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## Odd-skipped related 1 (Odd1) is an essential regulator of heart and urogenital development

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### Abstract

The *Odd-skipped related 1 (Odd1)* gene encodes a zinc finger protein homologous to the *Drosophila* Odd-skipped class transcription factors that play critical roles in embryonic patterning and tissue morphogenesis. We have generated mice carrying a targeted null mutation in the *Odd1* gene and show that Odd1 is essential for heart and intermediate mesoderm development. *Odd1*<sup>-/-</sup> mutant mouse embryos fail to form atrial septum, display dilated atria with hypoplastic venous valves, and exhibit blood backflow from the heart into systemic veins. In contrast to other transcription factors implicated in atrial septum development, *Odd1* mRNA expression is restricted to the central dorsal domain of the atrial myocardium during normal heart development. Moreover, expression patterns of known key regulatory genes of atrial septum development, including *Nkx2.5*, *Pitx2*, *Tbx5*, are unaltered in the developing heart in *Odd1*<sup>-/-</sup> mutants compared to that of the wildtype littermates. Furthermore, homozygous *Odd1*<sup>-/-</sup> mutant embryos exhibit complete agenesis of adrenal glands, metanephric kidneys, gonads and defects in pericardium formation. Detailed molecular marker analyses show that key regulators of early intermediate mesoderm development, including *Lhx1*, *Pax2*, and *Wt1*, are all down-regulated and nephrogenic mesenchyme undergoes massive apoptosis, resulting in disruption of nephric duct elongation and failure of metanephric induction in the *Odd1*<sup>-/-</sup> mutant embryos. These data provide new insights into the molecular mechanisms underlying heart morphogenesis and urogenital development.

### Keywords

adrenal gland; atrial septum; heart development; intermediate mesoderm; kidney development; *Odd-skipped*; *Osrl*; renal agenesis; venous valve

### Introduction

The *Drosophila* *Odd-skipped (odd)* gene was initially identified as a pair-rule gene because mutations at this locus cause loss of portions of the odd-numbered segments in the embryo (Nusslein-Volhard and Wieschaus, 1980; Coulter and Wieschaus, 1988). The *Odd* gene encodes a putative transcription factor containing four C2H2-type zinc finger motifs (Coulter et al., 1990). Gene expression and phenotypic analyses indicate that the Odd protein can either activate or repress the expression of downstream segmentation genes during *Drosophila* embryogenesis (DiNardo and O'Farrell, 1987; Mullen and DiNardo, 1995; Saulier-Le Drean et al., 1998). Two other *Drosophila* genes, *brother of odd with*

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*entrails limited (bowl)* and *sister of odd and bowl (sob)*, encoding proteins with highly similar zinc finger domains were isolated by sequence homology (Hart et al., 1996; Wang and Coulter, 1996). Mutations in *bowl* disrupt terminal segments and gut development during *Drosophila* embryogenesis (Wang and Coulter, 1996). A genetic screen for genes regulating *Drosophila* embryonic gut morphogenesis resulted in the identification of another gene with sequence homology to *odd*, named *drumstick (drm)*, which is essential for proventriculus formation and hindgut elongation (Green et al., 2002). It was demonstrated that the tissue-specifically expressed Drm protein blocks the function of a globally expressed transcription factor Lines by direct protein-protein interaction through the Drm zinc finger domain, which relieves inhibition of Bowl by Lines and leads to nuclear accumulation of the active Bowl protein (Green et al., 2002; Hatini et al., 2005). Recently, it was shown that the Drm-Lines-Bowl pathway links antagonistic Hedgehog and Wingless signaling inputs to regulate epidermal cell differentiation (Hatini et al., 2005). These data indicate that the Odd-skipped family transcription factors play essential roles in *Drosophila* embryonic development, cell differentiation and tissue morphogenesis.

By searching Expressed Sequence Tags Database for genes homologous to the *Drosophila* Odd-skipped family zinc finger proteins, So and Danielian (1999) isolated the first vertebrate homolog of *odd-skipped* and named it *odd-skipped related 1 (Osr1)*, renamed *Odd1* - Mouse Genome Informatics database). *Odd1* mRNA is expressed throughout the intermediate mesoderm at embryonic day (E) 8.5 during mouse embryogenesis and is activated in many other tissues, including the branchial arches and limb buds in later embryonic stages (So and Danielian, 1999). In mouse embryos lacking both *Foxc1* and *Foxc2*, paraxial mesoderm is respecified to intermediate mesoderm and *Odd1* mRNA expression is activated in this respecified tissue (Wilm et al., 2004), suggesting that *Odd1* mRNA expression may be important for intermediate mesoderm development. We previously characterized *Osr2*, another mouse *odd-skipped related* gene encoding a zinc finger protein with 65% amino acid sequence identity throughout the protein and 98% identity in the zinc finger region with *Odd1*, and showed that *Odd1* and *Osr2* displayed distinct expression patterns during mouse embryogenesis (Lan et al., 2001). Recently, a targeted null mutation in the *Osr2* gene was generated and characterized. The homozygous *Osr2* null mutants exhibited open eyelids and specific defects in secondary palate development but had no phenotypic abnormalities in several tissues including limb and kidney, which normally express both *Odd1* and *Osr2* mRNAs (Lan et al., 2001; 2004). Here we report that mouse embryos homozygous for a targeted null mutation in *Odd1* have specific defects in heart and kidney development. Our data identify *Odd1* as a new essential regulator of heart and urogenital development.

## Materials and Methods

### Generation of *Odd1*<sup>tm1RJ</sup> mutant mice

BAC clones containing the *Odd1* genomic region were isolated by screening the RPCI-22 129/SvEvTac mouse BAC library (BACPAC Resources, Children's Hospital of Oakland, Oakland, CA). Two adjacent BamHI fragments containing all three exons of the *Odd1* gene were subcloned from a BAC clone into the pBluescript II plasmid vectors (Fig. 1A). A targeting DNA construct, which contains a 2.1 kb 5' homology arm fused in-frame with a modified bacterial *lacZ* gene that encodes a nuclearly localized  $\beta$ -galactosidase, a loxP-flanked *neo* expression cassette, a 5.3 kb 3' homology arm, and a *PGK-DTA* expression cassette, was subcloned in the pBluescriptII KS(+) plasmid vector (Fig. 1A). Correct targeting of the *Odd1* locus with this construct would result in the replacement of 335 bp of the *Odd1* coding region with the *lacZ* gene, which is expected to produce a functional  $\beta$ -

galactosidase fusion protein containing the N-terminal 16 amino acid residues of the Odd1 protein.

The targeting vector was linearized and electroporated into CJ7 embryonic stem (ES) cells as previously described (Swiatek and Gridley, 1993). G418 resistant ES colonies were screened by Southern hybridization for homologous recombination. Correctly targeted ES clones were injected into blastocysts from C57BL/6J mice and the resultant chimeras bred with either C57BL/6J or ICR females. F1 mice were genotyped by Southern hybridization analysis of tail DNA. Mice and embryos from subsequent generations were genotyped by either Southern hybridization or PCR. PCR with Primer 1 (5'-GTC ATT CAC CCT AAG CCA GAG -3') and Primer 2 (5' GAA GCA TCA AGC CAA AGC AGC-3') amplifies a product of 450 bp from the wild-type *Odd1* allele. PCR with Primer 2 and Primer 3 (5'-CTT CTT GAC GAG TTC TTC TGA GG-3') amplifies a mutant allele-specific product of 760 bp. Heterozygous F1 mice were backcrossed to C57BL/6J and ICR mice, respectively to maintain the mutant mice in two different genetic backgrounds. Heterozygous mice from the backcross generations were intercrossed for analysis of homozygous phenotype.

### Histology, in situ hybridization and detection of $\beta$ -galactosidase activity

Embryos were dissected and DNA was prepared from yolk sac or tail biopsy samples for genotyping by PCR or Southern hybridization. For histology, embryos were fixed in Bouin's fixative, dehydrated through graded alcohols, embedded in paraffin, sectioned at 7  $\mu$ m thickness, and stained with hematoxylin and eosin. For in situ hybridization, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. Whole mount and sectioned in situ hybridization were performed as described previously (Lan et al., 2001). Staining for  $\beta$ -galactosidase activity in whole mount embryos and cryostat sections was performed using X-gal as described previously (Hogan et al., 1994). The sections were counterstained with eosin following X-gal staining.

### Detection of cell proliferation and apoptosis

For detection of cell proliferation in the intermediate mesoderm, timed pregnant *Odd1<sup>tm1R1/+</sup>* heterozygous females were injected intraperitoneally on gestational day 9.5 or 10.5 with BrdU (Roche) labeling reagent (3 mg/100g body weight). One hour after injection, embryos were dissected, fixed in Carnoy's fixative, dehydrated through graded alcohols, embedded in paraffin and sectioned at 5  $\mu$ m thickness. Immunodetection of BrdU was performed using the BrdU labeling and detection kit (Roche) according to manufacturer's instructions. Apoptotic cell death in the intermediate mesoderm was assessed by TUNEL labeling of either paraffin sections or cryostat sections using the in situ cell death detection kit (Roche) following manufacturer's instructions.

For detection of activated Caspase-3, paraffin sections were deparaffinized in xylene and hydrated through an ethanol series to distilled water. Antigen retrieval was carried out by boiling in Trilogy reagent (Cell Margue, hot Springs, AR) for 5 minutes, and peroxidase activity blocked in 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes followed by incubation in Histostain blocking reagent (Zymed laboratories) for 30 minutes. The sections were then incubated overnight with a monoclonal antibody specific for activated Caspase-3 (BD PharMingen) at 500 $\times$  dilution in PBS containing 0.2% skim milk and 0.3% Triton X-100, followed by incubation with biotinylated anti-rabbit secondary antibody and streptavidin-HRP (Zymed Laboratories), followed by 200 $\times$  dilution of biotinylated tyramide (Perkin Elmer) in PBS containing 0.05% Tween-20 and detected by Cy3-conjugated tyramide substrate (500 $\times$  dilution, Perkin Elmer). Washes were performed with PBS containing 0.5% Tween-20 at each step after the primary antibody incubation. Counterstaining of nuclei was carried out

with DAPI (1  $\mu\text{g/ml}$  in water) for one minute. The sections were washed with PBS and mounted with 50% glycerol and 20 mg/ml n-propyl gallate for fluorescence microscopy.

### Detection of blood flow

Embryos were dissected at E11.5 from timed pregnant *Odd1<sup>tm1RJ/+</sup>* heterozygous females. Yellow casting dye (Connecticut Valley Biological Supply Co. Inc., South Hampton, MA) or Indian ink was injected into the left ventricle of freshly dissected embryos by using pulled glass needles. Injected embryos were immediately fixed in 4% paraformaldehyde overnight, dehydrated in graded methanols, cleared in benzyl benzoate and benzyl alcohol (2:1) and photographed. Genotyping was carried out by allele-specific PCR using yolk sac genomic DNA as templates.

