Mouse lacking *COUP-TFII* as an animal model of Bochdalek-type congenital diaphragmatic hernia

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Congenital diaphragmatic hernia (CDH), a life-threatening anomaly, is a major cause of pediatric mortality. Although the disease was described >350 years ago, the etiology of CDH is poorly understood. Here, we show that tissue-specific null mutants of *COUP-TFII* exhibit Bochdalek-type CDH, the most common form of CDH. *COUP-TFII*, a member of orphan nuclear receptors, is expressed in regions critical for the formation of the diaphragm during embryonic development. Ablation of *COUP-TFII* in the foregut mesenchyme, including the posthepatic mesenchymal plate (PHMP), results in the malformation of the diaphragm and the failure of appropriate attachment of the PHMP to the body wall. Thus, both the stomach and liver enter the thoracic cavity, leading to lung hypoplasia and neonatal death. Recently a minimally deleted region for CDH has been identified on chromosome 15q26.1-26.2 by CGH array and FISH analysis. *COUP-TFII* is one of the four known genes residing within this critical region. Our finding suggests that *COUP-TFII* is a likely contributor to the formation of CDH in individuals with 15q deletions, and it may also be a potential contributor to some other Bochdalek-type of CDH.

nuclear orphan receptor | NR2F2

Congenital diaphragmatic hernia (CDH) occurs in ≈1 in every 3,000 live births (1). CDH is associated with a reported mortality of ≈30–60% of live-born patients, and morbidity is high among survivors (2, 3). The pathological anomalies are characterized by the inappropriate formation of the diaphragm, with the viscera invading the thoracic cavity. Protrusion of the abdominal contents, which may include the stomach, liver, intestines, and spleen, into the thoracic cavity impairs lung growth and development (4, 5). Thus, newborns with CDH suffer from pulmonary hypoplasia and pulmonary hypertension in addition to other anomalies, leading to a high rate of neonatal deaths. The mechanism underlying this serious and costly clinical problem is largely undefined.

Several types of CDH have been identified. The most common form is the Bochdalek-type CDH, which occurs in the dorsolateral region of the diaphragm and accounts for >70% of the diaphragmatic defects occurring in humans (6). The Morgagni-type CDH, which occurs anteriorly, is less common, and the central-type CDH is the rarest type and accounts for <2% of all cases (4). Central CDH has been documented in a mouse model lacking Slit3, in which the central tendon region fails to detach from the liver tissue because of abnormal morphogenesis of the diaphragm (7).

Recent advances in cytogenetic analysis have enabled the identification of various chromosomal aberrations and their association with CDH. Deletions of chromosome 15q have been associated with a number of congenital abnormalities, including Bochdalek-type CDH (8). By using array-based comparative genomic hybridization and FISH, Klaassens *et al.* (9) have recently defined an ≈5-mb minimal deletion region for CDH on chromosome 15q26.1-26.2. Only four known genes reside within this critical region, one of which is *COUP-TFII*.

COUP-TFII is a member of nuclear receptor superfamily that has been shown to play a critical role during mouse development. The *COUP-TFII*-null mutants exhibit defects in angiogenesis and heart development and die before embryonic day (E)10.5 (10). To circumvent the early embryonic lethality, we generated a floxed *COUP-TFII* mouse line and crossed this line with a tissue-specific Cre-recombinase mouse line to generate a conditional knockout of *COUP-TFII* in mice. Upon tissue-specific ablation of *COUP-TFII* in the mesentery by using *Nkx3-2* Cre recombinase, the anterior–posterior and radial patterning of the stomach is greatly altered (11). In this article, we demonstrate that a high percentage of *Nkx3-2* Cre-induced *COUP-TFII* conditional null mutants exhibit diaphragmatic hernia, in which the stomach and liver protrude into the thoracic cavity. This phenotype resembles that displayed by CDH patients. Because *COUP-TFII* resides in the CDH minimal deleted region on chromosome 15q, and ablation of *COUP-TFII* alone is capable of inducing a Bochdalek-type diaphragmatic hernia in mice, it is likely that *COUP-TFII* contributes to the formation of CDH in individuals with 15q deletions.

