# Supporting Information

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### SI Experimental Procedures

**Zebrafish Embryos and Microinjection.** Wild-type zebrafish embryos were obtained from natural crosses of fish with the AB/WT gentic background. A zebrafish GRK2 splicing gene silencer morpholino (5′ ATGGCTGGAAAGAGAGCAAACAAAT 3′; GRK2-MO) was designed to target the 62 bp third exon based on the zebrafish GRK2 sequence (Genbank Accession No. LOC569403) defined by Philipp et al. (1) 5-mismatched morpholino (5′ ATCGCTGCAAACAGACCAAAGAAAT 3′) was used as a control (con-MO).Morpholinos and mRNAs were diluted to appropriate concnetrations prior to injection. MOs and RNAs were injected into the yolk close to the animal pole at the one- or two-cell stage. Embryos were deposited into 24 well plates (≈25 per well) at indicated time points, and 200 embryos were counted for each group. The arrest morphants were scored based on lack of the germ ring formation at 6 hpf, or lack of elongate yolk at 19 hpf. Data obtained from independent injections were pooled. For whole-mount in situ hybridization, a zebrafish GRK2 probe was designed to target 265–1,465 bp of zebrafish GRK2 cDNA. DIG-labeled probes were generated for the following zebrafish genes as described for *ptch1* (2), *pax6a, otx2* and *gata1* (3–5). Whole-mount in situ hybridizations were performed essentially following a standard protocol with minor modifications.

**Yeast Two-Hybrid Screen.** A yeast two-hybrid screening of human brain cDNA library by using full-length bovine GRK2 as bait was performed with Matchmaker 3 Two-Hybrid System (Clontech). Two of 50 independent positive clones were identified to encode human PTCH1 C-terminal sequence (3,803–4,341 bp).

**Plasmid Construction.** Bovine GRK2 expression constructs, including GRK2-Flag, GRK2-GFP, and K220R-Flag have been previously described (6). Bovine GRK2 K220R with a GFP-tag on its C terminus (K220R-GFP), GRK2 catalytic domain with a Flag-tag on its C terminus (amino acid 186–543-Flag), GRK2-PTCH1 binding peptide (amino acids 262–379, BP), and GRK2 mutants Δ245–312, Δ312–379, Δ379–453, and Δ453–521, in which the corresponding residues in catalytic domain were deleted, were constructed by PCR mutagenesis. HA-tagged human cyclin B1 cDNA plasmid was purchased from GeneCopoeia. Cyclin B1 derivatives NLS-cyclin B1, cyc-C (NLS-CRS-Glu), and NLS-B1-Glu were constructed as described previously (7). PTCH1-myc expression plasmid was a kind gift from Dr. Dan J. Donoghue (University of California at San Diego, La Jolla, CA). Full-length zebrafish GRK2 cDNA was obtained by RACE PCR. mRNAs for injection were prepared with an in vitro transcription kit (Ambion). Expression plasmid construction incorporating cDNA templates for bovine GRK2, K220R, BP, D312–379, and NLS-cyclin B1 was carried out as described above.

**Cell Transfection and Immunoprecipitation.** Human embryonic kidney 293 (HEK293) cells were cultured in MEM (Modified Eagle's Medium, GIBCO) containing 10% FBS (FBS; HyClone). Cells were seeded in 60 mm tissue culture dishes at 1–3 × $10^6$/dish 20 h before transfection with 2–5 μg of each plasmid with calcium phosphate precipitation method. Assays were performed 44–48 h after transfection. For immunoprecipitation, cells were washed with ice-cold PBS and lysed in 800 μl Nonidet P-40 solubilization buffer (50 mM Hepes, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 2 mM EDTA, 1 mM NaF, plus 10 μg/ml aprotinin, 10 μg/ml benzamidine, and 0.2 mM PMSF). The following procedures are performed as described previously (6). Myc-tagged receptor was precipitated by using rabbit anti-myc antibody (Sigma)-coated protein A Sepharose beads. Flag-tagged GRK2 and its mutants were precipitated with M2-conjugated Sepharose (Sigma). Lysates and immunoprecipitation complexes were separated and detected by Western blotting. Samples for nuclear extraction were prepared as described previously (2). Rabbit anti-GRK2 antibody (Santa Cruz) was used to detect zebrafish GRK2 in homogenized zebrafish samples. Mouse anti-Flag antibody (Sigma), mouse anti-myc antibody (Invitrogen), and mouse anti-HA antibody (Sigma) were used to detect proteins with the corresponding tags. For immunoblot analysis, blots were incubated with IRDye 800CW-conjugated or 700CW-conjugated antibody (Rockland Biosciences) and infrared fluorescence images were obtained with the Odyssey infrared imaging system (Li-Cor Bioscience).

