COUP-TFII is essential for radial and anteroposterior patterning of the stomach

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Summary

***COUP-TFII*, an orphan member of the steroid receptor superfamily, has been implicated in mesenchymal-epithelial interaction during organogenesis. The generation of a *lacZ* knock-in allele in the *COUP-TFII* locus in mice allows us to use X-gal staining to follow the expression of *COUP-TFII* in the developing stomach. We found *COUP-TFII* is expressed in the mesenchyme and the epithelium of the developing stomach. Conditional ablation of floxed *COUP-TFII* by *Nkx3-2Cre* recombinase in the gastric mesenchyme results in dysmorphogenesis of the developing stomach manifested by major patterning defects in posteriorization and radial patterning. The epithelial outgrowth, the expansion of the circular smooth muscle layer and enteric neurons as well as the posteriorization of the stomach resemble phenotypes exhibited by inhibition of hedgehog signaling pathways. Using organ cultures and cyclopamine treatment, we showed downregulation of COUP-TFII level in the stomach, suggesting *COUP-TFII* as a target of hedgehog signaling in the stomach. Our results are consistent with a functional link between hedgehog proteins and COUP-TFII, factors that are vital for epithelial-mesenchymal interactions.**

Key words: Nuclear orphan receptor, Sonic hedgehog, Organogenesis, Stomach, Mouse, Nr2f1, Nr2f2

Development

Introduction

Gut development depends upon crosstalk between endoderm and splanchnic mesenchyme cell layers along the anteroposterior axis, resulting in the subdivision of the gut into foregut, midgut and hindgut (Kedinger et al., 1998b; Kedinger et al., 1998a; De Santa Barbara et al., 2002). The stomach becomes specified at the caudal end of foregut and emerges as a bulge of the developing GI tract (Kaufman and Bard, 1999). The stomach then undergoes progressive remodeling and differentiation in a regionally specific fashion. The detailed genetic and molecular pathways that direct gastric organogenesis are still unknown, but it is clear that patterning of the GI tract requires elaborate cellular communication between the epithelium and mesenchyme cell layers (Fukuda and Yasugi, 2002).

Sonic hedgehog (*Shh*) patterns a variety of embryonic tissues, including the fore-stomach epithelium by signaling to the mesenchyme prior to organ regionalization (Chiang et al., 1996). *Shh*-null mice exhibit inappropriate expression of alkaline-phosphatase (EAP) in the epithelium of the hind-stomach (Ramalho-Santos et al., 2000), which is consistent with the stomach epithelium acquiring an intestinal or ‘posteriorized’ character. Conversely, the stomach of the activin receptor IIA and B compound mutant (*ActRIIA/B* mutant; *Acvr2a*/*Acvr2b* – Mouse Genome Informatics) exhibits posterior extension of the fore-stomach epithelium with expansion of *Shh* expression (Kim et al., 2000), a process referred to as ‘anteriorization’. These data suggest that *Shh* expression in the fore-stomach acts to induce and/or maintain non-intestinal character, resulting in the development of gastric epithelium (Ramalho-Santos et al., 2000).

COUP-TF proteins are nuclear orphan receptors, highly conserved across species. Two members have been identified in mice, *COUP-TFI* (*Nr2f1* – Mouse Genome Informatics) and *COUP-TFII* (*Nr2f2* – Mouse Genome Informatics). The temporal and spatial expression pattern of *COUP-TFII* in mesenchyme led us to hypothesize that *COUP-TFII* plays a role in mesenchymal-epithelial interactions during organogenesis (Tsai and Tsai, 1997). In the developing neural tube, *Shh* has been shown to regulate *COUP-TFII* expression during the differentiation of motoneurons (Lutz et al., 1994). We have also identified a 5′-regulatory element that mediates *Shh* stimulation of *COUP-TFII* expression (Krishnan et al., 1997a; Krishnan et al., 1997b). *COUP-TFII* is likely to be a downstream target of *Shh* signaling, and the requirement for *Shh* in gastric organogenesis led us to infer that *COUP-TFII* may play a role in stomach development.

To circumvent the early embryonic lethality of the *COUP-TFII*-null mutation and to investigate its function during gastric organogenesis, we generated a conditional null mutant of *COUP-TFII* using the *Cre/LoxP* system. *Nkx3-2* (*Bapx1*) is a homeobox-containing gene (Tribioli et al., 1997) that is co-

expressed with *COUP-TFII* in the stomach primordium; thus, *Nkx3-2*^{Cre} knock-in recombinase was used to ablate *COUP-TFII* in the gastric mesenchyme. The stomachs of conditional mutant mice exhibited dysmorphogenesis accompanied by abnormalities of both compartmentalization and radial patterning, demonstrating a functional link between *Shh* and *COUP-TFII*.

Materials and methods

Generation of conditional mutant of *COUP-TFII*

Genomic DNA fragments, 7.2 kb *Bam*HI-*Sal*I, 1.4 kb *Sal*I-*Xba*I and 1.4 kb *Xba*I-*Eco*RI from Q#10 clone and a 2.6 kb *Eco*RI-*Bam*HI fragment from D-3.6RI clone containing *COUP-TFII* locus as described (Qiu et al., 1995) were subcloned into pBlueScriptII KS(+). The first loxP site was inserted into *Bgl*I site of 7.2 kb *Bam*HI-*Sal*I fragment and the *Nsi*I site on the 2.6 kb *Eco*RI-*Bam*HI fragment was modified with *Xho*I linker. These four genomic fragments were reconstructed, and *Xho*I site on the 2.6 kb *Eco*RI-*Bam*HI region was modified with linker containing *Cla*I site at the 3′ end. pNeoTKLoxP, a positive-negative selection cassette, was modified and ligated with *lacZ* from pDP46.21. This NeoTKLoxP-*lacZ* construct was inserted into *Xho*I-*Cla*I linker at 3′-UTR. 5′-*Sal*I site of NeoTKLoxP-*lacZ* was destroyed by ligation with *Xho*I, and *Cla*I site and *Not*I site were blunt-end-ligated after fill-in reaction. Targeting construct consists of 4 kb 5′-arm, 6.5 kb floxed *COUP-TFII* locus, 8.5 kb NeoTKLoxP-lacZ and 2 kb 3′-arm, a total size of ~24 kb was linearized with *Not*I at the 3′ end. Homologous recombination was performed in AB1.2 cells (Qiu et al., 1997) and ES cell clones were screened by two rounds of Southern blot analysis. Correctly targeted clones were first identified by screening *Xba*I digest with 5′-external and 3′-external probes (5′-ext and 3′-ext, respectively) and subsequently transfected with a Cre recombinase expression vector followed by screening *Hin*dIII digests with a 5′-internal probe (5′-int) and *Xba*I digest with a 3′-external probe. Chimeric animals were generated by microinjection and germline transmission was achieved by crossing with C57Bl/6 wild-type female. Thereafter, mutant mice carrying floxed allele were maintained in 129/B6 mixed background. Specific primers were designed for floxed *COUP-TFII* genotyping by PCR. The primer sequences were NT1 5′-CAGTCGCCTCTCCTTCTCCTTCTCC-3′, NT2 5′-CATCCGGGATATGTTACTGTCCGG-3′, NT3A 5′-TTCTG-GTCTTCACCCACCGGTACC-3′ and NT6A 5′-TGGGGAAGC-TAAGTGTTGTAGTGATTCC-3′. The sizes of the PCR product are 785 bp for wild type and 394 bp for floxed allele.

Nkx3-2^{Cre} knock-in animal was generated by homologous recombination in ES cells (K.M., W.E.Z. and R.J.S., unpublished). Cre recombinase was inserted in-frame into the exon1 of *Nkx3-2* locus. No overt phenotype was found in heterozygous animals.

