Supplementary Material

Targeted inactivation of murine *Ddx3x*: Essential roles of *Ddx3x* in placentation and embryogenesis

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Generation of Ddx3x targeting vector and conditional knockout mice

To generate conditional knockout allele, recombineering-based method (1) was used. A 20.8 kb genomic DNA fragments harboring *Ddx3x* gene, from RP23-459P19 (BAC clone, Invitrogen), was retrieved into plasmid PL253. The retrieved *Ddx3x* genomic DNA was placed at the 5' end of *MC1* herpes simplex virus thymidine kinase cassette (MC1-TK) which will be used for negative selection during embryonic stem (ES) cells screening. The first *lox*P site was inserted into intron 3 of *Ddx3x* and a new *Not* I site was introduced for later diagnosis. The *frt-PGK-neo-frt-loxP* cassette from plasmid PL451 was placed into exon 17 of *Ddx3x* genomic DNA. The result targeting construct consists of a 9.0 kb 5′-arm, floxed *Ddx3x* with a *frt-PGK-neo-frt* cassette, a 4.2 kb 3' arm and *MC1-TK*, the total size of ~28.1 kb. The targeting construct was linearized with *Sal* I at the 3' end and electroporated into R1 ES cells. Targeted clones were identified by Southern blot analysis using both 5' external and internal genomic probes. *Eco* RV-*Not* I-digested ES genomic DNA samples were analyzed by 5' external probe, the correct band size of the targeted allele is 16.9 kb whereas wild-type allele is 12.7 kb. *Bam* HI-*Stu* I digested ES genomic DNA samples were hybridized with internal probe, the correct band size of the targeted allele is 1.4 kb whereas wild-type allele is 3.0 kb. The positive ES clones were scheduled for C57BL/6 blastocysts injection to generate chimeric mice. Germline transmission of the chimeras was achieved by crossing with C57BL/6 wild-type females. Thereafter, $Ddx3x^{flox\text{-}neo/+}$ mutant mice were in 129/C57BL/6 mixed genetic background. To generate floxed *Ddx3x* allele, $Ddx3x^{flox\text{-}neo/+}$ mice were bred with the CAG-FLPe [Tg(*CAG-FLPe)36*, RIKEN Bioresources, BRC No. 01834] transgenic mouse line (2). The *frt-PGKneo-frt* cassette was removed to create $Ddx3x^{flox/+}$ mice upon FLPe-mediated recombination in the compound heterozygous (*CAG-FLPe/+;*$Ddx3x^{flox\text{-}neo}$*/Y* and *CAG-FLPe/+;*$Ddx3x^{flox\text{-}neo/+}$) mice. The resulted compound heterozygous mice were then bred with wild-type mice to obtain floxed *Ddx3x*

heterozygous (*Ddx3x*^{flox}*/Y* and *Ddx3x*$^{flox/+}$) mice. Genotypes of the mice were verified by polymerase chain reaction (PCR) analysis. The sizes of the PCR products are 361 bps for wild-type, 473 bps for floxed allele, and 254 bps null allele by using primer set 9431-F (5'- AGG CAA AAG GAA AGG AGC TT -3'), 9791-R (5'- ATA GCT GCG TTC TGC CAT TC -3') and 16888-R (5'- GGA AAT TTT TGC CAT CTT CC -3'). The primers for the PCR genotyping of Cre gene are Cre-F (5'-CGG TCG ATG CAA CGA GTG AT-3') and Cre-R (5'-CCA CCG TCA GTA CGT GAG AT-3'). Males were further confirmed by the specific primer set for the *Sry* gene located on the Y chromosome. The sizes of the PCR products are 271 bps by using primer set Sry-1 (5'-GAG AGC ATG GAG GGC CAT GTC AA-3') and Sry-2 (5'-AAA TGC CAC TCC TCT GTG ACA CT-3').

The antibodies used in immunohistochemistry

Antibodies against DDX3X (3), Cyclin D1 (Cell signaling), cleaved Caspase-3 (Asp175) (Cell signaling), GATA4 (Santa Cruz Biotechnology), γH2AX (Millipore), γH2AX (Abcam), Ki-67 (Cell signaling), Oct3/4 (Cell signaling), phospho-Histone H3 (Millipore), Placental lactogen I (PL1) (Santa Cruz Biotechnology), Proliferin (Santa Cruz Biotechnology), p21 (Santa Cruz Biotechnology), p-p53^{ser15} (Cell signaling), were used in this study. For immunostaining, Alexa Fluor-488 conjugated secondary antibodies (Molecular Probes) were used. To amplify the signal in immunohistochemistry, biotinylated secondary antibodies (Vector Laboratories) were used and Tyramide signal amplification kit (Molecular Probes) were subsequently applied according to the manufacturer's instructions. Nuclei were counterstained with DAPI (4',6-Diamidine-2'-phenylindole Dihydrochloride, Roche).

The primer sequences used for quantitative RT-PCR

Ccnd1-F (5'-CTG GCC ATG AAC TAC CTG GA-3') and Ccnd1-R (5'- GTC ACA CTT GAT CAC TCT GG-3'); Cdc25b-F (5'-CAG CCC CTG ACT TGA TGT G-3') and Cdc25b-R (5'-TCG ACA GGA GTC AAG GAA GAA-3'); Cdx2-F (5'-CAT CAC CAT CAG GAG GAA AAG-3') and Cdx2-R (5'-CTG CGG TTC TGA AAC CAA AT-3'); Ddx3x-F (5'-ATA TAA GGA AAA ACT CAA GTG GTA GCC-3') and Ddx3x-R (5'-ATC CAG AGC CCT AGG TGA GG-3'); Dlx3-F (5'-GGG GAT CCT ATA GGC AGT ACG-3') and Dlx3-R (5'-CCT CTT TCA CCG ACA CTG G-3'); Esrrb-F (5'- CGC TCC CAT ACG ATG ACA AGC-3') and Esrrb-R (5'-CGT GGC GCT GAC TCA GCT CAT-3'); Esx1-F (5'-AAC AAC TTT CTA CTC CGA CTT TGG-3') and Esx1-R (5'-TTG CTT CTT CCA GAA ACA TGG-3'); Gcm1-F (5'-TAC GGA TGA AGG GGA GAC C-3') and Gcm1-R (5'-CTT CCC TGA CTC GGG ATT T-3'); Hand1-F (5'-CAA GCG GAA AAG GGA GTT G-3') and Hand1-R (5'-GTG CGC CCT TTA ATC CTC TT-3'); Mash2-F (5'-GAG AGC TAA GCC CGA TGG AGC-3') and Mash2-R (5'-TAG GTC CAC CAG GAG TCA CC-3'); Mmp9-F (5'-CAG AGG TAA CCC ACG TCA GC-3') and Mmp9-R (5'-GGG ATC CAC CTT CTG AGA CTT-3'); Pcna-F (5'-CTA GCC ATG GGC GTG AAC-3') and Pcna-R (5'-GAA TAC TAG TGC TAA GGT GTC TGC AT-3'); Plk1-F (5'-TTG TAG TTT TGG AGC

