Supplemental Material

Fibroblast growth factor homologous factor 13 regulates Na^+ channels and conduction velocity in murine heart

Short title: Wang et al., FGF13 regulates $Na_V1.5$ in murine heart

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Materials and Methods

Mice and rats. Animals were handled according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Duke University Animal Care and Welfare Committee.

Plasmids and virus: 6xHis (His6) tagged human FGF13 isoforms, His6-FGF12B, and untagged FGF14B were subcloned into pIRES2-AcGFP1 (Clontech). The AdEasy System (ATCC) was used to generate recombinant adenoviruses expressing either FGF13 shRNA (FGF13 shRNA) or scrambled shRNA (scrambled). The sequence for FGF13 shRNA was: 5'-GCACTTACACTCTGTT-TAATTCAAGAGATTAAACAGAGTGTAAGTGC-3', which targets the end of exon 2 and the beginning of exon 3 (nucleotides 320-338; amino acids 107-113 using the numbering for FGF13-VY). The sequence for FGF13 scrambled shRNA was: 5'-GACCCTTAGTTTATACCTATTCAAGAGATAGGTATAAACTAAGGGTC-3', which does not correspond to any known sequence in the mouse genome. Briefly, single-stranded complementary DNA sequences with an H1 promoter were synthesized (Integrated DNA Technologies) with Sal I and Xho I sites and annealed under standard conditions. The product was subcloned into pAdTrack, which also expresses GFP under a CMV promoter. The resultant plasmid was packaged into a recombinant adenovirus, as described.[1] The recombinant virus was isolated by multiple freeze/thaw cycles, further amplified in HEK293 cells, and then purified and concentrated using Vivapure Adenopack 20 (Sartorius Stedim Biotech). The viral titer (optical particle units/ml) was determined at OD_{260}. All constructs were checked by direct sequencing.

Antibodies: Monoclonal anti-His6 antibody was from Qiagen; monoclonal anti-pan sodium channel antibody and anti-β-actin antibody were from Sigma; polyclonal anti-$Na_V1.5$ antibody was from Alomone; monoclonal anti-transferrin receptor antibody was from Invitrogen; monoclonal anti-GAPDH antibody was from Abcam; monoclonal ankyrin-G antibody was a generous gift from Vann Bennett (Duke University). Polyclonal FGF12 antibody (C-term) was from Abgent; monoclonal FGF11 and FGF14 antibodies were from NeuroMab. The anti-FGF13 antibody (raised in rabbit) was designed against a peptide (RSVSGVLNGGKSMSHNEST) in the C terminus of the protein and was affinity-purified (Yenzym). Rabbit IgG and goat-anti-rabbit IgG-HRP were from Santa Cruz. Goat-anti-mouse IgG-HRP was from Millipore. Cy3-conjugated goat-anti-rabbit IgG (H+L) was from Jackson Labs and Alexa Fluor488 goat-anti-mouse IgG (H+L) was from Invitrogen.

Isolation, Culture and Adenoviral Infection of Adult Mouse Ventricular Cardiomyocytes: C57/BL6 mice, 8 to 12 weeks old were anesthetized with tribromoethanol. The hearts were removed and perfused retrogradely on a Langendorff apparatus for approximately 10 minutes with perfusion solution containing Minimal Essential Medium (MEM), Joklik modified (Sigma, M8028) with the following additions (all Sigma, in mmol/L): $KHCO_3$ 10, HEPES 10, taurine 30, L-carnitine 2, creatine 2, D-glucose 20 and collagenase type 2 (150 units/ml, Worthington). The heart was removed from perfusion when soft and white. Left and right ventricle were minced into small pieces, and then allowed to digest for another 10 minutes in enzyme solution with frequent trituration at 37 °C. The solution was filtered through sterile 210 μm nylon and centrifuged at 1000 rpm for 3 minutes to pellet cells. The cells were resuspended in perfusion solution with bovine serum albumin (BSA, Sigma) at 5 mg/ml. Calcium tolerance was performed by gradually adding $CaCl_2$ to a final concentration of 1 mM. Cells were plated at high density on laminin (Sigma) coated coverslips in plating medium of MEM with Earle's salts and L-glutamine (Mediatech) plus 5% heat-inactivated FBS (Gibco), 1% penicillin/streptomycin, and 10 mM 2,3-

