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Supplemental Data

Mutations in Multidomain Protein MEGF8

Identify a Carpenter Syndrome Subtype

Associated with Defective Lateralization

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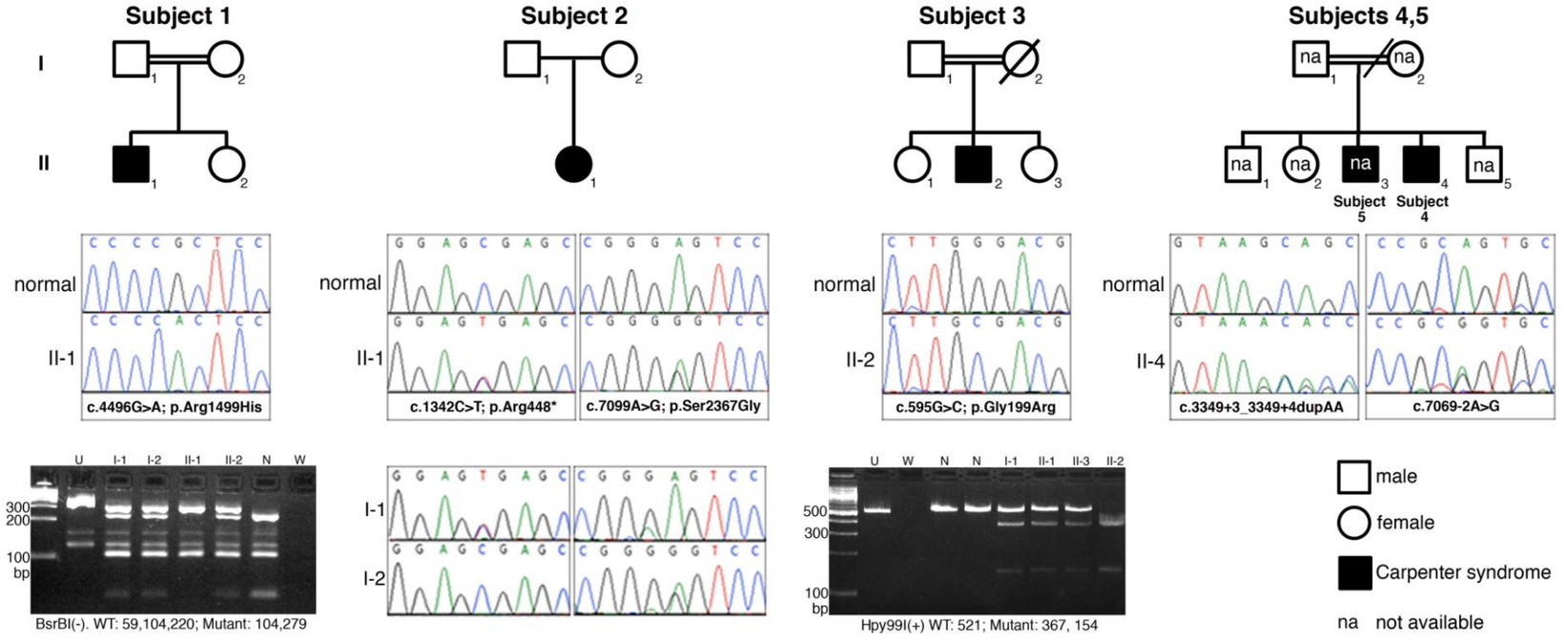

Figure S1. Pedigrees, DNA Sequence Analysis, and Mutation Segregation of Individuals with *MEGF8* Mutations

For each family the individuals analyzed in the pedigree are shown (top panel) together with a representative sequence chromatogram for a normal control and proband (middle panel). The lower panel shows either restriction digest or sequence analysis of all available family samples for Subjects 1-3. In family of Subject 1, I-1, I-2 and II-2 are heterozygous for the p.Arg1499His mutation, which is present in homozygous state in II-I. In family of Subject 2 the p.Arg448* and p.Ser2367Gly mutations present in the proband were inherited from I-1 and I-2, respectively. In family of Subject 3, individuals I-1, II-1 and II-3 are heterozygous for the p.Gly199Arg mutation, which is present in homozygous state in II-2. In family of Subjects 4 and 5, DNA was only available from Subject 4. U, undigested; N, normal control; W, water control.

