High throughput sequencing recovers the novel gene *Megf8* as the ENU induced mutation causing single ventricle cardiac defect associated with randomized left-right patterning

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Forward genetic screens can provide novel insights into gene function, but suffer from difficulties in gene recovery. Using high-throughput sequencing of 2.2 Mb mouse genomic interval, we successfully recovered an ENU induced mutation causing cardiac anomalies and laterality defects as a point mutation in the novel gene *Megf8*, a finding confirmed with zebrafish *Megf8* morpholino knockdown. Our studies suggest *Megf8* plays an essential role in Nodal signaling, possibly through chromatin regulation.

The remarkable success of mutagenesis in *Drosophila* in delineating the genetic pathways involved in specification of the segmental body plan elegantly showed the efficacy of mutagenesis in the systematic analysis of complex biological processes that regulate developmental patterning[1]. We previously showed mouse mutations causing congenital heart disease can be efficiently recovered from ethlynitrosourea (ENU) mutagenesis using in utero fetal echocardiography for cardiovascular phenotyping[2]. Despite the high phenotyping throughput, mutation identification remained a bottleneck. To explore the efficacy of high throughput sequencing technology for the recovery of ENU induced mutations, we conducted a pilot study on a mutation causing a single ventricle spectrum of complex heart defects associated with heterotaxy (the randomized left-right positioning of heart and visceral organ situs)[3]. The specific defects observed, which included right pulmonary isomerism, transposition of the great arteries, and asplenia/polysplenia, were similar to that of *Cryptic* and *Acvr2B* knockout mice and mice with hypomorphic *Nodal* alleles, indicating a disruption of Nodal signaling[4-6]- a pathway central for breaking symmetry at the node and propagation of left-right patterning to the lateral plate mesoderm (LPM)[7].

This mutation, initially mapped to a 3.3 Mb interval of mouse chromosome 7, was further narrowed to a critical 2.2 Mb region between markers D7Mit92-SNPrs13460395 (Supplementary Table S1). No candidate genes known to play a role in left-right patterning or Nodal signaling were found in the interval. As the interval is gene dense, traditional methods for exon sequencing would be prohibitively expensive and time consuming, as would efforts to further narrow the map interval with additional mouse breeding. To explore the feasibility of direct high throughput sequencing of the entire 2.2 Mb interval, a ten fold redundant BAC library was constructed using genomic DNA from a homozygous mutant animal. A BAC contig spanning the critical region was assembled (Supplementary Figure S1) and fifteen BACs comprising the minimum tiling path was analyzed by massive parallel sequencing with seventy fold coverage (Figure 1; Supplementary Figure S1). Analysis of the DNA sequencing data against the C57BL/6J reference sequence (strain background in which mutation was generated) revealed only two single base changes: C to A substitution in a non-conserved intergenic region and T to C substitution causing a missense mutation (C193R) in a novel gene *Megf8* (Figure 1A). This finding is remarkably in line with previous estimate of one ENU-induced mutation per Mb, with one in 1.82 Mb expected to alter function[8].

Megf8 is a novel gene with no known function, encoding a well conserved protein of 2780 amino acids with one predicted transmembrane domain (Genbank EU723517) (Figure 1B). The mutation replaces an invariant cysteine residue with arginine in a conserved EGF repeat (Figure 1B). In situ hybridization showed *Megf8* is ubiquitously expressed with no difference detected in its expression in wildtype vs. homozygote (*Megf8*$^{m/m}$) mutant embryos (Figure 2). Given the Megf8 expression pattern was not

informative as to its role in left-right specification, we used zebrafish morpholino knockdown to further examine Megf8 function. Two independent morpholinos designed against zebrafish Megf8 disrupted the normal laterality of heart looping (Figure 1C; Supplementary Figure 2S,Table 2S), with 75% of the *Megf8* morphants exhibiting discordant heart and gut situs, recapitulating the heterotaxy phenotype of the *Megf8*$^{m/m}$ mutants (Figure 1C; Supplementary Table S2).

Analysis of *Megf8*$^{m/m}$ embryos showed early defects in laterality, including randomization in embryonic turning and heart tube looping (Supplementary Figure S3 and Table S3). In situ hybridization analysis showed *Nodal* expression was intact at the node, but absent in the LPM (n=6, 3-4 somites, Figure 2). Disruption in expression of genes downstream of *Nodal* was observed. Thus *Lefty1* and *Lefty2* expression in the floor plate and LPM, respectively, were lost (n=5, 4-5 somites, Figure 2d,e), while LPM expression of *Pitx2* was either absent (n=2) or bilateral (n=5) (6-8 somites) (Figure 2g-i). In contrast, *Pitx2* expression in the head mesenchyme was unaffected. Some *Megf8*$^{m/m}$ embryos showed asymmetric node expression of *Nodal*, indicating the initial breaking of symmetry was preserved (Figure 2a,b). Node expression of *Gdf1*, also required for *Nodal* propagation to the LPM[9], was unchanged, as was the expression of *Cryptic* (Figure 2e,f). As motile function of monocilia at the node is essential for left-right patterning downstream from the node, we also analyzed nodal ciliary motion[10]. *Megf8*$^{m/m}$ embryos exhibited normal clockwise ciliary rotation with a beat frequency and flow indistinguishable from heterozygous and wildtype embryos (Supplementary Figure S4 and Supplementary Quicktime Movies).