butanedione monoxime (BDM). Cells were allowed to adhere for 1 hour before switching to culture medium of MEM with Earle's salts and L-glutamine plus (all from Sigma unless otherwise specified) 1% penicillin–streptomycin, 0.1 mg/ml BSA, 10 mM BDM, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml selenium (insulin-transferrin-selenium 100x solution, Gibco), 5 mM creatine, 5 mM taurine, 2 mM L-carnitine, 25 µM Blebbistatin (Toronto Research Chemicals). For adenoviral infection, virus was resuspended in culture medium and added to cells when they were switched from plating medium. Cells were checked for GFP fluorescence 36-48 hours post infection. Rod-shaped, striated cells were used for electrophysiological recording and image collection.

Immunostaining and confocal microscopy: For immunocytochemistry, tsA201 cells (48-72 hours after transfection), or cardiomyocytes (freshly isolated or 48 hours in culture) were fixed in 4% paraformaldehyde (PFA) for 15 min in room temperature (RT). Cells were washed 3 times with PBS and then permeabilized with 0.1% triton X-100 for 10 min at RT. To block non-specific antibody binding, cells were incubated 10% goat serum in PBS (blocking buffer) for 30-60 min at RT. Cells were incubated overnight with primary antibody in blocking buffer at 4 °C. After washing three times with blocking buffer, cells were stained with fluorophore-conjugated secondary antibodies for 1 hour at RT. As a control for specificity, the primary antibody was co-incubated with the peptide used for immunization or cells were incubated with secondary antibody only. Finally, cells on the coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories). For immunohistochemistry, adult mouse hearts were fixed with 4% PFA for 2 hours at room temperature, rinsed in PBS, cryoprotected in 30% sucrose over night and mounted in OCT. The hearts were then cryosectioned into 14 µm slices. The slices were stained for immunocytochemistry as above. All images were collected on a Zeiss 510 inverted confocal microscope (Carl Zeiss, Inc.) with a Zeiss 63x oil (NA, 1.4) or 40x dry (NA, .95) objective. The pinhole was set to 1.0 (Airy Disc) by using Carl Zeiss Imaging software (version 4.0 SP1). The emission signals from AlexaFluor488 and Cy3 were collected using 500 to 520 nm and LP560 nm filters, respectively. All cells were prepared identically, and imaged by using identical parameters (e.g. gain, offset, magnification, brightness, contrast, pinhole, scan time, resolution, etc.). Images were imported into Photoshop (Adobe) for processing.

RNA isolation, cDNA synthesis and quantitative real-time RT-PCR (qPCR): Total RNA was isolated from adult mouse heart. In brief, heart tissue was collected, weighed, homogenized, and processed for total RNA isolation at 4 °C using RNeasy Plus Mini Kit (Qiagen, Valencia, CA), following manufacturer's instructions. The concentration of total RNA for each sample was determined by Nanodrop ND-1000 Spectrophotometer (Thermo Scientific). The integrity of the extracted RNA was confirmed by electrophoresis under denaturing conditions. Reverse transcription (RT) was performed using iScript cDNA Synthesis Kit (Bio-Rad) for synthesis of single-stranded cDNA library according to manufacturer's protocol. qPCR was performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Each sample was run in triplicates. Three controls aimed at detecting DNA contamination in the RNA samples or during the RT or qPCR reactions were always included: an RT mixture without reverse transcriptase, an RT mixture including the enzyme reverse transcriptase but no RNA, and a water only control (reaction mixture with water instead of the cDNA template). **Online Table I** lists the primer pairs used for the amplification of each FGF13 isoform and GAPDH. PCR products were visualized on a 1.5% agarose gel. The data were collected and analyzed using iCycler Software (Bio-Rad). GAPDH was used as internal control. Relative quantification was performed using the

comparative threshold (CT) method ($\Delta\Delta$CT) after determining the CT values for the reference (GAPDH) and target genes (FGF11, 12, 13 and 14 isoforms) in each sample set.