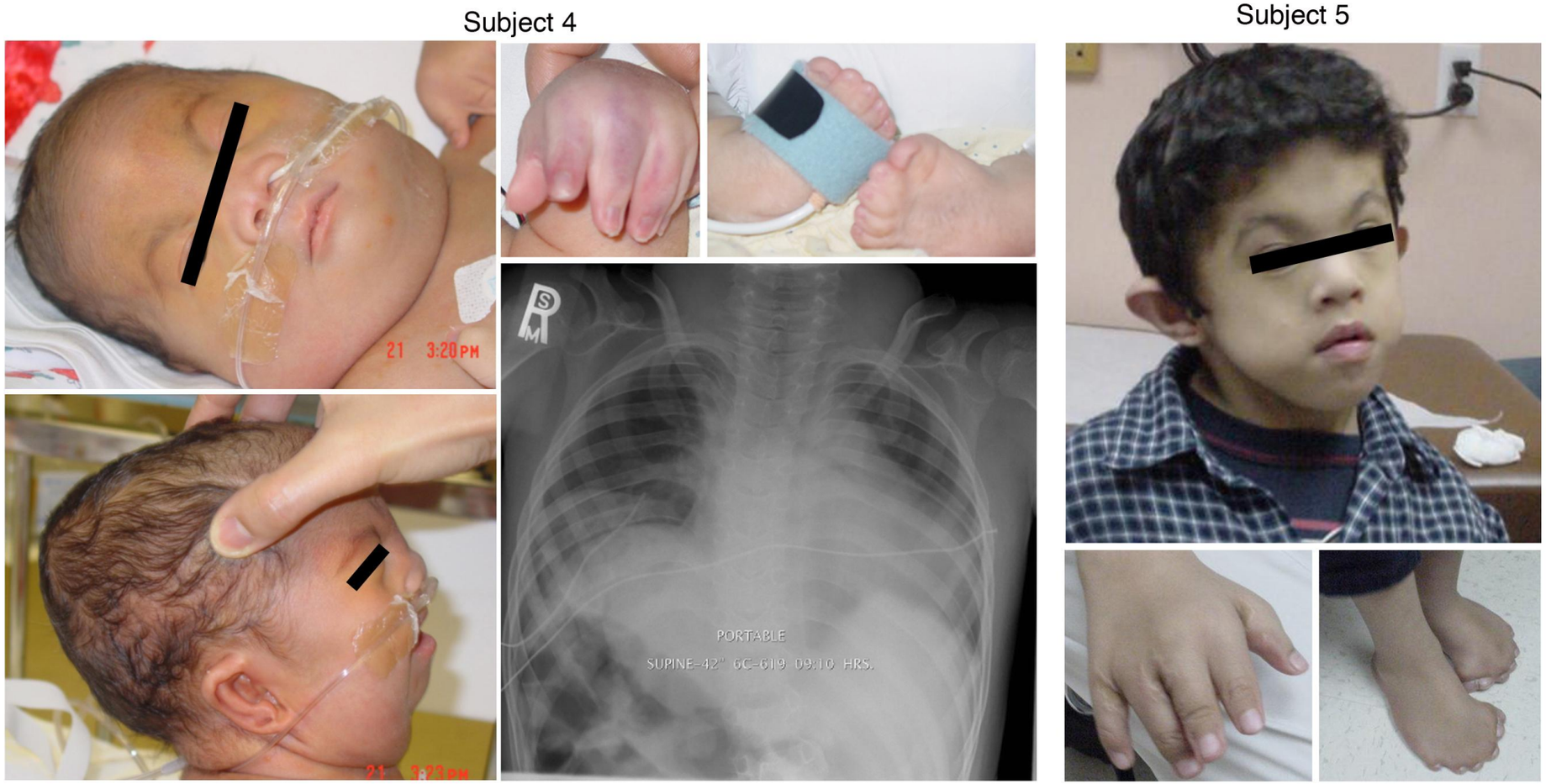

Figure S2. Clinical Features of Subjects 4 and 5

Left hand panel, Subject 4, appearance of face and limbs, at one week of age. A chest radiograph (age 3 years, 4 months) was reported as showing interposition of the colon on the right side of the chest and uncertain assignment of abdominal situs. Right hand panel, Subject 5, appearance of face and limbs aged 6 years, 6 months (hands are postoperative following syndactyly releases).