To explore the function of Megf8, we generated an antibody to Megf8. Surprisingly, immunostaining and confocal imaging showed prominent punctate dots in the nucleus, but no detectable cell surface localization (Figure 2l,m,n). Variable levels of cytoplasmic staining was also observed, with this being minimal in NIH3T3 cells (Figure 2l,m vs. n). The immunostaining in the nucleus and cytoplasm was significantly diminished with *Megf8* siRNA knockdown (Supplementary Figure S6). A previous yeast two hybrid screen had identified Megf8 as an interacting partner with Gfi1b[11], a protein localized in the nucleus and known to have transcription repressor activity and a role in heterochromatinization in erythroid cell lineages[12,13]. Immunostaining showed Megf8 and Gfi1b were coimmunolocalized in the nucleus as punctate dots (Figure 2). However, unlike the localization seen in erythroid progenitors, in MEFs and NIH3T3 cells, Gfi1b and Megf8 were not concentrated in heterochromatic regions delineated by strong DAPI staining. Moreover, unlike *Gfi1b* knockout mice[13], *Megf8*$^{m/m}$ mutants did not have defects in definitive erythropoeisis (Supplementary Figure S6), while *Gfi1b* knockout mice showed no laterality defects. Interestingly, another chromatin remodeling protein, Baf60C was previously shown to cause laterality defects with a single ventricle phenotype reminiscent of *Megf8*$^{m/m}$ mutants[14]. Confocal imaging showed Baf60C, Gfi1b, and Megf8 are colocalized in foci within the nucleus (Figure 2). However, Megf8 likely acts downstream of Baf60C, as Baf60C deficient embryos do not express *Nodal* at the embryonic node. These findings suggest genes involved in chromatin regulation may have important roles to play in congenital heart disease. Overall, these findings show the efficacy of high throughput sequencing for mutagenesis gene discovery efforts. In the

future, sequence capture arrays may further stream line such sequencing efforts by eliminating the need for BAC library constructions[5].

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Recovery of *Megf8* Mutation

(a). A BAC contig comprising of 15 BACs spanned the critical region containing the mutation in chromosome 7 (25, 837,390-28,028,384 Mb, NCBI m37 assembly). Sequencing showed T to C substitution, causing cysteine (C) to arginine (R) replacement in *Megf8*, and a C to A substitution in an intergenic region.

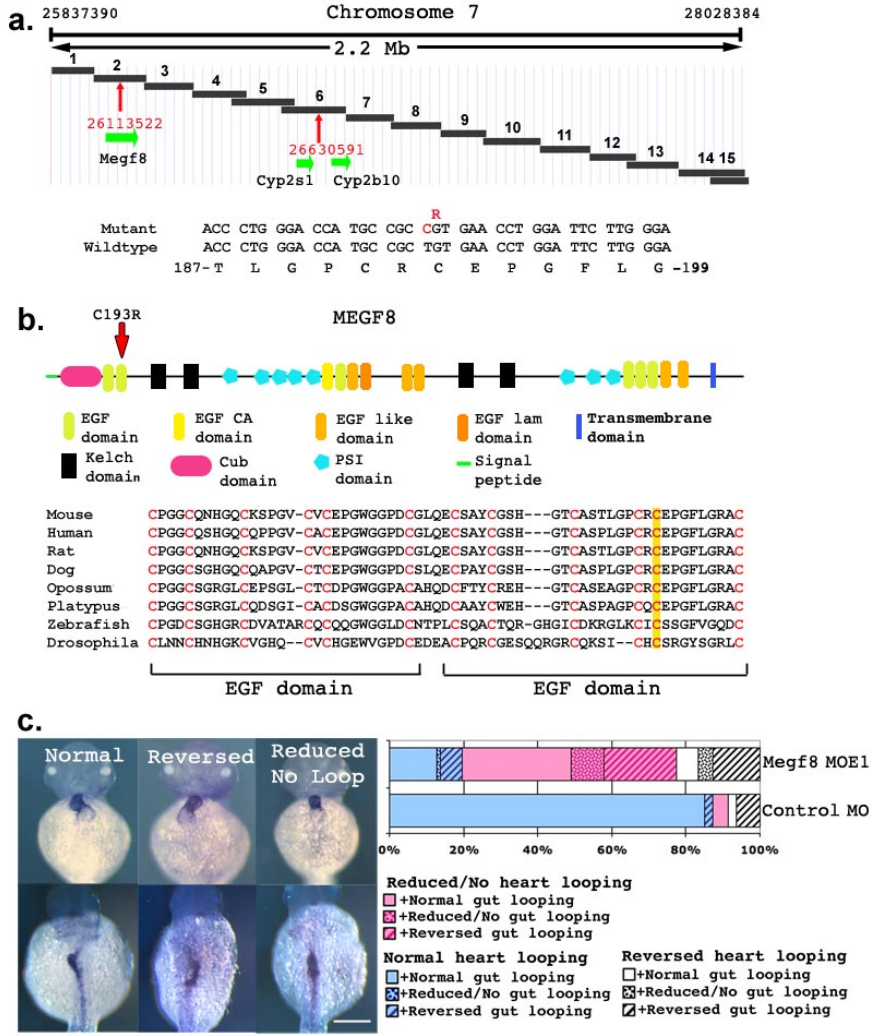
(b). *Megf8* encodes protein of 2,732 amino acids containing EGF, kelch, plexin, and CUB domains. The C193R substitution is in a highly conserved EGF domain.