Immunoblotting: Cell lysates were prepared by directly extracting cells in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, 1% Triton, 0.5% NP40 and a protease inhibitor cocktail (Roche). Following centrifugation at 10,000 rpm for 10 minutes, supernatants were collected and protein concentration quantified using a BCA Protein Assay Kit (Thermo). About 20 μg of protein were dissolved with 4x LDS sample buffer (Invitrogen) and separated on NuPAGE 4–12% Bis–Tris gels (Invitrogen). The blots were visualized by enhanced chemiluminescence and images were captured by using Kodak Image Station 4000 R and quantified using Kodak MI SE software.

Biotinylation: 48 hours after transfection, cultured mouse ventricular myocytes were washed with ice cold PBS and incubated in 1 mg/ml sulfo-NHS-SS-Biotin (Thermo Scientific) in PBS for 30 minutes at 4 °C. Cells were washed twice with 100 mM glycine to quench the reaction, and then resuspended in lysis buffer to rock for 30 minutes at 4 °C. Lysates were passed through 18G and then 25G needles for 25 passes each, then spun at 16,000 x *g* for 15 minutes at 4°C. Supernatants were collected and allowed to rock overnight with NeutrAvidin agarose resin (Thermo Scientific). The following day, the beads were washed three times with lysis buffer, resuspended in 4x LDS sample buffer (Invitrogen) containing 50 mM DTT and heated to 95 °C for 3 minutes. Samples were then subject to SDS page electrophoresis and western blotting as above.

GST pull down: The human $Na_V1.5$ C-terminus (aa 1773-2018) was fused to GST in pGEX-4T. The protein was expressed in BL21 cells and induced by isopropyl β-D-1-thiogalactopyranoside at OD_{600}=0.6 at 37 ºC for 4 hours. The fusion protein and the GST control protein were purified with glutathione sepharose 4B (GE Healthcare) according to the manufacturer's recommendations and retained on the beads. The cell lysate from tsA201 cells overexpressing His6-tagged FGF13-VY (total protein 200μg) was added and incubated overnight at 4°C in lysis buffer. The beads were washed extensively, and the bound proteins were eluted with SDS-PAGE sample buffer. Proteins were separated on a 12 % SDS-PAGE gel under reduced conditions and transferred to nitrocellulose membranes. The membrane was subsequently immunoblotted for the His6-tagged FGF13-VY as above.

Co-immunoprecipitation: Fresh adult mouse heart lysate was prepared by homogenizing tissue on ice in lysis buffer containing 50 mM NaCl, 20mM HEPES, 1% TritonX100, 0.5% NP40 and protease inhibitor cocktail (Roche). The crude heart lysate was centrifuged at 3500 rpm for 15 minutes and the supernatant retained. Protein concentration was determined using the BCA Protein Assay Kit. To pre-clear the lysate, supernatant was exposed to 30 µl of Protein A/G Agarose beads (Santa Cruz Biotechnology) for 30 minutes at 4° C. A total of ~1.5 µg of anti-$Na_V1.5$ or anti-FGF13 antibody was added to the pre-cleared lysates and the samples were rocked gently at 4° C for 1 hour. After adding 30 µl protein A/G agarose beads, the samples were incubated on a rocker for 12 hours at 4° C. The antibody-protein A/G agarose complex was spun down at 2500 rpm for 5 minutes and washed with lysis buffer three times. After the final spin, 100ul of lysis buffer and 20 µl of 6X sample buffer were added. Protein was eluted from the beads at 95° C for 5 minutes. 20 µl of samples were subjected to SDS-PAGE and co-immunoprecipitation was verified by immunoblot. As a negative control parallel reactions of pre-cleared lysate with Rabbit IgG as opposed to the antibody of interest were performed.

