Published in final edited form as:
Dev Biol. 2010 September 15; 345(2): 191–203. doi:10.1016/j.ydbio.2010.07.009.

Pinin modulates expression of an intestinal homeobox gene, *Cdx2*, and plays an essential role for small intestinal morphogenesis

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Abstract

Pinin (Pnn), a nuclear speckle-associated protein, has been shown to function in maintenance of epithelial integrity through altering expression of several key adhesion molecules. Here we demonstrate that Pnn plays a crucial role in small intestinal development by influencing expression of an intestinal homeobox gene, *Cdx2*. Conditional inactivation of *Pnn* within intestinal epithelia resulted in significant downregulation of a caudal type homeobox gene, *Cdx2*, leading to obvious villus dysmorphogenesis and severely disrupted epithelial differentiation. Additionally, in Pnn-deficient small intestine, we observed upregulated Tcf/Lef reporter activity, as well as misregulated expression/distribution of β-catenin and Tcf4. Since regulation of *Cdx* gene expression has been closely linked to Wnt/β-catenin signaling activity, we explored the possibility of Pnn's interaction with β-catenin, a major effector of the canonical Wnt signaling pathway. Co-immunoprecipitation assays revealed that Pnn, together with its interaction partner CtBP2, a transcriptional co-repressor, was in a complex with β-catenin. Moreover, both of these proteins were found to be recruited to the proximal promoter area of *Cdx2*. Taken together, our results suggest that Pnn is essential for tight regulation of Wnt signaling and Cdx2 expression during small intestinal development.

Keywords

Pinin; small intestine; villi; *Cdx2*; Wnt signaling; β-catenin

Introduction

The vertebrate small intestine is an ideal model system to study varied processes involved in biological regulation of development and carcinogenesis (Sancho et al., 2004; Wells and Melton, 1999). During small intestinal development, pseudostratified squamous epithelium

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of endodermally-derived tube undergoes a dramatic morphological and functional transition at about embryonic day 14.5 (E14.5) to generate the complex array of villi and crypts, both of which are lined with a simple columnar epithelium (Sancho et al., 2004; Wells and Melton, 1999). This dynamic and extensive morphogenesis of the gut tube requires close interactions between epithelial and mesenchymal cells to accomplish the precise regulation of cell fate specification, proliferation, migration, differentiation, cellular polarization, and the ultimate cell death and shedding in an error-free manner (Beck, 2002; Sancho et al., 2004). The gastrointestinal tract is composed of three interdependent tissue types including smooth muscle, stromal connective tissue, and epithelium. The epithelium itself is populated by enterocytes, enteroendocrine, and goblet cells on the villi, while Paneth cells, migrating progenitor cells, and intestinal epithelial stem cells are found within the crypts (Sancho et al., 2004).

Cdx parahox transcription factors, Cdx1 and Cdx2, have been shown to act as major regulatory proteins, governing expression of numerous intestine-specific genes, including intestinal alkaline phosphatase, cytokeratin 20, villin, and lactase-phlorizin hydrolase (Alkhoury et al., 2005; Chan et al., 2009; Troelsen et al., 1997; Yamamichi et al., 2009). Cdx2 expression becomes evident throughout the developing endoderm of the primitive gut at around E12, while Cdx1 shows apparent and homogenous endodermal expression at E14 (Guo et al., 2004b). Ectopic expression of either Cdx1 or Cdx2 in other gastrointestinal tissues, such as esophagus and stomach, results in intestinal cell metaplasia, demonstrating the potent ability of these homeogenes in epithelial cell fate specification (Mutoh et al., 2002; Silberg et al., 2002; Wong et al., 2005).

During mouse small intestinal development, Cdx2 plays key roles in patterning and differentiation of intestinal epithelium, while closely related Cdx1 does not appear to be required for small intestinal development (Freund et al., 1998; Gao et al., 2009; Grainger et al., 2010; Guo et al., 2004b; Subramanian et al., 1995). Although there exist a few variations in the observed consequence of *Cdx2* inactivation in developing mouse intestine, probably due to the different inactivation timing and/or Cre mouse line used, two recent studies clearly demonstrated major roles of Cdx2 in small intestinal development (Gao et al., 2009; Grainger et al., 2010). Disruption of Cdx2 in their studies consistently resulted in severe villus dysmorphogenesis, abnormal cytodifferentiation, and anterior transformation of small intestinal epithelial cell phenotype to that of esophagus and stomach, thus highlighting the profound impact of Cdx2 on small intestinal development.

Interestingly, a number of studies have reported somewhat conflicting observations on the regulation of *Cdx* gene expression by Wnt signaling. While Cdx2 was shown to be negatively regulated by Wnt/β-catenin activity (da Costa et al., 1999; Kim et al., 2007), activation of *Cdx2* promoter by β-catenin was also demonstrated (Saegusa et al., 2007). Similarly, consistent with its role as a tumor suppressor, downregulation of *Cdx1* was well documented in colon cancer cells which exhibit high β-catenin activity (Suh et al., 2002; Wong et al., 2004). However, other studies showed direct stimulation of *Cdx1* by Wnt/β-catenin signaling pathway (Domon-Dell and Freund, 2002; Lickert et al., 2000; Pilon et al., 2007; Prinos et al., 2001). Furthermore, both Cdx proteins exert inhibitory function on β-catenin activity, functioning as a negative feedback loop (Guo et al., 2004a; Saegusa et al., 2007) and they also can be controlled in an autoregulatory fashion (Prinos et al., 2001; Saegusa et al., 2007). Altogether, although the mechanism by which expression of Cdx1 and Cdx2 is regulated seems to be complex and context-specific, these studies indicate that expression of *Cdx* genes during intestinal morphogenesis may be intimately linked to the Wnt/β-catenin signaling pathway.

Subsequent to the discovery of mutations in *adenomatous polyposis coli* (*APC*) and β-*catenin* (*CTNNB1*) genes in hereditary colorectal cancer (Groden et al., 1991; Joslyn et al., 1991; Morin et al., 1997; Nishisho et al., 1991), particular attention has been paid to the Wnt signaling pathway in intestinal homeostasis, development, as well as tumorigenesis (Batlle et al., 2002; Korinek et al., 1998; Shibata et al., 1997). Indeed, numerous studies have demonstrated an essential role of Wnt signaling pathway in intestinal development (Andreu et al., 2005; Batlle et al., 2002; Blache et al., 2004; Lickert et al., 2000; Pinto et al., 2003; Sansom et al., 2007; Sansom et al., 2004; Theodosiou and Tabin, 2003). Wnt ligands activate the Wnt/β-catenin signaling pathway through binding to receptor complexes composed of the Frizzled proteins and low-density lipoprotein receptor-related proteins (LRP5/6) (Korinek et al., 1997; Schmidt and Patel, 2005). This ligand-receptor interaction leads to the activation of dishevelled (Dvl), which subsequently inhibits the phosphorylation of β-catenin by glycogen synthase kinase 3β (GSK-3β) and casein kinase I (CKI), allowing cytoplasmic accumulation and nuclear translocation of hypophosphorylated β-catenin. Nuclear β-catenin then interacts with T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of DNA-binding proteins to regulate the expression of numerous Wnt target genes. On the other hand, in the absence of ligand stimulation, free cytosolic β-catenin is rapidly degraded via phosphorylation-dependent ubiquitination and proteolysis by a multiprotein complex involving the tumor suppressor proteins APC, Axin, GSK-3β, and CKI. Consequently, Wnt target genes are thought to remain silenced by repressor proteins such as Groucho and CtBPs.