Materials and Methods

Generation of Conditional Deletion of *COUP-TFII* in the Mesentery. A floxed *COUP-TFII* mouse line having loxP sites flanking the *COUP-TFII* locus was generated as described in ref. 11. This *COUP-TFII* floxed mouse in 129/C57BL/6 background was crossed with *Nkx3-2 Cre* mice to generate *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ mice. Subsequently, the double heterozygous *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ mice were then crossed to *COUP-TFII*$^{flox/flox}$ mice to generate conditional *COUP-TFII*-null mice (*Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/flox}$). Diaphragms from E14.5–E18.5 embryos and postnatal day 0 (P0) mice were examined for evidence of CDH, and corresponding genotypes were determined by PCR, as described in ref. 11.

Histological Analysis of Conditional *COUP-TFII*-Null Mutants and Littermate Controls. Embryos were dissected and fixed in 4% paraformaldehyde (PFA)/PBS overnight, dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For frozen sections, embryos were dissected and fixed in 2% PFA/PBS, pH 7.2–7.4 for 30 min at 4°C, washed with PBS, cryoprotected by 20% sucrose/PBS, and embedded in optimal cutting temperature (OCT) medium (Tissue Tek, Torrance, CA). Serial sections at 7–10 mm were made for X-Gal staining performed as described in ref. 12 and counterstained with Nuclear Fast red (Vector Laboratories). For the immunohistochemistry analysis, monoclonal antibodies to *COUP-TFII* (H7147 at 1:6,000 dilution, Perseus Proteomics, Tokyo) were used. Biotinylated secondary antibodies (1:300, vector) and Alexa Fluor 488-conjugated Tyramide signal-amplification kit (Molecular Probes) was applied to amplify the signal. Nuclei were counterstained with DAPI.

Abbreviations: CDH, congenital diaphragmatic hernia; E*n*, embryonic day *n*; P*n*, postnatal day *n*; PHMP, posthepatic mesenchymal plate; PPF, pleuroperitoneal fold.

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Table 1. Perinatal mortality of conditional mutants

	COUP-TF$^{flox/+}$ (+/+)	*COUP-TF*$^{flox/flox}$ (+/+)	*Nkx3-2-Cre*/+; *COUP-TF*$^{flox/+}$	*Nkx3-2-Cre*/+; *COUP-TF*$^{flox/flox}$	Total
Prenatal (E14.5–E18.5)	14 (19.2)	17 (23.3)	18 (24.7)	24* (32.9)	73
Postnatal P0	9 (20.9)	10 (23.3)	15 (34.9)	9† (20.9)	43

Data in parentheses are percentages.

*Among E14.5–E18.5 embryos, 11 of 24 (45.8%) were complicated with CDH, whereas a spleen defect was found in 24 of 24 (100%).

†Seven of 9 (78%) were found dead with CDH; all of the mutants were asplenic.

Results and Discussion

Perinatal Lethality Caused by CDH Observed in Homozygous Conditional Null *COUP-TFII* Mutants. *COUP-TFII* is highly expressed in foregut mesentery during embryonic development. We have previously demonstrated that *COUP-TFII*-null mutants die before E10.0 because of defects in angiogenesis and heart development. To circumvent the early embryonic lethality and investigate the functional role of *COUP-TFII* in the mesentery, we chose to ablate the *COUP-TFII* gene in a tissue-specific manner by using the *Cre/loxP* system. We first generated floxed *COUP-TFII* targeting vector by placing the *loxP* sites flanking the *COUP-TFII* locus and then generated a floxed *COUP-TFII* allele in mouse through recombination (11). Subsequently, the floxed mice were crossed with *Nkx3-2* (*Bapx1*) Cre mice to delete *COUP-TFII* in the mesentery. *Nkx3-2* is a homeobox-containing gene (13) that is coexpressed with *COUP-TFII* in the foregut mesentery. We showed previously that deletion of *COUP-TFII* in the gastric mesenchyme results in dysmorphogenesis of the stomach, in which both anterior–posterior and radial patterning of the stomach are perturbed (11).

During analysis of the mutant phenotypes, we noted a significant decrease in the postnatal survival of homozygous conditional null mutants when compared with heterozygous null mutants. Anatomical analysis of homozygous conditional null mice revealed posteriolateral diaphragmatic hernias, with herniation of the liver and stomach within the thoracic cavity. This defect was not seen in heterozygous animals. From the breeding analysis summarized in Table 1, it is noted that 7 of 9 homozygous conditional mutants exhibited the anomalies at P0, all of which were found dead. Between E14.5 and E18.5, 11 of 24 homozygous mutants had CDH.

