Massively parallel sequencing identifies the gene \textit{Megf8} with ENU-induced mutation causing heterotaxy

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Forward genetic screens with ENU (N-ethyl-N-nitrosourea) mutagenesis can facilitate gene discovery, but mutation identification is often difficult. We present the first study in which an ENU-induced mutation was identified by massively parallel DNA sequencing. This mutation causes heterotaxy and complex congenital heart defects and was mapped to a 2.2-Mb interval on mouse chromosome 7. Massively parallel sequencing of the entire 2.2-Mb interval identified 2 single-base substitutions, one in an intergenic region and a second causing replacement of a highly conserved cysteine with arginine (C193R) in the gene \textit{Megf8}. \textit{Megf8} is evolutionarily conserved from human to fruit fly, and is observed to be ubiquitously expressed. Morpholino knockdown of \textit{Megf8} in zebrafish embryos resulted in a high incidence of heterotaxy, indicating a conserved role in laterality specification. \textit{Megf8}\textsuperscript{C193R} mouse mutants show normal breaking of symmetry at the node, but Nodal signaling failed to be propagated to the left lateral plate mesoderm. Videomicroscopy showed nodal cilia motility, which is required for left–right patterning, is unaffected. Although this protein is predicted to have receptor function based on its amino acid sequence, surprisingly confocal imaging showed it was translated into the nucleus, where it is colocalized with Gfi1b and Baf60C, two proteins involved in chromatin remodeling. Overall, through the recovery of an ENU-induced mutation, we uncovered \textit{Megf8} as an essential regulator of left–right patterning.

The power of forward genetic screens is well illustrated by the remarkable success of the \textit{Drosophila} chemical mutagenesis screen used to dissect the genetic pathways specifying the segmental body plan (1). This study elegantly showed the efficacy of phenotype driven mutagenesis screens in the systematic analysis of complex biological processes regulating developmental patterning. To elucidate the genetic basis for congenital heart disease, we pursued a high throughput mouse ENU mutagenesis screen using noninvasive fetal echocardiography for cardiovascular phenotyping. ENU is a potent mutagen ideally suited for the production of human disease models in mice, because it predominantly generates point mutations, which are often associated with human diseases. More than 13,000 mouse fetuses were ultrasound interrogated, with 4% of the fetuses showing some evidence of cardiac defects (2, 3). This highly efficient cardiovascular phenotyping protocol suggests the possibility of a saturation mutagenesis screen.

The recovery of ENU-induced mutations in mice traditionally entails breeding the mutation generated in one inbred strain into a different inbred strain background, thereby allowing the use of polymorphic DNA markers to map the mutation. Genome scanning of many meiotic recombinants with such polymorphic DNA markers can eventually reduce the map interval to < 1 Mb, when sequencing of candidate genes becomes more practical. This traditional approach for mutation recovery is costly and time consuming, and the mutation may be missed in regions with incomplete genome annotation. However, with rapid advances in a new generation of high throughput DNA sequencing technologies (4–6), rapid and low cost sequencing may greatly facilitate mutation recovery from mutagenesis screens. These new sequencing technologies have already proven useful for addressing a wide range of biological questions, from de novo sequencing of microorganisms (7), cancer mutation discovery (8), gene expression profiling (9) to epigenetic regulation (10).

In this study, we used massively parallel sequencing to recover an ENU-induced mutation causing a single-ventricle spectrum of complex structural heart defects recovered in our mouse fetal echocardiography screen (11). This mutant exhibits transposition of the great arteries, randomized left–right cardiopulmonary and visceral organ situs, a constellation of phenotype referred to as heterotaxy. This mutant also exhibits preaxial polydactyly. We mapped this mutation to a 2.2-Mb interval on mouse chromosome 7. Massively parallel DNA sequencing of the entire 2.2-Mb critical region revealed the underlying genetic lesion as a point mutation in a highly conserved gene, \textit{Megf8}. \textit{Megf8} is expressed ubiquitously, and plays an essential role in left–right patterning through the regulation of Nodal signaling. Overall, through the recovery of an ENU-induced mutation, we uncovered \textit{Megf8} as an essential regulator of left–right patterning.

