Supp. Materials and Methods

Patient

The infant was a full-term, 3140-gram female born via spontaneous vaginal delivery to a 32-year-old gravida 2, para 1 mother. The couple's previous pregnancy resulted in a healthy full-term female with no current medical issues at 2 years of age. The mother's pregnancy was unremarkable with negative serologies, including group B streptococcus colonization status. She denied the use of medications other than prenatal vitamins, including non-steroidal anti-inflammatory drugs (NSAIDs) or selective serotonin reuptake inhibitors (SSRIs) during the pregnancy. The delivery was unremarkable with Apgar scores of 8 and 9 at 1 and 5 minutes, respectively. The neonate initially went to the newborn nursery but was transported to the neonatal intensive care unit (NICU) at 7 hours postnatal age after she was noted to be cyanotic during breastfeeding. Upon assessment, she had an oxygen saturation of only 60% despite receiving FiO_2 of 1.0. She was intubated and treated with conventional ventilation and inhaled nitric oxide for presumed persistent pulmonary hypertension of the neonate (PPHN). Her chest radiograph did not reveal any apparent parenchymal or interstitial lung disease. She underwent a sepsis evaluation and treatment with ampicillin and gentamicin. She was subsequently transferred to a level III NICU for further evaluation and management. There, additional treatment strategies included high frequency oscillatory ventilation (HFOV), surfactant administration, dopamine infusion, sedation, and neuromuscular blockade. An echocardiogram revealed significant pulmonary hypertension and a partial atrioventricular canal defect. Despite increased support she was unable to be effectively oxygenated and ventilated and was transferred to a level IV NICU for possible extracorporeal membrane oxygenation (ECMO) support. Shortly after arrival, she was placed on venoarterial ECMO for idiopathic pulmonary hypertension and respiratory failure. On bypass, her chest radiograph remained clear and her lung compliance excellent. However, weaning trials at days 7 and 10 of ECMO revealed continued suprasystemic right ventricular pressures and an inability to effectively oxygenate. Without evidence of improvement, the assessment of more severe underlying processes such as ACDMPV was pursued. The family was approached and consented for *FOXF1* gene testing but did not give consent for lung biopsy while on ECMO. After 13 days on ECMO with no evidence of clinical or echocardiographic recovery, the decision was made to decannulate to conventional mechanical ventilation. The infant developed progressive hypoxemia over the next few hours

and care was withdrawn. Her parents consented to an autopsy limited to the examination of the lung. At gross examination, the left lung showed an incomplete lobation. Microscopically the lung parenchyma showed marked underdevelopment with simplification of the lobular architecture. The pulmonary arteries, including smaller peripheral arteries showed increase in medial smooth muscle. The veins were "malpositioned" and located near to the pulmonary arteries both centrally and in the peripheral parenchyma (Fig. 1A). There was a diffuse paucity of normally positioned capillaries with presence of dilated "dysplastic" capillaries in the interstitium (Fig. 1B). The constellation of these pathologic findings was consistent with the diagnosis of ACDMPV.

DNA and RNA isolation

DNA samples were obtained from the proband and her parents after informed consents approved by the Institutional Review Board for Human Subject Research at Baylor College of Medicine. DNA sample and lung tissue specimen were collected both pre- and post-mortem. Peripheral blood DNA was extracted using Gentra Puregene Blood Kit (Qiagen). DNA and RNA from FFPE lung tissue were extracted using MasterPure Complete DNA and RNA Purification Kit (Epicentre). RNA from transfected lymphoblasts, normal fetal lung fibroblasts, IMR-90, and lung tissues was extracted using RNeasy Protect Mini Kit (Invitrogen). All RNA preparations were treated with DNase using DNA-*free* Kit (Ambion).

Screening FOXF1 for mutations

The entire coding region of *FOXF1* (mRNA reference sequence: NM_001451.2) and its splice donor, acceptor and branch-point sites were PCR amplified from genomic DNA of the ACDMPV proband and sequenced. Direct sequencing of PCR products was performed in both the forward and reverse directions using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence data were compared with reference sequence NT_010498.15 using Mutation Surveyor v3.24 (Softgenetics).