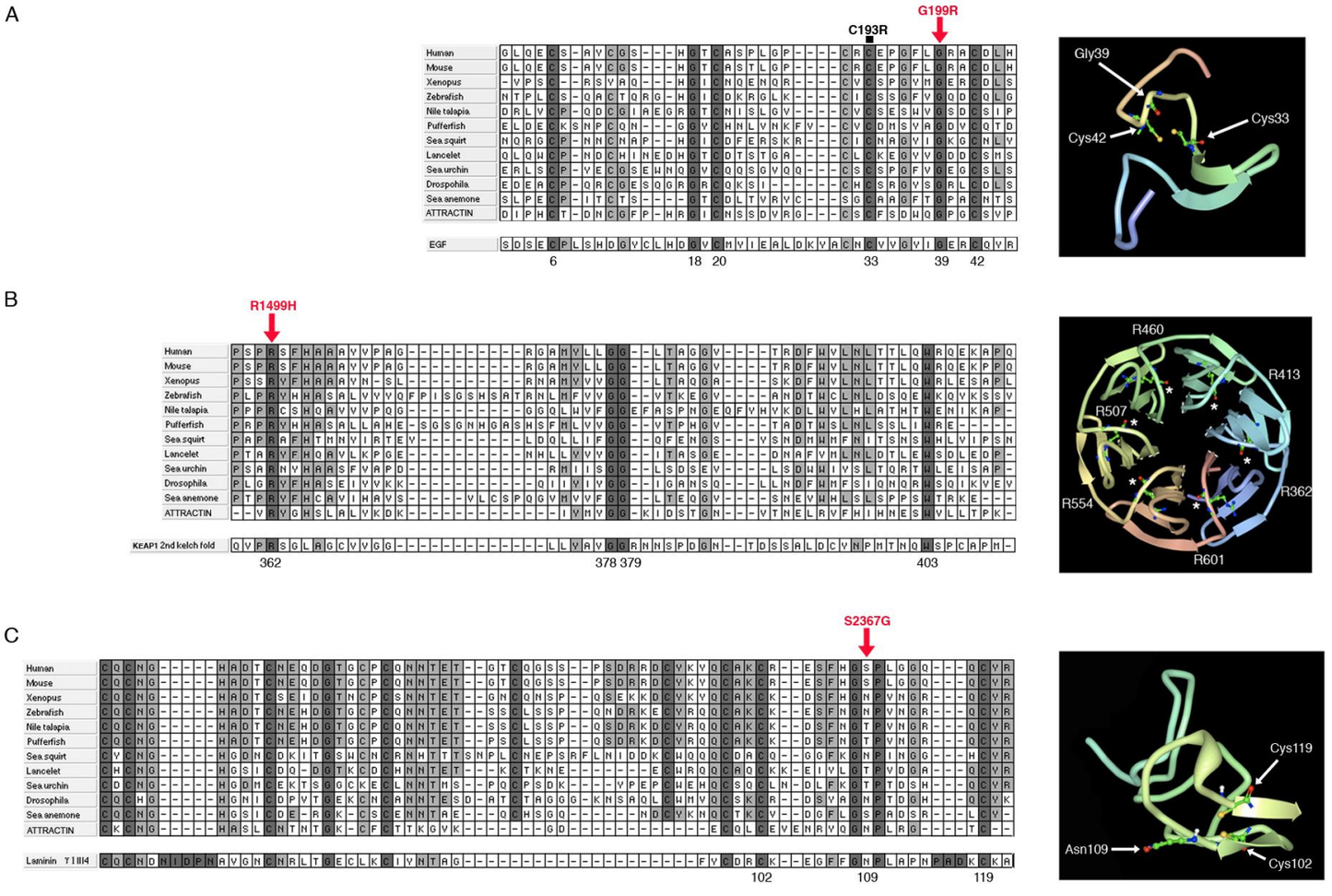

Figure S3. Analysis of Amino Acid Sequence Conservation and Structural Homology of Three Missense Substitutions in MEGF8

Each panel comprises a sequence alignment of the conserved domain on the left and a relevant crystal structure on the right. The sequence alignments are arranged according to a uniform scheme with human MEGF8 at the top, followed by homologs from ten other metazoan species shown in order of increasing evolutionary distance. At the bottom of the alignment is human ATTRACTIN, a distantly related protein, and (separated by a gap) the sequence of the molecule illustrated in the structure on the right. Fully and partially conserved residues are indicated by dark and light gray fill, respectively. Panel **A** shows the sequence of the EGF domain containing the p.Gly199Arg mutation reported here (red arrow) and the pCys193Arg mutation previously reported in the mouse.[29] In the crystal structure of human EGF,[33] note that a pair of cysteines (marked as Cys33 and Cys42) form a disulfide bridge. Between them, the Gly39 residue is located at a sharp turn in the main chain backbone. The position of Cys33 is equivalent to that of the murine Megf8 mutation, and the Gly39 is equivalent to the p.Gly199 mutated in patient 3. Panel **B** shows the sequence of one of the blades of a kelch domain, which is predicted to form a propeller-like structure as shown for the six-bladed structure of the human KEAP1 protein.[35] In KEAP1, note the six arginine residues (R362, R413, R460, R507, R554, R601), marked by white asterisks, one present in each blade of the protein, that contribute to the structural integrity of the blade. The p.Arg1499 mutated in patient 1 occurs at the equivalent position in MEGF8 to this series of arginine residues. Panel **C** shows the sequence of the EGF-laminin domain and its alignment with mouse laminin γ1, the structure of which is shown on the right.[37] Like the EGF domain in A, this structure includes a pair of disulfide-bonded cysteine residues, labelled as Cys102 and Cys119. The position mutated in Subject 2, Ser2367, lies between two fully conserved amino acids, and itself shows a limited repertoire of residues, alternatively serine, threonine or asparagine (shown as Asn109 in the laminin γ1 structure), but never glycine. This sequence conservation implies functional constraint, however since the Asn109 residue is surface-exposed, the consequences of substituting to glycine are difficult to predict. Accession numbers of structures used for modeling are EGF, 1JL9; KEAP1, 1ZGK; laminin γ1, 1KLO.

