SUPPLEMENTAL MATERIAL for Burkhalter *et al.*

Manuscript Title: GRK5 Controls Heart Development by Limiting mTOR Signaling During Symmetry Breaking

Extended Experimental Procedures

Zebrafish manipulation

Fertilized zebrafish eggs were injected with MO to generate loss-of-function embryos and synthetically prepared mRNAs for rescue experiments. Microinjections were performed at the one cell stage to achieve KD in the whole embryo or at the 1k cell stage to target the KV, respectively. MOs were designed and synthesized by Gene Tools (USA) based on submitted mRNA sequences. The sequences for MOs were as following: Grk5 MO: 5'- TCGCTGCCATTGTTTCGATCTCCAT, Grk5 CTRL MO: 5'- TCGGTGCGATTCTTTCCATCTGCAT, Grk5l MO: 5'- ATCCAAAGAATAGATACAGCTCCTG, Grk5l MO2: 5'- TTTGCTTCTTCCAGCCGAGAACGCG, Grk5l MO3: 5'- TGCAACAATATTCTCCAGCTCCATC, CTRL MO: 5'- AGCCCAATAATACATCACGATCCTA, Grk2/3 MO: 5'- AGGTCCGCCATCTTCGCCCTCTGGG. 3 to 4 nl of a 0.5mM dilution were injected of the Grk5l MO, Grk5l MO3 as well as the CTRL MO, while only 2 nl of Grk5l MO2 at 0.05 mM were applied. The Grk5 and Grk2/3 MO were used as previously (Chen et al., 2009; Philipp et al., 2008). mRNA for rescue experiments was prepared from linearized plasmids using the mMessage mMachine SP6 Kit (Ambion, USA).

Morpholino binding test

The efficiency of the Grk5l MOs was tested in an in vitro translation assay using the TNT SP6 Coupled Reticulolysate System (Promega, USA) and 0.2 µM of each MO. A Grk5l-GFP construct containing parts oft he 5'-UTR was used as template.

Drug treatment of zebrafish

Zebrafish embryos were immersed in egg water containing 1% DMSO or 1% DMSO and 500 nM Rapamycin (LLC Labs, USA) or 50 µM PF-4708671 (Sigma, Germany) at the indicated developmental stages. To block α_{1b}-adrenergic receptors, embryos were treated with 10 µM WB-4101 (Sigma, Germany) from 5 hpf on.

Whole mount *in situ* hybridization (WMISH)

Embryos were allowed to develop to the indicated stages. For embryos older than 22 somites, 0.003% 1-phenyl-2-thiourea (PTU, Sigma, Germany) was added to the egg water to prevent pigmentation. Embryos were dechorionated and fixed in 4% paraformaldehyde at 4 ºC overnight. Finally, *in situ* hybridizations were carried out following standard protocols (Thisse and Thisse, 2008). DIG-labeled

in situ probes were prepared by *in vitro* transcription from linearized plasmids encoding for the following mRNAs or parts thereof:

Atrial myosin heavy chain (*amhc*) (NM_198823.1), *Bone morphogenetic protein 4* (*bmp4*) (NM_131342.2), *cardiac myosin light chain 2* (*cmlc2*) (AF425743.1), *chondroitin sulfate proteoglycane 2a* (*cspg2a*) (BI326807), *cyclops* (NM_139133), *cytochrome P450 26A1* (*cyp26a1*) (NM_131146), *fibroblast growth factor 8* (*fgf8*) (NM_131281.2), *forkhead box A3* (*foxa3*) (NM_131299), *goosecoid (gsc)* (NM_131017.1) *grk5l* (transcript ID: OTTDART00000035943), *insulin* (NM_131056), *kinase insert domain receptor* (*kdr*) (NM_131472), *lefty1* (NM_130960.1), *lefty2* (cb720, ZIRC), *no tail* (*ntl*) (cb240, ZIRC), *notch1b* (NM_131302.2), *patched1* (BC139606.1), *pax2* (NM_131184.2), *pitx2* (NM_130975.1), *raptor* (XM_001919253.1), *southpaw* (*spaw*) (NM_180967), *ventricular myosin heavy chain* (*vmhc*) (AF114427).