Results

Recovery of \textit{Megf8} Mutation. A recessive mutation was previously recovered exhibiting a single-ventricle spectrum of complex congenital heart defects associated with heterotaxy. Typically these mutants exhibited thoracoabdominal organ situs anomalies that included dextrocardia/mesocardia, right pulmonary isomerism, transposition of the great arteries, abnormal pulmonary venous connections, right-sided stomach and asplenia/polysplenia (Fig. S1 A–C) (11). We reported the mapping of this mutation to a 3.3-Mb interval on mouse chromosome 7. With analysis of a total of 142 meiotic recombinants, we narrowed the map interval to a 2.2-Mb critical region between markers D7Mit192-SNP rs13460395 (Table S1). Exon sequencing of a number of potential candidates showed no mutation. Because the mapped genomic interval is gene dense, systematic resequencing of all coding exons was impractical.

To examine the feasibility of using massively parallel DNA sequencing to recover the mutation in this genomic interval, we


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constructed a BAC library containing genomic DNA from the mutant and assembled a 10-fold redundant BAC contig spanning the 2.2-Mb critical region (Fig. S2). From this BAC contig, a minimum tiling path of 15 BACs was delineated (Fig. 1A and Fig. S2). These were pooled and sequenced with 70-fold coverage by massively parallel sequencing. A total of 303 Mb of sequences were generated. Reads were aligned using BLAT to the C57BL/6J reference sequence, the strain background in which the mutation was generated, and potential SNPs were 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 variants. All 10 putative mutations were independently assessed by Sanger sequencing, with 2 (26113522 and 26630591) subsequently being sequence confirmed. One mutation was a single base change of a C to A substitution in a noncoding intergenic region, and a second T to C substitution causing a missense mutation (C193R) in the gene Megf8 (Fig. 1B).

The point mutation in the intergenic region resides in a repetitive element that is not conserved, even between mouse and rat. Hence, this intergenic mutation is unlikely to be deleterious. In contrast, Megf8 encodes a well-conserved protein of 2,789 aa (GenBank EU723517). Orthologs are found in many other species, including human, zebrafish and Drosophila (Fig. 1B). The SMART domain tool (12) predicts Megf8 contains multiple EGF, EGF-like, calcium-binding EGF-like and laminin-type EGF-like repeats, kelch domains, plexin repeats, and a CUB and transmembrane domain (Fig. 1B). The missense mutation eliminates an invariant cysteine situated in the second putative EGF domain, and could disrupt formation of a disulfide bond required for proper protein folding. Thus, this second mutation is a good candidate as the genetic lesion causing the defect phenotype in this mutant line. Consistent with this, the systematic analysis of nearly 200 embryos showed the mutant phenotype was strictly associated with the 42 homozygote Megf8<sup>C193R</sup> (Megf8<sup>m/m</sup>) mutant embryos.

**Megf8 Morpholino Knockdown in Zebrafish Causes Heterotaxy.** To evaluate the biological function of Megf8, we carried out morpholino knockdown in zebrafish embryos. Megf8 knockdown recapitulated the heterotaxy phenotype of Megf8<sup>m/m</sup> mutants, with discordant heart and gut situs observed in 75% of zebrafish Megf8 morphants (Fig. 2I and Tables S2 and S3). Among the zebrafish embryos with discordant situs, 10% showed reverse heart looping, and 59% had abnormal or no looping. The latter embryos often exhibited a shortened heart tube (Fig. 2C), indicative of a role for Megf8 in cardiac morphogenesis (11). Approximately half of the embryos with abnormal heart looping showed reverse looping of the gut. This same constellation of situs defects was observed with 2 independent splice-blocking morpholinos (Fig. S3). These results confirm Megf8 is indispensable for left–right patterning. The recapitulation of the mouse heterotaxy phenotype with Megf8 morpholino knockdown would suggest this C193R mutation might be a loss of function mutation.

