal survival, β Arr1/2 are unquestionably dispensable for the survival of cultured cells in vitro, as are apparently the entire set of heterotrimeric G α proteins (100). Clearly β Arr1/2 play some vital role(s) in development, but the nature of those roles, or how biased drugs may affect them, cannot be readily elucidated using cells that have adapted to survive without them.

Despite the remarkable contrast in our study between the consistent results we obtained from multiple HEK293 cell clones using siRNA-mediated knockdown of β Arr1/2 compared to the variability observed between independently-derived CRISPR β Arr1/2 clones, it is important to emphasize that no engineered cell line is a perfect model system. Both CRISPR/Cas9 and siRNA approaches are susceptible to off-target effects that can complicate the interpretation of results (102,103). Complementary gain-of-function and loss-of-function approaches and the use of multiple different siRNA sequences or independent CRISPR/Cas9 clones, as done here, may provide confidence that the observed effects are the consequence of the intended manipulation. Most studies to date of the involvement of β -arrestins in GPCR regulation of ERK1/2 using siRNAs have not included such rescue approaches, just as most studies using CRISPR β Arr1/2 KO have been performed using CRISPR/Cas9 clones derived from a single parental background (41,42). Whereas an abrupt reduction in the abundance of target proteins using siRNAs may reduce the likelihood that compensatory rewiring of intracellular signaling networks will obscure the function of the missing protein, siRNA approaches are limited in that knockdown is invariably incomplete. In this case, functions that do not require large amounts of the targeted protein may be unaffected and important roles may be missed. The β -arrestins, most of whose functions involve stoichiometric interaction with binding partners, may be somewhat less susceptible to this phenomenon than are proteins with catalytic activity, for example, kinases, but the potential for misleading results from experiments with siRNA-mediated knockdown must be highlighted.

Given the many caveats our data raise with respect to the effects of signal rewiring in CRISPR/Cas9 genome-edited cells, it is difficult to justify the claim that they are superior platforms for biased drug screening (41,49). Studies of ERK1/2 activation in β -arrestin-replete cells using inverse agonists for the β_2 AR, V2R, and PTH1R (27,64,73), are not easily reconciled with CRISPR/Cas9 cell data showing a universal requirement for

heterotrimeric G protein signaling. Whether the requirement for G protein signaling observed in CRISPR “G zero” cells (41) indicates that some background level of constitutive G protein activity or some threshold level of GPCR-dependent G protein activation is required for β -arrestin scaffolds to activate ERK1/2 is unclear. That arrestin-biased agonists can activate ERK1/2 in β Arr1/2 KO CRISPR cells or fail to do so in “G-zero” CRISPR cells may have as much to do with the nature of the genome-edited cells as with the actions of ligands in native backgrounds. GPCR overexpression and β Arr1/2 deletion both favor promiscuous coupling to nonphysiologic effectors (104,105). Deletion of $G\alpha$ subunits may lead to unknown effects on $G\beta\gamma$ subunit abundance, function, or both, and potentially alter the abundance or function of the $G\beta\gamma$ -dependent GRKs (GRK2/3) that promote desensitization versus the $G\beta\gamma$ -independent GRKs (GRK 5/6) that favor β -arrestin signaling (65,96). Ultimately, whether or not background G protein activity or a low level of G protein activation is required for β -arrestin to scaffold the ERK1/2 module is largely immaterial with respect to the basic paradigms of biased signaling. Hence, strategies such as acute knockdown of proteins by siRNA, inducible shRNA, or CRISPR/Cas9 transient epigenetic modification technology, wherein cells need not survive long term in the absence of the targeted protein, may be more reliable and interpretable. This is particularly relevant to drug development where disease states are more likely due to altered physiology from a modest reduction in the function of a protein rather than its long-term absence. At the end of the day, the choice of model system must be based on the experimental questions being asked, and no data should be over-interpreted or over-generalized. Engineered HEK293 cells offer many advantages for signal transduction research and as drug discovery platforms, but overexpressed receptors and genetic manipulations inevitably raise concerns about the physiologic relevance of the processes being studied. These systems are invaluable tools for hypothesis generation, but studies conducted in primary cells, organoids, or intact animals are ultimately necessary to fully understand the nuances of GPCR signaling.

MATERIALS AND METHODS

Antibodies and reagents

Arginine vasopressin (AVP), (–)-isoproterenol (+)-bitartrate, carvedilol, G418, poly-L-lysine, and *o*-phenylenediamine dihydrochloride were from Sigma-Aldrich (St Louis, MO). [¹²⁵I](–)-iodocyanopindolol was from Perkin Elmer Life Sciences (Waltham, MA). The PKA inhibitor 6–22 was from Tocris Bioscience (Minneapolis, MN). Vybrant DyeCycle orange stain and salmon sperm DNA were from Invitrogen (Carlsbad, CA). Coelenterazine 400a was from NanoLight Technology (Pinetop, AZ). recombinant human FSH was a gift from F. Gonla of Merck-Serono (Darmstadt, Germany). Lipofectamine 2000 was from Thermo Fisher (Waltham, MA); FuGene6 was from Promega Life Sciences (Madison, WI); Metafectene was from Biontex Laboratories (München, Germany); polyethylenimine linear 25K was from Polysciences (Warrington, PA); and GeneSilencer was from Gelantis (San Diego, CA). Mouse monoclonal anti-phospho-p44/42 ERK1/2 (cat no 9106) and rabbit polyclonal anti-phospho-p44/42 ERK1/2 (cat no 9101) were from Cell Signaling Technology (Danvers, MA and Leiden, The Netherlands). Rabbit polyclonal anti-ERK1/2 (cat no 9102) was from Cell Signaling Technology (Danvers, MA) or EMD Millipore (cat no ABS44; Burlington, MA). Rabbit polyclonal anti- β Arr1/2 antibody (A1CT), which

recognizes both β -arrestin1 and β -arrestin2 isoforms, was generated in the laboratory of Dr. Robert J. Lefkowitz (Duke University, Durham, NC) (106). Anti- β -actin mouse monoclonal antibody (clone AC-15) was from Sigma Aldrich (St Louis, MO). Anti-GAPDH mouse monoclonal antibody (clone 6C5) was from Santa Cruz Biotechnology (Dallas, TX). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were from GE/Amersham Biosciences (Chicago, IL), Rockland Immunochemicals (Pottstown, PA), Bio-Rad (Hercules, CA), or Jackson ImmunoResearch Europe (Cambridgeshire, UK). IRDye Alexa Fluor 680 and 800 secondary antibodies were from Li-Cor Biosystems (Lincoln, NE). FITC-conjugated anti-FLAG antibody was from Sigma-Aldrich (St Louis, MO), terbium-conjugated anti-FLAG antibody was from Cisbio (Codolet, France), and HRP-conjugated anti-HA IgG was from Roche Diagnostics (Indianapolis, IN).

Derivation of CRISPR/Cas9 HEK293 cell lines

The AI-CRISPR β Arr1/2 KO and corresponding AI-parental HEK293A cell lines were a gift from Asuka Inoue (Tohoku University, Sendai, Miyagi, Japan) and derived as previously described in detail (42). This is the CRISPR β Arr1/2 KO-parental HEK293 pair used by O'Hayre *et al.* and Grundmann *et al.* (42). The SL-CRISPR β Arr1/2 KO line was generated in the Laporte laboratory (McGill University, Montreal, Quebec, Canada) by successive deletion of β Arr1 and β Arr2 from a subclone of HEK293 cells (56) using the CRISPR/Cas9 method. *ARRB1* deletion was performed first using the vector PspCas9(BB)-2A-Puro (Px459v2) (Addgene plasmid #62988; deposited by F. Zhang) (107) and the two gRNA guide sequences 5'-CACCGTGTGGACCACATCGACCTCG-3' and 5'-CACCGCAACGTACAGTCGTTCCAC-3' for the human *ARRB1* gene exons 3 and 5. Each gRNA was cloned into the Px459v2 vector and cells were transfected with both constructs using Lipofectamine 2000 according to the manufacturer's instructions. After 24 hours, medium supplemented with 2 μ g/ml puromycin was added for 48 hours and the cells were serially diluted and plated in 96-well plates to generate individual colonies. Deletion of *ARRB1* was verified by Western blotting for β Arr1 and PCR genotyping using primers flanking both deleted and undeleted regions, and a positive clone was selected. One *ARRB1*-deleted cell line (clone 13) was subsequently used for *ARRB2* deletion using the same method with the two gRNA guide sequences 5'-CACCGAAGTCGAGCCCTAACTGCA-3' and 5'-CACCGCCTGTTCATCGCCACCTACC-3' for the human *ARRB2* gene exons 2 and 5. Selection was performed as described for the *ARRB1* deletion, and a positive *ARRB1/2* deleted clone was selected (clone 19). This clone is referred to as the SL-CRISPR β Arr1/2 KO line used herein. The HAR-CRISPR β Arr1/2 KO line was generated in the Rockman laboratory (Duke University, Durham, NC) as described previously (61), using the pSpCas9(BB)-2A-Puro (Px459) vector (Addgene plasmid #48139). *ARRB1* was targeted using the gRNA guide sequences 5'-CACCGCATCG ACCTCGTGGACCCTG-3' and 5'-AACCAGGGTCCACGAGGTCGATGC-3'. *ARRB2* was targeted using the gRNA guide sequences 5'-CACCGCGTAGATCACCTGGACAAAG-3' and 5'-AAACCTTTGTCCAGGTGATCTACGC-3'. The guide sequence oligos were cloned into pSpCas9(BB)-2A-Puro and plasmids and HEK293 cells were transfected with them using Eugene6 according to the manufacturer's instructions. Seventy-two hours after transfection, the cells were harvested to check genome insertion/deletion using the Surveyor Nuclease assay. Puromycin (2.5 μ g/ml) was added to the medium of Surveyor-positive cells to select

clones containing the puromycin resistance plasmid along with gRNA and Cas9. Knockout of both β -arrestins was confirmed by Western blotting. One clone referred to as the HAR-CRISPR β Arr1/2 KO line was used herein.