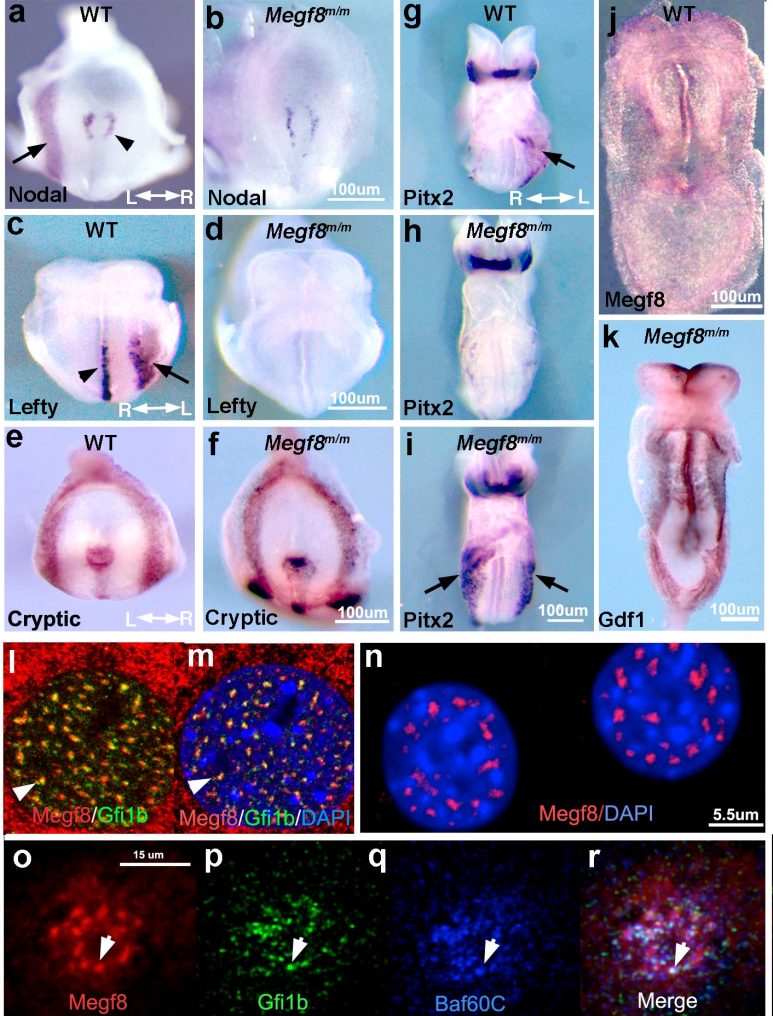
(c). Embryos injected with *Megf8* morpholinos show heart (Nkx2.5 expressing, ventral view-upper row) and foregut (Foxa3 expressing, dorsal view-bottom row) situs discordance. Results summarized in bar graphs.

Figure 2. Disruption of nodal signaling in *Megf8*$^{m/m}$ embryos and Megf8 immunolocalization.

(a-k). Expression of *Nodal (a,b)*, *Lefty1* and *2* (c,d), *Cryptic* (e,f), *Pitx2* (g,h,i), and *Gdf1* (k) in *Megf8*$^{m/m}$ and wildtype embryos were examined by in situ hybridization analysis. Arrowhead denote the node in (a), and the floor plate in (c). Arrow denotes the LPM. Posterior view. (c,d,g-i) Anterior view. (j, k) Ventral view.

(l-o). Confocal imaging show punctate nuclear localization of Megf8 (red) in MEFs (l,m) and NIH3T3 cells (n). Double and triple immunostaining show extensive colocalization of Megf8 with Gfi1b (green) (l,m) and Baf60c (blue) (o).