TCT GTC G-3') and Plk1-R (5'-CAG TGC CTT CCT CCT CTT GT-3'); Pgf-F (5'-CTG GGT TGG CTG TGC ATT-3') and Pgf-R (5'-GGC ACC ACT TCC ACT TCT GT-3'); Pparg-F (5'-GAA AGA CAA CGG ACA AAT CAC C-3') and Pparg-R (5'-GGG GGT GAT ATG TTT GAA CTT G-3'); Sp1-F (5'-GCT ATA GCA AAC ACC CCA GGT-3') and Sp1-R (5'-TCC ACC TGC TGT CTC ATC AT-3'); p15-F (5'- AAT AAC TTC CTA CGC ATT TTC TGC-3') and p15-R (5'- CCC TTG GCT TCA AGG TGA G-3'); p21-F (5'-AAC ATC TCA GGG CCG AAA-3') and p21-R (5'-TGC GCT TGG AGT GAT AGA AA -3'); p53-F (5'-GCG TTG CTC TGA TGG TGA-3') and p53-R (5'-CAG CGT GAT GAT GGT AAG GA-3'); Tfeb-F (5'-CCA CCC CAG CCA TCA ACA C-3') and Tfeb-R (5'-CAG ACA GAT ACT CCC GAA CCT T-3'); Tpbpa-F (5'-TTC CTA GTC ATC CTA TGC CTG G-3') and Tpbpa-R (5'-GGT CAT TTT CGC TAC TGT GAA GT-3'); Hprt-F (5'-TCC TCC TCA GAC CGC TTT T-3') and Hprt-R (5'-CCT GGT TCA TCA TCG CTA ATC-3').

Periodic acid-Schiff (PAS) staining

PAS staining was performed according to the manufacture's instruction (Muto Pure Chemicals Co., Ltd.). Paraffin sections from E12.5, E14.5 and E16.5 wild-type and *Ddx3x*$^{-/+}$ mutant placentas were deparaffinized and rehydrated. Sections were then treated with 1% periodic acid for 30 minutes, washed, and incubated in Schiff's reagent for 1.5 hours. After reaction, sections were rinsed in running tap water, counterstained with hematoxylin, dehydrated, and mounted by Permount.

Cell culture, transfection, immunofluorescence staining, cell synchronization, and flow cytometry

C3H10T1/2 mouse mesenchymal cells were cultured at 37℃ under 5% CO_2 and maintained in high-glucose (4.5 g/L) DMEM containing 10% FBS, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM non-essential amino acids, 100 U/ml penicillin-100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (Invitrogen). Cells were transfected with siRNAs (Non-TARGET siRNA pool and On-TARGETplus mouse *Ddx3x* siRNA pool; Thermo Scientific) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's instructions. For immunofluorescence staining, cells were harvested 96 hours after siRNA transfection. Antibodies against α-tubulin (Santa Cruz Biotechnology), γ-Tubulin (Abcam), and γH2AX (Abcam) were used. To monitor cell cycle progression, cells were synchronized at the G_1/S transition by a double thymidine block 48 hours after siRNA transfection. The *siCtrl* and *siDdx3x* cells were incubated in culture media containing 2 mM thymidine (Sigma) for 16 hours, and released in thymidine-free media for 8 hours. These cells were then reseeded in 6 cm dishes (3.5×10^5 cells/dish), and 2 mM thymidine was added for an additional 16 hours. The cells were harvested at 0, 1.5, 3, 6, 12, and 24 hours after release from the second thymidine block for flow cytometry and Western blotting analysis. For flow cytometry analysis, cells were fixed with chilled 70% ethanol at –30°C overnight. Cells were then rehydrated in 1×PBS and stained with propidium iodide (PI, 20 μg/ml, Sigma) containing 2% Triton X-100 and RNase A (400 μg/ml, Sigma) at room temperature for 30 minutes. Cells were sorted using a BD FACSCalibur flow cytometer (BD

Biosciences, Franklin Lakes, NJ, USA). The percentages of cells in G_1, S, and G_2/M phases were analyzed using ModFit software. Student's *t*-test was used to determine the *P* values.

MTS assay for cell proliferation

CellTiter 96 AQ_{ueous} One solution cell proliferation assay (MTS) was used to detect viable cells according to the manufacturer's instructions (Promega). The siRNA-transfected C3H10T1/2 cells were seeded in a 96-well plate at a density of 0.625×10^4 cells/well, and cell proliferation was analyzed at indicated time points. Measurements were performed in triplicate. Data are presented as mean±SEM from three independent experiments. Student's *t*-test was used to determine the *P* values.

Western blotting

Preparation of tissues, cell extracts, and Western blotting were performed as described previously (4). Antibodies against DDX3X, p21 (Santa Cruz Biotechnology), Cyclin D1 (Cell Signaling), p53 (Cell Signaling), phospho-Histone H3 (Millipore), Plk1 (Abcam), and GAPDH (Santa Cruz Biotechnology) were used.

References

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2 Kanki, H., Suzuki, H. and Itohara, S. (2006) High-efficiency CAG-FLPe deleter mice in C57BL/6J background. *Exp Anim*, **55**, 137-141.

3 Chao, C.H., Chen, C.M., Cheng, P.L., Shih, J.W., Tsou, A.P. and Lee, Y.H. (2006) DDX3, a DEAD box RNA helicase with tumor growth-suppressive property and transcriptional regulation activity of the p21waf1/cip1 promoter, is a candidate tumor suppressor. *Cancer Res*, **66**, 6579-6588.

