Supporting Information

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SI Materials and Methods

Clinical Evaluation and DNA Collection. Genomic DNA was prepared from peripheral lymphocytes and lymphoblastoid cell lines transformed by Epstein-Barr virus, as described previously (1, 2). Extraction of genomic DNA samples were performed as described previously (1, 2). In all, 21 samples were collected from patients with PTA. Phenotype data for the affected individuals and their family members were obtained from detailed clinical evaluations based on echocardiogram, cardiac catheterization, and/or surgical findings, and are summarized in Table 1. Clinical evaluations and genetic studies of the patients and their families were approved by the Internal Ethics Committee of Tokyo Women's Medical University, and were undertaken only after informed consent had been obtained.

Sequencing and Mutation Analysis. All exons and flanking introns of *GATA6* were amplified using PCR. For PCR amplification, we divided exon 2 into three amplicons (exon 2A–C) because the exon was large. Amplification of genomic DNA was performed in a reaction volume of 20 μL, using: (*i*) 50 ng DNA template, 0.1 μL AccuPrime TaqDNA Polymerase High Fidelity (Invitrogen), 2 μM deoxynucleotide triphosphate, 0.5 μM each primer, and 10 μL of 2× GC Buffer I (TAKARA) for exons 1, 2A, and 3; (*ii*) 50 ng DNA template, 0.25 μL TAKARA Ex Taq (TAKARA), 2 μM deoxynucleotide triphosphate, 0.5 μM each primer, and 10 μL of 2× GC Buffer II (TAKARA) for exons 2B and 2C; or (*iii*) 50 ng DNA template, 0.1 μL AccuPrime TaqDNA Polymerase High Fidelity, 0.5 μM each primer, and 2 μL of 10 × AccuPrime PCR Buffer II (Invitrogen) for exons 4, 5, 6, and 7. All reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems). Typical PCR cycling parameters were: (*i*) 94 °C for 30 s, followed by 35 cycles at 94 °C, annealing at 54 °C, and extension at 68 °C for 45 s, with a final extension step of 68 °C for 6 min for exons 1, 2A, and 3–7; or (*ii*) 94 °C for 1 min, followed by 40 cycles at 94 °C, annealing at 56 °C, and extension at 72 °C for 50 s, with a final extension step of 72 °C for 5 min for exons 2B and C. The PCR products were diluted by 10–50% and used for direct direct, bidirectional sequencing to search for mutations with a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3130xl genetic analyzer (Applied Biosystems). Primer sequences are given in Table S2. After screening using our genomic bank system, identified genomic changes were confirmed by original genomes collected from peripheral bloods of cases. Genome samples from available family members and the healthy 182 Japanese control volunteers with no CHD were analyzed using the same protocol.

Determination of Parentage. Paternity was confirmed by microsatellite marker analysis using the ABI Prism Linkage Mapping Set v2.5 MD10 Panel 23 and GeneMapper (Applied Biosystems).

