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Phenotypic Regulation of the Sphingosine 1-Phosphate Receptor Miles Apart by G Protein-Coupled Receptor Kinase 2

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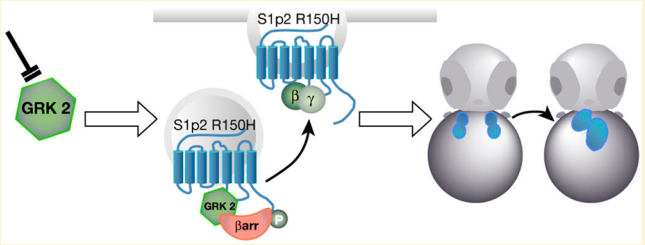
Supporting Information

ABSTRACT: The evolutionarily conserved DRY motif at the end of the third helix of rhodopsin-like, class-A G protein-coupled receptors (GPCRs) is a major regulator of receptor stability, signaling activity, and β-arrestin-mediated internalization. Substitution of the DRY arginine with histidine in the human vasopressin receptor results in a loss-of-function phenotype associated with diabetes insipidus. The analogous R150H substitution of the DRY motif in zebrafish sphingosine-1 phosphate receptor 2 (S1p2) produces a mutation, miles apart m^{93} (mil^{m93}), that not only disrupts signaling but also impairs heart field migration. We hypothesized that constitutive S1p2 desensitization is the underlying cause of this strong zebrafish developmental defect. We observed in cell assays that the wild-type S1p2 receptor is at the cell surface whereas in distinct contrast the S1p2 R150H receptor is found in intracellular vesicles, blocking G protein but not arrestin signaling activity. Surface S1p2 R150H expression could be restored by inhibition of G protein-coupled receptor kinase 2 (GRK2). Moreover, we observed that β-arrestin 2 and GRK2 colocalize with S1p2 in developing zebrafish embryos and depletion of GRK2 in the S1p2 R150H miles apart zebrafish partially rescued cardia bifida. The ability of reduced GRK2 activity to reverse a developmental phenotype associated with constitutive desensitization supports efforts to genetically or pharmacologically target this kinase in diseases involving biased GPCR signaling.



G protein-coupled receptors (GPCRs) form the largest class of targets for marketed therapeutic drugs, and most if not all of these GPCR drugs were developed to modulate G protein signaling. In the past 10 years, however, it has become evident that GPCRs also signal through β-arrestin-dependent cascades, which may explain many of the positive or adverse effects of drug treatment that were not easily explained by G protein considerations alone.[1] As a consequence of the multiplex signaling functions of GPCRs, so-called biased agonists that preferentially target only one GPCR signaling mode are now highly desired as laboratory tool compounds for dissecting biochemical pathways and as potential innovations for novel therapeutics (for reviews, see refs 2−4). The approach toward biased compounds in therapeutics is a highly important strategic change that has been validated by a number of physiological observations. For instance, the vitamin niacin is an agonist for GPR109A that lowers elevated levels of triglycerides through heterotrimeric G proteins. However, β-arrestin 1-mediated signaling via GPR109A mediates nicotinic acid-induced flushing, which is an inconvenient and limiting side effect.[5] β-Arrestin signaling also mediates parathyroid hormone's positive anabolic effects on bone mineralization, whereas its G protein activation accounts for unwanted bone resorption.[6] Similarly, diabetes insipidus can result from a mutation in the DRY motif arginine of the human vasopressin receptor (V2R) that selectively prevents the G protein signal while facilitating receptor−β-arrestin interaction.[7]

The zebrafish miles apart m^{93} mutant (Mil or mil^{m93}), identified in the course of a large mutagenesis screen for genes regulating cardiovascular development, contains the same arginine to histidine DRY motif substitution in sphingosine 1-phosphate receptor 2 (S1p2, formerly known as EDG5) that is found in the V2R.[8,9] The miles apart receptor S1p2 displays deficiencies in the activation of MAP kinases,[9] and on the physiological level, the mutation in the zebrafish prevents the lateral migration of cardiac precursor cells to the midline.[10] The two heart fields do not fuse as a result, producing a so-called cardia bifida in which two heart tubes form rather than a single one. Similar phenotypes exist in zebrafish transporter mutants for extracellular matrix sphingolipids, precursors of the endogenous ligand of S1p2, sphingosine 1-phosphate

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(S1P).[11,12] Taken together, these data indicate that the Mil phenotype is the result of a nonfunctional receptor.

The third transmembrane DRY motif is one of the most conserved GPCR domains, and mutations within it alter receptor stability and signaling. Aspartic acid substitution can produce a constitutively active G protein signaling mutant,[13−15] and arginine substitution can result in other unanticipated biochemical behaviors. This is because the DRY motif arginine forms an ionic lock with the sixth transmembrane domain[16] and by stabilizing receptors in an inactive conformation[13,17] uncouples them from their heterotrimeric G protein.[7,13,18] Quite unexpectedly, arginine to histidine substitution can induce basal receptor internalization associated with diabetes insipidus in the V2R,[7,19−21] and the R122C allele of the human P2Y12 receptor is associated with chronic bleeding.[20] Furthermore, the DRY motif mutation of drosophila Tre1 receptors impairs germ cell migration.[22]

An inability of DRY motif receptor mutants to couple to heterotrimeric G proteins may potentially result from constitutive agonist-independent receptor hyperphosphorylation,[19] and likely kinase candidates underlying this behavior are G protein-coupled receptor kinase (GRK) family members. The seven vertebrate GRKs fall into three groups, the visual kinases GRK1 and -7, and the subfamilies composed of GRK4−6 and GRK2 and -3.[23] GRK2 is the most widely expressed GRK and is an important regulator of adult cardiac physiology[24] and vertebrate heart development.[25,26] In this study, we show that GRK2-regulated S1p2-biased signaling influences the cardia bifida found in Mil zebrafish. We show that the S1p2 R150H receptor mutant is a constitutively desensitized and internalized receptor that is uncoupled from G proteins but not β-arrestins. Inhibition of GRK2 in Mil zebrafish can restore S1p2 R150H surface localization and partly rescue the associated cardia bifida phenotype.