4 Wei, T.C., Lin, H.Y., Lu, C.C., Chen, C.M. and You, L.R. (2011) Expression of Crip2, a LIM-domain-only protein, in the mouse cardiovascular system under physiological and pathological conditions. *Gene Expr Patterns*, **11**, 384-394.

- **Supplementary Tables**

Table S1 Genotypes of the offspring from *Ddx3x*$^{+/-}$ females and *Ddx3x*$^{+}$*/Y* males crosses.

Age	Number of animals/litters	*Ddx3x*$^{+/+}$		*Ddx3x*$^{+}$*/Y*		*Ddx3x*$^{-/+}$		*Ddx3x*$^{-}$*/Y*		Absorbed	
E6.5	99+6$^{\#}$/14	24	(22.8%)	17	(16.2%)	19	(18.1%)	0	(0%)	39	(37.1%)
E7.5	12/2	5	(41.7%)	2	(16.7%)	3	(25%)	0	(0%)	2	(16.7%)
E9.5	80/10	27	(33.8%)	20	(25%)	16+2*	(22.5%)	1*	(1.3%)	14	(17.5%)
E10.5-E12.5	38/5	12	(31.6%)	13	(34.2%)	5	(13.2%)	0	(0%)	8	(21.1%)
E13.5	28/4	6	(21.4%)	8	(28.6%)	3+2*	(17.9%)	0	(0%)	9	(32.1%)
E14.5	62/8	10	(16.1%)	12	(19.4%)	11	(17.7%)	0	(0%)	29	(46.8%)
E16.5	35/5	9	(25.7%)	14	(40%)	2	(5.7%)	0	(0%)	10	(28.6%)
E17.5-E18.5	29/4	8	(27.6%)	8	(27.6%)	3	(10.3%)	0	(0%)	10	(34.5%)
Weaning	78/20	36	(46%)	42	(54%)	0	(0%)	0	(0%)	-	
Expected percentage			25%		25%		25%		25%		

Numbers represent mice, with percentages in brackets.

Embryos were lost during the immunostaining process.

* Embryos not viable.

Absorbed: embryos were absorbed completely and could not be genotyped.

Table S2 Genotypes of the offspring from *Prm-cre/+;Ddx3x*$^{+/-}$ females and *Ddx3x*$^{+}$*/Y* males crosses.

Age	Number of animals/litters	*Prm-cre/+; Ddx3x*$^{+/+}$	*Prm-cre/+; Ddx3x*$^{+}$*/Y*	*Prm-cre/+; Ddx3x*$^{-/+}$	*Prm-cre/+; Ddx3x*$^{-}$*/Y*	*+/+; Ddx3x*$^{+/+}$	*+/+; Ddx3x*$^{+}$*/Y*	*+/+; Ddx3x*$^{-/+}$	*+/+; Ddx3x*$^{-}$*/Y*	Absorbed
E6.5	121+8$^{\#}$/15	6 (4.7%)	4 (3.1%)	4 (3.1%)	1 (0.1%)	24 (18.6%)	20 (15.5%)	12 (9.3%)	1 (0.1%)	49 (38.0%)
Expected percentage		12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	

Numbers represent embryos, with percentages in brackets.

Embryos were lost during the immunostaining process.

* Embryos not viable.

Absorbed: embryos were absorbed completely and could not be genotyped.

Table S3 Genotypes of the offspring from *Ddx3x*$^{flox/flox}$ females and *Sox2-cre*/+ males crosses.

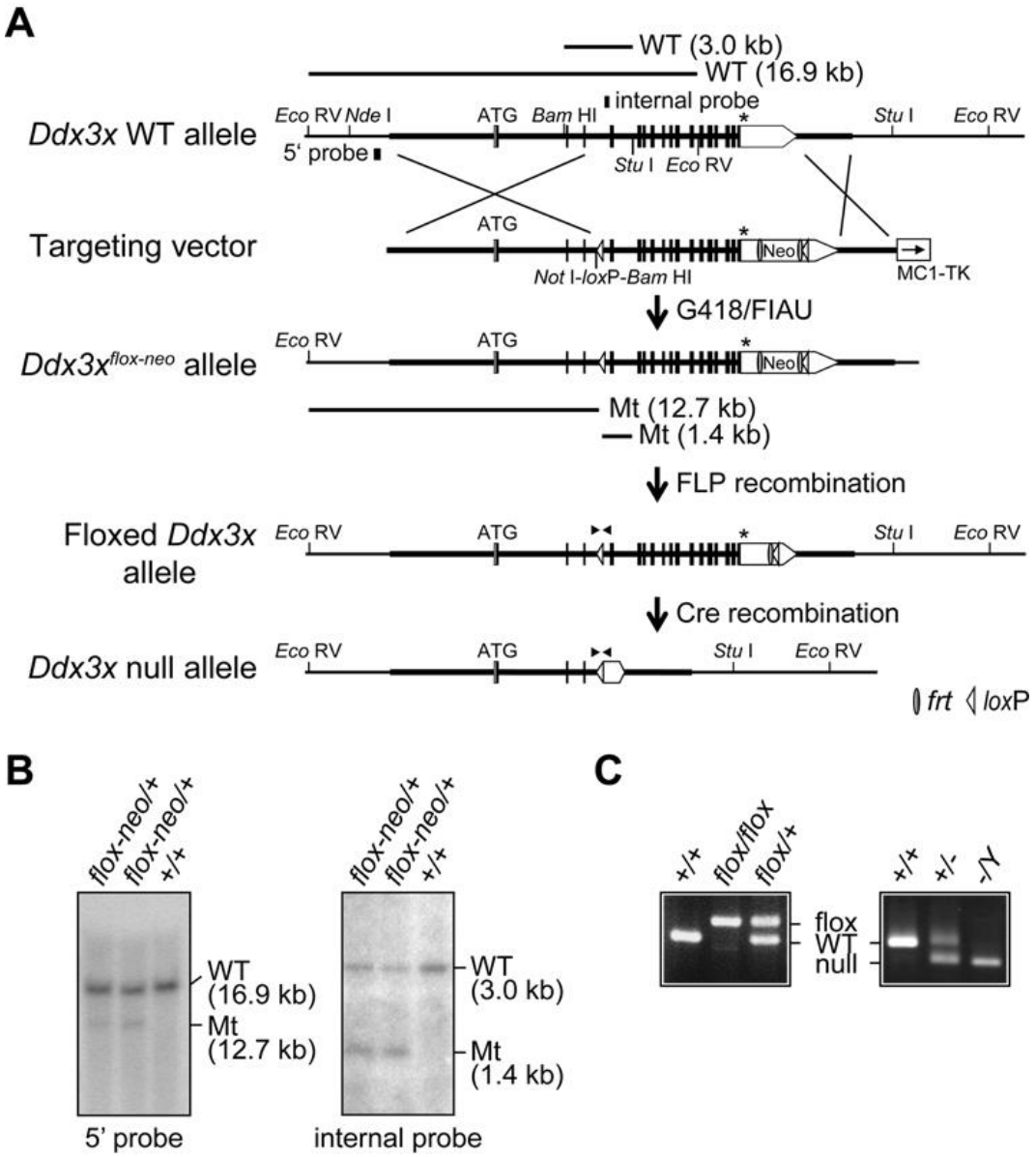
Age	Number of animals/litters	+/+; *Ddx3x*$^{flox/+}$		+/+; *Ddx3x*^{flox}/*Y*		*Sox2-cre*/+; *Ddx3x*$^{flox/+}$		*Sox2-cre*/+; *Ddx3x*^{flox}/*Y*		Absorbed	
E8.5	536/65	120	(22.4%)	120+1*	(22.6%)	128+2*	(24.3%)	112+1*	(21.1%)	52	(9.7%)
E9.5	233/21	62	(26.6%)	41	(17.6%)	52	(22.3%)	51	(21.9%)	27	(11.6%)
E10.5	41/6	6	(14.6%)	3	(7.3%)	16	(39.0%)	8+3*	(26.8%)	5	(12.2%)
E11.5	26/3	7	(26.9%)	8	(30.8%)	4	(15.4%)	2+4*	(23.1%)	1	(3.8%)
weaning	104/20	29	(27.9%)	46	(44.2%)	29	(27.9%)	0	(%)	-	
Expected percentage			25%		25%		25%		25%		

