Developmental Cell, Volume *24*

# Supplemental Information

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

**Phillip Grote, Lars Wittler, David Hendrix, Frederic Koch, Sandra Währisch, Arica Beisaw, Karol Macura, Gaby Bläss, Manolis Kellis, Martin Werber, and Bernhard G. Herrmann**

## Inventory of Supplemental Materials

### Supplemental Figures S1-S3

Figure S1, related to Figure 2. Mutant embryos lacking the *Fendrr* transcript die after E12.5 with heart defects and can be rescued using a BAC transgene expressing *Fendrr*

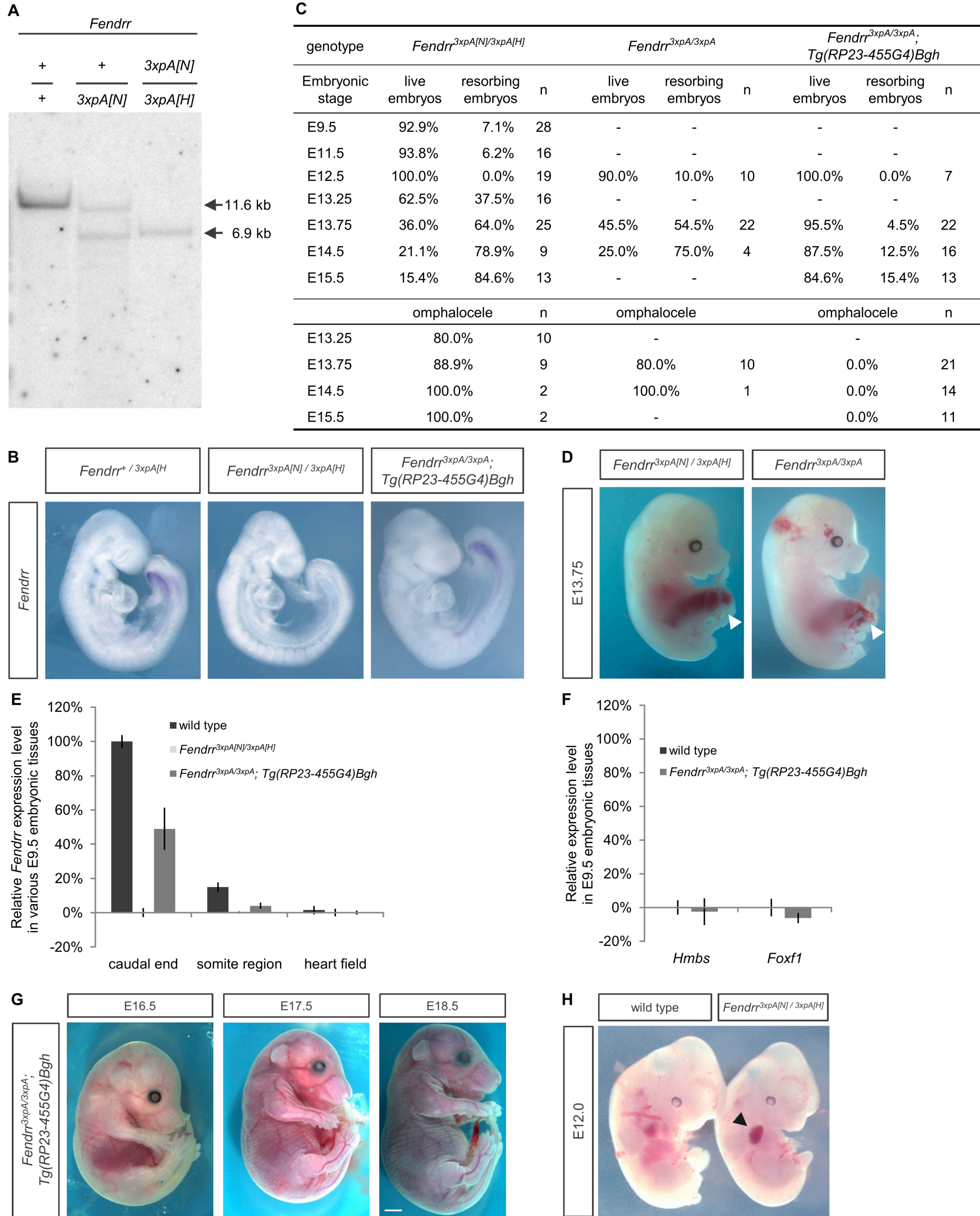
Figure S2, related to Figure 3. Occupancy of PRC2 and TrxG/MLL complex components on promoters in E8.5 embryo tissue

