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The Tissue-Specific IncRNA Fendrr Is an Essential Regulator of Heart and Body Wall Development in the Mouse

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INTRODUCTION

Embryonic development commences with the formation of a group of pluripotent stem cells, which give rise to all cell types of the body. Development then proceeds through the coordinated action of cellular proliferation, patterning, lineage commitment, and differentiation, which are controlled by transcriptional regulators acting in a cell type-specific manner. The activity of genes encoding such regulatory proteins depends largely on the chromatin structure at their promoters and the associated regulatory elements. The histone-modifying Polycomb repressive complexes (PRC1/PRC2) and the Trithorax group/MLL protein complexes (TrxG/MLL) play pivotal roles in the control of chromatin structure and, hence, gene activity of a subset of developmentally important regulators (Schuettengruber et al., 2007). In particular, PRC2 catalyzes the methylation of histone H3 at lysine 27 (H3K27me3), which is repressive to gene activity, while the TrxG/MLL complex catalyzes the methylation of histone H3 at lysine 4 (H3K4me3), which acts as an activating mark (Margueron and Reinberg, 2011; Schuettengruber et al., 2011). Thus, PRC2 and TrxG/MLL have opposing activities, and both are essential for embryonic development (O’Carroll et al., 2001; Yu et al., 1995).

It has been shown that components of both the PRC2 and TrxG/MLL complexes are able to interact with long noncoding RNAs (lncRNAs) (Zhao et al., 2008, 2010; Wang et al., 2011). Recent reports have suggested that lncRNAs may target PRC2 or TrxG/MLL to specific genomic loci, and thus contribute to the histone modification status and activity level of target genes (Rinn et al., 2007; Khalil et al., 2009; Bertani et al., 2011; Guttman and Rinn, 2012). In vitro knockdown experiments have revealed the involvement of IncRNAs in gene regulatory networks controlling embryonic stem (ES) cell differentiation (Guttman et al., 2011), and functional studies have provided evidence for the roles of several IncRNAs (Wang et al., 2011; Hu et al., 2011; Kretz et al., 2012; Rinn et al., 2007; Gupta et al., 2010; Ulltsik et al., 2011). For instance, the knockdown of HOTTIP by an RCAS-shRNA in the developing chick limb resulted in a shortening of the distal bony elements of the limb, and morpholino-mediated knockdown of megamind and cyrano in zebrafish embryos revealed important roles for these IncRNAs in organogenesis (Wang et al., 2011; Ulltsik et al., 2011). However, stringent genetic approaches for probing the role of IncRNAs in mouse embryogenesis using loss-of-function analyses have not yet been reported.

Here we have identified an IncRNA, which we have termed Fendrr, that is specifically expressed in nascent lateral plate mesoderm. We inactivated Fendrr by gene targeting in ES cells and show that Fendrr is essential for proper development of tissues derived from lateral mesoderm, specifically the heart and the body wall. We illustrate that Fendrr acts by modifying the chromatin signatures of genes involved in the formation and differentiation of the lateral mesoderm lineage through binding to both the PRC2 and TrxG/MLL complexes. Furthermore, we provide evidence that an oligonucleotide corresponding to part of the Fendrr transcript can bind to dsDNA in target promoters.
RESULTS

Fendrr Expression Is Restricted to Nascent Lateral Plate Mesoderm

We searched for differentially expressed lncRNAs in six different tissues dissected from early somite-stage mouse embryos (TS12, E8.25, three to six somites) using RNA-seq and ChIP-seq analyses. From this data set, we identified a gene that is specifically transcribed in the posterior mesoderm. We isolated it by RACE-PCR from embryonic cDNA and determined its 2,397 base pair (bp) sequence and gene structure (Figure 1 A). The gene consists of seven exons and is transcribed divergently from the transcription factor-coding gene Foxf1. Its transcriptional start site is located 1,250 bp upstream of the 5′-end of Foxf1. We termed this lncRNA Fendrr (Fetal-lethal noncoding developmental regulatory RNA).