**In Vitro Interaction Assay.** GST-PTCH1-N (1–502 amino acids), GST-PTCH1-M (503–1,121 amino acids), and GST-PTCH1-C (1,180–1,447 amino acids), based on pGEX-4T-1 and expressed in *Escherichia coli* BL21 (induced with IPTG), were purified by Glutathione Sepharose 4B medium (Amersham Pharmacia) according to the manufacturer's instructions. Cell lysis of HEK293T cells transfected with HA-Flag-GRK2 was incubated with anti-Flag M2 Affinity Gel (Sigma) for 8 h at 4 °C. Beads and captured protein complexes were washed 6 times using TBS (50 mM Tris-HCl, Ph 7.4, 150 mM NaCl), and supernatant with HA-Flag-GRK2 was collected after incubation with 0.1 mM Flag-peptide (Sigma) for 1 h at 4 °C and isolated by using Amicon Ultra-4 centrifugal filter devices (30000NMWL, Millipore). For pull-down assay, equal amounts of the GST fusion proteins were incubated with the Sepharose 4B medium in PBS (PBS) for 1 h at 4 °C, and the beads were washed 4 times with buffer C (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EGTA, 0.1% Nonidet P-40) and resuspended in 200 μl buffer C with 1 μg HA-Flag-GRK2. After 2 h at 4 °C, the beads were washed for 4 times with buffer C, resuspended in SDS/PAGE sample buffer, boiled for 8 min, and analyzed by Western blot.

**In Vivo Cell Cycle Assay.** For phospho-histone H3 (P-H3) and BrdU assays, HEK293 cells were incubated in MEM with 3 μg/ml BrdU (BD Biosciences) for 4 h. After fixation and permeabilization as described above, cells were treated with 2 N HCl at room temperature for half an hour and neutralized with 0.1M pH 8.7 Tris-HCl buffer. Cells were incubated with rabbit anti-*p*-H3 antibody (Santa Cruz) overnight and finally stained with Cy3-conjugated goat anti-rabbit antibody and FITC-conjugated mouse anti-BrdU antibody (BD Biosciences). For in situ cell cycle assays, 24-hpf zebrafish embryos were harvested, dechorionated, and then incubated in 10 mM BrdU in culture medium on ice for 25 min; embryos were quickly rinsed 3 times with BrdU-free medium and placed in BrdU-free medium at culture temperature for 10 min.

1. Philipp M, et al. (2008) Smoothened signaling in vertebrates is facilitated by a G protein-coupled receptor kinase. *Mol Biol Cell* 19:5478–5489.

2. Hogan BM, et al. (2005) Duplicate zebrafish pth genes are expressed along the lateral line and in the central nervous system during embryogenesis. *Endocrinology* 146:547–551.

3. Puschel AW, Gruss P, Westerfield M (1992) Sequence and expression pattern of pax-6 are highly conserved between zebrafish and mice. *Development* 114:643–651.
4. Mori H, Miyazaki Y, Morita T, Nitta H, Mishina M (1994) Different spatio-temporal expressions of three otx homeoprotein transcripts during zebrafish embryogenesis. *Brain Res Mol Brain Res* 27:221–231.
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6. Chen Y, et al. (2008) EGF transregulates opioid receptors through EGFR-mediated GRK2 phosphorylation and activation. *Mol Biol Cell* 19:2973–2983.
7. Barnes EA, Kong M, Ollendorff V, Donoghue DJ (2001) Patched1 interacts with cyclin B1 to regulate cell cycle progression. *EMBO J* 20:2214–2223.