X-gal staining

X-gal staining was performed according to the published methods (Hogan et al., 1994). For whole-mount staining, embryos (up to E10.5) were dissected and fixed in 4% paraformaldehyde, then stained in X-gal staining solution at room temperature. Histological sections of whole-mount stained embryos were processed in Histoclear, embedded in paraffin, and sectioned at 10-12 μm. Sections were cleared with Histoclear, rehydrated and counterstained with Eosin, and mounted with Permount. Larger embryos (~E12.5 or later) and postnatal samples were cryoprotected with 20% sucrose/PBS solution after fixation with 2% paraformaldehyde and embedded in OCT compound. Cryostat sections (16 μm) were briefly fixed in 2% paraformaldehyde, stained in X-gal staining solution and counterstained with Eosin.

Histological analysis

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 5 or 7 μm. Hematoxylin and Eosin staining was carried out according to the regular protocol. For immunohistochemistry, paraffin sections were dewaxed, rehydrated and incubated with primary antibodies. Antibodies against human CK14 (1:500) was a gift from Dr Roop (Baylor College of Medicine, Houston, TX). Polyclonal anti-serum against PGP9.5 was from Chemicon (1:1000). Antibodies against TUJ1 (Convance, 1:1000), GATA4 (Santa Crutz, 1:400), H^+/K^+-ATPase β-subunit (Affinity BioReagents, 1:500) were used in immunostaining. Texas Red-tagged *Griffonia simplicifolia* II (GSII) lectin was used to stain the stomach tissue. Positive staining for CK14 and PGP9.5 was visualized by using biotinylated secondary antibody and streptavidin-horseraddish peroxidase conjugate, and NovaRed (Vector) as a chromogen. SMA-α, TUJ1, GATA4 and H^+/K^+-ATPase β-subunit were visualized by using biotinylated secondary antibody and streptavidin-horseraddish peroxidase conjugate, and DAB (Vector) as a chromogen. Monoclonal anti-α-smooth muscle actin (SMA-α, 1A4, 1:1000) was purchased from Sigma-Aldrich and immunostaining was performed using Mouse-On-Mouse kit (Vector) according to manufacturers instruction, and detected with AlxaFluor488 (Molecular Probes). For ultrastructural study of the disorganized smooth muscle layer, 0.5 μm semi-thin sections were stained with Toluidine Blue. Endogenous alkaline-phosphatase staining was performed as previously described (Ramalho-Santos et al., 2000; Aubin et al., 2002) and counterstained with Methyl Green. The glycoconjugated production in parietal cells of the stomach was marked by DBA lectin. Whole-mount in situ hybridization for *COUP-TFII* was carried out as described (Qiu et al., 1997; Pereira et al., 1999). Probe for *Shh* was a gift from Dr M. P. Scott (Stanford University, CA). For section in situ hybridization of *Shh*, E11.5 embryos were cross-sectioned and *Shh* expressions in the fore-stomach were detected using ^{35}S-UTP labeled probes. Positive signals were pseudo-colored (red) and overlaid on bright field image of Hematoxylin staining as described (Pereira et al., 1999).

Explants culture of embryonic foregut

Foreguts were dissected from E10.5 *lacZ* knock-in heterozygous embryos and cultured on tissue-culture insert (Corning) with defined serum-free medium (Opti-MEMI, Invitrogen Life Technologies). Explants were incubated with vehicle (DMSO), 1, 5 and 10 μM of cyclopamine (Toronto Research Chemicals) for 48 hours, and fixed with 2% paraformaldehyde for whole-mount *lacZ* staining. Explants from wild-type embryos were fixed with 4% paraformaldehyde and used for whole-mount in situ hybridization.

Results

Expression of COUP-TFII in the stomach

To facilitate the profiling of *COUP-TFII* gene expression during development, we generated a targeted vector according to a scheme diagramed in Fig. 1A. This targeting vector was introduced into ES cells together with Cre recombinase. Upon recombination between the first and the last *LoxP* sites, *COUP-TFII* was deleted and the expression of *lacZ* reporter, inserted into the 5′ untranslated region of *COUP-TFII* locus, was activated. The ES cells were subsequently injected into blastocysts to generate *lacZ* knock-in alleles in mice. *lacZ* gene expression is under the control of *COUP-TFII* promoter and its expression recapitulates the expression of the endogenous *COUP-TFII* gene. Using this *lacZ* knock-in allele, we examined the expression pattern of *COUP-TFII* during gut development.

At embryonic day (E) 12.5, X-gal staining was detected in both the epithelium and mesenchyme of the stomach. Brief staining of cryostat sections revealed that mesenchymal cells adjacent to the endoderm had higher expression of *COUP-TFII*

Development

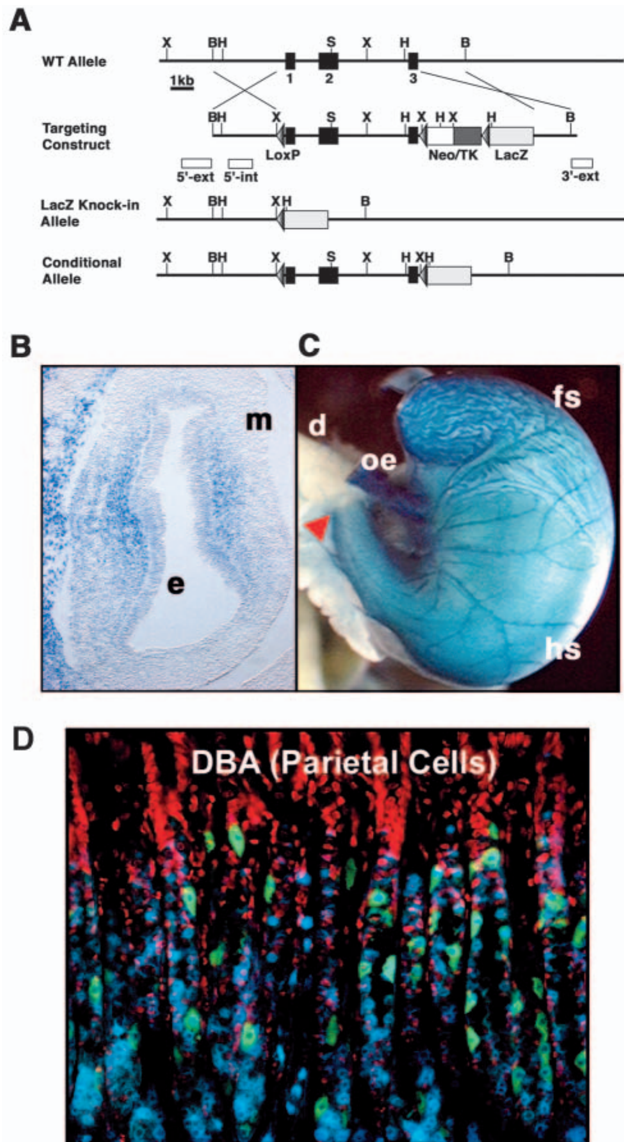

Fig. 1. Expression of *COUP-TFII* in the stomach using *lacZ* knock-in model. (A). Generation of the *lacZ* knock-in allele and generation of floxed *COUP-TFII* allele. Using homologous recombination, a targeting construct containing nuclear *lacZ*, *Neo/TK* and *LoxP* sites were inserted into the genomic *COUP-TFII* locus, generating a targeted allele in ES cells. Treatment with Cre recombinase and FIAU selection resulted in a *lacZ* knock-in allele in which *lacZ* gene expression was controlled by the endogenous *COUP-TFII* promoter when recombination took place between the first and the third loxP sites of the targeted allele. In addition, floxed *COUP-TFII ES* clones that retains *COUP*-TFII locus but lacks selection markers were generated when recombination took place between the second and the third loxP sites. B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; X, *Xba*I. (B) Cryostat sections of E12.5 heterozygous *COUP-TFII*/lacZ knock-in embryo were stained (for 2 hours) for *lacZ* activity. There is relatively high expression in the mesenchymal cells just adjacent to the epithelium. (C). The stomach from a 3-day-old heterozygous knock-in animal was dissected and whole-mount X-gal staining was performed. The boundary between stomach and duodenum is indicated by arrowhead. (D) A cryostat section of stomach from adult heterozygous knock-in animal was stained for *lacZ* activity (blue) and counterstained with propidium iodide (red). DBA lectin immunostaining denotes the parietal cells (green). There is strong X-gal staining in the base layer and negligible staining in the surface pit layer of the adult Zymogenic unit. m, mesenchyme; e, epithelium; fs, fore-stomach; hs, hind-stomach; d, duodenum; oe, esophagus.