Whole-mount in situ hybridization showed that Fendrr is confined to the caudal end of the lateral plate mesoderm (LPM) of midgestation embryos, which gives rise to ventral structures such as the heart and body wall (Figures 1B and 1C). We could not detect Fendrr expression in other tissues or in organs of later stage embryos using qPCR analysis (data not shown). In the caudal LPM it is coexpressed with Foxf1, while in more anterior LPM cells that are undergoing differentiation, Fendrr is downregulated, whereas Foxf1 expression is maintained in the splanchnic mesoderm (Peterson et al., 1997; Mahlapuu et al., 2001a). Quantitative PCR analysis of RNA extracted from the nuclear or cytosolic faction of E9.5 embryo caudal ends showed that Fendrr RNA is predominantly localized in the nucleus, consistent with its human ortholog in cultured cells (Figure 1D) (Khall et al., 2009).

Loss of Fendrr Causes Embryonic Lethality

To investigate the function of Fendrr in mouse development, we first knocked down Fendrr transcripts using a method for shRNAmir-mediated RNA interference in vivo (Vidigal et al., 2010). A reduction in Fendrr transcripts to 40% of the wild-type level caused no phenotype (data not shown). Therefore, we consecutively replaced the first exon of Fendrr on both chromosomes in ES cells with a strong transcriptional stop signal.
(3xpA) by homologous recombination (Friedrich and Soriano, 1991), thereby generating a Fendrr null mutant (Figure 2A). Successful targeting of both alleles in ES cells was confirmed by Southern blot analysis (Figure S1A available online). We confirmed loss of Fendrr transcripts in the caudal ends of Fendrr null embryos generated by tetraploid complementation (Gertsenstein, 2011) using whole-mount in situ hybridization (Figure S1B).

Homozygous mutants (Fendrr3xpA[A/H]) were found to be embryonic lethal around E13.75 (Figures 2B and S1C). At this stage they appeared pale and displayed a prominent omphalocele, wherein parts of the developing liver and all umbilical

ventricles compared to wild-type (values set to zero; Pmm2 and Hmbs are housekeeping genes); mean ± SD, n = 3.

(K) Gating of EOMES-positive cells from a single cell suspension of E6.5 wild-type embryo proper, and qPCR analysis of RNA derived from EOMES-positive and EOMES-negative cells. See also Figure S1.

Figure 2. Heart and Body Wall Development Are Impaired in Mutants Lacking Fendrr Transcripts

(A) Schematic showing the wild-type (upper) and targeted (lower) Fendrr-Foxf1 genomic regions; the first exon of Fendrr was replaced with a 3xpA stop cassette. Arrows indicate primers for qPCR.

(B) E13.75 embryos derived from wild-type or homozygous Fendrr mutant ES cells (Fendrr3xpA[A/H]), or from mutant cells containing a modified rescue BAC transgene (TgRP23-455G4Bgh). The tail and limbs have been removed. Scale bar, 1 mm.

(C) Eosin-stained sagittal sections of E13.75 wild-type and Fendrr mutant embryos showing liver protrusion in the latter (arrowhead).

(D and G) Transverse histological sections of E12.5 wild-type and mutant embryos at the midtrunk (D) or chest level (G). rv, right ventricle; ia, interventricular septum; cu, atrio-ventricular endocardial cushion; la, left atrium; lv, left ventricle. Scale bar, 200 μm.

(E) Tissue thickness measured from Eosin-stained transverse sections of E12.5 wild-type and homozygous mutant embryos; values from both sides were combined for paired t test analysis. Mean ± SD, (n = 3).

(F) Fluorescent imaging of E12.5 wild-type (n = 3) or mutant (n = 5) embryo hearts injected with FluorSpheres into the right atrium (white arrowhead); images were taken at 75 s of injection (see also Movie S1). ra, right atrium; rv, right ventricle; lv, left ventricle; la, left atrium.

(H) Percentage of mitotic cells (H3S10P) in heart ventricles determined on two distinct sections each of three different embryos. Paired t test analysis and mean ± SD, n = 3.