## Results

### Generation of *Odd1<sup>tm1RJ</sup>* mutant mice

To examine the function of the *Odd1* gene in mouse embryonic development, we inserted a *lacZ* gene into the first coding exon of the *Odd1* gene through homologous recombination in ES cells (Fig. 1A). The targeted allele, named *Odd1<sup>tm1RJ</sup>*, is expected to encode a functional  $\beta$ -galactosidase fusion protein containing the N-terminal 16 amino acid residues of the *Odd1* protein and result in loss of *Odd1* gene function. Chimeric animals were generated from correctly targeted ES cells and germ line transmission of the targeted *Odd1<sup>tm1RJ</sup>* allele was obtained. Southern hybridization analysis confirmed the expected structure of the targeted allele in mutant mice (Fig. 1B). Embryos heterozygous (*Odd1<sup>+/-</sup>*) for the *Odd1<sup>tm1RJ</sup>* allele were isolated and stained with X-gal to reveal domains of  $\beta$ -galactosidase activity. As shown in Fig. 2,  $\beta$ -galactosidase activity was detected specifically in the intermediate mesoderm cells at E7.5, shortly after they migrated away from the primitive streak (Fig. 2A). At E8.5,  $\beta$ -galactosidase expression is detected throughout the intermediate mesoderm caudal to the cardiac crescent (Fig. 2B). This intermediate mesoderm-specific expression of  $\beta$ -galactosidase activity in the *Odd1<sup>+/-</sup>* embryos is identical to the *Odd1* mRNA expression pattern at this stage (Fig. 2C, also see So and Danielian, 1999). By E9.5, expression of the  $\beta$ -galactosidase fusion protein in the caudal intermediate mesoderm is expanded to the somitic mesoderm (Fig. 2D, E). In addition, strong  $\beta$ -galactosidase activity is detected in the fore- and hindgut endoderm, in the lung bud mesenchyme as well as in the myocardial cells in the dorsal wall of the common atrial chamber at this stage (Fig. 2E, F). These expression patterns of the  $\beta$ -galactosidase fusion protein in the *Odd1<sup>+/-</sup>* embryos exactly match that of the *Odd1* mRNA detected by in situ hybridization of embryo sections (Fig. 6 and data not shown). By E10.5, expression of both *Odd1* mRNA and  $\beta$ -galactosidase fusion protein is activated in the developing branchial arches and limb buds in the heterozygous embryos (Fig. 2G, H). These data indicate that expression of the  $\beta$ -galactosidase fusion protein from the *Odd1<sup>tm1RJ</sup>* allele recapitulates the wild-type *Odd1* gene expression pattern and provides a good reporter of spatiotemporal *Odd1* gene activity during mouse embryogenesis.

### *Odd1<sup>-/-</sup>* mutants die prenatally with severe heart defects

Mice heterozygous for the *Odd1<sup>tm1RJ</sup>* allele are normal and fertile. Heterozygous animals were intercrossed and genotypes of their progeny were determined two weeks after birth. No mice homozygous for the mutation were found. Careful examination of staged embryos and newborn mice from heterozygous intercrosses revealed that most of the *Odd1<sup>-/-</sup>* homozygous mutants died between E11.5 and E12.5, judging from their tail somite numbers and limb bud morphology (Kaufman, 1992). Analyses of embryos at E12.0 showed that homozygous mutant embryos have blood pooled in the liver and major veins while their yolk sac lacked blood circulation (Fig. 3B, D). A few *Odd1<sup>-/-</sup>* mutant embryos (approximately 5% of all homozygotes) survived to late embryonic stages and all of these

exhibited generalized edema (Fig. 3F), indicating that these embryos were suffering from circulatory distress. All *Odd1*<sup>-/-</sup> mutant embryos examined at E11.5 by using histological analyses had severely dilated atria, lack of septum primum formation, and hypoplastic venous valves (Fig. 4B, D). All *Odd1*<sup>-/-</sup> mutant embryos that survived to late term also lacked the primary atrial septum and had dilated atria (Fig. 4F). These mutant embryos formed venous valves but the left leaflet of these valves appeared stumped and significantly reduced in size compared to that of heterozygous or wildtype littermates (Fig. 4H). In addition, these *Odd1*<sup>-/-</sup> embryos had malformed atrioventricular junction and ventricular septum (Fig. 4F), as well as clumped lung tissues and failure of mesothelium to form a complete parietal pericardium (Fig. 4F, H).

To investigate the cause of circulation defects and embryonic lethality, we injected yellow casting dye into the left ventricles of newly dissected E11.5 embryos to trace blood flow and found that the *Odd1*<sup>-/-</sup> mutant embryos exhibited blood backflow from the ventricle into the atria and the inflow tract (Fig. 5). We also injected diluted Indian ink into E11.5 embryonic ventricles to trace blood flow and found that all homozygous mutant embryos had the blood backflow problem whereas control wildtype and heterozygous embryos rarely exhibited backflow of either the yellow dye or the Indian ink into the inflow tract (data not shown). These data indicate that the defective atrioventricular junction and venous valves in the *Odd1*<sup>-/-</sup> mutants are the most likely cause of circulation distress and embryonic lethality.

### Defects in heart development in *Odd1*<sup>-/-</sup> mutant embryos correlate with the normal developmental pattern of *Odd1* mRNA expression

It has not previously been reported whether *Odd1* mRNA is expressed during heart development. To investigate whether the heart defects in the homozygous mutant embryos are due to primary roles of *Odd1* in heart development, we analyzed *Odd1* mRNA expression at different stages of heart morphogenesis by using in situ hybridization analyses of serial sections. As shown in Fig. 6, *Odd1* mRNA is highly expressed in the dorsal atrial myocardium as early as E9.5 during normal mouse embryogenesis (Fig. 6A). By E10.5, the septum primum grows into the common atrial chamber from the dorsal midline. *Odd1* mRNA is strongly expressed in the developing septum primum and the flanking dorsal atrial wall, with the lateral boundary of expression coinciding with the entrance of the cardinal vein (Fig. 6B, C). By E11.5, the venous valve leaflets are clearly visible on transverse and sagittal sections through the atrial chamber in normal embryos. *Odd1* mRNA is strongly expressed in the septum primum and the flanking dorsal wall, including the left leaflet of the venous valves (Fig. 6D, E). Strong *Odd1* mRNA expression is also detected in the outflow tract mesenchyme at this stage (Fig. 6E). At E13.5, in addition to expression in the dorsal atrial wall and the outflow tract, strong *Odd1* mRNA expression is observed throughout the mesothelium lining the thoracic cavity, including the newly formed parietal pericardium (Fig. 6F). *Odd1* mRNA expression also persists in the developing lung mesenchyme but at reduced levels compared with earlier stages (Fig. 6F). Thus, the defects in atrial septum and venous valve formation in the *Odd1*<sup>-/-</sup> mutants correlate well with the spatiotemporal pattern of *Odd1* mRNA expression during normal heart development.

Defects in atrial septum development have been reported in association with disruptions of left-right asymmetry of the heart (Icardo and Sanchez de Vega, 1991; Gage et al., 1999). In particular, *Pitx2* mRNA is normally expressed only in the left but not in the right atrial myocardium and mouse embryos homozygous for a null mutation in *Pitx2* lack atrial septum formation and exhibit venous valve defects (Gage et al., 1999). The atrial septum myocardium also expresses *Pitx2* mRNA, indicating that it is a left atrial structure (Fig. 7A). Whereas the *Odd1*<sup>-/-</sup> mutant heart lacks atrial septum, *Pitx2* mRNA is correctly expressed

in the left but not in the right atrial myocardium (Fig. 7B). Several other transcription factors, including *Nkx2.5* and *Tbx5*, have been previously shown to play essential roles in human and mouse atrial septum development (Basson et al., 1997; Li et al., 1997; Schott et al., 1998; Biben et al., 2000). *Nkx2.5* mRNA is expressed throughout the myocardial cells, including in the atrial septum and venous valves (Fig. 7C). *Nkx2* mRNA expression pattern is unchanged in the *Odd1*<sup>-/-</sup> mutant embryos except for the lack of the atrial septum structure (Fig. 7D). Similarly, expression patterns of *Gata4* and *Tbx5* mRNAs are unaltered in *Odd1*<sup>-/-</sup> mutant hearts compared with that of the wildtype littermates (data not shown). These data indicate that *Odd1* acts in a novel molecular pathway to regulate atrial septum formation. We also examined the expression pattern of *Tbx18*, an early epicardial marker, and found that its expression is unaltered either during heart development in the *Odd1*<sup>-/-</sup> mutants compared with that of the wildtype littermates (Fig. 7E, F), indicating that the parietal pericardial defect seen at later embryonic stages is not a result of early epicardial defect.

### Odd1 is required for intermediate mesoderm and kidney development

*Odd1* mRNA expression is the earliest molecular marker of the intermediate mesoderm compartment in the mouse embryo. The intermediate mesoderm gives rise to the kidneys and gonads. From anterior to posterior, the intermediate mesoderm differentiates into three types of nephric tissues: the pronephros, mesonephros, and metanephros. We carried out detailed histological analyses of five *Odd1*<sup>-/-</sup> mutant embryos that survived to late gestation (15.5 – 17.5 dpc). All five *Odd1*<sup>-/-</sup> mutants completely lacked metanephric kidneys and gonads (Fig. 8B, D). Detailed histological analyses of embryos from E10.5 to E11.5 showed that the *Odd1*<sup>-/-</sup> mutants never initiated metanephric kidney formation: no signs of metanephric mesenchyme condensation or ureteric bud formation were observed in *Odd1*<sup>-/-</sup> mutants (Fig. 9B, D). Compared with wildtype and heterozygous littermates, there are also fewer mesonephric tubules and the gonadal ridge is retarded in the mutant embryos at E11.5 (Fig. 9F). In addition, the Wolffian duct is often malformed or absent in the posterior trunk region of the mutant embryos (Fig. 9D, F). To investigate further the etiology of kidney developmental defects in the *Odd1*<sup>-/-</sup> mutants, we carried out detailed molecular marker expression analyses of embryos at E9.5 and E10.5, before the *Odd1*<sup>-/-</sup> mutant embryos exhibited any signs of circulatory distress. At E10.5, the ureteric buds, which are specifically marked by *Ret* mRNA expression, branch off from the caudal ends of the Wolffian ducts and grow into the metanephrogenic mesenchyme on both sides of the wild-type embryos (Fig. 10A, C). In contrast, no comparable *Ret*-expressing ureteric bud structures form in the *Odd1*<sup>-/-</sup> mutant embryos (Fig. 10B, D), consistent with the histology data (Fig. 9D). Both *Lhx1* (also known as *Lim1*) and *Pax2* are expressed in the Wolffian duct and mesonephric vesicles in wildtype and heterozygous embryos at E10.5 (Fig. 10E, G, I, K). In contrast, the *Odd1*<sup>-/-</sup> mutant embryos at this stage show much fewer *Lhx1*- and *Pax2*-expressing mesonephric vesicles and disrupted or posteriorly truncated Wolffian ducts (Fig. 10F, H, J, L). Examination of expression of *Ret*, *Lhx1*, and *Pax2*, which are all expressed in the Wolffian duct epithelial cells at E9.5, showed that Wolffian duct development is already disrupted in the mutant embryos at this early stage compared with wildtype and heterozygous littermates (Fig. 11). Interestingly, the Wolffian duct defect is always more severe on the left side than on the right side of the *Odd1*<sup>-/-</sup> mutant embryo such that the left Wolffian duct is often truncated posteriorly (Fig. 11B, F, J). On the other hand, expression of *Wtl*, which is normally expressed throughout the nephrogenic mesenchyme as early as E9.0 and its loss of function also causes agenesis of adrenal glands, kidneys and gonads as well as defective parietal pericardium (Armstrong et al., 1993; Kreidberg et al., 1993; Moore et al., 1999), is significantly down-regulated throughout the anteroposterior axis of the *Odd1*<sup>-/-</sup> mutant embryos but its expression domain is not truncated posteriorly and does not show left-right asymmetry (Fig. 11N, P). The overlaps in developmental expression patterns

and in mutant phenotypes indicate that *Odd1* and *Wt1* normally function in a common molecular pathway to regulate urogenital development.