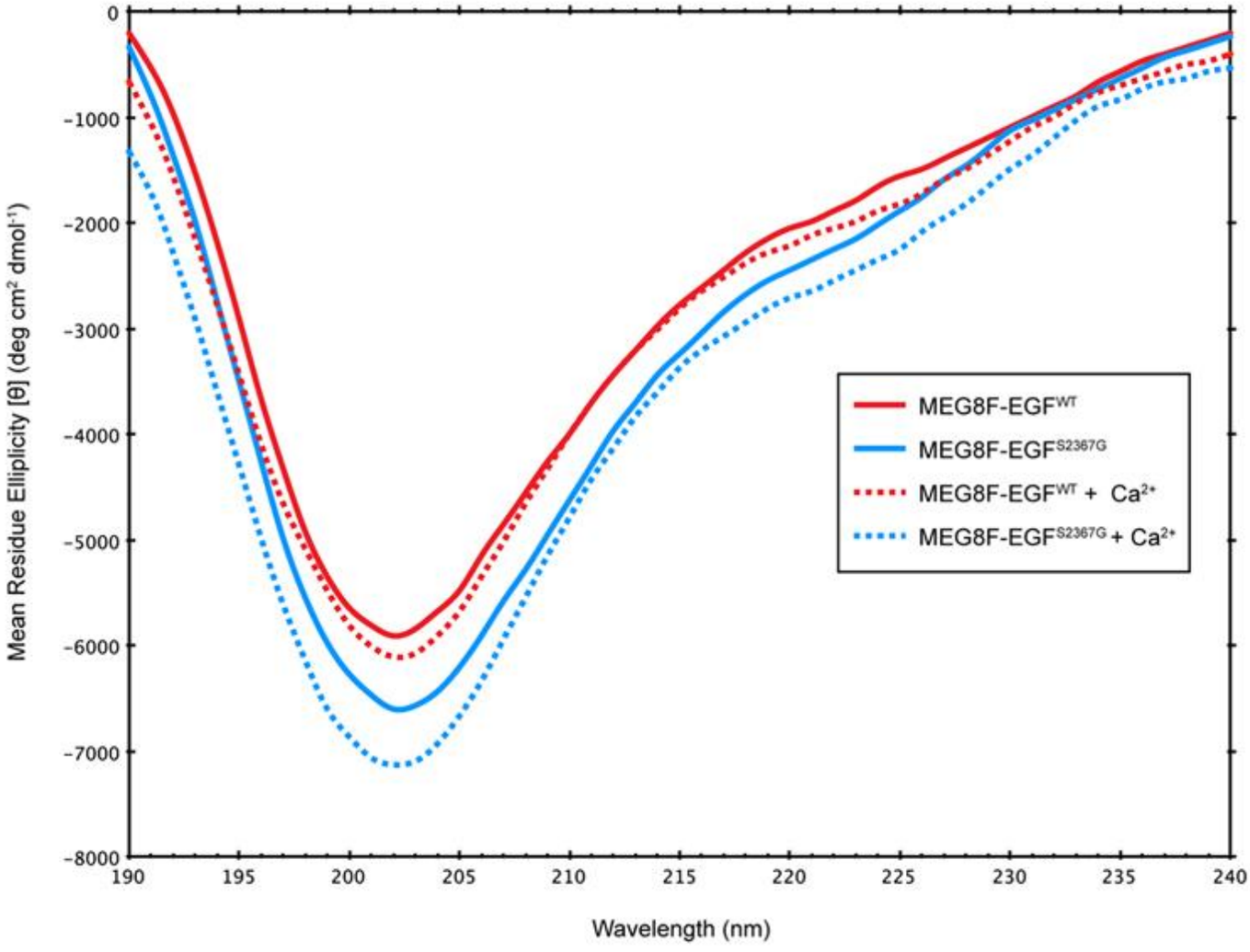

Figure S4. Far-UV Circular Dichroism Spectroscopy of EGF-Laminin Domain Containing the p.Ser2367Gly MEGF8 Missense Substitution

The spectra show that the normal and mutant domains are disordered to similar extents, and this was not affected by addition of equimolar calcium (CaCl2).