Knockdown of β Arr1/2 in parental HEK293 cells using siRNA

AI-parental and HAR-parental HEK293 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin or 20 μ g/mL gentamicin. SL-parental cells were maintained in Dulbecco's modified Eagle's medium (DMEM) without pyruvate supplemented with 10% FBS and 1% penicillin/streptomycin or 20 μ g/mL gentamicin. ERK1/2 activation assays in response to β_2 AR stimulation were performed in the Shenoy and Rockman laboratories (Duke University, Durham, NC). For experiments comparing the effects of receptor density on β_2 AR-stimulated ERK1/2 activation, stable lines of AI-parental HEK293 cells and AI-CRISPR β Arr1/2 KO cells were generated by transfecting cells with plasmid DNA encoding FLAG epitope-tagged β_2 AR (pcDNA3.1-FLAG- β_2 AR) and selecting positive clones against 1 μ g/mL G418. Stable selection was maintained by the inclusion of 400 ng/mL G418 in the growth medium. Knockdown of β -arrestins in parental HEK293 cells was performed using chemically synthesized, double-stranded siRNAs purchased from GE/Dharmacon (Lafayette, CO). The siRNA sequences used were as follows: human β Arr2: 5'-CTAAATCACTAGAAGAGAA-3' (53,102); human β Arr1/2: 5'-ACCTGCGCCTTCCGCTATG-3' (27,108); and the non-targeting control siRNA sequence was: 5'-AATTCTCCGAACGTGTACGT-3'. For siRNA experiments, parental HEK-293 cells in 10-cm dishes at 40 to 50% confluence were transfected with 20 μ g siRNA using the GeneSilencer transfection reagent according to the manufacturer's protocols. Forty-eight hours after transfection, cells were split into 6-well dishes for ERK1/2 activation assays. Before stimulation, the cells were incubated for 4 hours in serum-free medium containing 10 mM HEPES (pH 7.5) and 0.1% bovine serum albumin (BSA). Western blotting analysis of whole-cell lysates with the A1CT anti- β Arr1/2 antibody was performed to assess the extent of β Arr2 or β Arr1/2 knockdown. Assays assessing β_1 AR responses were performed in the Rockman laboratory (Duke University, Durham, NC). Before experimentation, the cells were transiently co-transfected with plasmid DNA encoding FLAG epitope-tagged murine β_1 AR (pcDNA3.1-FLAG- β_1 AR) and either control siRNA or siRNA targeting β Arr1/2 (27,52,61). β Arr1/2 were targeted by combining three siRNAs: 5'-AAA CCTGCGCCTTCCGCTATG-3', 5'-AAAGCCTTCTGCGCGGAGAAT-3', and 5'-AAGGACCGC AAAGTGTGTTGTG-3' in a 2:1:1 ratio, respectively. The nonsilencing RNA duplex 5'-AATTCTCCGAACGTGTACGT-3' was used as a control. HEK293 cells were seeded into 10-cm dishes and allowed to reach 30 to 40% confluence at the time of transfection. Co-transfection was performed using the GeneSilencer Transfection Reagent according to the manufacturer's protocols, with 4 μ g of pcDNA3.1-FLAG- β_1 AR plasmid and 20 μ g of pooled siRNA per dish. Twenty-four hours after transfection, the cells were split into 6-well dishes. Stimulations were performed 72 hours after transfection following overnight incubation in serum-free medium supplemented with 10 mM HEPES (pH 7.5), 0.1% BSA, and 1% penicillin/streptomycin. Cell surface β_1 AR abundance was assessed by FITC-conjugated anti-FLAG antibody binding and quantified with a BD LSRII flow cytometer (BD Biosciences; San Jose, CA). Western blotting analysis of whole-cell lysates

with the A1CT anti- β Arr1/2 antibody was performed to assess the extent of β Arr2 or β Arr1/2 knockdown. Assays assessing the V₂R response were performed in the Laporte laboratory (McGill University, Montreal Quebec, Canada). Before experimentation, the cells were transiently co-transfected with plasmid DNA encoding HA epitope-tagged human V₂R (pcDNA3.1-HA-V₂R) (104) and either control siRNA or siRNA targeting β Arr1/2. The siRNA sequences used were as follows: human β Arr1/2: 5'-ACCTGCGCCTTCCGCTATG-3'; non-targeting control siRNA: 5'-TAAGGCTATGAAGA GATAC-3'. HEK293 cells were seeded in poly-L-lysine-coated 12-well plates at a density of 0.7×10^5 cells/well. Forty-eight hours later, the cells were co-transfected with 250 ng of HA-V₂R plasmid and 100 nM of either control or β Arr1/2 targeted siRNA using the Lipofectamine 2000 transfection reagent according to the manufacturer's protocols and allowed to grow for an additional 48 hours. Before stimulation, the cells were incubated for 30 min in serum-free medium containing 20 mM HEPES (pH 7.5). Western blotting analysis of whole-cell lysates with the A1CT anti- β Arr1/2 antibody was used to assess the extent of β Arr2 or β Arr1/2 knockdown. Assays to assess FSHR responses were performed in the Reiter laboratory (INRA, CNRS, Université du Tours, Nouzilly, France). Before experimentation, the cells were transiently co-transfected with plasmid DNA encoding FLAG epitope-tagged human FSHR (pcDNA3-FLAG-FSHR) (107) and either control siRNA or siRNA targeting β Arr1/2. The siRNA sequences used were as follows: human β Arr1/2: 5'-AAACCTGCGCCTTCCGCTATG-3'; non-targeting control siRNA: 5'-TTCTCCGAACGTGTACAGT-3' (109). For siRNA experiments, parental HEK-293 cells in 10-cm dishes at 30% confluence were transfected with 2 μ g of pRK5-FLAG-FSHR and 20 μ g of either control or β Arr1/2-targeted siRNA using the GeneSilencer transfection reagent according to the manufacturer's protocols. Twenty-four hours after transfection, the cells were split into 12-well dishes. Assays were performed 72 hours after transfection following 4 hours of incubation in serum-free medium containing 10 mM HEPES (pH 7.5) and 0.1% BSA. Plasma membrane FSHR abundance was assessed on live cells using Terbium-conjugated anti-FLAG antibody measured with a TriStar² S LB942 microplate reader (Berthold Technologies; Bad Wildbad, Germany). Western blotting analysis of whole-cell lysates with the A1CT anti- β Arr1/2 antibody was performed to assess the extent of β Arr2 or β Arr1/2 knockdown.

Reconstitution of β Arr1/2 in CRISPR/Cas9 HEK293 cells by transient transfection

AI-CRISPR and HAR-CRISPR β Arr1/2 KO cells were maintained in MEM supplemented with 10% FBS and 1% penicillin/streptomycin or 20 μ g/mL gentamicin. SL-CRISPR β Arr1/2 KO cells were maintained in DMEM without pyruvate supplemented with 10% FBS and 1% penicillin/streptomycin or 20 μ g/mL gentamicin. For ERK1/2 activation assays of cells transiently expressing β_2 AR, CRISPR β Arr1/2 KO cells in 10-cm dishes at 40 to 50% confluence were transiently co-transfected with 2 μ g of pcDNA3.1-FLAG- β_2 AR and 1 μ g of plasmid encoding HA epitope-tagged β Arr2 (pcDNA3.1-HA- β Arr2) or 1 μ g each of pcDNA3-HA- β Arr2 and a plasmid encoding HA epitope-tagged β Arr1 (pcDNA3.1-HA- β Arr1) using Lipofectamine 2000 according to the manufacturer's protocol. Equal amounts of empty vector were used for mock-transfected controls. Twenty-four hours after transfection, cells were plated onto 6-well dishes. Stimulations were performed 16 to 24 hours later following 4 hours of serum deprivation. The cell surface abundance of β_2 AR was

