Supporting Information

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SI Materials and Methods

Examination of Malformations. To examine the incidence of various types of malformations, near-term fetuses were delivered by caesarean section at embryonic day (E) 18. The head length, crown–rump length, tail length, and weight were measured. External malformations of the eye (open or rudimentary eyelids, microphthalmia, and exophthalmia), ear (anotia, microtia, ectopic positioning, and abnormal/unfused auricular hillocks), jaw (micrognathia of the upper and/or lower jaws), limb (inward turning of hindlimbs and clubfeet), and anus (imperforate anus) were examined by gross morphology. The mouth was opened for inspect for cleft palate. Defects of internal organs affecting the diaphragm (diaphragmatic hernia), heart (small heart, persistent truncus arteriosus, and transposition of great vessels), testis (undescended testis), and kidney (renal agenesis and nonagenesis renal malformations including hypoplastic, dysplastic, hydronephrotic, and cystic kidneys) were determined by gross morphological examination after exposing the thorax and abdomen. The number of litters and fetuses examined in each treatment group are given in Table S1.

In Situ Hybridization. To study the expression pattern of the *Raldh* and *Cyp26* genes, embryos at different time points after retinoic acid (RA) treatment and age-matched controls were dissected free of all extraembryonic tissues and fixed in 4% (wt/vol) paraformaldehyde at 4 °C overnight. They were subjected to whole-mount in situ hybridization (ISH) for *Raldh1*, *Raldh2*, *Raldh3*, *Cyp26a1*, *Cyp26b1*, and *Cyp26c1* using digoxigenin-labeled probes according to the protocol of Wilkinson (1). Three to four litters of embryos were examined in each treatment group. To examine further the expression pattern of *Raldh2* in the metanephros, hybridized E11.0 embryos were embedded in gelatin and cut transversely into 20-μm–thick vibratome sections at the level of the cloacal region.

Real-Time Quantitative RT-PCR. To quantify gene-expression levels, each litter of whole embryos or the metanephroi isolated from embryos of one litter were pooled together as one sample. The total RNA was extracted using the Tissue Total RNA Mini Kit (Favorgen) and was synthesized into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. The cDNA then was amplified by real-time quantitative PCR using iTaq SYBR Green Supermix with ROX (Bio-Rad) with the 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR conditions included initiation at 95 °C for 10 min, followed by 40 cycles comprising denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for another 30 s. Primers used for amplification of various genes were

Raldh1: forward: 5′-CAT TGC TGT TGA GTT TGC AC-3′; reverse: 5′-TTG ATT TAT TCC TGG GGT CA-3′
Raldh2: forward: 5′-TTG CAG ATG CTG ACT TGG AC-3′; reverse: 5′-TCT GAG GAC CCT GCT CAG TT-3′
Raldh3: forward: 5′-GAT AAA GTT GGG CTG AGC AA-3′; reverse: 5′-CCA AAA TTC AGT GTC CGA AG-3′
Cyp26a1: forward: 5′-CAG TGC TAC CTG CTC GTG AT-3′; reverse: 5′-AGA GAA GAG ATT GCG GGT CA-3′
Cyp26b1: forward: 5′-TTC AGT GAG GCA AGA AGA CA-3′; reverse: 5′-CTG GGA GGA GGT GCT AAG TA-3′
Cyp26c1: forward: 5′-GGG ACC AGT TGT ATG AGC AC-3′; reverse: 5′-AGC CAA CTC CTT CAG CTC TT-3′
Wt1: forward: 5′-CAG ATG TAA TTC TAC AGG CGA TTG-3′; reverse: 5′-TCC CTA CAT ACT TGC AGA TTC AAA-3′
Ret: forward: 5′-TGG CAC ACC TCT GCT CTA TG-3′; reverse: 5′-GAT GCG GAT CCA GTC ATT CT-3′
β-actin: forward: 5′-TGT TAC CAA CTG GGA CGA CA-3′; reverse: 5′-GGG GTG TTG AAG GTC TCA AA-3′, as internal control

The standard curve was prepared by using the threshold cycle (C_T values) from serial dilutions of known concentrations of DNA obtained from respective cDNA plasmids or genomic DNA. Three separate trials of PCR were performed for each sample. Expression levels in five or six litters of whole embryos or pooled metanephroi in each group were examined.

