

The long non-coding RNA *Fendrr* links epigenetic control mechanisms to gene regulatory networks in mammalian embryogenesis

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Epigenetic control mechanisms determine active and silenced regions of the genome. It is known that the Polycomb Repressive Complex 2 (PRC2) and the Trithorax group/Mixed lineage leukemia (TrxG/Mll) complex are able to set repressive and active histone marks, respectively. Long non-coding RNAs (lncRNAs) can interact with either of these complexes and guide them to regulatory elements, thereby modifying the expression levels of target genes. The lncRNA *Fendrr* is transiently expressed in lateral mesoderm of mid-gestational mouse embryos and was shown to interact with both PRC2 and TrxG/Mll complexes *in vivo*. Gene targeting revealed that loss of *Fendrr* results in impaired differentiation of tissues derived from lateral mesoderm, the heart and the body wall, ultimately leading to embryonic death. Molecular data suggests that *Fendrr* acts via dsDNA/RNA triplex formation at target regulatory elements, and directly increases PRC2 occupancy at these sites. This, in turn, modifies the ratio of repressive to active marks, adjusting the expression levels of *Fendrr* target genes in lateral mesoderm. We propose that *Fendrr* also mediates long-term epigenetic marks to define expression levels of its target genes within the descendants of lateral mesoderm cells. Here we discuss approaches for lncRNA gene knockouts in the mouse, and suggest a model how *Fendrr* and possibly other lncRNAs act during embryogenesis.

Introduction

The process of gastrulation generates the three germ layers: (neuro-) ectoderm,

mesoderm, and (gut-) endoderm, which give rise to all organs of the body. In the mouse, this process takes place over six days and consecutively forms the anlage of the head, trunk, and tail. The lineage choice made by cells during gastrulation is the most crucial one for determining their fate and further differentiation of all of their descendants. Following the first lineage choice, cells can be recruited to various sub-lineages. For instance, the lateral mesoderm subdivides into the somatic and splanchnic mesoderm. In this manner, with each consecutive lineage decision, cellular plasticity becomes more and more constrained. Thus, it is evident that the molecular activities controlling the first lineage decision during gastrulation have the most impact on cell fate.

An example of an essential regulator involved in early lineage decisions is the transcription factor *Foxf1*. The targeted disruption of the *Foxf1* gene prevents the partition of lateral mesoderm into splanchnic and somatic mesoderm lineages, resulting in embryonic death.¹ Moreover, the *Foxf1* mutant is haploinsufficient, i.e., embryos expressing *Foxf1* from a single active allele exhibit perinatal lethality with variable abnormalities, including lung defects. This underpins the crucial role of this transcription factor during embryonic development.²

Many other protein-coding genes, which play essential and specific roles during embryonic development, have been described over the past two decades. The discovery of microRNAs (miRNAs) as essential regulators of developmental programs has shifted the focus toward genes

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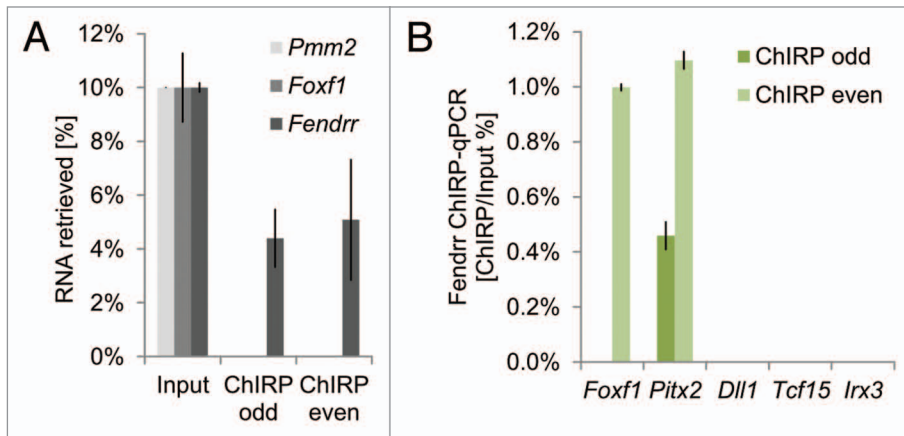