determined by [125 I](–)-iodocyanopindolol binding. Western blotting analysis of whole-cell lysates with the A1CT anti- β Arr1/2 antibody was used to assess the abundance of β Arr2 or β Arr1/2. For assays of cells transiently expressing β_1 AR, CRISPR β Arr1/2 KO cells in 10-cm dishes were transiently co-transfected with 4 μ g of pcDNA3-FLAG- β_1 AR and 50 ng of pcDNA3.1-HA- β Arr1 and 250 ng of pcDNA3.1-HA- β Arr2 using Fugene6 according to the manufacturer's protocol. Equal amounts of empty vector were used for mock-transfected controls. Twenty-four hours after transfection, cells were split into 6-well dishes and serum-deprived overnight before stimulation. β_1 AR cell surface abundance was assessed by FITC-conjugated anti-FLAG labeling and β Arr1/2 abundance was assessed by Western blotting analysis with the A1CT anti- β Arr1/2 antibody. For assays of cells transiently expressing V₂R, CRISPR β Arr1/2 KO cells were seeded in 10-cm dishes at an initial density of 7.5×10^5 cells/dish. Twenty-four hours later, the cells were transfected with 3 μ g of pcDNA3.1-HA-V₂R with or without 200 ng of pcDNA3.1-FLAG- β Arr1 or pcDNA3.1-FLAG- β Arr2 (77) using the calcium phosphate DNA precipitation method as previously described (110). Equal amounts of empty vector were used for mock-transfected controls. The next day, cells were detached with phosphate buffered saline (PBS), 5 mM EDTA and plated onto poly-L-lysine-coated 12-well plates. Stimulations were performed 24 hours later, following 30 min of serum deprivation. Western blotting analysis of whole-cell lysates with the A1CT anti- β Arr1/2 antibody was used to assess the abundance of β Arr2 or β Arr1/2. For assays of cells transiently expressing FSHR, CRISPR β Arr1/2 KO cells in 10-cm dishes at 30% confluence were co-transfected with 2 μ g of pcDNA3-FLAG-FSHR and 200 ng each of pcDNA3.1-HA- β Arr1 and pcDNA3.1-HA- β Arr2 or 400 ng of empty vector using Metafectene transfection reagent according to the manufacturer's protocol. Twenty-four hours after transfection, cells were split into 12-well dishes. After a further 24 hours, cells were serum-starved for 4 to 6 hours before stimulation. Plasma membrane FSHR abundance was assessed using Terbium-conjugated anti-FLAG antibody, whereas β Arr1/2 abundance was assessed by Western blotting analysis of whole-cell lysates with the A1CT anti- β Arr1/2 antibody.

Assessment of ERK1/2 activation by Western blotting analysis

Appropriately transfected and serum-deprived parental and CRISPR β Arr1/2 KO cells were stimulated at 37°C with the ligand concentrations and for the times described in the figure legends. After stimulation, the cells were lysed at 4°C in 1% NP-40 lysis buffer [20 mM Tris-HCL (pH 7.4), 137 mM NaCl, 20% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, 10 mM sodium fluoride, 10 μ g/ml aprotinin, 5 μ g/ml leupeptin and phosphatase inhibitors], protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), and equal amounts of cell lysate were mixed with 2X Laemmli sample buffer. Alternatively, stimulation was stopped by directly adding 2X Laemmli buffer [250 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), 0.01% bromophenol blue (w/v), 5% β -mercaptoethanol (v/v)] to solubilize the cells. Lysates containing equal amounts of total cell protein were resolved by SDS-PAGE on 10% or 4 to 20% gradient Tris-Glycine polyacrylamide gels (Invitrogen; Carlsbad, CA), transferred to nitrocellulose or polyvinylidene difluoride membranes, and incubated with rabbit polyclonal or mouse monoclonal anti-phospho-44/42 ERK1/2 and anti-total-ERK1/2, followed by HRP-conjugated anti-rabbit IgG or IRDye-conjugates as secondary antibodies. Protein bands were detected with Pierce SuperSignal West Pico

Enhanced Chemiluminescence Reagent (Thermo Fisher; Waltham, MA), and captured using a Chemidoc-XRS charge-coupled device camera system (Bio-Rad Laboratories; Hercules, CA), or either a G:Box (Syngene; Cambridge, UK) or a Li-Cor Odyssey CLX far infra-red scanner (Li-Cor Biosystems; Lincoln, NE). Bands were quantified by densitometry using ImageLab (Bio-Rad Laboratories, Hercules, CA) or Image J software.

BRET assay of G α s activation and β_2 AR internalization

BRET assays of β_2 AR-mediated G α s performed in the Bouvier laboratory activation and receptor internalization were (University of Montreal, Montreal, Quebec, Canada). Parental HEK293 and CRISPR β Arr1/2 KO cells were maintained in DMEM supplemented with 10% FBS, 100 Units/mL of penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine. Transient transfections were performed at a density of 0.3 to 0.7 $\times 10^6$ cells/mL using a 3:1 ratio of 25 kDa linear polyethylenimine per μ g DNA as described previously (111). A maximum of 1.2 μ g of plasmid DNA was used to transfect 1.2 mL of cells and the total amount of DNA was maintained constant with salmon sperm DNA. β_2 AR-mediated G α s activation was assessed by measuring changes in BRET efficiency between an *RlucII*-tagged G α s subunit and GFP10-tagged G γ 1 subunit upon stimulation (55). Cells were co-transfected with plasmids encoding HA epitope-tagged β_2 AR, untagged G β_1 , G α s tagged with *RlucII* at amino acid position 117 (*RlucII*-117-G α s), and G γ 1 tagged with GFP10 at its N-terminus (GFP10-G γ 1). Transfected cells (100 μ L/well) were seeded directly into 96-well white wall microplates. For reconstitution experiments, plasmid DNA encoding human β Arr1 or β Arr2 was included in the transfection. Assays were performed 48 hours after transfection. On the day of the assay, the culture medium was removed, cells were washed with phenol red-free Dulbecco's phosphate-buffered saline (DPBS), and the medium was replaced by Tyrode's buffer [137 mM NaCl, 0.9 mM KCl, 1 mM MgCl₂, 11.9 mM NaHCO₃, 3.6 mM NaH₂PO₄, 5.5 mM glucose, 1 mM CaCl₂, 25 mM Hepes (pH 7.4)]. After the addition of agonist or vehicle, cells were incubated at 37°C for 15 min. The luciferase substrate coelenterazine 400a was added to a final concentration of 2.5 μ M 5 min before reading BRET in a Synergy Neo microplate reader (BioTek; Winooski, VT) equipped with 515 $\pm$ 30 nm acceptor and 410 $\pm$ 80 nm donor filters. The BRET signal was determined as the ratio of the light emitted by the GFP-tagged biosensor (energy acceptor) and the light emitted by the *RlucII*-tagged biosensor (energy donor). The agonist-promoted BRET signal (BRET) is the difference in BRET recorded from cells treated with agonist and cells treated with vehicle. β_2 AR internalization was assessed by ebBRET, which monitors BRET between an *Rluc*-tagged receptor and rGFP anchored at the plasma membrane by a prenylated CAAX motif (56). To measure receptor internalization, cells were cotransfected with plasmid encoding β_2 AR tagged with *RlucII* at its C-terminus (β_2 AR-*RlucII*) and with a plasmid encoding the prenylated CAAX domain of K-Ras fused to *Renilla* GFP (rGFP-CAAX) in the absence or presence of human β -arr1 or β -arr2. Transfected cells (100 μ L/well) were seeded directly into 96-well white wall microplates and assayed 48 hours after transfection. On the day of the assay, the culture medium was removed, the cells were washed with DPBS, and the medium was replaced by Tyrode's buffer. After addition of agonist or vehicle, cells were incubated at 37°C for 15 min and the luciferase substrate coelenterazine 400a was added 5 min before reading BRET in a Synergy Neo microplate

reader. The BRET signal was determined as described for the Gαs activation assay and the results are expressed as BRET.

SureFire ERK1/2 activation assay

In the Bouvier laboratory, β_2 AR-stimulated ERK1/2 phosphorylation in live cells was assayed using the AlphaLISA SureFire Ultra kit (Perkin Elmer Life Sciences; Waltham, MA), a FRET assay based on a sandwich ELISA in which the bridging of donor and acceptor beads by the activated pThr²⁰²/pTyr²⁰⁴ ERK1/2 analyte produces an increase in fluorescence emission at 520 to 620 nm upon excitation at 680 nm (59). For this assay, cells were transfected with plasmid encoding HA- β_2 AR and the transfected cells were seeded (100 μ l/well) into 96-well white microplates pre-coated with poly-D-lysine hydrobromide. Twenty-four hours after transfection, the cells were serum-deprived for 16 hours with or without 0.1 μ g/ml PTX as appropriate. ERK1/2 activation was assayed 48 hours after transfection. The culture medium was first replaced by fresh serum-free medium with or without 100 nM 6–22 (PKA inhibitor) as appropriate and the cells were incubated at 37°C for 1 hour. After 2 min of stimulation with vehicle or 1 μ M isoproterenol, the cells were lysed at 4°C in the lysis buffer provided by the manufacturer and frozen overnight at –20°C. Thereafter, 10 μ l of thawed lysates were transferred to a 384-well white AlphaPlate and ERK1/2 activity was measured according to the manufacturer's protocol using an EnVision Multilabel plate reader (Perkin Elmer Life Sciences; Waltham, MA). For each experiment, stimulated ERK1/2 values were normalized to isoproterenol-stimulated controls.