than did distal mesenchymal cells, suggesting that a potential diffusible molecule, secreted from the epithelium might regulate *COUP-TFII* expression in the mesenchyme (Fig. 1B). At postnatal day 3, *COUP-TFII/lacZ* was detected throughout the stomach (Fig. 1C), and was sharply downregulated in duodenum (red arrowhead indicates junction between stomach and duodenum). *COUP-TFII/lacZ* was also expressed in the glandular epithelium of adult stomach. Adult glandular epithelium forms tubular structures, each of which is referred to as a zymogenic unit, which can be subdivided into four regions; a surface pit, an isthmus, a neck and a base that is located proximal to the sub mucosa (Karam and Leblond, 1993). Strong lacZ staining was evident at the base, and much lower at surface pit layer (Fig. 1D). To ensure COUP-TFII is also expressed in the parietal cells, we used DBA lectin for immunostaining. As shown in Fig. 1D, X-gal and lectin-positive green cells colocalized, indicating that COUP-TFII is expressed in the parietal cells of the Zymogenic unit. The expression pattern of COUP-TFII in the Zymogenic unit mimics the reported expression pattern of *Shh* in mouse stomach (van den Brink et al., 2001). Thus, *COUP-TFII* is abundantly expressed from an early developmental stage in both epithelium and mesenchyme of the stomach in parallel with *Shh*.

Nkx3-2Cre recombinase ablates COUP-TFII in the gastric mesenchyme

To examine the role of *COUP-TFII* in gastric development, we chose to ablate the *COUP-TFII* gene in a tissue-specific manner using the *Cre/LoxP* system. The targeting vector depicted in Fig. 1A, was introduced into ES cells. After excision of the Neo-TK cassette by Cre-based recombination, ES cells harboring the conditional allele were generated as depicted in Fig. 1A and used to generate floxed *COUP-TFII* mice. An *Nkx3-2*^{Cre} mouse line was also generated (K.M., W.E.Z. and R.J.S., unpublished), which was crossed with the floxed *COUP-TFII* mice. As *lacZ* is only turned on in cells with *COUP-TFII* locus recombined or deleted, X-gal staining can be used as a marker for successful *COUP-TFII* recombined or deleted cells. As shown in Fig. 2B, X-gal staining of an E12.5 stomach from *Nkx3-2*$^{Cre/+}$; COUP-TFII$^{flox/+}$ embryo demonstrates that *COUP-TFII* was ablated specifically in the gastric mesenchyme (Fig. 2C). This is expected because X-gal staining of *lacZ* knock-in mice is detected only in the stomach primordium (Fig. 2A) which is similar to *Nkx3-2-Cre* expression at E9.5, as demonstrated by the product of intercrossing the *Nkx3-2*^{Cre} with *ROSA26* reporter strain (Fig. 2B).

Conditional ablation of COUP-TFII in the gastric mesenchyme results in dysmorphogenesis and radial patterning defects of the developing stomach

The size of the mutant stomach is slightly smaller when compared with the control littermate at E12.5. Histological analysis of Hematoxylin and Eosin-stained sagittal sections of

the mutant stomach showed that the epithelium is considerably thicker than the controls at E12.5 (Fig. 3A,B). In addition, the circular smooth layer, which is stained with α-smooth muscle actin antibody, is disorganized in comparison with the controls at E13.5 (Fig. 3C,D). These abnormal morphological changes were again seen at E14.5 (data not shown), suggesting radial patterning of the stomach might be perturbed.

To further assess the potential radial patterning defects exhibited by the conditional *COUP-TFII*-null mutants, we examined the differentiation of smooth muscle and enteric neurons of the mutant stomach at later development. Immunostaining of α-smooth muscle actin confirmed the presence of a thickened circular smooth muscle layer formation in both the fore-stomach (Fig. 3E,F) and hind- stomach of conditional mutant mice (Fig. 3G,H). Moreover, submucosal

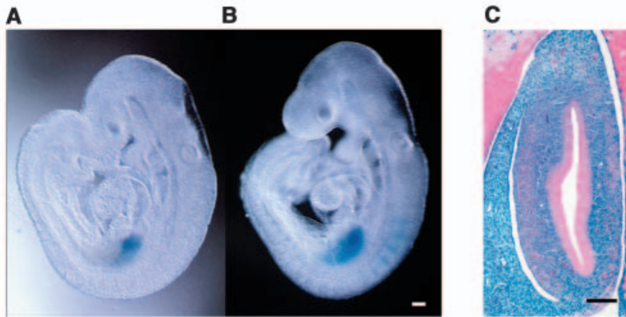

Fig. 2. Ablation of *COUP-TFII* in the mesenchyme of the developing stomach using *Nkx3-2^Cre^* and *COUP-TFII* floxed mice. (A) Whole-mount X-gal staining of *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ embryo, demonstrating the ablation of *COUP-TFII* by Nkx3-2Cre at E9.5 is shown. *lacZ* expression represents *Cre*-mediated recombination of floxed *COUP-TFII*. (B) The expression of Nkx3-2Cre at E9.5 was detected by whole-mount X-gal staining of *Nkx3-2*$^{Cre/+}$; *ROSA26R/+* embryo. X-gal staining represents Cre-mediated ablation of an interfering stop sequence in the *ROSA26* reporter gene. Specific staining was observed in the developing stomach of both embryos. (C) A partially dissected *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ embryo was stained by whole-mount X-gal staining, then paraffin embedded and serially sectioned. X-gal staining indicates Cre-mediated recombination and was detected throughout the gastric mesenchyme, but not in the epithelium, demonstrating the ablation of *COUP-TFII* in the gastric mesenchyme at E12.5. Scale bars: in B, 100 μm for A,B; in C, 100 μm for C.