Disruptions of early kidney development often cause apoptosis of the nephrogenic mesenchyme (e.g., Fernandez-Teran et al., 1997; Bouchard et al., 2000; 2002; Ostrom et al., 2000). Thus, we analyzed patterns of apoptosis in E9.5 and E10.5 embryos. Compared with wildtype littermates, the *Odd1*<sup>-/-</sup> mutant embryos showed massive cell death in the nephrogenic mesenchyme by E10.5 as detected by TUNEL assay (Fig. 12A, B). Moreover, we detected significant numbers of cells expressing activated Caspase-3, a marker for early stages of apoptosis, specifically in the nephrogenic mesenchyme in *Odd1*<sup>-/-</sup> mutant embryos at E9.5 (Fig. 12D). Together with the data showing down-regulation of early kidney developmental genes in the homozygous mutants (Fig. 10, Fig. 11), these data indicate that *Odd1* deficiency prevents the intermediate mesoderm from following the normal nephrogenic differentiation program and leads to apoptosis specifically in this tissue.

## Discussion

Defects in heart development account for the most common birth defects and are the leading cause of birth defect-related deaths. Whereas several transcription factors essential for heart development have been identified recently through genetic studies, the molecular mechanisms controlling atrial septum and venous valve development are not well understood (Vaughan and Basson, 2001). During mammalian heart development, the systemic venous tributaries initially join with the primary atrium through the sinus venosus from both sides of the embryo. During human embryogenesis, the left horn of the sinus venosus diminishes in size and becomes incorporated into the left atrioventricular junction as the coronary sinus in subsequent developmental stages while left anterior cardinal vein becomes connected to the right horn of sinus venosus via the newly formed brachiocephalic vein to drain into the right side of the primary atrium (Anderson et al., 2002; Kaufman and Richardson, 2005). In mice, however, the brachiocephalic vein does not form and the left horn of the sinus venosus persists and drains via the coronary sinus into the post-hepatic part of the inferior vena cava (Kaufman and Richardson, 2005). Despite the species differences, the venous system rearrangement in both species results in the entire systemic venous compartment draining into the right side of the atrium. As the venous system rearrangements occur, venous valves form at the junction between the venous compartment and the primary atrium to prevent blood backflow (Anderson et al., 2002). As heart growth and development continue, the primary atrial septum, also called septum primum, grows into the atrial chamber from the dorsal midline. In this paper, we show that mouse embryos homozygous for a targeted null mutation in the *Odd1* gene lack the primary atrial septum and have hypoplastic venous valves. These heart defects correlate with specific *Odd1* mRNA expression in the dorsal atrial wall, including the developing primary atrial septum and the venous valve, indicating that *Odd1* plays critical roles during normal atrial septum and venous valve morphogenesis. However, examination of the expression patterns of several transcription factor genes known to be essential for atrial septum development, including *Nkx2.5*, *Pitx2*, and *Tbx5*, showed that atrial myocardial differentiation and left-right patterning are unaltered, indicating that *Odd1* does not regulate the expression of these known atrial septum regulators. *Odd1* is a mouse homolog of the *Drosophila* Odd-skipped family transcription factors, which include *Odd*, *Bowl*, *Sob*, and *Drm* (Coulter et al., 1990; Hart et al., 1996; Wang and Coulter, 1996; So and Danielian, 1999; Lan et al., 2001; Green et al., 2002). Interestingly, both *Bowl* and *Drm* are required for the development of the proventriculus, a valve-like structure at the foregut-midgut junction that regulates food passage in the *Drosophila* embryo (Pankratz and Hoch, 1995; Wang and Coulter, 1996; Campos-Ortega and Hartenstein, 1997; Liu et al., 1999; Green et al., 2002; Johansen et al., 2003, reviewed by Myat, 2005). Proventriculus development begins when cells at the

posterior boundary of the foregut tube evaginate to form a keyhole structure. In subsequent developmental stages, the keyhole epithelium folds back over the foregut to form the valve-like structure (Pankratz and Hoch, 1995; Campos-Ortega and Hartenstein, 1997; Johansen et al., 2003; Myat, 2005). All four members of the *Drosophila odd-skipped* family genes are expressed in the keyhole epithelium and mutations in either *bowl* or *drm* disrupt keyhole formation (Wang and Coulter, 1996; Johansen et al., 2003), suggesting that Bowl and Drm normally regulates the cellular processes of foregut evagination during proventriculus development. Further evidence that the Odd-skipped family transcription factors regulate morphogenetic processes came from studies of *Drosophila* leg joint formation. Hao et al. (2003) showed that *bowl* mutant clones disrupted epithelial invagination processes during leg joint formation and that ectopic expression of any one of the four *odd-skipped* family genes induced invaginations in the leg disc epithelium characteristic of endogenous invaginating leg joint cells. Remarkably, formation of both the atrial septum and venous valves in mammals involve morphogenetic tissue folding: the atrial septum is initiated by protrusion of the dorsal mesocardium, the extracardiac mesoderm at the dorsal midline, into the primary atrial chamber whereas formation of the venous valves involves protrusion of the sinuatrial foramen into the lumen of the atrium during heart development in both mice and human (Webb et al., 1998; Anderson et al., 1999; Wessels et al., 2000; Lamers and Moorman, 2002). The expression pattern of *Odd1* mRNA in the dorsal atrial wall and extracardiac mesoderm as well as in the developing venous valve, together with the defects in the development of these structures in the *Odd1*<sup>-/-</sup> mutants suggest that the Odd-skipped family transcription factors act in an evolutionarily conserved molecular pathway in valve formation, including the morphogenesis of proventriculus in insects and the atrial septum and venous valves in vertebrates.

The identification of Odd1 as an essential regulator of atrial septum development provides a new foundation for characterization of the molecular pathways regulating atrial morphogenesis. One particularly disputed area in studies of atrial development is with regard to the role of extracardiac mesoderm in atrial septum formation. van Gils (1981) described the leading edge of the atrial septum is formed from extracardiac source as “an ingrowth of tissue from the dorsal mesocardium”. However, Arrechedera et al. (1987) and Gerety and Watanabe (1997), through structural and histochemical analyses, suggested that the septum primum mesenchymal tissue originated from epitheliomesenchymal transformation from endocardial cells, similar to that seen for the atrioventricular cushions. Wessels et al. (2000) carried out detailed spatiotemporal immunohistochemical analyses of human atrial development and showed that the dorsal mesocardium protrudes into the dorsal wall of the primary atrium and is continuous with the mesenchymal cap on the leading edge of the developing primary atrial septum. Although it was shown that neither the mesenchymal tissues of the dorsal protrusion nor the mesenchymal cap of the primary atrial septum expressed a specific endocardial tissue antigen at all developmental stages investigated, Wessels et al (2000) suggested that the cells in the dorsal protrusion could originate from either endocardial or extracardiac tissues. Since Odd1 is expressed in both the dorsal atrial myocardium and the extracardiac somatic mesoderm before atrial septum formation, failure of atrial septum formation in the *Odd1*<sup>-/-</sup> mutant embryos may result from defects in either the extracardiac dorsal mesocardium or the dorsal atrial myocardium. Future studies using tissue-specific gene inactivation approaches will enable characterization of the roles of the Odd1 molecular pathway in cardiac and extracardiac mesenchyme, respectively during atrial septum development.

We show that *Odd1* gene expression is first activated in the nascent intermediate mesoderm shortly after their migration from the primitive streak at the midgastrulation stage during mouse embryonic development. The intermediate mesoderm lies between the paraxial and lateral mesoderms and undergoes extensive epithelial transformation to generate the ducts



and tubules of the urogenital tracts and kidneys (Saxen, 1987). Previously, it was demonstrated that *Odd1* mRNA expression marks the intermediate mesoderm fate in normal and mutant mice (Wilm et al., 2004). Our mutation analysis indicates that *Odd1* is not required for intermediate mesoderm specification. Rather, it is involved in the control of intermediate mesoderm differentiation, as derivatives of the intermediate mesoderm including the nephric ducts, mesonephros, metanephros and gonads are either defective or absent in the *Odd1*<sup>-/-</sup> mutants. Gene targeting studies in mice have shown that many transcription factors, including *Lhx1*, *Pax2*, *Wt1*, *Emx2*, *Sall1* and the *Hox11* paralogs (Kreidberg et al., 1993; Shawlot and Behringer, 1995; Torres et al., 1995; Miyamoto et al., 1997; Tsang et al., 2000; Nishinakamura et al., 2001; Bouchard et al., 2002; Wellik et al., 2002), as well as several signaling molecules, such as the GDNF-Ret ligand-receptor pair, *Wnt4*, and *Wnt11* (Stark et al., 1994; Schuchardt et al., 1994; 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Majumdar et al., 2003), are essential for normal kidney development. We show that the expression of *Lhx1*, *Pax2*, and *Wt1* is each significantly reduced in the *Odd1*<sup>-/-</sup> mutant embryos as early as E9.5, indicating that *Odd1* acts upstream of these known transcription factors in regulating early urogenital development. At E10.5, while wildtype and heterozygous littermates have initiated ureteric bud outgrowth into the metanephric mesenchyme, the *Odd1*<sup>-/-</sup> homozygous mutant embryos lacked metanephric mesenchyme condensation and ureteric bud induction. Instead, the *Odd1*<sup>-/-</sup> mutant nephrogenic mesenchyme initiates Caspase-3 activation at E9.5 and undergoes massive apoptosis by E10.5. It has been reported that disruption of nephric duct elongation results in nephrogenic mesenchyme cell death due to lack of induction (Fernandez-Teran et al., 1997). Furthermore, a two-fold reduced expression of *Pax2* in *Pax2*<sup>+/-</sup> embryos caused significant nephrogenic mesenchyme death whereas complete absence of *Pax2* and *Pax8* resulted in massive death of the intermediate mesoderm as early as E9.5 (Bouchard et al., 2000; 2002; Ostrom et al., 2000). Thus, the cell death of the nephrogenic mesenchyme in the *Odd1*<sup>-/-</sup> mutants is most likely an indirect effect of reduced *Pax2* gene expression and/or disrupted nephric duct development. Our data, demonstrating that *Odd1* is expressed from the earliest stage of intermediate development and required for nephrogenic mesenchyme differentiation, suggest that *Odd1* acts as a competence factor in the control of normal intermediate mesoderm development.