Figure 1. ChIRP (Chromatin Isolation by RNA purification) analysis shows that *Fendrr* interacts with target promoters in embryonic stem cells differentiated in vitro. Fourteen biotinylated oligonucleotides tiling the *Fendrr* transcript were combined in even and odd numbered pools. (A) Each oligo-pool specifically precipitated the *Fendrr* transcript. (B) At least one pool of tiling oligos co-precipitated the target promoters of *Foxf1* and *Pitx2*, but not the control promoters of *Dll1*, *Tcf15*, or *Irx3*.

transcribed into non-coding RNA molecules.^{3–6} MiRNAs are a class of non-coding RNAs, which are processed into single-stranded RNAs of ~22 nucleotides in length, and form functional units with the RNA-induced silencing complex (RISC). They are known to be involved in post-transcriptional control of gene expression. LncRNAs constitute a distinct class of RNA molecules, which are typically over 200 nucleotides in length, highly heterogeneous, and associated with a plethora of different functions, but in particular epigenetic gene control (for review, see refs. 7 and 8).

Recent data from the human ENCODE project identified 13 333 genes encoding 22 631 transcripts in the human genome belonging to the lncRNA class (www.encodegenes.org).⁹ They preferentially localize to the nucleus and, therefore, are thought to be involved in genome regulation.¹⁰ Although many of these lncRNAs contain short open reading frames and can be found associated with ribosomes, the majority of them are assumed to be non-coding.^{11,12}

The identification and functional analysis of lncRNA transcripts encoded by the murine *Xist* (Xi-specific transcript) locus within the X-inactivation center (*XIC*) first revealed the importance of nuclear lncRNAs in genome regulation and embryonic development.^{13,14} The lncRNAs *Xist* and *RepA*, a PRC2

binding lncRNA transcribed from the *Xist* locus, were shown to play a role in X-chromosome inactivation.¹⁵ Moreover, the PRC2 component Enhancer of Zeste Homolog 2 (EZH2) was found to bind thousands of lncRNAs, further supporting a functional link between lncRNAs and histone-modifying protein complexes.¹⁶ Further importance of lncRNAs as factors regulating genome activity through histone-modifying complexes came from the discovery of *HOTAIR*, which is expressed from the *HOXC* locus and enhances PRC2 activity at the *HOXD* locus.¹⁷ *HOTAIR* binds to and might serve as a molecular scaffold for the histone-modifying complexes PRC2 and LSD1/CoREST/REST.¹⁸ In addition, the lncRNAs *HOTTIP*, *Mistral*, and *NeST* have been shown to associate with the TrxG/Mll component WDR5.^{19–21}

The ability to survey the transcriptional landscape through the technique of RNA-seq allows screening for lncRNAs, which are differentially expressed during embryogenesis. We analyzed six tissues from mid-gestational mouse embryos (embryonic day [E] 8.25) for the presence of lncRNAs, and identified several hundred which are expressed in a tissue-restricted manner (unpublished). This data set provided the basis for determining whether lncRNAs are able to play an essential role in development.

Functional Analysis of lncRNAs in Embryonic Development

The functional analysis of numerous embryonic control genes has shown that the expression pattern of essential regulators in the embryo is often highly restricted. Thus, we presumed that restricted expression may also serve as a useful criterion for selecting lncRNA genes for functional analyses. We analyzed a number of lncRNAs showing differential expression in the RNA-seq data in more detail by whole-mount in situ hybridization. One of these, *Fendrr*, showed a remarkably restricted and specific expression in the nascent lateral mesoderm. It is divergently transcribed from the *Foxf1* promoter and is co-expressed with *Foxf1*.²² Since divergently transcribed long non-coding/mRNA gene pairs are thought to be functionally linked and represent an intriguing gene class,^{23,24} we decided to proceed to a functional analysis of *Fendrr* in the mouse.