MATERIALS AND METHODS

Drugs and Antibodies. S1P was purchased from Cayman Chemicals and JTE-013 from Sigma-Aldrich. Mouse anti-ERK 1/2 (1:1000, clone 3A7), rabbit anti-phospho-ERK1/2 (T202/Y204) (1:1000, clone D13.14.4E), rabbit anti-GFP (1:200, clone D5.1), rabbit anti-HA tag (1:1000, clone C29F4), rabbit anti-p38 (1:1000, catalog no. 9212), rabbit anti-phospho-p38 (T180/Y192) (1:1000, clone D3F9), rabbit anti-AKT (1:1000, clone C67E7), and rabbit anti-phospho-AKT (S473) (1:1000, clone D9E) antibodies were obtained from Cell Signaling. The mouse anti-myc antibody (1:800, clone 9E10) was from Santa Cruz Biotechnology, and the mouse anti-HA antibody (1:1000, clone 12CA5) was from Roche. Rabbit anti-Flag (1:1000, clone F-7425), mouse anti-β-actin (1:10000, clone A1978), and rabbit anti-β-catenin (1:1000, clone C2206) antibodies were from Sigma-Aldrich. Goat anti-mouse Alexa 488 (1:1000), goat anti-rabbit Alexa 488 (1:1000), and goat anti-rabbit Alexa 568 (1:1000) antibodies were purchased from Life Technologies. Donkey anti-mouse IRDye800CW (1:20000) and donkey anti-mouse IRDye680LT (1:20000) were bought from LI-COR Biosciences.

Plasmids. Zebrafish S1p2 was amplified from cDNA of 24 hpf (hours postfertilization) zebrafish embryos and cloned into pCS2+ via ClaI and StuI restriction sites with a HA tag at the N-terminus of S1p2. To obtain S1p2 R150H as well as S1p2 R167C, site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). C-Terminally truncated versions of S1p2 and S1p2 R150H were cloned into pCS2+ from polymerase chain reaction (PCR) products resembling amino acids 1−308. β-Arrestin 1 was amplified from mouse cDNA and cloned with an additional Flag tag using the pcDNA3.1 TOPO TA Expression Kit (Life Technologies). Strawberry-tagged β-arrestin 2 and myc-tagged βARKct were kind gifts of S. Marion and B. Moepps, respectively.

Cell Culture. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, and a 0.75% $NaHCO_3$ (all obtained from Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO_2.

Internalization Experiments. Transiently transfected HEK 293T cells (as indicated in the figures) were seeded onto glass bottom dishes for subsequent antibody staining and confocal or epifluorescence microscopy (Greiner). RNAi against β-arrestin 2 was achieved through transfection of an siRNA against human β-arrestin 2 (FlexiTube siRNA no. SI02776928, Qiagen) and Lipofectamine RNAiMax (Life Technologies). In brief, cells were fixed for 30 min at room temperature using 4% buffered paraformaldehyde. After a short rinse in PBS, HA-tagged receptors were visualized using a rabbit anti-HA antibody (1:1000, Cell Signaling) and an Alexa-labeled secondary antibody (1:1000, Life Technologies). Flag-tagged β-arrestin 1 was probed with a rabbit anti-Flag tag antibody (1:1000, Sigma-Aldrich). To prevent bleaching, cells were mounted in Vectashield containing Dapi (Vectorlabs). Subcellular localization of receptors was assessed on a Leica SP5 confocal microscope or a Zeiss Axiophot equipped with a Hamamatsu ORCA-03G camera. Arrestin recruitment was quantified using ImageJ.[27]

Western Blot Analysis. HEK 293T cells were transiently transfected with plasmids encoding wild-type S1p2 and S1p2 R150H using Attractene (Qiagen). Twenty-four hours after transfection, cells were serum-starved overnight and then stimulated for 2−90 min using 0.5−1 μM S1P. Lysis was performed using a sodium dodecyl sulfate (SDS)-based buffer [2% SDS and 50 mM Tris (pH 6.8)] containing protease and phosphatase inhibitors (Roche). Equal amounts of protein were separated using NuPage 4 to 12% Bis-Tris gels (Life Technologies) and transferred to nitrocellulose membranes (Bio-Rad Laboratories). After being blocked in 5% nonfat milk in TBS containing 0.05% Tween 20, blots were incubated with primary antibodies overnight. Secondary antibodies that were used contain labels for near-infrared detection with a LI-COR Odyssey SA system. Signals were quantitated using LI-COR Studio-Lite version 3.1.

RhoA Activation Assay. HEK 293T cells were transfected with S1p2 and S1p2 R150H. One day after transfection, cells were serum-starved for 42 h. The RhoA activation assay was performed after stimulation for 3 min with 1 μM S1P according to the manufacturer's instructions (Cell Signaling).

Zebrafish Husbandry and Manipulation. Zebrafish were kept under standardized conditions in a circulating water tank system (Tecniplast). All husbandry and the experiments described were approved by the local authorities at Duke University and Ulm University. Wild-type strains used were AB and EK. The miles apart m^{93} mutant was a kind gift of D. Stainier.[8] Sox17-GFP transgenic fish for visualizing the endoderm have been described previously.[28] Embryos homozygous for the miles apart locus were generated by heterozygous mating and genotyped after processing. To do so, a 397 bp fragment was amplified from genomic DNA using

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Table 1. qPCR Primers and Probes

transcript	species	left primer (5′−3′)	right primer (5′−3′)	universal probe
ARR B2	*Homo sapiens*	gtggctcaactcgaacaaga	gagcagtggggttatggtgt	14
CTGF	*H. sapiens*	cctgcaggctagagaagcag	tggagattttgggagtacgg	85
CYR61	*H. sapiens*	aagaaacccggatttgtgag	gctgcatttcttgcccttt	66
GAPDH	*H. sapiens*	ccccggtttctataaattgagc	caccttccccatggtgtct	63
ctgf	*Danio rerio*	ccacaagaagacaccttccag	caggctggagaccgtgag	5
eef1a1	*D. rerio*	ccttcgtcccaatttcagg	ccttgaaccagcccatgt	67
fibronectin	*D. rerio*	ggagaacgctctacgcaaac	aggtctggtctcaggcactc	47
gapdh	*D. rerio*	caggcataatggttaaagttggta	catgtaatcaaggtcaatgaatgg	147
itgb1a	*D. rerio*	tccaagatcacctcagacttca	tccagtgcagggatttagaag	19
itgb1b	*D. rerio*	ccatcatgcaagtggctgt	cgtccgtggaaaacacaag	19
itgav	*D. rerio*	tctggctggactgctgct	ccgaacgcgattgaagaa	21
itga11	*D. rerio*	agagaggccggtcagacata	gataatccagatgggaacacg	101