Anatomical analysis conducted on homozygous conditional null mouse embryos at E18.5 revealed that the heart was pushed laterally to the right thorax, displaying dextroposition (Fig. 1 *D* and *E*), when compared with the littermate controls (Fig. 1 *A* and *B*). Frontal views of the mutant internal organs showed that the abdominal organs, both the stomach and the liver, protruded through the diaphragm into the thoracic cavity (Fig. 1 *E* and *F*), whereas the liver and stomach were located in the normal position beneath the diaphragm in the controls (Fig. 1 *B* and *C*). Herniation of the liver resulted in compression of the left lung in homozygous animals (Fig. 1*E*).

Similar abnormal development of the mutant mice at P0 is also shown in Fig. 2. The frontal view of the CDH mutant again illustrates that the stomach has protruded through the diaphragm and is located in the thoracic cavity, and the liver is herniated and the left lung is severely compressed, in comparison with the controls (Fig. 2 *A* and *B*). It is likely that lung hypoplasia and the resulting respiratory insufficiency is the underlying cause of the premature demise of these animals. As previously noted in the E18.5 embryos, the hearts of the homozygous null mice were located to the right of midline, displaying dextroposition (Fig. 2*B*).

Evaluation of the diaphragms of conditional *COUP-TFII*-null mice revealed holes located in the left dorsolateral region of the diaphragm (Fig. 2*D*) that were not present in control littermates (Fig. 2*C*). The unaffected portion of the diaphragm appeared well muscularized, suggesting that the diaphragmatic defect might arise from disruption of an early developmental event before muscularization of the diaphragm. This assumption is consistent with the recent finding that amuscular substratum could form appropriately in the absence of myoblast migration in *c-met*-null mice (14).

The diaphragmatic defects observed in conditional *COUP-TFII*-null mutants resemble the posteriolateral defect seen in individuals with Bochdalek-type CDH. Although Bochdalek-type hernias can be either right- or left-sided, left-sided hernias are more common. Similarly, the diaphragmatic defects seen in the conditional *COUP-TFII*-null mice were all left-sided. An interesting question that needs to be addressed is whether the formation of the amuscular substratum requires a functional foregut mesentery, in which *COUP-TFII* is highly expressed and is, presumably, ablated in the conditional null mutant.

Expression of *COUP-TFII* in Regions Necessary for Diaphragm Formation. The embryonic diaphragm forms from four major structural components: the central tendon, derived from the septum transversum; a dorsal unpaired midline portion, derived from the foregut mesentery; two dorsolateral shelves of tissues, derived from the pleuroperitoneal membrane; and a peripheral component, derived from the body wall (15). The central tendon initially adheres to the pericardium and liver at E12.5, but by E14.5, the central tendon is largely separated from these two tissues, and, by E17.5, the separation is complete. The dorsal and ventral mesentery of the foregut acts as a "strut" toward which other components, like the pleuro-

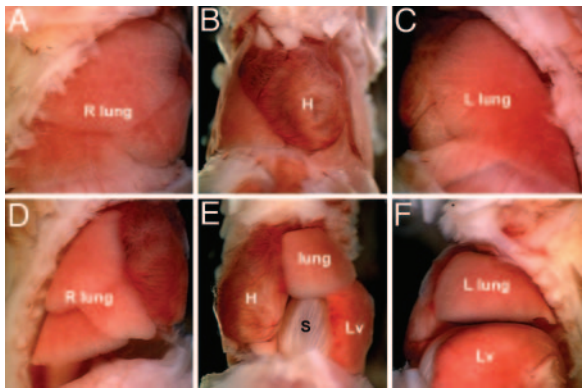

Fig. 1. CDH of E18.5 mutant mice. Control (+/+; *COUP-TFII*$^{flox/+}$) animal shows normal development of each organ with right-side, frontal, or left-side view (*A–C*). Each organ was located in the appropriate position and was of normal size; however, at E18.5, the mutant fetus exhibited CDH (*D–F*). Heart dextropostion, hypoplastic lung, and herniated stomach are visible in the central view (*E*). H, heart; L lung, left lung; R lung, right lung; Lv, liver.

