Targeted Mutagenesis of the *Hira* Gene Results in Gastrulation Defects and Patterning Abnormalities of Mesoendodermal Derivatives Prior to Early Embryonic Lethality

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The *Hira* gene encodes a nuclear WD40 domain protein homologous to the yeast transcriptional corepressors Hir1p and Hir2p. Using targeted mutagenesis we demonstrate that *Hira* is essential for murine embryogenesis. Analysis of inbred 129Sv embryos carrying the null mutation revealed an initial requirement during gastrulation, with many mutant embryos having a distorted primitive streak. Mutant embryos recovered at later stages have a range of malformations with axial and paraxial mesendoderm being particularly affected, a finding consistent with the disruption of gastrulation seen earlier in development. This phenotype could be partially rescued by a CD1 genetic background, although the homozygous mutation was always lethal by embryonic day 11, with death probably resulting from abnormal placentation and failure of cardiac morphogenesis.

The *Hira* gene was originally isolated during the positional cloning of the DiGeorge syndrome chromosome deletion region at human chromosome 22q11 (21). *Hira* homologues exist as strongly conserved single genes in most eukaryotic species (35, 36, 45) and as two related genes *Hir1* and *Hir2* in *Saccharomyces cerevisiae* (49). The *Hira* family of proteins have seven N-terminal WD40 domains predicted to form a β -propeller structure which may be involved in protein-protein interactions (43) and two potential nuclear localization domains. The region C-terminal to the WD40 domains has no strong matches to known motifs but is responsible for the biochemical interaction of *Hira* with paired homeodomains (39), *Hirip3* (38), and core histones (38), the functional significance of which remains unknown.

Structurally, the N-terminal half of *Hira* is most similar to *Hir1p*, and the C-terminal half most similar to *Hir2p* (31, 35, 36). *Hir1p* and *Hir2p* interact with one another and with as-yet-unknown members of a protein complex to mediate cell cycle-dependent transcriptional repression of six of the eight yeast histones (including HTA1-HTB1) via a negative element in the respective promoters (50). *Hir1p* mutants also have an *Spt* (suppressor of Ty) phenotype, and *Hir1p* has been shown to interact functionally with the *SPT4*, *SPT5*, and *SPT6* gene products and directly with the *SPT4* gene product. Recently, biochemical and genetic studies demonstrated that transcrip-

tion at the HTA1 or HTB1 locus is Swi-Snf dependent via interaction of the Swi-Snf complex and *Hir* proteins (expression at HTA1 is absent in *Snf5* and *Snf2* mutants [18]). *Hir2* interacts genetically and biochemically with chromatin assembly factor 1 in yeast (27); in humans CAF1p60 is structurally similar to the N terminus of *HIRA*, with sequence matches at the DNA level, suggesting that they may be derived from an ancestral gene.

The role of *Hira* in higher eukaryotes is not known, but the protein is localized to the nucleus where a proportion is associated with the nuclear matrix (15). *Hira* is a target for cdk2/cyclin E phosphorylation, and ectopic expression of *Hira* can block cells in the S phase of the cell cycle (22). Taken together, existing data suggest that *Hira* may be involved in cell cycle-dependent transcriptional regulation of a wide range of target genes, possibly acting via altered chromatin structure.

Hira displays a dynamic expression pattern as embryogenesis proceeds. In both mice (59) and chicks (45), expression is seen in postgastrulation stages. At mouse embryonic day 8 (E8) and E9, chick stage 6 to 12, expression is detected in the neuroepithelium, premigratory and migratory neural crest, and head mesenchyme. At E9.5, stages 18 to 23, expression is seen in neural-crest-derived regions of the head and branchial arches and in a number of other tissues, including the somites and forelimb bud.

The chromosomal location, expression, and Pax3 interaction made *Hira* a good candidate for DiGeorge syndrome (47). However, *Hira* maps outside targeted deletions of mouse chromosome 16 which model the heart defects seen in 22q11DS (33, 34). This phenotype is now known to be secondary to *Tbx1* haploinsufficiency (26, 34, 41). Thus, hemizyosity for *Hira* is not necessary for the production of this phenotype, although a murine deletion incorporating *Hira* has greater perinatal lethality (41), so it is possible that *Hira* hemizyosity contributes

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FIG. 1. Targeted disruption of the *Hira* gene. (a) Genomic wild-type restriction map for targeted region of the *Hira* gene with numbered exons. (b) Targeting vector. (c) Targeted *Hira*^{neo} allele. (d) Targeted *Hira*^{neoloxp} allele. The position and size of the expected restriction fragments are indicated by double-headed arrows. For the *Hira*^{neo} allele the HH350 probe (shaded) detects a 7-kb band from an *EcoRI* digest in the wild type and an 8.5-kb band when homologous recombination has taken place. For the *Hira*^{neoloxp} allele, an *SstI* digest generates a wild-type band of 6.5 kb and a targeted band of 10 kb. Abbreviations: R, *EcoRI*; S, *SstI*; B, *BamHI*; H, *HindIII*. (e) Southern blot of mice derived from targeted ES cell lines. Expected band sizes are as described for panel d. (f) Western blot of *Hira*^{-/-} embryos and heterozygous embryos. *Hira* migrating at 112 kDa is seen in the heterozygous lane but is lost in the *Hira*^{-/-} lane.

to the human phenotype as part of a multigene haploinsufficiency.

In order to examine the potential role of *Hira* in embryonic development, a mutation was generated in the gene by homologous recombination in embryonic stem (ES) cells. Homozygous mutants on an inbred background die by E10. When the mutation is crossed to an outbred background, embryos survive to E11. A wide range of phenotypes is seen on both genetic backgrounds, although embryonic lethality occurs at too early a stage to assess the morphogenesis of structures affected in 22q11DS. All embryos display growth retardation, with severely affected embryos being balls of disorganized tissue and more mildly affected embryos displaying recognizable head structures but lacking more caudal structures. All embryos demonstrate abnormal patterning in various degrees in most tissues, with mesendodermal tissues being the worst affected. We demonstrate that *Hira* is required for normal embryonic development and survival and suggest that the phenotype seen is the result of defective gastrulation.

MATERIALS AND METHODS

Construction of the targeting vectors. Genomic DNA encoding the *Hira* gene was cloned from a bacteriophage library prepared from 129/Sv ES cells by using a full-length murine *Hira* cDNA probe. A 14-kb clone encompassing exon 8 was isolated. The phage insert was subcloned into pBluescript in order to shuttle fragments to pTBPNS3, which contains *neo* and *Leu2* selectable markers (8). The *Hira*^{neo} construct was created by subcloning a 3.55-kb *HindIII*-*BamHI* fragment as the left-hand targeting arm, and a 1.5-kb *BamHI* fragment as the right-hand arm (Fig. 1). The *BamHI* site is located at nucleotide 795 of the murine *Hira* cDNA sequence accession X75295, and insertion of the *neo/Leu2* sequences interrupts WD40 domain 4. Skipping of the targeted exon 8 to exon 9

or 10 would yield an out-of-frame protein. For *Hira*^{neoloxp}, the cloning vector pPNTlox was used. pPNTlox was constructed by Shinji Hirotsune, and pPNT provided the backbone (16). The left-hand arm was created by cloning a 3.4-kb *XbaI*-*BamHI* fragment out of pBluescript into pPNTlox. The right arm was derived by first subcloning a 5.6-kb *BamHI*-*NotI* fragment (the *NotI* fragment originating from the lambda DASH [Stratagene] vector) into pTBPNS3 and subcloning a *Sall*-*NotI* fragment into the *XhoI*-*NotI* sites of pPNTlox (Fig. 1). This vector-mediated targeting deletes 1.5 kb from the 3' end of exon 8 and part of intron 8.