Chromosomal microarray analysis

Copy Number Variation (CNV) was studied by array comparative genomic hybridization (array CGH) using custom-designed 16q24.1 region-specific (2 Mb regions flanking *FOXF1*)

720K oligonucleotide microarrays (Roche-NimbleGen). Microarrays were scanned on Roche-NimbleGen MS 200 Microarray Scanner. Scanned images of the arrays were processed using NimbleScan v2.5 (Roche-NimbleGen) and analyzed using SignalMap v1.9 (Roche-NimbleGen).

Amplification and sequencing across the deletion breakpoints

PCR primers flanking the deletion were designed using Primer3 v0.4.0 (http://frodo.wi.mit.edu/primer3). Amplification of a junction fragment was performed using LA Taq polymerase (TaKaRa Bio USA) and primers FOXF1_F1 5'-AAGAGTGACTACCGCTCTTGACCCTAGTTT-3' and FOXF1_R2 5'-AAAGAGAAGACAAACTCCTTTCGGTCACACAT-3'. PCR conditions included 30 cycles of incubation at 94°C for 30 s and 68°C for 1 min. The deletion junction fragment was deposited in the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/) with the accession number AB830301.

Parental origin of the deletion

Parental origin of the *FOXF1* intronic deletion was determined following identification by sequencing of SNPs within the deleted fragment in the proband's and parental DNA samples. Primers used to amplify the SNPs were FOXF1_F1 and FOXF1_R2.

Quantitative RT-PCR analysis of the *FOXF1* transcript

RNA from normal and ACDMPV lung samples was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan primers and probes to quantify *FOXF1* and *GAPDH* (internal control) transcripts were synthesized by Applied Biosystems. Primers for *FOXF1* were: 5'-CGAGCTGCAAGGCATCCCGCGGTAT-3' and 5'-CAAGAGGAAGAGAGAGACCCTCACT-3'. qPCR was repeated four times using TaqMan Universal PCR Master Mix (Applied Biosystems). qPCR conditions included 40 cycles of 95°C for 15 s and 60°C for 1 min. For relative quantification, the $\Delta\Delta C_T$ method was utilized.

Splicing assay

Normal and deleted copies of the *FOXF1* intron, together with flanking exon sequences (chr16:86,545,022-86,546,787), were amplified from DNA prepared from FFPE lung tissue,

using LA Taq Polymerase (TaKaRa) in the presence of 8% DMSO. Primers used were: FOXF1_intr_del_F2 5'-AGAATTCTTATATCAAGCAGCAGCCCCTGTC-3' and FOXF1_intr_del_R2 5'-CCGACGGTTATACCTCGAGAAGAAAGCA-3'. PCR conditions included 30 cycles of incubation at 94°C for 30 s and 68°C for 1 min. PCR products were separated by agarose gel electrophoresis and inserted into *Eco*RI and *Xho*I sites of the multiple cloning site of the pcDNA3 vector, 108 bases downstream of the putative transcription start site, generating constructs pNI (normal intron) and pDI (intron with deletion).

Transient transfection of normal human lung fibroblasts, IMR-90 (ATCC), and lymphoblasts with pNI and pDI vectors was done using Lipofectamine LTX with PLUS Reagent (Invitrogen). Fibroblasts were cultured in EMEM medium supplemented with 10% FBS. Lymphoblasts were cultured in RPMI medium supplemented with 15% FBS. $5x10^5$ cells per well were transfected with 2.5 micrograms of pNI or pDI DNA in 6-well plate setting. Splicing of the cloned *FOXF1* intron was analyzed after 36 hrs by RT-PCR of RNA isolated from the transfected lymphoblasts using pcDNA-*FOXF1* chimeric primers specific around their 5'ends to the pcDNA3 multiple cloning site (FOXF1_intr_del_F3 and FOXF1_intr_del_R3 5'-CCTCTAGATGCATGCTCGAGAAGAAAGCA-3').