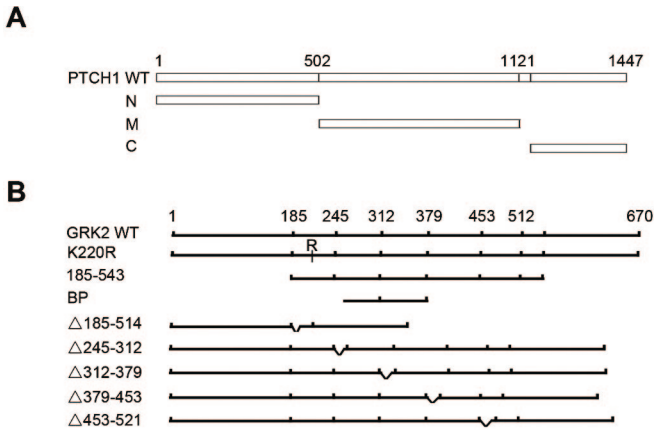


**Fig. S1.** Diagram of PTCH1 and GRK2. (*A*) Diagram of human PTCH1, listing the amino acid residues that define the N-terminal (N), middle (M) and C-terminal (C) segments. (*B*) Diagrams of wild type GRK2, kinase defective GRK2 (K220R) and various deletion constructs used in co-immunoprecipitation experiments.

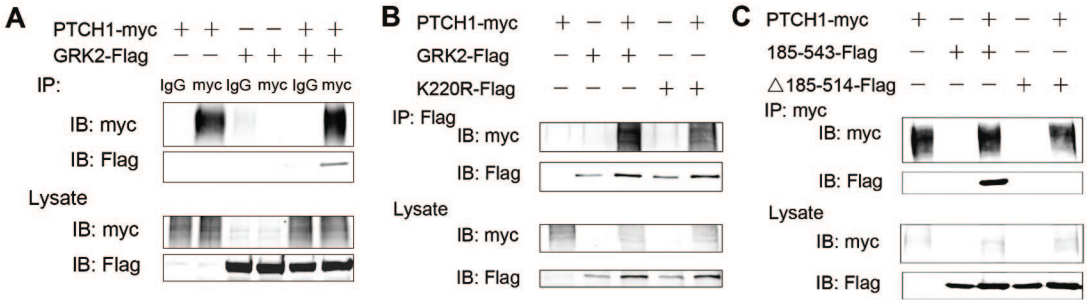


**Fig. S2.** Interaction between GRK2 and PTCH1. (*A*) Interaction between PTCH1 and GRK2 was detected in HEK293 cells transfected with human PTCH1-myc and/or bovine GRK2-Flag cDNA. Lysates were immunoprecipitated with anti-myc antibody. The immunocomplex (*upper panels*) and the input cell lysate (*lower panels*) were detected by Western blotting using the indicated antibodies. (*B*) Cells cotransfected with PTCH1-myc, and GRK2-Flag, or K220R-Flag, were immunoprecipitated with anti-PTCH1 antibody. Interaction of PTCH1 with GRK2 and K220R was tesed by immunoprecipitation. (*C*) Interactions between PTCH1 and the GRK2 truncation mutants 185-543-Flag and Δ185-543-Flag were tested by immunoprecipitation.