The *Odd1*<sup>-/-</sup> mutant phenotype is strikingly similar to that of the *Wt1*<sup>-/-</sup> mutant embryos. In addition to lack of ureteric bud induction and gonad development, both *Odd1*<sup>-/-</sup> and *Wt1*<sup>-/-</sup> mutant embryos have fewer mesonephric tubules at E11.5 as well as generalized edema, clumped lungs, failure of mesothelium to form a complete parietal pericardium, and lack of adrenal glands at later stages of embryonic development (Kreidberg et al., 1993; Moore et al., 1999; Hammer et al., 2005; and this study). *Wt1*, originally identified as a Wilms' tumor suppressor gene, encodes a transcription factor with four zinc finger domains (Call et al., 1990; Gessler et al., 1990). The earliest expression of *Wt1* mRNA during mouse embryogenesis was detected in the intermediate mesoderm at E9.0 and it is subsequently expressed in many tissues, including nephrogenic condensations, spinal cord, brain, and the differentiating mesothelium (Armstrong et al., 1993; Moore et al., 1999). We showed that *Wt1* mRNA expression is significantly down-regulated throughout the intermediate mesoderm in *Odd1*<sup>-/-</sup> mutant embryos at E9.5. Since *Odd1* expression is activated prior to the earliest stage of *Wt1* in the intermediate mesoderm (Armstrong et al., 1993, and this study), the down-regulation of *Wt1* mRNA expression in the *Odd1*<sup>-/-</sup> mutant embryos and the phenotypic similarities in urogenital, pericardial and adrenal gland development between the *Odd1*<sup>-/-</sup> and *Wt1*<sup>-/-</sup> mutants suggest that *Odd1* acts upstream of *Wt1* in the transcriptional network controlling normal intermediate mesoderm differentiation. The fact that nephrogenesis is blocked earlier in the *Odd1*<sup>-/-</sup> mutants than that reported for the *Wt1*<sup>-/-</sup> mutants, for instance metanephric mesenchyme condensation occurred in the *Wt1*<sup>-/-</sup> mutants but not in the *Odd1*<sup>-/-</sup> mutants, is consistent with this hypothesis. Alternatively, it

is possible that Odd1 and Wt1 normally interact with each other to regulate intermediate mesoderm differentiation such that lack of either factor results in similar defects in urogenital and pericardial development. The fact that two *Drosophila* homologs, Drm and Bowl interact with other transcription factors through their first zinc finger domain that is conserved in all invertebrate and vertebrate Odd-skipped family members suggest that the vertebrate Odd-skipped class transcription factors may also function through protein-protein interactions with other transcription factors (Green et al., 2002; Buckley et al., 2004; Hatini et al., 2005). Since mutations in *WT1* have been associated with multiple urogenital malformations and Wilms' tumors in humans (reviewed in Pritchard-Jones, 1999; Chesney, 2002, and references therein), further characterization of the role of Odd1 and its interaction with Wt1 will provide new insights into the molecular mechanisms underlying normal urogenital development as well as tumor formation.

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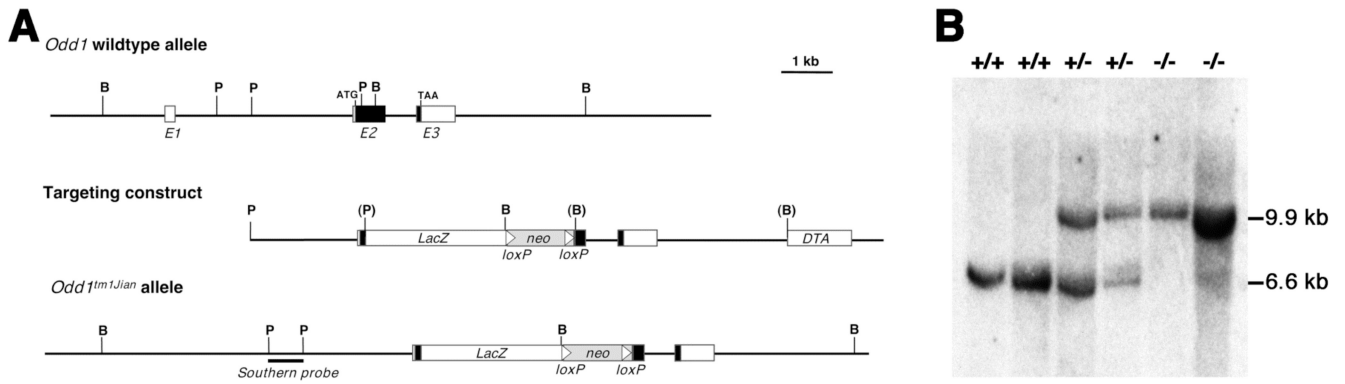
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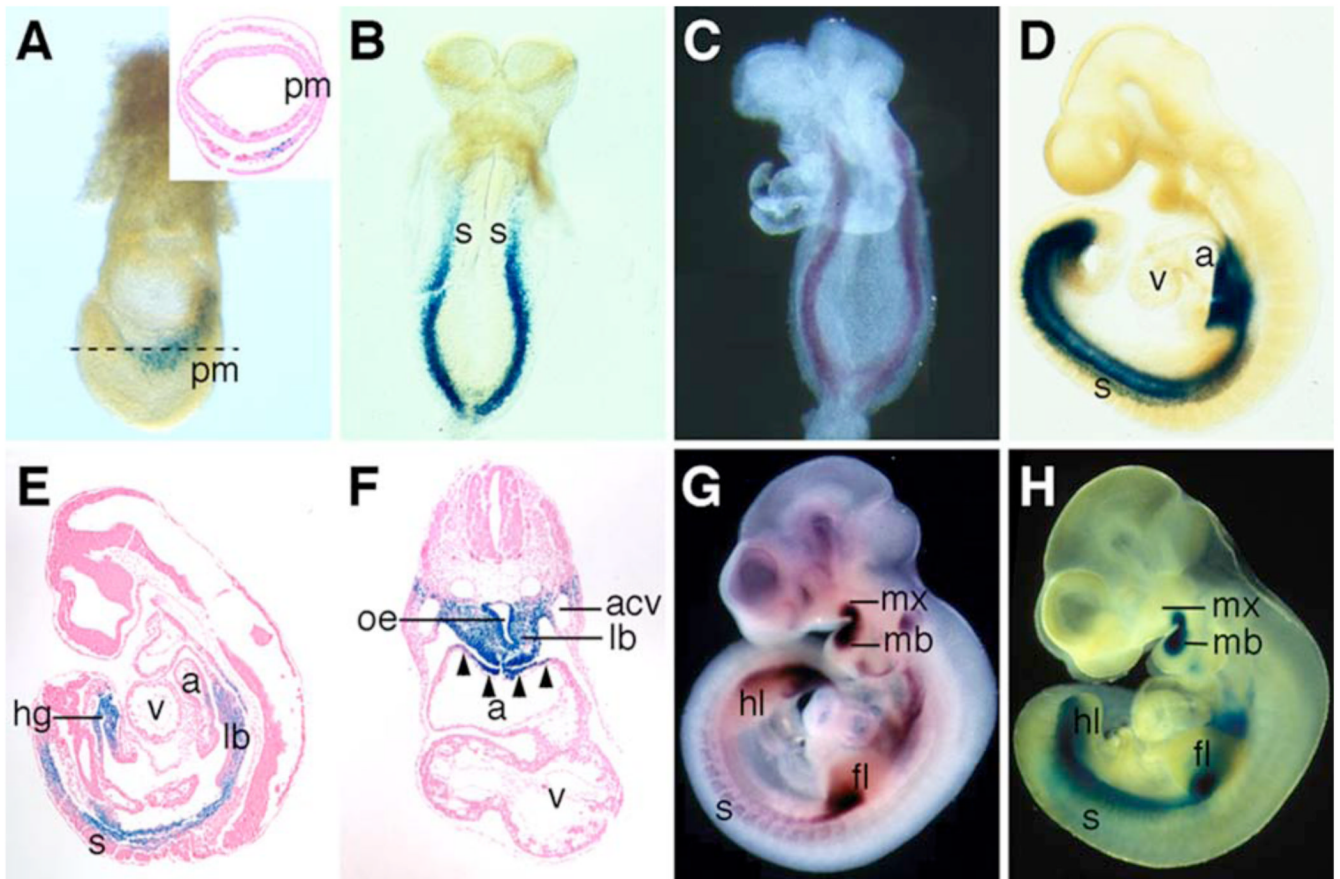
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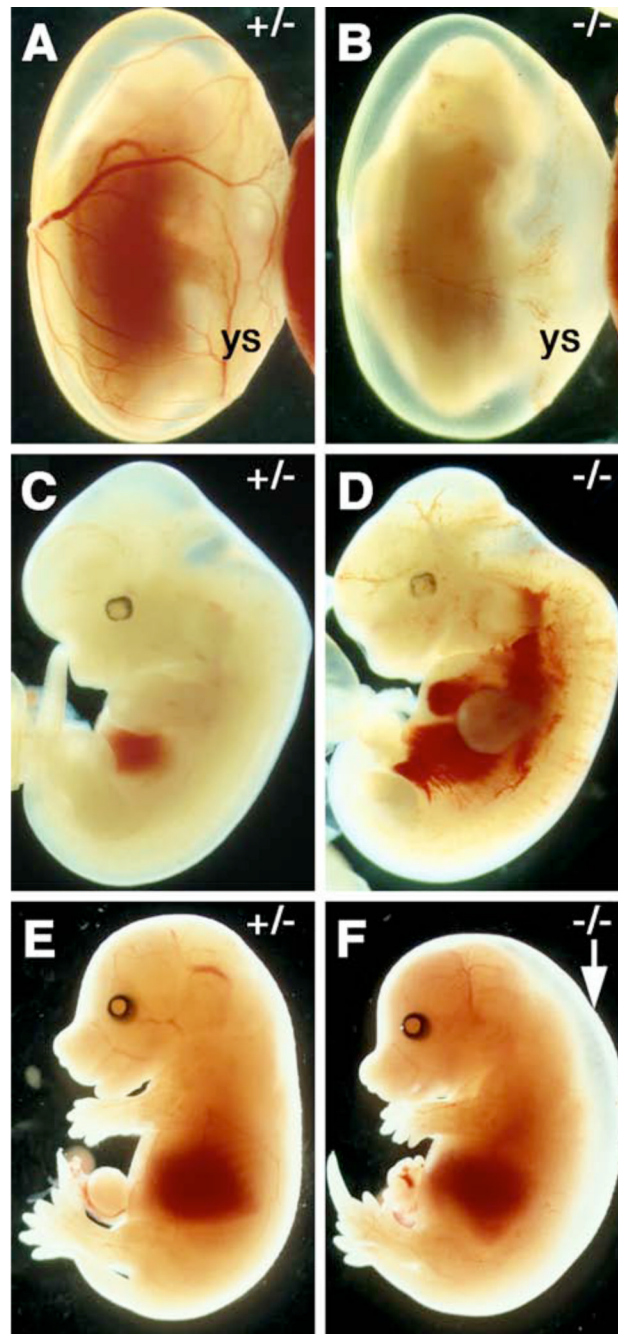
**Fig. 1.**

Targeted disruption of the mouse *Odd1* gene. (A) The *Odd1* gene consists of three exons spanning approximately 6 kb of genomic DNA. Boxes indicate exons, with the protein-coding region marked in black. The positions of the translation start (ATG) and stop (TAA) codons are also indicated. Restriction sites are: B, BamHI; P, PstI. The targeting vector used the 2.1 kb PstI fragment containing the intron 1/exon 2 junction as the 5' arm and the 5.3 kb BamHI fragment located 335 bp downstream as the 3' arm. A modified bacterial *lacZ* gene and a loxP-flanked *neo* expression cassette were inserted in between the arms and a diphtheria toxin A (*DTA*) expression cassette was cloned 3' to the 3' arm for negative selection against random integration. (B) Southern hybridization analysis of E11.5 embryonic DNA samples from a litter of F1 heterozygous intercross. The genomic DNA samples were digested with BamHI, separated by electrophoresis through a 0.8% agarose gel, transferred onto a Zetaprobe nylon memberane (BioRad), and hybridized with random prime-labeled probes made from the 700 bp PstI fragment isolated from the *Odd1* genomic region 5' to the targeted region. The 6.6 kb BamHI fragment corresponding to the wildtype allele was detected in wildtype and heterozygous embryos, while the 9.9 kb mutant allele-specific fragment was only detected in heterozygous and homozygous mutants. +/+, wildtype; +/-, heterozygous; -/-, homozygous mutant.