Optical Mapping of Action Potential Propagation: Neonatal rat (Sprague Dawley) ventricular myocytes (NRVMs) were isolated and seeded onto fibronectin-coated coverslips to create confluent isotropic monolayers [2]. On the second day of culture, the culture media (DMEM/F12 supplemented with 10% calf serum, 10% horse serum, and penicillin; Gibco) was exchanged with a reduced serum media (containing 2% horse serum) and experimental cultures were transduced with adenoviral constructs (scrambled or FGF13 shRNA). Four days later, on culture day 6, action potential propagation was accessed by optical mapping as previously described [3]. Specifically, monolayers were stained with the voltage-sensitive dye, Di-4 ANEPPS (15 μM, 5 minutes at room temperature), placed into a heated recording chamber perfused with Tyrode solution, and transmembrane voltage was optically mapped with 504 optical fibers arranged into a 20 mm diameter hexagonal bundle (Redshirt Imaging). Fluorescence signals were converted to voltage by photodiodes and acquired at a 2.4 kHz sampling rate with a 750 μm spatial resolution. A bipolar point electrode was used to stimulate the cultures at 1.2x threshold to initiate impulse propagation. Data was processed, displayed, and analyzed using custom-made MATLAB software. Conduction velocity (CV), action potential duration at 80% repolarization (APD80), and maximum capture rate (MCR) were derived as previously described.[3]

Cell Culture and Transfections: tsA201 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 4 mM L-glutamine, 10% fetal bovine serum (Gibco), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin and incubated at 37° with 5% CO_2. tsA201 cells were transfected at 80%-90% confluency using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of DNA for all transfections was kept constant. All experiments were done 48-72 hours post-transfection.

Electrophysiology: Na^+ currents (I_{Na}) were recorded using the whole-cell patch-clamp technique as previously described.[4-6] In brief, patch pipettes were fabricated from borosilicate glass (Warner Instrument Co.) by a P-97 Flaming-Brown micropipette puller (Sutter Instruments) and fire polished by using a microforge (MF 830, Narishige, Japan). Pipette resistance was between 1.0 and 2.0 MΩ. Voltage-clamp experiments were performed with an Axopatch 200B amplifier (Axon Instruments). All recordings were performed at room temperature (20-22 ºC) 48–72 hours after infection of cultured adult cardiomyocytes with adenovirus. I_{Na} was recorded in bath solution containing (in mmol/L): NaCl 20, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 20, CsCl 55, CsOH 10, Glucose 10, 4-AP 2, $CdCl_2$ 0.5, TEA-Cl 50, pH 7.35 (adjusted with HCl). The pipette solution contained the following (in mmol/L): NaCl 5, CsF 135, EGTA 10, MgATP 5, HEPES 5, TEA-Cl 20, pH 7.35 (adjusted with CsOH). Osmolarity was adjusted to 310 mOsm with sucrose for all solutions. Recordings were filtered at 5 kHz and digitally sampled at 40 kHz. The pulse protocol cycle time was 3 seconds to ensure full Na^+ channel recovery. Seal resistances exceeded 8 GΩ. Whole-cell membrane capacitance was calculated by integrating the capacitive transient elicited by a 10 mV voltage step from -120 mV to -130 mV. The average time constants for fast decay of the capacitative current transient were 973.8±75.9 μs (n=14), 1035.8±143.0 μs (n=10) and 995.0±94.7μs (n=14) for Control, Scramble and shRNA respectively (p=0.86 for scramble vs Control and p=0.68 for shRNA *vs* Control, respectively). Junction potential and pipette capacitance were corrected, and whole-cell capacitance and series resistance (Table 1) were ~80% compensated to assure that the command potential was reached within microseconds with a voltage error < 2 mV. The leakage current was small, and in most experiments, no leakage correction was necessary. To minimize time-dependent drift in gating parameters, all protocols were initiated 2-5 minutes after whole-cell configuration was obtained. Current amplitude data for each cell were normalized to its cell capacitance (current density, pA/pF). To determine the voltage-dependence of steady-state activation, currents were elicited by a 40 ms pulse from a holding potential of -120 mV to test potentials between -100 mV and +60 mV in 5