Plasmid Construction and Site-Directed Mutagenesis. Human *GATA6* cDNA was generated from the human embryo heart cDNA library (Clontech) by PCR using the primers 5′-AGAGAGGGATCCATGGCCTTGACTGACGG-3′ and 5′-AGAGAGCTCGAGTCAGGCCAGGGCCAGG-3′ and was subcloned into PT7blue T-vector (Novagen). Site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Full-length wild-type and mutant *GATA6* cDNAs were subcloned into the *Bam*HI/*Xho*I site of pcDNA3.1 (Invitrogen) or pCMV-tag2B expression vector (Stratagene). The *NPPA* promoter sequence containing a 1-kb fragment of the *NPPA* 5′-UTR was subcloned into pGL3-Basic (Promega), as described previously (3). The *WNT2* promoter-*luc* has been described elsewhere (4). The *SEMA3C* promoter sequence containing a 3.2-kb genomic fragment of human *SEMA3C*, including the 0.9-kb 5′-UTR sequence, exon 1, intron 1, and the first 94 bp of exon 2, was amplified by PCR using the primer pairs 5′-ACACACGGTACCAGCTCATTTCTCCCGATGT-3′ and 5′-CGAAGATAGTCTACAGGGCAA-3′, as well as 5′-GTTGAGTATTACAGTGCCTTCA-3′ and 5′-ACACACA-GATCTCCTTTAAGAGAGAGACAACATGT-3′, which were subcloned into the *Kpn*I/*Eco*RI and *Eco*RI/*Bgl*II sites of pGL3-basic, respectively. The *PLXNA2* promoter sequence containing a 1.2-kb genomic fragment of human *PLXNA2*, including the 1-kb 5′-UTR sequence and 198 bp of exon 1, was amplified by PCR using the primers 5′-TCTCTCGGTACCGGGGTC-CAGCTTGCAGG-3′ and 5′-ACACACGGTACCTGGCTT-TCCAGATCTATTTCGAGG-3′ and was subcloned into the *Kpn*I/*Bgl*II site of pGL3-Basic. The *lacZ* gene fragment from the pSV-beta-galactosidase control vector (Promega) was subcloned downstream of a 4.2-kb mouse genomic fragment containing the 2.2-kb putative *Sema3c* promoter sequence, exon 1, intron 1, and the first 38 bp of exon 2, and was amplified by PCR using the primers 5′-AGAGAGGCTAGCTTGTATTTGCTGGAAC-GTGTAGTG-3′ and 5′-AGAGAGAAGCTTCTTCTGATTT-GGGAATTAATATCCAAGG-3′ before being subcloned into a pT7blue T-vector. A 1.2-kb mouse genomic fragment containing the putative *Plxna2* promoter and exon 1, amplified by PCR using primers 5′-AGAGAGCTTAAGTCTCTCCTACT-GAGAATTCAGTCAAC-3′ and 5′-AGAGAGAAGCTTT-GCTCTGACATCAGCGATCC-3′, was subcloned into a pSV-beta-galactosidase control vector. All vectors constructed were verified by sequencing.

Western Blotting. FLAG-tagged GATA6 proteins were detected by western blotting after SDS-polyacrylamide gel electrophoresis, using anti-FLAG M2 antibodies (SIGMA) and ECL plus western blotting detection reagents (GE Healthcare).

Immunocytochemistry. Forty-eight hours after transient transfection with Lipofectamine LTX and Plus reagent (Invitrogen), COS-1 cells grown on cover slips were fixed with 2% formaldehyde/Opti-MEMI (Invitrogen), permeabilized with 0.1% TritonX-100/PBS, incubated with monoclonal anti-FLAG M2 antibody and DAPI (WAKO), and detected using anti-mouse Alexa Fluor 488 (Invitrogen).

Luciferase Assays. HeLa cells were transfected using Lipofectamine LTX and Plus reagent with 400 ng reporter vector, 800 ng expression vectors, and 0.25 ng pSV-*Rluc* internal control vector constructed from a backbone of pSV-beta-galactosidase control vector and the *Rluc* gene in pRL-CMV vector (Promega). Luciferase activity was measured 48 h after transient transfection according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared from pCMV, human GATA6 wild-type, and several mutant pCMV- and GATA4-pcDNA transfected COS-1 cells using the NE-PER kit (Pierce). Annealed oligonucleotides (Site1: 5′-TGGCAGATAACTGATTCC-3′; Site2: 5′-CCTC-TATGATAATGAGC-3′) representing the GATA *cis*-element within intron 1 of *SEMA3C* or upstream of *PLXNA2* were

labeled with [γ^{32}P] dATP using a T4 polynucleotide kinase (New England BioLabs). The DNA binding reaction and detection were performed as described previously (4).

Promoter Activity Assays. HeLa cells were cotransfected with 400 ng of several *SEMA3C* promoter-pGL3 vectors or *PLXNA2* promoter-pGL3 vectors and 0.25 ng pSV-*Rluc* internal control vector, together with 800 ng pcDNA3.1 or GATA6 wild-type expression vector. The luciferase assay was performed as described previously (4).