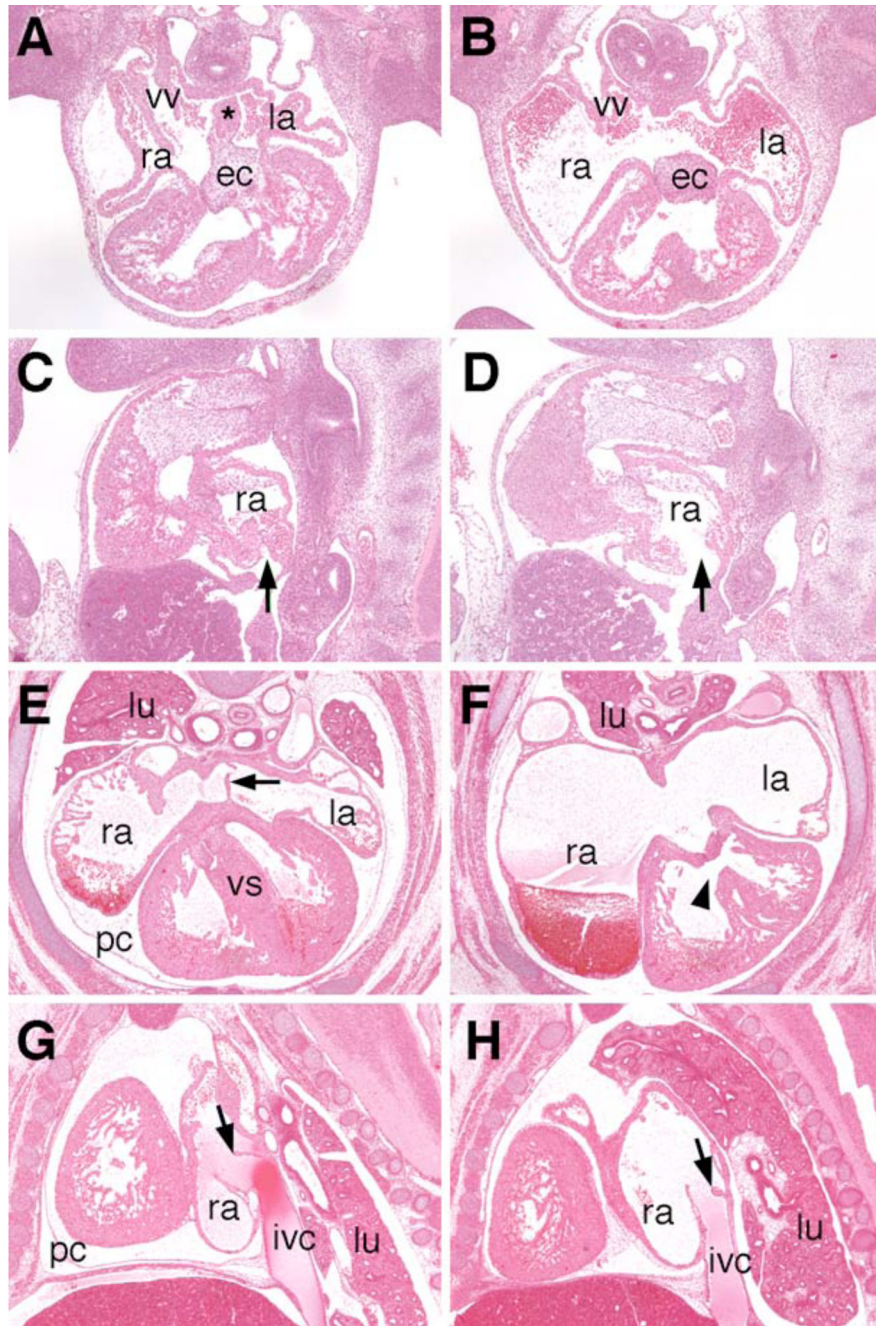
**Fig. 2.**

$\beta$ -galactosidase fusion protein expression from the *Odd1<sup>tm1Jian</sup>* allele recapitulates *Odd1* mRNA expression pattern during mouse embryogenesis. (A) Whole mount X-gal staining shows strong *Odd1*-LacZ expression (blue color) in a 7.5 dpc mouse embryo. The inset is a transverse section of a X-gal stained E7.5 embryo, which shows *Odd1*-LacZ expression in a few cells mixed with non-expressing cells in the intermediate region of the mesoderm layer. No staining is detected in the primitive streak cells. (B) At E8.5, *Odd1*-LacZ is expressed throughout the intermediate mesoderm caudal to the cardiac crescent. The expression pattern is identical to *Odd1* mRNA expression pattern at this stage (C). (D) By E9.5, *Odd1*-LacZ expression is detected, in addition to the intermediate mesoderm, at the dorsal region of the atrium and in the caudal somites. (E,F) Sagittal and transverse sections, respectively of E9.5 heterozygous embryos stained with X-gal. Strong *Odd1*-LacZ expression is detected in the lung bud mesenchyme, the fore- and hindgut endoderm, and in the dorsal atrial wall. (G,H) By E10.5, both *Odd1*-LacZ (G) and *Odd1* mRNA are expressed in identical patterns in the first branchial arches, limb buds, and somites. a, atrium; acv, anterior cardinal vein; fl, forelimb bud; hg, hindgut; hl, hindlimb bud; lb, lung bud; mb, mandibular process; mx, maxillary process; oe, oesophagus; pm, primitive streak; s, somite; v, ventricle.



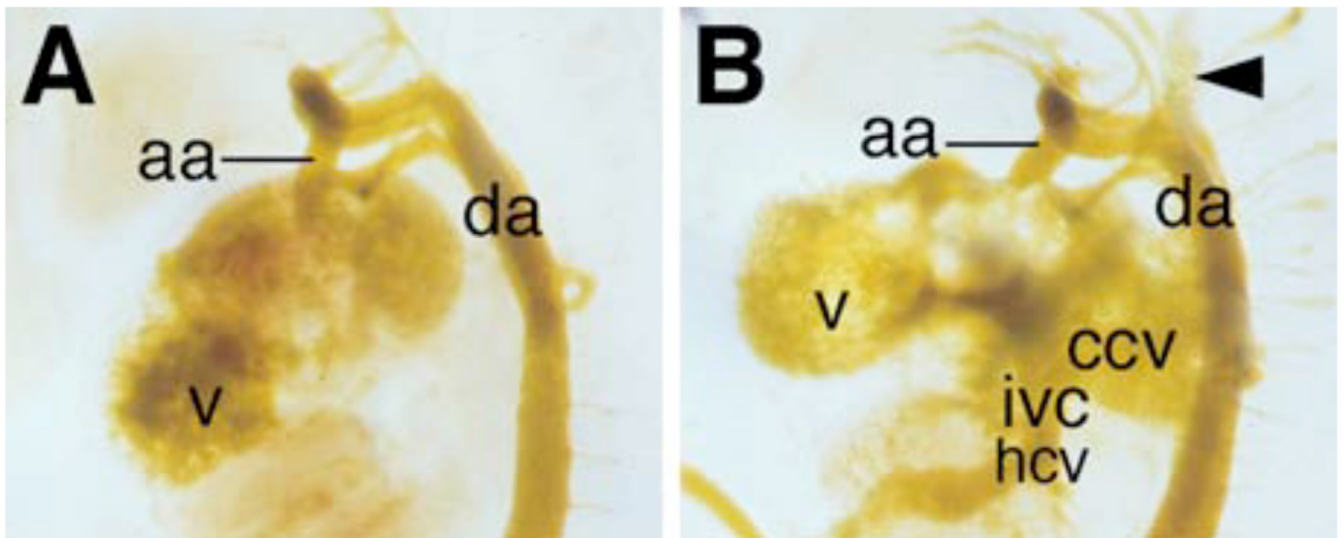
**Fig. 3.** *Odd1* homozygous mutant embryos die prenatally. (A, C, E) newly dissected *Odd1*<sup>+/+</sup> and (B, D, F) *Odd1*<sup>-/-</sup> mutant embryos. Most *Odd1*<sup>-/-</sup> mutant embryos die at E12.0 (B) with blood pooled in the heart and major veins (D). (F) An E15.5 *Odd1*<sup>-/-</sup> mutant embryo showing generalized edema (arrow). ys, yolk sac.