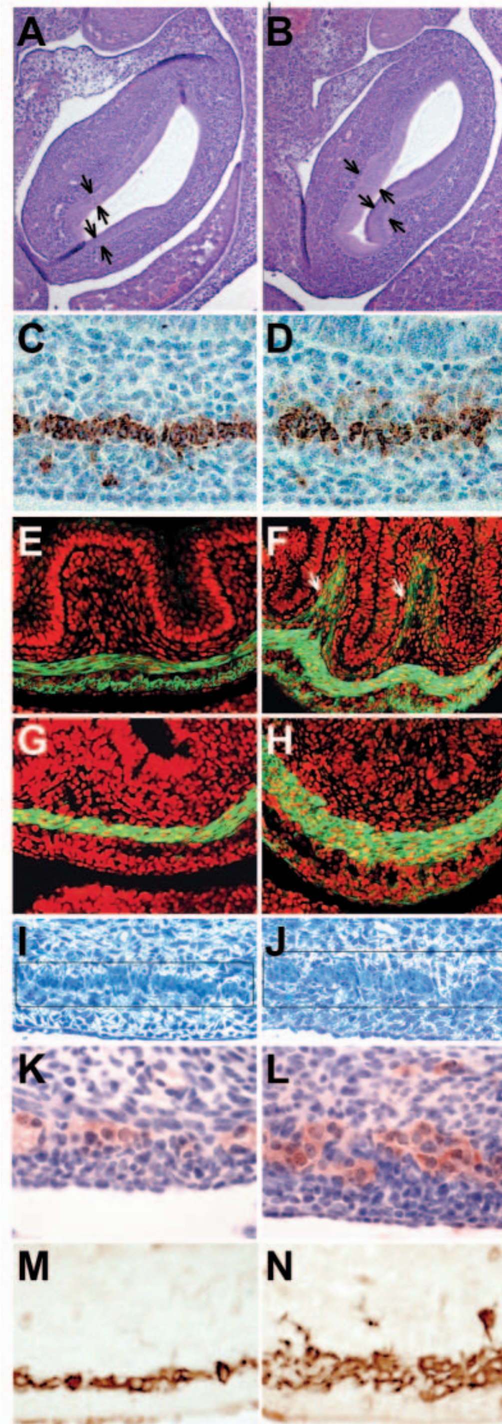

Fig. 3. Expansion of smooth muscle layers and enteric motoneurons in conditional mutant stomach. (A-D) Embryos were obtained by mating with *COUP-TFII* floxed homozygous males and *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ females. *COUP-TFII* floxed homozygote served as controls, and *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/flox}$ were designated as conditional mutants. Histological analysis of stomach dissected from E12.5 controls (A) and conditional mutants (B) showed that the epithelium of the conditional mutant is thicker (marked by the black arrows) in Hematoxylin and Eosin stained sagittal sections. At E13.5, the smooth muscle layers of the mutant stomach are disorganized in comparison with the controls, as seen by α-smooth muscle actin immunostaining (brown) (compare C with D). (E-H) α-smooth muscle actin staining of sagittal sections of E16.5 stomach. Smooth muscle cells were immunoassayed for α-smooth muscle actin (green), and counterstained with propidium iodide (red). White arrows indicate the extension of α-smooth muscle actin staining in the submucosal mesenchyme of the conditional mutant (F). (E,G,H) The thickened circular smooth muscle layer formation was observed in both the fore-stomach (F) and the hind-stomach (H) of the conditional mutant in comparison with the control (E,G). (I,J) Semi-thin section semi-thin sections of the stomachs from E15.5 embryos were examined. The circular smooth muscle layer of the conditional mutant stomach (J) is disorganized in comparison with the control (I). (K,L) PGP9.5 staining of E16.5 stomach. Enteric neurons were stained by protein gene product 9.5 (PGP9.5) antiserum (brown) and counterstained with Hematoxylin. (M,N) TUJ1 staining of E13.5 stomach. Anti-TUJ1 antibody was employed in immunostaining. An increase of TUJ1-positive cells is shown in the conditional mutant (N) in comparison with the littermate control (M).

extension of α-smooth muscle actin positive cells in the mutant fore-stomach (Fig. 3F, white arrows) indicated precocious differentiation of smooth muscle cells. To further demonstrate that the smooth muscle layer in the conditional mutant stomach is disorganized, semi-thin sections (Fig. 3I,J) and the ultrastructure (data not shown) of the stomachs from E15.5 embryos were examined. Again, circular smooth muscle layer of the conditional mutant stomach is disorganized (Fig. 3J) in comparison with the controls (Fig. 3I) at E15.5 as revealed by semi-thin sections. However, cellular defects, other than the disorganization of cell layers, have not been observed (data not shown). Immunostaining for PGP9.5, an enteric neuron marker, showed an increased number of positively stained cells in the conditional mutant mice (Fig. 3L). To ensure there is expansion of enteric neurons in the mutant stomach, an additional neuronal marker, TUJ1 (neuronal class III tubulin-β) was employed in immunostaining. An increase of TUJ1-positive cells is shown in the conditional mutant (Fig. 3N) in comparison with the littermate control (Fig. 3M). Taken together, conditional ablation of *COUP-TFII* in the mesenchyme resulted in at least two patterning abnormalities, epithelium outgrowth and expansion of circular smooth muscle and enteric neuron, both radial-patterning defects of the stomach. The fact that COUP-TFII is only deleted in the mesenchyme compartment but the epithelium of the mutant stomach is expanded, suggesting signals from the mesenchyme is essential for proper growth of the epithelium.

Anteroposterior patterning of the stomach is altered in the COUP-TFII conditional null mutant

The abnormal radial patterning of the stomach manifested by the *COUP-TFII* mutants prompted us to assess if other patterning defects were also present in the developing mutant stomach. A whole-mount view revealed dysmorphogenesis in the stomach of conditional mutants (Fig. 4A,B). The anatomical demarcation between the stratifying fore-stomach epithelium and the columnar hind-stomach epithelium, referred to as 'the limiting ridge' as indicated by broken line, begins to develop, forming a presumptive margin between the fore-stomach and hind-stomach. The size of the fore-stomach compartment and of the entire organ was noticeably reduced in the conditional mutants (Fig. 4C,D, white arrowheads indicate the junction of fore- and hind-stomach). Detailed histological examination reveals abnormalities in both the epithelium and mesenchyme. The glandular epithelium was thicker in mutants when compared with controls (Fig. 4E-H). In control mice, the epithelium was thickest at the pyloric region (to the left of Fig. 4E), and the thickness of the epithelium progressively decreased anteriorly (towards the right). By contrast, the epithelium of conditional mutant mice remained thick even in more anterior regions (Fig. 4F). The mesenchyme of the conditional mutant mice was also thickened. The hind-stomach epithelium showed several invaginations in the control mice (Fig. 4G), while more extensive invagination associated with the thickened epithelial layer in the conditional mutant mice (Fig. 4H). The morphological reduction of the fore-stomach and the extension of the hind-stomach suggest aberrant compartmentation of the stomach, a possible patterning defect in the anteroposterior axis.

Histological analysis of whole embryo sagittal sections were carried out to examine if indeed patterning of anteroposterior axis in the conditional mutant is perturbed, Analysis of E16.5 conditional mutants revealed an alteration in epithelial characteristics along the AP axis (Fig. 5A-H). The proximal duodenum exhibited typical characteristics for this stage in both control (Fig. 5A) and conditional mutant mice (Fig. 5E). However, the pylorus of conditional mutant mice exhibited a disorganized thickened circular smooth muscle layer, with an epithelium that resembled the epithelium of the duodenum and was devoid of vacuoles (Fig. 5F), unlike the highly vacuolated control (Fig. 5B). The hind-stomach of controls had a simple columnar epithelium with few invaginations, and a tight circular smooth muscle layer (Fig. 5C). By contrast, the hind-stomach of conditional mutant mice exhibited a vacuolated epithelium (Fig. 5G), resembling the pyloric region of control mice (Fig. 5B), with numerous invaginations and a thickened but disorganized circular smooth muscle layer. In addition, a