Figure S3, related to Figure 4. RIP from caudal ends of wild type E9.5 embryos and binding probability of *Fendrr* to the promoters of *Dll1* and *Tcf15*.

### Supplemental Experimental Procedures

### Supplemental References

## Figure S1, related to Figure 2



| genotype | *Fendrr*$^{3xpA[N]/3xpA[H]}$ | | | *Fendrr*$^{3xpA/3xpA}$ | | | *Fendrr*$^{3xpA/3xpA}$; *Tg(RP23-455G4)Bgh* | | |
|---|---|---|---|---|---|---|---|---|---|
| Embryonic stage | live embryos | resorbing embryos | n | live embryos | resorbing embryos | n | live embryos | resorbing embryos | n |
| E9.5 | 92.9% | 7.1% | 28 | - | - | | - | - | |
| E11.5 | 93.8% | 6.2% | 16 | - | - | | - | - | |
| E12.5 | 100.0% | 0.0% | 19 | 90.0% | 10.0% | 10 | 100.0% | 0.0% | 7 |
| E13.25 | 62.5% | 37.5% | 16 | - | - | | - | - | |
| E13.75 | 36.0% | 64.0% | 25 | 45.5% | 54.5% | 22 | 95.5% | 4.5% | 22 |
| E14.5 | 21.1% | 78.9% | 9 | 25.0% | 75.0% | 4 | 87.5% | 12.5% | 16 |
| E15.5 | 15.4% | 84.6% | 13 | - | - | | 84.6% | 15.4% | 13 |

| | omphalocele | n | omphalocele | n | omphalocele | n |
|---|---|---|---|---|---|---|
| E13.25 | 80.0% | 10 | - | | - | |
| E13.75 | 88.9% | 9 | 80.0% | 10 | 0.0% | 21 |
| E14.5 | 100.0% | 2 | 100.0% | 1 | 0.0% | 14 |
| E15.5 | 100.0% | 2 | - | | 0.0% | 11 |

**Figure S1, related to Figure 2. Mutant embryos lacking the *Fendrr* transcript die after E12.5 with heart defects and can be rescued using a BAC transgene expressing *Fendrr***

**(A)** Southern blot hybridization of SpeI-digested DNA derived from wild-type (+/+), *Fendrr* heterozygous (*Fendrr*$^{+/3xpA[N]}$) and homozygous (*Fendrr*$^{3xpA[N]/3xpA[H]}$) ES cells. Replacement of the first exon of *Fendrr* with the stop cassette 3xpA shifts the wild-type 11.6 kb fragment to a fragment of 6.9 kb. **(B)** Spatial and temporal expression of *Fendrr* in rescued E9.5 embryos, compared to heterozygous and *Fendrr* null embryos. **(C)** Survival rates and percentages of live embryos displaying an omphalocele phenotype in *Fendrr*$^{3xpA[N]/3xpA[H]}$ or *Fendrr*$^{3xpA/3xpA}$ mutant embryos, as compared to homozygous mutants, rescued by integration of a BAC transgene construct (*Tg(RP23-455G4)Bgh*) expressing *Fendrr* but not *Foxf1*. **(D)** E13.75 embryos derived by tetraploid complementation from wild-type ES cells or homozygous *Fendrr* mutants, either including the neighburing selection cassettes (*Fendrr*$^{3xpA(N)/3xpA(H)}$) or following removal of the selection cassettes by Flp recombinase (*Fendrr*$^{3xpA/3xpA}$). Limbs and tail have been removed. **(E)** *Fendrr* expression levels in different tissues from E9.5 embryos. The expression level is as expected from a single copy integration of the rescuing construct (Mean + s.d, n=3). **(F)** *Foxf1* expression does not change in the rescued embryos compared to wild type (Mean + s.d., n=2). **(G)** Embryos rescued by the BAC transgene. The rescue was monitored until E18.5, just prior to birth; at this stage a fraction of embryos showed omphalocele (not shown). **(H)** In *Fendrr* mutants (E12.0) blood accumulates in the right atrium (arrowhead). Limbs and tail have been removed.