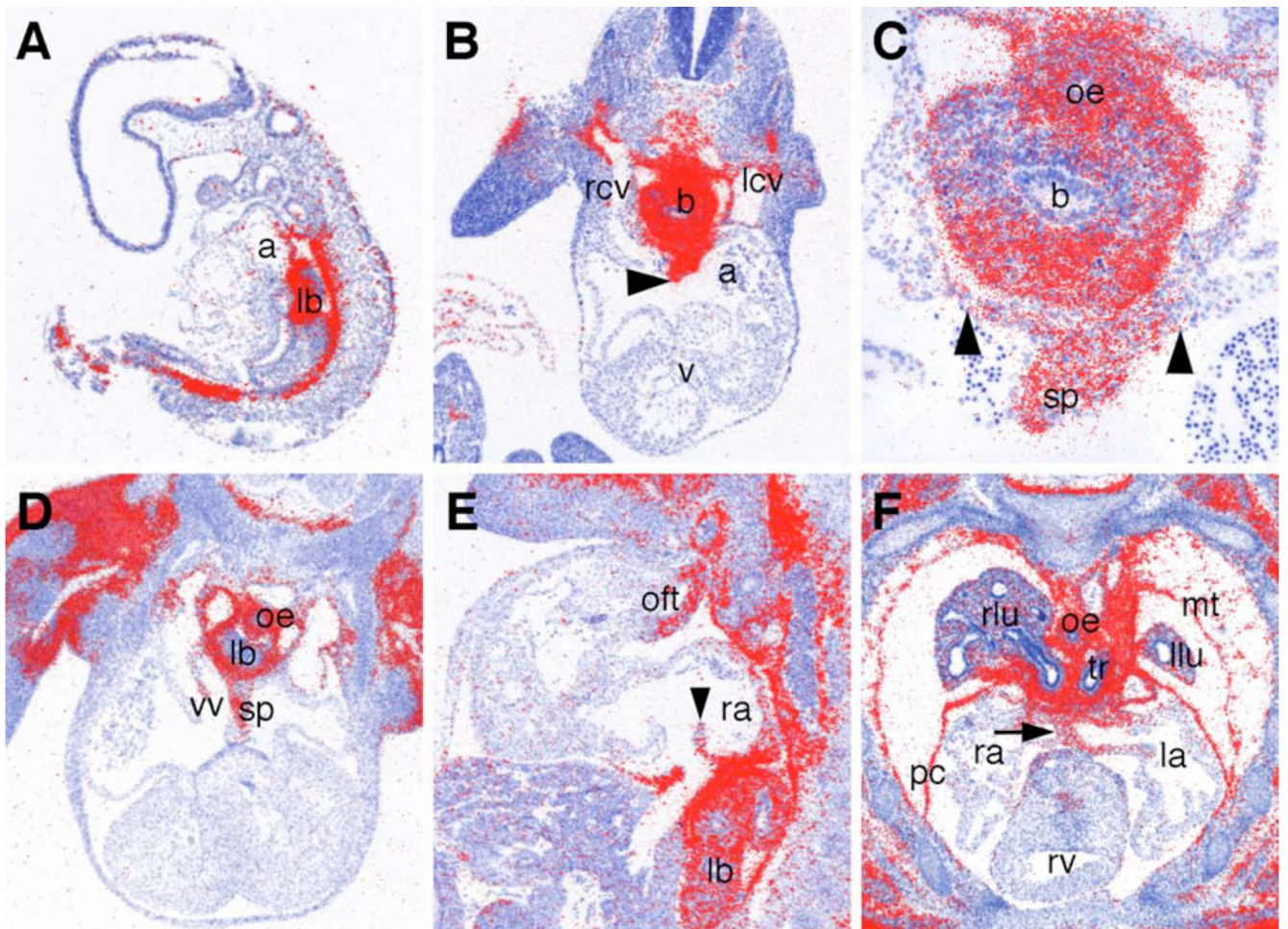


**Fig. 4.** Histological analyses of *Odd1* mutant heart defects. (A, B) Transverse sections of the wild-type and *Odd*<sup>-/-</sup> mutant embryos at E11.5 showing absence of septum primum (marked by the asterisk in A) and dilated atria in the *Odd*<sup>-/-</sup> mutant. (C, D) Sagittal sections of *Odd*<sup>+/-</sup> (C) and *Odd*<sup>-/-</sup> mutant (D) embryos at E11.5 show absence of venous valve in the homozygous mutant. The entrance of the inferior vena cava is wide open in the homozygous mutant (arrow in D). (E, F) Transverse sections of *Odd*<sup>+/-</sup> (E) and *Odd*<sup>-/-</sup> mutant (F) embryos at E15.5. Arrow in E points to the primary atrial septum, which is absent in the *Odd*<sup>-/-</sup> mutant heart (F). The *Odd*<sup>-/-</sup> mutant (F) also had severely dilated atria, defective

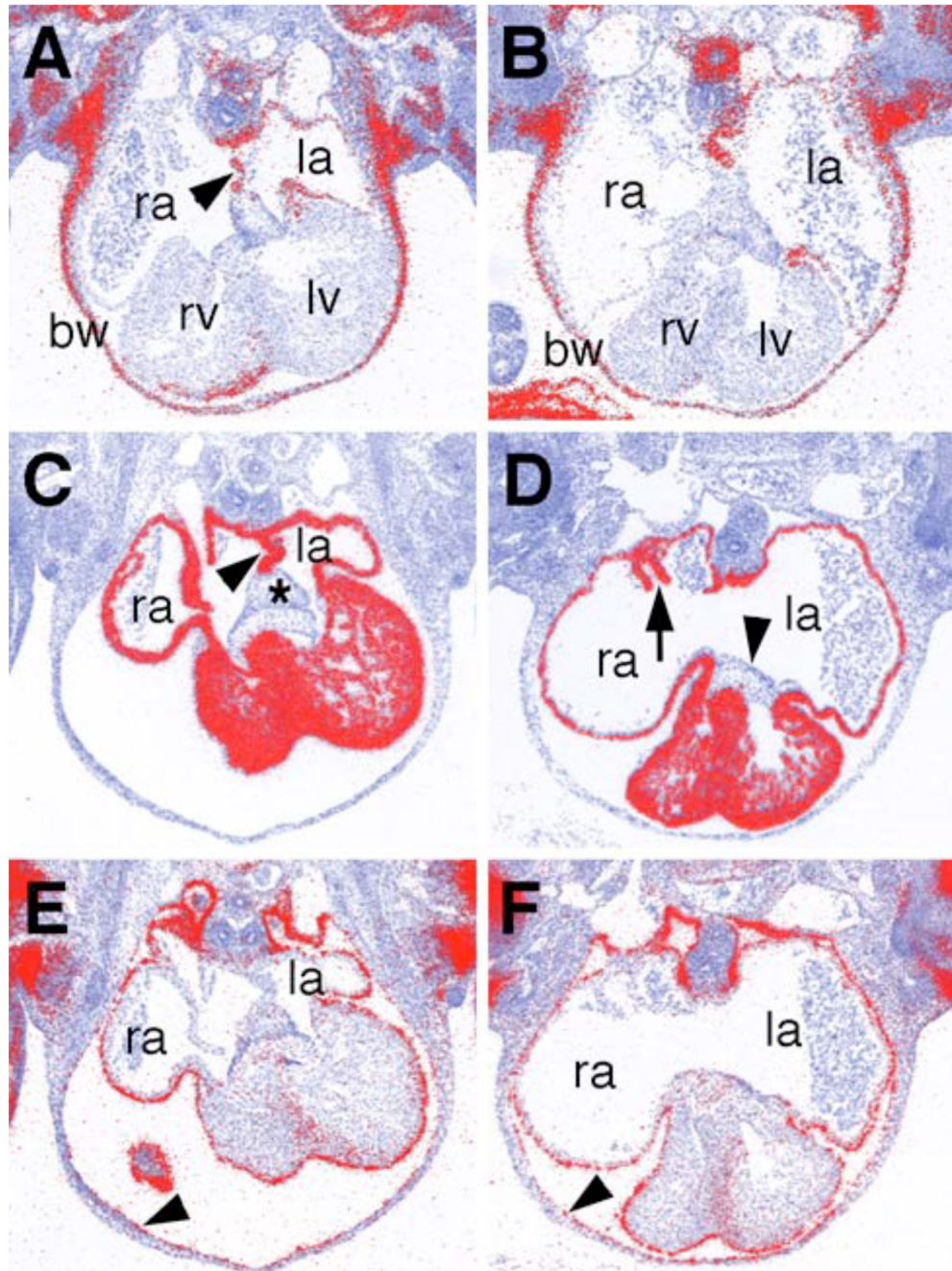
atrioventricular junction and ventricular septum (arrowhead), as well as lack of parietal pericardium surrounding the ventricles. (G,H) Sagittal sections of wildtype (G) and *Odd*<sup>-/-</sup> mutant (H) embryos show malformed venous valve at the entrance of the inferior vena cava. The left leaflet of the venous valve in the mutant heart is stumped and less than half the length of that in the control embryos (arrows in G and H). The lung tissues are clumped together in the homozygous mutants (F,H) compared to the well-extended left and right lungs in the wildtype (E) and heterozygous (G) embryos. ec, endocardial cushion; ivc, inferior vena cava; la, left atrium; lu, lung; pc, pericardium; ra, right atrium; vv, venous valve.



**Fig. 5.** *Odd1*<sup>-/-</sup> mutant embryos suffer blood backflow from the heart into the inflow tract. (A) An E11.5 wildtype embryo showing normal blood flow from the heart into the outflow tract as traced by yellow casting dye. (B) An *Odd1*<sup>-/-</sup> littermate of the embryo shown in A was similarly injected with yellow casting dye into the left ventricle. Although most of casting dye followed blood flow into the aortas, a significant amount of the dye backflowed into the common cardinal vein, the anterior cardinal vein (arrowhead), and hepato-cardiac vein. aa, ascending aorta; ccv, common cardinal vein; da, dorsal aorta; hcv, hepato-cardiac vein; ivc, inferior vena cava; v, ventricle.

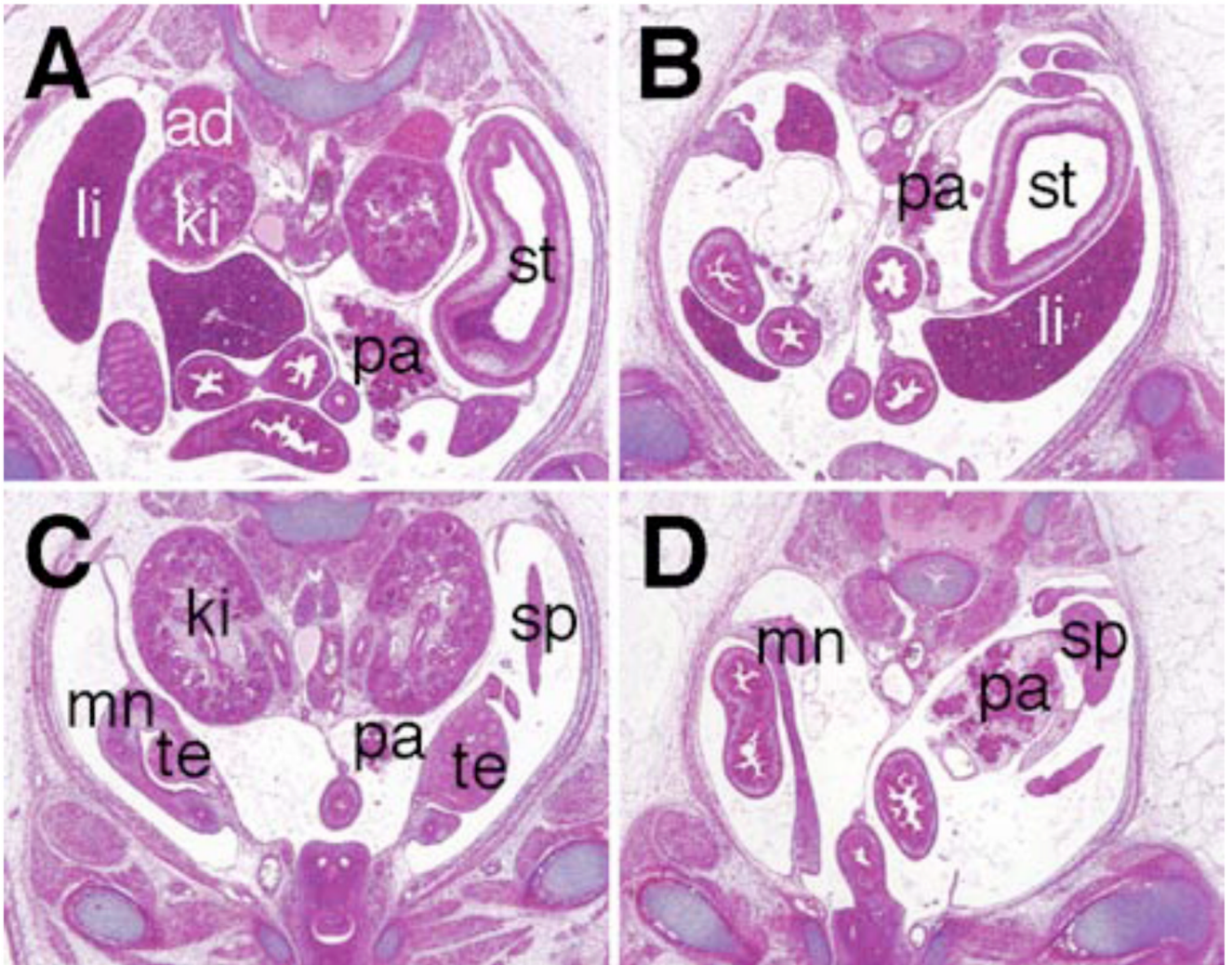


**Fig. 6.** *Odd1* mRNA expression during heart development. (A) Sagittal section of an E9.5 embryo showing *Odd1* mRNA expression (red color) in the dorsal atrial wall. (B) Transverse section of an E10.5 embryo showing abundant *Odd1* mRNA expression in the developing septum primum (arrowhead) and the dorsal atrial wall. *Odd1* mRNA is also highly expressed in the cardinal vein. (C) Higher magnification of the lung bud region of the section in B. *Odd1* mRNA is abundantly expressed in the oesophagus endoderm, the lung bud mesenchyme, the septum primum, and the dorsal atrial wall. Arrowheads mark the left and right boundaries of the dorsal atrial expression domain. (D, E) Transverse (D) and sagittal (E) sections of E11.5 embryos showing *Odd1* mRNA expression in the left, but not right leaflet of the newly formed venous valves. (F) Frontal section through the dorsal thoracic region an E13.5 embryo showing strong expression of *Odd1* mRNA in the dorsal atrial wall flanking the atrial septum (arrow) and in the mesothelium including the parietal pericardium. Low levels of *Odd1* mRNA is detected in the lung mesenchyme at this stage. a, atrium; b, main bronchus; la, left atrium; lb, lung bud; lcv, left cardinal vein; llu, left lung; mt, mesothelium; oe, oesophagus; oft, outflow tract; pc, parietal pericardium; ra, right atrium; rcv, right cardinal vein; rv, right ventricle; v, ventricle; vv, venous valve; sp, septum primum; tr, trachea.