Electroporation and selection of ES cells. The *Hira*^{neo} and *Hira*^{neoloxp} constructs were linearized with *SacII* and *NotI*, respectively, and DNA was electroporated into W9.5 and TC1ES cells, respectively, which were subject to positive or negative selection. Correct homologous recombination occurred at 2.5% frequency, as determined by Southern blotting analysis of ES cell DNA. DNA was digested with *SstI* (*Hira*^{neo}) or *EcoRI* (*Hira*^{neoloxp}) and analyzed by Southern analysis by using a 350-bp *HindIII* fragment. This probe detects a 6.5-kb wild-type and a 10-kb mutant allele for *Hira*^{neo} and a 7-kb normal and an 8.5-kb mutant allele for *Hira*^{neoloxp}. Correctly targeted ES clones derived with both constructs were injected into C57BL/6 blastocysts to derive chimeric mice as described elsewhere (24).

Mice were routinely genotyped by Southern analysis and PCR of tail tip DNA. Embryos were typed by Southern analysis or PCR of yolk sac DNA at E9.5 and later and by PCR at earlier stages. The sequences of the primers used for the *Hira* targeting events were as follows: *Hira*6F, AACCTGAGAGGTCATTCTGGC; *neo*, GGGACTGGCTGCTATTGGGC; *loxP*, GCTAGTCTAGACTGCAGGCCAGTTTAAAC; and *Hira*6R, ACTCATACAAGGCTTGGTG.

PCR comprised denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min. The targeted alleles were detected by using *Hira*6F and either the *neo* or *loxP* primers, which gave products of 600 and 180 bp, respectively. The normal allele was detected by using primers *Hira*6F and *Hira*6R under the same conditions, which gives a product of 150 bp.

The resulting seven chimeric males were identified by coat color and bred to C57BL/6 females. Heterozygous offspring identified by Southern analysis (Fig. 1) were then bred onto a 129/Sv inbred background, with successive generations being bred to wild-type 129/Sv females. Heterozygote 129/Sv mice were also crossed onto CD1 outbred mice in the same fashion. The *Hira*^{neoloxp} mutation was also bred onto a mixed C57BL/6/129/Sv genetic background. To collect embryos from all lines, heterozygous males and females were mated, and noon on the day of the plug was taken as 0.5 days post coitus (E0.5).

Reverse transcription-PCR (RT-PCR). CD1 background *Hira*^{neo} cross-embryos were collected and typed at E10 and then washed in phosphate-buffered saline (PBS), and RNA was prepared by using a Clontech total RNA isolation kit for ATLAS expression arrays, and cDNA was synthesized by using the Clontech SMART cDNA synthesis kit and Superscript II (Gibco-BRL). PCR conditions were as for typing, except the annealing temperature was 58°C, and three primer pairs were used: 1F-3R to amplify upstream of the *neo* insertion, 9F-10R to amplify just 3' to the *neo* insertion, and 23F-24R at the 3' end of the *Hira* transcript. The primer sequences were as follows: 1F, AGTTGATATTCACCC TGA; 3R, TGGTATCCAATGCATGTCCA; 9F, CTCACATGTTTGAAACG GCC; 10R, CTTTCTCTCTCACTCAGGG; 23F, TCCAGTTCAGTCTCCA CTG; and 24R, ATAGTGTGGTCTCTGCCCTTG.

Western analysis. CD1 background *Hira*^{neo} cross-embryos were collected and typed at E10, washed in PBS, and homogenized in Buffer A (10 mM HEPES, pH 7.6; 85 mM KCl; 5.5% sucrose; 0.5 mM spermidine; 0.5 mM EDTA; 0.5 mM dithiothreitol; protease inhibitors). An equal volume of Buffer B (Buffer A plus 0.7% NP-40) was added, and cells were incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 2,000 × g for 5 min at 4°C and then purified through a 0.8 M sucrose cushion in Buffer A. Purified nuclei were extracted in 10 mM HEPES (pH 7.6)–0.35 M KCl–1.5 mM MgCl₂–0.25% NP-40–0.2 mM EDTA–0.5 mM EGTA–0.5 mM dithiothreitol–protease inhibitors for 1 h at 4°C. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4°C. Nuclear extracts were separated on a sodium dodecyl sulfate–10% polyacrylamide gel and transferred to nitrocellulose. After being blocked with Tris-buffered saline containing 5% milk and 0.05% Tween 20, protein was detected with WC119 monoclonal antibody directed against *Hira*, with horseradish peroxidase-conjugated anti-mouse secondary antibody. The blots were washed in Tris-buffered saline containing 0.5% Tween 20 and developed by enhanced chemiluminescence reactions (Amersham). WC119 was a gift from Peter Adams (22).

Whole-mount in situ hybridization. Whole-mount hybridizations were performed essentially as described earlier (58) with minor modifications. CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} detergent (0.5%) was included in the hybridization buffer, and hybridization temperatures varied depending on the length of the probe, with the majority being 68°C.

Blocking buffer included 1% bovine serum albumin in addition to 10% serum. Boehringer-Mannheim purple-precipitating reagent was used in addition to nitroblue tetrazolium (NBT)-BCIP (5-bromo-4-chloro-3-indolylphosphate) as the coloring reagent. Digoxigenin-labeled antisense RNA probes were prepared as previously described (51).

Histologic analysis. Embryos were collected and wax embedded as described previously (57). Briefly, embryos were dissected in ice-cold PBS and fixed overnight in 4% paraformaldehyde-PBS. Embryos were then dehydrated through an ethanol series terminating in 100% ethanol. This was followed by two 30-min incubations in HistoClear, followed by one 20-min incubation in 50:50 HistoClear-wax at 60°C. After three changes of 100% wax, each for 20 min, embryos were placed in molds and wax and allowed to set. Sections were cut at 5 to 10 μ m and floated onto 3-aminopropyltriethoxysilane (TESPA)-treated slides before being stained with either methylene green or hematoxylin-eosin. Embryos sectioned after in situ hybridization were sectioned as described above and counterstained with eosin.