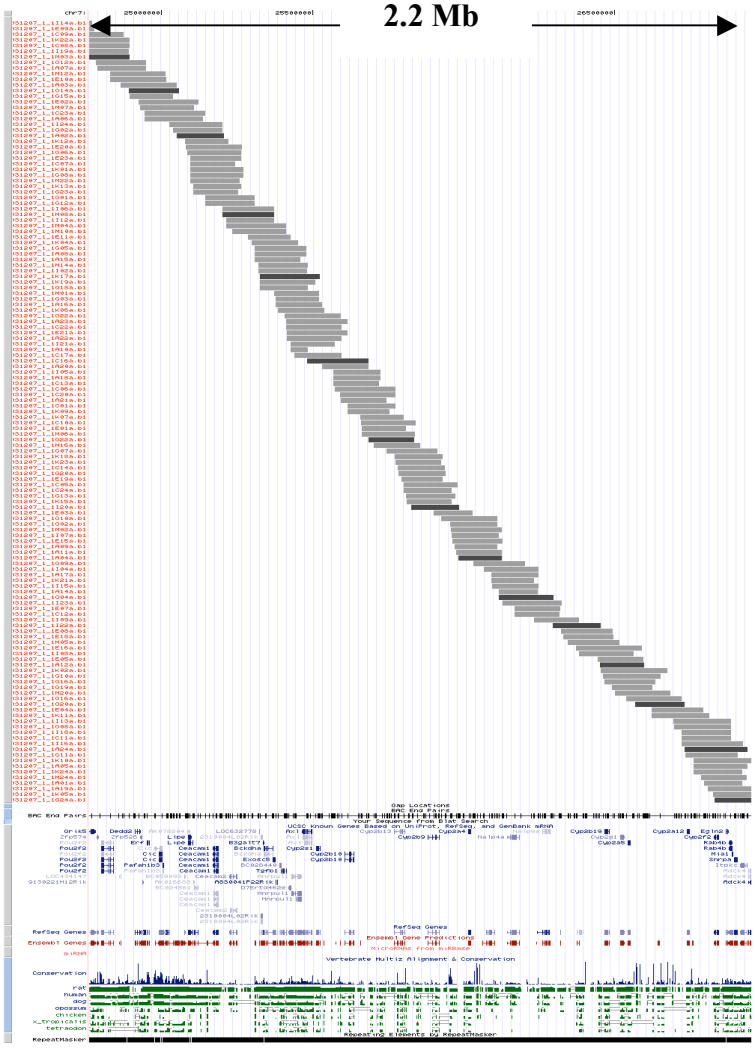

a.
25837390
Chromosome 7
28028384
2.2 Mb
1
2
3
4
5
6
7
8
9
10
11
12
13
14 15
26113522
Megf8
26630591
Cyp2s1
Cyp2b10
R
Mutant ACC CTG GGA CCA TGC CGC CGT GAA CCT GGA TTC TTG GGA
Wildtype ACC CTG GGA CCA TGC CGC TGT GAA CCT GGA TTC TTG GGA
187- T L G P C R C E P G F L G -199
b.
C193R
MEGF8
EGF domain
EGF CA domain
EGF like domain
EGF lam domain
Transmembrane domain
Kelch domain
Cub domain
PSI domain
Signal peptide
Mouse CPGGCQNHGQCKSPGV-CVCEPGWGGPDCGLQECSAYCGSH---GTCASTLGPCRCEPGFLGRAC
Human CPGGCQSHGQCQPPGV-CACEPGWGGPDCGLQECSAYCGSH---GTCASPLGPCRCEPGFLGRAC
Rat CPGGCQNHGQCKSPGV-CVCEPGWGGPDCGLQECSAYCGSH---GTCASTLGPCRCEPGFLGRAC
Dog CPGGCSGHGQCQAPGV-CTCEPGWGGPDCSLQECPAYCGSH---GTCASPLGPCRCEPGFLGRAC
Opossum CPGGCSGRGLCEPSGL-CTCDPGWGGPACAHQDCFTYCREH---GTCASEAGPCRCEPGFLGRAC
Platypus CPGGCSGRGLCQDSGI-CACDSGWGGPACAHQDCAAYCWEH---GTCASPAGPCQCEPGFLGRAC
Zebrafish CPGDCSGHGRCDVATARCQCQQGWGGLDCNTPLCSQACTQR-GHGICDKRGLKCICSSGFVGQDC
Drosophila CLNNCHNHGKCVGHQ--CVCHGEWVGPDCEDEACPQRCGESQQRGRCQKSI--CHCSRGYSGRLC
EGF domain
EGF domain
c.
Normal
Reversed
Reduced No Loop
Megf8 MOE1
Control MO
0%
20%
40%
60%
80%
100%
Reduced/No heart looping
+Normal gut looping
+Reduced/No gut looping
+Reversed gut looping
Normal heart looping
+Normal gut looping
+Reduced/No gut looping
+Reversed gut looping
Reversed heart looping
+Normal gut looping
+Reduced/No gut looping
+Reversed gut looping
1


a
WT
Nodal
L
R
b
Megf8m/m
Nodal
100um
g
WT
Pitx2
R
L
j
WT
Megf8
100um
c
WT
Lefty
R
L
d
Megf8m/m
Lefty
100um
h
Megf8m/m
Pitx2
k
Megf8m/m
Gdf1
100um
e
WT
Cryptic
L
R
f
Megf8m/m
Cryptic
100um
i
Megf8m/m
Pitx2
100um
l
m
Megf8/Gfi1b
Megf8/Gfi1b/DAPI
n
Megf8/DAPI
5.5um
o
15 um
Megf8
p
Gfi1b
q
Baf60C
r
Merge

2

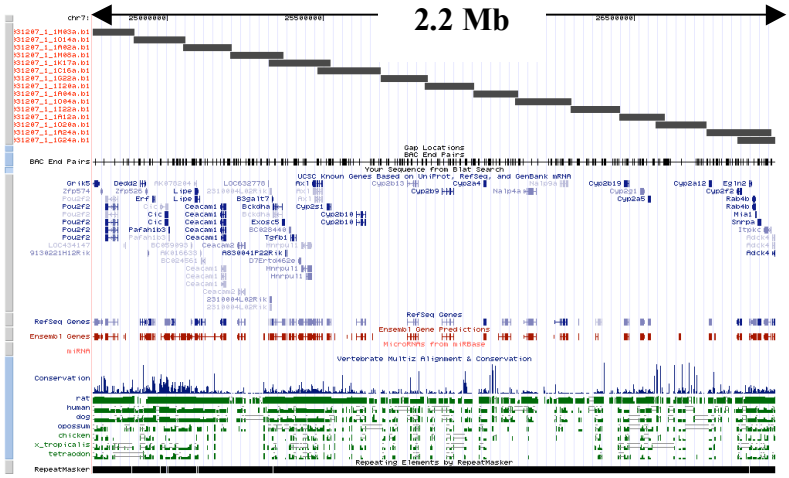
SUPPLEMENTARY FIGURES

Figure S1. BAC contig assembly spanning 2.2 Mb critical region of mouse chromosome 7



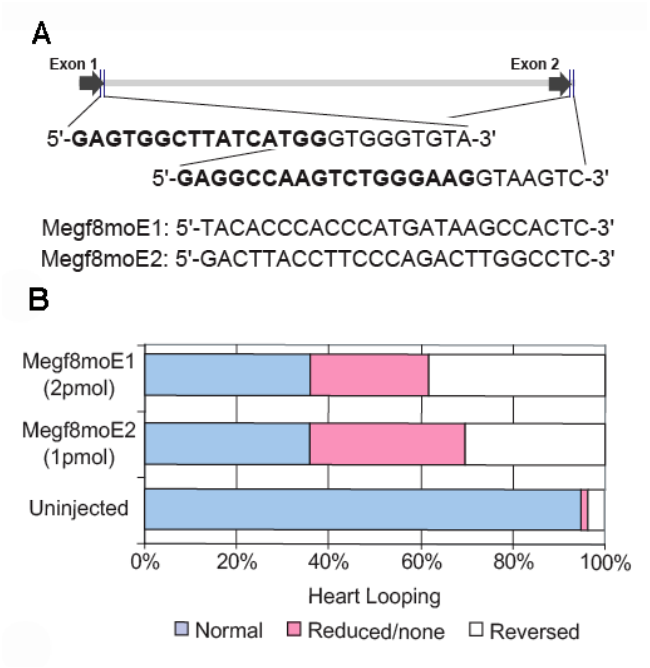
# BACs with both ends correct	139
Average insert length	167 kb
Region covered	2.2 Mb
Total length of genomic insert in BACs	23 Mb
Coverage	10.6 x