In many studies, the expression of Pnn has been found to correlate with greater epithelial and less mesenchymal characteristics. The expression of Pnn has been shown to enhance cell-cell adhesion, inhibit cell migration, suppress tumorigenesis, transcriptionally regulate expression of *E-cadherin* (*CDH1*) and $p21^{Waf1/Cip1}$ genes, and participate in alternative pre-mRNA splicing (Alpatov et al., 2004; Alpatov et al., 2008; Joo et al., 2005; Shi and Sugrue, 2000; Shi et al., 2000a; Shi et al., 2001; Shi et al., 2000b; Zimowska et al., 2003). Although the exact mechanism by which Pnn's varied activities are translated into enhanced epithelial phenotype is currently unclear, we have recently demonstrated Pnn's impact on Wnt/β-catenin signaling (Joo et al., 2010; Joo et al., 2007). In these previous studies, we reported that conditional inactivation of *Pnn* in developing ocular surface ectoderm led to metaplasia of corneal epithelium which was accompanied with increased β-catenin level (Joo et al., 2010), and that hypomorphic reduction of Pnn resulted in the markedly increased Tcf/Lef activity in mouse embryonic stem (ES) cells and in multiple embryonic tissues including somite, dorsal neural tube, apical ectodermal ridge of developing limbs, early metanephric kidney, and basal layer of the epidermis and underlying mesenchymal cells (Joo et al., 2007). This misregulation of Tcf activity in *Pnn* hypomorphic embryos appeared to cause severe defects in neural crest- and somite-derived tissues. Interestingly, *Pnn* hypomorphic embryos exhibited markedly similar phenotype to that found in ribs and sternum of $Cdx2^{+/-}$ heterozygotes, such as anterior homeotic transformation of thoracic vertebrae (Chawengsaksophak et al., 1997), suggesting possible functional interaction between Pnn and Cdx2.

In the present study, we employed the conditional inactivation of *Pnn* in developing mouse gastrointestinal tract, which revealed Pnn as an essential factor for small intestinal differentiation. In Pnn-deficient intestinal epithelial cells, we observed significant reduction in expression level of an intestinal master regulatory gene, *Cdx2*. Consistent with many previous studies demonstrating direct effect of Wnt/β-catenin signaling on expression of *Cdx2*, we found significant alterations in Wnt activity (Tcf/Lef activity) and aberrant distribution of β-catenin, Tcf4, and Axin2. Furthermore, PNN was found to form a complex with β-catenin and also located to the promoter area of *CDX2*. Thus, these data may suggest that Pnn modulates expression of *Cdx2* through its regulatory role in the Wnt signaling

pathway and its activity on *Cdx2* promoter regulation during mouse small intestinal development.

Materials and methods

Experimental animals

Specific methods pertaining to the generation of *Pnn*-floxed conditional mice were previously reported (Joo et al., 2007). *Pnn* conditional allele (Pnn^{2f}) contains two *loxP* sites flanking exons 3 to 8 and *Pnn* knockout allele (Pnn^{1f}) contains one remaining *loxP* site with exons 3 to 8 deleted. *Topgal* Tcf/Lef activity reporter transgenic mice (DasGupta and Fuchs, 1999) were purchased from Jackson Laboratories. *Shh-GFP-Cre* mice (Harfe et al., 2004) were kindly provided by Dr. Brian Harfe (Molecular Genetics and Microbiology, University of Florida). To increase the efficacy of the Cre-mediated *Pnn* deletion, we intercrossed *Shh-Cre;*$Pnn^{+/1f}$ males with $Pnn^{2f/2f}$ females and harvested embryos at various stages. For timed matings, the presence of a vaginal plug was checked in the morning (E0.5). All mice were housed in pathogen-free conditions and animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Histology, Electron Microscopy, Immunohistochemistry, and β-Galactosidase staining

Histological, electron microscopic, and immunohistochemical analyses were performed by standard methods as previously described (Joo et al., 2007; Ouyang and Sugrue, 1996). Commercially available antibodies used for immunostaining were rabbit polyclonal Cdx1 and Chromogranin A (Abcam Inc.), β-catenin (Cell Signaling), Axin2/Conductin (Santa Cruz Biotechnology), rabbit monoclonal Tcf4 (Cell Signaling), and mouse monoclonal Cdx2 (BioGenex).

Alkaline Phosphatase Activity was visualized with AP substrate kit (Vector Labs) according to the manufacturer's protocol and counterstained with Hematoxylin (Vector labs). Alcian Blue (pH 2.5) staining was carried out by standard methods and counterstained with Nuclear Fast Red (Vector Labs). Periodic Acid Schiff (PAS) staining was carried out with Periodic Acid-Schiff staining system (Sigma) as described by the manufacturer.

Whole-mount β-Galactosidase staining of embryonic intestines was performed as previously described (Joo et al., 2007) with X-Gal (5-Bromo-4-chloro-3-indolyl- β-D-galactopyranoside; Fisher) or Red-Gal (6-Chloro-3-indolyl-β-D-galactoside; Research Organics). Red-Gal stained intestines were embedded in paraffin, sectioned, and counterstained with Alcian Blue (Sigma).

Semi-quantitative and quantitative real-time RT-PCR

Total RNA was isolated from embryonic intestinal cells or mouse embryonic stem (ES) cells with NucleoSpin RNA II kit (Clontech) and treated with RNase-free DNase I. 1 μg of total RNA was reverse transcribed with Superscript III First-Strand Synthesis kit (Invitrogen Co.) using oligo-dT primers. Subsequent PCR steps were performed with GoTaq Flexi DNA Polymerase (Promega Co.) according to manufacturer's specifications at 55°C annealing temperature for 25 PCR cycles. Quantitative real-time RT-PCR assays were carried out by standard curve method with iQ SYBR Green Supermix on MyiQ single color real-time PCR detection system or CFX96 Real-Time PCR Detection System (BIO-RAD). The data were then analyzed with MyiQ Optical System Software or CFX Manager software (BIO-RAD). For primer sequences: for *mPnn*, 5'-GCTTCTGGAGCAGAAGGTTGA-3' and 5'-GATTACCTGTCTCCTCCAACT-3'; for *mCtnnb1*, 5'-GGAAGGAGCTAAAATGGCAGT-3' and 5'-GGTCCACAGAAGCTTCTCATA-3'; for *mLph*, 5'-GGGGAGTTGCTTCCATTACA-3' and 5'-CGTACAGCTTTGAGGGCTTC-3';