Whole-mount immunocytochemistry. For PECAM antibody staining, embryos were fixed for a minimum of 1 h in dimethyl sulfoxide-methanol (1:4) and then incubated in dimethyl sulfoxide-methanol-H₂O₂ (1:4:1) for 5 h at room temperature. The embryos were then rehydrated down through in 90, 75, 50, and 25% methanol-H₂O two times for 10 min each time and then washed three times for 15 min each time in PBS plus 0.3% Triton X-100, two times for 15 min each time in 0.5% Triton X-100-PBS, and finally two times 15 min each time in 0.3% Triton X-100-PBS. Embryos were then blocked in 2% Boehringer blocking reagent in 0.3% Triton X-100-PBS for 1 h and incubated overnight at 4°C in primary antibody 1:50 anti-PECAM rat anti-mouse immunoglobulin G (Pharmingen) in blocking buffer. Two rinses in 0.3% Triton X-100-PBS were then performed, followed by three 1-h washes in 0.5% Triton X-100-PBS, two 30-min washes in 0.3% Triton X-100-PBS, and finally one 1-h incubation in blocking buffer. Embryos were then incubated in secondary antibody: goat anti-rat alkaline phosphatase (Chemicon) 1:200 in blocking buffer 4°C overnight. Two rinses and the first three 1-h washes were performed as after the primary antibody incubations. Embryos were then washed two times for 15 min each time in 0.3% Triton X-100 in Tris buffer (100 mM Tris, pH 9.5; 150 mM NaCl; 25 mM MgCl₂) and developed in NBT (4.5 μ l/ml)-BCIP (3.5 μ l/ml) in 10% polyvinyl alcohol in Tris buffer.

Photography. Whole-mount embryos were photographed on a Nikon SMZ-U dissecting microscope, and sections were viewed on a Zeiss Axioskop 2 microscope.

RESULTS

Targeted disruption of the mouse *Hira* gene. In the targeted allele designated *Hira*^{neo} (Fig. 1), all of the coding sequence of exon 8 after amino acid position 183 is replaced with a neo/leu2 cassette. The *Hira*^{neoloxp} allele targets the same site and introduces a 1.5-kb deletion which encompasses the 3' sequences of exon 8, together with a loxP site for future chromosome engineering experiments. RT-PCR performed on homozygous *Hira*^{neo} embryos failed to detect transcripts (not shown), suggesting instability of the targeted mRNA, and Western analysis of proteins from the mutant embryos confirmed the absence of *Hira* at the protein level, indicating that the *Hira*^{neo} targeted allele is a true null allele (Fig. 1).

129Sv inbred *Hira*^{-/-} embryos die by E10. To investigate the phenotype of mice homozygous for the *Hira* targeted alleles, heterozygote intercrosses were performed, and offspring were examined at different developmental stages up to birth. No homozygous mutant mice were identified from 138 *Hira*^{neo} intercross liveborn offspring and 78 *Hira*^{neoloxp} intercross liveborn offspring, after genotyping from tail tip DNA by Southern blot and PCR. The null mutation is therefore embryonically lethal, and no live homozygous mutant embryos were detected after E9.5. At this stage, 122 embryos were genotyped by Southern blot analysis and, of these, 20 homozygous mutant embryos were identified (16%). However, a significant number

of reabsorbing embryos and empty decidua were also recovered from the litters analyzed ($n = 12$).

All normal embryos were homozygous wild type or heterozygous for the targeted allele. All homozygous *Hira*^{neo} and *Hira*^{neoloxp} embryos exhibited a complex phenotype, which was variable at all stages examined, even within a litter (see below). The *Hira*^{neo}/*Hira*^{neo} and *Hira*^{neoloxp}/*Hira*^{neoloxp} phenotypes were identical, so analysis was concentrated on *Hira*^{neo}/*Hira*^{neo} embryos (hereafter referred to as *Hira* mutant or *Hira*^{-/-} embryos). Since the abnormalities observed were consistent with a defect occurring earlier during development, we examined gastrulation-stage embryos.

Gastrulation-stage embryos display characteristics consistent with early mesodermal defects. The *Hira* gene was found to be first expressed at gastrulation stages at the extraembryonic-embryonic boundary and in the proximal ectoderm (Fig. 2a, i to iv). It was therefore possible that an early phenotype affecting these stages of development would be present.

To investigate this hypothesis, E6.5 to E7.5 embryos from *Hira* 129Sv heterozygous crosses were examined. Litters were assessed under the microscope and genotyped by PCR or else selected for in situ hybridization and subsequently genotyped by PCR. While post-in situ hybridization PCR was not successful in all cases; no embryo ever phenotyped as unaffected was found to be *Hira*^{-/-}, and no embryo phenotyped as a null was found to be *Hira*^{+/+}.

At the gastrulation stages studied, a range of mutant phenotypes was observed within litters. Some mutants were of relatively normal size compared to wild-type littermates but exhibited a distortion of the primitive streak so that it appeared to bulge out into the amniotic cavity (Fig. 2b, i to iii). Sections through these embryos revealed that the primitive streak contained many more cells than usual, resulting in the bulging out of the posterior side of the embryo into the amniotic cavity (Fig. 3a to j). The worst-affected embryos appeared as small relatively undifferentiated balls of cells compared to wild-type littermates (Fig. 2b, iv to vi). The phenotype of these embryos suggested that their development was severely delayed or arrested at early gastrulation. A subset of these embryos also had a constriction at the embryonic-extraembryonic junction (Fig. 2b, ii).

Whole-mount in situ on these mutant *Hira* embryos revealed abnormal expression of mesendodermal molecular markers. All *Hira* mutant embryos probed for *Brachyury*, a primitive streak marker, were developmentally delayed relative to wild-type littermates (Fig. 2c, i to viii). Mutant embryos from head-fold stage litters (E7.5 to 8.0) appeared to be E7.0 to 7.5 or younger. Embryos with distorted primitive streaks displayed abnormal displaced expression of *Brachyury*. In some embryos a large proportion of the expanded primitive streak appeared to express *Brachyury* and, occasionally, a thin stream of positive cells attempting to form a node- or notochord-like structure was evident (Fig. 2c, iv and v). Sections through these embryos revealed that the majority of cells with either epithelial or mesenchymal morphology within the expanded primitive streak were positive for *Brachyury* (with mesenchymal referring to cells with an ameboid appearance like those described by Sun et al. [52]). However, a central core of both cell types remained negative throughout most of the proximal-distal axis of the streak (Fig. 3o to q).