Mini-gene transcript levels from pNI and pDI constructs were quantitatively measured by qPCR using primers: qintdelF4 5'-CTACCAAGACATCAAGCCTTG-3' and qintdelR4 5'-CCTCTAGATGCATGCTCGAG-3'. pcDNA3 *AmpR* gene was used as an internal control and amplified with primers: qintdelF2 5'-CCGGGAAGCTAGAGTAAGTAGT-3' and qintdelR2 5'-GGAACCGGAGCTGAATGAA-3'. qPCR conditions were as described in the previous section.

Promoter constructs and reporter assay

The *FOXF1* promoter region, ranging from the first ATG translation initiation codon to 5.5 kb upstream (chr16: 86,538,679–86,544,175), was amplified from the normal human DNA using primers:

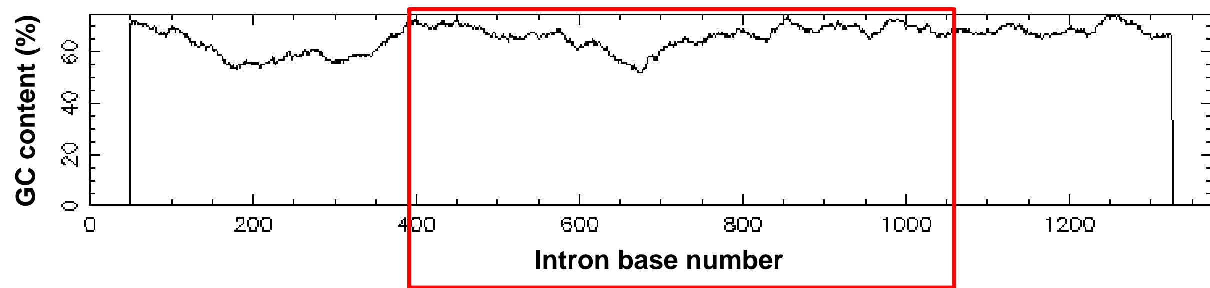
Foxp2F2 5`-CTAGCTAGCACATTTCCTCATATTCTGTGTAGAGAGCACCT-3` and FoxAUG2R 5`-TTGCGCCGATTCGAACGGGTGGCTGCTG-3` that included restriction sites for *Nhe*I and *Bst*BI, respectively. PCR was done using LA TaqDNA polymerase in the presence of 8% DMSO, applying 25 cycles of 30 sec at 94°C and 5 min at 68°C. The amplified *FOXF1* promoter was cut with *Bst*BI, blunt ended with Klenow, cut with *Nhe*I and cloned into *Eco*RV

and *Nhe*I sites of the multiple cloning site of the promoter-less vector pGL4.10 (Promega) to generate pGL4.10FOXF1p. The normal and truncated copies of the *FOXF1* intron were amplified from DNA extracted from the patient's FFPE lung tissue using primers:
intrDelNheF 5`-AGCTAGCTATCTGCACCAGAACAGCCACAAC-3` and intrDelNheR 5`-AGCTAGCGAAGACAAACTCCTTTCGGTCACA-3`, both containing the *Nhe*I sites. The deleted part of the intron was cloned using primers intrDelNheF2
5`-AGCTAGCTTTGTGTCCCTTAAGTCCCCTCAC-3` and intrDelNheR2
5`-AGCTAGCCACCCAAGGAAAAGACTCCAGAAC-3`, also containing the *Nhe*I sites. The amplified fragments were digested with *Nhe*I and cloned into *Nhe*I site of the multiple cloning site of the pGL4.10FOXF1p vector, upstream of the *FOXF1* promoter in the orientation as they appear on chr16 (Fig. 1C).