Measurement of cell surface β_2 AR abundance by ELISA

For BRET and Surefire ERK1/2 phosphorylation assays, the equivalence of HA- β_2 AR expression in parental HEK293 and CRISPR β Arr1/2 KO cells was determined by ELISA. For this assay, transfected cells in poly-D-lysine-coated, 96-well plates were fixed for 10 min in DPBS containing 3.7% formaldehyde. Cells were then washed three times with wash buffer (0.5% BSA in DPBS) and incubated for 1 hour with a 1:2000 dilution of HRP-conjugated anti-HA IgG in wash buffer. After three additional washes, the number of cells was measured by incubation for 30 min with 5 μ M Vybrant DyeCycle orange stain in wash buffer. Cells were again washed three times with DPBS, after which fluorescence was read at 563 nm with 519 nm excitation using a FlexStationII microplate reader (Molecular Devices; San Jose, CA). HRP activity, reflecting receptor number, was detected by incubating cells with *o*-phenylenediamine dihydrochloride. The addition of 0.6 M HCl stopped the reaction and the absorbance was read at 492 nm using a Tecan Infinite M200 Pro multifunction microplate reader (Tecan Inc.; Zurich, Switzerland).

Statistical analysis

To foster unbiased data collection and compare reproducibility between laboratories using different approaches to transfection and stimulation, the three independently derived HEK293 parental/CRISPR β Arr1/2 KO pairs were shared between the participating laboratories, each of which focused on a single GPCR using the common model systems. Each laboratory performed experiments independently using the protocols, reagents, and tools routinely used by that laboratory to investigate GPCR signaling. All of the raw data were subsequently collated at Duke University Medical Center and analyzed using

consistent methods. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software; Carlsbad, CA). The data were analyzed by two-way analysis of variance (ANOVA) with Sidak's multiple comparison test. The mean differences of a minimum of three independent experiments were considered to be statistically significant when $P < 0.05$.

Acknowledgements:

We thank Q. Lennon and J. Bisson for secretarial help, and W. Zou for excellent technical assistance. The AI-CRISPR β Arr1/2 KO and corresponding AI-parental HEK293 cell lines used in this study were generously provided by A. Inoue (Tohoku University, Sendai, Miyagi, Japan).

Funding: This work was supported in part by US National Institutes of Health grants R01 HL16037 to R.J.L.; R01 DK055524 and R35 GM126955 to L.M.L.; R01 HL056687 and P01 HL075443 to H.A.R.; K08 HL114643 and R01 GM122798 to S.R.; R01 HL118369 to S.K.S.; Canadian Institutes of Health Foundation Grant 14843 to M.B.; and Canadian Institutes of Health Research Grant MOP-74603 to S.A.L. E.R. is funded by the French National Research Agency Investissements d'avenir program, Grant Agreement LabEx MabImprove: ANR-10-LABX-53, and the Région Centre Val de Loire ARD2020 Biomédicaments program. L.Y. holds a postdoctoral internship from Mitacs Accelerate Canada; B.P. holds a postdoctoral fellowship from Diabetes Canada; S.R. holds a Burroughs Welcome Career Award for medical scientists; M.B. holds the Canada Research Chair in Signal Transduction and Molecular Pharmacology; R.J.L. is an investigator with the Howard Hughes Medical Institute.

REFERENCES and NOTES

1. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH, Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev* 22, 153–183 (2001). [PubMed: 11294822]
2. Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ, cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap-1-dependent pathway. *Cell* 89, 73–82 (1997). [PubMed: 9094716]
3. DeRooi J, Zwartkruis FL, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL, Epac is a Rap1 guanine nucleotide exchange factor directly activated by cAMP. *Nature* 396, 474–477 (1998). [PubMed: 9853756]
4. Lefkowitz RJ, Pierce KL, Luttrell LM, Dancing with different partners: PKA phosphorylation of seven membrane-spanning receptors regulates their G protein coupling specificity. *Mol Pharm* 62, 971–974 (2002).
5. Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW, Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science* 262, 1065–1068 (1993).
6. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D, Rapp UR, Protein kinase C α activates Raf-1 by direct phosphorylation. *Nature* 364, 249–255 (1993). [PubMed: 8321321]
7. Hawes BE, van Biesen T, Koch WJ, Luttrell LM, Lefkowitz RJ, (1995) Distinct pathways of Gi- and Gq-mediated mitogen activated protein kinase activation. *J Biol Chem* 270, 17148–17153 (1995). [PubMed: 7615510]
8. van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM, Lefkowitz RJ, Receptor-tyrosine-kinase- and Gbetagamma-mediated MAP kinase activation by a common signalling pathway. *Nature* 376, 781–784 (1995). [PubMed: 7651538]
9. Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ, Role of c-Src in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen activated protein kinases. *J Biol Chem* 271, 19443–19450 (1996). [PubMed: 8702633]
10. Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ, Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. *J Biol Chem* 272, 4637–4644 (1997). [PubMed: 9020193]
11. Daaka Y, Luttrell LM, Lefkowitz RJ, Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature* 390, 88–91 (1997). [PubMed: 9363896]

12. Zamah AM, Delahunty M, Luttrell LM, Lefkowitz RJ, Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system. *J Biol Chem* 277, 31249–31256 (2002). [PubMed: 12063255]
13. Ferguson SS SS, Evolving concepts in G protein-coupled receptor endocytosis: The role in receptor desensitization and signaling. *Pharmacol Rev* 53, 1–24 (2001). [PubMed: 11171937]
14. Peterson YK, Luttrell LM, The Diverse Roles of Arrestin Scaffolds in G Protein-Coupled Receptor Signaling. *Pharmacol Rev* 69, 256–297 (2017). [PubMed: 28626043]
15. Goodman OB, Jr., Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL, Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* 383, 447–450 (1996). [PubMed: 8837779]
16. Krupnick JG, Goodman OB, Jr., Keen JH, Benovic JL, Arrestin/clathrin interaction: Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus. *J Biol Chem* 272, 15011–15016 (1997). [PubMed: 9169476]
17. Laporte SA, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, Barak LS, The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* 96, 3712–3717 (1999). [PubMed: 10097102]
18. Laporte SA, Oakley RH, Holt JA, Barak LS, Caron MG, The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits. *J Biol Chem* 275, 23120–23126 (2000). [PubMed: 10770944]
19. Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ, Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci USA* 98, 2449–2454 (2001). [PubMed: 11226259]
20. Meng D, Lynch MJ, Huston E, Beyermann M, Eichhorst J, Adams DR, Klusmann E, Houslay MD, Baillie GS, MEK1 binds directly to beta-arrestin1, influencing both its phosphorylation by ERK and the timing of its isoprenaline-stimulated internalization. *J Biol Chem* 284, 11425–11435 (2009). [PubMed: 19153083]
21. Song X, Coffa S, Fu H, Gurevich VV, How does arrestin assemble MAPKs into a signaling complex? *J Biol Chem* 284, 685–695 (2009). [PubMed: 19001375]
22. Coffa S, Breitman M, Hanson SM, Callaway K, Kook S, Dalby KN, Gurevich VV, The effect of arrestin conformation on the recruitment of c-Raf1, MEK1, and ERK1/2 activation. *PLoS One* 6, e28723 (2011). [PubMed: 22174878]
23. Hanson SM, Cleghorn WM, Francis DJ, Vishnivetskiy SA, Raman D, Song X, Nair KS, Slepak VZ, Klug CS, Gurevich VV VV, Arrestin mobilizes signaling proteins to the cytoskeleton and redirects their activity. *J Mol Biol* 368, 375–387 (2007). [PubMed: 17359998]
24. DeFea KA KA, Zalevsky J J, Thoma MS MS, Dery O O, Mullins RD RD, Bunnett NW, beta-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 148, 1267–1281 (2000). [PubMed: 10725339]
25. Ahn S, Shenoy SK, Wei H, Lefkowitz RJ, Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279, 35518–35525 (2004). [PubMed: 15205453]
26. Wei H, Ahn S, Barnes WG, Lefkowitz RJ, Stable interaction between beta-arrestin 2 and angiotensin type 1A receptor is required for beta-arrestin 2-mediated activation of extracellular signal-regulated kinases 1 and 2. *J Biol Chem* 279, 48255–48261 (2004). [PubMed: 15355986]
27. Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, Eckhardt AE, Cowan CL, Spurney RF, Luttrell LM, Lefkowitz RJ, Distinct beta-arrestin-and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *J Biol Chem* 281, 10856–10864 (2006). [PubMed: 16492667]
28. Jafri F, El-Shewy HM, Lee M-H, Kelly M, Luttrell DK, Luttrell LM, Constitutive ERK1/2 activation by a chimeric neurokinin 1 receptor-beta-arrestin1 fusion protein. Probing the composition and function of the G protein-coupled receptor “signalsome”. *J Biol Chem* 281,19346–19357 (2006). [PubMed: 16670094]
29. Lin FT, Chen W, Shenoy S, Cong M, Exum ST, Lefkowitz RJ, Phosphorylation of beta-arrestin2 regulates its function in internalization of beta(2)-adrenergic receptors. *Biochemistry* 41, 10692–10699 (2002). [PubMed: 12186555]