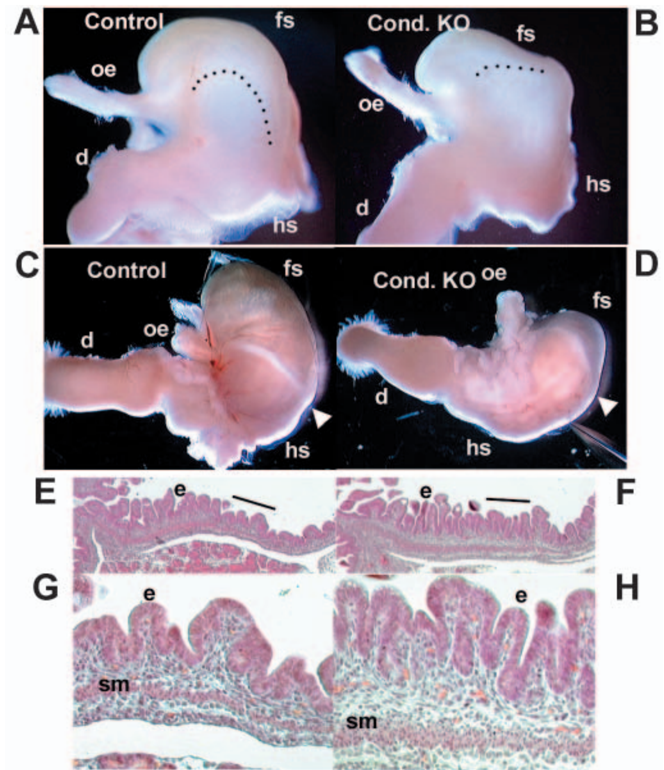

Fig. 4. Analysis of AP patterning defects in conditional *COUP-TFII* mutant stomach. Controls and conditional mutant embryos were obtained by mating with *COUP-TFII* floxed homozygous males and *Nkx3-2*$^{Cre/+}$; *COUP-TFII*$^{flox/+}$ females as described in Fig. 3. (A,B) Stomachs were dissected from E16.5 embryos and examined in whole mount under the dissecting microscope. The presumptive margin between fore-stomach and hind-stomach is indicated by dots and is shifted slightly anteriorly in the mutant. (C,D) Whole-mount postnatal day 28 stomachs were dissected and examined under the dissecting microscope. Position of limiting ridge is indicated by arrowhead. A clear anterior shift of the limited ridge is observed in the conditional mutant. (E,F) Dissected stomachs from E16.5 embryos were longitudinally sectioned and stained with Hematoxylin and Eosin. (G-H) Higher magnification of similar regions as indicated by lines in E,F. e, epithelium; sm, smooth muscle layer; fs, fore-stomach; hs, hind-stomach; oe, esophagus; d, duodenum.

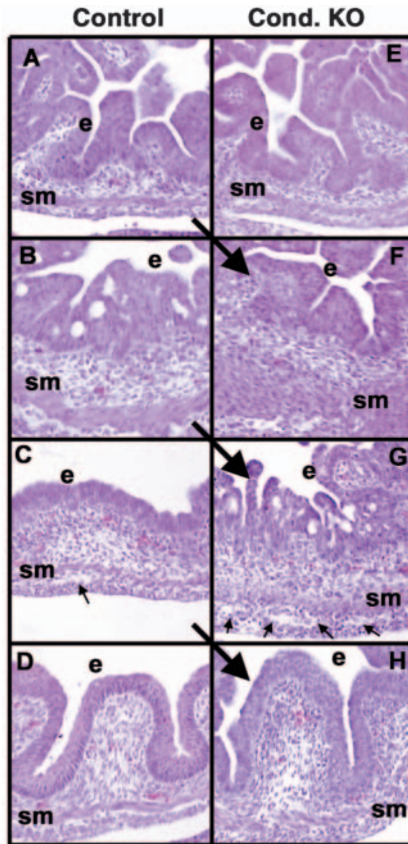

Fig. 5. Morphological changes in the posteriorized conditional mutant stomach: E16.5 whole embryos were sectioned sagittally and stained with Hematoxylin and Eosin for histological assessment. Corresponding regions in mutant and control were estimated by morphological characteristics of mesenchyme of each regions and relative location against other anatomical structures. (A,E) Duodenum. (B,F) Pyloric region. (C,G) Hind stomach. (D,H) Fore stomach. Oblique arrows across control and conditional mutant panels indicate changes of epithelial characteristics in the conditional mutants. e, epithelium; sm, smooth muscle layer.

cell layer located distal to the circular smooth muscle layer (Fig. 5G, indicated by small arrows at the bottom), where enteric neurons are normally located, was expanded in the conditional mutants. Finally, the fore-stomach epithelium begins to stratify at E16.5, with characteristic rough and deep infolding that was evident in the control mice (Fig. 5D). The conditional mutant mice showed similar infolding, but epithelium did not show clear signs of stratification (Fig. 5H). In fact, the fore-stomach epithelium of conditional mutant mice more closely resembled hind-stomach epithelium (Fig. 5C). These observable morphological changes in the compartmentation of different regions of the stomach are consistent with the notion that the stomach is posteriorized.

Alteration of AP patterning in the fore-stomach

If AP patterning of the stomach is indeed disrupted in the conditional mutant, it is anticipated that the specification/ differentiation of gastric epithelium might be affected. To assess if the fore-stomach is altered, we examined the fore-stomach regions using the stratified epithelial cell marker cytokeratin 14 (CK14). Immunostaining using CK14-specific antibody showed intense staining in the E16.5 control fore-stomach and the very anterior region of the mutant fore-stomach (data not shown). CK14 staining remained as intense throughout the fore-stomach of the control (Fig. 6A). However, it was barely detectable in the more caudal regions of the presumptive fore-stomach of the mutant (Fig. 6B), suggesting that the mutant stomach is posteriorized in which only the very anterior region of the stomach epithelium is stratified and expresses CK14, while the hind-stomach is morphologically expanded and is non-stratified.

Shh has been shown to be highly expressed in the fore-stomach and can serve as a fore-stomach marker. In the controls, Shh is highly expressed in the fore-stomach and the

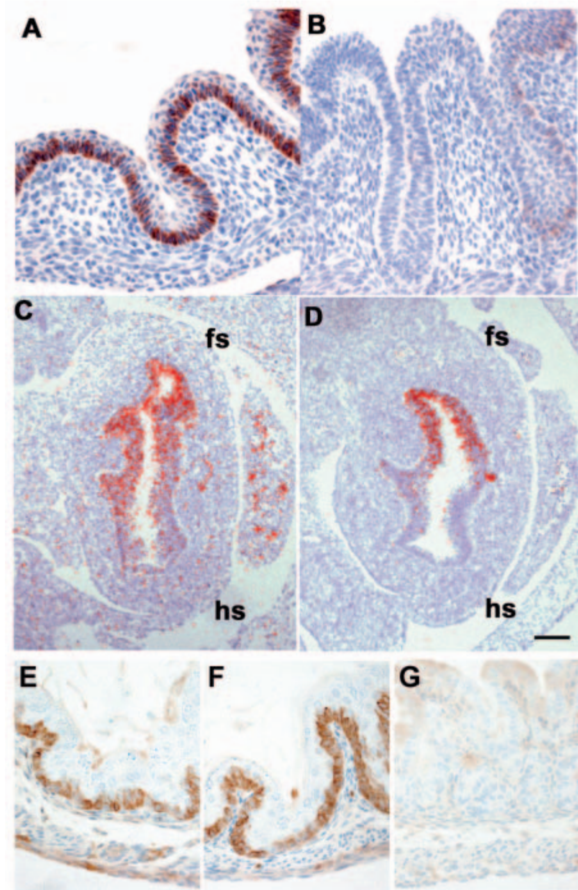

Fig. 6. Marker gene analyses confirm morphological changes in the conditional mutant stomach. (A,B) E16.5 sagittal sections were stained for stratified epithelial cell marker CK14. Intense staining was found in the basal cells in the control (A), while only faint staining was observed in the anterior edge of fore-stomach of the *COUP-TFII* conditional mutant (B). (C,D) Whole E11.5 embryos were cross-sectioned and *Shh* expressions in the fore-stomach were examined by section in situ hybridization using ^{35}S-UTP labeled probes. Positive signals were pseudo-colored (red) and overlaid on bright-field image of Hematoxylin staining. (E-G) Paraffin sections of E18.5 stomachs were stained with anti-CK14 antibody. CK14 staining (dark brown) remained as intense throughout the fore-stomach of the control (E) and mutant (F). Again, it was barely detectable in the more caudal regions of the stomach of the mutant (G) in comparison with similar anatomical position of fore-stomach of the control (E).

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expression extends into the hind-stomach (Fig. 6C). By contrast, the expression of *Shh* is anteriorly restricted in the conditional mutant (Fig. 6D). The anterior restriction of *Shh* expression observed in the conditional mutants is similar to that detected in the stomachs of *Hoxa5* mutant, indicating posteriorization of the fore-stomach (Aubin et al., 2002).