Minimum Tiling Path



# BACs	15
Total length of genomic insert in BACs	2.6 Mb
Overlap	269 kb
Region covered by BACs	2.2 Mb
Overlap	12%

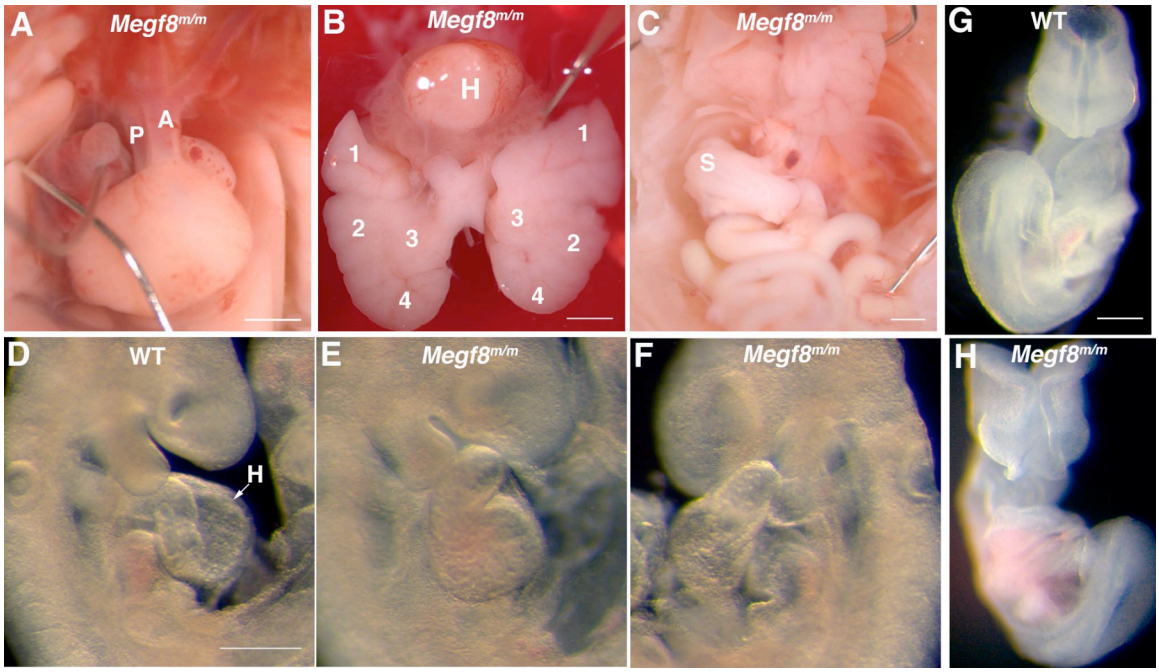
Figure S2. ***Megf8*** **regulates looping of the heart tube in zebrafish embryos.**



A) Two splice-blocking morpholino, Megf8moE1 and Megf8moE2, were designed to bind to the splice-donor sites of zebrafish *Megf8* exon 1 and exon2, respectively. Region 19356407-19359910 from chromosome 16 (Ensemble release 49) is illustrated, highlighting the sequence of the first two exon-intron boundaries of zMegf8 to which antisense morpholinos were designed. Exon sequence is in bold.

B) Both *Megf8* morpholinos disrupt heart looping. Normal orientation of heart looping was frequently observed in uninjected embryos (n=168), but not in embryos injected with either Megf8moE1 (n=92) or Megf8moE2 (n=123) morpholino.

Figure S3. Left-right asymmetric defects in *Megf8*$^{m/m}$ mutants.



(A) A Megf8$^{m/m}$ embryo has dextracardia as indicated by the heart apex pointing to the right side of the chest cavity. Transposition of the great arteries is also seen as the aorta (A) is positioned anterior to the pulmonary artery (P).

(B) Mutants typically exhibit four lung lobes bilaterally (viewed ventrally), indicating right pulmonary isomerism. H: heart

(C) A mutant with stomach (S) on the right side of the abdominal cavity.

(D) A normal D-loop heart tube (H) in a wildtype E9.0 embryo is viewed from the anatomical right.

(E,F) Megf8$^{m/m}$ embryos show randomized heart tube looping.

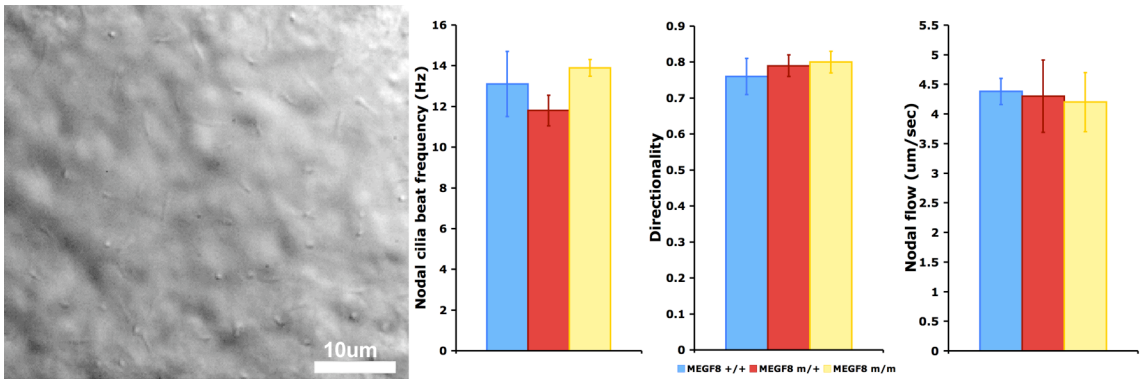
Shown are two Megf8$^{m/m}$ embryos, with one showing normal rightward heart tube looping (E), and a another embryo, viewed from the left, showing reversed leftward looping of the heart tube (F). Note its prospective left ventricle balloons to the right side of the chest cavity.

(G-H) Abnormal embryonic turning in *Megf8*$^{m/m}$ embryos.

Ventral view of E9 embryos show embryonic turning in a wildtype (G) and *Megf8*$^{m/m}$ (H) embryo. In wildtype embryo, embryonic turning positions the tail to the right of the head (G). In the *Megf8*$^{m/m}$ embryo, reversal in embryonic turning positions the tail positioned to the left of the head (H).