for *mApoa1*, 5’-CAGAGACTATGTGTCCCAGTTTGA-3’ and 5’-GGTGTGGTACTCGTTCAAGGTAG-3’; for *mFabp1*, 5’-TGCAGAGCCAGGAGAACTTT-3’ and 5’-CCCAGTCATGGTCTCCAGTT-3’; for *mFabp2*, 5’-GTAGACCGGAACGAGAACTATG-3’ and 5’-TAGCTTTGACAAGGCTGGAGAC-3’; for *Gapdh*, 5’-CTGCACCACCAACTGCTTAG-3’ and 5’-CCTGCTTCACCACCTTCTTG-3’; for *mCdx1* 5’-GGACAAGTACCGTGTGGTCTA-3’ and 5’-GCGGTTCTGGAACCAGATCTT-3’; for *mCdx2*, 5’-GGAGCTGGAGAAGGAGTTTCA-3’ and 5’-GGAGGAGAGGAATCTCTTCTG-3’; for *mAxin2*, 5’-CCACGGAAACAGCTGAAAACG-3’ and 5’-GGTAGAGACACTTGGCCATTG-3’; for *hCDX1*, 5’-GTAAGACTCGGACCAAGGACA-3’ and 5’-GCCGGTTTTGGAACCAGATCT-3’; for *hCDX2*, 5’-TATCGAGTGGTGTACACGGAC-3’ and 5’-CCTTTGCTCTGCGGTTCTGAA-3’.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were carried out as previously described (Crusselle-Davis et al., 2007). Briefly, cells or tissues were washed in ice-cold PBS containing protease inhibitors (Roche), crosslinked in 1.42% formaldehyde (Fisher) for 10–20 mins, and quenched with glycine. Crosslinked chromatin was fragmented by sonication to an average size of 500 bp with Branson Sonifier 250. Chromatin fragments were then incubated overnight at 4°C with primary antibodies; rabbit polyclonal anti-H3K4me3 and anti-GFP antibodies (Abcam Inc.), goat polyclonal anti-Tcf4 antibody (Santa Cruz Biotechnology), and mouse monoclonal anti-CtBP2 antibody (BD transduction Labs). Chromatin/antibody complexes were precipitated with Protein A- or G-Sepharose (GE Healthcare) and washed extensively. Purified DNA was analyzed by semi-quantitative PCR or real-time quantitative PCR system (Bio-Rad) using specific primer pairs for mouse and human *Cdx1*, *Cdx2* and *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) promoters: for *mCdx1* promoter, 5’-CGTTTGAAGTCAGCCTTGCTC-3’ and 5’-GGGTTACAGGTGGAGAAACTG-3’; for *mCdx1* exon3, 5’-CCCTACTAATGCTGGCCTTCT-3’ and 5’-CAACTCAGAACAGGTCCTTGG-3’; for *mCdx2* promoter 1, 5’-CTCTTTTGTAGAGGCCGAGTG-3’ and 5’-AGCTTTCTGACCAACCCAGGA-3’; for *mCdx2* promoter 2, 5’-GTTGGAAGGAGGAAGCTCGTA-3’ and 5’-TGACACAGACACCAATGGCTG-3’; for *mCdx2* exon3, 5’-CCTCAGGGGAAGACATGGTTT-3’ and 5’-CAAAGAGCTGGGGCAAAAGAG-3’; for *mGapdh*, 5’-GATGATGGAGGACGTGATGG-3’ and 5’-GGCTGCAGGAGAAGAAAATG-3’; for *hCDX1* promoter, 5’-CATCCACAGCTTCCATGACGA-3’ and 5’-CCATTTTACAGGCGGAGGAAC-3’; for *hCDX2* promoter, 5’-GGAGGTTAAAGTGCACCAGGT-3’ and 5’-GACACAGACACCAATGGTTGG-3’

BrdU incorporation

To detect cells in S-phase of mitosis, timed-pregnant females were injected intraperitoneally with BrdU (30 μg/gm body weight) in sterile PBS. Two hours after injection, female mice were sacrificed and embryos were collected. BrdU detection was carried out with Vectastain Elite ABC anti-rat Kit (Vector Labs) and rat anti-BrdU monoclonal antibody (Accurate chemicals).

Cell culture and stable cell lines

EcR293-PNNGFP cells were cultured and treated with Ponasterone A (Invitrogen) as previously reported (Shi et al., 2000a). Wild-type mouse embryonic stem cells were maintained as described previously (Joo et al., 2007). OE33 cells were maintained in RPMI1640 media supplemented with 10% fetal bovine serum and 200 U/ml each of

penicillin and streptomycin (Cellgro). For the stable transfection of PNNGFP construct (Shi et al., 2000a) into OE33 cells, cells were transfected using Lipofectamine 2000 reagent as described by the manufacturer (Invitrogen) and selected with 400 μg/ml G418 (Gibco). HET-1A and LoVo cells were cultured in LHC-9 (Gibco) and F12 (Cellgro) media respectively supplemented with 10% fetal bovine serum and 200 U/ml each of penicillin and streptomycin (Cellgro).

Co-Immunoprecipitation (Co-IP) Assay and immunoblotting

Co-IP assays were carried out with Profound™ Mammalian Co-IP kit (Pierce) according to manufacturer's protocol. Rabbit polyclonal anti-GFP antibody (Abcam Inc.) and mouse monoclonal anti-CtBP2 antibody (BD transduction Labs) were used for immunoprecipitation. Immunoblotting was performed as previously reported (Joo et al., 2005) with mouse monoclonal antibodies against CtBP1, CtBP2, PCNA (BD transduction Labs), goat polyclonal anti-p68 antibody (Abcam Inc.), rabbit polyclonal antibody against β-catenin (Cell Signaling). Monoclonal antibodies raised against PNN were mAb M2-96 and mAb 2-2–143.

Results

Deletion of Pnn in epithelium of the intestine leads to severe villus dysmorphogenesis

We have previously shown that *Pnn*-null embryos die at a peri-implantation stage, while *Pnn* hypomorphic mutants die at peri-natal stages with defects in the cardiac outflow tract, axial skeleton, and skin (Joo et al, 2007). To further define the role of Pnn in development, we have conditionally deleted *Pnn* by crossing the *Pnn* conditional knockout mice with the *Shh-Cre* mice (Harfe et al., 2004). *Pnn* conditional allele (Pnn^{2f}) contains two *loxP* sites flanking exons 3 to 8 and *Pnn* knockout allele (Pnn^{1f}) contains one remaining *loxP* site with exons 3 to 8 deleted. All *Shh-Cre;*$Pnn^{2f/1f}$ (hereafter termed "Pnn mutant") mice died shortly after birth with severe and specific defects in bronchial branching morphogenesis and small intestine differentiation. In this article, we specifically focus on the role of Pnn in small intestinal development.

As Cre recombinase is expressed in the endoderm of the gastrointestinal tract of *Shh-Cre* positive mice as early as E8.5 (Bitgood and McMahon, 1995; Harfe et al., 2004), *Pnn* gene is deleted in the early intestinal epithelium, but not in stromal or smooth muscle compartments of mutant intestine. X-Gal staining of control intestines positive for both *Shh-Cre* and *R26R* Cre reporter transgenes demonstrated specific Cre recombinase activity in the epithelia of developing intestine (Figs. 1A–C). The successful inactivation of *Pnn* in small intestinal epithelia of mutant mice was confirmed by quantitative RT-PCR (Fig. 1D), which showed significant reduction in transcript level of *Pnn* throughout the small intestine. Protein level of Pnn was also examined by immunostaining (Figs. 1E–H). As expected, mutant small intestine clearly displayed epithelial-specific depletion of Pnn.

The *Pnn*-deficiency within the intestinal epithelium did not result in observable gross or histological aberrations until E14 (data not shown). This might be due to the incomplete recombination of floxed *Pnn* allele in early endoderm, as recently shown to be the case in conditional *Cdx2* inactivation (Grainger et al., 2010). However, severe villus defects observed in directly following stages from Pnn-deficient small intestine is consistent with the possibility that Pnn is not required for the early endoderm development. On the other hand, beginning at E14.5, when the extensive remodeling of intestinal epithelium occurs, the small intestine of mutant embryos exhibited severe perturbations in the formation of villi (Figs. 2A–F). While control small intestine exhibited the typical arrangement of well-developed villi at late gestational stages (Figs. 2C, E), Pnn mutant showed markedly reduced

number of definitive villi that seemed to be replaced by abnormally-shaped short luminal protrusions (Figs. 2D, F). H&E staining of E18.5 small intestines revealed obvious irregular localization of nuclei of mutant villus epithelial cells, in contrast to control nuclei which characteristically localize to the basal side of villus epithelial cells (insets in Figs. 2E, F). Intervillus region of embryonic small intestine invaginates into the submucosa during the first weeks after birth, establishing the crypt, a highly proliferative epithelial compartment (Sancho et al., 2004). As shown in Figs. 2G, H, control intervillus area displayed regularly organized proliferating cells, however, mutant epithelium showed randomly localized (even at the tip of the mutant villi) BrdU-positive cells. While we did not observe significant change in the percentage of BrdU-positive epithelial cells in mutant epithelia (Fig. 2I), considerably increased portion (15.9%) of BrdU positive cells was detected within the mutant villus area at positions higher than 8, according to the position with the bottom of intervillus space as 0, as previously reported by Grainger et al. (Grainger et al., 2010). This observation may suggest disruption of cellular proliferation/differentiation program in Pnn mutant small intestine. Apoptotic cell death in mutant intestine was not noticeably changed from that seen in control epithelia (data not shown).

