Supporting Information

Coles and Ackerman 10.1073/pnas.1222797110

SI Materials and Methods

Kinesin family member 7 (*Kif7*) is required for the patterning and differentiation of the diaphragm in a model of syndromic congenital diaphragmatic hernia.

In Situ Hybridization. The *Scleraxis* plasmid was purchased from Addgene, and the *Kif7* plasmid was generated by cloning a 1.3-kb section of the 3′ UTR of *Kif7* cDNA into pGEM-T Easy vector using the following primer sequences: forward-AACCAGCAGCAGATCAGAG and reverse-AAGGAGCAGTCAAGTATTATAGC.

Primers used for generating a *Collagen type 1, α-1* probe were forward: 5′-TGG TGT GGT CGG TCT TCC-3′ and reverse: 5′-CTG TTG CCT TCG CCT CTG-3′.

Antibodies Used in This Report. The following primary antibodies were used: chicken ant-GFP (1:1,000; Invitrogen), rabbit anti-*Wilms tumor-1* (1:500; Abcam), rat anti-tenascin C (1:250; Sigma), goat anti-Tenomodulin (1:50; Santa Cruz), mouse anti-BrdU (1:250; BD), mouse anti-skeletal myosin heavy chain (1:1,000; Sigma), rabbit anti-Cyclin D1 (1:50; Thermofisher), rabbit anti-*retinaldehyde dehydrogenase* (*Aldh1a2*; 1:400; Sigma), rabbit anti-Actin (1:1,000; Santa Cruz), mouse anti-*Collagen type 1, α-1* (1:200; Sigma). Fluorescent secondary antibodies were obtained from Invitrogen. A goat anti-rat and goat anti-mouse IgG-HRP was used for whole-mount immunohistochemistry. Hoechst (AnaSpec) was used for immunofluorescent staining of nuclei.

Genotyping of Kif7 *disorganized diaphragm* Allele. Genotyping of Kif7 *disorganized diaphragm* (*dd*) allele is performed using the following primers: 5′-AGT CTC CTG GTC ATC CTC TC-3′, reverse: 5′-CGC TAC CGA CTG CTA CAA-3′.

PCR amplification of genomic DNA from WT and mutant mice generated a 582-bp product containing a unique restriction fragment-length polymorphism that was present in the mutant background but not the WT background. Digestion with restriction enzyme from Nocardia corallina (Nco1) cleaves the mutant sequence but not the WT sequence to give ∼100-, 180-, and 380-bp fragments.

RNA Isolation and Quantitative RT-PCR. RNA was isolated from either whole embryonic day (E) 13.5 diaphragms or primary cell diaphragmatic cultures using the RNeasy Micro Kit (Qiagen) as per the manufacturer's instructions. E13.5 $Kif7^{dd/dd}$ diaphragms possessed left-side posterior diaphragmatic hernias, and therefore, mutant diaphragms were normalized to control diaphragms by removing the posterior diaphragms from both WT and mutant embryos before the RNA isolation procedure. Diaphragms from three to five *dd*/*dd* mutant and WT littermate control embryos from three independent litters were used for quantitative RT-PCR analysis. Total RNA (1 μg) was reverse transcribed into cDNA using SuperScript III and primed with oligo(dt) (Invitrogen). Primer sequences were obtained from the Massachusetts General Hospital PrimerBank or designed using Beacon software (Biorad) and optimized to perform at 95–100% efficiency. Primer sequences are available on request. Quantitative real-time PCR was performed using Bio-Rad IQ Sybr Green supermix and analyzed on the Bio-Rad IQ5 icycler. Quantification of relative gene expression was performed using the Livak method. Gene expression was normalized to Ribosomal protein S13 (Rps13) expression levels and then normalized to control levels. *Rps13* was used as a reference gene in all experiments because of its limited variation in expression across all diaphragmatic samples.

Transfection and siRNA Treatment. siRNA oligonucleotides were purchased from Dharmacon. The siGenome system was used to target Retinoic acid receptor gamma (Rarg), and the On-target plus SMART pool system was used to target *Kif7*, Retinoic acid receptor alpha (Rara), and Retinoic acid receptor beta (Rarb). The sequences are as follows: *Rarg*-targeting sequence: GCGGAUCUGUACAAGGUAU; *Kif7*-targeting sequences: CCAAGGAGCUGCUGCAUGG, GAACUGCGGCUACGCCUAG, GGAACUAGGUCGACACAUG, and GAGAUUUCCUGGCGGCUUU; *Rara*-targeting sequences: GCAAGUACACUACGAACAA, AAGACAAAUCAUCCGGCUA, CGGUGCGAAACGAUCGAAA, and CGAAUCUGCACGCGGUACA; *Rarb*-targeting sequences: GAUAAGAACUGCGUCAUUA, GAAAGGGUGCCGAACGUGUA, GAUCUACACUUGCCAUCGA, and AAGAGUCUGUUAGGAAUGA. On-target plus nontargeting siRNA #2 was used as the control. Cells were cultured in 10% serum until confluent and then transfected with 100 nM control siRNA or 100 nm targeting siRNAs (listed above) using Dharmafect reagent 1. For the *Rara Rarb* double knockdown experiments, 75 nm *Rara* and 75 nm *Rarb* siRNA were cotransfected together, and 150 nm nontargeting siRNA was used as control. On the following day, media were replaced, and cells were treated with all transretinoic acid or left untreated for 24–48 h. Cells were then washed in PBS, and RNA was isolated as described above.