FIG. 2. Gastrulation-stage (E6.5 to E7.5) 129Sv wild-type and *Hira*^{-/-} embryos. Unless stated otherwise, posterior is to the right, and mutants and wild-type embryos are shown to the same scale. Constrictions at the embryonic-extraembryonic boundary are indicated with black arrowheads. (a, i to iv) Wild-type embryos hybridized with *Hira*. Expression is visible in ectoderm and the extraembryonic boundary and mesoderm. (i) E6.5 to E7.0. (ii) E7.0. (iii) E7.5 rotated 90° laterally to view the position of *Hira* expression within the ectodermal layers. (iv) E7.5. (b, i to vi) Phenotype of gastrulation stage *Hira*^{-/-} embryos. (i) Wild-type embryo, headfold stage (E7.5 to E8.0). (ii and iii) *Hira*^{-/-} mild-phenotype embryo at same stage as panel i. (iv) Wild-type embryo (E7.0). (v and vi) *Hira*^{-/-} severe-phenotype embryos (E7.0 to E7.5). (c, i to viii) In situ hybridization with *Brachyury*. (i) Wild-type embryo (E7.75 to E8.0). (ii) Wild-type embryo (E7.0 to E7.5). (iii) Wild-type embryo (E6.5). (iv to viii) Littermate *Hira*^{-/-} embryos of panel c, i. (iv) Embryo has been rotated 90° laterally to display the full extent of the expansion of *Brachyury* expression. (vii) Embryo has also been rotated 90° laterally. (iv, v, and vi) Embryos display expansion and/or displacement of *Brachyury* expression resulting from the primitive streak abnormalities. (vii and viii) Embryos express *Brachyury* in a manner consistent with extreme developmental delay/arrest. (d, i to iv) In situ hybridization with *Foxa2*. (i) Wild-type headfold-stage embryo (E7.5) rotated 90° laterally to show expression in node and notochord. (ii) *Hira*^{-/-} littermate with expanded expression in the bulging primitive streak. (iii) Wild-type embryo (E7.0 to E7.5), showing expression within the node and AVE (pink arrow). (iv) *Hira*^{-/-} embryo (E7.5) showing expanded expression in the primitive streak and loss of AVE expression (pink arrow). (e, i to iii) In situ hybridization of E7.5 embryos with *mCerberus*. (i) Wild-type embryo with expression in the AVE. (ii and iii) *Hira*^{-/-} embryos showing loss of expression. (f, i and ii) In situ hybridization of E7.0 embryos with *Hex*. (i) Wild-type embryo with expression in the AVE. (ii) *Hira*^{-/-} embryo without expression. (g, i to iv) In situ hybridization with *Bmp4*. (i) Wild-type E7.0 to E7.5 embryo with expression at the extraembryonic-embryonic boundary. (ii) Same-stage *Hira*^{-/-} embryo with downregulated expression. (iii) Wild-type E6.5 to E7.0 embryo expressing *Bmp4* at the extraembryonic-embryonic boundary. (iv) Same-stage *Hira*^{-/-} embryo showing loss of expression.

In other mutant embryos with distorted primitive streaks, expression was displaced but not expanded, being present only at the edge of the primitive streak bulging into the amniotic cavity (Fig. 2c, vi). Sections through these different embryos showed that expression of *Brachyury* was present in cells with epithelial morphology at the edge of the primitive streak and in a small number of cells of mesenchymal appearance directly adjacent (Fig. 3n).

Headfold-stage mutant embryos with the most severe early phenotype expressed *Brachyury* in a pattern consistent with

that seen at E6.5 at the start of gastrulation (Fig. 2c, vii), as did mutant embryos with constrictions from E7.0 to E7.5 litters (Fig. 2c, viii). *Fgf8* expression appeared to be appropriate for the stage of development of the embryo (not shown).

Foxa2 expression was also displaced or expanded in mutant embryos of headfold and earlier stages (Fig. 2d, i to iv), raising the possibility that axial mesendoderm is specified but cannot migrate out from the primitive streak. In addition, expression of *Foxa2* in the anterior visceral endoderm (AVE) was reduced or absent (Fig. 2d, iv). Other early markers for the AVE *Hex* and *mCerberus* appear to be downregulated in gastrulation-stage (E6.5 to E7.5) *Hira* mutant embryos, particularly in those with the more severe phenotype (Fig. 2e, i to iii, and f, i and ii).

FIG. 3. Sections through the primitive streak of gastrulation-stage embryos. (a and b) Transverse sections through wild-type E7.0 embryo at mid-streak level. Primitive streak is indicated by black arrowheads. The section in panel a is proximal to the section in panel b. (c and d) Transverse sections through wild-type E7.0 to E7.5 embryos at mid-streak level. The section in panel c is proximal to the section in panel d. (e to h) Transverse sections through E7.0 to E7.5 *Hira*^{-/-} embryos at mid-streak levels. Excess cells of mesenchymal morphology (yellow star) cause the ectoderm to collapse inward into the amniotic cavity producing the bulging primitive streak phenotype. The section in panel e is proximal to that in panel f, and the section in panel g is proximal to the section in panel h. (i to k) Sagittal sections through E7.0 embryos. (i) Wild-type embryo. (j) *Hira*^{-/-} embryo. The expanded mesenchymal portion of the primitive streak (yellow star) can be seen propelling the ectoderm out into the amniotic cavity in the characteristic bulge. (k) *Hira*^{-/-} embryo of the more-severe phenotype. The embryo displays developmental delay, consisting only of a simple cup of ectodermal cells. (l to q) High-power transverse sections through whole mount in situ E7.5 embryos hybridized with *Brachyury*. (l) Section through wild-type embryo showing very anterior primitive streak in which the strongly *Brachyury* expressing notochordal plate is clearly visible. (m) More-proximal section through mid-primitive streak level of wild-type embryo. Primitive streak cells are *Brachyury* positive. (n to q) Sections through *Hira*^{-/-} embryos. (n) Section through embryo seen in Fig. 2c, vi, at the mid-primitive streak level. *Brachyury* expression is present in the epithelium (red star) and adjacent mesenchyme (yellow star) at the edge of the primitive streak; however, a significant amount of tissue remains negative (green arrow). (o to q) Sections through the embryo seen in Fig. 2c, iv. Section o is the most distal; section q is the most proximal. The large number of mesodermal cells (yellow star) in the primitive streak are clearly visible. The majority of both mesodermal and ectoderm cells express *Brachyury*, but a central portion of both tissues remains negative for this transcript (green arrow).

FIG. 4. Phenotypes of *Hira*^{-/-} embryos at E9.5 to E10.5; mutants and wild-type embryos shown to the same scale. (a) Wild-type embryo (E10.5). (b to d) 129Sv *Hira*^{-/-} mild-class embryos (E9.5). (e) 129Sv *Hira*^{-/-} severe class embryo (E9.5). (f) Section through the forebrain of an E8.5 to E9 wild-type embryo. (g) Section through forebrain region of E9.5 *Hira*^{-/-} embryo. (h and i) Sections through the forebrain and hindbrain in E10.5 *Hira*^{-/-} embryos. Forebrain is at the bottom of the picture. (j and k) Whole *Hira*^{-/-} CD1 mild-class embryos (E10.5). Note the restoration of somites. (l and m) CD1 *Hira*^{neo/neo} severe-class embryos (E10.5). In panel m, the embryo displays the dumbbell heart phenotype. Abbreviations: a, allantois; h, heart; nf, fused neural folds; s, somites. Pink arrows indicate open neural folds, yellow arrows indicate the point from which caudal structures fail to form, and green arrows indicate pericardial edema.