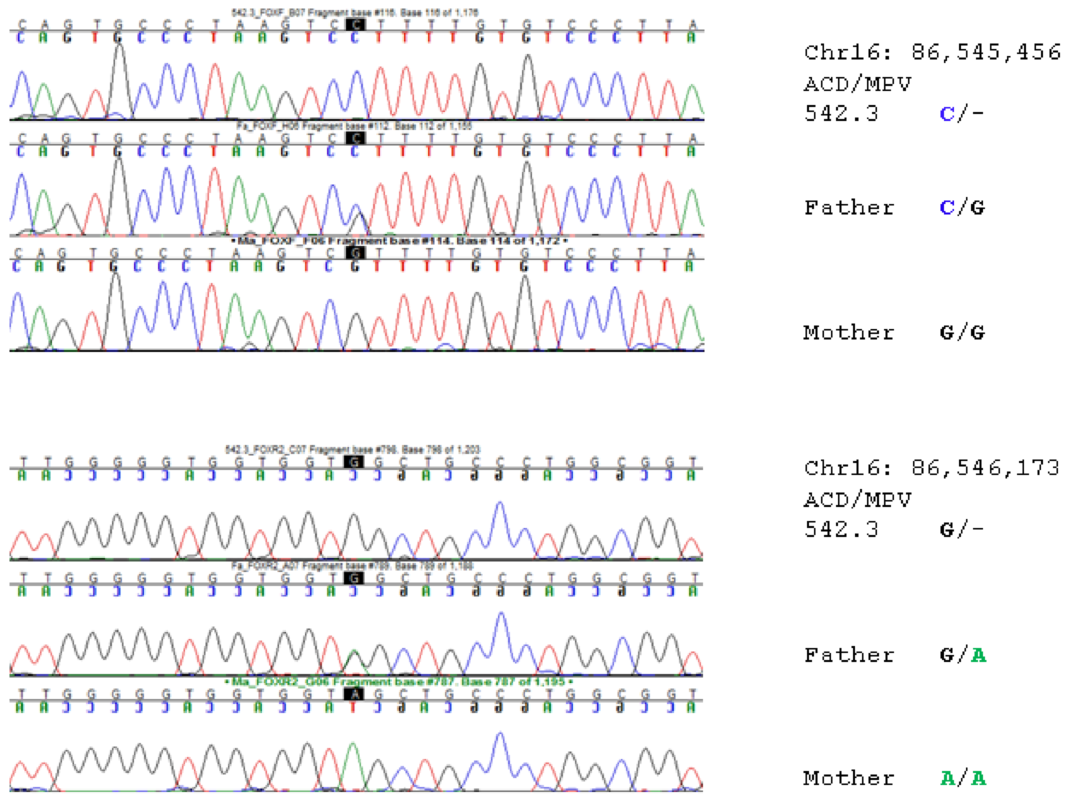
Transfections of normal fetal lung fibroblasts, IMR-90, were performed on sub-confluent cells grown on 12-well plates. The cells were co-transfected with 1 μg of a reporter plasmid and 0.1 μg of pGL4.75, constitutively expressing *Renilla* luciferase, using Lipofectamine LTX with PLUS reagent. The cells were lysed in reporter passive lysis buffer 24 hrs after transfection and assayed for activities of firefly and *Renilla* luciferases using the Dual-Luciferase Reporter Assay System (Promega). The activity of *Renilla* luciferase was used to normalize the transfection efficiency of each sample. The relative luciferase activity represents the ratio of firefly luciferase activity to *Renilla* luciferase activity. Transfections were conducted three times in triplicates. Statistical evaluation of the results was done with nonparametric Mann-Whitney test using SPC software (www.spcforexcel.com).