Numbers represent mice, with percentages in brackets.

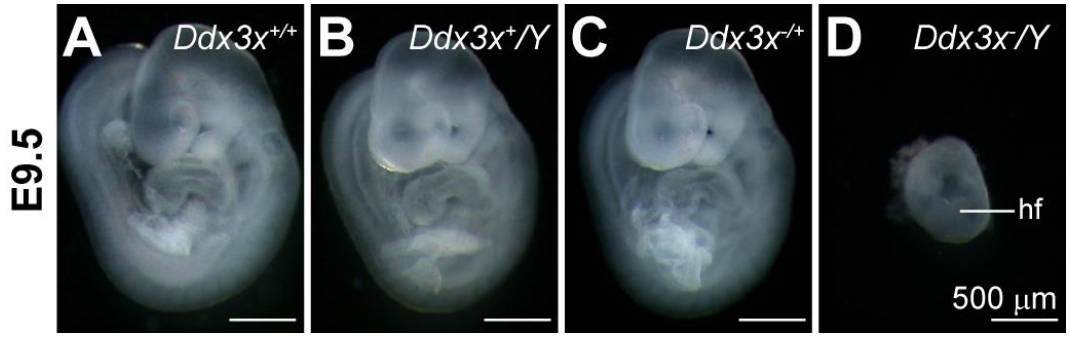
* Embryos not viable.

Absorbed: embryos were absorbed completely and could not be genotyped.