30. Khoury E, Nikolajev L, Simaan M, Namkung Y, Laporte SA SA, Differential regulation of endosomal GPCR/ β -arrestin complexes and trafficking by MAPK. *J Biol Chem* 289, 23302–23317 (2014). [PubMed: 25016018]
31. Ge L, Ly Y, Hollenberg M, DeFea K, A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. *J Biol Chem* 278, 34418–34426 (2003). [PubMed: 12821670]
32. Ge L, Shenoy SK, Lefkowitz RJ, DeFea K K, Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2. *J Biol Chem* 279, 55419–55424 (2004). [PubMed: 15489220]
33. Kim IM, Tilley DG, Chen J, Salazar NC, Whalen EJ, Violin JD, Rockman HA, Beta-blockers alprenolol and carvedilol stimulate beta-arrestin-mediated EGFR transactivation. *Proc Natl Acad Sci U S A* 105, 14555–14560 (2008). [PubMed: 18787115]
34. Tilley DG, Kim IM, Patel PA, Violin JD, Rockman HA, beta-Arrestin mediates beta1-adrenergic receptor-epidermal growth factor receptor interaction and downstream signaling. *J Biol Chem* 284, 20375–20386 (2009). [PubMed: 19509284]
35. Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corvoisier P, Violin JD, Wei H, Lefkowitz RJ, Rockman HA, beta-Arrestin-mediated beta1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J Clin Invest* 117, 2445–2458 (2007). [PubMed: 17786238]
36. DeWire SM, Kim J, Whalen EJ, Ahn S, Chen M, Lefkowitz RJ, Beta-arrestin-mediated signaling regulates protein synthesis. *J Biol Chem* 283, 10611–10620 (2008). [PubMed: 18276584]
37. Kendall RT, Lee M-H, Pleasant DL, Robinson K, Kuppuswamy D, McDermott PJ, Luttrell LM, Arrestin-dependent angiotensin AT1 receptor signaling regulates Akt and mTor-mediated protein synthesis. *J Biol Chem* 289, 26155–26166 (2014). [PubMed: 25081544]
38. Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM, eta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem* 277, 9429–9436 (2002). [PubMed: 11777902]
39. Lee M-H, El-Shewy HM, Luttrell DK, Luttrell LM LM, Role of beta-arrestin-mediated desensitization and signaling in the control of angiotensin AT1a receptor-stimulated transcription. *J Biol Chem* 283, 2088–2097 (2008). [PubMed: 18006496]
40. Gesty-Palmer D, El-Shewy H, Kohout TA, Luttrell LM, beta-Arrestin 2 expression determines the transcriptional response to lysophosphatidic acid stimulation in murine embryo fibroblasts. *J Biol Chem* 280, 32157–32167 (2005). [PubMed: 16027114]
41. Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, Rüttiger N, Ziegler N, Benkel T, Schmitt NK, Ishida S, Müller I, Reher R, Kawakami K, Inoue A, Rick U, Kühl T, Imhof D, Aoki J, König GM, Hoffmann C, Gomeza J, Wess J, Kostenis E, Lack of beta-arrestin signaling in the absence of active G proteins. *Nat Commun* 9, 341 (2018). [PubMed: 29362459]
42. O'Hayre M, Eichel K, Avino S, Zhao X, Steffen DJ, Feng X, Kawakami K, Aoki J, Messer K, Sunahara R, Inoue A, von Zastrow M M, Gutkind JS, Genetic evidence that β -arrestins are dispensable for the initiation of β 2-adrenergic receptor signaling to ERK. *Sci Signal* 10, pii: eaal3395 (2017).
43. Pawson T, Olivier P, Rozakis-Adcock M, McGlade J, Henkemeyer M, Proteins with SH2 and SH3 domains couple receptor tyrosine kinases to intracellular signalling pathways. *Philos Trans R Soc Lond B Biol Sci* 340, 279–285 (1993). [PubMed: 8103930]
44. Luttrell LM, Lefkowitz RJ, The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci* 115, 455–465 (2002). [PubMed: 11861753]
45. Lefkowitz RJ, Whalen EJ, beta-Arrestins: traffic cops of cell signaling. *Curr Opin Cell Biol* 16, 162–168 (2004). [PubMed: 15196559]
46. Shenoy SK, Lefkowitz RJ RJ, β -Arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci* 32, 521–533 (2011). [PubMed: 21680031]
47. Strungs EG, Luttrell LM, Arrestin-dependent activation of ERK and Src family kinases. *Handb Exp Pharmacol* 219, 225–257 (2014). [PubMed: 24292833]
48. Luttrell LM, Spiller BW, Arrestin-dependent ERK activation and its disruption. In: *The structural basis of arrestin functions* Springer 199–217 (2017).

49. Milligan G, Inoue A, Genome editing provides new insights into receptor-controlled signalling pathways. *Trends Pharmacol Sci* 39, 481–493 (2018). [PubMed: 29548548]
50. Kohout TA, Lin FS, Perry SJ, Conner DA, Lefkowitz RJ, beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Natl Acad Sci U S A* 98, 1601–1606 (2001). [PubMed: 11171997]
51. Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, Lefkowitz RJ, Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* 100, 10782–10787 (2003). [PubMed: 12949261]
52. Ahn S, Wei H, Garrison TR, Lefkowitz RJ, Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *J Biol Chem* 279, 7807–7811 (2004). [PubMed: 14711824]
53. Ahn S, Nelson CD, Garrison TR, Miller WE, Lefkowitz RJ, Desensitization, internalization, and signaling functions of beta-arrestins demonstrated by RNA interference. *Proc Natl Acad Sci U S A* 100, 1740–1744 (2003). [PubMed: 12582207]
54. Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ, beta-Arrestin-dependent G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem* 281, 1261–1273 (2006). [PubMed: 16280323]
55. Galés C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, Bouvier M, Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nat Struct Mol Biol* 13, 778–786 (2006). [PubMed: 16906158]
56. Namkung Y, Le Gouill C, Lukashova V, Kobayashi H, Hogue M, Khoury E, Song M, Bouvier M, Laporte SA, Monitoring G protein-coupled receptor and β -arrestin trafficking in live cells using enhanced bystander BRET. *Nat Commun* 7, 12178 (2016). [PubMed: 27397672]
57. Barak LS, Ferguson SS, Zhang J, Caron MG, A beta-arrestin/green fluorescent protein biosensor for detecting G protein-coupled receptor activation. *J Biol Chem* 272, 27497–27500 (1997). [PubMed: 9346876]
58. Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS, Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. *J Biol Chem* 275, 17201–17210 (2000). [PubMed: 10748214]
59. Osmond RI, Sheehan A, Borowicz R, Barnett E, Harvey G, Turner C, Brown A, Crouch MF, Dyer AR, GPCR screening via ERK 1/2: a novel platform for screening G protein-coupled receptors. *J Biomol Screen* 10, 730–737 (2005). [PubMed: 16129779]
60. Zhang J, Barak LS, Anborgh PH, Laporte SA, Caron MG, Ferguson SS, Cellular trafficking of G protein-coupled receptor/beta-arrestin endocytic complexes. *J Biol Chem* 274, 10999–11006 (1999). [PubMed: 10196181]
61. Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, Ahles A, Engelhardt S, Rockman HA, Gai is required for carvedilol-induced β_1 adrenergic receptor β -arrestin biased signaling *Nat Commun* 8:1706 (2017). [PubMed: 29167435]
62. Tohgo, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM, The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem* 278, 6258–6267 (2003). [PubMed: 12473660]
63. Charest PG, Bouvier M, Palmitoylation of the V2 vasopressin receptor carboxyl tail enhances beta-arrestin recruitment leading to efficient receptor endocytosis and ERK1/2 activation. *J Biol Chem* 278, 41541–41551 (2003). [PubMed: 12900404]
64. Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, Piñeyro G, Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci U S A* 100, 11406–11411 (2003). [PubMed: 13679574]
65. Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ, Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci U S A* 105, 1448–1453 (2005).