Similar result for CK14 staining was observed at the later stage of development. CK14 staining remained as intense throughout the fore-stomach of the control and mutant at E18.5 (Fig. 6E,F). Again, it was barely detectable in the more caudal regions of the stomach of the mutant (Fig. 6G), in comparison with similar anatomical position of fore-stomach of the control (Fig. 6E). This result further support the notion that the mutant stomach is posteriorized in which only the very anterior region of the stomach epithelium is stratified and expresses CK14, whereas the hind-stomach is expanded and is non-stratified.

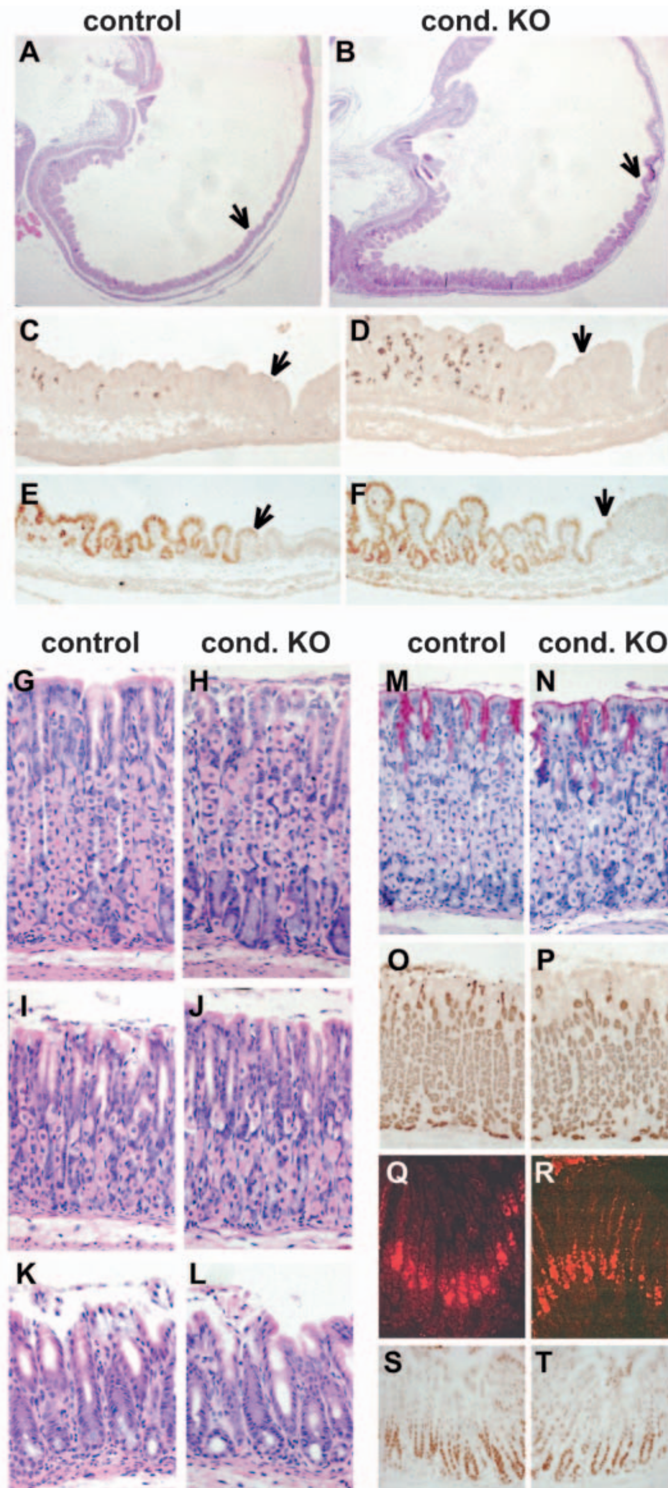

Fig. 7. (A,B) Dissected stomachs from E18.5 embryos were longitudinally sectioned and stained with Hematoxylin and Eosin. The thickness of the glandular epithelium is increased in the mutant stomach compared with the littermate control. (C-F) The adjacent tissue sections between fore-stomach and hind-stomach were stained by anti-H^+/K^+-ATPase β-subunit antibody (C,D) and anti-GATA4 (E,F) antibodies. The transition zone between oxyntic mucosa (os) and stratified squamous epithelium (sse) of the fore-stomach are indicated by arrows. (G-T) Marker gene analyses in the adult stomachs: (G-L) Sections (4 μm) prepared from the stomachs of 35-day-old mice were staining with Hematoxylin and Eosin. The histological morphology of zymogenic region (G,H), mucoparietal zone (I,J) and pure mucus zone (K,L) showed no obvious difference in the conditional mutant stomach (H,J,L) and littermate control (G,I,K). (M,N) The secreted glycoprotein of the zymogenic zone of stomachs was visualized by PAS staining and no discernable difference was observed in the conditional mutant stomach (N) and littermate control (M). (O,P) The gastric parietal cells were stained with anti-H^+/K^+-ATPase β-subunit antibody. (Q,R) The neck and pre-neck cells (red) at the gastric units showed no difference in the control (Q) and conditional mutant (R) stomachs. (S,T) Anti-GATA4 antibody was used to mark glandular gastric epithelium. Positive signals were found in base of glandular gastric epithelium at pure mucus zone in the control (S) and the mutant (T).

The expression of the differentiated hind-stomach markers remains unchanged in the conditional mutants

As shown earlier, the radial patterning and the AP patterning of the stomach are altered in the mutants at E13.5, leading to the respective thickening of the epithelium and the expansion of the hind-stomach (Figs 3-5). To examine if these phenotypes persist at later development, we examined the mutant stomach at E18.5. Similar to early development, it is clear that the epithelium is thickened and the hind-stomach of the mutant is considerably expanded (Fig. 7B) in comparison with the controls (Fig. 7A). To examine whether differentiation of the glandular epithelium is perturbed in the mutant, the expression of parietal cell marker H^+/K^+-ATPase β-subunit and of the glandular gastric epithelium marker GATA4 were analyzed at E18.5. Although H^+/K^+-ATPase β-subunit positive parietal cells are present in the glandular gastric epithelium of both controls (Fig. 7C) and conditional mutant mice (Fig. 7D), the number of the parietal cells were increased in the thickened epithelial layer of the conditional mutant mice (Fig. 7D). The expression of GATA4 in the thickened glandular epithelial layer of the mutant (Fig. 7F) is similar to that of the controls (Fig. 7E), suggesting differentiation of the glandular epithelium is unchanged in the mutant.

To further substantiate that differentiation of the hind-stomach is largely intact in the mutant, we examined the mutant hind-stomach at adulthood. Histological analysis of the stomachs from 35-day-old of mutant mice and littermate

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controls showed no obvious difference in the hind-stomachs at the regions of Zymogenic zone (Fig. 7G,H), mucoparietal zone (Fig. 7 I,J) and pure mucus zone (Fig. 7K,L) by Hematoxylin and Eosin. Mucins secretion was analyzed by periodic acid-Schiff (PAS) staining and similar PAS staining was observed in both control (Fig. 7M) and mutant (Fig. 7N) stomachs. To further examine if there is any changes in cell specification in the mutant hind-stomach, we employed H^+/K^+-ATPase β-subunit as the parietal cells marker (Fig. 7O,P), GSII as the neck and pre-neck cell marker (Fig. 7Q,R) and GATA4 as marker in the base of the pure mucus unit (Fig. 7S,T) in the analysis. The expression profiles of all these markers are similar in the mutants when compared with the controls. These results, taken together, support the notion that there are no cell type changes in the conditional mutant in comparison with littermate control throughout development even though there are observable patterning defects in the stomach.