Quantification of RA Levels. The amount of all-*trans* RA in whole embryos was quantified by HPLC. Control and RA-overexposed E9.25 and E9.5– E12.0 embryos, collected at half-day intervals, were dissected free of all extraembryonic tissues under dim yellow light. For control embryos at E9.25 and E9.5, three litters of embryos were pooled as one sample. For control embryos at E10.0 onwards and for RA-overexposed embryos at all stages, each litter of embryos was collected as one sample. Before chromatography, the embryo was extracted according to the protocol of Suh et al. (2). A small portion (10 μL) of each sample was used to determine protein concentrations by the Bradford protein assay (Bio-Rad) according to the manufacturer's protocol. Chromatography was performed on a 4.6 × 150 mm SunFire C18 Column containing 5-μm particles (Waters) with the Alliance HPLC System equipped with a 2996 photodiode array detector (Waters) under conditions according to Schmidt et al. (3). The amount of all-*trans* RA in each sample was calculated with reference to a calibration curve established with external standards of known all-*trans* RA concentrations. Five samples were examined for each treatment group. The amount of RA in metanephroi was quantified by a F9 teratocarcinoma reporter cell line stably transfected with a retinoic acid response element driving β-galactosidase expression (4). Metanephroi were isolated from E11.0, E11.5, and E12.0 control and RA-overexposed embryos under dim yellow light. Two metanephroi were pooled as one sample. The metanephroi samples were lysed in cell-culture medium and applied to the RA-responsive cells grown on a 96-well plate. After incubation in a 5% CO_2 incubator at 37 °C for 16 h, X-Gal staining was performed to determine β-galactosidase activity in each well. The intensity of the colored product was measured by a spectrophotometer at 650 nM. The amount of RA in each sample was determined by comparison with a standard curve prepared from serially diluted all-*trans* RA solutions. The amount of RA in 7–17 samples from two independent litters of embryos was examined for each group.

TUNEL Staining and Quantification of Apoptotic Cells. To identify apoptotic cells in kidneys of E12.0 embryos, embryos were fixed in 4% (wt/vol) paraformaldehyde at 4 °C overnight and then were prepared as 7-μm–thick serial paraffin sections. Apoptotic cells with DNA breaks were detected using the TUNEL assay with the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore) according to the manufacturer's instructions. The sections were counterstained with 0.5% methyl green. To quantify apoptotic cells, the number of apoptotic nuclei per sectional area of the metanephros was counted using the Stereo Investigator system (MBF Bioscience). To avoid counting signals from the same apoptotic nuclei across adjacent sections, quantification was performed in

one of every three sections covering the whole metanephros. For each treatment group, apoptotic signals in five to nine metanephroi from three independent litters of embryos were quantified.

Statistical Analysis. The expression level of various genes, the amount of RA, and the number of apoptotic nuclei in embryos or metanephroi samples were analyzed by independent samples *t* test. The frequency and severity of renal malformations in RA-overexposed fetuses supplemented with various dosages of RA were analyzed by the Jonckheere–Terpstra test. Correlation between the dose of supplemented RA and the frequency and severity of renal malformations was tested by Spearman's rank correlation. The head length, crown–rump length, and the incidence rate of extrarenal malformations were analyzed by independent samples *t* test. The significance level of all statistical analyses was set at $P < 0.05$.

1. Wilkinson DG (1992) Whole mount in situ hybridization of vertebrate embryos. *In Situ Hybridization: A Practical Approach* (IRL, Oxford, UK), pp 75–83.
2. Suh MJ, Tang XH, Gudas LJ (2006) Structure elucidation of retinoids in biological samples using postsource decay laser desorption/ionization mass spectrometry after high-performance liquid chromatography separation. *Anal Chem* 78:5719–5728.
3. Schmidt CK, Brouwer A, Nau H (2003) Chromatographic analysis of endogenous retinoids in tissues and serum. *Anal Biochem* 315:36–48.
4. Wagner M, Han B, Jessell TM (1992) Regional differences in retinoid release from embryonic neural tissue detected by an in vitro reporter assay. *Development* 116: 55–66.