(I) Scatter plot of transcript abundances (FPKM) derived from RNA-seq analysis of E12.5 mutant compared to wild-type ventricles with one value per gene; genes with an FPKM of less than 2 in both samples were omitted. The dotted lines represent a fold-change of 2 and 0.5. The samples show a high correlation, with a Pearson correlation coefficient of 0.969. Heart-specific markers and structural genes (Tbx5, Tbx20, Nkx2-5, Mef2c, Gata4, Gata6, Hand1, Hand2, Acta1, Acta2, Actc1, Actg2, Myh6, Myh7, Myhf1, Myh9, Tnn1c, Tnn1, Tnn2, and Tnn3) are indicated in red and show little variation between the two samples; yellow dots relate to Foxf1, Inx3, and Pitx2. RNA-seq data are available at GEO accession number GSE43078.

(J) Normalized expression levels of heart genes determined by qPCR analysis of E12.5 mutant ventricles compared to wild-type (values set to zero; Pmm2 and Hmbs are housekeeping genes); mean ± SD, n = 3.
vessels protruded from the ventral body (88.9%, n = 9) (Figures 2B and 2C). Omphalocele and embryonic death persisted after removal of the PKG-Neo and PKG-Hygro selection cassettes (genotype Fendrr<sup>3xpA/3xpA</sup>; Figures S1C and S1D).

To rule out that the phenotype of Fendrr<sup>3xpA/3xpA</sup> mutant embryos was due to compromised genetic integrity of the ES cells we performed a rescue experiment. We introduced a BAC clone containing a functional Fendrr gene next to an inactivated Foxf1 locus into Fendrr<sup>3xpA/3xpA</sup> mutant ES cells and generated embryos by tetraploid complementation. These Fendrr<sup>3xpA/3xpA</sup>Tg(RP23-455G4)Bgh embryos showed a normal expression pattern of Fendrr at E9.5 (Figure S1B) and expressed approximately half of the wild-type level of Fendrr RNA, as expected from a single functional allele (Figure S1E). The Foxf1 expression level was unchanged in rescued embryos in E9.5 caudal ends as compared to wild-type embryos (Figure S1F).

They appeared phenotypically normal until E17.5, while at E18.5 rescue was observed in the majority of embryos (Figures 2B, S1C, and S1G). Thus, the embryonic rescue confirmed the genetic integrity of the mutant ES cells used in the following experiments, and we conclude that the lethal phenotype of Fendrr<sup>3xpA/3xpA</sup> and Fendrr<sup>3xpA/3xpA</sup> embryos is entirely due to loss of Fendrr transcripts.

To determine the etiology of the mutant phenotype, we examined the morphology of embryos at stage E12.5, when mutants still appeared phenotypically normal. Measurements of the thickness of the ventral body wall, a derivative of the somatic LPM lineage, showed a severe reduction in homozygous mutant as compared to wild-type embryos (Figures 2D and 2E; n = 3). This suggests a possible cause for the observed omphalocele: that the weak body wall of the mutant is insufficient to resist the pressure from the growing liver (Figures 2B and 2C).

Besides an omphalocele, mutant embryos also displayed blood accumulation in the right heart chamber (Figure S1H). To assess functioning of the heart, which is a derivative of the mesoderm lineage (Costello et al., 2011), we determined the mitotic activity of the ventricular walls revealed a severe reduction in mutant as compared to wild-type hearts (Figure 2E). To examine the cellular basis for the hypoplasia, we counted the mitotic cells on sections of wild-type and mutant hearts at three developmental stages. No difference was found at E9.5 and E11.5, whereas E12.5 mutant hearts showed a marked decrease of mitotic cells (Figure 2H). We detected no significant apoptosis in E12.5 wild-type or mutant hearts by cleaved caspase-3 (Asp175) staining on sections (data not shown). These data indicate that the cardiac hypoplasia in mutants may be due to impaired proliferation of cardiac myocytes at later stages of heart development.

To investigate the changes in gene expression related to the heart phenotype on a genome-wide scale, we analyzed the transcriptomes of E12.5 wild-type and Fendrr mutant hearts by RNA-seq. We found no strong changes in the expression levels of any of the known heart control genes, nor of genes associated with cell proliferation control in mutant tissue (Figure 2I). Quantitative PCR analysis of a subset of heart-specific structural genes confirmed the RNA-seq data (Figures 2J and 2K) (Lin et al., 1997; Olson, 2006). Thus, the transcriptome data did not offer a molecular explanation for the heart phenotype of the Fendrr mutant.