Production and Analysis of Transgenic Mouse Embryos. DNA fragments were isolated from mouse *Plxna2* promoter–*lacZ* and mouse *Sema3c* promoter-*lacZ* plasmids using *Sal*I alone or in combination with *Nhe*I, respectively. These fragments were microinjected into fertilized oocyte pronuclei, eggs were transferred into the oviducts of pseudopregnant females, and transgenic embryos were identified by PCR analysis for each promoter-*lacZ* gene in yolk sac DNA. Embryos were harvested from E10.25 to E12.5 and stained for beta-galactosidase activity, as described previously (5). The experiment was approved by the Ethical Committee of Animal Experiments of Tokyo Women's Medical University.

Statistics. For luciferase assays, all experiments were performed at least in triplicate and data are reported as normalized relative light units (fold activation) together with the SEM. For promoter activity assays, all experiments were performed at least in triplicate and data are reported as the ratio of normalized relative light units for coexpression with GATA6 to that with mock (pcDNA3.1). Error bars show the SEM. Data were analyzed by two-tailed unpaired *t* test. A *P* value of 0.05 or less was considered significant.

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2. Yagi H, et al. (2003) Role of TBX1 in human del22q11.2 syndrome. *Lancet* 362:1366–1373.
3. Durocher D, Nemer M (1998) Combinatorial interactions regulating cardiac transcription. *Dev Genet* 22:250–262.
4. Alexandrovich A, et al. (2006) Wnt2 is a direct downstream target of GATA6 during early cardiogenesis. *Mech Dev* 123:297–311.
5. Yamagishi H, Olson EN, Srivastava D (2000) The basic helix-loop-helix transcription factor, dHAND, is required for vascular development. *J Clin Invest* 105:261–270.

A

SEMA3C-luc fold activation

B

PLXNA2-luc fold activation

GATA6WT - + + +

GATA6E486del - - + -

GATA6N466H - - - +

Fig. S1. Transactivation ability of GATA6 mutant proteins. (*A* and *B*) Relative luciferase activity in HeLa cells transfected with wild-type GATA6 (WT) with or without GATA6 mutant (E486del or N466H) expression constructs and *SEMA3C-luc* (*A*) or *PLXNA2-luc* (*B*). Cotransfection with same amount of wild-type GATA6 (WT) and E486del mutant resulted in significant inhibition of the transactivation of each promoter. The N466H mutant showed no significant interaction with WT on each promoter (*A*: WT vs. WT with E486del, $P = 0.0026$; WT vs. N466H, $P = 0.056$, $n = 3$; *B*: WT vs. WT with E486del, $P = 0.0063$; WT vs. WT with N466H, $P = 0.93$, $n = 4$; two-tailed unpaired *t* test). **, $P < 0.01$; ***, $P < 0.001$ compared with WT.

A

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                              *  *            *   *
GATA6(NM_005257)     GLSCANCHTTTTTLWRRNAEGEPVCNAC
GATA1(NM_002049)     .TQ.T..Q...........S.D......
GATA2(NM_032638)     .TQ.T..Q...........S.D......
GATA3(NM_001002295)  .T....Q...........N.D......
GATA4(NM_002052)     .......Q....................
GATA5(NM_080473)     ..C.T....N.......S.........
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B