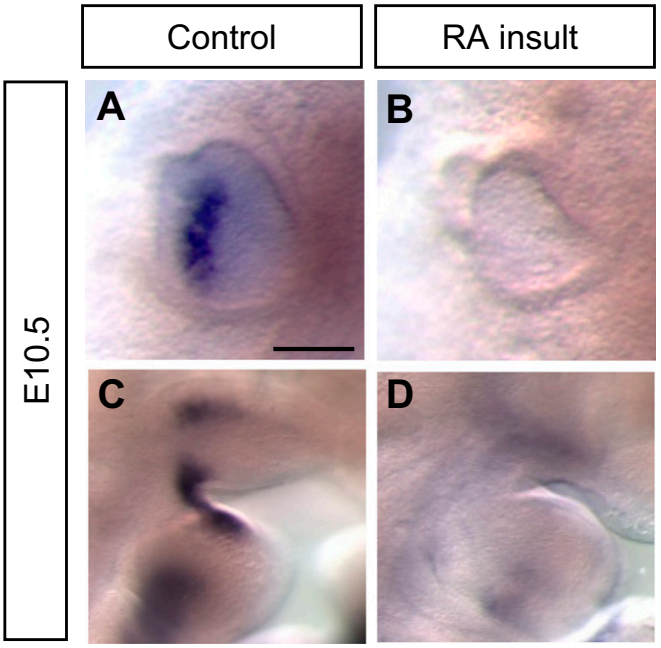

Fig. S1. Prolonged reduction of *Raldh2* expression in the embryo after teratogenic RA insult. (*A–D*) Whole-mount ISH patterns of *Raldh2* in control (*A* and *C*) and RA-overexposed embryos (*B* and *D*) at E10.5 show prominent reduction of *Raldh2* expression in the otic vesicle (*A* and *B*) and the maxillomandibular region (*C* and *D*). (Scale bar: *A* and *B*, 0.15 mm; *C* and *D*, 0.18 mm.)

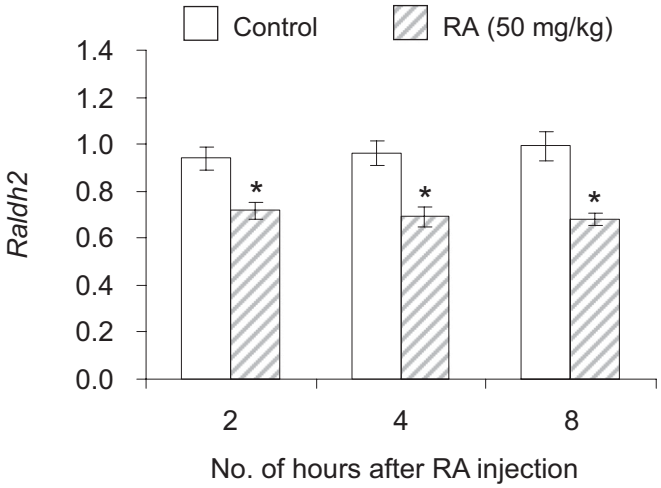

Fig. S2. Rapid down-regulation of *Raldh2* in the embryo after exposure to a teratogenic dose of RA. Real-time quantitative RT-PCR of gene-expression levels of *Raldh2* relative to *β-actin* in control and RA-overexposed embryos at 2, 4, and 8 h following maternal injection of 50 mg/kg b.w. of all-*trans* RA at E8.5. (Data are shown as mean ± SEM, $n = 4–5$; embryos in each litter were pooled as one sample.*$P < 0.001$ vs. time-matched control, independent samples *t* test.)

