Supporting Information

Methods

Study population. The subjects were comprised of 82 unrelated individuals (59 males and 23 females). The individuals were of varied ethnicity (61 European Americans, 10 Hispanics, 10 African Americans, and 1 Asian) and had either atrioventricular septal defect (AVSD) or hypoplastic left heart syndrome (HLHS). Of these patients, 7 had other birth anomalies including one with cryptorchidism. Subjects were prospectively recruited for genetic testing and informed consent obtained according to protocol as approved by the Institutional Review Board at the University of Texas Southwestern Medical Center and Nationwide Children's Hospital. Patients with known chromosomal abnormalities were excluded from the study. Patients underwent complete cardiac evaluation at Children's Medical Center of Dallas or Nationwide Children's Hospital, and echocardiogram, cardiac catheterization, and operative reports were reviewed. Genomic DNA was isolated using the PUREGENE kit (Gentra Systems, Minneapolis, MN) from affected subjects. Genomic DNA was obtained from a control population consisting of 287 individuals (96 Caucasian American, 95 Hispanic and 96 African American). The control population did not have known congenital heart defects, but subclinical cardiac malformations such as bicuspid aortic valve or patent foramen ovale were not excluded.

Sequencing of *FOXP1*. Exons 1 to 20 of *FOXP1*, which contain the complete protein coding sequence, were sequenced in the affected subjects and all sequence variations identified. The *FOXP1* mutation described is based on sequences from NM_032682.5 and has been submitted to the NCBI Clin Var database (http://www.ncbi.nlm.nih.gov/clinvar/). Only the rare c.1702C>T nucleotide variant was screened in the control population by direct sequencing. The sequencing primers were designed on the basis of the cDNA sequences available for *FOXP1*

[NM_032682.5] and are shown in Supplemental Table 1. PCR amplification was performed using the GoTaq ® DNA Polymerase following the manufacturer's instructions (Promega, Madison, WI).

Plasmid construction and site-directed mutagenesis. The mouse *Foxp1* expression vector was generously provided by Dr. Edward E. Morrisey.[Zhang, et al., 2010] The c.1786C>T point mutation was introduced into the plasmid containing the mouse *Foxp1* cDNA (Genbank Accession Number NM_053202.2) to generate the mouse *Foxp1* p.Pro596Ser mutant expression vector using the QuickChange II Site-Directed Mutagenesis Kit following the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). The mutation was verified by direct sequencing.

Immunoblotting. Murine atria and ventricles were isolated at different embryonic and postnatal timepoints and homogenized in RIPA buffer using a Dounce homogenizer. The cell lysate was obtained from tissue after incubation on ice for 30 minutes and centrifugation at 10,000xg for 10 minutes at 4°C (repeated twice). Forty micrograms of cell lysate was loaded per lane and separated using 10% SDS-acrylamide gels, and transferred to immune-blot PVDF membranes (Bio-rad, Hercules, CA). After blocking with 5% non-fat milk in PBST, the membrane was probed with primary monoclonal mouse anti-FOXP1 (1:500, ab32010, Abcam, Cambridge, MA) and rabbit anti-β-actin antibody (1:5000, ab1801, Abcam, Cambridge, MA). The membrane was further probed with horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG (PI-2000) and goat anti-rabbit IgG (PI-1000) (1:5000, Vector Labs, Burlingame, CA).

Transactivation assay. The mouse *Nkx2.5*-luciferase reporter was generously provided to us by Dr. Katherine Yutzey. For luciferase reporter studies, HEK293 cells were transiently transfected with 300 ng Nkx2.5-luciferase reporter plus 100 ng Hsp-lacZ plasmid, in combination with 150

ng of wildtype *Foxp1* or *Foxp1* p.P596S plasmid using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol. Luciferase activity was measured 48 hours after transfection using the Luciferase Reporter Assay (Promega, Madison, WI) according to the manufacturer's protocol. Mean luciferase activity was calculated after normalization to β–gal. Three independent experiments were performed in triplicate.

Cardiomyocyte proliferation assays. H9C2 rat cardiomyoblasts were plated in antibiotic/serum-free medium 2 hours before transfection. On the day of transfection, H9C2 cells were transfected by using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol with 200ng of wildtype *Foxp1* or *Foxp1* p.P596S expression plasmid. Five hours after transfection, antibiotic/serum-free medium was replaced by fully supplemented medium. Cells were cultured for 48 hours after transfection and fixed to perform immunofluorescence staining or RNA was extracted to perform qRT-PCR. HL-1 murine cardiomyocytes were graciously provided to us by Dr. William Claycomb and grown according to published protocols.[Claycomb, et al., 1998; Filipeanu, et al., 2006] HL-1 cardiomyocytes were plated in antibiotic-free medium the day before transfection. On the day of transfection, HL-1 cells were transfected by using Lipofectamine 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's protocol with 2μg of wildtype *Foxp1* or *Foxp1* p.P596S expression plasmid. Twenty four hours after transfection, antibiotic-free medium was replaced by fully supplemented medium. Cells were cultured for 48 hours after transfection and fixed to perform immunofluorescence staining or RNA was extracted to perform qRT-PCR.