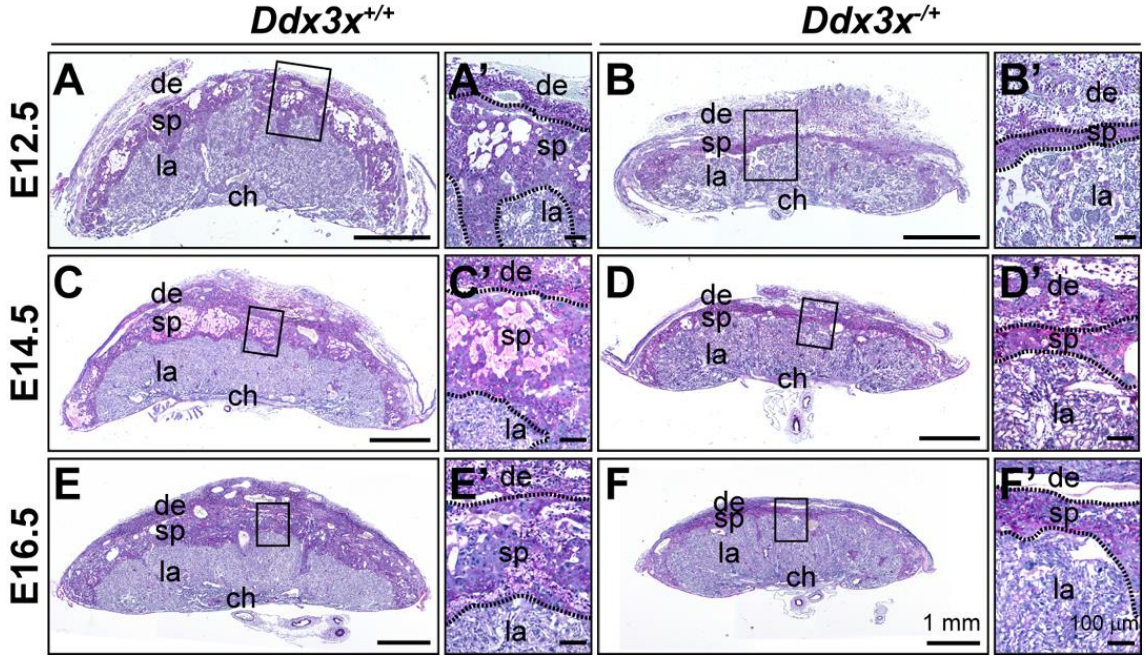
Supplementary Figures and Legends



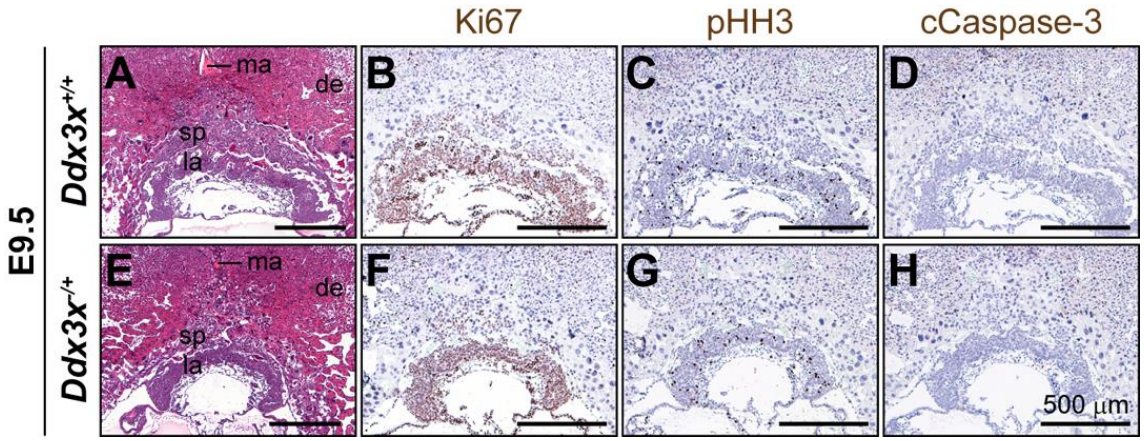
Supplementary Figure S1. Targeting of the mouse *Ddx3x* gene. (A) Schematic diagrams of the wild-type *Ddx3x* locus, the targeting vector, the floxed *Ddx3x* and null alleles. The *Ddx3x* gene has 17 exons, which are illustrated on the top line, with the non-coding and coding regions represented as white box and black shading, respectively. The lines above the wild-type *Ddx3x* allele and below that of the *Ddx3x*$^{flox\text{-}neo}$ allele depict the restriction fragments and expected DNA sizes detected by 5' and internal probes (heavy lines) in Southern blot analysis. Arrowheads represent the primers used in PCR reactions to distinguish the floxed and null alleles from the wild-type *Ddx3x* allele in genomic DNA. The translation stop codon is marked by an asterisk. Abbreviation used: neo, PGK-neo-poly(A) cassette. (B) Southern blot analysis of *Eco* RV-*Not* I and *Stu* I digested genomic DNA from wild-type and mutant ES cells with the 5' (left panel) and internal (right panel) probes, respectively. (C) Tail DNAs with indicated genotypes were analyzed by PCR.



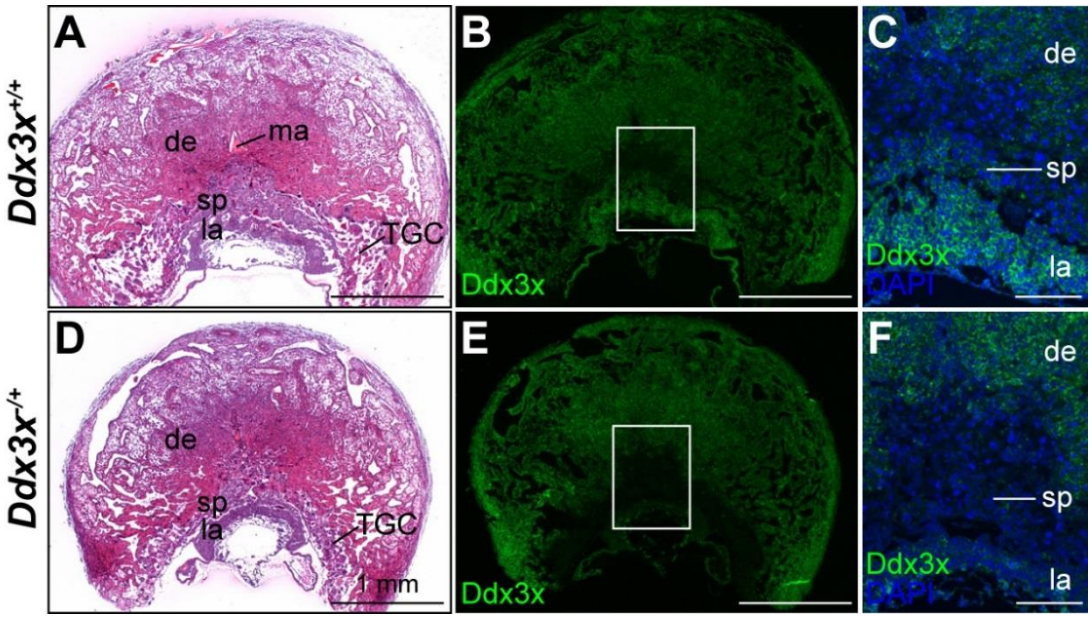
Supplementary Figure S2. Loss of *Ddx3x* results in early embryonic lethality. Whole mount morphology of littermate controls (A-C) with indicated genotypes and *Ddx3x*$^{-}$*/Y* mutant (D) at E9.5. Loss of *Ddx3x* led to severely growth retardation and embryonic lethality. hf, headfold.