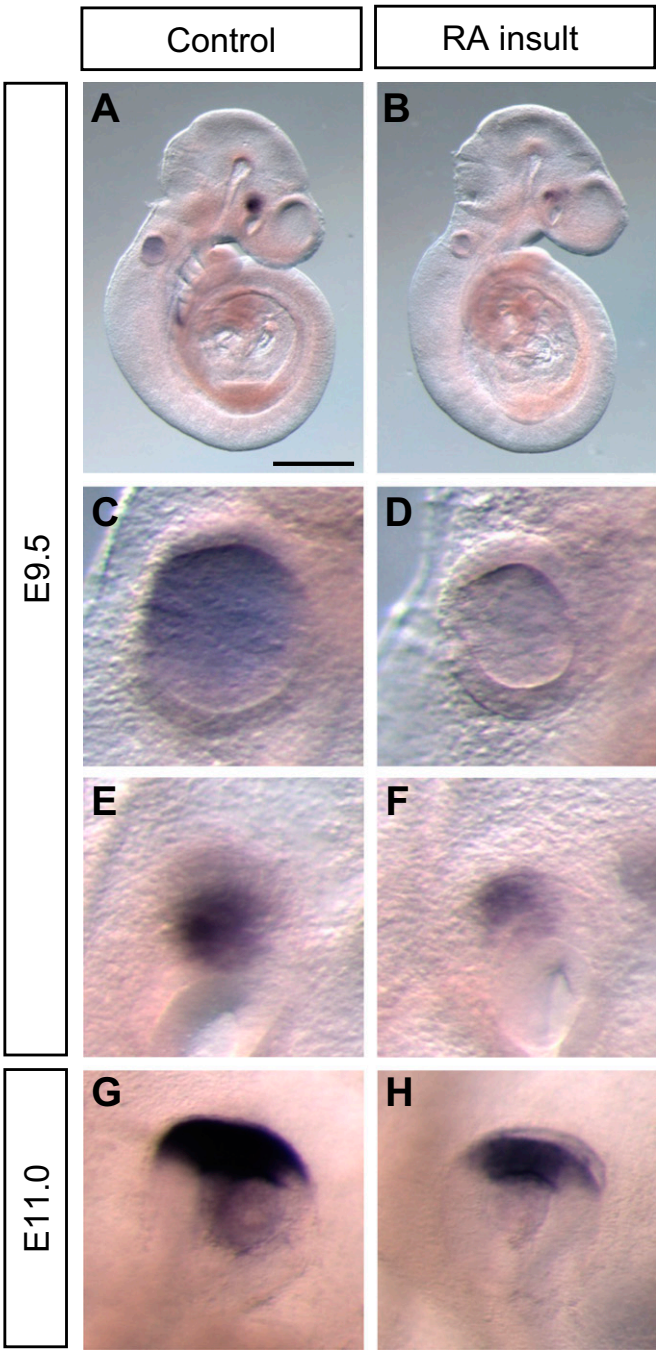

Fig. S3. Prolonged reduction of *Raldh1* expression in the embryo after teratogenic RA insult. (*A*–*H*) Whole-mount ISH patterns of *Raldh1* in control (*A*, *C*, *E*, and *G*) and RA-overexposed embryos (*B*, *D*, *F*, and *H*) at E9.5 (*A*–*F*) and E11.0 (*G* and *H*). At E9.5, a decrease in *Raldh1* expression was observed in the whole embryo (*A* and *B*) and especially in the otic vesicle (*C* and *D*) and the optic vesicle (*E* and *F*). Reduced expression was prolonged up to E11.0 in the optic vesicle (*G* and *H*). (Scale bar: *A* and *B*, 0.8 mm; *C*–*F*, 0.1 mm; *G* and *H*, 0.15 mm.)