mV increments. The sodium conductance (G) was calculated by dividing the peak current for each voltage step by the driving force (V_m-V_{rev}) then normalized to the peak conductance (G_{max}). Data were fitted with the Boltzmann relationship, $G/G_{max}=1/\{1+\exp[(V_{1/2}-V_m)/k]\}$ in which $V_{1/2}$ is the voltage at which half of $Na_V1.5$ channels is activated, k is the slope factor and V_m is the membrane potential. Standard two-pulse protocols were used to generate the steady-state inactivation curves: from the holding potential -120 mV, cells were stepped to 500-ms preconditioning potentials varying between -130 mV and -10 mV (prepulse), followed by a 20 ms test pulse to -30 mV. Currents (I) were normalized to I_{max} and fit to a Boltzmann function of the form $I/I_{max}=1/\{1+\exp[(V_m-V_{1/2})/k]\}$ in which $V_{1/2}$ is the voltage at which half of $Na_V1.5$ channels is inactivated, k is the slope factor and V_m is the membrane potential. Recovery from inactivation was analyzed by fitting data with the two exponential function: $I(t)/I_{max}=A_f\times[1-\exp(-t/\tau_f)]+A_s\times[1-\exp(-t/\tau_s)]$, where values for A and τ refer to amplitudes and time constants, respectively. Curve fitting and data analysis were performed using Clampfit 10.2 software (Axon Instruments) and Origin 8 (Originlab Corporation).

Statistical analyses: Results are presented as means ± standard error; the statistical significance of differences between groups was assessed using either a two-tailed Student's t test or one-way ANOVA and was set at $P< 0.05$.