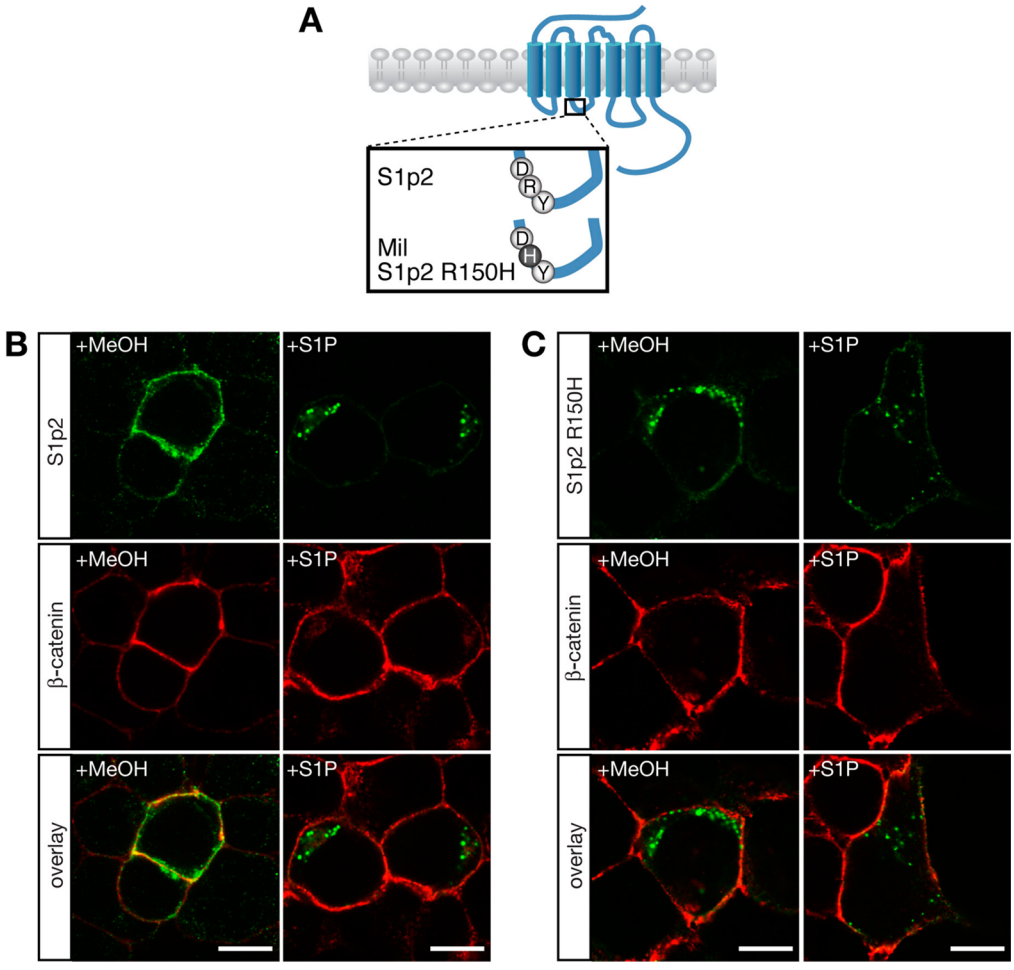

Figure 1. S1p2 R150H is internalized in the absence of agonist. (A) Cartoon illustrating the DRY motif mutation of the miles apart m[93] S1p2 Mil receptor, in which arginine 150 is substituted with histidine. (B) Confocal images of HEK 293T cells expressing HA-S1p2. The wild-type receptor (green) is internalized after stimulation for 20 min with 1 μM S1P, causing a punctate pattern within the cytoplasm. Control cells treated with vehicle (MeOH) still showed S1p2 expression at the membrane, which was counterstained for β-catenin (red). (C) The Mil receptor (green) is already localized in endocytotic vesicles under basal conditions, which does not change upon further stimulation with S1P. Representative images of two independent experiments are shown. Scale bars are 10 μm.

the following primers: 5′-CAA ACA AAG CTT GAG CTC CC and 5′-AAG AAT GGA CAT CAC CCA GC. The PCR product was subsequently digested with Bsp1286I, which yields bands of 324, 61, and 12 bp for wild-type alleles and 225, 99, 61, and 12 bp for m[93] alleles. For knockdown studies, fertilized eggs were injected with a translation blocking antisense morpholino oligonucleotide (MO) targeted against Grk2/3 (Grk2/3 MO). All experiments were controlled against embryos, which were injected with a five-base mismatch control MO (5mis MO). Both MOs were tested as described previously.[26,29,30]

Quantitative Polymerase Chain Reaction (qPCR). Transfected HEK cells for qPCR analysis were processed 60 h post-transfection. For Yap target analysis, transfected HEK

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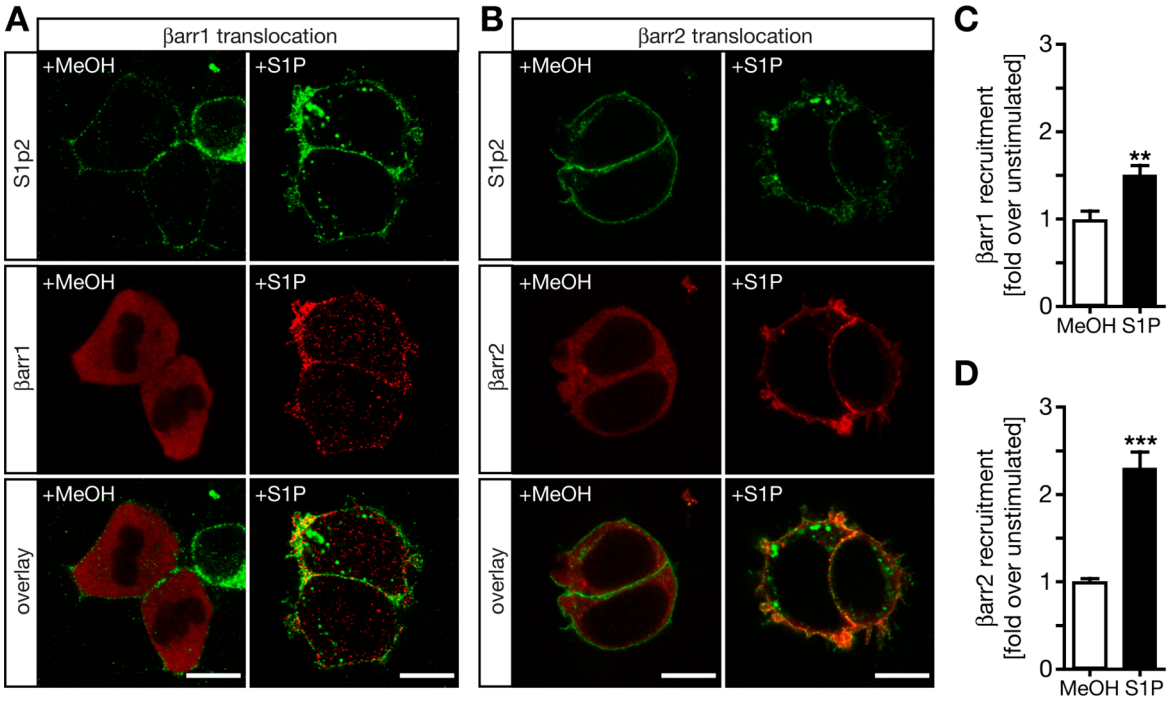