Methods: The normal and mutant laminin EGF domains of MEGF8 were amplified from sequence-verified cloned cDNA samples (MEG8F-EGFWT or MEG8F-EGFS2367G respectively), using oligonucleotides MEGF8A-f010 and MEGF8A-r003 (Table S2) and Pfx DNA polymerase (Invitrogen, Paisley, UK) and cloned into pNIC28-Bsa4. Protein expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cell pellets were resuspended in binding buffer (50 mM sodium phosphate [pH 7.5], 500 mM NaCl, 5% glycerol, 10 mM imidazole), with the addition of 1x protease inhibitor cocktail set VII (Merck, Darmstadt, Germany) and 15 U/ml Benzonase (Merck) in the absence of reducing agents.After lysis and centrifugation cells were passed through a 3 ml Ni-IDA (iminodiacetic acid) gravity-flow column, washed in 30 volumes binding buffer (30 mM imidazole) and eluted in 5 x 2 volumes elution buffer (300 mM imidazole). Pooled fractions were cleaved overnight with Tobacco Etch Virus protease and dialyzed into 0.5 x binding buffer. Samples were passed again through 1 ml Ni-IDA gravity-flow columns to remove uncleaved protein and the His6 tag. Column flow-through was fractionated by anion exchange chromatography using 1 ml HiTrap Capto-Q columns and a 30 column volume linear gradient of buffer A (20 mM Tris-Cl, 100 mM NaCl, 5% glycerol) to 50% buffer B (20 mM Tris-Cl, 2 M NaCl, 5% glycerol), on an ÄKTAxpress™ (GE Healthcare, Amersham, UK) system at 8 ºC. Column flow-through was concentrated and fractionated by size exclusion chromatography on a HiLoad 16/60 S75 column equilibrated in 10 mM potassium phosphate (pH 7.5), 250 mM NaCl. Protein identity was confirmed by electrospray ionization time-of-flight mass spectrometry, LC/ESI-TOF (Agilent Technologies, South Queensferry, UK) and tryptic digestion followed by MS/MS analysis on an ESI ion trap MS

(Brucker Daltonics, Billerica, MA, USA). Circular dichroism experiments were performed on proteins at 100 µg/ml in the presence and absence of calcium, with 10 mM potassium phosphate (pH 7.5) as the standard buffer, on a Jasco J-810 spectrophotometer (Jasco, Great Dunmow, Essex, UK). Data were smoothed with the Savitsky-Golay algorithm1 and smoothed buffer blank spectra were subtracted from the respective smoothed data. Data were converted to Mean Residue Ellipticity ([θ]) and deconvoluted with the CDSSTR algorithm,2 implemented in the DICHROWEB server.3

1. Savitsky, A., and Golay, M.J.E. Smoothing and differentiation of data by simplified least squares procedures. Anal. Chem. 36, 1627–1639 (1964).

2. Johnson, W.C. Analyzing protein circular dichroism spectra for accurate secondary structures. Proteins 35, 307-312 (1999).

3. Whitmore, L., and Wallace, B.A. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res. 32, W668-673 (2004).

Table S1. Classification of Cardiac Anomalies in Patients with Nonsyndromic Isomerism/Laterality Disturbances

Case	Cardiac Anomalies Present[a]
1	Atrial situs inversus, dextrocardia, (L) sided SVC, ventricle connects to (L) atrium, large inlet VSD, (L) sided morphological (R) ventricle connects to aorta, pulmonary atresia with VSD, (R) aortic arch, confluent pulmonary arteries supplied by PDA, hypoplastic (R) sided (L) ventricle
2	Dextrocardia with situs solitus, atrio-ventricular discordance with arterio-ventricular discordance (congenitally corrected TGA), VSD, pulmonary atresia
3	Pulmonary atresia, hypoplastic (R) ventricle, tricuspid valve, ASD, inlet VSD, atrial isomerism
4	Heterotaxy syndrome situs inversus, (L) sided IVC, bilateral SVCs (no innominate vein), TGA, pulmonary atresia, complete AVSD, asplenia
5	Common atrium (L) atrial isomerism, bilateral superior vena cava
6	Dextrocardia, DORV, ventricle with side by side arteries, pulmonary stenosis, (R) isomerism
7	Complex cyanotic CHD, (L) atrial isomerism, complete AVSD with small (L) ventricle, (L) AV valve component, DORV, mild pulmonary stenosis, (R) aortic arch, moderate AV valve regurgitation
8	(L) atrial isomerism, bilateral SVC, absent innominate vein, interrupted, IVC with azygos continuation to (L) SVC, common atrium, (R) pulmonary veins into (R) sided atrium, (L) pulmonary veins into (L) atrium, polysplenia
9	(R) atrial isomerism, dextrocardia, complete AVSD, pulmonary atresia, asplenia
10	DORV with subpulmonary and pulmonary stenosis, mitral atresia, VSD, hypoplastic (L) ventricle, (L) atrial isomerism
11	(L) Atrial isomerism, common atrium
12	(L) Atrial isomerism, common atrium, azygos continuation IVC to (R) SVC, IVC continuation to (R) side of atrium, (L) SVC to (L) side of atria, hypoplastic (R) AV valve, hypoplastic (R) ventricle, small VSD, pulmonary atresia, hepatic veins to IVC, (R) sided pulmonary veins to (R) side of atrium, (L) pulmonary veins to (L) side atrium
13	Situs inversus, mitral atresia, DORV
14	(R) atrial isomerism, (L) sided SVC, IVC to (L) sited atrium, ostium primum ASD, common AV valve, DORV, TGA, valvar and subvalvar stenosis, SVC connection to (R) pulmonary artery
15	Complex pulmonary atresia, heterotaxy, situs inversus