This may be linked to the extraembryonic-embryonic constriction observed in some embryos. Finally, *Bmp4* expression, required for normal mesodermal development, was also found to be downregulated in gastrulation-stage embryos (Fig. 2g, i to iv).

Mid-gestation embryos display a phenotype consistent with earlier gastrulation defects. At E9.5 all *Hira*^{-/-} embryos examined ($n = 167$) displayed a developmental delay of up to 24 h, were much smaller than wild-type littermates, and had rarely undergone embryonic turning (Fig. 4a to e). A total of 53% of embryos were small balls of disorganized tissue with little overt sign of differentiation (Fig. 2e). A small number of $-/-$ embryos (12%) had developed a little further than this, making attempts to develop an anterior-posterior axis (Fig. 6e and q). These embryos had disordered neuroepithelial vesicles anteriorly, associated with an elongated condensed tissue, which could be the embryonic trunk. These embryos are grouped under the term “severely affected class” and probably arise due to the failure of the embryo to gastrulate, thus not forming the mesendodermal layers required for further development.

The least-affected embryos (35%, termed “mildly affected class”), while still exhibiting developmental delay, had recognizable but abnormal head structures, branchial arches, and hearts (Fig. 4b to e). However, caudal to the level of the hindbrain-spinal cord boundary structures of mesodermal origin, such as somites and notochord, were rarely present, reflecting failure of the progenitors of the axial and paraxial mesoderm to pass through gastrulation normally. Any somites found were small and asymmetric and did not have clear

boundaries. The neural tube was also often missing and, when present, was either severely kinked or open. Histologic sections revealed that the neural plate was often extended laterally with abnormal folding beyond its normal boundaries, especially in more caudal regions, and that the mesenchyme was often deficient within the head (Fig. 4f to i). Anteriorly, commonly seen defects included failure of the brain to fuse resulting in an everted cap-like structure, left-right asymmetry in both the amount of tissue present and anatomical structures formed, and altered development of heart chambers (Fig. 4 to 7). These neural defects are in turn most likely the result of the failure of axial mesoderm to form.

A total of 67% of all *Hira*^{-/-} embryos also exhibited problems with extraembryonic mesoderm. These embryos had a relatively large allantois, which did not fuse with the chorion to form the chorioallantoic placenta as normal, and which therefore persisted posteriorly. The unfused allantois remained either as a vesicle, which was sometimes blood-filled, or as a solid structure of various shape (Fig. 4b and e).

CD1 outbred homozygous mutant embryos die by E11 and display a partially rescued phenotype. To investigate the possible effect of genetic background on the homozygous mutant phenotype, 129Sv *Hira*^{+/-} females were backcrossed to CD1 outbred males. Embryonic development was less badly affected than on the 129Sv inbred background, and the majority of *Hira*^{-/-} embryos survived one more day of gestation. E10.5 embryos were collected from heterozygous matings between fourth- and fifth-generation mice (93.8 and 96.9% CD1 genotype, respectively) and genotyped by Southern blot or PCR. The overall percentage of homozygous mutant embryos was 25% (77 of 307). All homozygous *Hira*^{neo} CD1 embryos were abnormal compared to heterozygous and wild-type littermates.

The phenotype of living *Hira*^{-/-} embryos at E10.5 ($n = 77$) remained variable on the CD1 outbred genetic background. Developmental delay and retarded growth were evident, but this was less apparent than on the inbred background for many embryos. A total of 7% were disorganized balls of tissue. The next most severely affected CD1 *Hira*^{-/-} embryos resembled the milder phenotype observed on the 129 background, and none of these embryos had undergone turning. All embryos in this class of phenotype had recognizable, though badly patterned head structures, and abnormal or absent caudal axial structures (Fig. 4l and m). These embryos accounted for 33% of those dissected and are referred to here as the CD1 severe phenotype.

The remaining 60% of embryos had a phenotype that was much milder than any observed on the inbred background. All of these embryos had turned and had relatively normal caudal structures, with notochord, neural tube, and somites all being present. In some cases the somites were small, malformed, or asymmetric (Fig. 4j and k). These “rescued” embryos continued to display head defects, particularly the failure of the brain to fuse, which resulted in the neuroepithelium of the brain forming large open everted cap-like structures (Fig. 4i). Distinct forebrain structures could not be discerned in some embryos (e.g., Fig. 4j). Defects of the allantois similar to those seen on the inbred background were also present across the phenotypic range (Fig. 4j to l), but only in 45% of embryos, implying that some recovery of the extraembryonic mesoderm had occurred.

A total of 52 embryos of the CD1 genetic background had formed a heart or heart tube. In six embryos no looping had taken place. Two chambers appeared to balloon out of the linear heart tube, resulting in a dumbbell-shaped appearance (Fig. 4m). This heart phenotype was always associated with severe caudal mesoderm disruption. However, failure of looping was not secondary to general developmental delay since the development of large blood vessels in the head was consistent with stage E10 embryos when looping should be well under way. Beating hearts were observed in some of these embryos, but in an irregular fashion, with blood being shuttled back and forth between the two chambers. Myocardial cells must therefore have differentiated within these mutant hearts. A small number of mild-phenotype 129Sv embryos were also observed to have hearts with similar defects. In embryos where looping had occurred normally (at least when developmental delay was taken into consideration) cardiac development was relatively normal by external morphology (69%), and these embryos were the best preserved generally. In other embryos with normal looping, chamber formation was disrupted, with a number of aberrantly formed chamber configurations being apparent (19%) (Fig. 7). These could be the result of delayed or defective folding of the heart relative to the ballooning of the primary myocardium. Severe pericardial edema was observed in some embryos (Fig. 4j, k, l, and particularly m) and was found more often in embryos with a linear heart tube.

Mesodermal markers show severe axial and paraxial defects consistent with early abnormal mesendodermal development. It was apparent from our analysis of E6.5 to E7.5 *Hira*^{-/-} embryos and the morphology of older mutant embryos that axial and paraxial mesodermal derivatives were particularly affected by the gastrulation defects resulting from the loss of the *Hira* gene. To analyze these abnormalities in older embryos in more detail and to examine the extent of the rescue of these defects provided by the outbred genetic background, a number of molecular markers were employed to examine mesodermal structures in E9.5 to E10.5 mutant embryos.

Somite development was assessed by using *Mox1* to detect presomitic and segmented somitic mesoderm. In all 129 and severe CD1 embryos, no segmentation was observed. Asymmetric stripes or patches of unsegmented somitic mesoderm were identified in these embryos (Fig. 5b to d). This phenotype was rescued in the mild CD1 class, for which segmented somites were clearly visible (Fig. 5d).