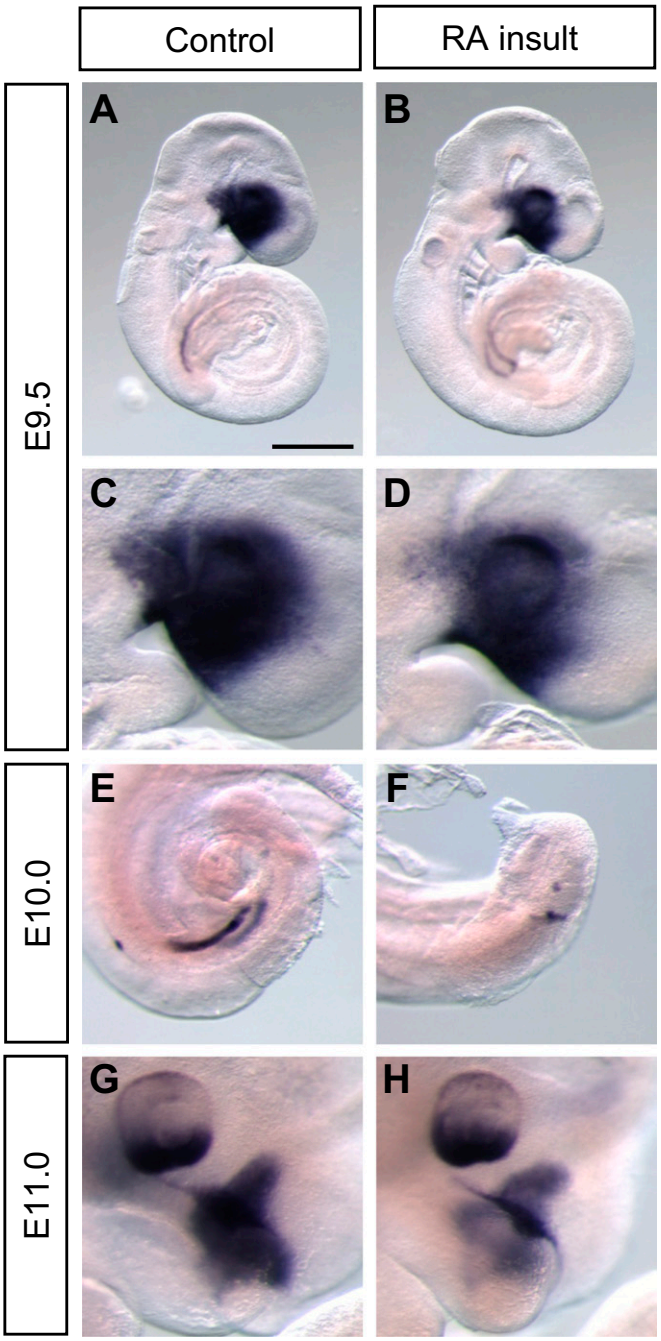

Fig. S4. Prolonged reduction of *Raldh3* expression in the embryo after teratogenic RA insult. (*A*–*H*) Whole-mount ISH patterns of *Raldh3* in control (*A*, *C*, *E*, and *G*) and RA-overexposed embryos (*B*, *D*, *F*, and *H*) at E9.5 (*A*–*D*), E10.0 (*E* and *F*), and E11.0 (*G* and *H*). At E9.5 reduced *Raldh3* expression was observed in the whole embryo (*A* and *B*) and especially in the surface ectoderm overlying the optic vesicle and the olfactory region (*C* and *D*). Down-regulation of *Raldh3* expression was observed in the nephrogenic cord at the caudal region (*E* and *F*). Reduced expression was prolonged up to E11.0 in the olfactory region (*G* and *H*). (Scale bar: *A* and *B*, 0.8 mm; *C*, *D*, *G*, and *H*, 0.3 mm; *E* and *F*, 0.5 mm.)