[a]Abbreviations: ASD, atrial septal defect; AV, atrio-ventricular; AVSD, atrio-ventricular septal defect; CHD, congenital heart disease; DORV, double outlet right ventricle; IVC, inferior vena cava; L, left; R, right; SVC, superior vena cava; TGA, transposition of great arteries; VSD, ventricular septal defect.

Table S2. Primers and Amplification Conditions for *MEGF8*

Screening				
	Primer Sequence 5′→3′ (M13 tags in lowercase)		Product Size (bp)	Amplification Conditions[a]
Amplicon	Forward	Reverse		
Ex1	GATCTACAAGGTCATGTTATGCCTATAGAG	GTGGTGAGTGGATGAATGCACACATGAATG	381	65
Ex2	GGGTTTGGTGTACAATTGTGGGAGGCTGCAGGG	CCTCTTACTGACTCTCTGTCCCTCCTTTC	329	65
Ex3	GGTGGAGAGAGGCAAAGGAAGGAGATGAGG	GGGGAGTTTCATGCAGGAGCAAGGTCAG	438	65
Ex4,5	CCCTTTGTGCCCTGTCTGTCTCATTCTG	CCCGGCTGGGTCTGATTGAGTGGAAAGG	521	65
Ex6	GTCTAAGCCTGGCTCTGGCTCTCTTGCTG	GCCTGGCCTGTGGAGCATGGGTTATGGG	521	65
Ex7,8	GCTCCTAAGAGCCTGGAGGAGGGAGAG	GACCCTCAGGAGCCCCTAGGGAAGAAC	512	65
Ex9	GCT GGG CTG TGG CCC AGG AGA ATC AG	CCTTTCCCCTCACCTGTGGAAACCCTGCTA	319	65
Ex10	GCTAAAGCAAGCGGGGACTTGGGAGG	CTATGCTAGGGAACAGAGGGTCTAGGG	254	65
Ex11,12	GATCTCTTGAGCTCCAGTTGACAGTGAG	CCTAGGATCAGGATCATCAACACCCCACCC	496	65
Ex 12A	CTGATGTGGCCTGTGAGTCCAGAAAGGG	GATGCTCTATACCACACCCCTAACTCCC	332	65
Ex13	GTCTGAGGAAGGAATGGGAAGGGTTCTGAGG	CAGAAAGCCCAGGTGATCTGAGGCTGGGG	328	65
Ex14	CTTTCAGTTAGCGCCAGACTCTGACCCC	CTTGACTGCCCATTGCCTGCTGTGGCTCCC	376	65
Ex15	CCAAAGGAAAGGGCTGAGTGGGGTTCTG	CCACAGCCAGCACACATCCCCAGGCAC	236	65
Ex16,17	gtaaaacgacggccagtCCCGCTCTGCAGCCAGTGAGTCA	agcggataacaatttcacacaggaCAGTACGGAAAAGGGAAGGTGGCCATG	570	65
Ex18	GTTTCTGTCTCTCCGCTCTCCCTTTCACTGCAT	CACAGGAGGGGACCCAGCGCCATG	572	65
Ex19	GGTGGAGATGATGGGGTGCTTTAGGGG	CTAGAGCTGCATGAGAGGACACTGAGG	386	65
Ex20	GGGGTCAGGGTTTAGCTGAGCCAGTAGG	CCACTGTGTCCCCAAGCACACCCTACC	349	65
Ex21	GGTAGGGTGTGCTTGGGGACACAGTGG	CAGGCTGGGTCAGGGGACACGGTTCTGG	387	65
Ex22	GTTCTCATCCTCATTGTCTCCTAATCCTC	CGGCAGAGCTGGGTCCTGAGCCCACTTC	279	65
Ex23	CTTAGCATCTGGGGGTGGAAGGGGCCAGG	CCCTGATCACCAGCCCTGTCCCACCAGAC	367	65
Ex24	GTAACCAGGTACAGGTGGGAGAGGGCAAGTC	ATTGGGGATCAGGCTAGGGTCAGGACAAAGTGC	319	65