**Nodal Signaling Fails to Propagate to the Left LPM.** To investigate the developmental origin of laterality defects in the Megf8<sup>m/m</sup> embryos, we examined the direction of embryonic turning and heart looping, two early indicators of left–right specification. Megf8<sup>m/m</sup>
The breaking of symmetry and specification of left–right patterning are largely orchestrated by the embryonic expression of the highly conserved Nodal, Lefty1/Lefty2 and Pitx2 signaling network early in embryogenesis, largely spanning E7.75–8.5 (13). Notably, Megf8 is not asymmetrically expressed, because RNA in situ hybridization analysis with 2 independent Megf8 probes showed Megf8 transcripts are ubiquitously expressed in the embryo (Fig. S4). This same pattern was observed in wild-type and Megf8m/m mutant embryo (Fig. S4). An early molecular evidence of left–right asymmetry in Nodal expression at the embryonic node. Analysis of Megf8m/m mutants showed 3 of 7 embryos with stronger left sided perinodal expression of Nodal (Fig. 3B). This compared with 3 of 5 wild-type/heterozygous embryos exhibiting stronger left sided Nodal expression at the node (Fig. 3A). These results suggest the breaking of symmetry at the node is unaffected. Consistent with this, motile function associated with monocilia at the node, which is required for breaking symmetry (14), was also preserved (Fig. S5 and Quicktime Movie S1 and Movie S2).

Thus, Megf8m/m embryos exhibited normal clockwise ciliary rotation with a beat frequency indistinguishable from heterozygous and wild-type embryos. Using fluorescent beads, we also examined cilia generated flow at the node and again, no change was detected (Fig. S5). These findings suggest Megf8 functions downstream of the node. Indeed, in situ hybridization analysis of Megf8m/m embryos showed Nodal expression, although intact at the node, was absent in the left LPM (n = 7, 3–4 somites) (Fig. 3B). Expression of genes downstream of Nodal was also disrupted, with Lefty1 and Lefty2 expression lost in the floor plate and LPM, respectively (n = 5, 4–5 somites, Fig. 3D). Pitx2 expression in the LPM was also either absent (n = 2) or bilateral (n = 5) (6–8 somites) (Fig. 3G–J). In contrast, Pitx2 expression in the head mesenchyme was unaffected.

Genetic analyses have suggested that Nodal expression at the node is required for the activation of left-determinant transcription cascade in the left LPM (15, 16). Our data, would suggest the molecular defect underlying the Megf8m/m phenotype is either an interruption of signal transfer from the node to left LPM and/or a disruption in the initiation and expansion of Nodal expression in the LPM. To further elucidate the underlying molecular mechanism, we examined the expression of Gdf1 and Cryptic. Gdf1 encodes a TGFβ superfamily ligand required for Nodal long-range action, including propagation of Nodal signaling from the embryonic node to the LPM (17), while Cryptic, a coreceptor required for Nodal signal reception, is also essential for Nodal expression in the left LPM (18). Knockout mutants of either gene have phenotypes that are very similar to the Megf8m/m mutants. However, in situ hybridization analysis showed no change in Gdf1 and Cryptic expression in Megf8m/m mutants (Fig. 4).

Fig. 2. Morpholino knockdown of Megf8 causes situs discordance of heart tube and foregut. Zebrafish embryos injected with control or Megf8 morpholinos were examined for heart and gut situs defects using RNA in situ hybridization analysis with Nkx2.5 (A–D) and Foxa3 (E–H) probes to visualize the heart tube and gut, respectively. In the majority of zebrafish embryos injected with control morpholino, the heart tube exhibited normal rightward looping delineating a 5-shaped heart tube (A), whereas the foregut showed normal leftward looping (E). Megf8 morpholino injection resulted in randomization of the direction of looping for both the heart tube (B–D) and foregut (F–H). Megf8 morphants show normal (B and F), reversed or no looping (C and G), or reversed looping (D and H). (I) The distribution of heart and foregut looping pattern observed in the control and Megf8 morphants is summarized in the bar graph. Note that 74% of Megf8 morphants show heterotaxy, as indicated by discordance in their heart and gut situs. (A–D) Embryos shown in ventral view with anterior at the top. (E–H) Embryos shown in ventral view with anterior at the top. (Scale bar: 100 μm.)
nucleus (Fig. 4) tion repressor activity and a role in heterochromatinization in a 2-hybrid screen that identified Megf8 as a potential interacting partner, particularly interesting in light of a previous report from a yeast flag immuno- 