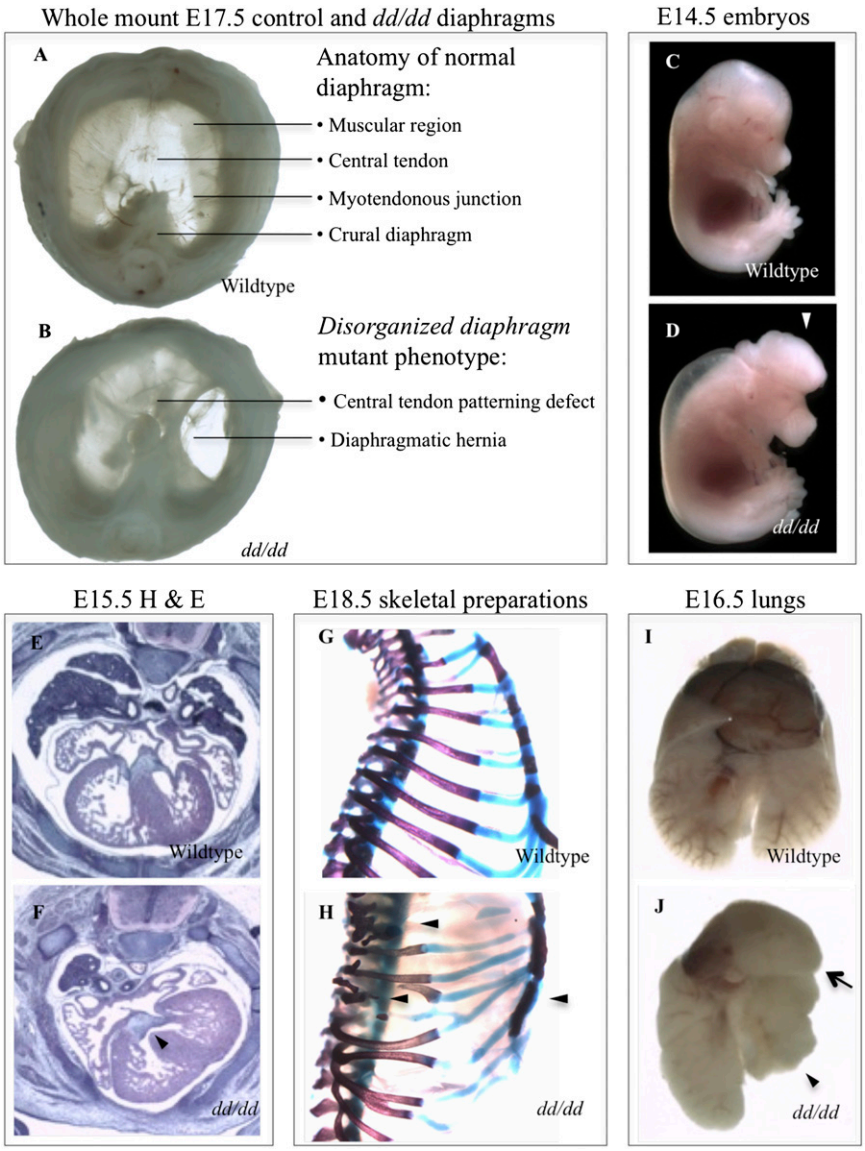

Fig. S1. The *dd* mutant embryos have multiple developmental defects associated with syndromic congenital diaphragmatic hernia. (*A* and *B*) Images of whole-mount E17.5 control and *dd/dd* mutant diaphragms with description of diaphragm anatomy. (*C* and *D*) Images of E14.5 embryos show that *dd/dd* embryos have exencephaly (arrowhead). (*E* and *F*) Transverse sections stained with H&E shows that E15.5 *dd/dd* embryos have ventricular septal defects (arrowhead). (*G* and *H*) Skeletal preparations stained with Alcian Blue (cartilage) and Alizarin red (bone) of E18.5 embryos reveal that *dd/dd* embryos have defects in rib and sternum development (arrowheads). (*I* and *J*) Images of lungs and heart indicate that *dd/dd* mutant embryos possess pulmonary hypoplasia (arrowheads) with abnormal cardiac position (arrow).