References

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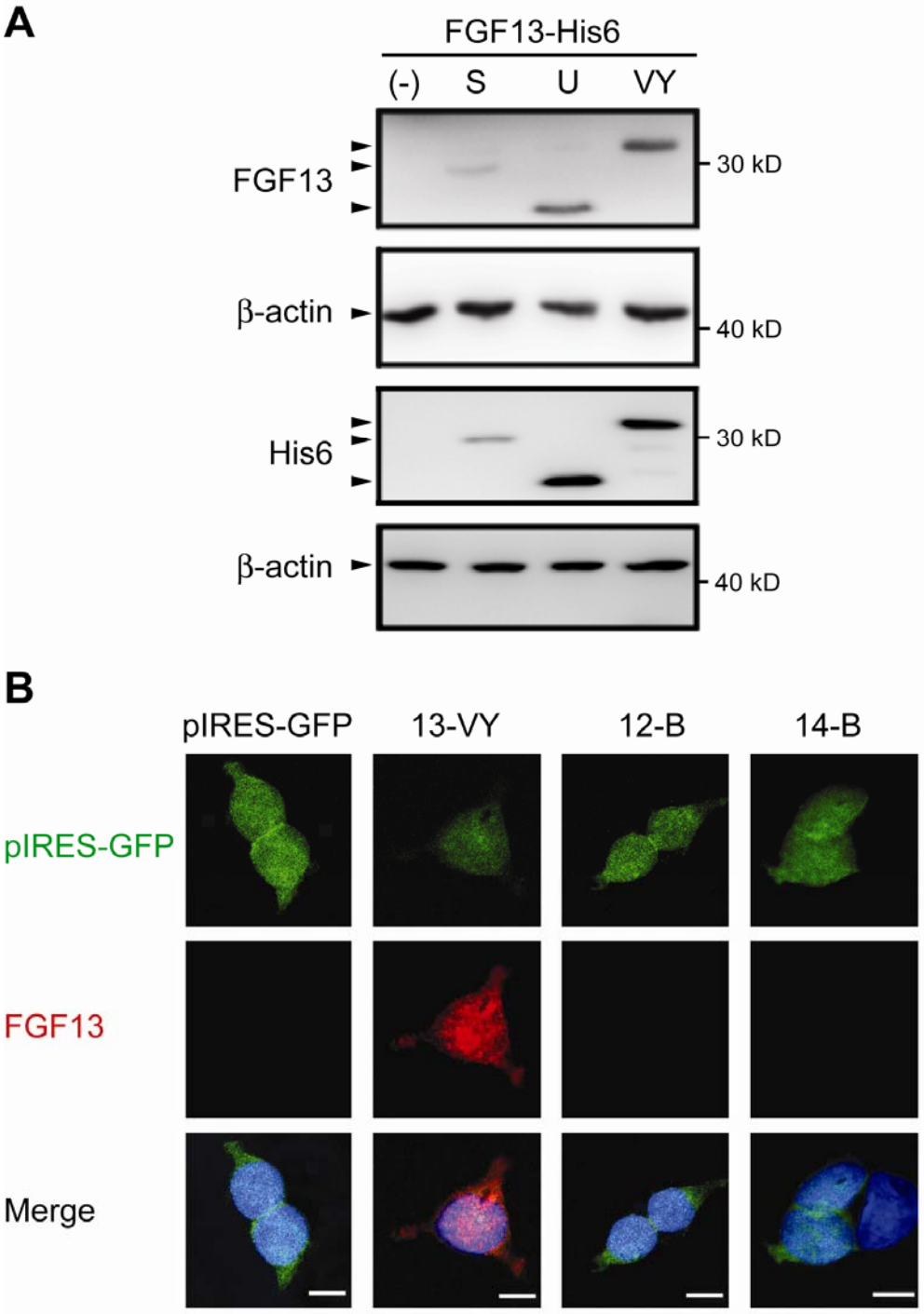
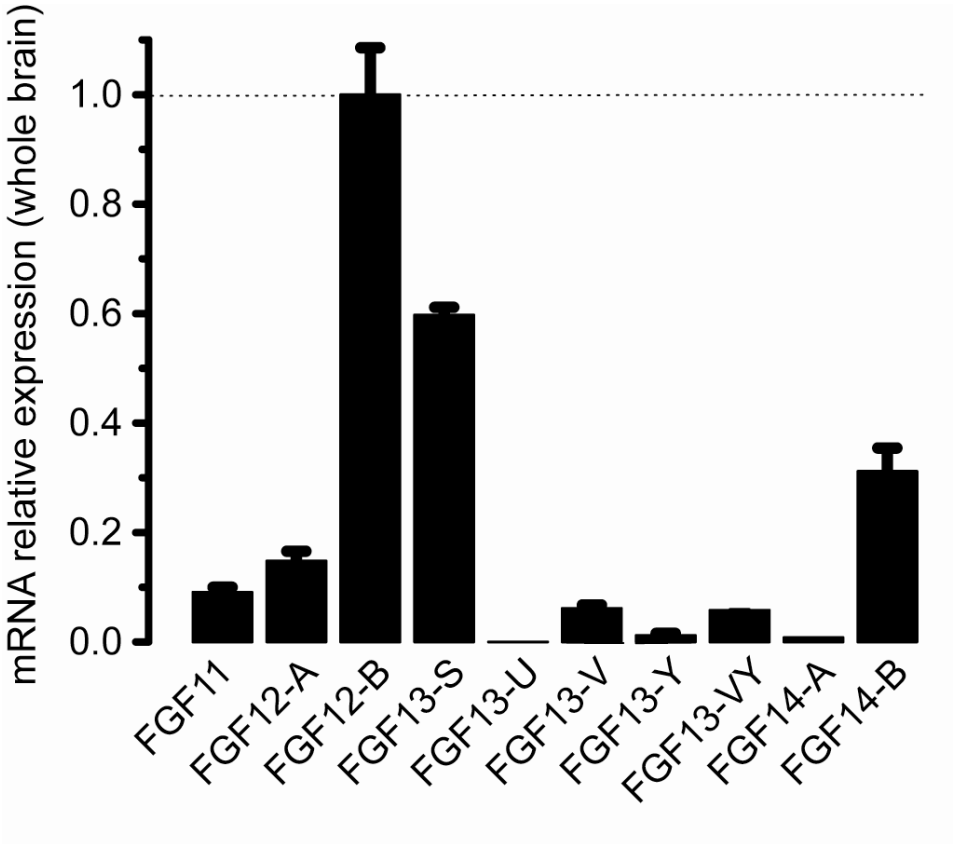
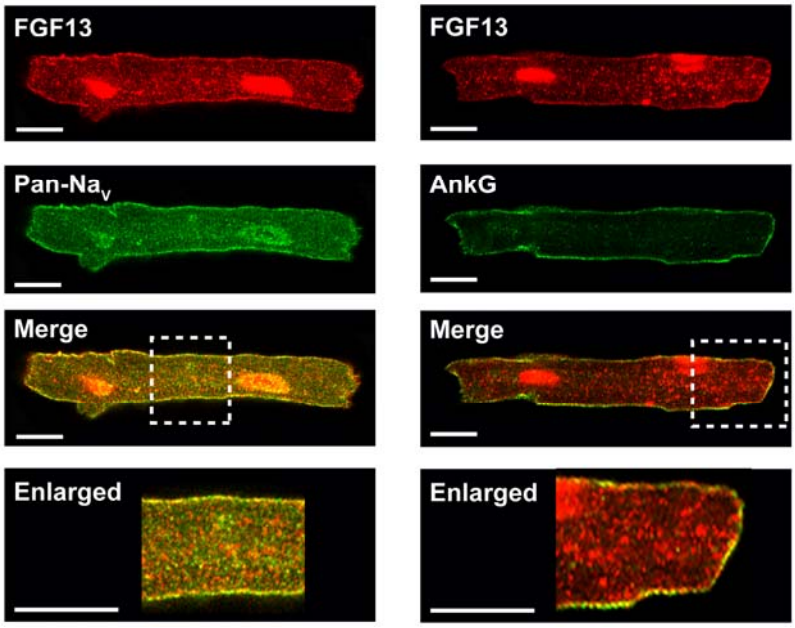