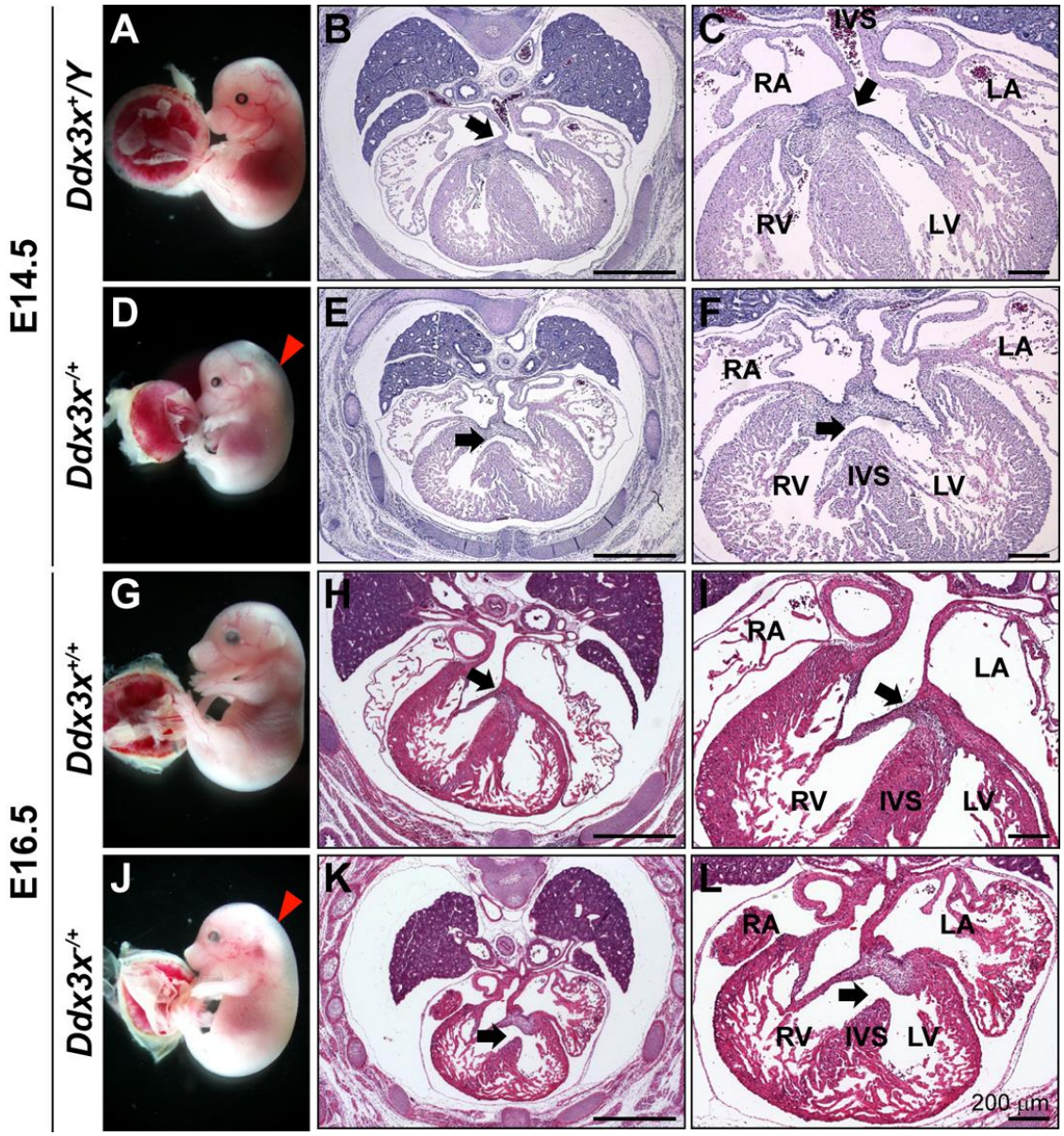
Supplementary Figure S3. Spongiotrophoblast layer is significantly reduced in F2 *Ddx3x*$^{-/+}$ placenta. (A-F') Representative mid-sagittal sections of E12.5 (A-B'), E14.5 (C-D') and E16.5 (E-F') placentas were stained with periodic acid-Schiff (PAS) to identify the glycogen containing cells. *Ddx3x*$^{+/+}$ placentas (A, C, and E) showed very intense PAS staining in the compacted spongiotrophoblast layers (purple red). In *Ddx3x*$^{-/+}$ placentas (B, D, and F), the spongiotrophoblast layers were decreased significantly in thickness. Boxed areas in A-F are shown at higher magnification in A'-F', respectively. Spongiotrophoblast boundary is indicated by the dotted lines. ch, chorion; de, decidua; la, labyrinthine; sp, spongiotrophoblast. Scale bar, 1 mm in A-F; 100 μm in A'-F'.



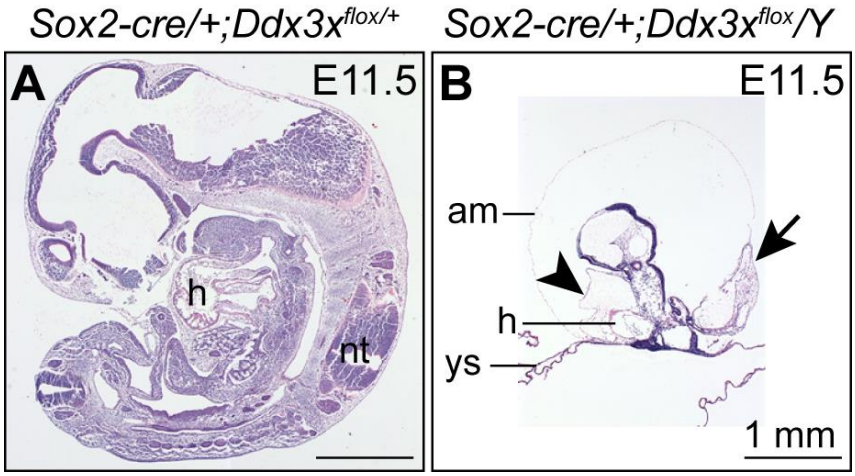
Supplementary Figure S4. Cell proliferation and apoptosis in F2 *Ddx3x*$^{-/+}$ placenta. Placental sections from E9.5 control littermate (A-D) and *Ddx3x*$^{-/+}$ (E-H) embryos were stained with H&E and antibodies against the proliferative marker Ki67, mitotic marker phospho-Histone H3 (pHH3), and apoptotic marker cleaved Caspase-3 (cCaspase-3) (brown). Sections were counterstained with hematoxylin. de, decidua; la, labyrinth; ma, maternal artery, sp, spongiotrophoblast. Scale bar, 500 μm.