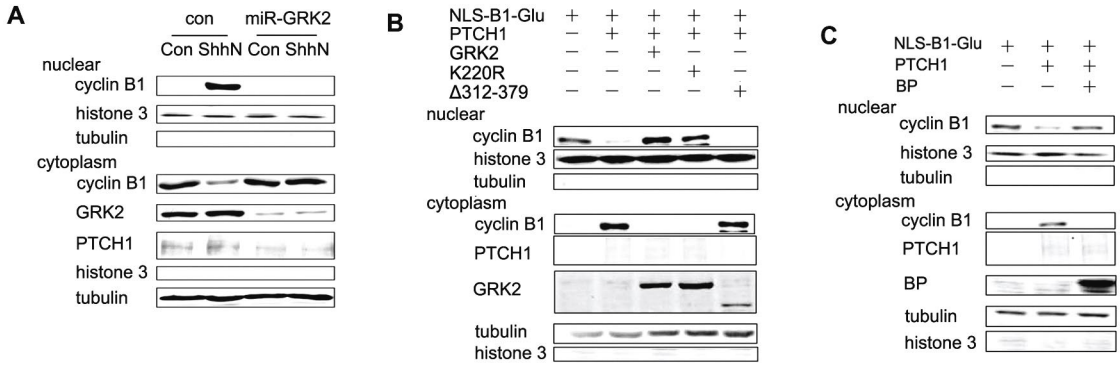


**Fig. S3.** GRK2 inhibits PTCH1-mediated inhibition of cyclin B1 nuclear translocation. (*A*) GRK2 is essential for ShhN-induced cyclin B1 nuclear translocation. HEK293 cells were transfected with the cyclin B1 derivative, NLS-B1-Glu alone, NLS-B1-Glu and PTCH1-myc, or NLS-B1-Glu, PTCH1-myc and miR-GRK2-EGFP. At 48 hours post-transfection, the cells were treated with vehicle (con = control) or 5 nM ShhN for 2 hrs and then analyzed by Western blotting of the nuclear extracts. (*B–C*) GRK2 promotes cyclin B1 nuclear translocation. Cells were co-transfected with NLS-B1-Glu and PTCH1-myc, in combination with GRK2 or its mutants. Samples were analyzed by Western blotting.

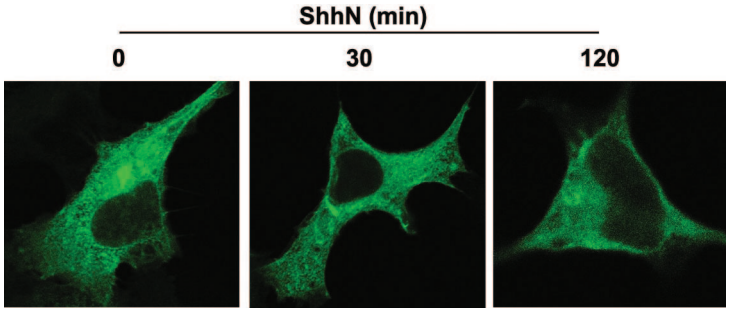


**Fig. S4.** Shh stimulation does not induce GRK2 translocation into the nuclei. HEK293 cells were transfected with GRK2-GFP and treated with ShhN for indicated times 48 h after transfection. Cells were fixed and analyzed by confocal microscopy.