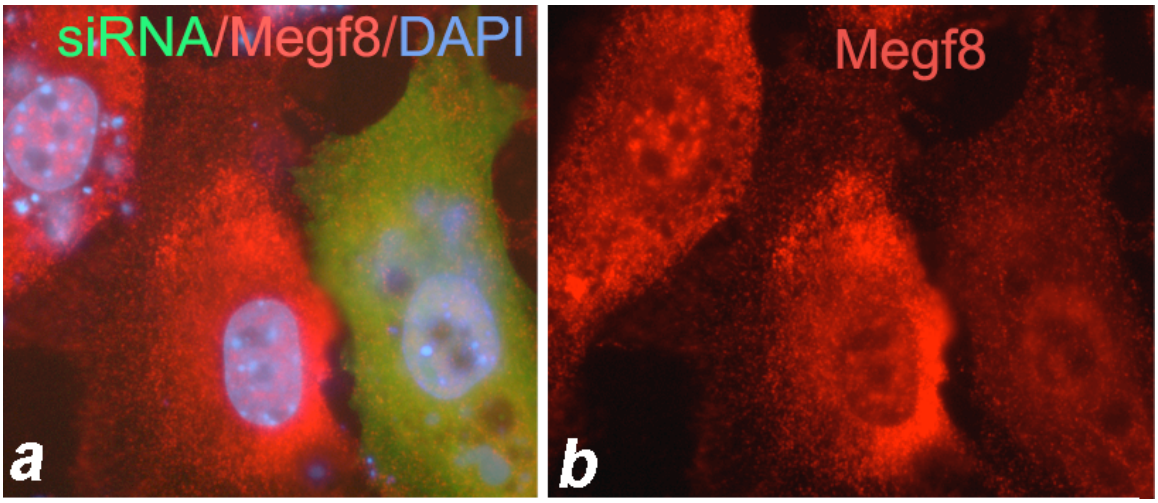
Scale bar = 500μm (A-C), = 100μm (D, G)

Figure S4. Analysis of ciliary motion at the embryonic node



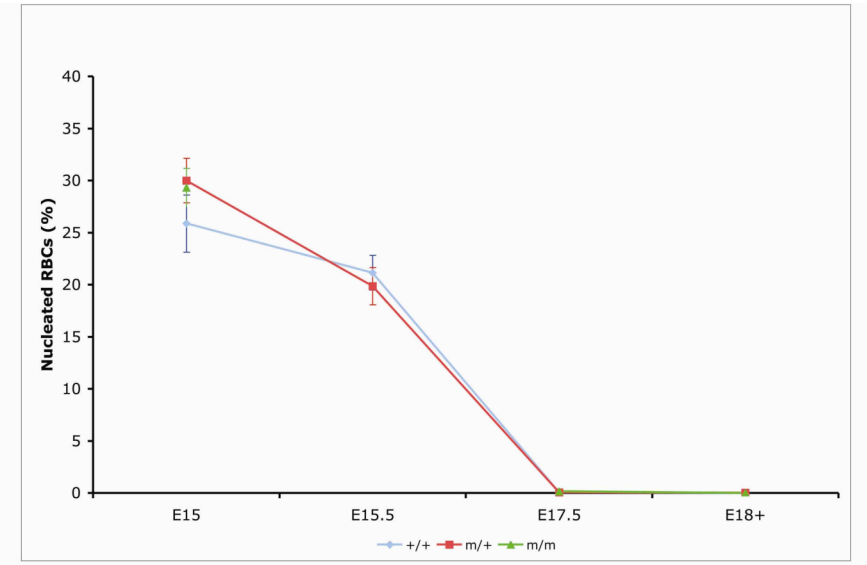
Movement of the nodal cilia at the embryonic node in wildtype, *Megf8*$^{m/+}$ and *Megf8*$^{m/m}$ embryos were examined by videomicroscopy. Quantitation of ciliary beat frequency, directionality and nodal flow showed no change in the motile function in the heterozygous or homozygous mutant embryos.

Figure S5. ***Megf8*** **siRNA knockdown eliminates Megf8 antibody staining**



Mouse embryonic fibrobasts were cotransfected with *Megf8* siRNA and GFP expressing constructs, then imunostained with a Megf8 antibody. In cells transfected with *Megf8* siRNA, indicated by expression of the GFP reporter (green), Megf8 expression (red) was significantly diminished.

Figure S6. Analysis of definitive erythropoeisis in *Megf8* mutant embryos.



Developmental Age	+/+	+/m	m/m
E15	25.9± 2.7 n=1	30.0±2.1 n=4	29.3±1.8 n=4
E15.5	21.2±1.7 n=4	19.9±1.8 n=3	-
E17.5	0.1±0.01 n=4	0.08±0.08 n=3	0.2±0.1 n=2
E18.5	0±0 n=5	0.03±0.03 n=5	0±0 n=1

To determine if *Megf8* mutant embryos have a defect in definitive erythropoiesis, blood smears were made with blood collected from the umbilical vessels of E15-E18 stage embryos. After air drying followed by 2 minute fixation in 100% methanol, the blood smears were mounted using DAPI containing Vectashield mounting medium (Vector Laboratories, CA) and the percentage of nucleated red blood cells (RBCs) was quantitated. The percentage of nucleated RBCs declined from E15 until none were detected near term at E17.5 and beyond. This was observed for all three genotypes (wildtype, heterozygous and homozygous *Megf8* mutants), consistent with normal transition to definitive erythropoiesis. These findings suggest *Megf8* mutants have no defect in definitive erythropoiesis.

SUPPLEMENTARY TABLES

Table S1. Chromosome 7 Microsatellite and SNP Markers

Microsatellite Markers		**SNP markers**	
D7Mit152	4646621	rs8254028	19677381
D7Mit75	6524930	rs6294145	20031893
D7Mit56	17488717	rs32238315	20654082
D7Mit191	19471933	rs31766853	22477135
D7Mit57	20283120	rs16792309	24127640
D7Mit192	25837390	rs3679003	24253209
D7Mit294	28074461	rs8276482	25088645
D7Mit77	28391392	rs16800724	25163948
D7Mit266	29086063	rs13479151	25218302
D7Mit227	37365609	rs13479152	25512658
D7Mit25	37653505	rs13479153	25722935
D7Mit247	37927844	rs32393147	26479896
D7Mit228	47279833	rs31194828	27915985
D7Mit229	52942350	rs3662508	28006891
D7Mit248	73484970	rs13460395	28028384
D7Mit350	90734599	rs31307939	28036213
D7Mit330	126800204		
D7Mit71	138168667		

To map and track the mutation, the founder animal was intercrossed with C3H and in later generations, intercrossed with consomic strain C57BL/6J-Chr7$^{A/J}$/NaJ. The mutation was initially mapped to mouse chromosome 7. For fine mapping the mutation, 142 mutants were analyzed using 18 microsatellite and 16 SNP markers that could differentiate between C57BL6/J and C3H or A/J genome (http://www.ncbi.nlm.nih.gov/projects/SNP/MouseSNP.cgi, http://www.informatics.jax.org/searches/polymorphism_report.cgi.). This allowed tracking of the mutation via the segregation of C57BL/6J markers, the strain background in which the mutation was generated. Markers used in this analysis are listed below with the position on chromosome 7 indicated.