**Figure S2, related to Figure 3**

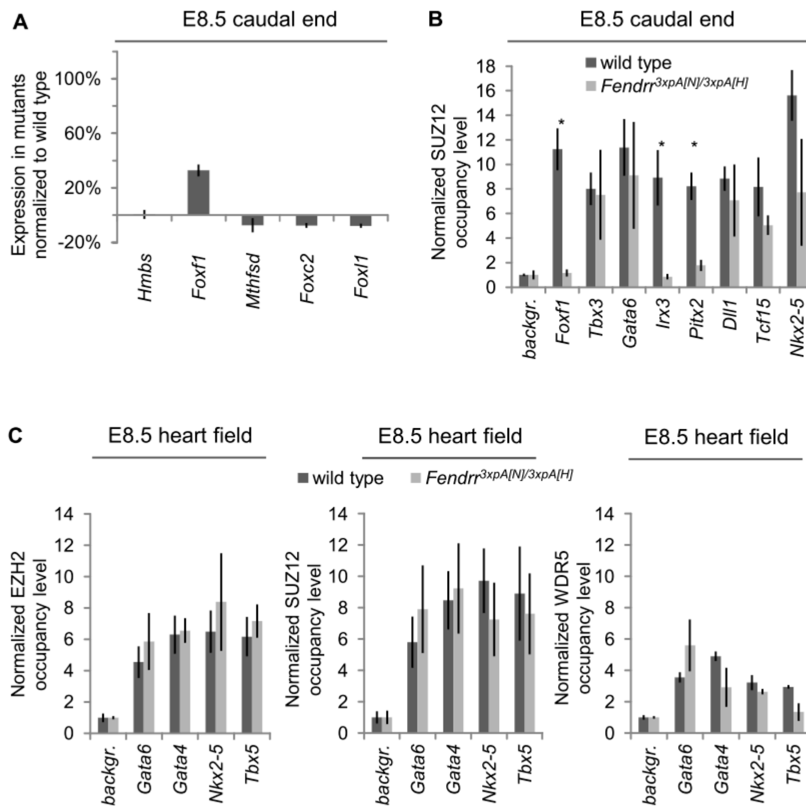



**Figure S2, related to Figure 3. Occupancy of PRC2 and TrxG/MLL complex components on promoters in E8.5 embryo tissues**

**(A)** Expression of *Foxf1* and neighboring genes in E8.5 caudal ends of *Fendrr* mutants normalized to wild type. Only *Foxf1* is up-regulated in the mutant indicating a specific effect of *Fendrr* on *Foxf1* (Mean + s.d, n=3). **(B, C)** Promoter occupancy was determined by ChIP of EZH2, SUZ12 and WDR5 followed by qPCR analysis. **(B)** Occupancy of SUZ12 on the *Foxf1, Irx3* and *Pitx2* promoters is drastically reduced in the caudal end of E8.5 *Fendrr* mutant embryos as compared to wild-type, whereas the other promoters tested were not affected. (Mean + s.d., n=3, * $p \leq 0.05$). **(C)** Occupancy of the PRC2 components EZH2 and SUZ12 or the TrxG/MLL component WDR5 on heart gene promoters is not altered in E8.5 mutant heart field tissue as compared to wild-type (Mean + s.d, n=2).