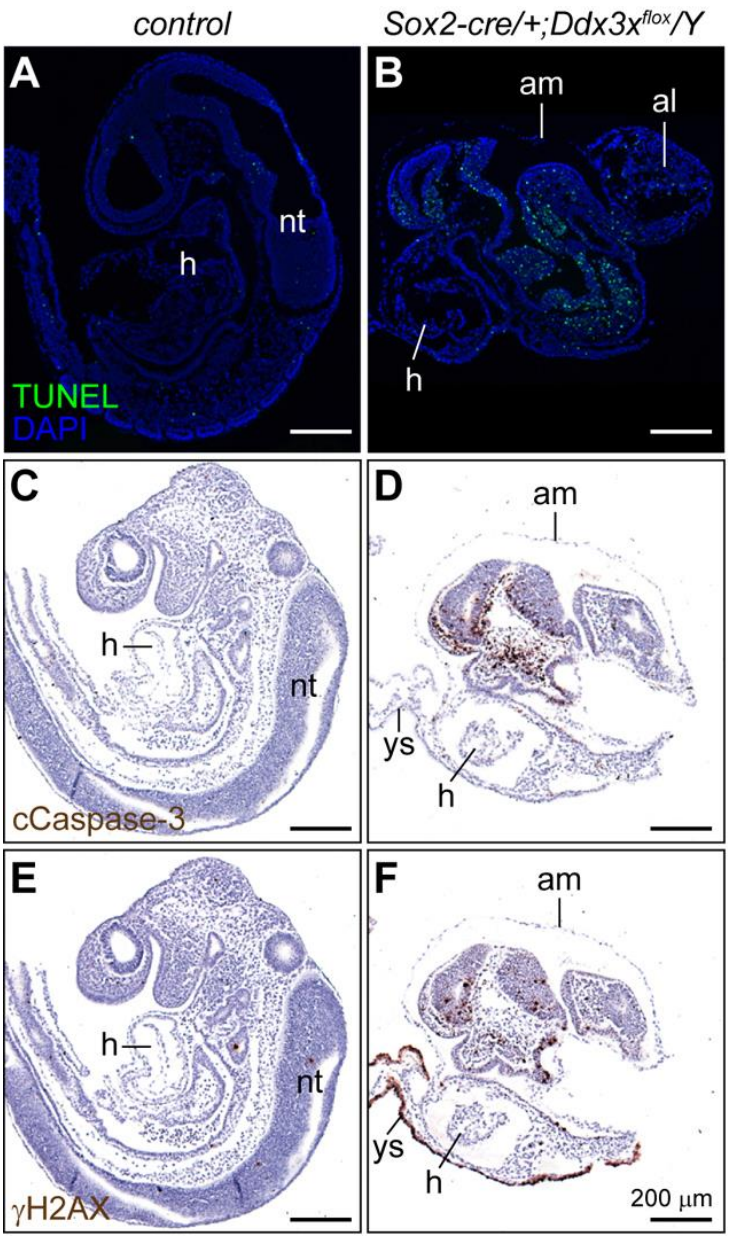
Supplementary Figure S5. Paternally-derived *Ddx3x* is preferentially inactivated in E9.5 placenta. Placental sections from E9.5 *Ddx3x*$^{+/+}$ littermate (A-C) and *Ddx3x*$^{-/+}$ (D-F) embryo were stained with H&E and antibody against Ddx3x (green). Ddx3x was broadly expressed in E9.5 placenta. Ddx3x level was moderate in labyrinth layer of developing placenta, with weak expression also detected in spongiotrophoblast, TGC, and decidua. The levels of Ddx3x in labyrinth, spongiotrophoblast, and TGC were significantly reduced in *Ddx3*$^{-/+}$ placenta compared to *Ddx3x*$^{+/+}$ placenta. Note the Ddx3x expression levels were comparable between *Ddx3x*$^{+/+}$ and *Ddx3*$^{-/+}$ decidual tissues. Higher magnification of the boxed areas in B and F are shown in C and F, respectively. Nuclei were counterstained with DAPI (blue). de, decidua; la, labyrinth; ma, maternal artery, sp, spongiotrophoblast. Scale bar, 1 mm in A, B, D, and E; 200 μm in C and F.