Bioinformatics analyses

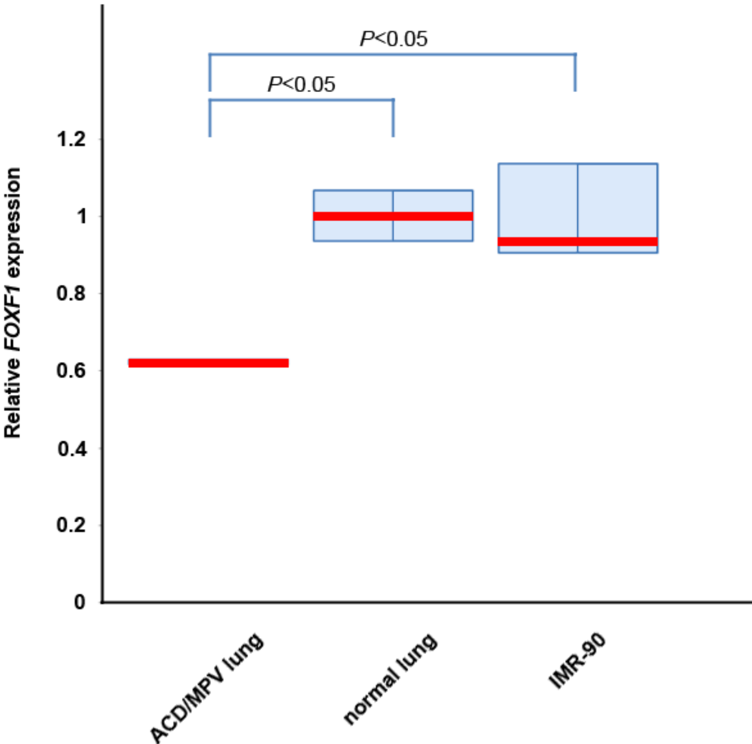
DNA sequences were assembled using Sequencher v4.8 (GeneCodes). Repetitive sequences were identified using RepeatMasker (http://repeatmasker.org). GC content was determined using CpGPlot (http://ebi.ac.uk/Tools/emboss/cpgplot). Splicing signal prediction was performed using Human Splicing Finder www interface accessible at http://umd.be/HSF. Reference sequences were downloaded from the UCSC Genome Browser (NCBI build 37/hg19, http://genome.ucsc.edu). Chromatin modification and ChIP-seq databases for selected transcription regulators were accessed using the UCSC Genome Browser. Expression data were access through a gene annotation portal BioGPS (http://biogps.com).



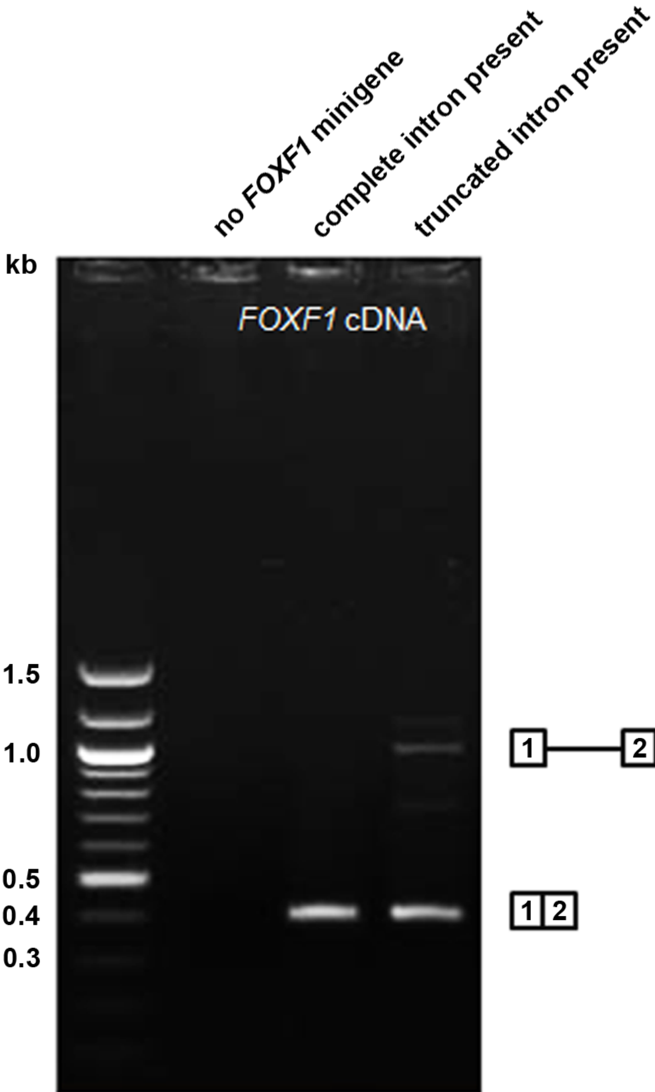
Supp. Figure S1. The deletion breakpoints map to locally GC-rich regions. The GC content of the entire *FOXF1* intron is shown. Deleted region is in red frame.