Alteration in the very caudal part of the mutant hind-stomach

As histological analysis indicated that the very caudal part of the hind-stomach might have acquired pylorus characteristics, we used alkaline phosphatase (EAP) to determine whether hind-stomach is indeed posteriorized. As shown in Fig. 8A, EAP is mainly expressed in the duodenum and pylorus region of the controls. By contrast, high level of EAP expression not just found in the duodenum and pylorus, but it was also found in the hind-stomach of conditional mutant mice using longitudinally sectioned E16.5 dissected stomach (Fig. 8B). Clear anterior extension of EAP-positive epithelium, as indicated by brackets, was found in the hind-stomach of conditional mutant mice. Although expression of the markers H^+/K^+-ATPase β-subunit (Fig. 8C,D) and GATA4 (Fig. 8E,F) is no different in the regions anterior to the junction of the pylorus and duodenum from E18.5 control (Fig. 8C,E) and mutant (Fig. 8D,F) stomachs, the higher level of EAP activity was again found in the mutant hind-stomach (Fig. 8H,J). Taken together, the extended and high levels of EAP expression in the hind-stomach again indicate that patterning of the AP axis is abnormal in the conditional mutants.

Does COUP-TFII mediate Hh signaling in the stomach?

The radial and AP patterning defects elicited by the *COUP-TFII*-null mutants bear resemblance to the dysmorphogenesis of the stomach in *Shh*-null mutant mice (Ramalho-Santos et al., 2000). Because of this similarity, we asked if *COUP-TFII* expression is altered in the Shh-null mutant. Whole-mount in situ hybridization showed that *COUP-TFII* expression in the caudal region of the stomach of the *Shh*-null mutant is downregulated in comparison with the control littermate. However, the expression of *COUP-TFII* in other regions of the stomach is only slightly downregulated (Fig. 9A,B). An anterior shifting of the caudal margin of *COUP-TFII* expression at the gastro-duodenal junction (indicated by broken lines; presumptive gastro-duodenal junction is indicated by arrowheads based on morphology and location of the dorsal mesogastrium) was evident in the *Shh*-null mutant. In addition, a significant size reduction, particularly in the fore-stomach was noted in the *Shh*-null mutant stomachs, even as early as E12.5 stage, using the esophagus as a guide (Fig. 9B, indicated by white lines).

As the presence of *Ihh* in the *Shh*-null mutant stomach (Bitgood and McMahon, 1995) might functionally compensate for *Shh* and influence *COUP-TFII* expression, we used cyclopamine to inhibit all *Hh* signals (Chen et al., 2002)

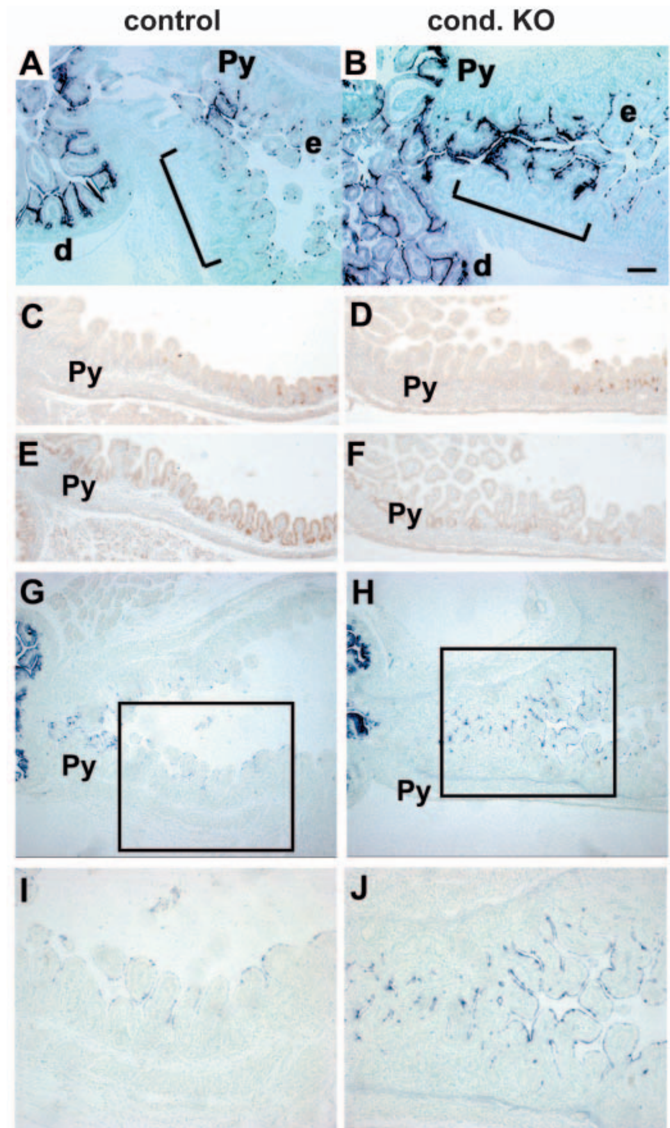

Fig. 8. (A,B) Paraffin sections of E16.5 stomachs were stained for EAP activity. The bracket indicates corresponding regions of the caudal end of control and mutant stomach. oe, esophagus; Py, pylorus; d, duodenum. (C,D) The junction region of the caudal end of the stomach and the duodenum show slight morphological differences. The gastric parietal cells were stained with anti-H^+/K^+-ATPase β-subunit antibody. (E,F) Anti-GATA4 antibody was used to mark glandular gastric epithelium. (G-J) Paraffin sections of E18.5 stomachs were stained for EAP activity. The bracket indicates corresponding regions of the caudal end of control and mutant stomach. Very low positive signals were found in glandular gastric epithelium of the control (G,I) while highly intense signals were seen in the mutant (H,J). High magnification of the boxed corresponding regions in the control (G) and mutant (H) were shown in I and J, respectively. Scale bar in B: 100 μm for A,B.

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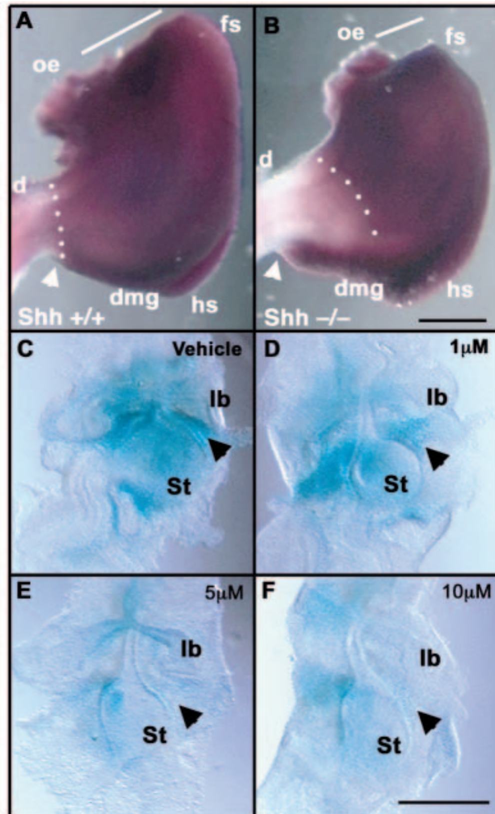

Fig. 9. Effect of inhibition of Hh signaling in *COUP-TFII* expression. (A,B) Developing stomachs were dissected from E12.5 *Shh* wild-type and null mutant, and expression of *COUP-TFII* mRNA was detected by whole-mount in situ hybridization. Caudal margins of *COUP-TFII* expression are indicated by dots, and presumptive anatomical boundaries between stomach and duodenum are indicated by arrowheads. The relative size of fore-stomach is indicated by a white line at the top of the stomach. (C-F) Foregut explants were dissected from E10.5 knock-in embryos and cultured for 48 hours in increasing concentrations of cyclopamine (0-10 μM, D-F). Strong *lacZ* expression was detected in the mesenchyme between lung bud and fore-stomach, as indicated by arrowhead, and cyclopamine inhibited *lacZ* expression in a dose-dependent manner. Scale bars: in B, 0.5 mm for A,B; in F, 0.5 mm for C-F. oe, esophagus; fs, fore-stomach; hs, hind-stomach; dmg, dorsalmesogastrium; lb, lung bud; St, stomach; Py, pylorus; d, duodenum; e, hind-stomach epithelium.