Ultrastructural analysis revealed that, while control epithelia demonstrated a well-differentiated brush border, the free surface of the Pnn mutant epithelial cells appeared disorganized and lacking differentiated qualities (Figs. 3A, B). Moreover, as shown in Figs. 3C–J, mutant epithelial cells displayed perturbed cellular differentiation into goblet and enteroendocrine cell lineages, and failed to exhibit comparable intestinal alkaline phosphatase (IAP) activity to the control epithelial cells. The expression levels of differentiation-dependent genes were also markedly reduced in Pnn mutant intestine, compared to those in control intestine (Figs. 3K, L), suggesting that Pnn-deficient epithelial cells of small intestine are impaired for proper differentiation.

Expression of Cdx2 is markedly reduced in Pnn-deficient small intestinal epithelium

To gain insight into the underlying mechanism of disrupted cytodifferentiation and villus morphogenesis in Pnn mutant small intestine, we examined expression level of Cdx2, which functions as a key regulator of small intestinal development (Beck, 2002; Gao et al., 2009; Grainger et al., 2010; Guo et al., 2004b), and whose inactivation led to very similar villus phenotypes to those described here in Pnn mutant small intestine. While control small intestine showed strong homogeneous expression of Cdx2 throughout the epithelial layer, mutant epithelial cells displayed significantly reduced Cdx2 immunoreactivity (Figs. 4A, B). Additionally, similar to the *Cdx1* downregulation following inactivation of *Cdx2* in small intestine (Gao et al., 2009), immunohistochemical study of Cdx1 revealed significant downregulation of *Cdx1* in Pnn-depleted small intestine as well (Figs. 4C, D). Control intestine showed a typical Cdx1 expression pattern with an obvious expression-gradient along the crypt-villus axis, whereas Pnn mutant epithelium demonstrated no detectable Cdx1 immunostaining at E16.5. Downregulation of both *Cdx2* and *Cdx1* in mutant small intestine was confirmed by real-time RT-PCR assays and the reduction was clearly apparent even as early as E14.5 (Fig. 4E).

Next, to determine whether decreased mRNA levels of *Cdx2* and *Cdx1* are due to the transcriptional repression on their promoters, we carried out ChIP assays. Consistent with their downregulation in Pnn mutant epithelium, the trimethylation on lysine 4 of histone H3 (H3K4me3), a general marker of transcriptional activation, was dramatically decreased in both *Cdx2* and *Cdx1* promoters of mutant intestine (Fig. 4G). Together, these data indicate that, in Pnn-deficient intestinal epithelial cells, expression of *Cdx2* and *Cdx1* is significantly downregulated probably through the mechanism of transcriptional repression.

Tcf/Lef activity is upregulated in Pnn-deficient small intestinal epithelium

An increasing number of studies have documented that *Cdx* homeogenes are responsive to Wnt/β-catenin signaling activity, especially during intestinal development (da Costa et al., 1999; Domon-Dell and Freund, 2002; Kim et al., 2007; Lickert et al., 2000; Pilon et al., 2007; Prinos et al., 2001; Saegusa et al., 2007). Our previous studies demonstrated upregulation of β-catenin level and TOPFLASH/*Topgal* reporters in Pnn-depleted tissues and ES cells, suggesting Pnn's possible involvement in the modulation of Wnt signaling activity (Joo et al., 2010; Joo et al., 2007). Thus, it is likely that Pnn's role in Wnt/β-catenin signaling may provide a link for its influence on expression of *Cdx* genes. To investigate the extent to which Pnn's impact on Cdx expression involves its ability to modulate Wnt signaling, we utilized *Topgal* transgenic mice. It has been previously reported that Topgal activity is first detectable around E16 during small intestine organogenesis (Kim et al., 2007). Interestingly, their study showed that only villus epithelial cells, not intervillus cells, display Topgal activity during late gestation and early postnatal period. The earliest stage that Topgal activity is detected from intervillus area is the third postnatal day. And, because non-specific β-galactosidase activity is frequently observed in the gastrointestinal tissues, especially after prolonged incubations with the X-Gal staining solution, we carefully monitored β-galactosidase staining across narrow time-course. In late gestational periods (E16.5 – E18.5), distinct ring-shaped X-Gal-positive areas were apparent in Pnn mutant *Topgal*-positive small intestine, whereas no such area was observed in control *Topgal*-positive intestine after a short period of incubation (Figs. 5A–D). Sectioning of intestines stained with Red-Gal for 1 hour revealed that the signal was most intense in the cells residing within the base area encircling mutant villi, while control small intestine was yet to display a detectable level of staining (Figs. 5E, F). This observation of strong ectopic Topgal activity from the area, which shows no activity in wild-type embryonic intestine, may indicate aberrant over-activation of Tcf/Lef activity in mutant small intestinal epithelium.

Expression or distribution of β-catenin, Tcf4, and Axin2 are altered in Pnn-deficient intestinal epithelia

Consistent with the elevated Topgal activity in Pnn mutant small intestinal epithelium, considerable increase in β-catenin immunoreactivity was observed in mutant small intestine (Figs. 6A, B). Mutant epithelial cells localizing at the basal area exhibited more prominent increase in cytoplasmic and nuclear immunoreactivity of β-catenin than mutant cells residing within the short projections. This increase did not seem to be due to the upregulated transcription of β-catenin, as quantitative RT-PCR assay revealed similar level of β-catenin transcript between control and mutant small intestine (Fig. 6G).

In addition, aberrant distribution of Tcf4, one of the predominant Tcf/Lef family transcription factors of the small intestine, was also detected in Pnn mutant intestine (Figs. 6C, D). At E16.5, a stage when control intestine showed mostly intervillus-restricted expression of Tcf4, mutant intestines exhibited virtually homogeneous Tcf4 expression across the epithelium.

Axin2, a direct Wnt target gene and negative feedback regulator of Wnt signaling (Leung et al., 2002), has been shown to be expressed primarily in the villus epithelial cells of wild-type intestines during late gestational periods (Kim et al., 2007). Axin2 has also been shown to localize mainly within the nuclei of crypt cells in normal colon (Anderson et al., 2002). In normal mouse embryonic small intestine, Axin2 was detected in both the nucleus and cytoplasm of epithelial cells with increased expression in villus area (Fig. 6E). Control intestines also exhibited very infrequent villus cells with strong nuclear Axin2 expression. In contrast, most of mutant intestinal epithelial cells displayed strong nuclear distribution of Axin2 with comparable cytoplasmic Axin2 level to control cells (Fig. 6F). To quantitatively

assess the expression level of *Axin2* in Pnn mutant intestine, we performed real-time RT-PCR assay with E14.5 and E17.5 small intestines. Transcript levels of *Axin2* appeared sustained or increased in mutant intestine at the stages tested (Fig. 6G). The upregulation of a Wnt target gene, *Axin2*, in Pnn mutant intestine is a strong indication of Wnt activation in the area and is consistent with the described upregulation of Tcf activity and increase in β-catenin level.