66. Charest PG, Oligny-Longpré G, Bonin H, Azzi M, Bouvier M, The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling. *Cell Signal* 19, 32–41 (2007). [PubMed: 16857342]
67. Kara E, Crépieux P, Gauthier C, Martinat M, Piketty V, Guillou F, Reiter E, A phosphorylation cluster of five serine and threonine residues in the C-terminus of the follicle-stimulating hormone receptor is important for desensitization but not for beta-arrestin-mediated ERK activation. *Mol Endocrinol* 20, 3014–3026 (2006). [PubMed: 16887887]
68. Wehbi V, Decourtaye J, Piketty V, Durand G, Reiter E, Maurel MC, Selective modulation of follicle-stimulating hormone signaling pathways with enhancing equine chorionic gonadotropin/antibody immune complexes. *Endocrinology* 151, 2788–2799 (2010). [PubMed: 20332198]
69. Ayoub MA, Landomiel F, Gallay N, Jégot G, Poupon A, Crépieux P, Reiter E, Assessing gonadotropin receptor function by resonance energy transfer-based assays. *Front Endocrinol (Lausanne)* 6,130 (2015). [PubMed: 26379624]
70. Jean-Alphonse F, Bowersox S, Chen S, Beard G, Puthenveedu MA, Hanyaloglu AC, Spatially restricted G protein-coupled receptor activity via divergent endocytic compartments. *J Biol Chem* 289, 3960–3977 (2014). [PubMed: 24375413]
71. Yue TL, Cheng HY, Lysko PG, McKenna PJ, Feuerstein R, Gu JL, Lysko KA, Davis LL, Feuerstein G, Carvedilol, a new vasodilator and beta adrenoceptor antagonist, is an antioxidant and free radical scavenger. *J Pharmacol Exp Ther* 263, 92–98 (1992). [PubMed: 1357162]
72. Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, Shusterman NH, The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group. *N Engl J Med* 334, 1349–1355 (1996). [PubMed: 8614419]
73. Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, Shenoy SK, Lefkowitz RJ, A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci U S A* 104, 16657–16662 (2007). [PubMed: 17925438]
74. Sung C-P, Arleth AJ, Eichman C, Truneh A, Ohlstein EH, Carvedilol, a multiple-action neurohumoral antagonist, inhibits mitogen-activated protein kinase and cell cycle progression in vascular smooth muscle cells. *J Pharm Exp Thera* 283, 910–917 (1997).
75. Cahill TJ, 3rd, Thomsen AR, Tarrasch JT, Plouffe B, Nguyen AH, Yang F, Huang LY, Kahsai AW, Bassoni DL, Gavino BJ, Lamerdin JE, Triest S, Shukla AK, Berger B, Little J, 4th, Antar A, Blanc A, Qu CX, Chen X, Kawakami K, Inoue A, Aoki J, Steyaert J, Sun JP, Bouvier M, Skiniotis G, Lefkowitz RJ, Distinct conformations of GPCR- β -arrestin complexes mediate desensitization, signaling, and endocytosis. *Proc Natl Acad Sci U S A* 114, 2562–2567 (2017). [PubMed: 28223524]
76. Coffa S, Breitman M, Spiller BW, Gurevich VV, A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding. *Biochemistry* 50, 6951–6958 (2011). [PubMed: 21732673]
77. Beaudrait A, Paradis JS, Zimmerman B, Giubilaro J, Nikolajev L, Armando S, Kobayashi H, Yamani L, Namkung Y, Heydenreich FM, Khoury E, Audet M, Roux PP, Vepintsev DB, Laporte SA, Bouvier M, A new inhibitor of the β -arrestin/AP2 endocytic complex reveals interplay between GPCR internalization and signalling. *Nat Commun* 8, 15054 (2017). [PubMed: 28416805]
78. de Mendoza A, Sebe-Pedros A, Ruiz-Trillo I, The evolution of the GPCR signaling system in eukaryotes: modularity, conservation, and the transition to metazoan multicellularity. *Genome Biol Evol* 6, 606–619 (2014). [PubMed: 24567306]
79. Jean-Charles PY, Rajiv V, Shenoy SK, Ubiquitin-related roles of β -arrestins in endocytic trafficking and signal transduction. *J Cell Physiol* 231, 2071–2080 (2016). [PubMed: 26790995]
80. Nair KS, Hanson SM, Kennedy MJ, Hurley JB, Gurevich VV, Slepak VZ, Direct binding of visual arrestin to microtubules determines the differential subcellular localization of its splice variants in rod photoreceptors. *J Biol Chem* 279, 41240–41248 (2004). [PubMed: 15272005]
81. Song X, Raman D, Gurevich EV, Vishnivetskiy SA, Gurevich VV, Visual and both non-visual arrestins in their “inactive” conformation bind JNK3 and Mdm2 and relocate them from the nucleus to the cytoplasm. *J Biol Chem* 281, 21491–21499 (2006). [PubMed: 16737965]

82. Hanson SM, Francis DJ, Vishnivetskiy SA, Klug CS, Gurevich VV, Visual arrestin binding to microtubules involves a distinct conformational change. *J Biol Chem* 281, 9765–9772 (2006). [PubMed: 16461350]
83. Lin A, DeFea KA, β -Arrestin-kinase scaffolds: turn them on or turn them off? *Wiley Interdiscip Rev Syst Biol Med* 5, 231–241 (2013). [PubMed: 23319470]
84. McDonald PH, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ, Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* 290, 1574–1577 (2000). [PubMed: 11090355]
85. Zhan X, Kaoud TS, Dalby KN, Gurevich VV, Nonvisual Arrestins Function as Simple Scaffolds Assembling the MKK4–JNK3 α 2 Signaling Complex. *Biochemistry* 50, 10520–10529 (2011). [PubMed: 22047447]
86. Zhan X, Stoy H, Kaoud TS, Perry NA, Chen Q, Perez A, Els-Heindl S, Slagis JV, Iverson TM, Beck-Sickinger AG, Gurevich EV, Dalby KN, Gurevich VV, Peptide mini-scaffold facilitates JNK3 activation in cells. *Sci Rep* 6:21025 (2016). [PubMed: 26868142]
87. Terrillon S, Bouvier M, Receptor activity-independent recruitment of beta-arrestin2 reveals specific signalling modes. *EMBO J* 23, 3950–3961 (2004). [PubMed: 15385966]
88. Miyatake M, Rubinstein TJ, McLennan GP, Belcheva MM, Coscia CJ, Inhibition of EGF-induced ERK/MAP kinase-mediated astrocyte proliferation by mu opioids: integration of G protein and beta-arrestin 2-dependent pathways. *J Neurochem* 110, 662–674 (2009). [PubMed: 19457093]
89. Saulière A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, Altié M-F, Seguelas M-H, Pathak A, Hansen JL, Sénard J-M, Galés C, Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. *Nat Chem Biol* 8, 622–630 (2012) [PubMed: 22634635]
90. Gardella TJ, Luck MD, Jensen GS, Schipani E, Potts JT, Jr, Juppner H. Inverse agonism of amino-terminally truncated parathyroid hormone (PTH) and PTH-related peptide (PTHrP) analogs revealed with constitutively active mutant PTH/PTHrP receptors. *Endocrinol* 137, 3936–3941 (1996).
91. Kumar P, Lau CS, Mathur M, Wang P, DeFea KA, Differential effects of beta-arrestins on the internalization, desensitization and ERK1/2 activation downstream of protease activated receptor-2. *Am J Physiol Cell Physiol* 293, C346–357 (2007). [PubMed: 17442737]
92. Lee M-H, Appleton KM, Strungs EG, Kwon JY, Morinelli TA, Peterson YK, Laporte SA, Luttrell LM, The conformational signature of activated arrestin3 predicts its trafficking and signaling functions. *Nature* 531, 665–668 (2016). [PubMed: 27007854]
93. Nuber S, Zabel U, Loreza K, Nuber A, Milligan G, Tobin AB, Lohse MJ, Hoffmann C, FRET-based β -arrestin biosensors reveal a rapid, receptor-dependent activation/deactivation cycle in living cells. *Nature* 531, 661–664 (2016). [PubMed: 27007855]
94. Kenakin TP, Functional selectivity through protean and biased agonism: who steers the ship? *Mol Pharmacol* 72, 1393–1401 (2007). [PubMed: 17901198]
95. Luttrell LM, Minireview: More than just a hammer: ligand “bias” and pharmaceutical discovery. *Mol. Endocrinol* 2014, me20131314 (2014).
96. Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ, Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci U S A* 102, 1442–1447 (2005). [PubMed: 15671181]
97. Tobin AB, Butcher AJ, Kong KC, Location, location, location... site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci* 29, 413–420 (2008). [PubMed: 18606460]
98. Nobles KN, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ, Distinct phosphorylation sites on the β (2)-adrenergic receptor establish a barcode that encodes differential functions of β -arrestin. *Sci Signal* 4, ra51 (2011).
99. Kommaddi RP, Shenoy SK, Arrestins and protein ubiquitination. *Prog Mol Biol Transl Sci* 118, 175–204 (2013). [PubMed: 23764054]
100. Zhang M, Liu X, Zhang Y, Zhao J, Loss of beta-arrestin1 and beta-arrestin2 contributes to pulmonary hypoplasia and neonatal lethality in mice. *Dev Biol* 339, 407–417(2010). [PubMed: 20060823]