The combined data show that Fendrr mutants exhibit myocardial dysfunction, which most likely is the cause of embryonic death. Moreover, the impaired development of both the heart and the body wall illustrates that loss of Fendrr transcripts in nascent LPM causes impaired development of derivatives of both the somatic and splanchnic LPM lineages.

**Loss of Fendrr Affects the Epigenetic Modification and Expression of Factors Controlling Lateral Mesoderm Differentiation**

Cardiac mesoderm derives from the first lateral mesoderm formed in the primitive streak at the early to midgestation stage (E6.5-7). To ensure that Fendrr is expressed in the prospective cardiac mesoderm at this early embryonic stage, we isolated Eomes expressing cells from E6.5 mouse embryos by fluorescence activated cell sorting using an EOMES-specific antibody (Figure 2K). Eomes and Myl7 are markers of the early cardiac mesoderm lineage (Costello et al., 2011). We determined the RNA expression level of Eomes, Myl7, and Fendrr in EOMES-positive and EOMES-negative cells in comparison to unlabeled control cells. We found coexpression of Myl7 and Fendrr exclusively in EOMES-positive cells, while neither transcript was detectable in EOMES-negative cells (Figure 2K). Thus, Fendrr is indeed expressed in cardiac mesoderm progenitor cells. Because formation of the heart tube occurs around E8.0, we asked whether the expression of four important regulators of heart development, Gata4, Gata6, Tbx5, and Nkx2-5 were affected at this stage (Olson, 2006; Watanabe and Buckingham, 2010). We found that Gata6 and Nkx2-5 expression in the heart field of E8.5 mutant embryos was significantly increased in comparison to wild-type tissue, while Gata4 and Tbx5 remain unchanged (Figure 3A).

Next, we asked whether these changes in gene expression were mirrored by alterations of the histone methylation status at the promoters of these genes. The histone methyltransferase complexes TrxG/MLL and PRC2 deposit activating H3K4me3 and repressive H3K27me3 marks, respectively, and both complexes have been shown to be involved in lineage commitment in vitro (Marqueron and Reinberg, 2011; Surface et al., 2010; Schuettengruber et al., 2011). We found no significant change in the status of H3K27me3 at the promoters of the heart control genes analyzed, while the H3K4me3 mark of the Gata6 and Nkx2-5, but not Gata4 or Tbx5 promoters was increased (Figure 3B). Thus, loss of Fendrr results in an increase of H3K4 trimethylation in a subset of heart control gene promoters, mirrored by an increased expression of these genes.
Because Gata6 is widely expressed in the LPM, including nascent LPM and heart progenitor cells, we wanted to determine whether changes in Gata6 expression and promoter histone modifications were already present in nascent LPM. In addition, we analyzed the expression of the LPM control genes Foxf1, Pitx2, and Irx3, which play important roles in determining the splanchnic and somatic LPM lineages, along with Tbx3 (Mahlapuu et al., 2001b; Rallis et al., 2005; Kitamura et al., 1999). The presomitic mesoderm (PSM) marker genes Dll1 and Tcf15 were included as controls.

The expression levels of Gata6 and Foxf1 were significantly increased in the caudal ends of E8.5 mutant embryos, while Irx3, Tbx3, and the PSM marker genes were not affected at this stage (Figure 3C). This increase in Gata6 and Foxf1 expression persisted in the nascent LPM of E9.5 mutant embryos. In addition, Irx3 and Pitx2 expression was also increased at this later stage, during which the progenitors of the ventral body wall are generated (Figure 3C). The expression of Foxc2, Fox1, and Mthfsd, located in close vicinity to Foxf1 and Fendrr, was unchanged, excluding unspecific effects of Fendrr gene locus alterations on neighboring genes in the knockout allele (Figure S2A).

Similarly to what was observed in the heart, changes in gene expression were accompanied by changes in the methylation status of the promoters. The Gata6 and Foxf1 promoters of mutant E8.5 embryos showed a strong increase in H3K4me3, and the Irx3 and Pitx2 promoters a slight increase in H3K4me3 (Figure 3D). In contrast to Gata6, which showed no difference in the repressive mark, the H3K27me3 levels at the Foxf1, Irx3, and Pitx2 promoters of mutant embryos were strongly reduced as compared to wild-type (Figure 3D). The methylation status of the control PSM marker gene promoters was not altered for either the H3K4me3 or the H3K27me3 marks.