**Figure S3, related to Figure 4**

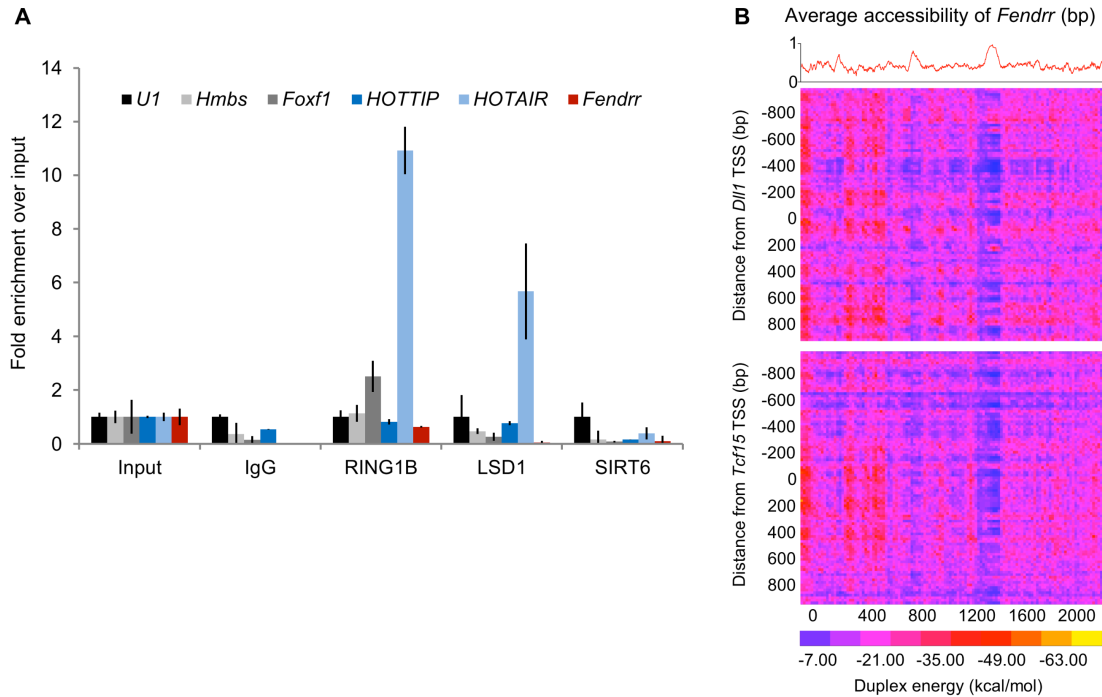



**Figure S3, related to Figure 4. RIP from caudal ends of wild type E9.5 embryos and binding probability of *Fendrr* to the promoters of *Dll1* and *Tcf15*.**

**(A)** *Fendrr* does not bind to RING1B, LSD1 or SIRT6 in RIP from E9.5 wild type embryos (Mean+ s.d., n=2). **(B)** Binding potential between *Fendrr* and genomic control regions. The red curve shows the average probability of single stranded RNA as computed by sfold with a length parameter of 200 and W=1 (Ding et al. 2004). The heat map represents the base-pairing energy for an RNA/RNA duplex model for 40bp regions along the *Fendrr* transcript and 2,000 bp around the TSS of *Dll1* (top) and *Tcf15* (bottom). The duplex energy is computed for each such region, staggered by 20bp.

## Supplemental Experimental Procedures

### Isolation of *Fendrr* and generation of transgene constructs

Full length *Fendrr* was isolated by standard rapid amplification of cDNA-ends with polymerase chain reaction (RACE-PCR) on RNA derived from the caudal ends of E9.5 C57BL/6J embryos. In order to generate a genetic null of *Fendrr*, we replaced the genomic region coding for the first exon of *Fendrr* with a stop cassette containing a strong transcriptional stop signal (3xpA) (Friedrich et al. 1991). For rescue a neomycin resistance cassette was inserted into the coding region of *Foxf1* to generate a *Foxf1* mutant BAC that contains a wild-type *Fendrr* locus. The BAC was linearized with PI-SceI (NEB) and stably integrated into *Fendrr*$^{3xpA/3xpA}$ ES cells.