Most current studies on the function of lncRNAs involve RNAi or similar mechanisms to reduce transcript levels.^{6,25–29} It is, however, important to note that transcripts that localize to the nucleus are less susceptible to knockdown by RNAi. In addition, many lncRNAs exhibit a very low expression level.^{10,30} Hence, it cannot be excluded that even a few residual transcripts per cell that escape knockdown might be functionally sufficient. Initially, we attempted the functional analysis of *Fendrr* by using an in vivo knockdown system, which we had previously applied to the inactivation of several protein-coding genes.³¹ However, an shRNAmir construct expressing two different hairpins against *Fendrr* (best of six individually tested hairpins) achieved a maximal knockdown of only 60%. We observed no apparent phenotype in knockdown embryos up to embryonic day E18.0. Thus, 40% of the total *Fendrr* transcript was sufficient for embryonic development. This finding also holds true for many, if not most, transcription factors.

The gold standard for analyzing gene function in the mouse is gene targeting, resulting in a loss-of-function mutation. A number of approaches have been developed for targeting protein-coding genes,

however, non-coding genes provide fewer options since an uncharacterized lncRNA sequence does not reveal the nucleotide stretch that is crucial for its function. Notably, it has been shown that nucleotides within the first 300 bases of the *HOTAIR* lncRNA transcript interact with EZH2.¹⁸ Thus, for a clear functional analysis of lncRNAs the complete transcript must be prevented from being expressed. One previously used option involves deletion of the complete gene.^{32,33} This approach is not ideal, since the deletion might also remove *cis*-acting regulatory sequences for neighboring genes.^{23,24,34} Another option is to integrate a strong transcriptional stop signal right at the start of the transcription unit. We consider this latter option superior, but it should be noted that possible alternative transcriptional start sites (TSS) downstream of the TSS that has been chosen for stop-cassette integration must not be overlooked. Also, since the insertion of genetic elements such as transcriptional stop signals can alter the chromatin landscape and, thereby, influence gene regulation at the site of insertion, it is crucial to rule out that the phenotype observed is due to a secondary effect. This can be done by a rescue experiment wherein the lncRNA transcript is expressed from a construct integrated randomly into the genome. If the phenotype is rescued it proves that the knockout phenotype was indeed caused by loss of the lncRNA transcript and, moreover, that the lncRNA acts in *trans*.

Two strong transcriptional stop signals have been previously used for gene knockout, a triple SV40 polyA signal and the chicken β -globin polyA signal.^{35,36} Both elements harbor an intron upstream of the polyadenylation signal that is presumed to increase their efficacy. Insertion of the triple SV40 polyA cassette into the first exon of *Fendrr* proved to be very efficient, but not absolutely transcriptionally tight. A residual transcript level of 0.1% was still detectable in *Fendrr* mutants following 3' SV40 pA insertion (data not shown). However, this residual activity was found to be insufficient for *Fendrr* function. *Fendrr* mutants presented with omphalocele and showed malfunctioning of the heart, which resulted in embryonic death by E13.75.²²