Next we asked whether the changes in histone modifications observed correlated with altered occupancy of the PRC2
and/or TrxG/MLL complexes at the respective promoters. We
analyzed the presence of PRC2 or TrxG/MLL by ChIP with anti-
bodies against EZH2, SUZ12 (PRC2 components), or WDR5
(TrxG/MLL component), followed by qPCR analysis of the
promoter regions. Immunoprecipitation of the
Foxf1, Irx3, and
Pitx2
promoter regions with EZH2 or SUZ12 antibodies from
mutant caudal end tissue was drastically reduced compared to
wild-type tissue, whereas all other promoters were not affected
(Figures 3 E and S2B). In contrast, promoter occupancy of
WDR5 in caudal end tissue was unchanged for all of the
promoters tested (Figure 3 E). In addition, no changes in EZH2,
SUZ12, or WDR5 occupancy were observed in heart tissue for
any of the promoters tested (Figure S2 C).

These combined data show that
Fendrr
has differential effects
on the histone modification of promoters for transcriptional
regulators in the lateral mesoderm and at least one of its deriva-
tives, the cardiac mesoderm. The data suggest that the primary
role of
Fendrr
is to promote occupancy of the PRC2 complex
on particular promoters for LPM control genes, resulting in an
increase of the repressive H3K27me3 mark, accompanied by a
reduction in gene expression. In addition, probably via a different
mechanism,
Fendrr
is involved in controlling the level of the
activating H3K4me3 mark on a subset of promoters, thereby
modifying the expression level of those genes.

We asked if the epigenetic changes caused by loss of
Fendrr
at the Foxf1, Pitx2, and
Irx3
promoters, which caused upregula-
tion of these genes in lateral mesoderm, had an effect on the
expression of these genes in E12.5 cardiac tissue. RNA-seq
data showed a moderate, ectopic expression of
Foxf1
in E12.5
mutant hearts, whereas no
Foxf1
transcripts were detected in
wild-type tissue (Figure 2 I). 
Irx3
and
Pitx2
expression, which
is active in the wild-type E12.5 heart, was not altered in the
mutant organ. These data suggest that the epigenetic changes
caused by
Fendrr
at its target promoters in lateral mesoderm
may persist through consecutive stages of differentiation and
thus also take effect in the descendants of the cells exposed
to
Fendrr
activity.

**Figure 4. Fendrr Binds to the PRC2 and TrxG/MLL Complexes and to Target Promoters**

(A and B) RNA coimmunoprecipitation (RIP) from forebrain (upper panels) and caudal end (lower panels) lysates from wild-type embryos using antibodies directed
against EZH2 and SUZ12 (A) or WDR5 (B); normal rabbit IgG was used as control. Fold enrichment has been normalized to nonenriched input sample and U1
rRNA, Foxf1 and Hmbs RNA served as negative control. Mean ± SD are shown (n = 3).

(C) Binding potential between
Fendrr
and genomic regions. The red curve shows the average probability of single-stranded RNA (Ding et al., 2004). The heat map
represents the base-pairing energy for an RNA/RNA duplex model for 40-bp regions along the
Fendrr
transcript and 2,000 bp around the TSS of
Foxf1
top) and
Pitx2
topbottom).

(D) Representation of the predicted interaction of the
Fendrr
RNA region and the promoter DNA region exhibiting the lowest free energy of approximately
−70 kcal/mol (see yellow spot in C).

(E) In vitro RNA/dsDNA binding assay utilizing biotin tagged RNA oligos as bait. Bars represent normalized enrichment of indicated 2,000-bp promoter fragment
over background using a control RNA oligonucleotide (Mean ± SD, n = 3).