Figure 2. S1p2 predominantly recruits β-arrestin 2. (A) Confocal images of HEK 293T cells cotransfected with HA-S1p2 (green) and flag-tagged β-arrestin 1 (red). Cells were stimulated for 20 min with 1 μM S1P to induce receptor internalization and β-arrestin translocation. Representative images of four experiments are shown. Scale bars are 10 μm. (B) β-Arrestin 2-strawberry (red) recruitment upon S1P stimulation of S1p2 (green). Both β-arrestins are recruited to the plasma membrane after stimulation for 20 min with 1 μM S1P, although the translocation was more profound in the case of β-arrestin 2. Representative images of five experiments are shown. Scale bars are 10 μm. (C) Quantification of β-arrestin 1 recruitment upon stimulation with 1 μM S1P. n = 16–17 cells. p = 0.0018 (two-tailed Student's t test). (D) Quantification of β-arrestin 2 recruitment after stimulation with 1 μM S1P. n = 16–19 cells. $p < 0.0001$ (two-tailed Student's t test).

cells were stimulated for 2 h with 0.5 μM S1P after overnight serum starvation. Grk2/3 knockdown and control embryos were allowed to develop at 28.5 °C to the desired stages before total RNA was isolated using RNeasy mini columns (Qiagen). Mil^{m93} embryos for Yap target gene analysis were singly genotyped, and RNA was isolated from pools of 10 embryos. Equal amounts of RNA were reverse transcribed into cDNA using oligodTTP primers and SuperScript III reverse transcriptase (Life Technologies). qPCR was performed in an ABI PRISM 7500 Sequence Detection system (Applied Biosystems) or a LightCycler 480 (Roche) with the Absolute QPCR ROX Mix from ABGene (Thermo Scientific) and the Universal Probe System (Roche). All primers and probes are given in Table 1.

***In Situ* Hybridization.** *In situ* hybridization was conducted according to standard protocols[31] with *in vitro*-transcribed, DIG-labeled RNA probes. Probes used were *cardiac myosin light chain 2* (*cmlc2*, kind gift of K. Poss), *grk2/3*,[30] *βarrestin 1* (*arrb1*, GenBank accession number NM_001159822.1), *βarrestin 2b* (*arrb2b*, GenBank accession number NM_201124.1), and *s1p2* (GenBank accession number NM_001159970.1). Stained embryos were photographed using a Leica MZ125 with an IC80 HD camera setup. To show endodermal expression of *s1p2*, *grk2/3*, *arrb1*, and *arrb2*, *in situ* hybridization was performed on Sox17-GFP embryos, which were subsequently cut into 20 μm thick sections on a Leica Jung Frigocut 2800N cryostate. The GFP fluorescence was enhanced by antibody staining, and images were taken on an Olympus BX 60 microscope equipped with an Olympus DP72 camera.

Statistical Analysis. All graphs display means ± the standard error of the mean (SEM). Data were analyzed using GraphPad Prism 4 and 5.

RESULTS

The S1p2 R150H Mil Receptor Is Constitutively Internalized. The Mil mutant contains a histidine substitution at position 150 (Figure 1A).[9] Some GPCRs carrying mutations of this arginine localize constitutively to endocytotic vesicles,[21] whereas receptors with an intact DRY motif normally are found at the cell surface under basal conditions. In fact, wild-type S1p2 for the most part localizes to the plasma membrane (Figure 1B and Figure S1A of the Supporting Information), and it internalizes when stimulated with its orthosteric agonist S1P. In line with the general paradigm of R to H DRY motif mutants, S1p2 R150H receptors are found predominantly in cytoplasm vesicles, and stimulation by S1P does not alter this distribution (Figure 1C and Figure S1B of the Supporting Information). In contrast to that, S1p2 R167C, another mutation rendering cardia bifida in zebrafish, localized to the plasma membrane and remained there even after agonist stimulation (Figure S2 of the Supporting Information).

Because β-arrestins are crucial mediators of GPCR internalization,[32] we wondered if S1p2 had a preference for β-arrestin 1 or 2. Wild-type S1p2 recruits both arrestins to the cell surface in a model system (Figure 2A,B and Figure S3 of the Supporting Information), but recruitment is more robust with β-arrestin 2 (Figure 2C,D and Figure S3B of the Supporting Information). We thus hypothesized that an arginine to histidine mutation resulting in constitutive receptor desensitization *in vivo* most probably involves β-arrestin 2.

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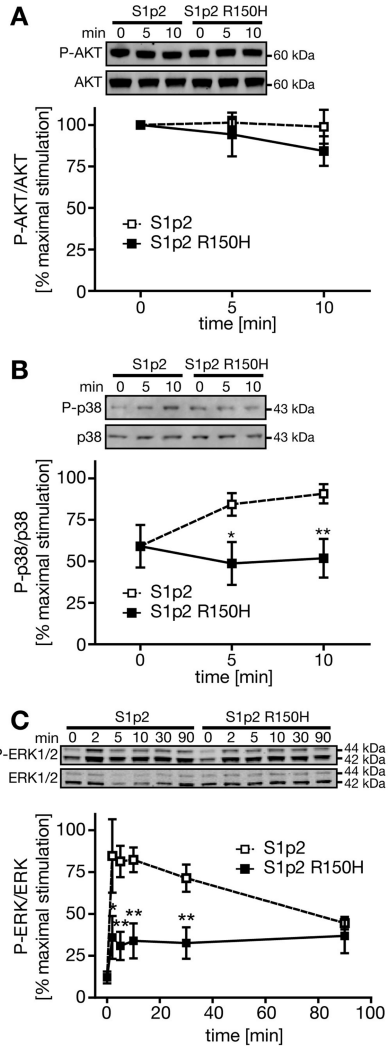

Figure 3. S1p2 R150H is still able to signal at reduced levels. (A) Western blot for AKT phosphorylation at serine 473. AKT phosphorylation could not be observed in either S1p2 or S1p2-transfected cells upon stimulation with 0.5 μM S1P. p = 0.3173 (5 min), and p = 0.1714 (10 min) (one-tailed Student's t test). The graph summarizes three independent experiments. (B) Time course of p38 MAPK phosphorylation in S1p2- and S1p2 R150H-expressing cells obtained by Western blotting. S1P (1 μM) mildly but robustly induces p38 MAPK phosphorylation at threonine 180 and tyrosine 192. p = 0.0203 (5 min), and p = 0.0086 (10 min) (one-tailed Student's t test). The graph displays means ± SEM of five independent experiments. (C) Western blot of ERK1/2 phosphorylation after stimulation for 2, 5, 10, 30, and 90 min with 0.5 μM S1P. S1p2-transfected cells show the expected initial peak and then sustained phosphorylation at threonine 202 and tyrosine 204. Cells transfected with S1p2 R150H lack the G protein-dependent initial rise in the level of ERK1/2 activation. The graph displays data as means ± SEM (n = 5). p = 0.0459 (2 min), p = 0.0020 (5 min), p = 0.0019 (10 min), p = 0.0072 (30 min), and p = 0.2545 (90 min) (one-tailed Student's t test). Representative blots for all experiments are shown.