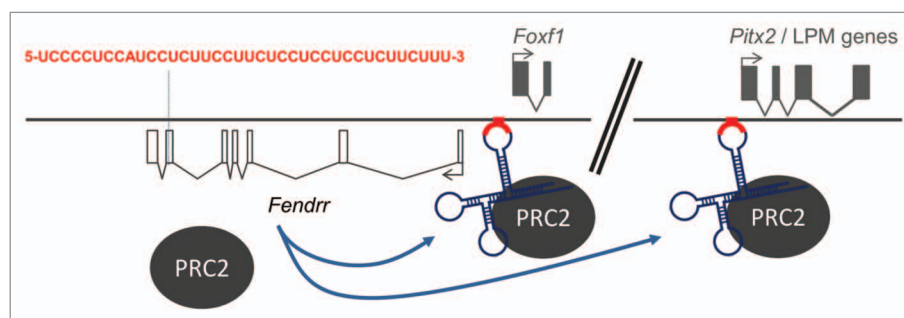


Figure 2. *Fendrr* anchors PRC2 to specific target sites. Schematic drawing illustrating binding of the *Fendrr*/PRC2 complex through a 40-nucleotide stretch (red) to specific recognition sequences in target promoters, e.g., that of *Foxf1* and *Pitx2*, thus increasing PRC2 occupancy and H3K27 trimethylation at these sites.

To rescue the *Fendrr*-knockout phenotype, we integrated a mini-gene construct expressing the *Fendrr* transcript under control of the human *ACTB* promoter randomly into the genome of *Fendrr*-null ES cells. Ubiquitous expression of *Fendrr* at approximately 20% of its wild-type level was observed in embryos derived from the transgenic ES cells. This level of *Fendrr* transcript was not sufficient for a complete rescue of the knockout phenotype. For instance, though surviving embryos showed no omphalocele at E14.5 (*Fendrr* null: 100% omphalocele), the survival rate only increased moderately (from 21% [n = 9] to 60% [n = 5]). This partial rescue is most likely due to low *Fendrr* expression in the lateral mesoderm, but we cannot exclude the possibility that ubiquitous ectopic expression of *Fendrr* in the embryo may have contributed to the observed lethality.

We reasoned that expressing *Fendrr* under control of its endogenous promoter, providing correct spatial and temporal control as well as correct expression levels, would be more adequate for achieving rescue of the knockout phenotype. Therefore, we randomly integrated a BAC (bacterial artificial chromosome) containing an intact *Fendrr* gene in the middle of a 172194 bp region of the C57Bl/6J mouse genome, into *Fendrr*-null ES cells. The BAC clone was genetically modified by insertion of a Neomycin selection cassette into the translational start site of *Foxf1*. This inactivated *Foxf1*, thus avoiding an increase of *Foxf1* activity, which might trigger unwanted effects in BAC transgenic embryos, while it left the *Fendrr*

transcription unit on the BAC unchanged, and also allowed for antibiotic selection of ES clones, which had integrated the BAC. This construct faithfully reproduced the wild-type expression pattern of *Fendrr*, and produced approximately 50% of the *Fendrr* transcript level observed in wild-type embryos, corresponding to expression of one allele.²² This construct rescued the *Fendrr*-null phenotype until E17.5 illustrating that the *Fendrr* lncRNA acts in *trans*, and that loss of the *Fendrr* transcript is responsible for the lethal phenotype.

The rescue experiment was also necessary as a test of the genetic integrity of the mutant ES cells that were used for embryo production via tetraploid complementation.³⁷ One advantage of tetraploid complementation over blastocyst injection for producing knockout mouse lines is the vast difference in the time required for obtaining homozygous embryos. While the standard approach takes 8–12 mo, tetraploid complementation allows the production of virtually pure ES cell-derived homozygous embryos directly from the genetically altered cells (consecutively mutated on both alleles, which takes around 2–3 mo). In a typical experiment, 8 to 12 genetically identical homozygous embryos can be obtained per mouse. Additional genetic manipulations, such as the introduction of a reporter, constructs for lineage tracing, rescue BAC, etc. can be performed in a reasonably short time frame. Importantly, the genetic background of the embryos produced for knockout and consecutive analyses can be kept virtually identical, which is highly desirable to avoid errors due to background effects. Therefore, the