3XFLAG-tagged Megf8 fusion protein gave an expected high molecular weight band that was detected by both the Megf8 and 3222/H20841/FLAG antibodies (Fig. S7).

immunoblotting of transfected cells expressing a C-terminal 3222/H20841/FLAG epitope allowed us to investigate the subcellular distribution of Megf8. Although Megf8 is predicted to have a transmembrane domain, immunostaining and confocal imaging showed no cell surface localization in either mouse embryonic fibroblasts (MEFs) or NIH 3T3 cells. Instead, we observed prominent punctate nuclear staining and varying levels of cytoplasmic staining (Fig. 4 D–F). Immunostaining of embryo cryosections showed a similar distribution, including punctate nuclear localization (Fig. 4 A–C). No obvious change in Megf8 expression level or distribution was observed in either Megf8membryos or MEFs derived from the mutant embryos (Fig. S6). The specificity of the Megf8 antibody was verified with Megf8 siRNA knockdown, which largely abolished the Megf8 antibody staining (Fig. S6). In addition, Western immunoblotting of transfected cells expressing a C-terminal 3XFLAG-tagged Megf8 fusion protein gave an expected high molecular weight band that was detected by both the Megf8 and FLAG antibodies (Fig. S7).

The finding of Megf8 localization in the nucleus was particularly interesting in light of a previous report from a yeast heterochromatinization model system (20, 21). Double immunostaining showed Megf8/Gfi1b are colocalized as punctate spots in the nucleus (Fig. 4 D and E). Unlike the localization seen in erythroid progenitors, in MEFs and NIH 3T3 cells, Gfi1b and Megf8 were not concentrated in heterochromatic regions delineated by strong DAPI staining (Fig. 4 E and F). Further analysis showed Megf8membryos, unlike Gfi1b knockout mouse embryos (20), do not have defects in definitive erythropoiesis (Fig. S8). Conversely, Gfi1b knockout mouse embryos, unlike Megf8membryos, did not exhibit laterality defects.

Interestingly, a chromatin remodeling protein, Baf60C, a component of the Swi/Snf-like BAF complex, also has been shown to play an essential role in left–right patterning, with the Baf60C knockdown mice exhibiting cardiac and laterality phenotypes very similar to the Megf8membryos (22). Double immunostaining showed Megf8 and Baf60C have regions of overlapping localization in the nucleus. Triple immunostaining and confocal imaging with antibodies to Baf60C, Gfi1b, and Megf8 showed regions in which all 3 proteins are colocalized in the nucleus (Fig. 4 G–J). Together these findings suggest Megf8 may regulate Nodal signaling through a role in chromatin remodeling.

Discussion

Recovery of ENU-Induced Mutations by Massively Parallel Sequencing.

Using massively parallel sequencing, we identified 2 single-base substitutions in a 2.2-Mb genomic interval containing an ENU-induced mutation causing heterotaxy. This is the first demonstration of the use of massively parallel sequencing in the recovery of ENU-induced mutations. We used a strategy combining both massively parallel sequencing and BAC contig construction to recover the ENU-induced mutations. This strategy obviated the presently prohibitive expense entailed in whole genome resequencing. By sequencing only a subset of the genome contained within the BAC contig, we also minimized potential complications from segmental duplications and repeat sequences in the mouse genome. Using BLAT analysis for sequence alignment, we obtained 10 mismatch calls in the 2.2-Mb genomic interval relative to the C57BL/6J reference sequence, only 2 of which were corroborated as real mutations by Sanger sequencing. These results indicate refinement of the calling algorithms is needed before base calling for whole genome resequencing can be automated. Significant technical challenges also remain in the development of sequence alignment algorithms that can automate the assembly of short reads in whole genome sequencing projects when repeat sequences and segmental duplications are unavoidable.