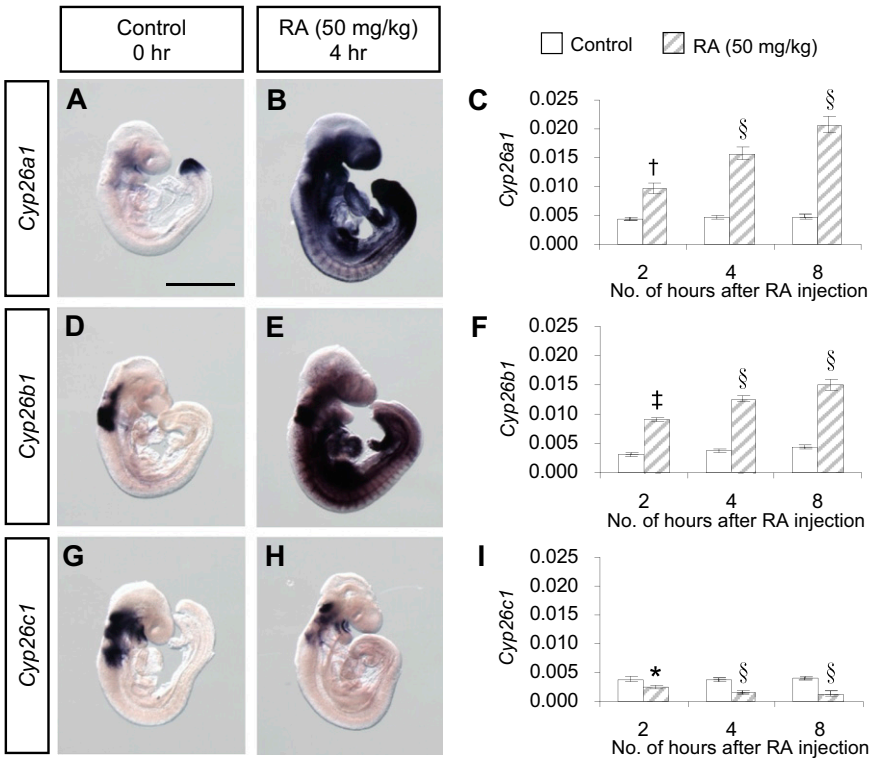

Fig. S5. Changes in *Cyp26s* expression in the embryo in response to maternal treatment with a teratogenic dose (50 mg/kg b.w.) of all-*trans* RA at E9.0. *Cyp26a1* and *Cyp26b1* showed prominent up-regulation, whereas *Cyp26c1* was down-regulated significantly. (*A*, *B*, *D*, *E*, *G,* and *H*) Whole-mount ISH patterns of *Cyp26a1* (*A* and *B*), *Cyp26b1* (*D* and *E*), and *Cyp26c1* (*G* and *H*) in embryos before (*A*, *D*, and G) or 4 h after (*B*, *E,* and *H*) RA treatment. The three *Cyp26* genes have tissue-specific expression domains. A few hours after exposure to exogenously applied RA, *Cyp26a1* and *Cyp26b1* became highly expressed at multiple ectopic sites throughout the embryo, whereas *Cyp26c1* showed notably reduced expression. (Scale bar: 1 mm.) (*C*, *F*, and *I*) Real-time quantitative RT-PCR of gene-expression levels of *Cyp26a1* (*C*), *Cyp26b1* (*F*), and *Cyp26c1* (*I*) relative to *β-actin* in control and RA-treated embryos. Significant changes in expression levels were detectable as soon as 2 h after RA treatment and persisted until at least 8 h posttreatment. (Data are shown as mean ± SEM, $n = 3–5$; embryos in each litter were pooled as one sample. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.005$; $^{\S}P < 0.001$ vs. time-matched control, independent samples *t* test.)

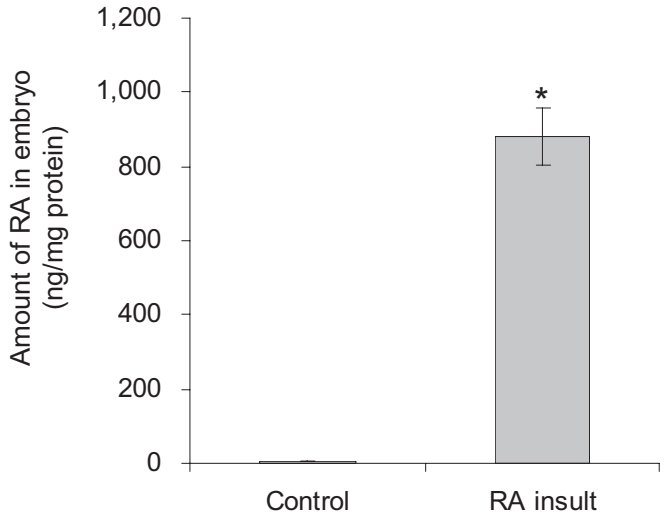

Fig. S6. Dramatic increase in RA levels in embryos at 6 h after teratogenic RA insult. Quantification of all-*trans* RA levels (mean ± SEM, $n = 5$; three litters of control embryos or one litter of RA-overexposed embryos were pooled as one sample) in control (0.487 ng/mg protein) and RA-overexposed embryos (878 ng/mg protein) by HPLC. ($^{*}P < 0.001$ vs. control, independent samples *t* test.)