### Whole mount *in situ* hybridization

Whole-mount *in situ* hybridization was carried out using standard procedures described on the MAMEP website (http://mamep.molgen.mpg.de/index.php). Probes were generated by PCR from E8.5 whole embryo cDNA and subcloned into pCRII-TOPO (Life Technologies) or pBluescript II SK(+) (Agilent Technologies). After verification of the probe templates by sequencing, antisense *in situ* probes were generated as described on the MAMEP website using T7 polymerase (Promega). The *in situ* probe against *Fendrr* covers 698 bases at the 3' end of the *Fendrr* transcript and the probe against *Foxf1* is complementary to nucleotides 80-885 (805 bases) of the *Foxf1* protein coding sequence.

### Genetic manipulation of ES cells and generation of embryos

The genetic background of all ES cells and embryos generated in this work is identical (129S6/C57BL6 (G4)) (George et al. 2007). ES cells were cultured and modified according to standard procedures. Briefly, $10x10^6$ ES cells were electroporated with 25µg of linearized targeting construct and cultivated with selection media containing 250 µg/ml G418 (Life Technologies) or 125 µg/ml Hygromycin B (Life Technologies) for the first and second targeting, respectively. Resistant clones were isolated, and successful gene targeting was confirmed by Southern blot analysis of the *Fendrr*/*Foxf1* locus (Figure S1A). Embryos and live animals were generated by tetraploid complementation (Gertsenstein 2011). Heterozygous *Fendrr*$^{+/3xpA[N]}$ ES cells generated 21 mice from four foster mothers, confirming their integrity and usability in subsequent developmental assays. The selection cassettes consisting of *PGK::Neo-SV40pA*

(abbreviated “N”) or *PGK::Hygro-SV40pA* (abbreviated “H”) were flanked by *FRT* sites. Absence of *Fendrr* expression in embryos was confirmed by *in situ* hybridization (Figure S1B).

Transient transfection of pCAGGS-flpE-puro using Lipofectamine 2000 (Life Technologies) into *Fendrr*$^{3xpA[N]/3xpA[H]}$ ES cells removed the selection cassettes (Beard et al. 2006), resulting in *Fendrr*$^{3xpA/3xpA}$ ES cells. Rescue of the *Fendrr*$^{3xpA/3xpA}$ embryos was achieved by stable random integration of the rescue BAC construct. Briefly, $6x10^6$ ES cells (*Fendrr*$^{3xpA/3xpA}$) were electroporated with 5 µg linearized BAC (RP23-455G4) DNA and cultivated in 250 µg/ml G418 (Life Technologies) selection media. Resistant clones were isolated and integration was confirmed by Southern blot analysis. The expression level of *Fendrr* was analysed by qPCR in embryos derived by tetraploid complementation (Figure S1E).

All animal procedures were conducted as approved by the local authorities (LAGeSo Berlin) under the license number G0368/08.

**Embryo preparation and histology**

Staged embryos were dissected from uteri into PBS and fixed in fresh 4% paraformaldehyde/PBS overnight at 4°C. For histology, embryos were embedded in paraffin. Sections (4-6 µm thickness) were mounted onto Superfrost® Plus microscope slides (Thermo scientific) and Eosin (Sigma) stained according to standard procedures.

Immunohistochemistry on sections were carried out using standard procedures. Antibodies used for the detection of mitotic cells was anti-H3S10P (Millipore) and cleaved caspase 3 (Asp175) (Cell Signaling) and slides were mounted in Vectashield with DAPI (Vector Laboratories). For quantification of mitotic cells, DAPI positive cells were counted on two sections each of three different embryos, and subsequently the number of H3S10P positive cells in the counted area was determined.