PNN forms a complex with β-catenin

To investigate the molecular mechanism by which Pnn affects the level/activity of β-catenin, we performed co-immunoprecipitation assay on EcR293-PNNGFP cells (Shi et al., 2000a), which stably express PNN upon Ponasterone A induction (Fig. 7A). Intriguingly, our co-immunoprecipitation data revealed that PNN is present in a complex with β-catenin (Fig. 7B). PNN's other previously known interaction proteins such as transcriptional co-repressors CtBP1and CtBP2, and RNA helicase p68 were also detected among PNN immunoprecipitates, while unrelated Proliferating Cell Nuclear Antigen (PCNA) was not found in PNN complex demonstrating the specificity of our assay. Moreover, the association of PNN with β-catenin was also observed in another cell line, OE33 human esophageal adenocarcinoma (Barrett's metaplasia) cells (Fig. 7C). This is the first report demonstrating PNN's association with β-catenin and, together with the abnormal upregulation of β-catenin in Pnn-deficient cells, raises exciting possibility of PNN's role in the regulation of β-catenin activity. Our co-IP data also demonstrates that CtBP2, a PNN-interacting protein and negative regulator of Wnt target gene expression, forms a complex with β-catenin (Fig. 7D). As expected from the heterodimer formation between CtBP1 and CtBP2 (Bergman et al., 2006), large amount of CtBP1 was co-immunoprecipitated with CtBP2. However, CtBP2 was associated with neither p68 nor PCNA. Taken together, the association of PNN and its interacting Protein CtBP2 with β-catenin implies possible functional interactions between these proteins, and may provide a solid framework for future investigation.

PNN is present at the promoter area of CDX2

As shown in Fig. 4G, *Cdx2* was transcriptionally downregulated in Pnn-depleted small intestine. Since PNN was previously shown to relieve transcriptional repression activity of CtBPs and thus lead to the derepression of *CDH1* (*E-Cadherin*) promoter (Alpatov et al., 2004;Alpatov et al., 2008), we examined whether PNN is directly involved in the promoter regulation of *Cdx2* in two human cell lines, OE33 and EcR293 cells, both expressing GFP-tagged PNN protein. Semi-quantitative RT-PCR assay revealed that both OE33 and EcR293 cells express *CDX2*, however neither of them expressed *CDX1* (Fig. 8A). Chromatin status of *CDX2* and *CDX1* promoter areas in EcR293 cells was consistent with our RT-PCR data (Fig. 8C), where active chromatin marker, H3K4me3, was enriched only on *CDX2* promoter, but not on *CDX1* promoter. Importantly, our ChIP analyses on both EcR293-PNNGFP and OE33-PNNGFP cells showed that PNN is specifically recruited to the active promoter of *CDX2*, but not to the inactive *CDX1* promoter (Figs. 8D, E). Additionally, we also found CtBP2, an interaction partner of PNN, on the promoter area of *CDX2* in OE33 cells and mouse ES cells (Figs. 8F, G). These observations imply that, as previously shown in *CDH1* promoter regulation (Alpatov et al., 2004;Alpatov et al., 2008), PNN and CtBP proteins are directly involved in the regulation of *CDX2* promoter activity.

Discussion

Here, we demonstrate that conditional deletion of a nuclear speckle-associated protein, Pnn, in epithelial cells of the developing small intestine results in severe dysmorphogenesis of intestinal villi. An intestinal homeobox gene, *Cdx2*, is significantly downregulated in the absence of Pnn, suggesting a pivotal role of Pnn in the expression of Cdx2 during intestinal

development. We also provide evidence of misregulated Wnt/β-catenin signaling in Pnn-depleted intestine, association of PNN with β-catenin, and recruitment of PNN to *CDX2* promoter, which may all signify the direct role of Pnn in the correct regulation of Wnt signaling and Cdx2 expression for differentiation of small intestinal epithelia and morphogenesis of intestinal villi during late gestational period.

In *Shh-Cre* mice, Cre recombinase is expressed in multiple tissues including notochord, floor plate of the neural tube, mesenchymal cells within the limb bud, and the epithelial cells of developing lung and intestine (Harfe et al., 2004). However, Pnn mutant embryos exhibited specific defects in lung epithelial branching morphogenesis and in the development of small intestinal villi, but not noticeably in other Shh-expressing tissues, such as limb, neural tube, and hair follicle. In addition, increase in cell death was not detected in Pnn-deficient epithelial cells. These results suggest that Pnn plays a role in specific epithelial developmental processes rather than a general housekeeping role required for cell vitality.

Our observation of significant reduction and/or delay in expression of intestinal differentiation marker genes in Pnn mutant intestine indicates that undifferentiated mutant gut endodermal cells are unable to begin the initial arrangement requisite for villi formation. Differentiation of gut endoderm into intestinal epithelium occurs around E14.5. Among the many transcription factors involved in intestinal differentiation, a caudal type homeobox gene, *Cdx2*, has been considered as a master regulator in intestinal development due to its ability to both direct proper intestinal patterning and expression of numerous intestine-specific genes, and induce gastrointestinal metaplasias (Beck, 2002; Gao et al., 2009; Grainger et al., 2010; Guo et al., 2004b). Thus, based on the downregulation of *Cdx2* in Pnn mutant intestine and the phenotypic similarity between Pnn- and Cdx2-deficient small intestines (Gao et al., 2009; Grainger et al., 2010), we postulate that Pnn deficiency-induced perturbation of Cdx2 expression may be integral to the disruption of endodermal differentiation. Importantly, our current studies identify Pnn as an important upstream factor for expression of Cdx2. Indeed, despite substantial amount of information pertaining to the function of Cdx proteins and their target genes, not many proteins have been discovered to be involved in the regulation of the Cdx proteins themselves. In cell culture system, expression of Cdx2 was shown to be positively regulated by Smads (1 and 4), NF-κB, and Cdx2 itself (Barros et al., 2008; Kim et al., 2002; Saegusa et al., 2007) and negatively by Sox9 (Blache et al., 2004). Thus, our study may provide valuable new *in vivo* evidence of an additional mode of Cdx regulation during small intestinal development. In summary, our data clearly demonstrate that Pnn may play a pivotal and direct role for small intestinal morphogenesis by influencing expression level of Cdx2, even though we are unable to provide specific molecular mechanism for Pnn's role in the regulation of *Cdx2* gene expression during small intestinal development.

It has been previously reported that inactivation of *Cdx2* or inhibition of Hedgehog signaling in developing intestine similarly disrupt epithelial-mesenchymal interaction, leading to the severe villus dysmorphogenesis and expansion of smooth muscle cells (SMCs) into villus cores (Gao et al., 2009; Madison et al., 2005). Interestingly, Pnn-depletion in intestinal epithelium did not seem to noticeably affect mesenchymal compartment of mutant intestines. We have not observed considerable changes in the expression levels of Hedgehog signaling-related genes nor expansion of SMCs (Supplemental Fig. S1). Thus, it is likely that epithelial-specific Pnn depletion does not lead to dysregulation of Hedgehog signaling in developing small intestine.

The data presented in this study, which are largely consistent with our previous studies (Joo et al., 2010; Joo et al., 2007), provide strong evidences of Wnt activation upon *Pnn*

inactivation. We successfully documented ectopic *Topgal* transgene activation, increased β-catenin level, disrupted Tcf4 distribution, and upregulation of a well-established Wnt target gene, *Axin2*, in Pnn-depleted small intestine, all of which strongly suggest that Pnn plays a critical role in the regulation of Wnt signaling. Furthermore, we were, for the first time, able to demonstrate that PNN is a part of β-catenin complex, which raises the possibility of PNN's direct role in the regulation of β-catenin activity.