Gene expression analysis. Cardiac: RNA was purified from hearts at embryonic day 15.5 of wildtype embryos along with H9C2 and HL-1 cells using Trizol (Invitrogen, Grand Island, NY). 500 ng of total RNA was used for reverse transcription using the SuperScript® VILO™ cDNA

Synthesis Kit (Invitrogen, Grand Island, NY) and quantitative real-time PCR was performed using Applied Biosystems 7500 real-time PCR machine. Commercially available SYBR Green (Applied Biosystems, Carlsbad, CA) PCR mix was utilized for the following genes: human FOXP1, mouse Foxp1, rat CyclinD1, CyclinD2, CyclinA2, Nkx2.5. The primer sequences are in Supplemental Table 2. Mean relative gene expression was calculated after normalization to human GAPDH RNA, mouse 18S ribosomal RNA or rat GAPDH RNA using the ΔΔCt method. Three independent experiments were performed in triplicate.

Gonadal: Human gonadal RNA from adults (Ambion, Austin, TX) and embryonic tissue from gestational week 18 (Advanced Bioscience Resources, Alameda, CA) was obtained. RNA was extracted from embryonic tissue via Trizol (Invitrogen, Grand Island, NY). 500 ng of total RNA was used for reverse transcription using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Grand Island, NY) and real-time PCR was performed using iCycler real-time PCR machine (Bio-Rad, Richmond, CA). Commercially available TaqMan Gene Expression Master Mix (Applied Biosystems, Carlsbad, CA) was utilized with probes for the following genes: FOXP1 (Hs00212860_m1) and CDKN1B (Hs00153277_m1). Mean relative gene expression was calculated after normalization to CDKN1B using the ΔΔCt method. Three independent experiments were performed in triplicate.

Immunofluorescence. Transfected H9C2 or HL-1 cells were fixed in 2.5% paraformaldehyde (PFA) at 4°C for 15 min. The cells were permeabilized in 0.1 Triton X-100 in PBS at room temperature for 3 min. Cells were then blocked by 1% BSA in PBS at room temperature for 30 min followed by incubation with rabbit anti-Ki67 (1:250, ab15580, Abcam, Cambridge, MA) antibody overnight at 4°C. Cells were then washed in PBS and stained with secondary antibody Alexa Fluor 594 anti-rabbit (1:500, A-21207, Invitrogen, Grand Island, NY) for 1 hour at room

temperature. Coverslips were placed and mounted by using mounting medium with DAPI (H-1500, Vector Labs, Burlingame, CA). The percentage of Ki67-stained H9C2 or HL-1 cells / total number of H9C2 or HL-1 cells, respectively, was calculated by analyzing 3 slides for each condition. Three independent experiments were performed in triplicate.

Statistical Analysis. Statistical comparisons were performed using Student's *t* test or Fisher's Exact test and a *p* value less than or equal to 0.05 was considered significant.

References

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Filipeanu CM, Zhou F, Lam ML, Kerut KE, Claycomb WC, Wu G. 2006. Enhancement of the recycling and activation of beta-adrenergic receptor by Rab4 GTPase in cardiac myocytes. J Biol Chem 281:11097-103.

Zhang Y, Li S, Yuan L, Tian Y, Weidenfeld J, Yang J, Liu F, Chokas AL, Morrisey EE. 2010. Foxp1 coordinates cardiomyocyte proliferation through both cell-autonomous and nonautonomous mechanisms. Genes Dev 24:1746-57.

Supplemental Figure Legends

Supplemental Figure 1. Cardiac phenotype of patient with deletion of *FOXP1.* (A) Illustration demonstrating the cardiac malformation in patient with microdeletion on chromosome 3p14. *, ASD secundum; #, AVSD; RA, right atria; LA, left atrium; RV, right ventricle; LV, left ventricle; AO, aorta; PA, pulmonary artery. Inset, schematic of normal heart. (B) Pedigree showing that chromosome 3p14 deletion was found in affected patient and unaffected mother.

Supplemental Figure 2. Cardiac expression of Foxp1 in mice. (A) Foxp1 is highly expressed in the embryonic heart as compared to other E15.5 murine tissues. *, p value<0.05.

Supplemental Figure 3. FOXP1 C1702T nucleotide variant alters highly conserved proline at codon 568 and the mutant protein has similar expression compared to wildtype. (A) Schematic of FOXP1 protein is shown with location of p.Pro568Ser missense mutation marked by (*). NLS, nuclear localization signal. (B) Alignment of human FOXP1 protein with orthologues from multiple species. Highly conserved proline at codon 568 is noted with (*). The NCBI GenBank Accession Numbers that were utilized for the alignment are human: NP_001231743.1; Cow: NP_001077158.1; Rat: NP_001029303.1; Mice: NP_444432.1; Chicken: NP_001019998.1; Frog: NP_001089002.1. (C) Immunoblot demonstrating protein expression of wildtype Foxp1 and Foxp1 p.P596S in H9C2 and HEK293 cells.

Supplemental Table 1. Primer sequences for exonic regions of FOXP1.

Supplemental Table 2. Primer sequences for gene expression.