Plasmids for cmlc2, fgf8, lefty1, notch1b and pitx2 were kind gifts of Prof. Ken Poss, Duke and Prof. Joe Yost, Utah, respectively. The plasmids obtained from ZIRC are indicated by their clone number.

Multiple protein alignment and sequence comparison

Amino acid sequences of various GRKs were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and edited using Jalview (Waterhouse et al., 2009) and Adobe Illustrator (Adobe, USA). Sequence comparison was further performed using the online tool at http://fasta.bioch.virginia.edu/fasta_www2/.

Immunocytochemistry

NIH3T3 cells were seeded on MatTek dishes. When cells had reached confluency they were fixed with 4% paraformaldehyde for 30 minutes at room temperature and blocked with 10% normal goat serum in PBS containing 0.01% Triton-X 100. Mouse anti acetylated tubulin antibody (Sigma Aldrich) was diluted 1:1000 in blocking solution. For detection, Alexa 561-coupled secondary antibody (Life Technologies, Germany) was used. After staining, cells were mounted in Vectashield mounting medium containing Dapi (Vectorlabs, UK) and analyzed by confocal analysis.

Analysis of cilia in zebrafish

Zebrafish embryos were fixed at the indicated stages and processed for cilia staining as described in Jaffe et al. (Jaffe et al., 2010). Antibodies used were mouse-anti acetylated tubulin (1:500, clone 6-11B-1, Sigma-Aldrich, Germany) and rabbit-anti-PKCζ (1:200, clone C-20, Santa Cruz, USA). For KV analysis tailbuds of stained embryos were dissected and flat-mounted between two coverslips in Vectashield mounting medium with Dapi (Vectorlabs, UK). Pronephric cilia were imaged from flat-mounted zebrafish tails. Confocal stacks were obtained in 0.4 µm increments using sequential laser excitation and converted into single plain projections. All measurements were performed using ImageJ.

Western blotting

Whole embryo lysates were prepared as described before (Philipp et al., 2008). In brief, embryos were dechorionized by pronase (Roche, Germany) treatment and washed several times using embryo water. Then, embryos were deyolked by manual pipetting up and down with a P200 pipet and yolk protein was washed out. The deyolked embryos were finally lysed in the presence of protease and phosphatase inhibitors.

Furthermore, HEK293T cells were seeded into 12 well plates and transfected transiently using Attractene transfection reagent (Qiagen, Germany). The next day, cells were exposed to starving conditions for 16 hours. Afterwards, cells were stimulated using 10% FCS for 90 minutes and lysed in the presence of protease and phosphatase inhibitors. Protein amounts were assessed using Bradford assay. Lysates were separated using precast Novex gradient gels (Life Technologies, Germany) and blotted onto nitrocellulose membranes. After blocking with 5% milk in TBST, blots were probed with antibodies against p-S6K1 (Thr389), S6K1, p-S6 (Ser240, 244), S6 clone 54D2 (all 1:1000, Cell Signaling Technologies, USA), GRK5 (1:500, Aviva, USA), Gapdh clone 6C5 (1:500, Millipore, Germany), followed by incubation with secondary IR-Dye (Licor, Germany) or HRP-coupled antibodies (Thermo Fisher, Germany). Western blot signals were detected by chemiluminescence (Super Signal West Femto, Thermo Fisher, Germany) or using a Licor Odyssey SA infrared western blot detection system. Quantitation was done using ImageJ or the Licor software.

Co-immunoprecipitation

HEK293T cells were lyzed in IP buffer (50 mM HEPES, 250 mM NaCl, 2 mM EDTA, 10 % Glycerol, 0.5% NP-40, 1 mM KF, 1 mM Sodium orthovanadate) containing protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail Tablets, EDTA-free, Roche, Germany). The lysates were cleared by centrifugation and incubated with Protein A Agarose beads, which had been pre-coupled to HA-Tag-, GRK5-antibody or control immunoglobulin, respectively. After 2 hours, beads were washed five times with lysis buffer. Immunoprecipitates were eluted from beads with 2x Lämmli (100 mM Tris pH 6.8, 2 % SDS, 20% Glycerol, 4% β-Mercaptoethanol) and heating to 85ºC for 5 minutes. 2% of the initial lysate was used to monitor protein expression in the whole extract.