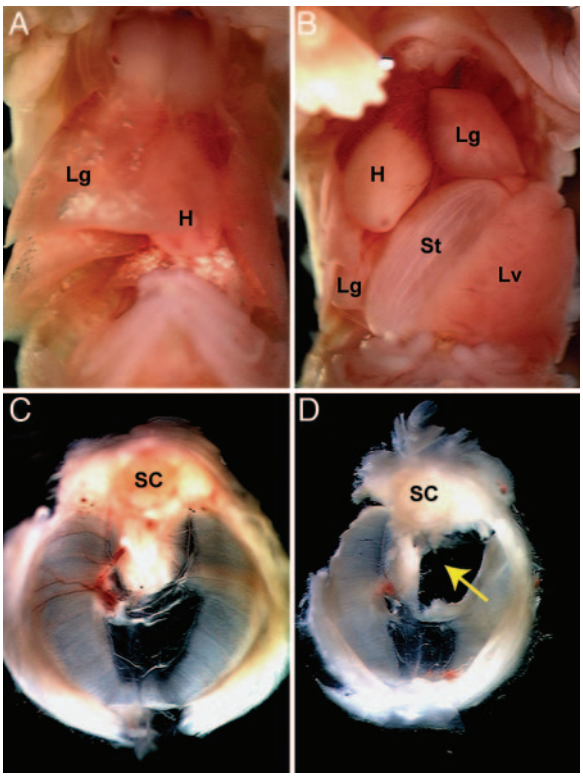

Fig. 2. CDH of P0 mutant mouse. (*A* and *B*) Frontal view of representative P0 newborn mice. (*A*) Control newborn. (*B*) Mutant CDH at P0. In the mutant, the stomach is located in the center of the thorax, just above the diaphragm, the liver is herniated, and the left lung is severely compressed. The heart is also mislocated to the right thorax, exhibiting dextroposition. (*C* and *D*) Dissected diaphragm illustrates the presence of a left-sided dorsolateral hernia in the mutant. The dissected diaphragm has been placed with the abdominal side facing the objective, with the dorsal side on top. Note the large left-sided dorsolateral defect of the diaphragm forming a hole (indicated by an arrow), which permitted the abdominal contents (e.g., stomach) to herniate into the thorax. H, heart; Lg, lung; SC, spinal cord; St, stomach; Lv, liver.

peritoneal membrane, grow to form the dorsolateral part of the diaphragm. The dorsolateral part of the diaphragm eventually fuses with the central tendon (15) and attaches to the body wall component, which forms a narrow rim around all but the midline unpaired region of the diaphragm by E13.5. The pleuroperitoneal membranes, also called the pleuroperitoneal fold (PPF), are invaded by the myoblast cells, derived from cervical somites and innervated by the phrenic nerves, whereas the body-wall-derived region of the diaphragm is innervated by the intercostal nerves. The definitive diaphragm functions not only to facilitate breathing; it also serves as barrier to separate the two pleural cavities from the peritoneal cavity.

In mouse, the mesenchymal tissue that appears dorsal to the liver and ventral to the peritoneal canal was defined by Iritani (16) as the posthepatic mesenchymal plate (PHMP). The same region is defined by Greer as PPF in rat and mouse (17). At E11.5, PHMP is divided into the left and right plates, with the cranial portion meeting the septum transversum at a right angle. The medial portion of the PHMP is contiguous to the mesenchymal tissue surrounding the esophagus, whereas the lateral portion grows to be contiguous to the body wall (16). In an attempt to assess how *COUP-TFII* contributes to the formation of the diaphragm during embryonic development, we determined the expression pattern of *COUP-TFII* in regions critical for diaphragm formation by immunostaining with a COUP-TFII-specific antibody. High *COUP-TFII* expression was found in the foregut mesenchyme at E10.5 (Fig. 3*A*). By E11.5, *COUP-TFII* expression was clearly seen in the wedge-shaped PHMP regions, and the expression levels were slightly higher in left side of the PHMP compared with the right (Fig. 3*B*). Higher *COUP-TFII* expression was detected at PHMP and the mesenchymal tissue contiguous to it at the region of the esophagus (Fig. 3*D*), as compared with PHMP in the more rostral part of the body axis (Fig. 3*C*) at E12.5. Also, a more pronounced difference in *COUP-TFII* expression between the left and the right PHMP was noted (Fig. 3*D*). A very high level of *COUP-TFII* was also detected in the central tendon region of septum transversum at E12.5 (Fig. 3*E*). The expression level of *COUP-TFII* in the diaphragm started to decrease at E13.5 (Fig. 3*F*). To further confirm that *COUP-TFII* is expressed in the developing lung and the PHMP region, we used FOG2 as marker for the lung and myogenin as a marker for the PHMP. As shown in Fig. 3 *G* and *H*, FOG2 is expressed in the developing lung, and myogenin is expressed in the PHMP region where *COUP-TFII* is expressed. Taken together, expression data indicate that *COUP-TFII* is expressed in the primitive foregut mesenchyme, the developing PHMP, developing lung, and the septum transversum, the components that are important for the formation of the diaphragm. In addition, low *COUP-TFII* expression was detected in the body-wall component by E12.5 (Fig. 3*E* and data not shown), and reduced expression was detected at E13.5 (data not shown).