We identified a single C to T base change (C195R) causing a cysteine to arginine substitution in the coding region of Megf8. In addition, a second C to A base change was found in a noncoding intergenic region in the mapped interval. These results are remarkably in line with previous estimate of one ENU-induced mutation per Mb, with one in 1.82 Mb expected to alter function (23). We note Megf8 was not yet annotated in the mouse genome when this mutation was first mapped to mouse chromosome 7. Thus, a candidate gene sequencing strategy at that time would have failed to recover the mutation. We note with the recent emergence of sequence capture technology, targeted genome sequencing may be feasible without the expense entailed in BAC library construction (24, 25). Moreover, with the rapid decline in the cost of massively parallel sequencing and the advent of real time single-molecule DNA sequencing technology (26), whole genome resequencing may be on the horizon, making the recovery of ENU-induced mutations affordable and straightforward.

Role of Megf8 in Left–Right Patterning. Our studies show an essential role for Megf8 in left–right patterning. Current model for left–right patterning suggests 4 discrete steps in left–right specification: first is the breaking of symmetry at the embryonic node, second is transfer of asymmetric signal from the node to the left LPM, third is Nodal-activated transcription of left-determinant genes such as Pitx2 and Lefty, and 4th is the final asymmetric elaboration of organogenesis (13, 27). Megf8membryos showed asymmetric expression of Nodal in the embryonic node, but neither Nodal nor Lefty is expressed.
in the LPM, and Pitx2 expression was either absent or bilateral. The discordance in Pitx2 and Nodal expression in our mutant is reminiscent of previous studies showing the uncoupling of Nodal and Pitx2 expression in zebrafish embryos (28) and in various vertebrates. These models, such as the polycystin-2 knockout mouse (29), is interesting four to note that polycystin-2, like Megf8 is also ubiquitously expressed, and polycystin-2 knockout mutants show normal node expression of Nodal, but largely absent Nodal expression in the LPM. These findings suggest additional levels of complexities beyond the central Nodal signaling cascade in the specification of laterality. Overall, these findings show Megf8 is required, not for the initial breaking of symmetry, but in subsequent steps to initiate Nodal expression in the left LPM.

The finding of prominent Megf8 colocalization with Gfi1b and Baf60C in the nucleus suggests a role in the regulation of gene expression in the left LPM through chromatin remodeling. Baf60c was shown to regulate left–right asymmetry breaking of symmetry, but in subsequent steps to initiate these findings show mutants show normal node expression of Nodal, but largely absent Nodal expression in the LPM. This suggests a regulatory role in gene expression in the left LPM, and Baf60c and Megf8 could potentially act in the same chromatin remodeling complex to promote Nodal-driven asymmetric transcription in the LPM, either downstream of or in a pathway parallel to Notch-dependent regulation of left–right patterning.

Alternatively, Megf8 may regulate left–right patterning by facilitating the transfer of Nodal from the embryonic node to the left LPM. The long-range action of Nodal has been shown to require the formation of Nodal-Gdf1 heterodimers (17). Recent studies also suggest the interaction of Nodal and sulfated glycosaminoglycans (GAGs) play a role in the long-range propagation of Nodal signaling (30). The predicted protein structure of Megf8, which includes a transmembrane domain and many EGF repeats in the presumptive extracellular domain, would suggest it might function as a protein scaffold for GAGs or directly interact with Nodal or Gdf1 to stabilize Nodal-Gdf1 heterodimers. Although we failed to observe Megf8 immunolocalization at the cell surface, we cannot exclude a shorter Megf8 isoform being localized to the cell surface, because the Megf8 antibody was made using a peptide toward the N terminus. Similarly, it is possible that cleaved and secreted N-terminal Megf8 might play a role in Nodal-Gdf1 heterodimer transfer. Further studies are needed in the future to define the precise function of Megf8, including the analysis of conditional knockout mouse models to define the cell lineage requirement for Megf8 and its role in cardiac morphogenesis. Because Megf8 mutants also exhibit preaxial polydactyly, it is possible that Megf8 may have additional independent roles in other aspects of embryonic development.