101. Alvarez-Curto E, Inoue A, Jenkins L, Raihan SZ, Prihandoko R, Tobin AB, Milligan G, Targeted elimination of G proteins and arrestins defines their specific contributions to both intensity and duration of G protein-coupled receptor signaling. *J Biol Chem* 291, 27147–27159 (2016). [PubMed: 27852822]
102. Kadam US, Shelake RM, Chavhan RL, Suprasanna P, Concerns regarding 'off-target' activity of genome editing endonucleases. *Plant Physiol Biochem pii: S0981–9428(18)30150–5* (2018).
103. Han SO, Kommaddi RP, Shenoy SK, Distinct roles for beta-arrestin2 and arrestin-domain-containing proteins in beta2 adrenergic receptor trafficking. *EMBO Rep* 14, 164–171 (2013). [PubMed: 23208550]
104. Cordeaux Y, Briddon SJ, Megson AE, McDonnell J, Dickenson JM, Hill SJ, Influence of receptor number on functional responses elicited by agonists acting at the human adenosine A(1) receptor: evidence for signaling pathway-dependent changes in agonist potency and relative intrinsic activity. *Mol Pharmacol* 58, 1075–1084 (2000) [PubMed: 11040056]
105. Zhu X, Gilbert S, Birnbaumer M, Birnbaumer L, Dual signaling potential is common among Gs-coupled receptors and dependent on receptor density. *Mol Pharmacol* 46, 460–469 (1994). [PubMed: 7935326]
106. Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ, Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *J Biol Chem* 267, 17882–17890 1992. [PubMed: 1517224]
107. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F, Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8, 2281–2308 (2013). [PubMed: 24157548]
108. Shenoy SK, Han S, Zhao YL, Hara MR, Oliver T, Cao Y, Dewhirst MW, beta-Arrestin1 mediates metastatic growth of breast cancer cells by facilitating HIF-1-dependent VEGF expression. *Oncogene* 31, 282–292 (2012). [PubMed: 21685944]
109. Tranchant T, Durand G, Gauthier C, Crépeux P, Ulloa-Aguirre A, Royère D, Reiter E, Preferential β -arrestin signalling at low receptor density revealed by functional characterization of the human FSH receptor A189 V mutation. *Mol Cell Endocrinol* 331, 109–118 (2011). [PubMed: 20801186]
110. Zimmerman B, Simaan M, Lee M-H, Luttrell LM, Laporte SA, c-Src-mediated phosphorylation of AP-2 reveals a general mechanism for receptors internalizing through the clathrin pathway. *Cell Signal* 21, 103–110 (2009). [PubMed: 18938240]
111. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 92, 7297–7301 (1995). [PubMed: 7638184]

Fig 1. Variable effects of CRISPR/Cas9 gene editing on β_2 AR-stimulated activation of ERK1/2 in three independently derived β Arr1/2 CRISPR KO HEK293 cell lines.

(A to D) Top: Parental and corresponding CRISPR β Arr1/2 KO HEK293 cell lines expressing endogenous β_2 ARs (A to C) or overexpressed FLAG- β_2 AR (D) were analyzed by Western blotting (IB) with antibody against β Arr1/2. β -Actin was used as a loading control. The abundance of endogenous β_2 AR determined by [125 I](–)-iodocyanopindolol binding was 15 ± 5 fmol/mg protein in all parental and CRISPR β Arr1/2 KO lines, and the abundance of FLAG- β_2 AR in the stably transfected AI-parental and CRISPR β Arr1/2 KO lines was 2.3 ± 0.01 and 4.7 ± 0.6 pmol/mg protein, respectively. Middle: Serum-deprived cells were stimulated with 100 nM isoproterenol (Iso) for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots for each parental-CRISPR pair are representative of four (B to D) or five (A) experiments. Bottom: For each parental-CRISPR β Arr1/2 KO pair, Iso-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the parental line and data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of four or five biological replicates. Statistical significance was determined by two-way ANOVA and Sidak's multiple comparison test. * $P < 0.05$ compared with parental cells; # $P < 0.05$ compared with parental cells at the corresponding time point; ns, not significant.

Fig 2. Consistent effects of siRNA-mediated knockdown of β Arr1/2 on β 2AR-stimulated ERK1/2 activation in three parental HEK-293 cell lines.

(A to H) Top: Parental HEK293 cell lines expressing endogenous β ₂AR (A to C) and (E to G) or overexpressed FLAG- β ₂AR (D and H) and transfected with siRNAs targeting no mRNA (CTL), β Arr2 (A to D), or both β Arr1 and β Arr2 (E to H) were analyzed by Western blotting (IB) with antibody against β Arr1/2. β -Actin was used as a loading control. Middle: Serum-deprived cells were stimulated with 100 nM isoproterenol (Iso) for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots for each pair of CTL siRNA- and β Arr siRNA-treated cells are representative of three (H), four (B to C and E to G), or five (A and B) experiments. Bottom: For each CTL siRNA- (black symbols) and β Arr siRNA-treated (red symbols) pair, Iso-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the CTL siRNA-treated cells and data expressed a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of four or five biological

replicates. Statistical significance was determined by two-way ANOVA and Sidak's multiple comparison test. * $P < 0.05$ compared with parental group (control); # $P < 0.05$ compared with control at the corresponding time point; ns, not significant.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig 3. Variable effects of restoring β Arr1/2 on β_2 AR-stimulated ERK1/2 activation in three CRISPR β Arr1/2 KO cell lines.

(A to F) Top: CRISPR β Arr1/2 KO cell lines were transiently transfected with plasmid encoding FLAG- β_2 AR together with either empty vector or plasmids encoding HA- β Arr2 (A to C) or both HA- β Arr1 and HA- β Arr2 (D to F). Parental HEK293 cells, vector-transfected CRISPR cells, and CRISPR cells expressing HA- β Arr1, HA- β Arr2, or HA- β Arr1/2 were analyzed by Western blotting (IB) with antibody against β Arr1/2. β -Actin was used as a loading control. Middle: Serum-deprived cells were stimulated with 100 nM isoproterenol (Iso) for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots for each pair of vector-transfected and HA- β Arr-expressing CRISPR cells are representative of three (B to C and E to F) or four (A and D) experiments. Bottom: For each vector-transfected (black symbols) and HA- β Arr-expressing (green symbols) CRISPR cell pair, Iso-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the vector-

transfected cells and data are expressed a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of three or four biological replicates. Statistical significance was determined by two-way ANOVA and Sidak's multiple comparison test. # $P < 0.05$ compared with control at the corresponding time point; ns, not significant.

Fig 4. Effects of β -arrestin expression on G α_s activation, receptor internalization, and ERK1/2 activation by the β_2 AR.

(A) Top: Schematic representation of the BRET biosensors used to monitor G α_s activation. Activation of G α_s induces dissociation of RlucII-G α_s from GFP10-G γ 1 resulting in a decrease in BRET signal (55). Bottom: Isoproterenol (Iso) concentration-response curves generated in each parental HEK293 cell line (black symbols; solid black line) together with those observed in the corresponding CRISPR β Arr1/2 KO line in the absence (open symbols; dashed black line) or presence of exogenous β Arr1 (open symbols; dashed blue line) or β Arr2 (open symbols; dashed red line). Data are means $\pm$ SEM of four to 11 biological replicates as follows: SL parental (n = 11), SL CRISPR (n = 11), SL CRISPR + β arr1 (n = 5), SL CRISPR + β arr2 (n = 5), AI parental (n = 10), AI CRISPR (n = 10), AI CRISPR + β arr1 (n = 4), CRISPR + β arr2 (n = 4), HAR parental (n = 8), HAR CRISPR (n = 8), CRISPR + β arr1 (n = 4), CRISPR + β arr2 (n = 4). (B) Top: Schematic representation of the BRET biosensors used to monitor loss of β_2 AR from the plasma membrane. Receptor

internalization was measured by the decrease in BRET between $\beta 2AR$ -*RlucII* and rGFP-CAAX labeling the plasma membrane (56). Bottom: Iso concentration-response curves generated in each parental HEK293 cell line (black symbols; solid black line) together with those observed in the corresponding CRISPR $\beta Arr1/2$ KO line in the absence (open symbols; dashed black line) or presence of exogenous $\beta Arr1$ (open symbols; dashed blue line) or $\beta Arr2$ (open symbols; dashed red line). Data are means $\pm$ SEM of four to six biological replicates as follows: SL parental (n = 6), SL CRISPR (n = 6), SL CRISPR + $\beta arr1$ (n = 5), SL CRISPR + $\beta arr2$ (n = 5), AI parental (n = 5), AI CRISPR (n = 5), AI CRISPR + $\beta arr1$ (n = 4), AI CRISPR + $\beta arr2$ (n = 4), HAR parental (n = 5), HAR CRISPR (n = 5), HAR CRISPR + $\beta arr1$ (n = 4), HAR CRISPR + $\beta arr2$ (n = 4). (C) Top: Schematic representation of the FRET-based assay used to monitor ERK1/2 phosphorylation. The AlphaLISA SureFire Ultra system is a sandwich ELISA in which bridging of donor and acceptor beads by the activated pThr²⁰²/pTyr²⁰⁴ ERK1/2 analyte produces an increase in fluorescence emission (59). Middle: The effect of pretreatment with PTX, the PKA inhibitor 6–22, or both, on 1 μ M Iso-stimulated ERK1/2 phosphorylation in SL-parental HEK293 cells. Bottom: Results of identical experiments performed using the corresponding SL-CRISPR $\beta Arr1/2$ KO line. In each graph, responses are expressed as a percentage of the maximal Iso-stimulated response in the absence of inhibitor (control). Data are means $\pm$ SEM of three to six biological replicates as follows: SL parental: control (n = 6), PTX (n = 5), 6–22 (n = 6), PTX + 6–22 (n = 3). SL CRISPR: control (n = 6), PTX (n = 5), 6–22 (n = 6), PTX + 6–22 (n = 3). Significance was assessed by two-way ANOVA. * P < 0.05; ** P < 0.01 for the indicated comparisons.