***COUP-TFII* Expression in Foregut Mesenchyme and PHMP Is Essential for Primitive Diaphragm Formation.** As alluded to earlier, central-type (septum transversum) CDH occurs in the midline of the septum transversum and accounts for 1–2% of the total cases of CDH. In Slit-null mouse mutants, thinning of the central tendon and inappropriate development of the falciform ligament allow the developing liver to intrude into the thoracic cavity, resulting in a central-type CDH (7). Although *COUP-TFII* is highly expressed in the septum transversum, with limited ablation in this region by the *Nkx3-2* Cre recombinase (see below), a central-type CDH has not been seen in these conditional null mutants. The *COUP-TFII* targeting construct used in the creation of the tissue-specific *COUP-TFII* knockout mice includes a *LacZ*-reporter element that is activated when *COUP-TFII* is excised upon recombination, and, thus, the expression of *LacZ* reporter is induced (11). Therefore, we used X-Gal staining to follow the excision of *COUP-TFII* and identified sites where *COUP-TFII* was ablated. Identifying the spatiotemporal pattern of *COUP-TFII* ablation in conditional null animals provides a second means of identifying developmental structures that may play a role in *COUP-TFII*-related CDH.

X-Gal staining was clearly seen in the mesenchyme surrounding the esophagus, but not in the wedged PHMP in the region of the rostral body axis at E12.5 (data not shown). However, X-Gal staining was prominently detected in the PHMP regions (Fig. 3*I*), particularly in the left PHMP region, where the growing PHMP begins to attach to the body wall at later stages of development (Fig. 3*I*). The pattern of X-Gal staining in the left PHMP region and the developing diaphragm is consistent with the expression pattern of *COUP-TFII* and with the propensity to develop left-sided dorsolateral CDH. Although limited X-Gal staining is observed in the diaphragm at the esophagus level (data not shown), the ablation of *COUP-TFII* was clearly obvious at the left dorsolateral side of the diaphragm at E13.5 (Fig. 3*J*). These results indicate that *COUP-TFII* is ablated in the foregut mesenchyme and the PHMP regions, but not in the central tendon, when the *Nkx3-2* Cre (Fig. 3 *I* and *J*) is used to delete it. To further determine the extent of *COUP-TFII* deletion in the foregut mesenchyme, PHMP, and developing lung, we carried out immunostaining with *COUP-TFII* antibody. As shown in Fig. 3 *K–P*, we found that the *COUP-TFII* signal is drastically