Materials and Methods

Mutation Mapping. The mutation was mapped to a 3-3-Mb region of chromosome 7 between markers D7mit192 and D7mit266 (11). Further fine mapping was carried out using SNPs or microsatellite markers within the mapped interval that are polymorphic between C57BL/6J and C3H or A/J, and tracking the segregation of C57BL6 markers.

BAC Library Construction, Screening, and Sequencing. CHORI-602 BAC library construction was conducted as described in ref. 31. Briefly, DNA from a single homozygous mouse embryo was partially digested with EcoRI and cloned into the BAC vector pTARBA2.1. Forty-four pairs of radiolabeled unique oligonucleotides (overgots) were used to screen the BAC library and 172 BAC clones were identified as having genomic fragments in the critical region. Positive BAC clones were end-sequenced to locate genomic fragment bound-


Supporting Information

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Fig. S1. Left–right asymmetric defects in Megf8<sup>m/m</sup> mutants. (A) A Megf8<sup>m/m</sup> embryo exhibits dextracardia, as indicated by the heart apex pointing to the right side of the chest cavity. Transposition of the great arteries is also seen with the aorta (A) positioned anterior to the pulmonary artery (P). (B) Mutants typically exhibit 4 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–F) A normal D-loop heart tube (H) in a wild-type E9.0 embryo is viewed from the anatomical right (D). Megf8<sup>m/m</sup> embryos show randomized heart tube looping. Shown are 2 Megf8<sup>m/m</sup> embryos, with one showing normal rightward heart tube looping (E), and another embryo, viewed from the left, showing reversed leftward looping of the heart tube (F). Note relatively shorter outflow tracts demarcated by white lines in mutants. (G and H) Abnormal embryonic turning in Megf8<sup>m/m</sup> embryos. Ventral view of E9 embryos show embryonic turning in a wild-type (G) and Megf8<sup>m/m</sup> (H) embryo. In wild-type embryo, embryonic turning positions the tail to the right of the head (G). In the Megf8<sup>m/m</sup> embryo, reversal in embryonic turning positions the tail to the left of the head (H). [Scale bars: 500 μm (A–C); 100 μm (D and G).]
Fig. S2. BAC contig assembly spanning 2.2-Mb critical region of mouse chromosome 7.
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 2 exon-intron boundaries of zMegf8 to which antisense morpholinos were designed. Exon sequence is in bold. (B) Both Megf8 morpholinos disrupt heart looping. Injected and uninjected embryos were visually scored for the direction of 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. These injections were not included in the results shown in the bar graph of Fig. 1C, for which heart and gut looping were scored using in situ hybridization markers.

Fig. S3. 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 2 exon-intron boundaries of zMegf8 to which antisense morpholinos were designed. Exon sequence is in bold. (B) Both Megf8 morpholinos disrupt heart looping. Injected and uninjected embryos were visually scored for the direction of 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. These injections were not included in the results shown in the bar graph of Fig. 1C, for which heart and gut looping were scored using in situ hybridization markers.
Fig. S4. *Megf8* is ubiquitously expressed and the mutation doesn’t affect *Notch1* and *Dll1* expression. (A, C, E) Wild-type embryos. (B, D, F) *Megf8*<sup>m/m</sup> embryos. *Megf8* (A, B), *Dll1* (C, D), and *Notch1* (E, F) expression are not affected in *Megf8*<sup>m/m</sup> embryos. Scale bar = 100 μm.
Fig. S5. Analysis of ciliary motion at the embryonic node. Movement of the nodal cilia at the embryonic node in wild-type, Megf8<sup>m/+</sup> and Megf8<sup>m/m</sup> 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.