Supp. Figure S2. Maternal origin of the *FOXF1* intronic deletion. Chromatopherograms across informative SNPs are shown.



Supp. Figure S3. *FOXF1* mRNA levels in ACDMPV and normal fetal lungs, and normal fetal lung fibroblasts, IMR-90. The red bar within each box represents the median. Mann-Whitney *P* values are indicated for relevant comparisons.



Supp. Figure S4. Splicing pattern in periferal blood lymphoblasts of *FOXF1* minigenes bearing normal and truncated copies of the *FOXF1* intron. Boxes 1 and 2 represent intron-flanking regions of the *FOXF1* exon 1 and 2, respectively. The predominant 0.4 kb band represents RT-PCR product of correctly spliced mRNA of minigenes from plasmids transfected into cells.

Supp. Figure S5

A

CTCF binding sites

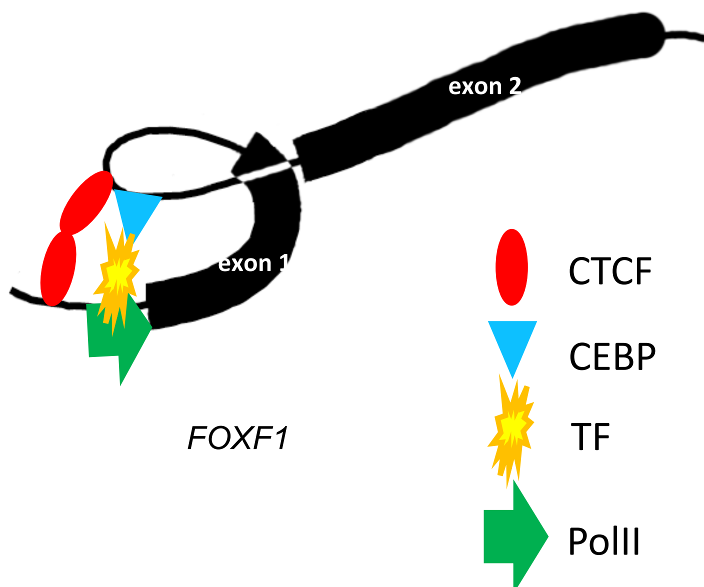
CC*tcttatatcaagcagcagcccctgtc*CCCCTGTAACCCCGCGGCCAACCCCCTGTCCGGCAGCCTCTC
CACGCACTCCCTGGAGCAGCCGTATCTGCACCAGAACAGCCACAACGCCCCAGCCGAGCTGCAAGGTGAG
TGGGGAGGCCGAGGGCGCCCTGGTCCCCGGGAAGTCGAGTCTGAGTGGCAGCGGGACCCAGCTGGGGCGA
GCCCCTCCACTTCTGTGGTCGGAACCCCAAGGCTGAGGGGAGGCACCAGCTCCCCAAGGTGTCTCTTGGC
CCCACCTCTCCCCCTTCGAAGAGTGACTACCGCTCTTGACCCTAGTTTGGGCCAATCTGTTTCTCTTTCT
GTTCGAACTCCAGCTGCCAGCTGCTCCAGGCCTGACCAGGTAGGCTGGTCCTGAGCCAGATGTCCCAGAC
CCCAGTGCCCTAAGTCCTTTTGTGTCCCTTAAGTCCCCTCACAGCTTGGAAAGATCCT**G**GGATGGACCCA