Table S2. *Megf8* Morpholino Knockdown in Zebrafish Embryos

A. *Megf8* Morpholino Knockdown Causes Heterotaxy

	Normal Situs	Reverse Situs	Discordant Situs
Control MO (n=47)	85% (40)	6% (3)	9% (4)
***Megf8* MO* (n=102)**	13% (13)	13% (13)	75% (76)
$p<0.0001$			

*p values obtained by Chi square analysis.

B. Embryos with Discordant Situs

Heart Tube	**Reduced or No Looping**			**Reverse Looping**		**Normal Looping**	
Gut Position	**Normal**	**Reverse**	**Reduced**	**Normal**	**Reduced**	**Reverse**	**Reduced**
Control MO	4% (2)	0%	0%	2% (1)	0%	2% (1)	0%
	Total = 4%			**Total = 2%**		**Total = 2%**	
Megf8 MO	29% (30)	20% (20)	9% (9)	6% (6)	4% (4)	6% (6)	1% (1)
	Total = 59%			**Total = 10%**		**Total = 6%**	

Table S3. Randomized heart tube looping and direction of embryonic turning

Heart Looping	Right		Left	
Embryonic Turning	Right	Left	Right	Left
Megf8$^{m/m}$	3	3	2	4

Supplementary Materials and Methods

Mutation Mapping

Previously we mapped the mutation to a 3.3 Mb region of chromosome 7 between markers D7mit192 and D7mit266[1]. Further fine mapping was done with 33 mutants by tracking the segregation of C57BL6/J genome with the phenotype using SNPs in the mapped region that could differentiate between C57BL6/J and C3H or A/J genome.

BAC Library Construction and Screening

CHORI-602 BAC library construction was constructed as described in details[2]. A single mouse embryo was grinded to powder under liquid nitrogen using mortar and pestle, resuspended in a chromatin-extraction buffer (20 mM NaCl, 80 mM KCl, 15 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 0.2 mM Spermine, 0.5 mM Spermidine, 18 mM β-Mercaptoethanol, pH7.2) and homogenized in a glass Douncer. The resulting cell and tissue suspension was embedded in 0.5% InCert agarose (Cambrex) for high molecular weight DNA extraction. Agarose embedded DNA was partially digested with EcoRI restriction enzyme and size fractionated by pulsed-field electrophoresis. The appropriate size fractions were isolated and used for ligation reactions with the BAC vector (pTARBAC2.1) that has been linearized using EcoRI restriction enzyme. The ligation products were used to transform DH10B E.coli cells (Invitrogen). The library was arrayed into 528 384-well microtiter dishes and subsequently gridded onto 11 22x22cm nylon high-density membranes for screening by probe hybridization. Each hybridization membrane represented over 18,000 distinct BAC clones, stamped in duplicate. 44 pairs of unique overlapping oligonucleotides (overgos) were selected to screen the library for the critical region based on Build 36.1 "essentially complete" assembly by NCBI and the

Mouse Genome Sequencing Consortium (2.2 Mb region of chromosome 7 position 24,761,131 - 26,952,125). Each pair of overgos was annealed to each other, ends were filled in with Klenow polymerase fragment in a presence of ^{32}P-ATP and ^{32}P-CTP to produce the 40-basepaire double stranded probe. Probes were pooled together, purified through a Sepharose column, heat denatured and hybridized against high-density membranes. 172 BAC clones were identified as carrying genomic fragments within critical region based on hybridization results. Positive BAC clones were end-sequenced to locate genomic fragment boundary and 139 BACs were positively mapped to the critical region by both end sequences. A total of 15 overlapping BAC clones comprising of a minimal tiling path over the critical region were selected for high throughput sequencing.

BAC Sequencing and Mutation Detection

Each BAC was independently isolated by standard alkaline lysis (Qiagen) and equimolar amounts of each BAC were pooled for standard Illumina Genome Analyzer I sequencing. A total of 303 Mb were generated. Reads were aligned to the reference using BLAT and potential SNPs identified as mismatches within the alignments. Filtering for a minimum depth of 5 reads and at least 70% of aligned reads containing the mismatch identified 10 putative variatns. All 10 putative mutations were independently assessed by Sanger sequencing, two (X and Y) of which were confirmed to be real.

Mouse Breeding and Genotyping

Megf8$^{m/+}$ mice were maintained in the C57BL/6J and C3H/HeJ mixed background. Genotyping PCR was performed with forward primer (TCCTTGAACATTCCCTTCCTG) and reward primer

(CCCATCTGCTTACCTCCAAAG). PCR product was digested with MspA1I. As the *Megf8* mutation eliminates a MspA1l site in PCR product, the mutant allele generates two bands at 259 and 58 bps while the wildtype allele generates three bands at141, 119, and 58 bps.