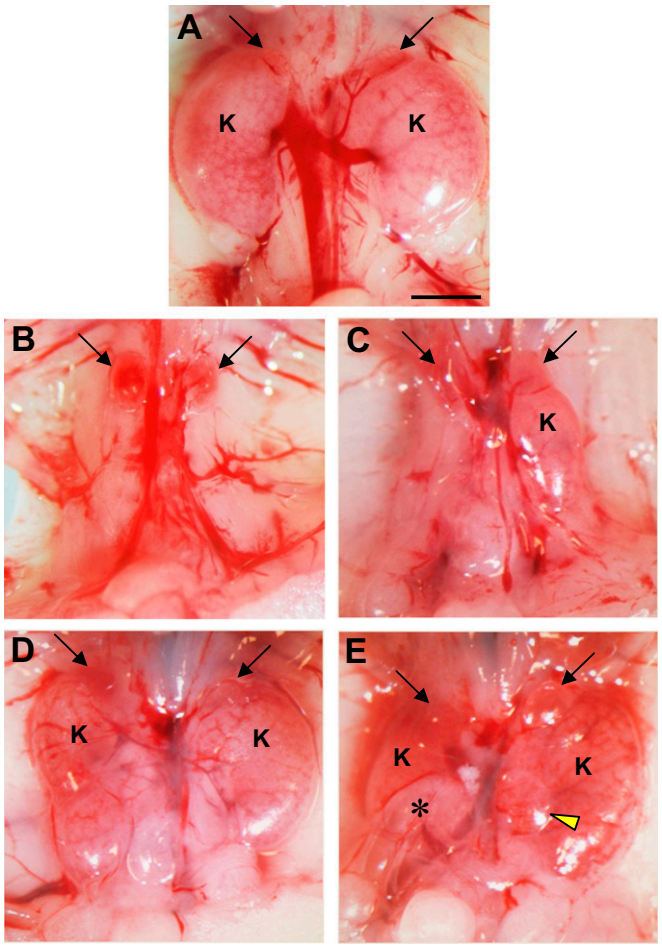

Fig. S7. Ventral view of abdominal cavities of E18 fetuses with various renal phenotypes. (*A*) A pair of normal, bean-shaped kidneys (K). The adrenal glands (arrows in all frames) are situated above the kidneys. (*B*) Absence of the kidney on both sides (bilateral renal agenesis); note that the adrenal glands are still present. (*C*) Absence of kidney on one side (unilateral renal agenesis); the contralateral, solitary kidney is smaller than normal (hypoplastic). (*D*) The right kidney (on the left side of this image) is dysmorphic (dysplastic) and had an abnormal contour; the left kidney is normal in size and shape. (*E*) The renal pelvis of the right kidney is distended with fluid (hydronephrosis) (asterisk), and cyst-like structures (arrowhead) were noted in the left kidney. (Scale bar: 1 mm.)