and asked if *COUP-TFII* expression is affected in the explants organ culture. Explants dissected from E10.5 *lacZ* knock-in embryos were cultured for 48 hours in the presence of increasing amounts of cyclopamine. Whole-mount X-gal staining of *COUP-TFII* illustrated a significant dose-dependent reduction of *COUP-TFII/lacZ* expression, especially in the mesenchyme between lung bud and fore-stomach (indicated by arrowheads) (Fig. 9C-F). At 1 μM of cyclopamine, a slight reduction of *COUP-TFII* mRNA, as represented by X-gal staining, was detected in the stomach explants from E11.5 embryos. At 5 μM and 10 μM of cyclopamine, the reduction of X-gal staining was more pronounced in the stomach when compared with the controls (Fig. 9E,F), while *COUP-TFII* mRNA was not affected in lung explants (not shown). The downregulation of COUP-TFII expression when Hh signaling is inhibited by cyclopamine substantiates the notion that *Hh* signaling regulates *COUP-TFII* expression in the stomach.

Discussion

The *lacZ* knock-in mice allow us to follow the expression of *COUP-TFII* during gastric development. It is apparent that COUP-TFII is expressed in both the mesenchyme and the epithelium of the developing stomach. The graded mesenchymal expression profile, with highest levels in mesenchyme subjacent to the epithelium, suggests a diffusible molecule secreted by the epithelium may regulate the expression of *COUP-TFII*. *COUP-TFII* is also expressed in the glandular epithelium of the stomach, with highest expression in the base where the zymogenic cells are localized, but is not expressed in the surface pit cells. The expression patterns in the zymogenic unit resemble that of Shh (van den Brink et al., 2001). Taken together of the above findings, we speculate that Shh might regulate *COUP-TFII* in the stomach in a similar manner as previously shown in the motoneurons (Krishnan et al., 1997a).

Conditional ablation of *COUP-TFII* in the mesenchyme of the developing stomach by *Nkx3.2* Cre recombinase firmly established that *COUP-TFII* is essential for radial patterning of the stomach. At E12.5, it is already apparent that the circular smooth muscles layers and the enteric neurons layers are expanded and disorganized in the conditional mutants. These defects persist at later stages of development. Although *COUP-TFII* in the epithelial compartment has not been deleted, the epithelium is considerably thicker in comparison with the control littermates, indicating signals from the mesenchyme are required for appropriate epithelium growth. The defects display by the *COUP-TFII* conditional mutants indicate that *COUP-TFII* is essential for radial patterning of the developing stomach and these defects are not seen in the compound heterozygote of *Nkx3.2* and *COUP-TFII*. Instead, the inappropriate differentiation of the smooth muscles cells and enteric neurons in the mesenchyme has been shown when Hh signaling is disrupted by treatment with cyclopamine (Sukegawa et al., 2000; van den Brink et al., 2001). Shh is believed to be the epithelial signal that inhibits the gut mesenchyme to differentiate into smooth cells and the neural crest cells to differentiate into enteric neurons. Our findings indicate that COUP-TFII participates in the negative regulation of differentiation of smooth muscle cells and enteric neurons in the gut mesenchyme. COUP-TFII can either serve as a downstream target of Hh to mediate its negative function in the gut mesenchyme or exert its effect in a pathway parallel to Hh to regulate mesenchymal differentiation.

In addition to radial patterning of the stomach, COUP-TFII is also required for AP patterning of the stomach. The anterior shift of the limited ridge that divides the fore- and hind-stomach, the reduced size of the fore-stomach, the expansion of the hind-stomach and the expansion of EAP expression into the hind-stomach are all consistent with disruption of the AP axis patterning in the stomach. However, the differentiation of the epithelium of the fore- and hind-stomach in the adult mutant remains unchanged; with all the molecular markers analyzed, even the mutant stomach is posteriorized. The posteriorization and ectopic extended expression of EAP in the

hind-stomach of the conditional mutant have been demonstrated in the *Shh*-null mutant mice (Ramalho-Santos et al., 2000). The striking similarity of the phenotypes exhibited by conditional *COUP-TFII*-null mutants and animals, chick and mouse, when Hh signaling is disrupted further implicates that Hh and COUP-TFII act in the same and/or parallel pathways.

Although the general slight reduction of expression of *COUP-TFII* in the *Shh*-null mutant is a little surprising, the high expression of Ihh in the expanded hind-stomach of the *Shh*-null mutant could have compensated for the missing Shh to induce *COUP-TFII* expression. Indeed, the downregulation of COUP-TFII expression in the mesenchyme of the stomach is more pronounced in the explants treated with cyclopamine, supporting the notion that *COUP-TFII* is a downstream target of Hh signaling. In addition, Shh signaling can directly activate *COUP-TFII* expression and Shh-N activates *COUP-TFII* expression in P19 cells without de novo protein synthesis (Krishnan et al., 1997a; Krishnan et al., 1997b). Furthermore, analysis of the *COUP-TFII* promoter has identified an ShhRE that is distinct from the defined Gli-binding site (Krishnan et al., 1997a). Thus, all the above results indicate that COUP-TFII mediates Hh signaling in the stomach. Taken together, our results established that COUP-TFII is essential for radial and AP patterning of the stomach. Restriction of anterior *Shh* expression and attenuation of *Shh* action in the epithelium of the *COUP-TFII* conditional mutant, in turn, suggests a potential role for COUP-TFII in the stimulation or maintenance of *Shh* expression.

Shh derived from the epithelium signals the mesenchyme of the stomach, but how mesenchymal factors influence endodermally expressed *Shh* is unclear. The *Hoxa5*-null mutant phenotype provides evidence for mesenchyme-mediated regulation of *Shh* expression in the developing stomach (Aubin et al., 2002). Similarly in chick, it was suggested that adjacent mesenchyme regulates *Shh* expression (Narita et al., 1998). In our study, *COUP-TFII* was ablated in the mesenchyme and the observed dysmorphogenesis suggests that *COUP-TFII* potentially affects such mesenchymal factor(s). One such candidate, *Bmp4*, which belongs to the TGFβ superfamily, is expressed in the mesenchyme of developing murine stomach, while the chick ortholog has been demonstrated to play a role in gizzard patterning. *Bmp4* is important for mesoderm development, but because of early lethality in *Bmp4*-null mutants, no specific role for *Bmp4* in gastric development has been elucidated (Winnier et al., 1995). Interestingly, it has been shown that *COUP-TFII* binds to the mouse *Bmp4* promoter and regulates *Bmp4* promoter activity in transient transfection assays (Feng et al., 1995), suggesting COUP-TFII might function through BMP signaling. Another member of TGFβ super family, activin, governs embryonic axial patterning, and restricts *Shh* expression in Hensen's node (Monsoro-Burq and Le Douarin, 2001). Furthermore, the expansion of *Shh* expression domain within the stomach of *Acvr2a*/*Acvr2b* mutant mice indicates a restriction of *Shh* in the foregut that is potentially regulated by TGFβ/Bmp signaling. As Acvr2 is able to interact with BMPs (Kim et al., 2000), mesenchymal expressed Bmp4 may restrict *Shh* expression. Alternatively, it is possible that COUP-TFII may modulate the expression of activin/activin receptor themselves. Enhanced activin signaling may restrict the expression domain of Shh to the anterior border and perturb the epithelial growth, as seen in the conditional mutant stomach. Further analysis of TGFβ/Bmp/activin signaling in the *COUP-TFII* conditional mutant stomach may shed light on which mesenchymal factor(s) that regulate *Shh* expression are affected by the absence of *COUP-TFII*.

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