The R150H Mutation Specifically Abrogates G Protein-Mediated Signaling. GPCRs can initiate MAP kinase phosphorylation cascades upon ligand activation. Research over the past decade indicates this activation occurs in at least two ways. Classically, GPCRs provoke a rapid spike of a short duration in MAP kinase activation via heterotrimeric G proteins.[33] Alternatively, arrestins can initiate the MAP kinase cascade noncanonically and independently of G protein signaling (for a review, see ref 34), which, however, is relatively prolonged. S1P through the S1p2 is reported to activate p38 MAPK as well as p42/44 ERK, but not AKT.[34,35] We thus tested whether S1p2 R150H would be fully functional in these pathways. S1P stimulation did not trigger AKT phosphorylation in cells transfected with either wild-type receptor S1p2 or S1p2 R150H (Figure 3A), but it induced a robust phosphorylation of p38 MAP kinase in cells transfected with the wild-type receptor. In contrast, we saw a significant decrease

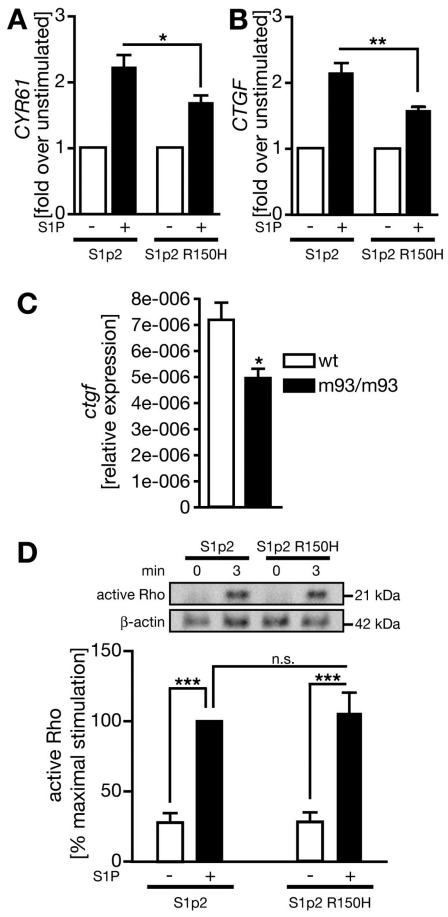

Figure 4. S1p2 R150H displays reduced Hippo signaling despite normal activation of RhoA. (A) The relative level of expression of Yap target gene *CYR61* is lower in S1p2 R150H-expressing cells. HEK cells were transfected with the wild-type S1p2 or S1p2 R150H receptor, serum-starved overnight, and stimulated for 2 h with 0.5 μM S1P. Gene expression was analyzed using qPCR. The graph displays data as means ± SEM (n = 4). p = 0.0201 (one-tailed Student's t test). (B) Relative expression of Yap target gene *CTGF* in HEK cells treated and analyzed as described for panel A. The graph shows means ± SEM (n = 4). p = 0.0048 (one-tailed Student's t test). (C) Bar graph depicting the expression of *ctgf* normalized to *eukaryotic elongation factor 1A1* (*eef1a1*) in 17−20 ss zebrafish embryos. *ctgf* was significantly reduced in embryos homozygous for the mil^{m93} mutation. Data are shown as means ± SEM (n = 4). p = 0.0106 (one-tailed Student's t test). (D) RhoA activation assay upon stimulation with 1 μM S1P. HEK cells were transfected with either receptor variant. Twenty-four hours post-transfection, cells were serum-starved for an additional 42 h before they were stimulated and processed for the RhoA activation assay. Representative blots of four independent experiments are shown. The bar graph displays RhoA signals normalized to β-actin as means ± SEM. Three asterisks indicate a p value of <0.001, and n.s. denotes a p value of >0.05. One-way ANOVA with Bonferroni post-test.

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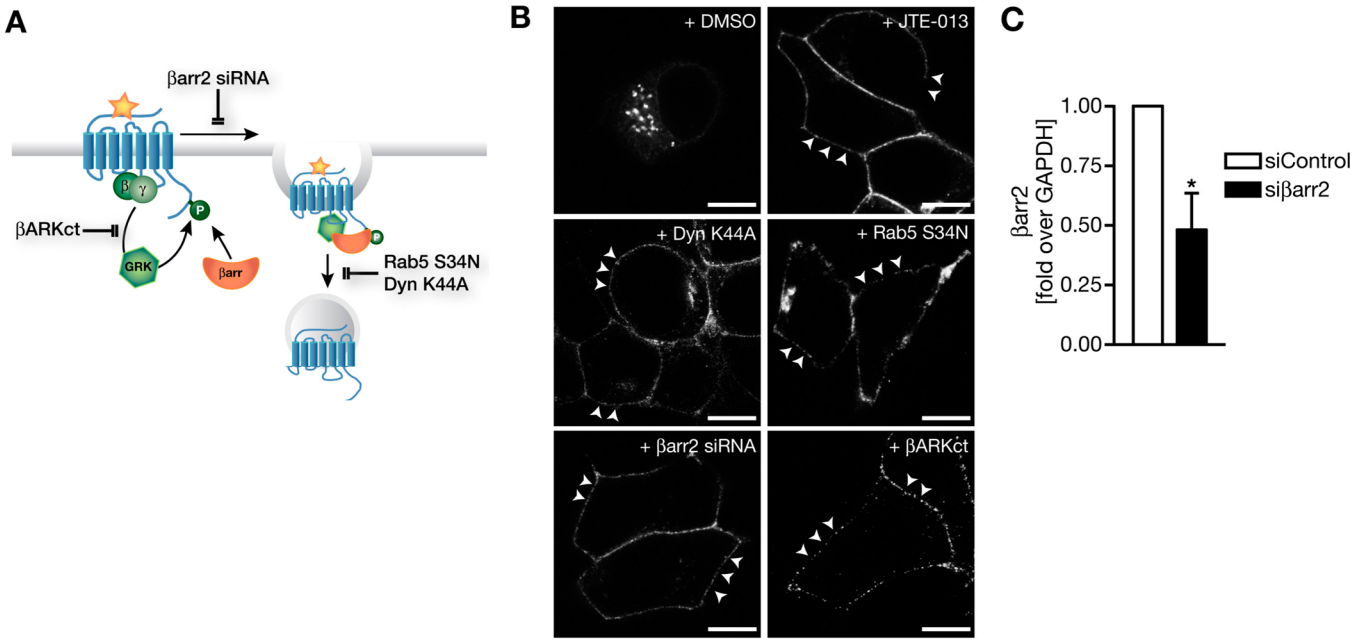