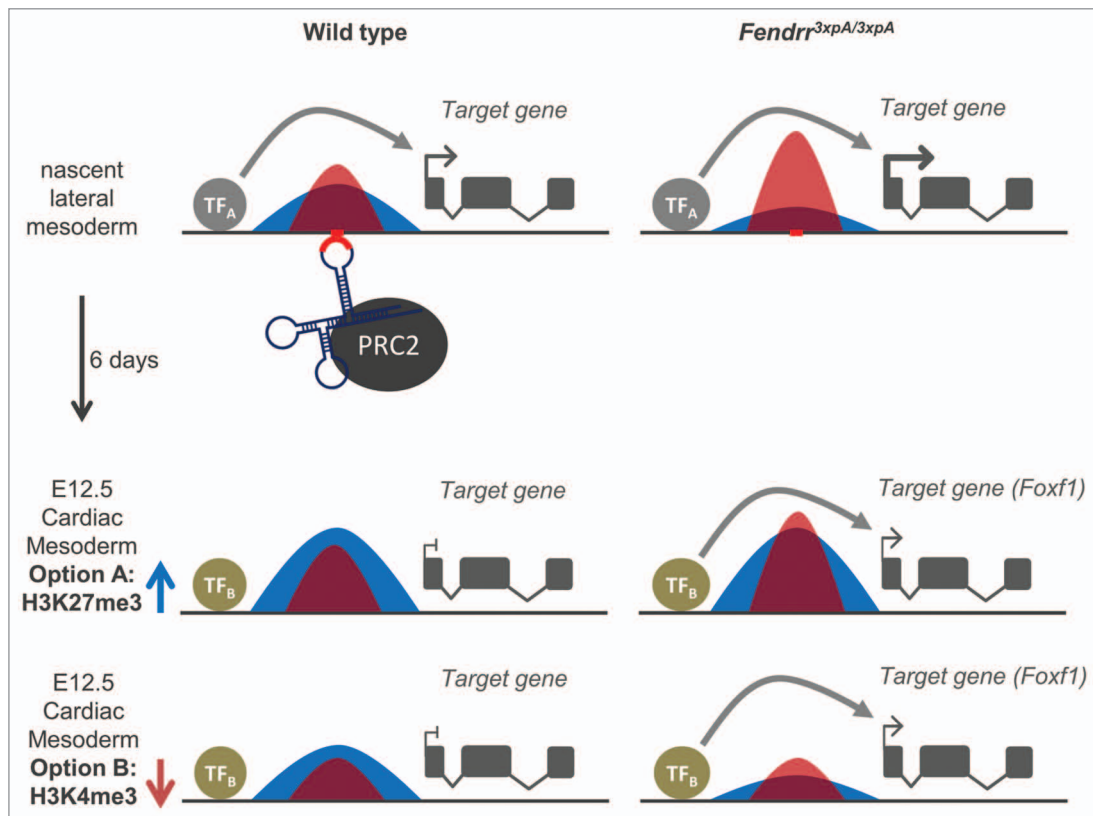


Figure 3. Model of immediate and long-term effects of *Fendrr* acting via PRC2 on epigenetic modification and gene expression. The ratio of repressive H3K27me3 (blue) to active H3K4me3 (red) marks determines the level of gene expression. Histone marks set in the lateral mesoderm are maintained in the descendants of these cells until they are modified by other site-specific regulators resulting, for instance, in an increase in the repressive or a reduction in the active marks within cardiac mesoderm (indicated by arrows). In the case of *Foxf1*, this leads to a dominating repressive mark and silencing of the gene in wild-type cardiac mesoderm. In *Fendrr* mutant cells, anchoring of PRC2 to the *Foxf1* promoter fails, thus skewing the H3K27me3/H3K4me3 ratio and leading to an increased *Foxf1* expression level. The subsequent histone modifications which normally take place in cardiac mesoderm are not able to correct the false prior epigenetic marks set in the lateral mesoderm cells and thus, *Foxf1* cannot be silenced.

starting ES cell line does not need to be derived from an inbred strain.