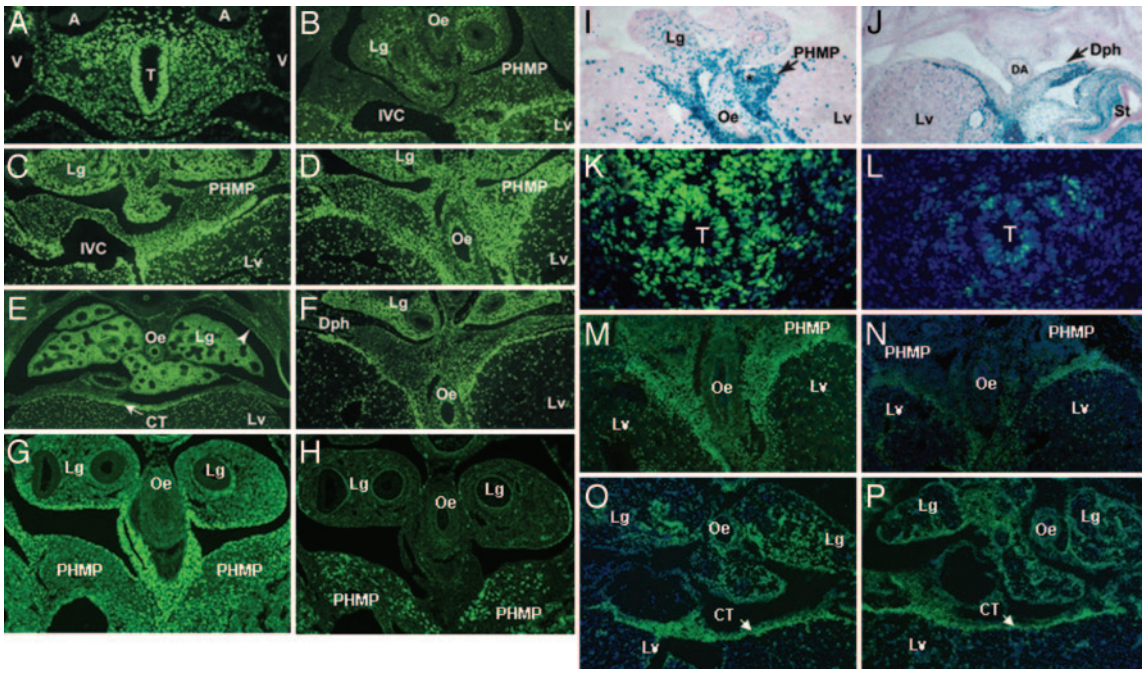

Fig. 3. Developmental expression pattern and *Nkx3-2* Cre-mediated excision of *COUP-TFII* during diaphragm formation. (*A–E*) Using immunostaining, we observed the strong expression of *COUP-TFII* (green) in the mesenchyme surrounding foregut at E10.5 (*A*) and a moderate level of expression in the wedge-shaped PHMP at E11.5 (*B*) and in the rostral PHMP at E12.5 (*C*). The strong expression of *COUP-TFII* was detected in PHMP surrounding the esophagus in the caudal region at E12.5 (*D*). *COUP-TFII* expression was higher in the left side of the PHMP compared with the right side. The high expression level of *COUP-TFII* was also observed in the central tendon (indicated by an arrow) at E12.5 (*E*). The *COUP-TFII* expression level was gradually reduced in the diaphragm at E13.5 (*F*). (*G* and *H*) FOG2 and myogenin were used as markers for the developing lung and PHMP. As shown, FOG2 is expressed in the developing lung and PHMP (*G*), whereas myogenin is expressed only in the PHMP (*H*) of E12.5 embryos. (*I* and *J*) X-Gal-stained transverse sections from a *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ embryo demonstrated the Cre-excision pattern in the diaphragm. *COUP-TFII* was clearly ablated in the mesenchyme surrounding the esophagus and the PHMP at the region of the caudal body axis at E12.5 (*I*). The phrenic nerve is marked by an asterisk (*I*). By E13.5, obvious deletion of *COUP-TFII* was seen in the left side of the diaphragm at E13.5 (*J*). (*K–P*) Immunostaining to determine the extent of *COUP-TFII* deletion demonstrates efficient deletion of *COUP-TFII*, as depicted by the substantial reduction of *COUP-TFII* expression in the foregut mesenchyme (*L*) and PHMP (*N*) of the conditional null mutants in comparison with the corresponding regions of the control littermates (*K* and *M*). In contrast, slight differences in *COUP-TFII* expression are seen in the developing lung and central tendon of the mutant (*P*) and the control (*O*), indicating low excision of *COUP-TFII* in these region. Ages of embryos used are E10.5 (*K* and *L*) and E12.5 (*M–P*). Data of *COUP-TFII*$^{flox/flox}$ control (*K*, *M*, and *O*) and *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/flox}$ knockout (*L*, *N*, and *P*) embryos are shown. Immunostaining is shown in green, and DAPI counterstaining is shown in blue. A, aorta; CT, central tendon; Dph, diaphragm; IVC, inferior vena cava; Lg, lung; Lv, liver; Oe, esophagus; St, stomach; T, trachea; V, vein.

reduced in the foregut mesenchyme of the mutant (Fig. 3*L*) as compared with the controls (Fig. 3*K*). Similar reduction is also seen in the PHMP of the mutant (Fig. 3*N*) in comparison with controls (Fig. 3*M*). However, the *COUP-TFII* signal is only slightly reduced in the developing lung and the central tendon region of the mutant (compare Fig. 3*P* with 3*O*). Our results indicate that, whereas *COUP-TFII* is expressed highly in the gut mesenchyme, central tendon, PHMP, developing lung, and, at low level, on the body wall, Nkx 3.2 Cre-induced deletion of *COUP-TFII* is quite complete at the caudal PHMP and gut mesenchyme but not complete at the rostral PHMP, central tendon, and developing lung, all of which are important for the formation of the diaphragm. Together, these results strongly indicate that *COUP-TFII* in the foregut mesenchyme and PHMP is essential for the proper formation of the diaphragm. The incompleteness of deletion in the central tendon raises a question whether complete ablation of *COUP-TFII* in this region could result in diaphragmatic defects within the central portion of the diaphragm, similar to those seen in Slit-null mice.