Cell size measurements

HEK293T cells were transiently transfected with pCS2+ only or pCS2+-Grk5l or mock transfected using the calcium phosphate method. The following day, cells were trypsinized and mean cell size was assessed using a Vi-Cell XR cell viability analyzer (Beckman Coulter, USA). To control for expression of *grk5l*, total RNA was extracted from transfected cells using the Qiagen RNeasy kit (Qiagen, Germany). Reverse transcription was performed using the GoScript Reverse Transcription System (Promega). Expression levels of *grk5l* were analyzed as described in the next section and

quantified in reference to human *GAPDH* (Pre-developed TaqMan Assay Reagent, Applied Biosystem, USA).

qPCR analysis Grk5l

Total RNA from embryos was extracted using the Qiagen RNeasy kit (Qiagen, Germany) and transcribed into cDNA with SuperScript III Reverse Transcriptase (Life Technologies, Germany). To analyze expression levels, the Universal Probe Library (Roche) was used. mRNAs were quantitated in reference to *gapdh* with following probes and primers: probe no. 147: *gapdh* Fw: 5'-CAGGCATAATGGTTAAAGTTGGTA, *gapdh* Rev: 5'-CATGTAATCAAGGTCAATGAATGG; probe no. 89: *grk5l* Fw: 5'-TCAGAAGACACCAAAGCCATC, *grk5l* Rev: 5'-GAGCCTCTGCTTGGGATCTT; probe no. 10: *raptor* Fw: 5'-AAACAGTTTGCATTGCAGAGG, *raptor* Rev: 5'-TGGGTGGTTTGGGTTTATTG. All PCRs were carried out in triplicate in the ABI PRISM 7300 Sequence Detection system (Applied Biosystems) using the Absolute QPCR ROX Mix from ABGene (Thermo Fisher, Germany).

Imaging and statistical analysis

Whole mount embryos were imaged using a Leica M125 equipped with a QImaging Micropublisher 3.3 camera or a Leica IC80 HD camera. Confocal images were acquired on a Leica TCS SP5II.

Bar graphs represent means ± SEM, if not otherwise noted. Data was analyzed using one-or two-tailed Student's t-test and in the case of stacked bar graphs using Fisher's exact test, respectively. All statistical analyses were done using Prism4 (GraphPad Software Inc).

Supplemental References

Thisse, C., and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat Protoc *3*, 59-69.

Waterhouse A.M., Procter J.B., Martin D.M., Clamp M., and Barton G.J. (2009) Jalview version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics *25*,1189-1191

Legends to Supplemental Figures

Figure S1, related to Figure 1: Grk5l is the closer homolog of human GRK5

A, Multiple protein sequence alignment of both zebrafish Grk5 variants to human GRK5 (CAI15804.1) indicates that Grk5l (OTTDARP00000028829) is the variant with more homology to human GRK5. Amino acids conserved between both zebrafish proteins and human GRK5 are coloured in blue. Residues identical between zebrafish Grk5 (OTTDARP00000033957) and human GRK5 are indicated in light blue, whereas those conserved between Grk5l and its human homolog are

marked in orange. drGrk5, *danio rerio* Grk5; drGrk5l, *danio rerio* Grk5l; hsGRK5, *homo sapiens* GRK5.

B, Table displaying the percentage of amino acids identical or similar (in brackets) between zebrafish and human GRKs. Human GRK2 (NP_001610.2), zebrafish Grk2/3 (ACH56535.1).

C, Western blot of whole embryo lysates (24hpf). Zebrafish were left either uninjected (NI) or were control injected (CTRL MO) or injected with a translation blocking MO (Grk5l MO). Probing with an anti-GRK5 antibody revealed a decrease in Grk5l protein in morphant embryos. Gapdh was used as loading control.