The caudal defects of *Hira*^{-/-} embryos prompted investigation of notochord development using the axial mesodermal markers *Foxa2* (*Hnf3β*), *Shh*, *Tbx6*, *Fgf8*, and *Brachyury* (*T*). Each of these notochord markers gave similar results across the phenotypic range (Fig. 5). Some axial mesoderm is formed in both mild and severe embryos on the 129Sv background, but normal axial structures were never present. In many severe embryos, axial mesodermal markers (*Foxa2*, *T*, and *Fgf8*) were highly expressed centrally over a disproportionately large area of the embryo and surrounded by nonexpressing tissue (Fig. 5g and l). In other severe embryos, these markers were expressed in small scattered patches in the embryonic disk or were not detected at all.

In milder inbred null embryos the prechordal plate or notochord, as delineated by staining for axial mesodermal markers (*Foxa2*, *Shh*, and *Gsc*), was often absent (Fig. 4p and q) or

FIG. 5. Whole-mount in situ hybridization *Hira*^{-/-} embryos with mesodermal molecular markers (E9.5 to E10.5); mutants and wild-type embryos are shown to the same scale. (a) *Mox1*, wild-type embryo (E9.5). (b and c) Mild- and severe-phenotype *Mox1*, 129Sv *Hira*^{-/-} embryos (E9.5), respectively, both displaying loss of segmented somite expression. (d and e) *Mox1*, CD1 *Hira*^{-/-} mild-phenotype embryos, both displaying rescued segmented somitic expression at E10.5. (f) *Brachyury*, wild-type embryo (E9.5). (g and h) Mild- and severe-class *Brachyury*, 129Sv *Hira*^{-/-} embryos (E9.5), respectively, with extensive *Brachyury* expression. (i) *Foxa2*, wild-type embryo (E9.5). (j and k) *Foxa2*, 129Sv *Hira*^{-/-} embryos (E9.5). (j) Mild-phenotype embryo. Note the duplicate notochordal structure ventrally (blue arrow). (k) Severe-phenotype embryo, heavily expressing *Foxa2*. (l and m) *Foxa2*, CD1 *Hira*^{-/-} embryos (E10.5). (l) Severe-class embryo with truncated notochord. (m) Mild-class embryo with rescued notochordal structures. (n) *Shh*, wild-type embryo, ventral view (E8.5). (o and p) *Shh*, 129Sv *Hira*^{-/-} embryos (E9.5), both mild-phenotype embryos with truncated notochord expression. (q) *Gsc*, wild-type embryo (E9.5). (r) *Gsc*, CD1 *Hira*^{-/-} severe-phenotype embryo (E9.5).

else detected only at the level of the mid- or hindbrain (Fig. 5j and o). Expression to the level of the heart was often accompanied by ventral shifting (Fig. 5h). In the embryos in which the notochord extended caudal to the heart, expression of the marker gene was reduced in patches (Fig. 5k). Ventral duplication of the notochord was seen in a few embryos (Fig. 5k). On the outbred background, less severely affected embryos demonstrated normal notochordal expression of *Foxa2* extending along the midline over the entire length of the embryo (Fig. 5i). This suggests that, as for paraxial mesoderm, the CD1 genetic background mitigated axial mesodermal defects resulting from the *Hira* mutation.

Expression analysis of *Hand1*, a marker for the left ventricle and lateral plate mesoderm, revealed that, unlike axial mesoderm, lateral plate mesoderm was relatively unaffected in *Hira*^{-/-} embryos (not shown).

Neural markers indicate that the neuroepithelium is disorganized and abnormally patterned, a result of disrupted mesodermal development. Markers expressed along the entire anterior-posterior axis of the neuroepithelium (*Pax3*) and in the neural crest (*cadherin-6*) and regional brain markers such as *Otx-1* and *-2*, *En2*, *Krox20*, *Eph4A*, and *Fgf8* were examined (Fig. 6). In general, the expression of these markers in homozygous mutant embryos reflected the various levels of disorganization and developmental delay seen from embryo to embryo.

FIG. 6. Whole-mount in situ hybridization of *Hira*^{-/-} embryos with neural molecular markers at E9.5 to E10.5; mutants and wild-type embryos shown to the same scale. (a) *Pax3* wild-type embryo (E9.5). (b) *Pax3* 129Sv *Hira*^{-/-} mild-phenotype embryo (E9.5). Expression is maintained in the neural tube. (c) *Otx2* wild-type embryo (E9.5). (d and e) Mild- and severe-phenotype *Otx2* CD1 *Hira*^{-/-} embryos, respectively (E10 to E10.5). A decreased forebrain expression is observed in the mild phenotype, with low or undetectable levels in the severely affected embryo. (f) *En2* wild-type embryo (E9.5). (g to i) *En2* *Hira*^{-/-} embryos (E9.5). (g) 129Sv mild phenotype. An essentially normal expression pattern is evident. (h) 129Sv severe phenotype. Expression is present but not in any anatomically recognizable domain. (i) CD1 mild phenotype (E9.5). Normal expression is detected. (j) and (k) *Krox20* wild type (E9.5 and E8.5, respectively). (l to n) *Krox20* 129Sv *Hira*^{-/-} embryos (E9.5). (l) Mild phenotype. Expression in rhombomeres is detectable in the mild-phenotype mutants but at a reduced level. (o) *EphA4* wild-type embryo (E9.5). (p and q) Mild- and severe-class *EphA4* 129Sv *Hira*^{-/-} embryos, respectively (E9.5). Asymmetric expression is observed in the mutant embryos.

This disorganization of neural patterning most probably results from the disruption of the axial mesoderm.

Pax3, which is normally expressed along the length of the nervous system in the dorsal neuroepithelium, is relatively well preserved in more mildly affected embryos, and the restriction to dorsal neuroepithelium was maintained in these embryos (Fig. 6b). Similarly, low level expression of *cadherin-6* in most mutant types indicated some maintenance of dorsoventral patterning (data not shown).

Anterior-posterior regional neural markers showed variation in their expression across all phenotypic classes, illustrating the differing levels of pattern and tissue disorganization, both between and within these groups. *Otx2* (forebrain marker) was found to be downregulated in the telencephalon in mild 129 and CD1 embryos and lost entirely in more severely affected embryos (Fig. 6c to e). However, normal *En2* was present in all mutant embryos but the most severely affected (Fig. 6f, g, and i). Here aberrant spatial expression domains indicated the disruption of the development of the midbrain-hindbrain border (Fig. 6h). Hindbrain development was affected to various degrees, as illustrated by the expression of *Krox20*/*EphA4* (Fig. 6j to q). Downregulation and asymmetry of expression were evident, with reduced expression in the center and lateral edges of rhombomeres 3 and 5 in some embryos and the loss of all transcripts from single rhom-

bomeres apparent in others (Fig. 6l and m). In the more severely affected embryos, expression was either absent or not correlated to any known anatomical structure (Fig. 6m, n, and q).