The excision of *COUP-TFII* in PHMP suggests that abnormal development of the PHMP might play a critical role in the development of the diaphragm. To assess more closely whether morphogenesis of PHMP is perturbed in the conditional null mutants during diaphragm development, we performed a histological examination of the PHMP from control and homozygous conditional null embryos at E14.5. In the control embryos, the PHMP/PPF properly joined the lateral body wall at ≈E14.5 (Fig. 4*A*). In contrast, the left side of the PHMP in the conditional null embryos was not attached to the body wall, leaving a hole in the left side of the developing diaphragm, whereas the right side of the diaphragm appeared normal (Fig. 4 *B* and *C*). The inappropriate growth of the left PHMP resulted in herniation of the stomach into the thoracic cavity. The liver, however, remained in the abdominal cavity at this stage of development (Fig. 4 *B* and *C*).

Our results are consistent with the hypothesis that inappropriate formation of PHMP underlies the formation of Bochdalek CDH, which accounts for >70% of the CDH in patients (6). The predominance of left-sided defects in the developing diaphragm of the conditional null mutant might arise from the need for higher levels of *COUP-TFII* expression in the left PHMP. It is possible that deletion of *COUP-TFII* in the PHMP disrupts the signaling between the PHMP and the body wall, altering the growth and differentiation of these two compartments, and confers the predominant left-sided defects of the Bochdalek-type CDH.

The defects exhibited by the homozygous *COUP-TFII* conditional null mice resemble the type of CDH generated by exposing mouse or rat embryos to the herbicide nitrofen (16–19, 32). This animal model demonstrates that hypoplastic growth of PHMP/PPF could affect the formation of the

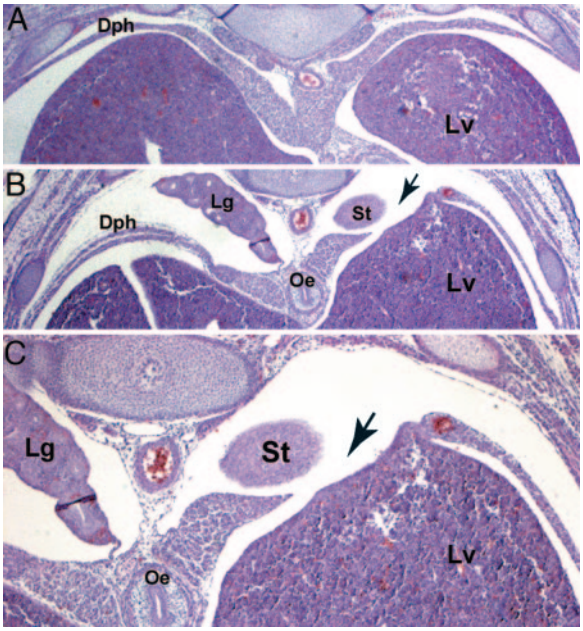

Fig. 4. CDH in *COUP-TFII* conditional null mutants. Normal diaphragm formation at E14.5 in a control littermate (*A*). Diaphragm herniation was detected in mutant (*Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/flox}$) embryos. Hematoxylin and eosin-stained transverse section shows the stomach and liver in the thoracic cavity of a *COUP-TFII* conditional mutant embryo at E14.5 (*B*). High magnification view of *B* is shown in *C*. Arrows mark the discontinuous diaphragm. Abbreviations are as in Fig. 3.

primitive diaphragm, which subsequently affects diaphragmatic muscular malformation, resulting in formation of a hole in the dorsolateral region of the diaphragm (16, 17, 20). Because nitrofen has been shown to be a potent inhibitor of retinal dehydrogenase (RALDH-2), the result leads to the hypothesis that abnormal retinoid signaling contributes to the etiology of CDH (21). Because *COUP-TFII* has been shown to be a downstream target of retinoid signaling, it could serve as a mediator of retinoid signaling to modulate embryonic diaphragm formation (22).