AGGCTCCCAGCGCTGGCCAGATGGCTGTCCCTCCCTGGTGGTGGCCCTCAGGCTGCCTGGAGGGCTGCCT
CTGCCTGGGGCTG**GGCGGAACGGAAGGTGTTAGGCCCAAAGCCGCTGGGCCAGCGCTCAGAGGCTCAG**CC
CCGGCCTTTCAGGGGACCAGACCTGGCAGACCCACAGCTGAGTCCAGGGGCATTTTGTGGTGACTGAGGC
AGGAGGGTGCCGTGGCCAGGCCCCGGAGGGTCAGAGCTGCAGACCGGGCCATGGGGGCTCCTTGTTTTTT
AGGATCCAAGTTTTCAGGTCCCCACACCCCCAACACCCCTGATACCCCAATACCCCGAACATGCAGAAGC
ATCTGCCAAGGGAGATGCAGCAGCCTCGCCTCCTGATTCTGCCCCAGGCCCCCCGCAGCCTGCAGGCCCA
GCTCTGGAAAAAGCTGGAGTGAGCTTGCCAGGGCCAGTCCAGGGTCAGCTGTCAGCAGCCCCAGGAGGCA
GGAGCCAGGCCTGGAGGCCTTAGGCAGCCTCTCTGCGGGGGACTCCCCGGCTCCGGGGCCTGTTCTCTGG
AGAAGCTTCTTCTCACCCCTGCTGCTGGCCCGGCGCTGGCCCTGCACTGGCCTCTCCTTCAACCCCGCCG
GCAGTCGTAGCCTCTGAGGTTGGGGGTGGTGGTGGCTGCCCTGGCGGTGACGGCCATGGGCGTTAGGGGC
CCTCCACTTGGTCTCCCTCACCACTTCCCCTATGGCTCTGGAAGCCCTGGGCCCCTGCACGGTGTCCGAG
GCTGGACAGGCACGCAGCAGGCAGGCAGGCAGGCAGGCAGGCAGGCCGTGGCAAGTGTTCTGGAGTCTTT
TCCTTGGGTGCGCAGCTGGAAGGCCGGGACTCGGGGAGGAGAGCGGGGAGGCGGCCGTGCTCTGGAGCCA
GGCTGACAGGCCCTGTGCGCCCCACGGG**AGCACTGCTCCTCTGCCTGAACTCTGAGCCACCGTGGCTAAC**
TCTTCTGCTCCCCCAACCCCTCCTGTCGCCTCGCCTTGCAGGCATCCCGCGGTATCACTCGCAGTCGCCC
AGCATGTGTGACCGAAAGGAGTTTGTCTTCTCTTTCAACGCCATGGCGTCCTCTTCCATGCACTCGGCCG
GCGGGGGCTCCTAC**TACCACCAGCAGGTCACCTACCAAGACATCAAGCCTTGCGTGATGTGAGGCTGCCG**
CCGCAGGCCCTCCTGGTGCAGGCAGGCGGGTCACAGGGACCCTGGACCGGCACAAGAAAC*tgctttcttc*
***tcgaggtataaccgtcgg*CAGAAGAAAAGGGTTCCACCTCTC**