CTL siRNA– and β Arr1/2 siRNA–treated cells are representative of four experiments. Bottom: For each CTL siRNA– (black symbols) and β Arr1/2 siRNA–treated (red symbols) pair, Iso-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the CTL siRNA–treated cells and data are expressed a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of four biological replicates. (D to F) Top: CRISPR β Arr1/2 KO cell lines transiently transfected with plasmid encoding FLAG- β 1AR together with either empty vector or plasmids encoding HA- β Arr1 and HA- β Arr2 were analyzed by Western blotting (IB) with antibody against β Arr1/2. GAPDH was used as a loading control. Middle: Serum-deprived cells were stimulated with 10 μ M Iso for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots for each pair of vector-transfected and HA- β Arr1/2–expressing CRISPR cells are representative of three experiments. Bottom: For each vector-transfected (black symbols) and HA- β Arr1/2– expressing (green symbols) CRISPR cell pair, Iso-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the vector-transfected cells and the data are expressed a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of three biological replicates. In all panels, statistical significance was determined by two-way ANOVA and Sidak’s multiple comparison test. # $P < 0.05$ compared with control at the corresponding time point; ns, not significant.

Fig 6. Reciprocal effects of β Arr1/2 knockdown in parental HEK293 cells and β Arr1/2 reconstitution in CRISPR β Arr1/2 KO cells on ERK1/2 activation by the V₂R.

(A to C) Top: Parental HEK293 cell lines co-transfected with plasmid encoding HA-V₂R and siRNAs targeting no mRNA (CTL) or β Arr1/2 were analyzed by Western blotting (IB) with antibody against β Arr1/2 (top row). Total ERK1/2 was used as a loading control. Serum-deprived cells were stimulated with 1 μ M arginine-vasopressin (AVP) for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) (middle row) and total ERK1/2 (ERK) (bottom row). Western blots for each pair of

CTL siRNA– and β Arr1/2 siRNA–treated cells are representative of three experiments. Bottom: For each CTL siRNA– (black symbols) and β Arr1/2 siRNA–treated (red symbols) pair, AVP-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the CTL siRNA–treated cells and the data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of three biological replicates. (D to F) Top: CRISPR β Arr1/2 KO cell lines transiently transfected with plasmid encoding HA-V₂R together with either empty vector or plasmids encoding FLAG- β Arr1 or FLAG- β Arr2 were analyzed by Western blotting (IB) with antibody against β Arr1/2 (top rows). Total ERK1/2 was used as a loading control. Serum-deprived cells were stimulated with 1 μ M AVP for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) (middle rows) and total ERK1/2 (ERK) (bottom rows). Western blots for each pair of vector-transfected and FLAG- β Arr– expressing CRISPR cells are representative of three experiments. Bottom: For vector-transfected (black symbols), FLAG- β Arr1–expressing (purple symbols), and FLAG- β Arr2–expressing (green symbols) cell comparisons, AVP-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the vector-transfected cells and the data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of three biological replicates. In all panels, statistical significance was determined by two-way ANOVA and Sidak’s multiple comparison test. & P < 0.05 for β Arr1 reconstitution compared with control at the corresponding time point; # P < 0.05 for β Arr2 reconstitution compared with control at the corresponding time point; ns, not significant.

Fig 7. Reciprocal effects of β Arr1/2 knockdown in parental HEK293 cells and β Arr1/2 reconstitution in CRISPR β Arr1/2 KO cells on ERK1/2 activation by the FSHR.

(A to C) Top: Parental HEK293 cell lines co-transfected with plasmid encoding FLAG-FSHR and siRNAs targeting no mRNA (CTL) or β Arr1/2 were analyzed by Western blotting (IB) with antibody against β Arr1/2. Total ERK1/2 was used as a loading control. Middle: Serum-deprived cells were stimulated with 100 ng/mL recombinant human FSH for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots for each pair of CTL siRNA- and β Arr1/2

siRNA-treated cells are representative of five experiments. Bottom: For each CTL siRNA- (black symbols) and β Arr1/2 siRNA-treated (red symbols) pair, FSH-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the CTL siRNA-treated cells and the data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of five biological replicates. (D to F) Top: CRISPR β Arr1/2 KO cell lines transiently transfected with plasmid encoding FLAG-FSHR together with either empty vector or plasmids encoding HA- β Arr1 and HA- β Arr2 were analyzed by Western blotting (IB) with antibody against β Arr1/2. Total ERK1/2 was used as a loading control. Middle: Serum-deprived cells were stimulated with 100 ng/mL FSH for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots for each pair of vector-transfected and FLAG- β Arr1/2-expressing CRISPR cells are representative of four (D and F) or five (E) experiments. Bottom: For each vector-transfected (black symbols) and HA- β Arr1/2-expressing (green symbols) CRISPR cell pair, FSH-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the vector-transfected cells and the data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of four or five biological replicates. In all panels, statistical significance was determined by two-way ANOVA and Sidak's multiple comparison test. # $P < 0.05$ for β Arr1/2 reconstitution compared with the control at the corresponding time point; ns, not significant.

Fig 8. Reciprocal effects of β Arr1/2 knockdown in parental HEK293 cells and β Arr1/2 reconstitution in CRISPR β Arr1/2 KO cells on ERK1/2 activation by the arrestin pathway-selective biased agonist carvedilol.

(A) Top: SL-Parental HEK293 cells co-transfected with plasmid encoding FLAG- β_2 AR and siRNAs targeting no mRNA (CTL) or β Arr1/2 were analyzed by Western blotting (IB) with antibody against β Arr1/2. β -Actin was used as a loading control. Middle: Serum-deprived cells were stimulated with 10 μ M carvedilol (Carv) for the indicated times and cell lysates analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots of CTL siRNA- and β Arr1/2 siRNA-treated cells are representative of three experiments. Bottom: For CTL siRNA- (black symbols) and β Arr1/2 siRNA-treated (red symbols) cells, Carv-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the CTL siRNA-treated cells and the data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of three biological replicates. (B) Top: SL-CRISPR β Arr1/2 KO cells transiently transfected with plasmid encoding FLAG- β_2 AR together with either empty vector or plasmids encoding HA- β Arr1 and HA- β Arr2 were analyzed by Western blotting (IB) with antibody against β Arr1/2. β -Actin was used as a loading control. Middle: Serum-deprived cells were stimulated with 10 μ M Carv for the indicated times and cell lysates were analyzed by Western blotting sequentially for pERK1/2 (pERK) and total ERK1/2 (ERK). Western blots of vector-transfected and FLAG- β Arr1/2-expressing CRISPR cells are representative of four experiments. Bottom: For vector-transfected (black symbols) and HA- β Arr1/2-expressing (green symbols) CRISPR cells, Carv-stimulated pERK/ERK ratios were normalized to the maximum signal observed in the vector-transfected cells and the data are expressed as a percentage of the maximum control response (% max control). Data are means $\pm$ SEM of four biological replicates. (C and D) Analogous set of experiments to those described in (A) and (B) were performed in AI-parental HEK293 and AI-CRISPR β Arr1/2 KO cells

expressing the β_1 AR. Western blots and densitometry data represent three (D) or four (C) biological replicates. In all panels, statistical significance was determined by two-way ANOVA and Sidak's multiple comparison test. # $P < 0.05$ for β Arr1/2 reconstitution compared with the control at the corresponding time point; ns, not significant.

Fig 9. Model depicting the dual roles of β-arrestins in the GPCR-dependent stimulation of ERK1/2.

(A) In native cells, ERK1/2 activation reflects a balance between G protein-dependent pathways that are attenuated by β-arrestin-dependent desensitization and β-arrestin-dependent pathways that are augmented by its scaffolding function. (B) In the CRISPR/Cas9 βArr1/2 KO background, G protein-dependent ERK1/2 activation is unrestrained and β-arrestin-dependent ERK1/2 activation is absent. The resulting increase in signal strength in the G protein pathways may or may not offset the loss of β-arrestin-mediated signaling, leading to a net increase, decrease, or no change in GPCR-stimulated ERK1/2 activity. (C) β-Arrestin-biased ligands that possess little or no G protein efficacy rely predominantly on β-arrestin scaffolds to support ERK1/2 activation.