See also Figure S3.
in the mouse embryo, confirming data previously obtained in cultured human foreskin fibroblasts for the human orthologous IncRNA (Knaill et al., 2009). Next, we tested whether Fendrr transcripts could interact with the TrxG/MLL component WDR5. The IncRNA HOTTIP served as positive control (Wang et al., 2011). Again, both Fendrr and HOTTIP transcripts were coimmunoprecipitated with WDR5 from the caudal end, but not from forebrain tissue (Figure 4B). In contrast, Fendrr RNA was not coprecipitated with the PRC1 component RING1B, the NuRD complex component LSD1, or SIRT6 (Figure S3A). Thus, Fendrr RNA binds to PRC2 and TrxG/MLL and discriminates between various histone-modifying complexes.

**Fendrr Binds to the Foxf1 and Pitx2 Promoters In Vitro**

Because Fendrr binds to the PRC2 complex and loss of Fendrr resulted in a strong reduction of PRC2 occupancy at the Foxf1, Pitx2, and Irx3 promoters, we asked whether Fendrr is able to bind directly to any of these promoters. We calculated the binding potential of Fendrr to fragments covering 1 kb upstream to 1 kb downstream of the transcriptional start site of each of the three genes. The heat map revealed a short stretch in the Fendrr RNA predicted to bind to a complementary region in the Foxf1 and Pitx2 promoters (Figures 4C and 4D). The Irx3 promoter was negative within the region analyzed, just as the promoters ofDll1 and Tcf15, which served as negative controls (Figure S3B). We used a synthetic RNA oligonucleotide coupled to psoralen and biotin at either end and performed an in vitro binding assay (Besch et al., 2004; Schmitz et al., 2010). The promoter fragments of both Foxf1 and Pitx2, but not of Dll1, co-coprecipitated with this RNA oligomer (Figure 4E). Coprecipitation occurred in the presence of RNaseH, but was prevented by RNAseV1 treatment. The former enzyme specifically cuts RNA in DNA/RNA heteroduplexes, while the latter cleaves base-paired nucleotides. The data show that Fendrr can bind to double-stranded Foxf1 and Pitx2 promoter fragments, and suggest triplex formation at the complementary region (Buske et al., 2012). The data in combination with findings discussed above confirm that Fendrr acts in cis (at the Foxf1) and in trans (at the Pitx2 and possibly other promoters).

The combined data suggest that Fendrr anchors PRC2 at its target promoters, thereby increasing PRC2 occupancy and H3K27 trimethylation, which consequently leads to attenuation of target gene expression. Moreover, the coexpression of the transcriptional regulator Foxf1 and the IncRNA Fendrr in lateral mesoderm links the transcriptional regulatory network with the epigenetic regulatory network acting in this tissue.

**DISCUSSION**

Fendrr is a regulatory RNA, which mediates the modification of the epigenetic landscape of target promoters thereby causing attenuation of the expression of transcription factors that are important in lateral mesoderm differentiation.

Our data suggest that changes in epigenetic modifications within promoters of genes involved in a gene regulatory network (GRN) can cause deleterious effects, which are similar to those seen following the loss of a single crucial transcription factor. For instance, Foxf1 is essential for the separation of splanchic and somatic mesoderm (Mahlapuu et al., 2001b), where it is required to inhibit Irx3 expression in the splanchnic mesoderm and direct it to the somatic mesoderm lineage (Mahlapuu et al., 2001b). Pitx2 is required for heart and ventral body wall development (Kitamura et al., 1999). However, at this point we cannot conclude that the mutant phenotype observed is indeed caused by increased expression of several transcriptional regulators, nor can we exclude that failure of Fendrr binding to TrxG/MLL at presently unidentified promoters leading to downregulation of target genes may contribute to the mutant phenotype.

Compound heterozygosity of Gata4 and Gata6 has been shown to result in embryonic lethality demonstrating that a threshold of both genes is required to support cardiovascular development (Xin et al., 2006). Moreover, heterozygosity for Foxf1 or Pitx2 results in haplo-insufficiency phenotypes, indicating that a threshold level of each of these regulators is critical for proper embryonic development (Mahlapuu et al., 2001a; Liu et al., 2003). While on the basis of current knowledge this is well conceivable, it is harder to envision how an excess of transcriptional regulators might perturb embryonic processes.