Online Figure I. FGF13 antibody specifically recognizes FGF13 isoforms in tsA201 overexpressing cells. (A) Immunoblot with either FGF13 antibody or anti-His6 antibody on lysates from untransfected tsA201 cells or cells expressing the indicated His6-tagged FGF13 isoforms. (B) Immunocytochemistry on tsA201 cells expressing GFP and the indicated FHFs. Nuclei were stained with DAPI (blue). Scale bar, 10 μm.



Online Figure II. Relative mRNA expression of FGF11-14 in adult mouse whole brain using the same primers as for mouse heart (Figure 1). All data were corrected with GAPDH and normalized to FGF12-B. FGF13-Y relative levels were calculated by subtracting FGF13-VY from the sum of both FGF13-VY and FGF13-Y. Results were averaged from three different experiments.



Online Figure III. Lack of transmural gradient of FGF13 across the left ventricle. Confocal images of immunocytochemistry of FGF13 and $Na_V1.5$ (left panels) or FGF13 and ankyrin-G (AnkG) (right panels) in adult mouse ventricular myocytes. Enlarged images (bottom panels) represent the boxed regions in the images above. Scale bar, 25 μm.

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Online Table I: qPCR primer pairs used for detecting FGF11-14 and SCN5A mRNA level in adult mouse heart and brain

FGF isoforms	Forward primer (5' – 3')	Reverse primer (5' – 3')	Product length (bp)
FGF11	CCAAGGTGCGACTGTGCG	CGACGCTGACGGTAGAGAG	354
FGF12-A	CCGCAAGAGGCCAGTGAG	CACCACACGCAGTCCTACAG	177
FGF12-B	GGAGAGCAAAGAACCCCAG	CACCACACGCAGTCCTACAG	159
FGF13-core	CAGCCGACAAGGCTACCAC	GTTCCGAGGTGTACAAGTATCC	184
FGF13-S	CGAGAAATCCAATGCCTGC	CACCACCCGAAGACCCACAG	279
FGF13-U	GTTAAGGAAGTCATATTCAGAGC	CACCACCCGAAGACCCACAG	155
FGF13-V	GCTTCTAAGGAGCCTCAGC	CACCACCCGAAGACCCACAG	158
FGF13-VY	GCTTCTAAGGTTCTGGATGAC	CACCACCCGAAGACCCACAG	326
FGF13-VY/Y	CACAGAACCCGAAGAGCCTCAG	CACCACCCGAAGACCCACAG	162
FGF14-A	GAGCAGCCCCAGCAAGAAC	GTGGAATTGGTGCTGTCATC	215
FGF14-B	CCCAAATCAATGTGGTTTC	GTGGAATTGGTGCTGTCATC	211
SCN5A	CAACAGCTGGAACATCTTCG	CCGAAGATGGAGTAGATGAAC	398
GAPDH	TGTCAGCAATGCATCCTGCA	CCGTTCAGCTCTGGGATGAC	220

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