All image documentation was carried out on a Zeiss SteREO Discovery.V12 microscope and captured with Zeiss AxioVision 4.8.2 software.

**Isolation of EOMES-positive cells by FACS**

30 wild type embryos of stage E6.5 were isolated, washed once with PBS and trypsinized on ice for 5 minutes to obtain a single cell suspension. The cells were washed three times with 1% BSA/PBS and fixed in 4% paraformaldehyde/PBS for 10 minutes on ice. Cells were washed again three times with 1% BSA/PBS, followed by a 30 minute incubation with 1% BSA/1%

FCS/PBST (PBS+0.1% Tween-20). Cells were split in two equal fractions and incubated 30 minutes with or without the primary (anti-EOMES, Abcam, 1:250) antibody, washed 3 times with PBST and incubated another 30 minutes with anti-rabbit-Cy3 (GE Healthcare, 1:250) antibody. Cells were washed three times with PBST and sorted on a FACS Aria II SORP (BD Bioscience). EOMES positive cell (582) and EOMES negative cells (8,042) were sorted directly into RLT buffer (Qiagen). RNA was isolated (Qiagen), reverse transcribed with random hexamers and subjected to real-time qPCR analysis.

**Real-time quantitative PCR analysis**

RNA was isolated using the Qiagen RNAeasy mini kit according to the manufacturer's protocol. Quantitative PCR (qPCR) analysis was carried out on a StepOnePlus$^{TM}$ Real-Time PCR System (Life Technologies) using Power SYBR® Green PCR Master Mix (Promega). RNA levels were normalized to housekeeping genes and chromatin immunoprecipitation (ChIP) levels were normalized to intergenic genomic control regions. Quantification was calculated using the ΔΔCt method (Muller et al. 2002). *Pmm2* served as housekeeping control gene for qPCR, and an intergenic region between *Msgn1* and *Kcns3*, devoid of any specific histone methylation signatures, served as a reference for ChIP. Error bars indicate the standard error from biological replicates, each consisting of technical triplicates. A list of oligonucleotides can be found in Table S1. Oligonucleotide primers used in Figure 2K were a gift from S. Sperling (Charité, Berlin) and sequences are available upon request.

**Chromatin immunoprecipitation (ChIP)**

E8.5 embryos (8-12 somites) were dissected, and tissue from eight embryos were pooled for ChIP analysis. Samples were treated with 0.75% formaldehyde at room temperature (RT) for 10 minutes, and crosslinking was stopped by adding 1/10 volume of 1.25 M Glycin/PBS solution for five minutes at RT. Tissues were washed twice with ice-cold PBS and 200 µl of ChIP lysis buffer was added (50 mM TrisHCl pH8.1, 10 mM EDTA, 1% SDS, Roche Complete Protease Inhibitor Cocktail). In case of ChIP with antibodies against EZH2 (Active Motif), SUZ12 (Abcam) and WDR5 (Bethyl Laboratories, Inc.) the nuclei were isolated from cells in cell lysis buffer (5 mM PIPES-KOH, pH8.0, 85 mM KCl, 0.5% IGEPAL). Chromatin was fragmented with a Branson Digital Sonifier II W-450 (3 mm tip) using 6x10 second pulses with 50 second breaks, and fragmentation was verified on an agarose gel (mean size ~250-350bp). ChIP lysate was diluted in dilution buffer (16.7 mM TrisHCl pH8.1, 155 mM NaCl, 1.1% Triton X-100, 1.2 mM EDTA, 0.01% SDS) containing 1:1 Dynabeads® Protein A/Dynabeads® Protein G (40 µl each per