Wnt signaling has been firmly linked to regulation of intestinal *Cdx* gene expression (Guo et al., 2004b; Pilon et al., 2007; Prinos et al., 2001; Saegusa et al., 2007). While more clarification on the exact mechanism is needed, *Cdx* genes are clearly shown to be affected, both positively and negatively, by Wnt/β-catenin activity. Interestingly, in Pnn mutant intestine, which exhibits severe villus morphogenesis defect, we discovered that downregulation of *Cdx2* is accompanied with significant upregulation of Wnt/β-catenin activity. Thus, it is tempting to speculate that increased Wnt/β-catenin activity following inactivation of *Pnn* might play a causal role in the downregulation of *Cdx2*. Indeed, Kim *et al*. reported that downregulation of *Cdx2* is specifically correlated with decreased differentiation potential of small intestinal epithelial cells of mouse expressing constitutively active β-catenin under the control of *Shh* promoter (Kim et al., 2007). However, the exact mechanism by which increased β-catenin level/activity leads to the downregulation of Cdx2 and factors involved in the process are yet to be determined. In this regard, although we recognize that our current study cannot rule out other possibilities, we propose that Pnn might function as one of essential factors involved in β-catenin-mediated regulation of Cdx2 expression by modulating β-catenin level/activity through its interaction with β-catenin to ensure proper expression timing of key regulatory *Cdx2* gene during small intestinal development.

In addition, recruitment of PNN to the proximal promoter of *CDX2* near the Tcf-binding element may extend PNN's role to the promoter regulation of a Wnt-regulated gene, *Cdx2*. In fact, PNN was previously shown to relieve CtBP-mediated repression on *CDH1* promoter, which is shown to be directly downregulated by Wnt activity (Alpatov et al., 2004; Jamora et al., 2003). Therefore, our study suggests that PNN may also function as a part of transcriptional complexes assembled on the target genes of Wnt/β-catenin signaling.

Conversely, Cdx2 has been shown to possess an ability to inhibit β-catenin activity (Guo et al., 2010; Guo et al., 2004a; Saegusa et al., 2007). Moreover, conditional inactivation of *Cdx2* in developing intestine by *Foxa3* promoter-driven Cre expression resulted in upregulation of multiple Wnt ligands and Wnt target genes (Gao et al., 2009). Thus, direct and preceding downregulation of *Cdx2* caused by perturbed promoter regulation due to the absence of Pnn may, in turn, result in the abnormal activation of Wnt signaling and defective villus development. These may also work in a feedback loop fashion as depicted in a diagram shown in Fig. 9. However, since we have observed elevated Wnt/β-catenin activity in other Pnn-depleted tissues, which do not express Cdx2 (Joo et al., 2010; Joo et al., 2007), we propose Pnn's direct role on Wnt/β-catenin signaling as the causal element of disrupted Cdx2 expression and subsequent intestinal maldevelopment in Pnn mutant mice. We believe that more careful examination of changes in their expression and/or activity will be required to resolve this complicated functional cascade.

In summary, our data clearly demonstrate that Pnn exerts an essential role in small intestinal differentiation by regulating expression of Cdx2. We also present Pnn-deficiency-induced upregulation of Wnt activity as a possible explanation for observed *Cdx2* suppression. Further detailed work will enable us to gain more mechanistic insights into this fundamental developmental step and essential elements involved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Brian Harfe, University of Florida, for the *Shh-GFP-Cre* mouse line. We also thank Debra Akin for her technical assistance and Todd A. Barnash for his computational help. This work was supported by grants to S.P.S. (R01 EY07883) from the NIH.

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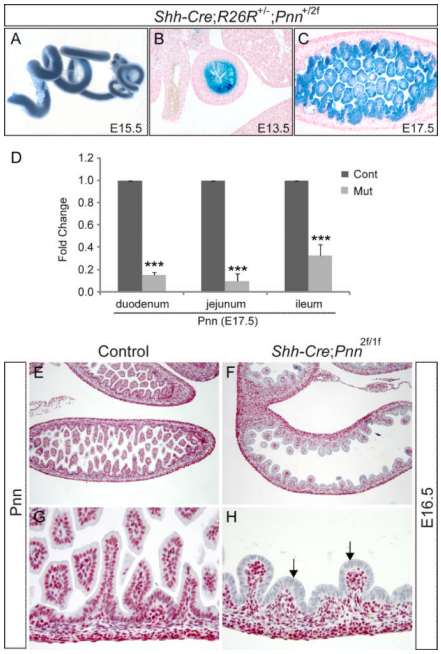

Fig. 1.

Deletion of *Pnn* in developing small intestinal epithelium. (A) Whole-mount X-Gal staining of E15.5 control intestine positive for both *Shh-Cre* and *R26R* demonstrates strong Cre activity in full length of developing mouse intestine. (B, C) Section images of X-Gal stained control *Shh-Cre;R26R*$^{+/-}$;*Pnn*$^{+/2f}$ intestine reveal specific Cre activity in epithelial cells of small intestine. (D) Inactivation of *Pnn* was analyzed by quantitative RT-PCR assays with different parts of small intestine (duodenum, jejunum, and ileum). *Pnn* transcript level was significantly reduced in all parts of mutant small intestine at E17.5. Expression levels are normalized to *Gapdh*. Error bars represent standard deviation. All *p* values are compared to each control intestine. ***: $p < 0.001$ (two-tailed unpaired Student's t-tests, n=3). (E–H) Pnn immunostaining (red signal within the nuclei) shows successful and specific inactivation of *Pnn* in epithelial cells of mutant small intestine (arrows in H). Note similar Pnn immunostaining of stromal and muscle cells of control and mutant intestines. Original magnification: (A) 10X; (B, C) 200X; (E, F) 100X; (G, H) 400X.

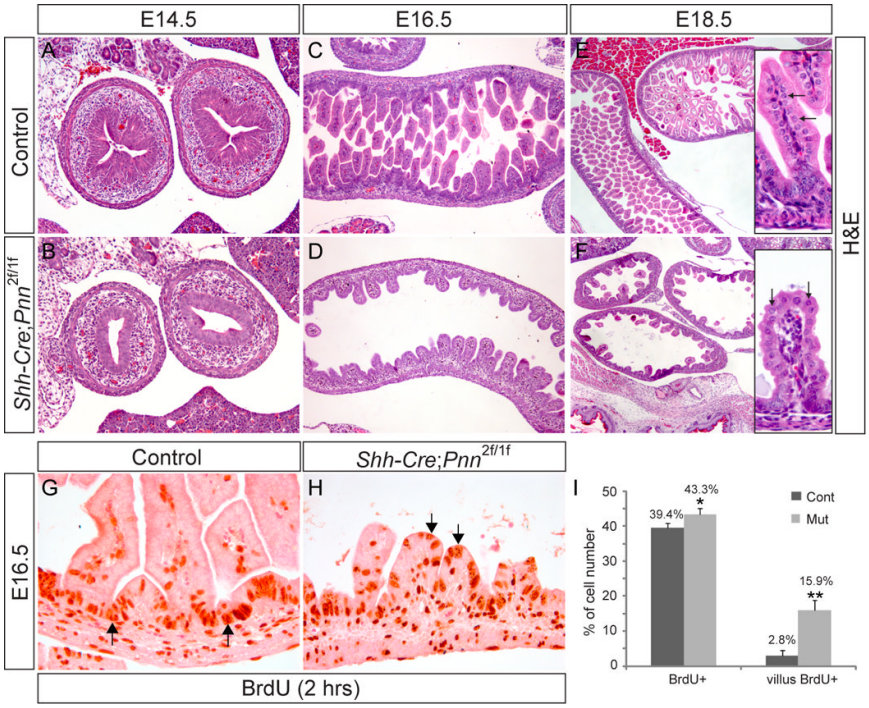

Fig. 2.