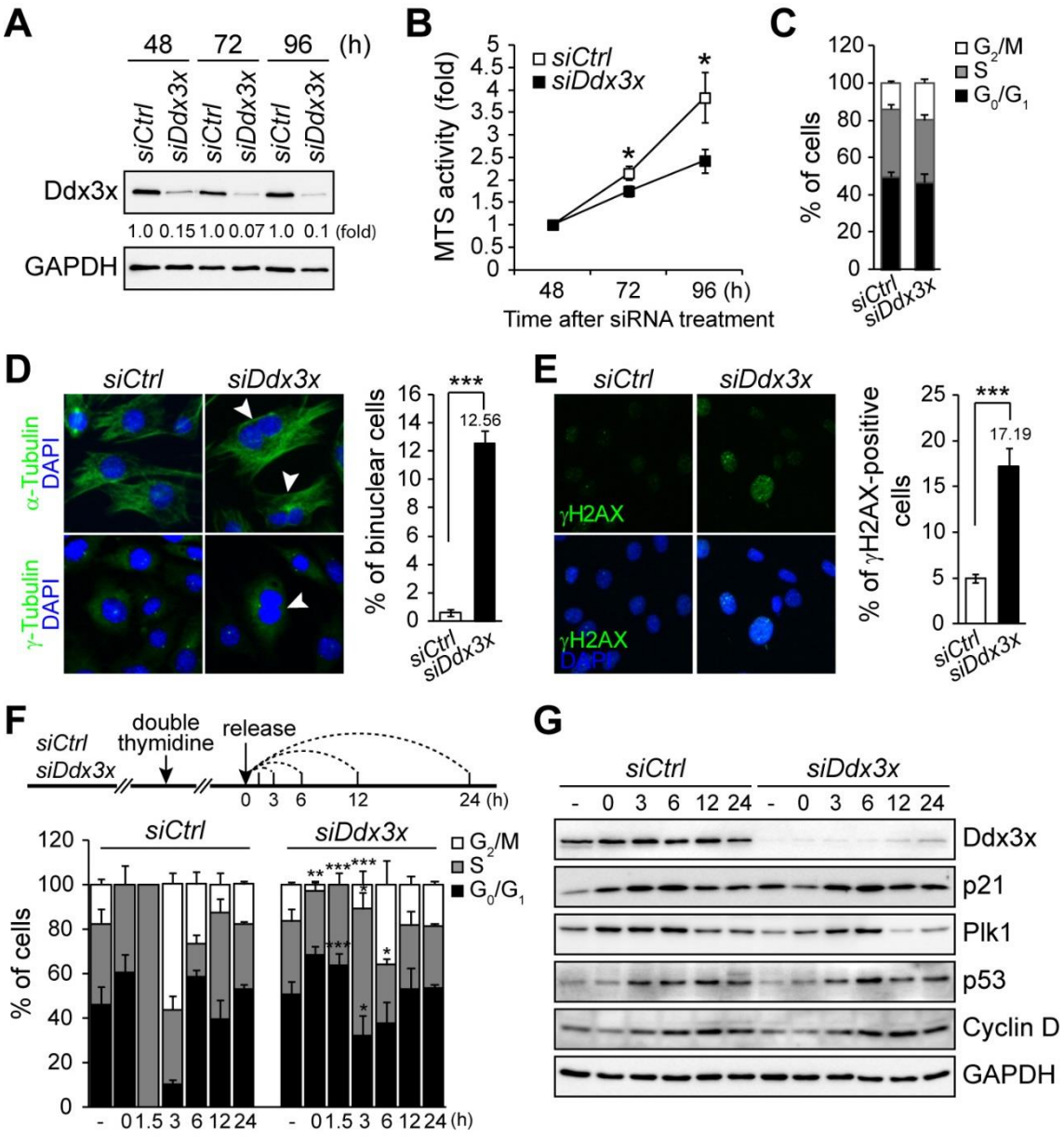
Supplementary Figure S6. Ventricular septal defect (VSD) in F2 *Ddx3x*$^{-/+}$ embryo. (A-L) Representative gross appearance of embryos and transverse sections of the hearts at atrioventricular valve level from control littermates (A-C and G-I) and *Ddx3x*$^{-/+}$ embryos (D-F and J-L) at E14.5 and E16.5. Red arrowheads indicate whole body edema in *Ddx3x*$^{-/+}$ mutants (D and J). Histological analysis revealed that ventricular septation was completed in E14.5 (B and C) and E16.5 (H and I) control embryos, whereas ventricular septal defects (arrows) were presented in E14.5 (E and F) and E16.5 (K and L) *Ddx3x*$^{-/+}$ embryos. LA, left atrium; RV, right atrium; IVS, interventricular septum; LV, left ventricle; RV, right ventricle. Scale bar, 200 μm.



Supplementary Figure S7. Severe developmental defects in *Sox2-cre/+;Ddx3x^{flox}/Y* mutant. H&E stained sagittal sections of E11.5 control littermate (A) and *Sox2-cre/+;Ddx3x^{flox}/Y* mutant (B). *Sox2-cre/+;Ddx3x^{flox}/Y* mutant was apparently smaller in size with abnormal headfolds, pericardial effusion (arrowhead), and defective body turning and caudal embryonic structure (arrow) compared to control littermate. am, amnion; h, heart; nt, neural tube, ys, yolk sac. Scale bar, 1 mm.



Supplementary Figure S8. TUNEL-, cCaspase-3- and γH2AX-positive cells were widespread in *Sox2-cre/+;Ddx3x^{flox}/Y* mutant. When control littermates had completed turning at late-stage E8.5, *Sox2-cre/+;Ddx3x^{flox}/Y* mutants remained unturned with an abnormal allantois. Representative TUNEL (A and B, green), cCaspase-3 (C and D, brown) and γH2AX immunostaining in control and *Sox2-cre/+*;*Ddx3x^{flox}/Y* mutant. Significantly greater numbers of TUNEL-, cCaspase-3- and γH2AX-positive cells were detected in *Sox2-cre/+*;*Ddx3x^{flox}/Y* mutants than in controls. al, allantois; am, amnion; h, heart; nt, neural tube; ys, yolk sac. Scale bar, 200 μm.



Supplementary Figure S9. *Ddx3x* knockdown in C3H10T1/2 cells induces DNA damage and affects cell cycle progression. (A) Total Ddx3x protein levels in *siCtrl* control and *siDdx3x* C3H10T1/2 cells at 48–96 hours after transfection were analyzed by Western blotting. GAPDH was used as the loading control (lower panel). Ddx3x levels were quantified in cell extracts, and normalized and expressed with respect to GAPDH levels. (B) *Ddx3x* knockdown in C3H10T1/2 cells affected cell proliferation. Equal numbers of siRNA-treated cells were seeded at 48 hours after transfection, and cell proliferation was analyzed by MTS assay at 72 and 96 hours after transfection. (C) *Ddx3x* knockdown did not significantly alter cell cycle progression in asynchronous C3H10T1/2 cells as monitored by DNA content. (D and E) Asynchronous *siCtrl* and *siDdx3x* cells were immunostained with anti-α-tubulin, anti-γ-tubulin (green, D), and γH2AX (green, E) antibodies at 96 hours after transfection. Representative images are shown. Percentages of binuclear (γ-tubulin positive, indicated by arrowheads in D) and γH2AX-positive (foci, E) cells were scored. (F) Loss of Ddx3x in C3H10T1/2 cells slows cell cycle progression. Schematic diagram of the

experimental protocol is shown in the upper panel. The *siCtrl* and *siDdx3x* C3H10T1/2 cells were synchronized by a double thymidine (DT) block, released at the indicated times, and analyzed by flow cytometry. Although *siCtrl* cells were synchronized in both G_0/G_1 and S phases, some *siDdx3x* cells remained in the G_2/M phase after DT treatment. At 1.5 hours after release, all *siCtrl* cells progressed into S phase. The *siDdx3x* cells displayed longer cell cycle times and markedly accumulated in G_0/G_1 and S phases. At 3 hours after DT release, a skewed distribution of all cell cycle phases was observed in *siDdx3x* cells compared with that in *siCtrl* cells. (G) Expression levels of indicated proteins in *siCtrl* and *siDdx3x* cells at different time points after DT block were detected by Western blotting. A p21 increase and a Plk1 decrease were detected in asynchronous *siDdx3x* cells compared with those in *siCtrl* cells. A total of 30 μg protein per lane was loaded. Quantitative results are presented as means±SEM of at least three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.005.