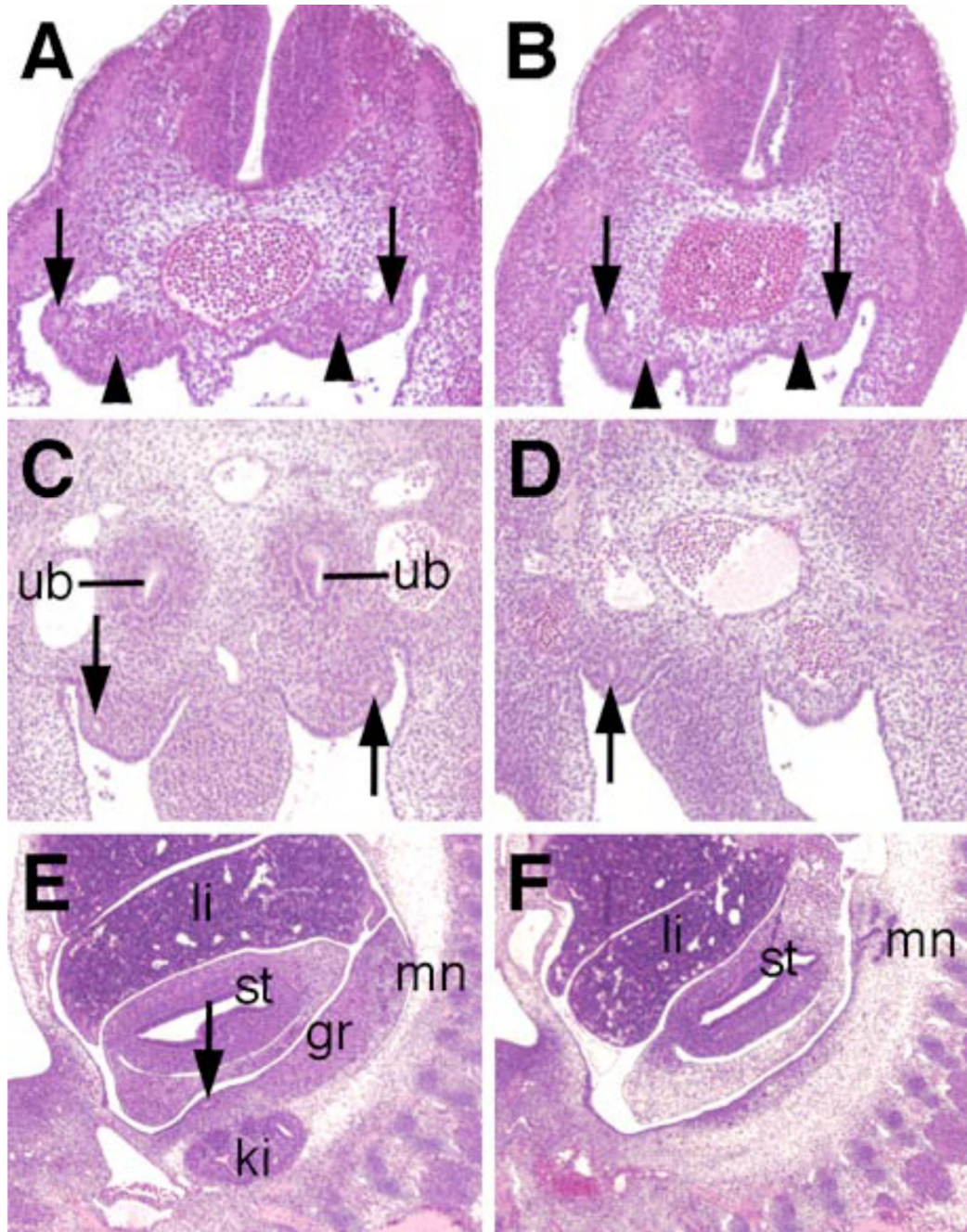


**Fig. 7.** Comparison of molecular marker expression during heart development in the *Odd1*<sup>-/-</sup> mutant (B, D, F) and wildtype control (A, C, E) embryos at E11.5. (A, B) *Pitx2* mRNA expression is restricted to the left atria in both wildtype (A) and *Odd1*<sup>-/-</sup> mutant embryos. Arrowhead in A points to the primary atrial septum, which is absent in the *Odd1*<sup>-/-</sup> mutant embryo. (C, D) *Nkx2.5* mRNA is expressed throughout the myocardium in both the wildtype (C) and the mutant (D) embryos. Arrowhead in C points to the primary atrial septum, which is absent in the mutant embryos. The asterisk in C marks the mesodermal cap of the atrial septum, which is also absent in the mutant embryos. Arrow in D points to the short stomped

venous valve in the mutant atrium whereas arrowhead in D points to the endocardial cushion, which is present at the atrioventricular junction in both wildtype and mutant embryos. (E, F) *Tbx18* mRNA is expressed in the epicardium in both wildtype (E) and mutant (F) embryos. Arrowheads in E and F point to the mesothelial inner lining of the body wall, which expresses *Tbx18* mRNA similarly in wildtype and mutant embryos. bw, body wall; la, left atrium, lv, left ventricle; ra, right atrium; rv, right ventricle.



**Fig. 8.** *Odd1*<sup>-/-</sup> mutant embryos lack metanephric kidneys. Representative transverse sections of E15.5 wildtype (A, C) and *Odd1*<sup>-/-</sup> mutant (B, D) littermates show the absence of kidneys, gonads, and adrenal glands in the mutant embryo. Due to the absence of kidneys, the internal organs such as pancreas and spleens were displaced in the mutant embryos but they were morphologically normal compared to the wildtype littermates. ad, adrenal gland; ki, metanephric kidney; li, liver; mn, mesonephros; pa, pancreas; sp, spleen; st, stomach; te, testis.

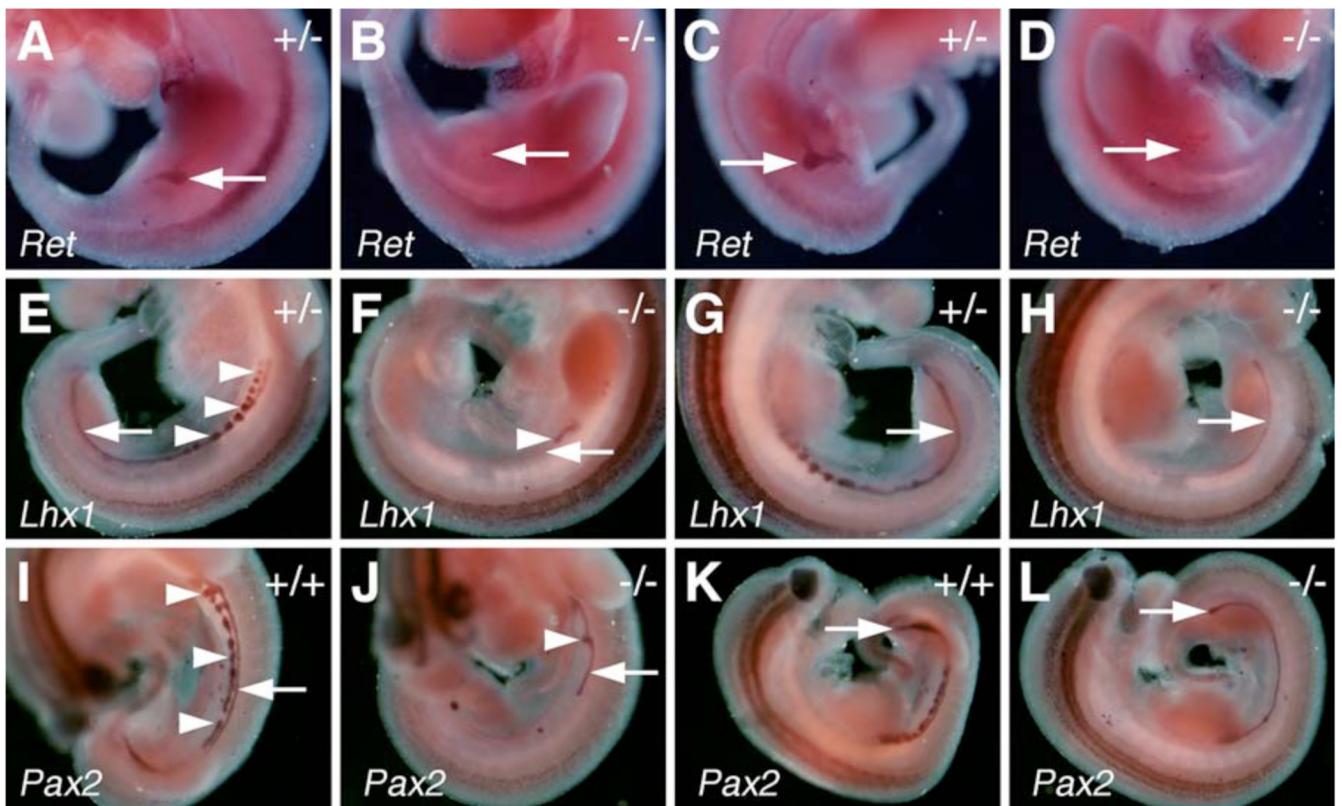


**Fig. 9.**

Defects in kidney development in *Odd1*<sup>-/-</sup> mutants. (A, B) Transverse sections of E10.0 wildtype (A) and *Odd1*<sup>-/-</sup> mutant embryos at the levels just rostral to the hindlimb buds. The wildtype embryo (A) shows condensation of the metanephric mesenchyme (arrowhead) next to the nephric duct (arrow) whereas the nephrogenic mesenchyme does not condense in the mutant embryo (B). (C) At E10.5, ureteric bud invades the condensed metanephric mesenchyme in the wildtype embryo. (D) A *Odd1*<sup>-/-</sup> mutant littermate of the embryo in C do not have ureteric bud structure and lack nephric duct (arrow) on the left side of the caudal trunk. (E) Sagittal section of an E11.5 wildtype embryo showing the