ChIP), pre-adsorbed with 1 µg (5 µg in case of anti-EZH2 and anti-WDR5) of antibody. The following antibodies were used: anti-H3K4me3 (Abcam), anti-H3K27me3 (Upstate). ChIP lysates were incubated for 4h at 4°C and washed 8 minutes at 4°C in the following order: 2x Low salt wash buffer (20 mM Tris-HCl, pH 8.1, 140 mM NaCl, 0.1% SDS, 1% Triton X-100, 1 % Na-Deoxycholate, 2 mM EDTA), 1x High salt wash buffer (as Low salt wash buffer, but with 500 mM NaCl) and 1x LiCl wash buffer (20 mM TrisHCl pH8.1, 250 mM LiCl, 0.5% IGEPAL, 0.5% Na-Deoxycholate, 2 mM EDTA) and 1x TE. Bound DNA/histone complexes were eluted by incubating the beads twice for 15 minutes at 37°C with 10 mM TrisHCl pH 8.1, 1 mM EDTA, 140 mM NaCl, 5 mM DTT, 2% SDS. DNA was purified using the Qiagen PCR Cleanup Kit.

**RNA-Seq analysis**

RNA was extracted from 4 pooled ventricles each dissected from E12.5 wild-type or *Fendrr*$^{3xpA[N]/3xpA[H}$ mutant embryos using the RNAeasy Mini Kit (Qiagen). Ribosomal RNA was depleted from 500ng of total RNA using the Ribo-Zero Magnetic Kit (Epicentre) according to manufacturer's instructions with minor modifications for input amounts lower than 1µg. In brief, we used 90µl of beads per reaction, washed them with an equal amount of $H_2O$ and resuspended them in 35µl of resuspension buffer with 0.5µl of RNase inhibitor. We added 2µl of rRNA removal solution and 2µl of reaction buffer to 16µl of the total RNA (corresponding to 500ng). The final eluate was cleaned-up with RNeasy MinElute columns (Qiagen) and used to prepare sequencing libraries utilizing the Script-Seq v2 RNA-Seq Library Preparation Kit (Epicentre) according to manufacturer's instructions. The cDNA was purified with DNA MinElute columns (Qiagen), 15 cycles of PCR were used to amplify the library with index primers before purification with AMPure XP beads (Beckman Coulter). The libraries were independently sequenced on the MiSeq v2 (Illumina) running at 1x100bp cycles. The sequences, approximately $16x10^6$ for wild-type and $18x10^6$ for *Fendrr* mutant RNA, were aligned against the mouse genome (mm9) using Tophat (Trapnell et al. 2009) supplied with the UCSC refseq transcriptome annotation. We used Cufflinks to normalize the samples, calculate transcript coverage and obtain transcript abundances (FPKM) (Trapnell et al. 2010). Different isoforms per gene were combined into one metagene for analysis. The data has been deposited to GEO under the accession number GSE43078.