Molecular markers delineate cardiovascular defects in *Hira*^{neo}/*Hira*^{neo} embryos. *MLC-2a* and *MLC-2v* are markers for presumptive atrial and ventricular tissue, respectively. Neither gene is expressed in severe *Hira*^{-/-} embryos on the 129Sv background. In milder inbred *Hira*^{-/-} embryos, some heart tubes were morphologically relatively normal. In some of these *MLC-2a* was expressed throughout the heart at E9.5 (Fig. 7a). Other embryos of this class had clearly developed abnormally. For example, at least one heart consisted of two apparently separate chambers, both expressing *MLC-2a* at very high levels (Fig. 7b). In similar embryos probed with *MLC-2v*, a number of embryos had staining in paired bilateral structures presumably corresponding to the developing ventricle (Fig. 7f). On a CD-1 background, expression of *MLC-2a* varied from absence (not shown), expression at similar levels throughout the heart tube, to the more normal situation of down regulated expression in the presumptive ventricular region (Fig. 7c to e). *MLC-2v* was expressed relatively normally in presumptive ventricular regions on the CD1 background (Fig. 7g and h).

As all *Hira*^{-/-} embryos, regardless of genetic background, are dead or dying by E10.5 or E11, and allantoic development

FIG. 7. Whole-mount in situ hybridization of *Hira*^{-/-} embryos with cardiac and endothelial molecular markers. (a to e) In situ hybridization with *MLC-2A*. (a and b) 129Sv embryos (E9.5). (c to e) CD1 embryos (E10.5). In panel a, the wild-type embryo is to the left and the *Hira*^{-/-} mild-phenotype embryo is to the right. (b) *Hira*^{-/-} mild phenotype. (c) *Hira*^{-/-} embryo with dumbbell type heart. (d and e) *Hira*^{-/-} mild phenotype. (f to h) In situ localization with *MLC-2V*. (f) 129Sv embryos at E9.5, with the wild type to the left and the *Hira*^{-/-} mild phenotype to the right. (g and h) CD1 *Hira*^{-/-} embryos, mild phenotype (E10.5). (i and j) High-power photomicrographs of blood vessels connecting the embryo and yolk sac or vitelline membrane. Compared to the wild type, the mutant displays very few interconnecting vessels between the embryo and the extraembryonic membranes. (i) CD1 wild-type embryo (E10.5). (j) CD1 *Hira*^{-/-} embryo, mild phenotype (E10.5). (k and l) PECAM antibody staining of the yolk sac from 129Sv embryos (E9.5). (k) Wild-type embryo. (l) *Hira*^{-/-} embryo, mild phenotype. In the mutant, the number of branching vessels is reduced and the fine branching of vessels is less evident.

is abnormal in a high percentage of mutant embryos we examined the vascular system that would normally connect the embryo to the yolk sac and placenta. It was clear that even in the most mildly affected embryos the vasculature was compromised, with very few of the large blood vessels connecting the embryo to the placenta or to the yolk sac being apparent in mutant embryos (Fig. 7i and j). Closer examination of the vascularization of the yolk sac by PECAM staining, a marker for endothelial cells, revealed that, although high levels of staining were evident in mutant embryos, vascularization was abnormal. The number of large vessels branching off the main trunks was markedly decreased and the interlocking network of small blood vessels was disorganized (Fig. 7k and l). The complex vascularization of the embryo was also disrupted, with fewer vessels evident and as larger patches of staining rather than a fine network (data not shown).

DISCUSSION

***Hira* is essential for normal embryonic development and survival.** *Hira* encodes a nuclear protein of unknown function, although structural homologies, transfection, and protein interaction studies suggest a role in cell cycle-regulated transcriptional regulation. To assess the requirement for *Hira* during murine embryogenesis, we created a targeted mutation of the *Hira* gene. The targeted mutation created resulted in a null allele, as determined by RT-PCR and Western analysis. Mice heterozygous for the mutation are normally viable and fertile, but no homozygote mutant liveborn mice were identified. *Hira* is first expressed at E6.5 to E7.0 at the extraembryonic-embryonic junction and in the proximal ectoderm. This expression pattern overlaps with domains of expression of genes known to be required for normal mesodermal development such as *Bmp4* (55, 60) and *Fgf8* (52).

Targeted mutation of the *Hira* gene results in gastrulation defects, specifically the expansion of the primitive streak into the amniotic cavity, which is suggestive of a failure of cells to exit the primitive streak normally. This leads to the loss of axial and paraxial mesendodermal structures such as the notochord and somites later in development, and this further leads to disorganization of neuroepithelial patterning. How the loss of *Hira* expression early in development could lead to the complex phenotypes seen later is discussed in more detail below.

In the absence of a good understanding of the cellular function of *Hira*, it is difficult to correlate the phenotype with defects at the level of the cell. However, given the regulation of *Hira* by cdk2/cyclin E and the role of the yeast Hir1p and Hir2p in cell cycle-regulated transcription there may be an underlying cellular proliferation defect responsible for the profound growth delay. TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining of embryos (unpublished data) does not reveal any excess apoptosis, at least not at E8.5. Our unpublished work suggests *Hira*^{-/-} ES cells show no gross differences in flow cytometry profiles, indicating altered cell cycling, and no retardation of population doubling time (in fact, cell division may be increased in -/- cells). Cell proliferation studies and blastocyst hatching studies will be of value in analyzing this further.

The mesendodermal defects in *Hira* mutants may be the

result of a gastrulation block. The phenotype described above for the *Hira*^{-/-} embryos bears a remarkable resemblance to those reported for *Fgf8* and *Fgfr1*. Features in common at E9.5 include small, flat, disorganized embryonic disks that exhibit an expansion of axial mesendoderm or primitive streak markers, recognizable but disorganized head structures, truncated posterior regions, loss of axial and paraxial mesendodermal derivatives such as notochord and somites, and open extended neural plates with excess folding (12, 17, 52, 60). These parallels between the phenotypes of the different knockouts continue at gastrulation stages. The *Hira* mutant phenotype in which embryos arrest at early gastrulation was very similar to some *Fgfr1*^{-/-} embryo phenotypes, both displaying developmental retardation, a shorter egg cylinder, and smaller proamniotic cavity. Constrictions at the extraembryonic-embryonic boundary were also present. The expanded primitive streak phenotype, which results in the posterior part of the embryo bulging into the amniotic cavity, was found in both *Fgf8*^{-/-} and *Fgfr1*^{-/-} embryos, as well as in *Hira* mutants.

In both the *Fgf8* and *Fgfr1* knockouts, the underlying defect is suggested to be the failure of primitive streak cells to migrate out of the streak to form the mesendodermal derivatives of the older embryo. Given the many phenotype similarities between *Fgf8*, *Fgfr1*, and *Hira* targeted mutations, this explanation may be extended to the *Hira*^{-/-} phenotype at both gastrulation and neurulation. Our finding that extraembryonic mesoderm derivatives, such as the allantois, yolk sac, blood, and endothelial cells are present in *Hira* mutants indicates that the passage of cells through the proximal (posterior) end of the streak is relatively normal. The same is also true of the presumptive cardiac mesoderm that passes through the anterior streak relatively early at mid-streak (E7.0) stages. However, prospective axial and paraxial mesendoderm cells arising from the node and anterior primitive streak (notochord, prechordal plate, and somites) appear to be unable to exit the streak, resulting in the loss of these structures at postgastrulation stages.