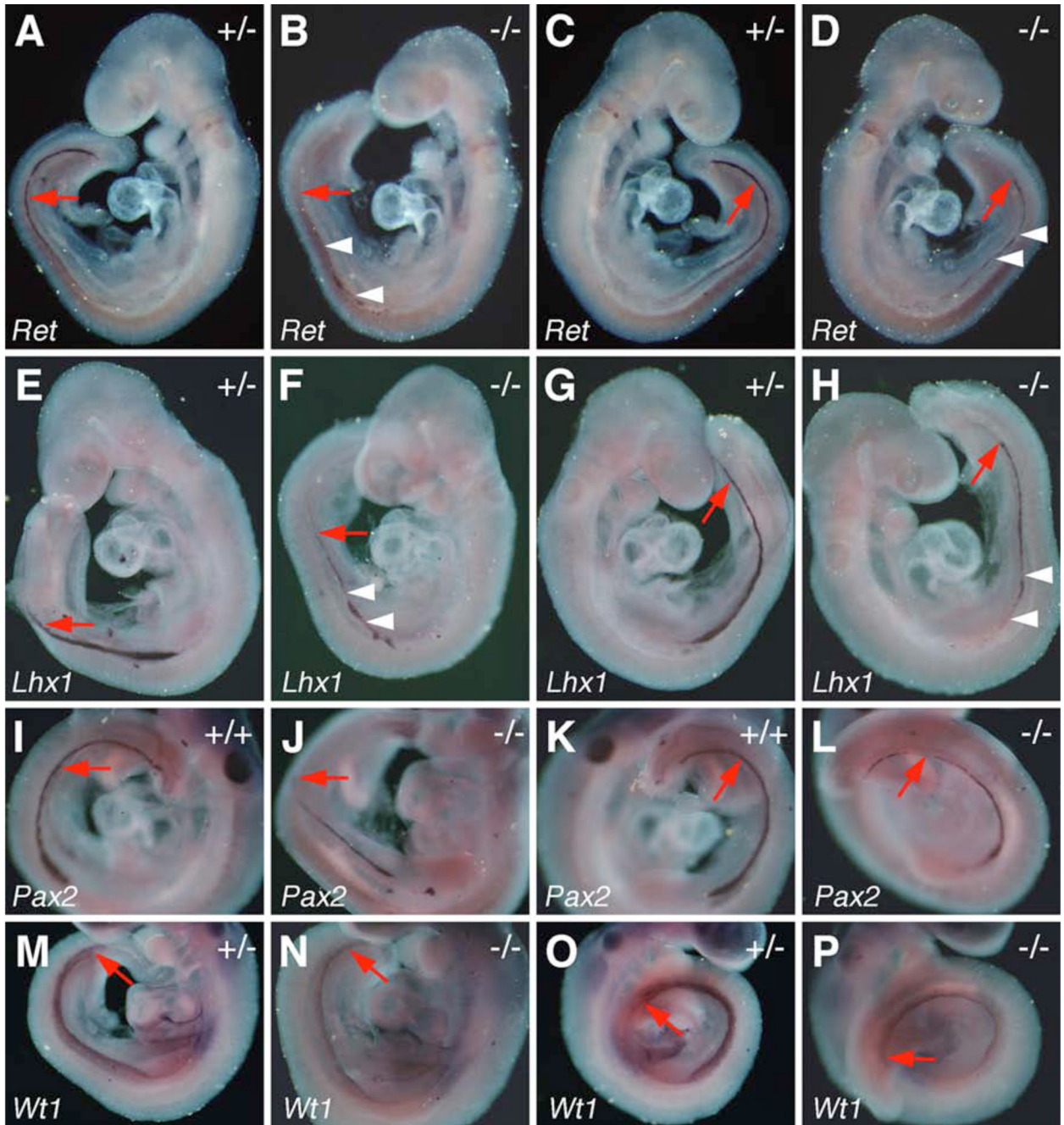


mesonephros, gonadal ridge, and metanephric kidney. (F) Sagittal section of an *Odd1*<sup>-/-</sup> mutant littermate showing lack of metanephric kidney and hypoplastic gonadal ridge. gr, gonadal ridge; ki, metanephric kidney; li, liver; mn, mesonephros; st, stomach; ub, ureteric bud.



**Fig. 10.**

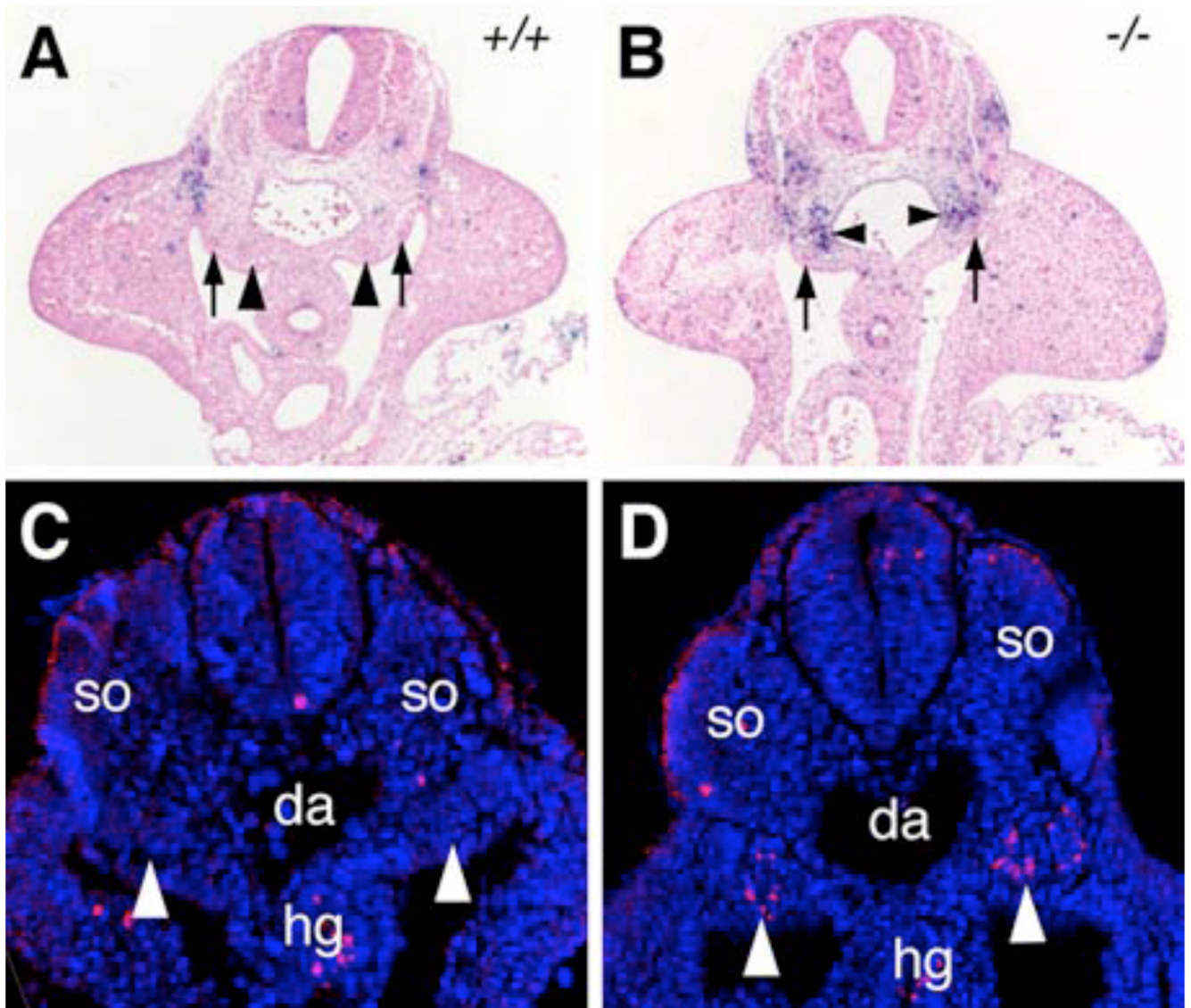
In situ hybridization analyses of molecular marker gene expression during kidney development in E10.5 control and *Odd1*<sup>-/-</sup> mutant embryos. (A, C) Left and right sides, respectively of an E10.5 *Odd1*<sup>+/-</sup> embryo showing normal patterns of *Ret* mRNA expression in the newly initiated ureteric bud structures (arrows). (B, D) *Odd1*<sup>-/-</sup> mutant embryos at E10.5 lack *Ret*-expressing ureteric bud structures (red arrow). (E, G) *Odd1*<sup>+/-</sup> embryos show normal patterns of *Lhx1* mRNA expression in the nephric duct (arrow) and mesonephric vesicles (arrowheads). (F, H) *Odd1*<sup>-/-</sup> mutant littermates of the embryos shown in E and G have significantly reduced *Lhx1* mRNA expression in regions corresponding to mesonephros and nephric ducts. (I – L) Similarly, *Pax2* mRNA is expressed in the nephric ducts (arrow) and mesonephric vesicles (arrowheads) in wildtype embryos (I, K) and is significantly reduced in the *Odd1*<sup>-/-</sup> mutant embryos (J, L). The *Lhx1* and *Pax2* expression patterns indicate that the left nephric duct is absent in the posterior trunk of mutant embryos (F, J), consistent with histology data (Fig. 8D). +/+, wildtype; +/-, heterozygous; -/-, homozygous mutant.



**Fig. 11.**

In situ hybridization of expression of kidney development marker genes in E9.5 control and *Odd1*<sup>-/-</sup> mutant embryos. Both left and right sides of the embryos are shown. Expression of *Ret* (A-D), *Lhx1* (E-H), and *Pax2* (I-L) mRNAs is significantly reduced in the *Odd1*<sup>-/-</sup> mutant embryo nephric ducts compared to their wildtype and heterozygous littermates. Expression domains of all three mRNAs are posteriorly truncated on the left side of the homozygous mutant embryos (B, F, J). Arrowhead point to additional areas of patchy disruption of the gene expression patterns in the mutant embryos. (M-P) *Wt1* mRNA expression in the nephrogenic mesenchyme is significantly reduced throughout the

anteroposterior axis in the homozygous mutants (N, P) compared to the heterozygous control littermates (M, O). +/+, wildtype; +/-, heterozygous; -/-, homozygous mutant.



**Fig. 12.**

Increased nephrogenic mesenchyme cell death in the *Odd1*<sup>-/-</sup> mutant embryos. (A, B) Transverse sections at hindlimb bud levels of E10.5 wildtype (A) and *Odd1*<sup>-/-</sup> mutant (B) embryos were stained for apoptotic cells using TUNEL assay. Arrows point to the nephric ducts and arrowheads point to metanephric mesenchyme. (C, D) Transverse sections of lower trunk regions of E9.5 wildtype (C) and *Odd1*<sup>-/-</sup> mutant (D) embryos were stained for Caspase-3 expression (red). The sections were counterstained with DAPI (blue) to show cell nuclei. Arrowheads point to the nephrogenic mesenchyme, which contains significant numbers of Caspase-3 positive cells in the *Odd1*<sup>-/-</sup> mutant embryo (D). da, dorsal aorta; hg, hindgut; so, somite; +/+, wildtype; -/- homozygous mutant.