Mode of *Fendrr* Action in Embryonic Development

Fendrr mutant embryos appear normal until E12.5, but by E14.5 most are dead. Both sub-lineages of the lateral plate mesoderm are affected: the heart, a derivative of the splanchnic mesoderm, and the ventral body wall, a product of the somatic mesoderm. The heart progenitors express *Fendrr* transiently at E6.5–7.0, and the progenitors of the ventral body wall at around E9.5 of development. We showed that proliferation of cardiomyocytes is impaired in *Fendrr* mutants, but not before E12.5.¹⁸ Thus, there is a delay of 6 d between the time of *Fendrr* expression in wild-type embryos and malfunctioning of the tissue derived from *Fendrr*-null

cells in the mutant. How can this delay be explained? The molecular analysis provided some important hints toward answering this question.

Analogous to the human *FENDRR* (previously *FOXFI-ASI*) transcript, mouse *Fendrr* binds to the PRC2 complex.^{22,38} We showed that the occupancy of PRC2 was severely reduced at three putative target promoters in the mutant, which led to decreased trimethylation of lysine 27 on histone 3 (H3K27me3), the footprint of PRC2.²² For two of these targets, *Foxf1* and *Pitx2*, we provided evidence that *Fendrr* interacts directly with the promoter DNA. Using an in silico approach, we identified a 40-nucleotide stretch of the *Fendrr* RNA, which is able to precipitate the *Foxf1* and *Pitx2* promoters in vitro.²² This interaction is not destroyed by RNaseH treatment, excluding heteroduplex formation as a binding mechanism

between *Fendrr* and the promoter DNA. The 40-base binding sequence also closely resembles a purine-pyrimidine motif that has been previously shown to favor triplex formation.^{39,40}

We confirmed by ChIRP (Chromatin isolation by RNA purification) analysis⁴¹ that interaction of *Fendrr* with the *Foxf1* and *Pitx2* promoters exists in a cellular context. For this we differentiated mouse ES cells to mesoderm in vitro for 4 d to reach peak expression of endogenous *Fendrr*. We then precipitated *Fendrr* RNA from cellular extracts with two pools of tiling oligonucleotides complementary to *Fendrr*. We found that *Foxf1* and *Pitx2* promoter DNA co-precipitated with at least one of the pools (Fig. 1).

Taken together, these data suggest that *Fendrr* binds to target dsDNA sequences via formation of a dsDNA:RNA triplex structure. *Fendrr* binds to specific sites

at target promoters as well as to PRC2. This increases PRC2 occupancy at the promoters, allowing for enhanced H3K27 trimethylation. Therefore, at least in the context of PRC2 binding, *Fendrr* acts as an anchor to promote repressive H3K27 trimethylation at target promoters (Fig. 2).

We were able to show that *Fendrr* also interacts with the TrxG/Mll component WDR5. TrxG/Mll sets active marks by methylating lysine 4 on histone 3 (H3K4me3). We found that all genes showing higher mRNA expression in the *Fendrr* mutant in our analysis also displayed increased H3K4me3 levels at their promoters. However, WDR5 occupancy was not enhanced in parallel, suggesting an indirect effect of *Fendrr* loss on H3K4 trimethylation at these promoters. Indeed, the H3K4me3 active mark can be set by numerous methyl-transferase-containing complexes. Besides the MLL family of methyl-transferases from the TrxG/Mll complexes, SETD1A, SETD1B, SETD7, SMYD1/2/3, and ASH1L have also been shown to methylate H3K4.^{42–47} Moreover, WDR5 has also been found as a component of the histone acetylating complexes NSL/MSL1v and ATAC.^{48,49} Hence, at present it cannot be absolutely excluded that *Fendrr* might interact with any of these latter complexes involving WDR5.