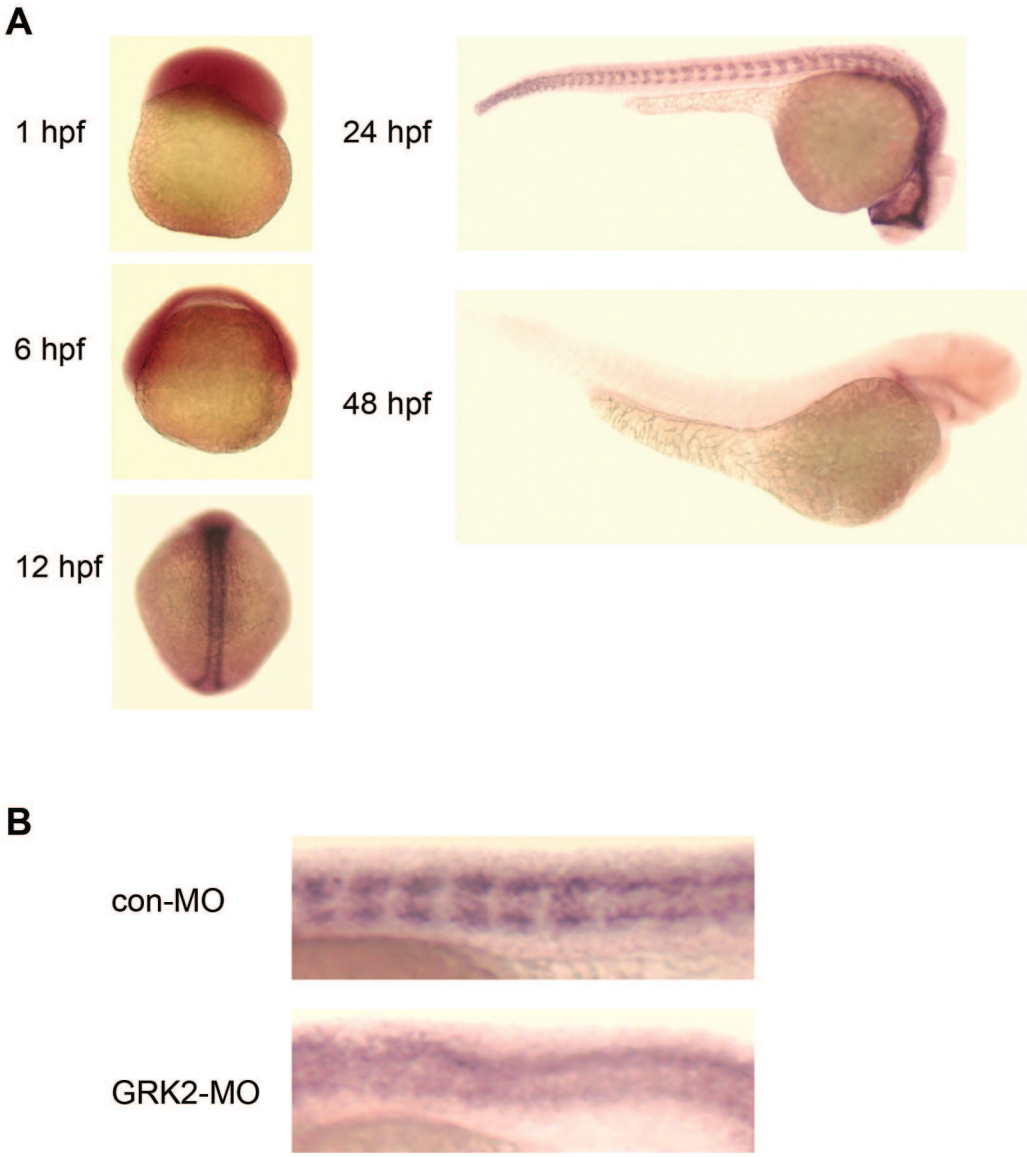


**Fig. S5.** Expression pattern of PTCH1 in zebrafish embryos. (*A*) Distribution of zebrafish Ptch1 transcript during early developmental stages shared some similarities with that of GRK2. Wild-type embryos were collected at the indicated time points and WISH was performed by using the Ptch1 riboprobe as described by Hogan et al. [Hogan BM, et al. (2005) Duplicate zebrafish pth genes are expressed along the lateral line and in the central nervous system during embryogenesis. *Endocrinology* 146:547–551]. (*B*) In situ hybridization result of zebrafish Ptch1 in 24-hpf control and GRK2 morphants.

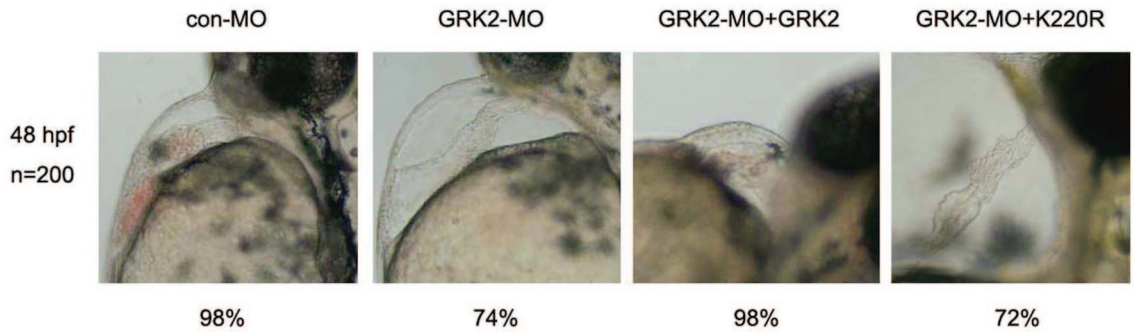


**Fig. S6.** Knockdown of GRK2 in zebrafish results in the cardiac malformation. Embryos were injected with 2-ng con-MO, GRK2-MO, or coinjected with 0.15-ng bovine GRK2 or K220R mRNA. Photos were taken at 48 hpf and 200 embryos were counted for each group as a pool. GRK2-MO-treated embryos displayed stretched heart tube, incomplete heart loops, and pericardial edema. Hearts of GRK2 morphants beat weakly, and the blood in circulation was deficient.