Villus dysmorphogenesis in Pnn mutant small intestine. (A–F) H&E staining of small intestines demonstrates severe villus defect in mutant. At E14.5, mutant small intestine shows slight delay in intestinal epithelial morphogenesis. However, at later stages, villus defect of mutant intestine becomes more apparent, exhibiting much shorter and fewer villi. Insets in E, F show high magnification images of control and mutant villi, respectively. Arrows indicate nuclei. (G, H) BrdU incorporation assay shows a regularly organized pattern of BrdU-positive epithelial cells in E16.5 control intervillus area (arrows in G), however, randomly distributed BrdU-positive epithelial cells (arrows in H) are observed in Pnn mutant small intestine. (I) Quantification of BrdU-positive cells in small intestinal epithelia after 2hr *in vivo* BrdU labeling reveals increased mislocalization of BrdU-positive epithelial cells in mutant intestine. The number of total epithelial cells and BrdU-positive epithelial cells were determined by counting cells from the same magnification images (n=9) of each control and mutant small intestine (E16.5) and presented as percentage of cell numbers. The position of cells was determined according to the cell at the bottom of intervillus space as 0. *: $p < 0.05$, **: $p < 0.01$ (two-tailed unpaired Student's t-tests). All *p* values are compared to control samples. Original magnification: (A, B) 200X; (C, D) 100X; (E, F) 50X; (Insets in E, F) 400X; (G, H) 400X.

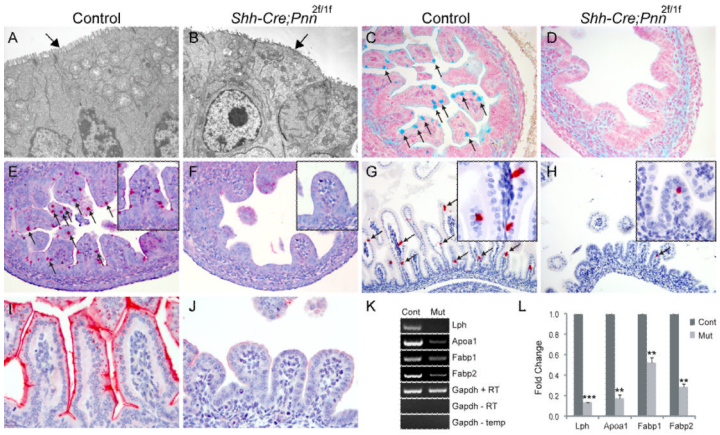

Fig. 3.

Disrupted differentiation of Pnn mutant small intestinal epithelial cells. (A, B) Electron microscopy of E16.5 small intestine reveals disorganized brush border formation in Pnn mutant small intestine (arrows in A, B). (C, D) Alcian blue staining demonstrates differentiation of sialomucin-producing goblet cells in control intestine (blue signal, arrows in C), while the absence of developing goblet cells is obvious in mutant villi at E16.5. (E, F) Representative images of PAS staining on E16.5 control and mutant small intestine also reveal interrupted differentiation of goblet cell lineage in Pnn mutant small intestine. While control small intestine shows numerous PAS stain-positive cells (arrows in E), mutant intestine failed to exhibit staining positive goblet cells. Insets are higher magnification images of an individual villus. (G, H) Immunostaining analysis of chromogranin A reveals impaired differentiation of enteroendocrine cell lineage in Pnn mutant small intestine at E18.5. Chromogranin A-positive cells are shown in red and marked with arrows. Insets in G, H are higher magnification images showing chromogranin A-positive cells. (I, J) Control enterocytes show strong alkaline phosphatase activity (red reaction in I), while mutant villus epithelial cells display marginal IAP activity at E17.5. (K) Semi-quantitative RT-PCR assays reveal significantly reduced expression of cytodifferentiation markers in E17.5 mutant small intestine. The experiment was carried out with three or more mice for each control and mutant, and all mutant intestines examined consistently showed significant reduction in the transcript levels of genes shown. (L) Quantitative real-time RT-PCR analyses of E18.5 control and mutant small intestines confirm downregulation of marker genes. Expression levels for all tested genes are normalized to *Gapdh* level. Statistical analysis was performed using two-tailed unpaired Student's t-tests (n=3). **: $p < 0.01$ vs. control, ***: $p < 0.001$ vs. control. Error bars represent standard deviation. Original magnification: (A, B) 5300X; (C, D) 400X; (E, F) 200X; (Insets in E, F) 400X; (G, H) 200X; (Insets in G, H) 400X; (I, J) 400X.

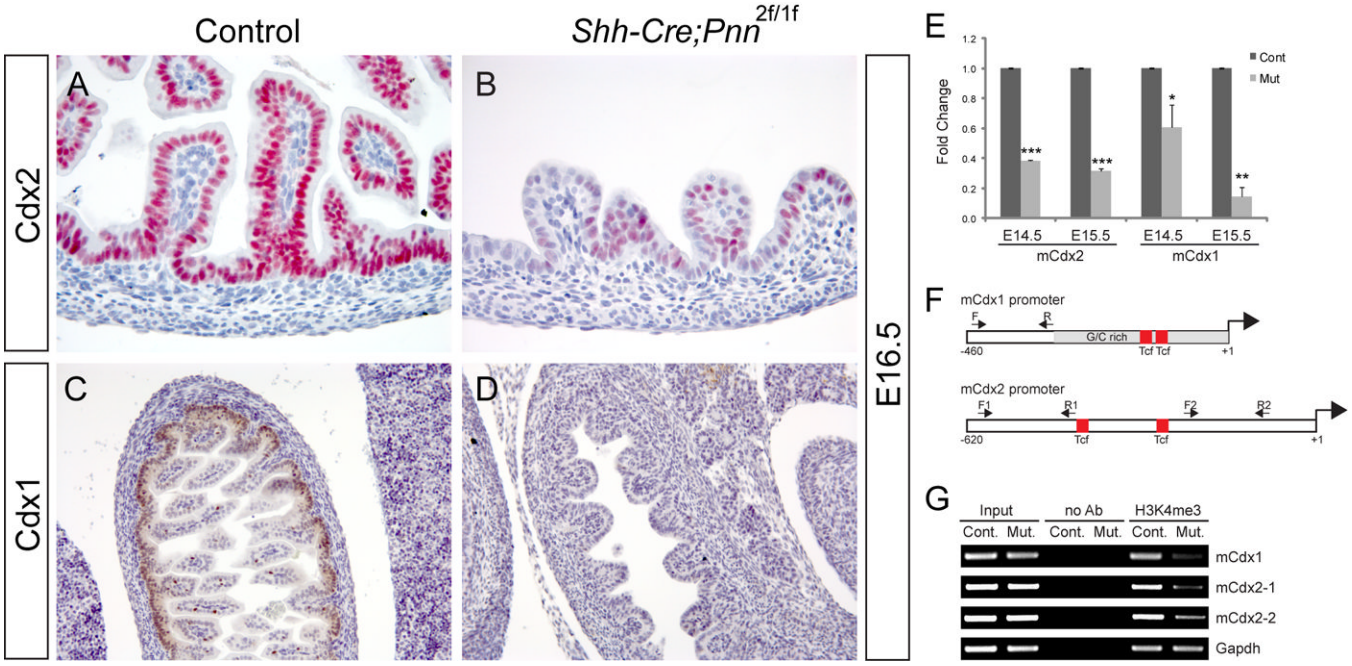

Fig. 4.