D, Autoradiogram showing the binding efficiency of the Grk5l MO 1, 2 and 3. In vitro translation of GFP-tagged Grk5l was performed in the presence of CTRL and translation blocking MOs using a cell-free reticulocyte lysate and 35S-labeled methionine for detection. As template, pCS2+GFP containing parts of the 5'UTR fused to the ORF of Grk5l was used.

E, Knockdown of Grk5l does not affect *grk5* levels in zebrafish embryos. Bar graph displays relative levels of *grk5* normalized to *gapdh* as mean ± SEM. n=4-6. p=0.45, two-tailed *t*-test.

F, Live images of 48 hpf embryos, which were left uninjected (NI) or were injected with either CTRL MO, Grk5l MO, GRK5l MO2 or Grk5l MO3 at the one cell stage. Arrows indicate pericardiac edema developing in Grk5l morphants. Arrowheads point towards curled tails, which can be an indication of a ciliary defect. Bar graph summarizes 3 to 8 experiments and displays as means ± SEM. Grk5 CTRL MO vs. Grk5l MO: $p<0.0001$, two-tailed *t*-test, Grk5 CTRL MO vs. Grk5l MO2: $p<0.0001$, one-tailed *t*-test, Grk5 CTRL MO vs. Grk5l MO3: $p<0.0001$, one-tailed *t*-test. n=76 to 274 embryos in total.

G, Heart looping is also disturbed, when Grk5 alone or along with Grk5l is knocked down. WMISH for *cmlc2*. CTRL MO here describes a 5 bases mismatch MO for Grk5.

H, Bar graph summarizing heart looping experiments as shown in (F). D, D-loop, N, no loop, L, L-loop. Grk5 CTRL MO vs. Grk5 MO: $p<0.0001$, Grk5 CTRL MO vs Grk5 MO combined with Grk5l MO: $p=0.0002$; Fisher's exact test.

Figure S2, related to Figures 3 and 4: *grk5l* and *raptor* are similarly spatially expressed

Expression analysis by *in situ* hybridization reveals an overlapping pattern for *grk5l* and *raptor* during early development.

A, *grk5l* is already maternally supplied. Picture shows expression at the 4 cell stage.

B, Maternal expression of *grk5l* persists at the 16 cell stage.

C, *grk5l* expression at the 256 cell stage. Animal view.

D, At 10 hpf, *grk5l* is expressed in the tailbud region. Dorsal view.

E, Flatmount of 4 ss embryo showing stronger *grk5l* expression in the tailbud region. Anterior to the left.

F, Closeup of tailbud expression (4 ss).

G, During somitogenesis, *grk5l* is ubiquitously transcribed at low levels. However, stronger expression can be detected in the tailbud region (12 ss).

H, *grk5l* is enriched in the otic placode.

I, At 24 hpf, *grk5l* is most highly expressed in the head.

J, From 2 dpf on, *grk5l* expression becomes more restricted and can also be detected in the pronephros.

K, Higher magnification of the tail of a 2 dpf embryo highlighting the expression of *grk5l* in the neural tube, the notochord and the pronephros.

L, Weak, but ubiquitous expression of *raptor* at the tailbud stage. Lateral view, dorsal to the right.

M, Spatially unrestricted expression of *raptor* during somitogenesis (8 ss).

N, At 24 hpf, *raptor* is most highly expressed in the head and the tail.

O, Overlapping with *grk5l*, *raptor* transcripts can be detected in the head and also weakly in the tail of 2 dpf embryos.

P, Higher magnification of the tail of a 2 dpf embryo highlighting the expression of *raptor*. Although the expression appears to be generally lower than *grk5l*, it is similar in the neural tube, the notochord and the pronephros.

Figure S3, related to Figure 4: Heterotaxy phenotype is not due to altered α_1-adrenergic GPCR signaling or changed expression of key components of hedgehog (Hh) and retinoic acid (RA) signaling.