B

CEBPB binding sites

CC*tcttatatcaagcagcagcccctgtc*CCCCTGTAACCCCGCGGCCAACCCCCTGTCCGGCAGCCTCTC
CACGCACTCCCTGGAGCAGCCGTATCTGCACCAGAACAGCCACAACGCCCCAGCCGAGCTGCAAGGTGAG
TGGGGAGGCCGAGGGCGCCCTGGTCCCCGGGAAGTCGAGTCTGAGTGGCAGCGGGACCCAGCTGGGGCGA
GCCCCTCCACTTCTGTGGTCGGAACCCCAAGGCTGAGGGGAGGCACCAGCTCCCCAAGGTGTCTCTTGGC
CCCACCTCTCCCCCTTCGAAGAGTGACTACCGCTCTTGACCCTAGTTTGGGCCAATCTGTTTCTCTTTCT
GTTCGAACTCCAGCTGCCAGCTGCTCCAGGCCTGACCAGGTAGGCTGGTCCTGAGCCAGATGTCCCAGAC
CCCAGTGCCCTAAGTCCTTTTGTGTCCCTTAAGTCCCCTCACAGCTTGGAAAGATCCTGGGATGGACCCA
AGGCTCCCAGCGCTGGCCAGATGGCTGTCCCTCCCTGGTGGTGGCCCTCAGGCTGCCTGGAGGGCTGCCT
CTGCCTGGGGCTGGGCGGAACGGAAGGTGTTAGGCCCAAAGCCGCTGGGCCAGCGCTCAGAGGCTCAGCC
CCGGCCTTTCAGGGGACCAGACCTGGCAGACCCACAGCTGAGTCCAGGGGCATTTTGTGGTGACTGAGGC
AGGAGGGTGCCGTGGCCAGGCCCCGGAGGGTCAGAGCTGCAGACCGGGCCATGGGGGCTCCTTGTTTTTT
AGGATCCAAGTTTTCAGGTCCCCACACCCCCAACACCCCTGATACCCCAATACCCCGAACATGCAGAAGC
ATCTGCCAAGGGAGATGCAGCAGCCTCGCCTCCTGATTCTGCCCCAGGCCCCCCGCAGCCTGCAGGCCCA
GCTCTGGAAAAAGCTGGAGTGAGCTTGCCAGGGCCAGTCCAGGGTCAGCTGTCAGCAGCCCCAGGAGGCA
GGAGCCAGGCCTGGAGGCCTTAGGCAGCCTCTCTGCGGGGGACTCCCCGGCTCCGGGGCCTGTTCTCTGG
AGAAGCTTCTTCTCACCCCTGCTGCTGGCCCGGCGCTGGCCCTGCACTGGCCTCTCCTTCAACCCCGCCG
GCAGTCGTAGCCTCTGAGGTTGGGGGTGGTGGTGGCTGCCCTGGCGGTGACGGCCATGGGCGTTAGGGGC
CCTCCACTTGGTCTCCCTCACCACTTCCCCTATGGCTCTGGAAGCCCTGGGCCCCTGCACGGTGTCCGAG
GCTGGACAGGCACGCAGCAGGCAGGCAGGCAGGCAGGCAGGCAGGCCGTGGCAAGTGTTCTGGAGTCTTT
TCCTTGGGTGCGCAGCTGGAAGGCCGGGACTCGGGGAGGAGAGCGGGGAGGCGGCCGTGCTCTGGAGCCA
GGCTGACAGGCCCTGTGCGCCCCACGGGAGCACTGCTCCTCTGCCTGAACTCTGAGCCACCGTGGCTAAC
TCTTCTGCTCCCCCAACCCCTCCTGTCGCCTCGCCTTGCAGGCATCCCGCGGTATCACTCGCAGTCGCCC
AGCATGTGTGACCGAAAGGAGTTTGTCTTCTCTTTCAACGCCATGGCGTCCTCTTCCATGCACTCGGCCG
GCGGGGGCTCCTACTACCACCAGCAGGTCACCTACCAAGACATCAAGCCTTGCGTGATGTGAGGCTGCCG
CCGCAGGCCCTCCTGGTGCAGGCAGGCGGGTCACAGGGACCCTGGACCGGCACAAGAAAC*tgctttcttc*
*tcgaggtataaccgtcgg*CAGAAGAAAAGGTTCCACCTCTC

Supp. Figure S5. Location of selected regulatory elements within the deletion region of the *FOXF1* intron. The part of the intron deleted in ACDMPV case (chr16:86,545,491-86,546,265) is highlighted red. The remaining parts of the intron are marked green. Sequences corresponding to primers used to amplify the intron for mini-gene constructs are in lower case letters and in italics. (A) *In silico* predicted CTCF binding sites are highlighted black (CTCFBSDB 2.0, http://insulatordb.uthsc.edu/home_new.php, score > 5). CTCF binding sites identified by ChIP-seq in fetal lung fibroblasts, IMR-90 (UCSC Genome Browser), are highlighted gray. (B) The *in silico* identified CAAT boxes for binding of CEBPB are highlighted yellow. CEBPB binding sites identified by ChIP-seq in IMR-90 cells (UCSC Genome Browser) are highlighted gray.



Supp. Figure S6. A model of interactions between the *FOXF1* promoter and its intronic enhancer.