Quantitation of Nodal Ciliary Motion by Videomicroscopy

E7.5-E7.75 embryos were obtained from heterozygous matings. The bottom section of each embryo was dissected and transferred nodal side down onto a 35mm glass bottomed culture dish with a few drops of L-15 medium (+ 10% FBS). A round glass coverslip (FisherBrand, USA), covered with a silicone sheet 0.012" thick (AAA Acme Rubber Co, AZ) with a small window cut out to make a thin walled chamber, was placed over the embryo to help secure the node facing the bottom of the dish. Cilia beat dynamics were subsequently captured at room temperature using a Leica inverted microscope (Leica DMIRE2) with oil x100 objective and DIC optics. To quantify cilia beat frequency High Speed AVI movies (200 fps) were collected using a Phantom v4.2 camera (Vision Research, NJ) and later analyzed using Openlab software (Openlab 3.1.7, Improvision, UK). To quantify nodal flow a small quantity of 0.20µm Fluoresbrite microspheres (Polysciences, PA) were added to the media bathing the node and fluorescent movies were collected using FITC filter prisms (excitation 425/60nm, emission 480nm) and a high-speed CCD camera (Hamamatsu, C9100-12). Movies were subsequently analyzed using Volocity 3.5.1 software (Improvision, UK) to manually track microsphere speed and directionality. Directionality was defined as the net displacement achieved divided by the total distance traveled, with a particle moving in a straight line having a directionality of one.

Zebrafish Morpholino Injections

Two antisense morpholino oligonucleotides (Megf8moE1 and Megf8moE2, Gene Tools) to *Megf8* were designed as illustrated in supplementary Figure S2A. The Standard negative control morpholino was provided by Gene Tools. Morpholinos were resuspended in 1x danieau (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM (CaNO3)2, 5.0 mM HEPES pH 7.6), and increasing doses (0.5 pmol– 4.0 pmol) were injected into one-cell-stage embryos to determine optimal concentrations. At 48hpf, embryos were scored for the direction of heart looping or collected and fixed for in situ hybridization. Data in Figure 1c were from experimental groups injected with 1pmol Megf8moE1 (n=107) and 1pmol control morpholino (n=47). Working doses for other experiments are listed in supplementary Figure S2B.

In Situ Hybridization Analysis

Whole mount RNA in situ hybridization was performed as previously described[3], using an in situ hybridization robot (Intavis AG). Hybridization was carried out using DIG-labeled probes for zebrafish *Nkx2.5* and *Foxa3*, and mouse *Nodal, Lefty1, Lefty2 and Pitx2*.

Megf8 Antibody Production

Polyclonal antibody to Megf8 was raised in chickens by immunization with the synthetic peptide QQEKETRRLQRPGSDR, which corresponded to a N-terminal region of the protein (Aves Labs, Tigard, OR). At the N-terminal side of the peptide a cysteine residue was incorporated for crosslinking, followed by Z (N-aminohexanoic acid), which was added as a spacer. The antibodies were affinity purified and analyzed by ELISA.

Immunohistochemistry

Immunocytochemistry was performed on Megf8$^{m/m}$ and Megf8^{WT} MEFs, as well as NIH3T3 cells grown on 12mm coverslip for 24 h using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% Penicillin/Streptomycin (Gibco). Cells were fixed in 4% PFA at RT for 10 min, washed 3X in PBS then incubated in BlockHen (Aves Labs) diluted 1:10 in PBST for 1 hour at RT. Primary antibodies were diluted in 3% BSA and incubated for 2 h at RT. Following 3X washes in 1% BSA and 3X PBS for 30 min total, secondary antibodies diluted in 3% BSA were incubated for 30 min at RT. Coverslips were washed 3X in PBS and mounted in Vectashield with DAPI (Vector Labs, Burlingame, CA). Primary antibodies: were chicken anti-Megf8 (1:1000, Aves Labs), Goat anti-Gfi-1B (Clone D-19, 1:50, Santa Cruz, CA), and mouse anti-SMARCD3 (1:100, clone 1G6, Abnova, Walnut, CA). Secondary antibodies: Alexa Flour 488 conjugated donkey anti-goat IgG and Donkey anti-mouse IgG (1:1000, Molecular Probes), Cy5 and Texas Red conjugated donkey anti-chicken IgY and Goat anti-mouse IgG (1:1000, Jackson Immunoresearch, Westgrove, PA). Coverslips were imaged using 40X oil emersion lens on the Zeiss LSM 510 META Laser Scanning Confocal Microscope. Images were further processed using Velocity software (Improvision Inc A Perkin-Elmer Company, Waltham, MA)

Generation of Mouse Embryonic Fibroblasts (MEFs)

Pregnant females staged at E13.5 were euthanized using CO2. Embryos were dissected out of the female under sterile conditions and washed in D-PBS (Gibco). The head and internal organs of each embryo were removed leaving only the outer body including both fore and hind limbs. The remaining tissue was disaggregated and passaged through a 21 gauge syringe 15X or until it was thoroughly homogenized. Cells were spun down at 200

x g for 10 minutes at RT and the pellet was re-suspended in DMEM with 10% FBS and 1% P/S. Cells were then plated onto 10cm dishes and incubated at 37 degrees at 5% CO_2. Medium was changed the following day to prevent cell toxicity.

siRNA knockdown of *Megf8*

Wild type MEFs were seeded into 24 well plates containing 12mm coverslips at a density of $5X10^4$ cells/well. Cultures were maintained for 24 hours before transfection in DMEM with 10% FBS in a 37 degree incubator with 5% CO2. MEFs were transiently transfected with a combination of SureSilencing ^{TM} ShRNA plasmids for mouse Megf8 containing a GFP reporter (SuperArray, Fredrick, MD) using Lipofectamine LTX (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. The sequences of the four plasmids including a GFP Control are: Clone sequence 1: ACAGGCTACACCATGGACAAT, Clone sequence 2: TCACCGCCTGGGACATACTAT, Clone sequence 3: CTAGGTTGGTGTGTGCACAAT, Clone sequence 4: ACCCTTGAGCCCACAGAAGAT and control: GGAATCTCATTCGATGCATAC. Coverslips were fixed in 4% PFA and immunostained using the chicken anti-Megf8 antibody as previously described above. Coverslips were imaged using a Hamamatsu ORCA-ER camera mounted on the Leica DMRA (Leica Microsystems GmbH, Wetzlar, Germany). Images were captured and analyzed using Open Lab 3.1.7 software (Improvision Inc A Perkin-Elmer Company, Waltham, MA)