Figure 5. Inhibition of internalization restores S1p2 R150H surface expression. (A) General mechanism of the desensitization process. Upon receptor activation, $\beta\gamma$ subunits of the heterotrimeric G protein recruit GRKs that phosphorylate the C-terminus of the receptor. β-Arrestins translocate to the phosphorylated receptor and initiate internalization. This receptor desensitization can be blocked by blocking GRKs or β-arrestins, or by introducing dominant negative mutants of proteins involved in the internalization process such as Rab5 S34N and DynK44A. (B) Fluorescence images of HEK 293T cells showing the relocation of HA-tagged S1p2 R150H upon different treatments as described in panel A. As a control, intracellular localization of S1p2 R150H treated with 1% DMSO is shown in the top left panel. Treatment with the S1p2 specific inhibitor JTE-013 at 10 μM induced relocation of the receptor to the cell surface (n = 3) (top right). Similarly, cotransfection of either dynamin K44A (n = 4) or Rab5 S34N-GFP (n = 4) resulted in cells with S1p2 R150H being localized at the plasma membrane (middle). siRNA-mediated knockdown of β-arrestin 2 also caused relocation back to the membrane (n = 4) (bottom left). Control cells, which were transfected with scrambled siRNA, displayed S1p2 R150H predominantly in vesicles (data not shown). Transfection of βARKct, which functions as a scavenger for $\beta\gamma$ subunits and thus prevents recruitment of endogenous GRK2, also caused relocalization of S1p2 R150H to the membrane. Arrowheads always indicate surface expression of S1p2 R150H. Scale bars are 10 μm. (C) Verification of β-arrestin 2 knockdown using qPCR. The bar graph displays means ± SEM and summarizes three independent experiments. p = 0.0216 (one-tailed Student's t test).

in the ability of mutant receptor to induce p38 MAP kinase phosphorylation (Figure 3B), but it still supported a reduced level of activation of p42/44 ERK. Time course analysis further revealed the initial short duration G protein component was absent (Figure 3C), whereas the longer duration β-arrestin-mediated portion remained, indicating that S1p2 R150H ERK signaling persists (via mechanisms that should include β-arrestins).

The R150H Mutation Partially Abrogates Hippo Signaling. Another way to assess G protein-dependent signaling of S1p2 is through the analysis of Hippo pathway activity, which depends on S1p2-mediated activation of RhoA via G12/13 proteins.[36,37] This induces the release of the transcriptional activator Yap from blockage by Lats1/2 kinases for subsequent translocation to the nucleus. Here, it induces the transcription of Hippo target genes such as *connective tissue growth factor* (*ctgf*) and *cysteine-rich angiogenic inducer 61* (*cyr61*).[37,38] Very recently, it was shown that functional Yap and induction of CTGF are required for cardiac precursor migration.[36,39] We thus hypothesized that S1p2 R150H-transfected cells are likely to show a reduction in Hippo pathway activity if the Mil mutation is unable to execute G protein-dependent signal transduction. In fact, when we analyzed the expression of *CTGF* and *CYR61* in cells, we found a significant reduction in the level of Hippo signaling (Figure 4A,B). Additionally, a similar decrease in *ctgf* could be detected in mil^{m93} embryos (Figure 4C). However, the effects in cells were not due a reduction in the level of RhoA activation (Figure 4D), which may be explained by the fact that many GPCRs can activate RhoA also through arrestin.[40−42]

Restoration of S1p2 R150H Surface Expression. Receptor internalization is a multistep process that begins with ligand-activated receptor phosphorylation. Arrestins recognize phosphorylated receptors and target them to clathrin-coated pits for internalization.[43] This process can be inhibited on several levels as shown in Figure 5A. Previous studies by us and by our colleagues demonstrate that arginine DRY motif mutants can be rescued back to the cell surface by treatment with antagonists and inverse agonists.[19,44]

To verify if this occurred for the Mil, we exposed cells containing the mutant receptor to the S1P2 antagonist JTE-013 and observed a significant though incomplete receptor redistribution to the plasma membrane (Figure 5B). To test that the intracellular localization of S1p2 R150H occurred with plasma membrane transit, we utilized approaches involving dynamin and Rab5. Dynamin is a small GTPase that regulates scission of clathrin vesicles from the plasma membrane,[45] and expression of dynamin K44A, a dominant negative mutant, impedes scission and blocks endocytosis.[46,47] Rab5 mediates trafficking of the receptor into early endosomes,[48] and overexpressed dominant negative Rab5 (Rab5 S34N) blocks this step.[49] Co-expression of either dominant negative protein restored surface expression of S1p2 R150H (Figure 5B, middle).

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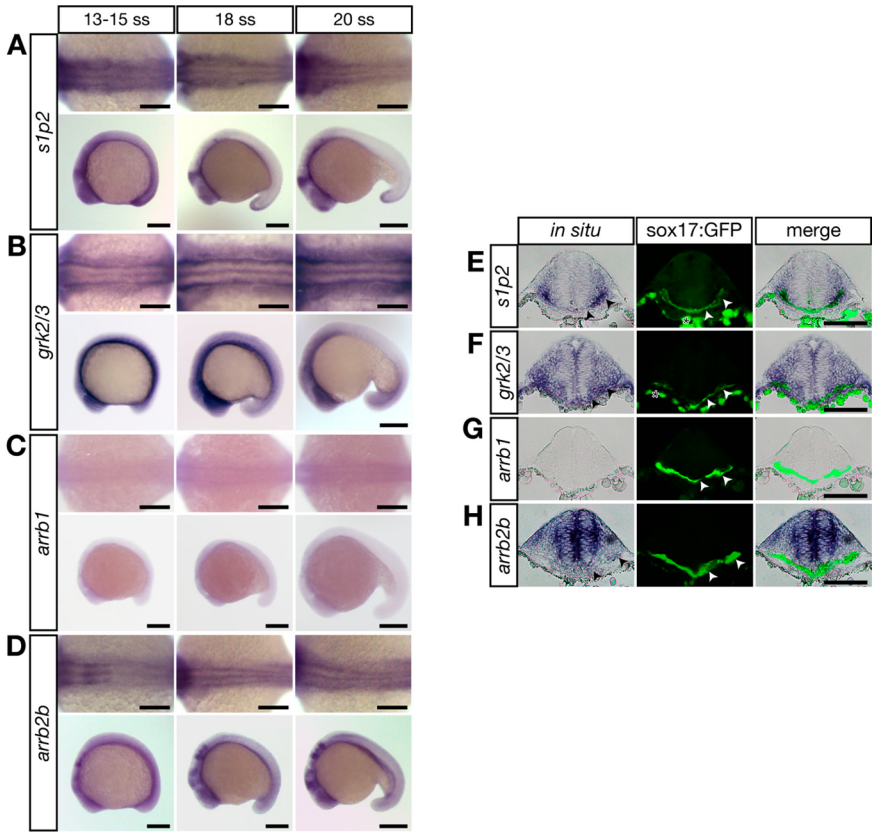