An important feature of Fendrr is its long-term effect. In general, the action of transcription factors is restricted to the cells in which they are expressed. In contrast, the epigenetic signatures of regulatory elements set early in a differentiation process can persist through several stages of differentiation. The ectopic upregulation of Foxf1 in E12.5 Fendrr mutant hearts indicates that Fendrr is involved in epigenetic modifications affecting activation or repression of its target genes in descendants of the cells in which Fendrr was, but no longer is, active. Thus, disturbances in the epigenetic prepattern within the early precursor cells of organs and tissues may have far-reaching consequences on subsequent cell proliferation, patterning or differentiation processes in the descendants of those cells. Cardiac myocyte proliferation in Fendrr mutant hearts is not affected before E12.5, 6 days after Fendrr expression in the cardiac progenitor cells of the lateral mesoderm has been lacking. Future work has to address how this late effect in cardiac tissue is triggered by Fendrr loss in cardiac progenitor cells.

The gene pairing of Fendrr and Foxf1 highlights the intriguing transcriptional and functional coupling of a divergent IncRNA with an adjacent regulatory protein, both of which are essential for development of the same embryonic tissue. Such IncRNA:protein-coding gene neighbors are found throughout the genome (Cabili et al., 2011) and this functional link may reveal a more general mechanism for the control of patterning and lineage commitment (Cabili et al., 2011; Ulltisky et al., 2011; Mar-gueron and Reinberg, 2011; Órom et al., 2010).

**EXPERIMENTAL PROCEDURES**

**Fluorescent Imaging**

All animal procedures were conducted as approved by the local authorities (LAGeSo Berlin) under the license number G0358/08. Embryos were dissected at E12.5 into warm M2 medium, and kept at 37°C. Embryos were fixed onto a Leica MZ 16FA microscope equipped with a GFP3 filter and videos recorded using the Leica LAS AF software.
RNA coimmunoprecipitation
RNA coimmunoprecipitation was carried out as previously described (Galgano et al., 2008). Magnetic Protein A and G beads (Life Technologies) were used for isolation of antibody-bound protein/RNA complexes. Coprecipitated RNA was reverse transcribed using random hexamers, and cDNA content quantified by qPCR. Antibodies used were anti-EZH2 (Active Motif), anti-SUZ12 (Abcam), anti-WDR5 (Bethyl Laboratories, Inc.), anti-RING1B (Abcam), anti-RBP2 (Abcam), and anti-SIRT6 (Abcam). For oligonucleotide sequences, see the Supplemental Experimental Procedures (Zhao et al., 2008).

Binding Potential Calculations and RNA/dsDNA Interaction Assay
The average probability of single-stranded RNA was computed by sfold with a length parameter of 200 and W = 1 (Ding et al., 2004). Base-pairing energy for an RNA/RNA duplex model for 40-bp regions along the Fendrr transcript and 2,000 bp around the TSS of Foxf1 and Pitx2 was calculated (Lorenz et al., 2011). The duplex energy is computed for each such region, staggered by 20 bp, and displayed in a heatmap. Probabilities are then averaged for a sliding window of 40 bp to give the average RNA accessibility of the region that is binding.

The RNA/RNA interaction assay was essentially carried out as described (Besch et al., 2004). Briefly, 1 pmol of promoter fragment (~1 kb to +1 kb from the transcriptional start site) were incubated at 37°C for 30 min with 100 pmol of Psoralen-C6-UCCCUCUCUCUCCUCUCUCUCUCUCUCUC UCUCUCUU-BiotinTTEG (Fendrr) or unspecific Psoralen-C6-UCCCUCUGUGG UGGGGUGGGUGGUCCUCUU-U-BiotinTTEG RNA oligonucleotides (Biomers) (Schmitz et al., 2010). The reaction was UV (265 nm) treated as described. Preblocked M270 Streptavidin beads (Life Technologies) were used to precipitate bound DNA in the presence or absence of RNase H or V1. Fold enrichment was determined by the ratio of specific to unspecific DNA precipitate obtained from three replicates.

ACCESSION NUMBERS
The GenBank accession number for the Fendrr full-length cDNA sequence reported in this paper is JN0973641. The GEO accession number for the RNA-seq data reported in this paper is GSE43078.

SUPPLEMENTAL INFORMATION
Supplemental Information includes three figures, one movie, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2012.12.012.

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