Ex25	AGCCGTGAGTTGTGGGTACCCGCTGTCTAG	CTCTGAGGTGACTCTTAGCATCCCGAGGTGAC	339	65
Ex26	GGTCACCTTGAAGGATGCTGGGATGACTG	GTGTGAGGGCTGGGGAGTGGGCTGGGG	309	65
Ex27	CTTCTACCCACAAGGTGACCCCTGACCTC	GAGCCCACAGGAAGCCCAGATGCCC	314	65
Ex28	GGTCGGCGGGGTCAGTGCTGTTGTCAG	CTCCCTCCTGCCCCATACATCCTTGGGTC	273	65
Ex29,30	CTTGGAGGCAGGGGGCTAGAAGCAAGAGACT	GATGGGGCTCCTCCTGGATGTCCCTCAG	582	65
Ex31	CCTGGAGTCTCCTGCTCTCTATCTGTCTG	CACCCCTTTCCTGAACCAGACGGTATACTCC	398	65
Ex32	CAGGGTTGGGGGCCTGCAGGACAGT	GATGGGACCCTCAGCACCCCTCCT GA	361	65
Ex33	CAGGGTCTCAGGAACCACCGAGTTCTCAG	CAT AGG TGG GCT GCA CGG AGA GGA TGC	784	65*
Ex34	GCTGGGCTTGCGATCTCCTGGGTCTGAGG	GGAGGGTGGAGGAGATGGTGGAAGAAAGG	371	65
Ex35	GTGTGGTCTGGGAAGATACCCAGAATGTGTTTGTTTAAGCT	GGTCTGGCCCAGTATCCCTATGTTCACTGTATCAG	386	65
Ex36	TCAGAGGAGGCAGGAGGGAGGGCCTAG	TGCTCCCCACAGACTCTCCCACCCTAGC	303	65
Ex37	GTGGGGTAGTTGGTTGGGTGCTAGGCCATG	GGCTCAGGATGAGATGCGAGCCGCCTGA	369	65
Ex38	CCCTCCCGTGGGCTCTCAGAACCTGCCCC	CAGCCCCTCCTCCCTCAGACCCAGGCATC	316	65
Ex39	GGTTGGTCCAGGCCTTTCTATGATCACACTG	GGCTCCAGAGCTACTCCCAGTGAGAAGGG	262	65[b]
Ex40	GTTGCACAGAGTTGAGCTCACATATGGGG	CTACCTCATCCCAGCCCTGACCTCCAC	292	65
Ex41-1	GGCTACTTAGCAGTGGGTATAGAGTATTCGTCACTG	CCCCGCGGCCATACCTCCCGTAC	551	65
Ex41-2	gtaaaacgacggccagtACCACCTCCACCACCCCCTGCA	agcggataacaatttcacacaggaGAGGCCGGGGCAGTAGGGTCA	543	65
Ex41-3	CAGCGCCGGCACTTGCAGGAGATGAC	CCCCAGGTGGAGGGACCCCAAGTC	572	65

Mutation Confirmation

	Primer Sequence 5′→3′		Product Size (bp)	Amplification Conditions[a]	Digest
Mutation	Forward	Reverse			
c.595G>C	CCCTTTGTGCCCTGTCTGTCTCATTCTG	CCCGGCTGGGTCTGATTGAGTGGAAAGG	521	65°C	Hpy99I
c.1342C>T	GCTCCTAAGAGCCTGGAGGAGGGAGAG	GACCCTCAGGAGCCCCTAGGGAAGAAC	512	65°C	SEQ[c]
c.3349+3_3349+4dupAA	GGTGGAGATGATGGGGTGCTTTAGGGG	CTAGAGCTGCATGAGAGGACACTGAGG	386	65°C	SEQ[c]
c.4496G>A	GGTCACCTTGAAGGATGCTGGGATGACTG	GTGTGAGGGCTGGGGAGTGGGCTGGGG	309	65°C	BsrBI

c.7069-2A>G	GGCTACTTAGCAGTGGGTATAGAGTATTCGTCACTG	CCCCGCGGCCATACCTCCCGTAC	551	65°C	SEQ[c]
c.7099A>G	GGCTACTTAGCAGTGGGTATAGAGTATTCGTCACTG	CCCCGCGGCCATACCTCCCGTAC	551	65°C	SEQ[c]

Primers Used for cDNA Mutagenesis[d]

	Primer Sequence 5′→3′	
	Forward	Reverse
c.595G>C	TGAGCCTGGCTTCTTGCGACGTGCCTGTGACCT	AGGTCACAGGCACGTCGCAAGAAGCCAGGCTCA
c.4496G>A	CCAGGCCCATCGCCCCACTCCTTCCATGCAGCCG	CGGCTGCATGGAAGGAGTGGGGCGATGGGCCTGG
c.7099A>G	GGGAATCATTTCACGGGGGTCCGCTGGGCGGCCAGC	GCTGGCCGCCCAGCGGACCCCCGTGAAATGATTCCC