A Overstimulation of α_{1b}-adrenergic receptors was shown to be causative for situs inversus in a rat model. As GRKs are considered the terminating enzymes in the signaling of GPCRs, loss of GRK activity could theoretically result in a situation similar to receptor overstimulation. However, as measured by *lefty2 in situ* hybridization at 22 ss, the phenotype upon loss of function of Grk5l is not caused by increased activity of the α_{1b}-adrenergic receptor. Subtype-specific inhibition of α_{1b}-adrenoceptors by treatment with WB-4101 from pregastrulation stages (5 hpf) on did not rescue the Grk5l depletion phenotype. Bar graph displays summary of three independent experiments. CTRL MO^{DMSO} vs. Grk5l MO^{DMSO}: $p<0.0001$; Grk5l MO^{DMSO} vs. Grk5l $MO^{WB-4101}$: $p=0.6699$; Fisher's exact test.

B, B', B'' Expression of the Hh target gene *patched 1* (*ptc1*) remains unchanged after KD of Grk5l. B noninjected; B' injected with control MO; B'' injected with Grk5l MO.

C, C', C'' Expression pattern of *cytochrome P450 family 26 subfamily A polypeptide 1* (*cyp26a1*), a key component of RA signaling, is unchanged after KD of Grk5l. (C, noninjected; C' injected with control MO; C'' injected with Grk5l MO.)

D, D', D'' Expression of *fibroblast growth factor 8* (*fgf8*), a target of retinoic acid signaling, is not changed upon KD of Grk5l. (D, noninjected; D' injected with control MO; D'' injected with Grk5l MO.)

E, E', E'' Similar to Grk5, Grk5l regulates canonical wnt signaling in a positive fashion. Loss of either zebrafish Grk5 variant causes downregulation of the wnt target gene *axin2*.

Figure S4, related to Figure 4: Rapamycin rescues lateralization phenotype also during ciliogenesis stages and upon ablation of Grk5l in the KV

A, Rapamycin treatment from tailbud stage (10 hpf) rescues the Grk5l phenotype similarly to the treatment during gastrulation. Same day clutches of NI, CTRL MO and Grk5l MO injected embryos were treated with rapamycin from 10 hpf on. Bar graph of *spaw* misexpression. CTRL MO^{DMSO} vs. Grk5l MO^{DMSO}: $p<0.0001$; Grk5l MO^{DMSO} vs. Grk5l $MO^{Rapamycin}$: $p=0.0152$; Fisher's exact test.

B, Coimmunoprecipitation of endogenous GRK5 with endogenous Raptor from HEK293T cells. Asterisks indicate unspecific background staining in the rabbit IgG control, which originates from crossreaction with the secondary anti-rabbit antibody. IP, immunoprecipitation; WCL, whole cell lysate.

C, Randomized *spaw* expression caused by KV-specific depletion of Grk5l can be normalized by rapamycin treatment from 6 to 10 hpf. CTRL MO^{DMSO} vs. Grk5l MO^{DMSO}: $p<0.0001$; Grk5l MO^{DMSO} vs. Grk5l $MO^{Rapamycin}$: $p=0.0017$; Fisher's exact test.

Supplemental Tables

Table S1, related to Figure 1: Simultaneous loss of GRK5 and GRK6 is embryonically lethal in mice

Number of mice	**$GRK5^{+/-}$ $GRK6^{+/-}$ x $GRK5^{+/-}$ $GRK6^{+/-}$**		**$GRK5^{-/-}$ $GRK6^{+/-}$ x $GRK5^{+/-}$ $GRK6^{-/-}$**	
Total	**56**		**14**	
Expected number / percentage	**3 to 4**	**6.25%**	**3 to 4**	**25%**
Actual	**0**	**0%**	**0**	**0%**

Mice lacking both, GRK5 and GRK6 are embryonically lethal. From 56 born mice of a double heterozygous crossing 3 to 4 mice corresponding to 6.25% were expected to be double homozygous. However, no living double homozygous mouse could be found. A second mating strategy, which should produce one double homozygous in every 4 mice, also yielded no GRK5/GRK6 double KO.