In *Fgfr1* mutants this problem is thought to occur because primitive streak cells do not undergo the required epithelial-mesenchymal transition (EMT) (12). However, *Fgf8* primitive streak cells do undergo EMT but still fail to emigrate (52). Cells with a mesenchymal morphology are evident in *Hira* mutant streaks, suggesting that EMT has taken place and that the migration defect is therefore more analogous to that seen in the *Fgf8*^{-/-} embryos, although we did not detect any gross abnormality of *Fgf8* expression itself.

Corroboration for this idea comes from the expression of *Foxa2*, a marker for the node and later the notochord. Expression is expanded in *Hira* mutant primitive streaks (Fig. 7) but is lost at caudal levels at E9.5 concomitant with the truncation of the notochord, suggesting that although node and anterior streak cells are produced they cannot move away from the streak in the normal fashion.

A further contribution to the phenotype seen in *Hira* mutants may come from the downregulation of several genes known to be important in early patterning of the embryo. The AVE is known to be required for proper anterior patterning in the early mouse embryo. *Hira* mutants display constrictions of the extraembryonic-embryonic boundary similar to those seen in a number of knockout mice in which the AVE is defective, including *Otx2*, *Lim1*, and *Foxa2* (1, 3, 6, 48, 56). *Hex* and

mCerberus and *Foxa2*, markers for the AVE, are also down-regulated in *Hira* mutant embryos, suggesting that the AVE may also be affected in these knockout mice. Chimera analysis and DiI labeling of mutant primitive streak cells could answer this question.

Disorganization of the neuroepithelium may be the result of mesendodermal defects in *Hira* mutants. The axial mesendoderm in the mouse comprises the notochord and prechordal plate and is known to be required for the normal anterior-posterior position of the neural tube (2, 3, 5, 9, 53, 54). As might be expected, the severity of the neuroepithelial abnormalities seen in *Hira*^{-/-} embryos correlated with the severity of the axial mesendoderm defects observed. In the most mildly affected *Hira*^{-/-} mutants (CD1 mild) markers for the notochord such as *Foxa2* showed normal ventral midline expression, with the overlying neural tissue being normally patterned. In the next class of severity (mild 129Sv, severe CD11), *Hira*^{-/-} embryos had reduced or absent notochordal expression of *Foxa2/Shh*. The overlying neuroepithelium in these embryos was severely affected, displaying a variety of patterning defects such as loss or misexpression of anterior-posterior markers.

In the majority of 129 mild/CD1 severe *Hira* mutant embryos that formed a recognizable nervous system, both the cranial and trunk neural folds failed to fuse and were poorly elevated. In CD1 mild embryos the trunk phenotype was rescued but cranial fusion was not. The closure of the cranial neural folds depends upon interactions with the underlying head mesenchyme (10). This tissue underlying the mid- and hindbrain is derived from cells from the anterior and mid-level streak at E7.0. Head mesenchyme found below the forebrain may emanate from the primitive streak earlier than this (29). Histologic analysis of cranial mesenchyme in *Hira* mutants revealed that in many embryos it was significantly diminished and did not populate the region below the neural folds in a normal fashion. Somitic mesoderm that normally lies beneath the trunk neural plate also failed to form, possibly also affecting neural tube elevation and fusion.

***Hira* mutants show failure of embryonic turning and heart looping.** Normal mouse embryos undergo a clockwise axial rotation early in development (E8.5 to E9) which results in the establishment of the normal fetal position (20, 28). This process accompanies the formation and right-sided looping of the heart. In all *Hira* mutant embryos, except the CD1 mild class, embryonic turning failed to take place. In some embryos, heart looping did not take place or failed to complete. A number of mouse knockouts with phenotypes similar to that of the *Hira* mutant mouse and for which the phenotype was ascribed to abnormal mesoderm development also exhibit laterality defects such as abnormal heart looping and embryonic turning, e.g., *Fgf8* (42), *Bmp4* (32, 60), *Brachyury* (30), and *Foxa2* (13, 19).

Intact midline structures such as the notochord and floor plate are essential for the development of left-right asymmetry (14, 37). Mutants such as *no tail* and *floating head* (zebra fish) and *SIL* and *no turning* (mouse), which have compromised notochordal development, also display aberrant heart looping and again altered expression of left-right transcripts (14, 25, 40, 44, 46). Our results demonstrate that in all but the least-affected *Hira* mutant embryos the notochord does not develop

normally. These embryos fail to undergo embryonic turning, and the majority also have abnormal looping of the heart.

Some of the *Hira* mutant embryos that fail to undergo heart looping also display a second characteristic, which is interesting with regard to the process of heart development. Current theory suggests that cardiogenic mesodermal sheets form a crescent which folds toward the ventral midline, forming a linear primitive heart tube composed of primary myocardium. This primary myocardial tube then loops rightward and, concomitantly, secondary working myocardium balloons out from the outer curvature (ventral side linear heart tube) at sites defined by the expression of a specific gene pattern (11, 23). Both processes are crucial for the formation of the four-chambered heart in which all chambers and major connections to blood vessels are correctly juxtaposed. In some *Hira* mutants, although the hearts have not looped, the ballooning out of secondary myocardium to form two distinct chambers is visible. This suggests that under some conditions looping of the primary heart tube and the ballooning out of the working chambers can be uncoupled. This phenomenon may be linked to the decreased *Bmp4* expression seen in *Hira* mutant embryos. Breckenridge et al. (7) have recently demonstrated that inhibition of *Bmp4* in the heart can prevent looping but allow normal chamber and anterior-posterior development.

Embryonic lethality in *Hira*^{-/-} embryos. Homozygous mutant *Hira* embryos probably die due to the failure of normal placentation, compounded by abnormal heart development. In half to two-thirds of embryos the allantois is formed but is abnormally large and often blood filled. It does not fuse with the chorion, so the chorioallantoic placenta and umbilical connection do not form. This leads to death due to failure of proper nutrition when the embryo switches from nutrients supplied by the yolk sac to that supplied by the placenta at E10.5 to E11.

In some *Hira*^{-/-} embryos chorioallantoic fusion and the subsequent development of the placenta and its vascular connections occurred relatively normally. However, all *Hira* mutants die by E10 or E11. A possible explanation is that in embryos in which fusion has occurred a more subtle defect is present in the allantoic mesoderm, such as that seen in other mutants, e.g., *Gcm1* (4), which die at E10.

In conclusion, *Hira* inactivation leads to a midgestational embryonic lethality. The wide variety of malformations seen in midgestation embryos reflect earlier defects seen in gastrulation stage embryos, in particular defects of primitive streak elongation. The cellular role of *Hira* has yet to be fully elucidated, but it may involve regulation of cell cycle-dependent aspects of transcription. Cell lines derived from *Hira*^{-/-} embryos will be useful in investigating this topic further.

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