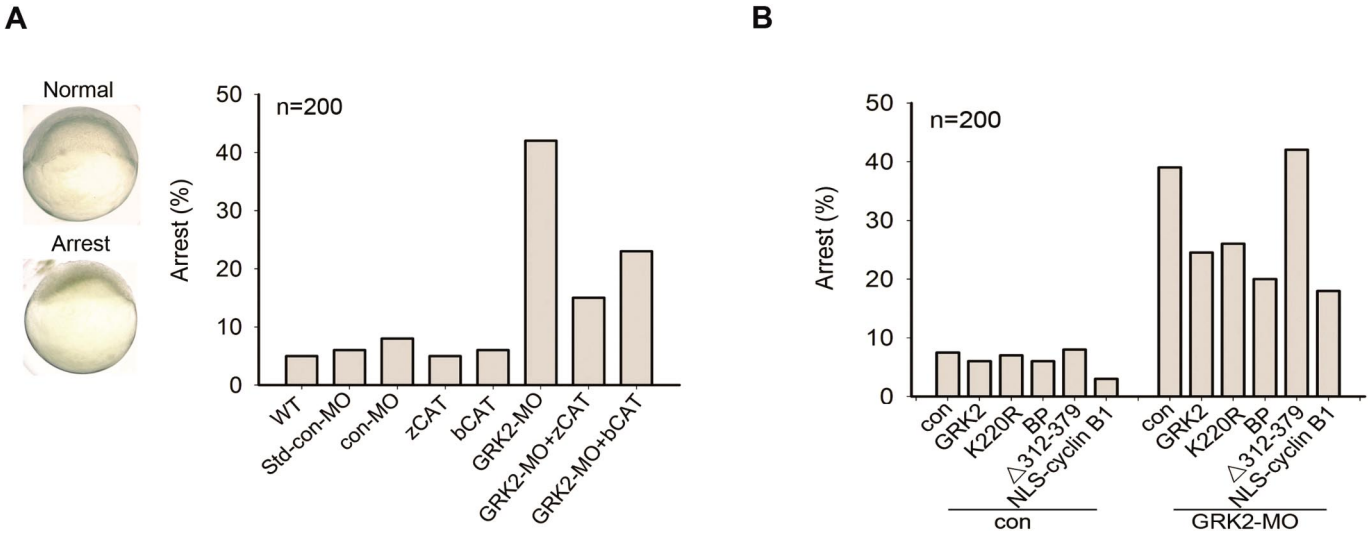


**Fig. S7.** Regulation of PTCH1-cyclin B1 pathway by GRK2 is involved in gastrulation of zebrafish embryos. (*A*) Delayed gastrulation in GRK2 morphants which could be rescued by both zebrafish and bovine GRK2 catalytic domain. Each embryo was injected at the one- or two-cell stage with 2 ng con-MO, or 2 ng GRK2-MO alone or with 0.15 ng zebrafish or bovine GRK2 catalytic domain mRNA. Embryos were categorized, counted, and photographed at 6 hpf. The percentages of embryos in high-cell stage are shown. 200 embryos were counted for each bar. (*B*) GRK2, K220R, BP, or NLS-cyclin B1 rescues the arrest phenotype of GRK2 knockdown embryos. Embryos were injected with control morpholino (con-MO) or co-injected with 2 ng GRK2 morpholino (GRK2-MO) plus 0.15 ng wild type GRK2, one of the indicated GRK2 constructs, or 0.2 ng NLS-cyclin B1 mRNA. 200 embryos were counted for each bar. Percentage of arrest at 6 hpf was shown.