Primers for cDNA Clone Sequencing

1R	GGGGTACCATCACTGAAGAGGTGCAGCAGCATCTTGCC	SEQ 8	GGACCCGATGCCACCTTGTGG
1F	GGCTAGCGAATTCATGGCCCTGGGCAAGGTTCTGGCCATGGC	DLSEQ 1F	GGGTCCTCAACCTCACCACCCTGCA
2F	GGGCCGCTGCTTGCCAGTCTAAGTGGGAG	SEQ 9	GCACTGTCCTGACCGCACCTGG
SEQ 1	GGGGTCCTGACTGTGGCCTGCAGGAG	6F	CCCCTGCCGCCTGCTGTCCTCACCTGAGC
SEQ 2	GCCTTGACAGCACCAGCGGGGGCTATTGGG	4F	GGAATGTCGACGTCTCCGGACCTGCAGTGAG
SEQ 3	GTGGGGACTTGATGGCGTACAAGGT	SEQ 11	GGAGCAGCAGCCTGCAGCAGTGT
SEQ 4	GGCGGGACCACAAGTATGCAGTAGAG	SEQ 12	GGTGTTGGGGCGCGTGACCACTG
SEQ 5-2 F	CCCACGCCGTGGCCACTGCGAC	SEQ 14	GTAACGAGCAGGATGGGACGGGCTGTCCATG
SEQ 5-2 R	GTCGCAGTGGCCACGGCGTGGG	SEQ 13	GGTGGAGCAGGAGTGCTGCCTGG
SEQ 7 REV	CCATTGGCCTCCCAGTGCAGCAC	42F2	GTCGGCAGTGCTGGTGGTCCGCGGCGTG
SEQ 8 REV	GGGCAGTAAGGGTGTGACCAGCAACGG	42F3	CTCCCACCTCCCGCCTTCCGCCGCTC
SEQ 7	GCTGCACTGGGAGGCCAATGG		

Multiplex Ligation-Dependent Probe Amplification (MLPA)[e]

	Primer Sequence 5′→3′		Size (bp)
Probe	5’	3’	
Exon 4-1	gggttccctaagggttggaGTCTCTCTGTCCTTGTCTCTCCCTCTCTTATCTCTGCCTTT	ATGTCTCCTTTCCCGCAGCCCCTGGGACCATGCtctagattggatcttgctggcac	116
Exon 4-2	gggttccctaagggttggaCTTTCTTTTTTCTTCTTCCTCTTCCTTTATCTGCCTTTGTGCATCTTc	ATTTTTCCATGTGTCTCCTGTTGCTCAATCTGTCTCTGCtctagattggatcttgctggcac	128
Exon 4-3	gggttccctaagggttggaCACTCATTCATTCATTCACCCACTCACACATTCTCTGATGTTTT	ATTCAATCTCTTGGTGAATTGCCTTCCACACACTCCCatctagattggatcttgctggcac	124

Synthesis of Laminin-EGF Domain

MEGF8A-f010	MEGF8A-r003
TACTTCCAAT CCATGGATGGGACGGGCTGTCCATGTCAGAATAAC	TATCCACCTTTACTGTCATTTGGGCTCATGGAAGCAGTTG

Note.— Primers for *MEGF8* analysis were designed using NM_000019.9. [a]DNA was obtained from whole blood samples by phenol-chloroform extraction and was amplified in a total volume of 20 µl containing 15 mM TrisHCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM each dNTP, 0.4 µM primers, and 0.5 units of Amplitaq Gold polymerase (Applied Biosystems) with or without 10% DMSO. Cycling conditions consisted of an 8 min denaturation step at 95°C, followed by 35 cycles of 95°C for 30 s, 65°C for 30 s (except * 40 s) and 72°C for 30 s, with a final extension at 72°C for 10 min. [b]Amplification was as above except the PCR buffer consisted of: 160 mM $(NH_4)_2SO_4$, 500 mM Tris.HCl pH 9.2, 22.5 mM $MgCl_2$, 5% Tween. Mutation confirmation was carried out by PCR using the above conditions and indicated primers, followed by restriction digest of 8 µl of PCR product or [c]Sanger sequencing alone. [d] Site-directed mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutant nucleotides are highlighted in red. [e]Multiplex-ligation-dependent probe amplification was performed using synthetic oligonucleotide probes designed to *MEGF8* according to protocols available from MRC-Holland: http://www.mrc-holland.com/pages/indexpag.html. Fragments were analysed by capillary electrophoresis using an ABI 3130 containing POP-7 polymer. Peaks were visualized using Gene Mapper v3.7 (Applied Biosystems). Common PCR primer annealing sequences are shown in lower case, hybridizing sequences are shown in upper case and the 3' probe sequence is 5' phosphorylated.