**Oligos used for various qPCR analysis**

| | target | Forward | Reverse |
|---|---|---|---|
| cDNA | *Pmm2* | AGGGAAAGGCCTCACGTTCT | AATACCGCTTATCCCATCCTTCA |
| | *Hmbs* | CCTGGGCGGAGTCATGTC | ACTCGAATCACCCTCATCTTTGA |
| | *Foxf1* | GGCCTCCTACATCAAGCAAC | CCGTTGTGACTGTTTTGGTG |
| | *Fendrr* | CTGCCCGTGTGGTTATAATG | TGACTCTCAAGTGGGTGCTG |
| | *Xist* | GCCACGGATACCTGTGTGTC | CCGATGGGCTAAGGAGAAGA |
| | *Gata4* | TGCAATGCGGAAGGAGGGGA | AGGCACCACTGGAGGGAGGG |
| | *Gata6* | GCAGTGGCTCTGTCCCTATG | TCTCCCACTGCAGACATCAC |
| | *Tbx3* | CGTCTCAGGCCTAGAATCCA | GAAAGGCGACATAACCAAGC |
| | *Tbx5* | GGCAGTGATGACCTGGAGTT | GCTGTGGTTGGAGGTGACTT |
| | *Nkx2-5* | CAAGTGCTCTCCTGCTTTCC | GTCCAGCTCCACTGCCTTCT |
| | *Irx3* | TGCCGAGCCGGAGAGTGGAA | ACCAGAGCAGCGTCCAGATGGT |
| | *Dll1* | CCTCCCGGAGAAGCCAGCCT | TGGATCTCTGCGGCTCTTCCCC |
| | Pitx2 | CGCGAAGAAATCGCCGTGTGG | TGCTGGTTGCGTTCCCGCTT |
| | *Tcf15* | TCTCAGCAACCAGCGCAAGGG | CCTCGGAGCGGGGCTACACC |
| | *MmU1* | ACGAAGGTGGTTTTCCCAG | GTCCCCCACTACCACAAA |
| | *HOTTIP* | TGGGTCCCTCCGGCTCCAAAAAT | ATCTCTTCCCGATGGCTGGCTCC |
| | *HOTAIR* | AGGACCGACGCCTTCCTTAT | CCTTTTGGTCTACGTTGGCG |
| | *Eomes* | AGAGACACAGTTCATCGCTGT | TTGGCGAAGGGGTTATGGTC |
| | *Myl7* | GGGGTGGTGAACAAGGAAGA | GGTGTCAGCGCAAACAGTTG |
| genomic | *backgr.* | GGAACTTTGCACAGGAGGAG | TGGGTAACGGCTTCCTAATG |
| | *Gata4* | GTCCATGCGCGCGGAACTCT | TGGCAGAGCAGCAAACCGCA |
| | *Gata6* | GCCGGGAGCACCAGTACAGC | TGCTGGCCAGGCAGGAGTCA |
| | *Nkx2-5* | CGCTGCTGCTGCTCCAGGTT | TGCGTCGCCACCATGTTCCC |
| | *Tbx5* | GCCCCCACCTCGGTTCTCCT | CCAGGCGAACGAGCCCCAAA |
| | *Foxf1* | ACCCGGCAAGGGCCACTACT | CAGGCTTGAGCGCCTGGCAT |
| | *Tbx3* | GGAGCGGAGGCACCCAGAGA | TGCCGCTCTGGGCTTGGAGA |
| | *Irx3* | GCGTTGGCGAACCAGGTGGA | CGAGCACCGCAAGAACCCGT |
| | *Dll1* | GCATCTGACCTTGTTTCTTTTCTT | AAACAGTCCCTGTCCTTTGTAAAC |
| | Pitx2 | GCCCGCTAGCTTTAGCAGCCC | CGGGTCTGGGTGGAACTGCG |
| | *Tcf15* | CCGAGCCCGTGGACCGAAAG | AAACACGGCTGCCCGTCGTC |

**Supplemental References**


Beard, C., Hochedlinger, K., Plath, K., Wutz, A., and Jaenisch, R. (2006). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis 44, 23–8.

Ding, Y., Chan, C. Y., and Lawrence, C. E. (2004). Sfold web server for statistical folding and rational design of nucleic acids. Nucleic acids research 32, W135–41.

Friedrich, G., and Soriano, P. (1991). Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes & Development 5, 1513–23.

George, S. H. L., Gertsenstein, M., Vintersten, K., Korets-Smith, E., Murphy, J., Stevens, M. E., Haigh, J. J., and Nagy, A. (2007). Developmental and adult phenotyping directly from mutant embryonic stem cells. Proc Natl Acad Sci USA 104, 4455–60.

Gertsenstein, M. (2011). Tetraploid Complementation Assay 1st Editio. S. Pease and T. L. Saunders, eds. (Berlin, Heidelberg: Springer Berlin Heidelberg).

Muller, P., Janovjak, H., Miserez, A., and Dobbie, Z. (2002). Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32, 1372–1378.

Trapnell, C., Pachter, L., and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics (Oxford, England) 25, 1105–11.

Trapnell, C., Williams, B. a, Pertea, G., Mortazavi, A., Kwan, G., Baren, M. J. van, Salzberg, S. L., Wold, B. J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nature Biotechnology 28, 511–515.