Figure 6. S1p2, Grk2/3, and β-arrestin 2 are expressed in a similar fashion during heart migration stages. (A–D) Dorsal and lateral views of expression of *s1p2*, *grk2/3*, *arrb1*, and *arrb2b* in zebrafish embryos. Transcripts were labeled by whole mount *in situ* hybridization (*in situ*) at the 13–15 somite stage (ss), the 18 ss, and the 20 ss. For all embryos in panels A–D, the anterior is to the left. Scale bars are 200 μm. (A) *s1p2* is expressed in cardiogenic tissues lateral to the midline at all three stages analyzed. In addition, *s1p2* is most abundant in the encephalic region at the 13–15 ss and the forebrain as well as midbrain–hindbrain boundary at later stages. (B) *grk2/3* is characterized by a low level of ubiquitous expression, which is stronger laterally to the neural tube. In addition, higher levels can be observed in the developing eye. (C) *β-arrestin 1* appears to be expressed only weakly, if at all. (D) *β-arrestin 2b* displays an expression pattern similar to those of *s1p2* and *grk2/3*. In the dorsal view, ubiquitous expression also lateral to the midline can be detected as well as in several areas of the developing brain such as hindbrain rhombomeres, forebrain, and midbrain. (E) Transverse section showing *s1p2* expression in sox17-GFP embryos, in which the endoderm is colored green. Arrows indicate the endoderm. Asterisks indicate remnants of the yolk that shows autofluorescence. Eighteen ss. The scale bar is 100 μm. (F) *grk2/3* mRNA is strongly expressed in the endoderm. Eighteen ss. The scale bar is 100 μm. (G) *β-arrestin 1* is absent from the endoderm. Eighteen ss. The scale bar is 100 μm. (H) *β-arrestin 2b* is strongly expressed in the neural tube but can be also detected in endodermal cells. Eighteen ss. The scale bar is 100 μm.

We next tested whether decreased β-arrestin activity would similarly increase the level of Mil surface localization (Figure 5B, bottom left), and because the translocation of β-arrestin 2 to S1p2 is more robust than β-arrestin 1 recruitment, we knocked down β-arrestin 2 using siRNA (Figure 5C). This resulted in the rescue of a substantial amount of mutated receptor back to the plasma membrane (Figure 5B, bottom left). Co-expression of βARKct, which functions as a scavenger for βγ subunits[50] and lowers the level of receptor phosphorylation,[51] acted in a manner similar to reduction of arrestin activity (Figure 5B, bottom right). Thus, the cytosolic localization of S1p2 R150H is dependent on GRK2 and β-arrestin 2. Finally, we tried to delete the C tail of the receptor to prevent GRK-mediated phosphorylation of S1p2 R150H; however, we did not observe membrane localization. As neither C-terminally truncated wild-type receptor localized to the cell surface, we concluded that the truncation also prevented correct trafficking of S1p2 receptors (Figure S4).

Similar Expression of GRK2 and β-Arrestin 2 with S1p2 in Zebrafish. To investigate whether the Mil cellular results extended to zebrafish, we compared the expression pattern of S1p2 (Figure 6A), Grk2/3 (which is the combined homologue of mammalian GRK2 and -3) (Figure 6B), and the two β-arrestins (Figure 6C,D). We monitored the expression during developmental stages, when heart field migration takes place,[52] because the miles apart mutant develops cardia bifida because of a deficiency in the lateral movements of cardiac precursors.[10] Both Grk2/3 and β-arrestin 2 show a distribution similar to that of S1p2, and all three genes are expressed bilaterally to the midline in the region of the heart fields. Furthermore, as S1p2 functions in the endoderm rather than in the migrating heart progenitors,[36] we tested for endodermal expression of Grk2/3 and β-arrestin. With the help of a transgenic line expressing GFP in the endoderm, we confirmed that S1p2, Grk2/3, and β-arrestin 2 are expressed in the endodermal cells, which provide the basis for heart field migration[10,36,53] (Figure 6E,F,H). In contrast, β-arrestin 1 is barely detectable (Figure 6C,G). This result and our cell culture experiments indicate that β-arrestin 1 is an unlikely candidate for mediating abnormal S1p2 R150H internalization.

Loss of Grk2/3 Partially Restores Heart Tube Formation in Miles Apart Zebrafish. Our results to this point indicated that rescue of the mil^{m93} zebrafish phenotype may be achieved by inhibiting receptor phosphorylation. We

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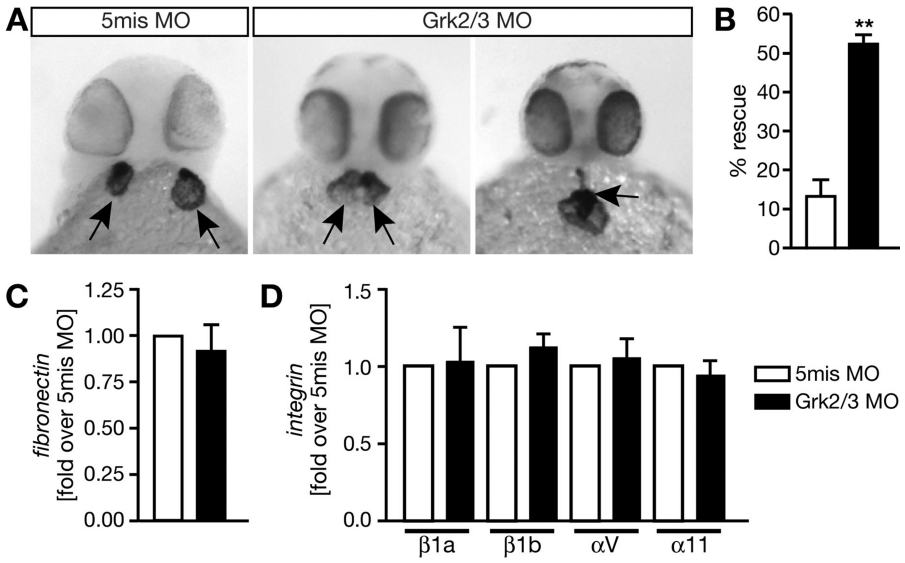

Figure 7. Loss of Grk2/3 ameliorates cardia bifida in miles apart zebrafish. (A) MO-mediated knockdown of Grk2/3 produces miles apart hearts, which are very close to being or have partially fused. Arrows indicate hearts, which were stained for *cardiac myosin light chain 2* (*cmlc2*). The scale bar is 100 μm. (B) Quantitative analysis of Grk2/3 knockdown experiments. Hearts, which were either fused or spatially very close, were counted as rescue. The bar graph displays means ± SEM and summarizes three independent experiments. p = 0.0013 (two-tailed Student's t test). (C) Knockdown of Grk2/3 does not alter *fibronectin* mRNA levels. qPCR of wild-type embryos shows no difference between control and knockdown conditions. n = 3. p = 0.6142 (two-tailed Student's t test). (D) Knockdown of Grk2/3 does not change the expression of integrin subunits, which serve as receptors for fibronectin. Integrin levels were measured in wild-type embryos injected with either 5mis MO or Grk2/3 MO using qPCR. n = 3−8. p = 0. 0.8931 (*itgb1a*), p = 0.0879 (*itgb1b*), p = 0.6668 (*itgav*), and p = 0.5415 (*itga11*) (one-tailed Student's t test). In panels B−D, the white bar always indicates injection with 5mis MO whereas the black bar illustrates Grk2/3 MO injections.