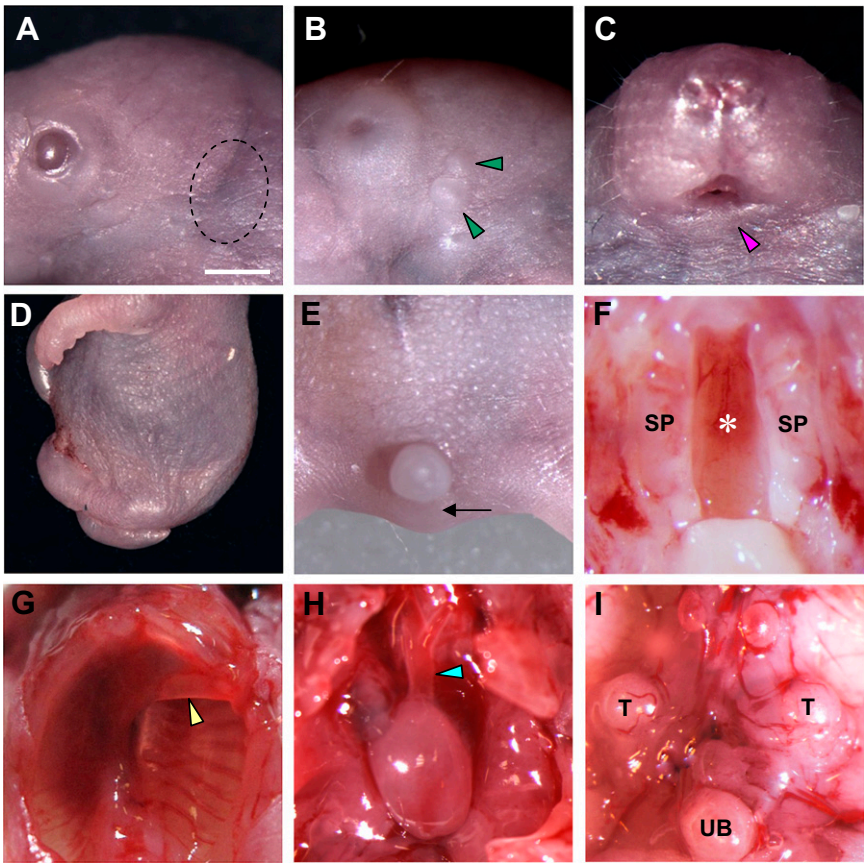

Fig. S8. RA-overexposed E18 fetuses with various types of malformations. (*A*) Open eyelids and absence of external ear. (The normal position of the external ear is shown by the dotted circle). (*B*) Rudimentary eyelids and abnormal fusion of auricular hillocks (green arrowheads) in an ectopic position. (*C*) Micrognathia. The lower jaw (pink arrowhead) was reduced markedly in size. (*D*) Inward turning of hindlimbs and clubfeet. (*E*) Imperforate anus (the arrow indicates the normal position of the anal opening) and absence of tail. (*F*) Cleft palate. There is a wide gap (asterisk) between the unfused secondary palatal shelves (SP). (*G*) Diaphragmatic hernia. There is a large opening in the left diaphragm (yellow arrowhead). (*H*) Small heart with persistent truncus arteriosus. The truncus arteriosus failed to divide properly into the pulmonary trunk and aorta (blue arrowhead). (*I*) Undescended testes. A pair of testes (T), normally lying close to the urinary bladder (UB), is located ectopically in a more superior position. (Scale bar: *A*, *B*, *C*, and *E*, 14 mm; *D*, 35 mm; *F*, 7 mm; *G*, 25 mm; *H* and *I*, 20 mm.)

Table S1. Effect of oral supplementation with low doses of all-*trans* RA after teratogenic RA insult on various growth parameters in E18 fetuses

Parameter	Control (no insult)	RA insult (125 mg/kg body weight)			
RA supplement (mg/kg)	0	0	0.625	1.25	2.5
No. of litters	5	12	7	14	11
No. of live fetuses	64	107	64	150	72
Mean weight (g) ± SEM	1.29 ± 0.01	0.90 ± 0.01	0.96 ± 0.01†	0.98 ± 0.01‡	0.97 ± 0.02*
Mean head length (mm) ± SEM	11.38 ± 0.06	10.04 ± 0.05	10.26 ± 0.06*	10.67 ± 0.04‡	10.47 ± 0.06‡
Mean crown-rump length (mm) ± SEM	23.85 ± 0.12	20.01 ± 0.10	20.42 ± 0.11*	21.14 ± 0.09‡	20.65 ± 0.12‡
Mean tail length (mm) ± SEM	12.17 ± 0.06	0	0	0	0

Statistical significance: *$P < 0.05$; $^{\dagger}P < 0.005$; $^{\ddagger}P < 0.001$ vs. RA insult without RA supplementation analyzed by independent samples *t* test.