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Fig. S6. Megf8 antibody staining has no obvious difference between wild-type and homozygous mutant MEFs, but can be knocked down by Megf8 siRNA. (A, B) Immunofluorescent staining of mouse embryonic fibroblast cells created from wild-type embryos (B) and Megf8<sup>mut/mut</sup> embryos (A) using the chicken antimouse Megf8 N-terminal antibody. (C, D) Mouse embryonic fibroblasts were cotransfected with a GFP expression vector and Megf8 shRNA-expressing plasmid, then immunostained with a Megf8 antibody. In cells transfected with the Megf8 shRNA plasmid, indicated by expression of the GFP reporter (green), Megf8 expression (red) was significantly diminished in 31% of these GFP<sup>+</sup> cells.
Western Immunodetection of 3XFlag-Megf8 fusion protein. Western immunoblot of NIH 3T3 cells transfected with a construct expressing full length Megf8 with a C-terminal 3XFLAG tag showed a band > 300 kDa (see arrow), in the size range expected for the Megf8 fusion protein. This same band was detected simultaneously with both the FLAG (red) and Megf8 (green) antibodies using 2 color Western immunodetection.
Fig. S8. Analysis of definitive erythropoiesis in \textit{Megf8} mutant embryos. To determine if \textit{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), and the percentage of nucleated 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 3 genotypes (WT, heterozygous, and homozygous \textit{Megf8} mutants), consistent with normal transition to definitive erythropoiesis. These findings suggest \textit{Megf8} mutants have no defect in definitive erythropoiesis.
Movie S1. Rotational movement associated with the nodal cilia in an E7.5 homozygous Megf8<sup>C193R</sup> mutant embryo. Movies were collected at 200 fps. Movie playback is 5 fps.
Movie S2.  Rotational movement associated with nodal cilia in an E7.5 wild-type embryo. Movies were collected at 200 fps. Movie playback is 5 fps.
Table S1. Chromosome 7 microsatellite and SNP markers

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<td>27915985</td>
</tr>
<tr>
<td>D7Mit229</td>
<td>52942350</td>
<td>rs3662508</td>
<td>28006891</td>
</tr>
<tr>
<td>D7Mit248</td>
<td>73484970</td>
<td>rs13460395</td>
<td>28028384</td>
</tr>
<tr>
<td>D7Mit350</td>
<td>90734599</td>
<td>rs31307939</td>
<td>28036213</td>
</tr>
<tr>
<td>D7Mit330</td>
<td>126800204</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D7Mit71</td>
<td>138168667</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To map and track the mutation, the founder animal was intercrossed with C3H and in later generations, intercrossed with consomic strain C57BL/6J-Chr7A/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 (www.ncbi.nlm.nih.gov/projects/SNP/MouseSNP.cgi, www.informatics.jax.org/searches/polymorphism_report.cgi.). This allowed tracking of the mutation via the segregation of C57BL6/J 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 as mm37.1.
Table S2. *Megf8* morpholino knockdown in zebrafish embryos causes heterotaxy

<table>
<thead>
<tr>
<th>Embryo</th>
<th>n</th>
<th>Normal situs</th>
<th>Reverse situs</th>
<th>Discordant situs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control MO</td>
<td>47</td>
<td>85% (40)</td>
<td>6% (3)</td>
<td>9% (4)</td>
</tr>
<tr>
<td><em>Megf8</em> MO</td>
<td>102</td>
<td>13% (13)</td>
<td>13% (13)</td>
<td>75% (76)</td>
</tr>
</tbody>
</table>

*P* values were obtained by $\chi^2$ analysis.
Table S3. *Megf8* morpholino in embryos with discordant situs causes heterotaxy

<table>
<thead>
<tr>
<th>Heart Tube</th>
<th>Reduced or no looping</th>
<th>Reverse looping</th>
<th>Normal looping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Reverse</td>
<td>Reduced</td>
</tr>
<tr>
<td>Control MO</td>
<td>4% (2)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Megf8 MO</td>
<td>29% (30)</td>
<td>20% (20)</td>
<td>9% (9)</td>
</tr>
<tr>
<td>Total</td>
<td>59%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S4. Randomized heart tube looping and direction of embryonic turning

<table>
<thead>
<tr>
<th>Heart Looping</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic Turning</td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Megf8&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>