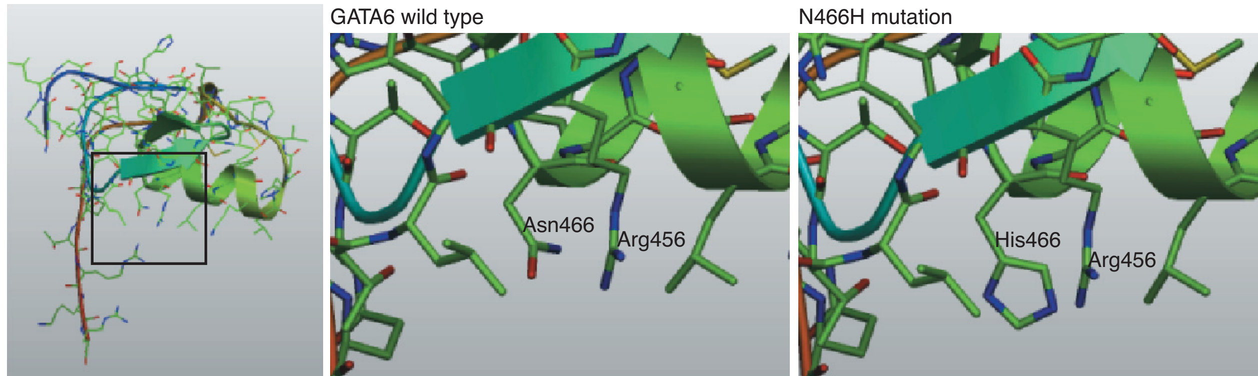

Fig. S2. Three-dimensional structure of conserved C-terminal zinc finger domain in the GATA family. (*A*) An alignment of C-terminal zinc fingers among members of the GATA transcription factor family. Asterisks show cystein residues that are necessary for the capture of zinc ions. Arrows indicate the residues making up the core zinc module that is essential for hydrogen bonds and van der Waals contacts with DNA bases of the GATA consensus sequence. Asn-466 in the GATA6 c-zinc finger is highlighted in red. (*B*) Protein model of C-terminal zinc finger domain in GATA6 and magnified images of the core module (boxed area) essential for DNA binding. The Arg-456 and Asn-466 in the core module are required for the recognition of the first three nucleotides (GAT) in the binding site. The N466H mutation inhibits DNA binding by loss of hydrogen bonding between the GATA6 Arg-456-Asn-466 domain and the three nucleotides (GAT). The plots were created using the program SWISS-MODEL Version 8.05 (Swiss Institute of Bioinformatics, Basel, Switzerland).

Table S1. Non-synonymous GATA6 nucleotide changes

Nucleotide change	Amino acid change	Location	Number of alleles with nucleotide changes		Note
			Samples from patients with PTA	Samples from controls (without CHD)	
43G > C	G15R	NTD	3/42	17/364	found in this study
482C > T	A161V	NTD	0/42	0/364	previously reported at www.ensembl.org
979C > A	H327N	NTD	0/42	0/364	previously reported at www.ensembl.org
1705T > C	Y569H	CTD	0/42	0/364	previously reported at www.ensembl.org

NTD, N-terminal domain; CTD, C-terminal domain; PTA, persistent truncus arteriosus; CHD, congenital heart diseases.

Table S2. Human GATA6 primers for PCR and sequence (SEQ) reactions

Exon	Forward primer (5′-)	Reverse primer (5′-)
1	CTCCTCCCCTCGATCCCT	CAAATCGCCTCGGCTCATC
2A	TAACCCGTCGATCTCCTACCA	CGGCTTGGTCGAGGTCA
2B	GCTGCTCAGTTCCTACGCTTC	TGGTGGTACGTCCCGTTCA
2C	PCR; GCGTGGGTTCCATGCTG	CAGCGCTACCGGACTCT
	SEQ; CTCCATACGGCAGCGGA	
3	GGCCAAGGAGAAAAGCTCA	PCR; GTTTCGGGGTAACTTCTACTTGG
		SEQ; GGTGGGCGTTGGAACAG
4	CAGATACATACTTGTTGATGACAGGG	GGAATGTGTCTTAAATGAACGTTTGC
5 and 6	GCCGCCAAATTCTTTTAAATGAGAG	AGAGCAGCCCAGTAATTTAGGAG
7	ACAGACCCCGTTCATTAGCTC	AGTCCTGGCTTCTGGAAGTG