thus injected clutches of heterozygous mil^{m93} crossings with a translation-blocking morpholino targeted against Grk2/3 (Grk2/3 MO), previously validated for its efficiency in blocking Grk2/3 translation.[29,30] As a control, we utilized a five-base mismatch MO (5mis MO). *In situ* hybridization to visualize the heart and subsequent genotyping revealed that Grk2/3 depletion is able to partially re-establish heart field migration. Half of the homozygous mil^{m93} mutants under this Grk2/3 MO treatment displayed either two hearts, which were very close together or even fused (Figure 7A,B). Embryos treated with the 5mis MO still developed two hearts that appeared miles apart.

It was reported that miles apart mutants can be rescued by injection of fibronectin into the area between the migrating heart fields.[53] Thus, the rescue obtained by GRK2 inhibition could potentially result from an increase in fibronectin content or better usage of it. qPCR analysis of wild-type embryos injected with Grk2/3 MO, however, did not show upregulation of *fibronectin* transcription (Figure 7C). We also did not see a significant change in integrin subunits (Figure 7D), which are expressed during stages, when cardiac progenitor cells migrate.[54−56] These data support our hypothesis that constitutive receptor desensitization underlies cardia bifida in mil^{m93} embryos.

DISCUSSION

Cardia bifida is a severe developmental defect that is incompatible with early life, and Mil embryos die between 4 and 6 days postfertilization. In the process, they develop two separate heart tubes that are most probably not connected to the appropriate vasculature.[8−10,53] This abnormal morphology results from a failure of the bilateral heart primordia to migrate straight to the midline while simultaneously heart progenitor cells remain competent to undergo angular movements.[10] A number of excellent studies address possible underlying molecular mechanisms that contribute to the phenotype and present elegant strategies for rescuing the S1p2 R150H mutants.[11,53,57] Nevertheless, no one has attempted to directly restore the wild-type phenotype at the level of the mutated receptor. Our data demonstrate that Mil zebrafish S1p2 R150H displays three classical hallmarks of a constitutively desensitized receptor. Foremost, it resides in endocytic vesicles in the absence of an agonist, and this inappropriate localization can be reversed by treatment with an antagonist.[21] Second, S1p2 R150H is unable to initiate rapid G protein-dependent signaling, as cells expressing it lack early G protein activation of ERK1/2. Last, the Mil receptor is most probably hyperphosphorylated, and inhibition or depletion of the endogenous GRK2 pool restores S1p2 R150H surface expression and ameliorates cardia bifida in homozygous zebrafish mutants.

However, we found that S1p2 R150H is not completely nonfunctional in signaling. While verifying a Jurkat cell finding in HEK 293T cells that it leads to the loss of p38 MAPK activation,[9] we were able to establish that it still can activate ERK1/2,[35] though less well than wild-type S1p2. This is intriguing, as receptor internalization is considered to be an efficient way to terminate receptor signaling and only a handful of reports support G protein-mediated GPCR signaling from intracellular compartments.[58−61] The level of ERK1/2 activation through G proteins increases and decreases rapidly, limiting the duration of ERK1/2 signaling to only a few minutes. β-Arrestin-induced ERK1/2 activity in general, however, occurs more slowly and is much more prolonged,[28,47−49] similar to what we observed for S1p2 R150H and characteristic of an arrestin bias in the mutated receptor that coincides with extended receptor scaffolding of accessory proteins.[62] Dopamine D3 with an analogous DRY motif mutation also displays a similar β-arrestin phenotypic bias.[63]

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Furthermore, Ye et al. demonstrate that simultaneous knockdown of two Gα13 subunits, to which S1p2 most probably couples, interferes with heart progenitor migration. Their data together with the very recent report of an endodermal requirement of S1p2-mediated, G protein-dependent Hippo signaling[36] nicely emphasize the importance of the G protein branch of S1p2 signaling for the formation of a single heart tube.[57]

Another hallmark of arginine DRY motif mutants is a tendency toward an increased level of phosphorylation by GRKs. Here, the DRY motif mutant of the V2R provides a prime example of phosphorylation occurring in the absence of agonist to receptor conformations that would not otherwise be available except for the mutation.[7,44] This suggested that inhibition or depletion of GRKs should rescue the Mil receptor-associated phenotype. In fact, MO-mediated knockdown of the combined Grk2/3 zebrafish homologue did partially rescue heart formation. GRK2 had been linked to migratory events before, although in different ways depending on the target protein. It has been reported that in the presence of CCR2 GRK2 inhibition enhances monocyte migration,[64] whereas GRK2 phosphorylation of nonreceptor substrates is typically pro-migratory.[65] In the case of cardiac progenitor migration, the endoderm plays a crucial role,[10,36,57] and in particular, extracellular matrix fibronectin that resides between the migrating cells and the endoderm is required for proper migration.[53] Our data indicate that the desensitization proteins β-arrestin 2 and GRK2 function in the endoderm, but most probably, the beneficial effects of zebrafish Grk2/3 knockdown are due to a reduction in the level of receptor desensitization rather than fibronectin, because we did not observe any changes in either fibronectin or the integrins that bind fibronectins. Thus, we propose that constitutive receptor desensitization originating in the GRK/β-arrestin signaling pathway is the underlying cause of the development of cardia bifida in mil^{m93} zebrafish embryos.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Subcellular localization of S1p2 and S1p2 R150H (Figure S1), subcellular distribution of S1p2 R167C (Figure S2), β-arrestin translocation upon S1p2 stimulation (Figure S3), and subcellular localization of C-terminally truncated S1p2 and S1p2 R150H (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.B., M.D.B., T.B., S.M., and M.P. performed experiments. M.B., M.D.B., L.S.B., and M.P. analyzed data. M.G.C., L.S.B., and M.P. designed experiments. M.B., L.S.B., and M.P. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GPCR, G protein-coupled receptor; GRK, GPCR kinase; S1P, sphingosine 1-phosphate; S1p2, sphingosine 1-phosphate receptor 2; mil^{m93}, miles apart m^{93} mutant; Dyn K44A, dynamin K44A; βARKct, C-terminus of GRK2; hpf, hours postfertilization; MO, antisense morpholino oligonucleotide; 5mis MO, five-base mismatch MO; cmlc2, cardiac myosin light chain 2; itgb1a, integrin β1a; itgb1b, integrin β1b; itgav, integrin αv; itga11, integrin α11.

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