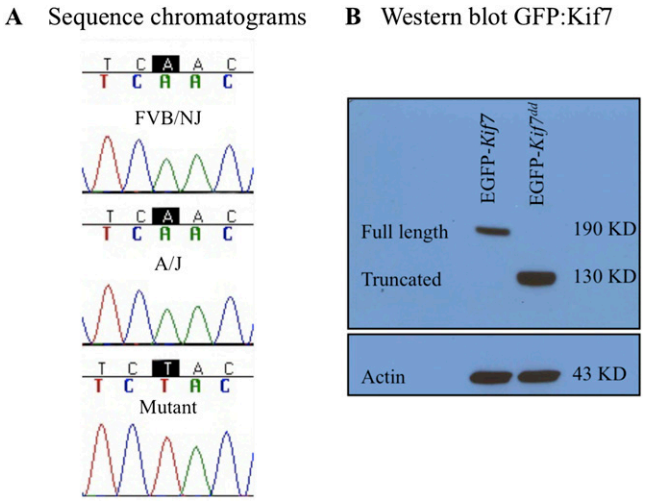

Fig. S2. Analysis of the Kif7^{dd} nonsense mutation. (*A*) Chromatograms showing sequence of PCR-amplified genomic DNA from exon 8 of mouse *Kif7* from the FVB/NJ strain, A/J mutagenized strain, and *Kif7*$^{dd/dd}$ mutant. *N*-ethyl-*N*-nitrosourea induced an A to T transversion. (*B*) Western blot of transfected WT *GFP:Kif7* and *GFP:Kif7*^{dd} HEK293T cell extracts shows that the *Kif7*^{dd} mutation generates a truncated protein of the predicted size.

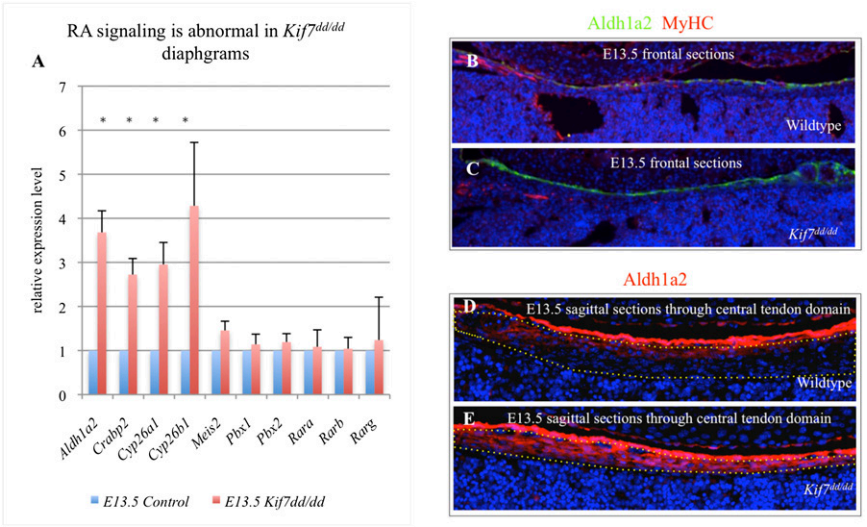

Fig. S3. Retinoic acid (RA) signaling is abnormal in *Kif7*$^{dd/dd}$ mutant diaphragms. (*A*) Quantitative RT-PCR analysis of RA-signaling molecules shows that RA signaling is abnormal in E13.5 *Kif7*$^{dd/dd}$ diaphragms. Data are means, with error bars representing SE; $n = 3$ independent experiments, each performed in triplicate (*$P < 0.05$, *t* test). (*B* and *C*) Aldh1a2 (green) and myosin heavy chain (MyHC; red) coimmunofluorescence staining of frontal sections through the diaphragms of control and E13.5 *Kif7*$^{dd/dd}$ diaphragms. Note that Aldh1a2 is expressed strongly throughout the mesothelium of the diaphragm. (*D* and *E*) Aldh1a2 (red) immunofluorescent staining of sagittal sections through the central tendon domain of control and E13.5 *Kif7*$^{dd/dd}$ diaphragms. Note that Aldh1a2 expression is elevated in the nonmesothelial mesenchymal central tendon domain of E13.5 *Kif7*$^{dd/dd}$ diaphragms (region delineated by dotted yellow lines).