***COUP-TFII*, a Likely Candidate Gene for Bochdalek CDH.** Chromosomal aberrations are a relatively common cause of CDH (23). One of the more frequent chromosomal anomalies associated with CDH is deletion of a distal portion of chromosome 15q (24). Cytogenetic analysis suggests that the region between 15q24 and 15q26 plays a crucial role in the development of CDH, and patients with deletion in 15q24–26 have a poor prognosis (8). By using array-based comparative genomic hybridization and FISH, Klaassens *et al.* (9) have recently defined a minimal deletion region for CDH of ≈5 Mb on chromosome 15q26.1–26.2. Interestingly, clinical evaluation of patients with deletion of this region of chromosome 15q reveals left-sided Bochdalek-type hernias similar to those seen in the *COUP-TFII* conditional knockout mice. Four known genes reside within this minimal region. They are *COUP-TFII* (*NR2F2*), *CHD2* (a chromodomain helicase 2 gene) (25), *RGMA* (a repulsive guidance molecule) (26, and sialyltransferase (a cell-adhesion molecule) (27).

The fact that the *COUP-TFII* gene is located within the minimal deleted regions of the CDH patients, and the conditional null mutants described above display the Bochdalek-type of hernia strongly implicates *COUP-TFII* as the most likely candidate gene for CDH associated with 15q deletions. It is also possible that *de novo* mutation in *COUP-TFII* may be the etiology for some cases of Bochdalek-type CDH.

Patients with deletions of 15q commonly exhibit intrauterine growth retardation and other multiple anomalies, including edema, short limbs, atrial and ventricular septal defects, vascular defects, and renal malformations (8, 9, 28). Some of these nondiaphragmatic defects have also been observed in other *COUP-TFII* mouse models. Heterozygotes of the *COUP-TFII* conventional mutant mice all exhibit intrauterine growth retardation (29). Also, hypomorphic *COUP-TFII* mutants exhibit both atrial and ventricular septum defect and thin myocardium (L.-R.Y., M.-J.T., and S.Y.T., unpublished results). Furthermore, conditional and conventional *COUP-TFII*-deletion mutants have severe edema, vascular defects, cryptorchidism, and short limbs (10, 30, 31). These similar defects lend further support to *COUP-TFII*'s role in developing CDH in some patients.

Our data indicate that deletion of the *COUP-TFII* gene contributes to the development of CDH. The major question still remains: Why didn't we detect CDH in our heterozygous *COUP-TFII* mutants? It should be noted that not all 15q deletions have CDH (9), and, as far as we know, there are no data available on the percentage of 15q deletion in humans that develop CDH. Consequently, the penetrance of CDH phenotype by *COUP-TFII* deletion or mutation in humans could be low. Therefore, it is possible that additional mutations, such as point mutation in the other *COUP-TFII* allele or molecules important for *COUP-TFII* function (such as FOG2) are needed to develop CDH. It is also possible that genetic background may contribute to the development of CDH, because of the genetic background of different mouse strains or the differences between human and mouse. Furthermore, whereas our data are consistent with the hypothesis that malformation of the diaphragm contributes to the development of CDH, we cannot rule out that malformation of the lung may also contribute to the formation of CDH. In our mice, there is an incomplete deletion of *COUP-TFII* in the developing lung and central tendon, even though it is well deleted in the gut mesenchyme and PHMP (Fig. 3). Consistent with this finding, our mice have no obvious lung hypoplasia in the early stage of development. Therefore, had we been able to delete *COUP-TFII* completely in the developing lung and in the diaphragm, we might have had a higher frequency of CDH in our heterozygous mice. Finally, by the same token, because our mice are conditional deletion, we do not have "complete" deletion of *COUP-TFII* in all areas important for diaphragm formation, as shown in Fig. 3. Removal of the residual amount of *COUP-TFII* (≈10%) in the PHMP and gut mesenchyme and complete deletion in the central tendon might enhance the frequency of CDH in heterozygous *COUP-TFII* mutants. Taken together, these possibilities may explain why our *COUP-TFII* heterozygous mice do not develop CDH or develop at a very low frequency.

We also want to emphasize that not all CDH cases are due to *COUP-TFII* mutation. Actually, the frequency of CDH patients because 15q deletion is low (3 of 200) (9). Therefore, mutation in other genes or even point mutation of *COUP-TFII* must also contribute to these complex CDH diseases. Indeed, recently, FOG2 point mutation has been shown to associate with CDH (33). In any event, these mouse models will provide valuable tools through which the *in vivo* physiological roles of *COUP-TFII* and other genes responsible for CDH and associated congenital anomalies can be better understood.

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