The increase in target gene expression observed in *Fendrr* mutants is rather mild—on the order of 30–40% over the wild-type level. Is it conceivable that the altered expression of numerous (presumed) *Fendrr* target genes together causes sufficient cellular imbalance to lead to a phenotype? Some transcriptional regulators exhibit haploinsufficiency phenotypes, demonstrating that expression of a single allele can be insufficient for proper embryonic development, and the *Fendrr* targets *Foxf1* and *Pitx2* both belong to this group of genes.^{2,50} However, can a mild overexpression also cause impaired cellular function, or is the *Fendrr* phenotype due to a different mechanism?

Foxf1 is completely silenced in the cardiac tissue of E12.5 wild-type embryos, but is ectopically expressed (at a low level) in the cardiac tissue of E12.5 *Fendrr* mutants.²² This finding, combined with the delay between *Fendrr* expression in the lateral plate mesoderm and the onset of

heart dysfunction in *Fendrr* mutants, suggests a long-term effect of *Fendrr* action, a characteristic of epigenetic control mechanisms. In the mutants, failure to increase the level of repressive H3K27me3 marks, in combination with an increase in the active H3K4me3 mark at the *Foxf1* promoter in the lateral mesoderm apparently affects the ability of descendants of these cells in the cardiac mesoderm to silence *Foxf1*. It is conceivable that, in wild-type cells, an increase of the H3K27me3/H3K4me3 ratio mediated by *Fendrr* at target promoters in nascent lateral mesoderm at a later stage of cardiac mesoderm differentiation is followed by a reduction of the active or an enhancement of the repressive mark, leading to a further increase of the H3K27me3/H3K4me3 ratio followed by gene silencing (Fig. 3). Since the H3K27me3/H3K4me3 ratio in lateral mesoderm of *Fendrr* mutants is reduced in favor of the active mark, a reduction of the resulting high H3K4me3 level or increase of the repressive marks in cardiac mesoderm might not be sufficient anymore to permit silencing of the *Foxf1* promoter (Fig. 3). In this manner, setting and removal of active and repressive marks mediated by promoter-specific factors at consecutive stages of cell differentiation might control the activities of many genes, including important regulatory proteins, in a differential manner. Consecutive epigenetic modifications at particular promoters might be separated by hours or days and, thus, changes set early in a cell lineage may become effective much later in descendants of that lineage. This mode-of-action is quite different from the way transcription factors work, where a loss usually becomes effective right away in the very cells they are expressed in.

In summary, we propose that *Fendrr* functions in the lateral mesoderm via epigenetic modification of regulatory elements, thereby adjusting the expression level of target genes in this tissue and setting long-term marks, which allow for the proper control of *Fendrr* target genes in the descendants of lateral mesoderm cells.

In addition to its role in embryogenesis, the human *FENDRR* gene has been found to be expressed in the lung, and to be associated with a lethal lung developmental disorder called ACD/MPV (alveolar

capillary dysplasia with misalignment of pulmonary veins).⁵¹ In one ACD/MPV case (#41.1) an 11 kb deletion removed the distal portion of the *FOXF1* promoter and a portion of *FENDRR* (*FOXF1-ASI*).³⁴ Notably, knockdown of *FENDRR* by siRNA in Human Pulmonary Microvascular Endothelial Cells (HPMEC) caused a weak increase of *FOXF1* transcription, supporting the finding that *FENDRR* acts as transcriptional repressor.³⁴

Perspectives

Fendrr is one of the first lncRNAs that has been shown to play an essential role in mammalian embryogenesis. Our preliminary data obtained by deep sequencing of the transcriptomes of six tissues dissected from E8.25 mouse embryos revealed several hundred differentially expressed lncRNAs, and many more are expected to arise from high-resolution RNA-seq analyses of different developmental stages. Current available information suggests that lncRNAs play a pivotal role in epigenetic control mechanisms acting in cells and on cell lineages. However, detailed functional analyses of many such genes are needed in order to derive a clearer picture of the roles of lncRNAs in epigenetic regulatory networks.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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