Downregulation of *Cdx* homeobox genes in Pnn mutant small intestine. (A, B) Immunohistochemical analysis of Cdx2 expression demonstrates homogenous epithelial-specific Cdx2 expression (red signal) in control small intestine. However, significant downregulation of Cdx2 is observed in mutant small intestine at E16.5. (C, D) While control intestine shows a typical Cdx1 expression pattern (brown signal in C) with gradient along the crypt-villus axis, the Pnn mutant intestinal epithelium exhibits undetectable Cdx1 immunoreactivity. (E) Quantitative real-time RT-PCR assays reveal that mRNA levels of *Cdx2* and *Cdx1* are markedly decreased in Pnn mutant small intestines. Expression levels are normalized to *Gapdh*. Error bars represent standard deviation. All *p* values are compared to the control samples. *: $p < 0.1$, **: $p < 0.01$, ***: $p < 0.001$ (two-tailed unpaired Student's t-tests, n=3). (F) A schematic diagram shows primer location used for ChIP assays on the promoter area of *Cdx* genes in mouse. Numbers are relative to the transcription start site (+1). Tcf binding elements are shown as red blocks. (G) ChIP assays demonstrate significantly reduced level of H3K4me3, a general marker for active chromatin, on both of *Cdx2* and *Cdx1* promoters in E17.5 mutant small intestine. The analyses were performed three times and the results shown were seen in all experiments. Original magnification: (A, B) 400X; (C, D) 200X.

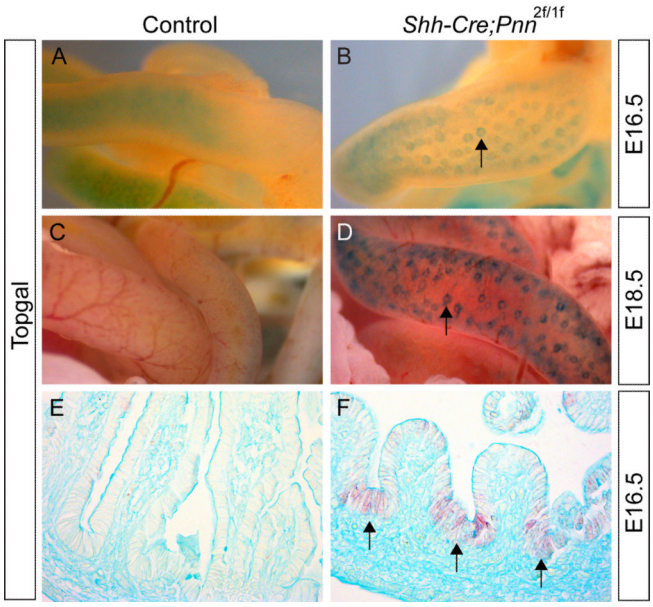

Fig. 5.

Upregulation of Topgal reporter activity in Pnn mutant small intestine. (A–D) Whole-mount X-Gal staining of E16.5 and E18.5 Topgal-positive small intestines shows distinct rings of β-galactosidase activity only in Pnn mutant small intestine (arrows in B, D). Control and mutant intestines were incubated in X-Gal staining solution for only 1 hour for A, B and for 30 minutes for C, D. (E, F) E16.5 small intestines were Red-Gal stained for 1 hour, sectioned, and counterstained with Alcian Blue. Topgal reporter activity is mainly detected in basal area of mutant villi (arrows in F). Original magnification: (A–D) 50X; (E, F) 400X.

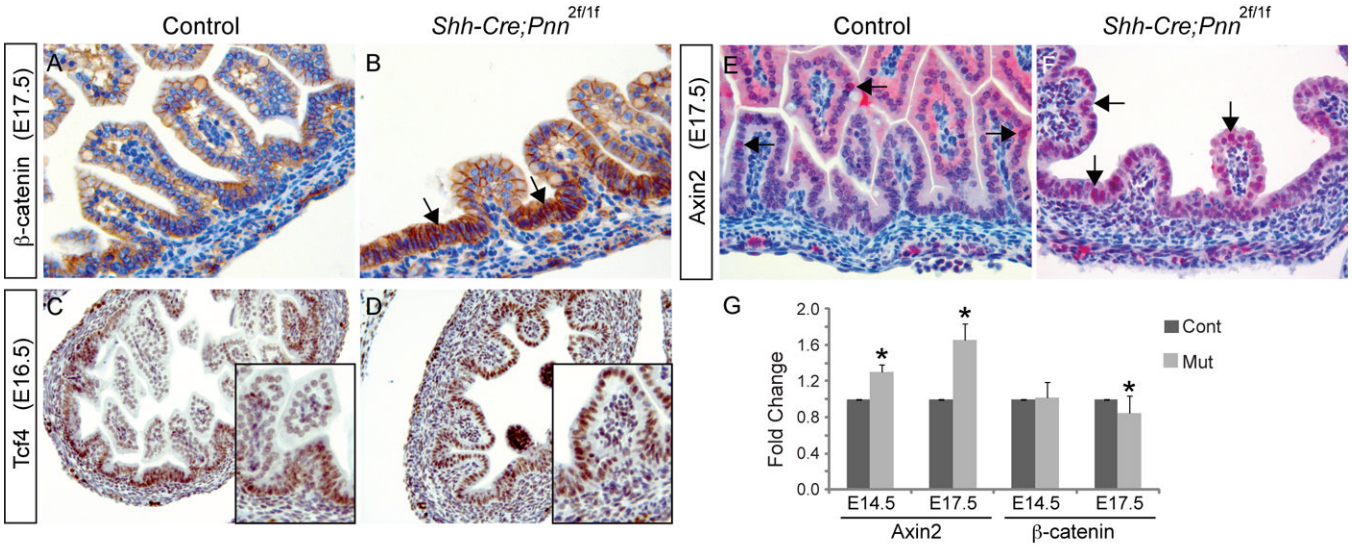

Fig. 6.

Misregulation of β-catenin, Tcf4, and Axin2 in mutant intestine. (A, B) Immunohistochemical staining of E17.5 intestines reveals considerable accumulation of β-catenin in Pnn mutant intestinal epithelial cells (arrows in B). Note that the increased β-catenin level is detected mostly in the basal area showing elevated Topgal activity in mutant small intestine (shown in Fig. 5F). (C, D) Aberrant expression pattern of Tcf4 is also detected in Pnn mutant intestinal epithelium. At E16.5, control intestine shows typical intervillus-restricted expression of Tcf4, however, Pnn mutant intestines present nearly uniform Tcf4 expression. (E, F) Axin2 immunostaining shows obvious Axin2 nuclear localization (arrows) in only a few cells of control villus epithelial cells, but the majority of cells of Pnn mutant intestine demonstrate positive nuclear Axin2 staining. (G) Quantitative RT-PCR assays demonstrate increased expression of *Axin2* in E14.5 and E17.5 mutant small intestine, while transcript level of β-catenin in mutant small intestine remains similar to that of controls. The demonstrated extent to which Axin2 is increased in the epithelial cells of Pnn mutant intestine may be underestimated due to the inclusion of non-epithelial cells in the sample preparation. *: $p < 0.05$ vs. control. (two-tailed unpaired Student's t-tests, n=3). Original magnification: (A, B) 400X; (C, D) 200X; (E, F) 400X.

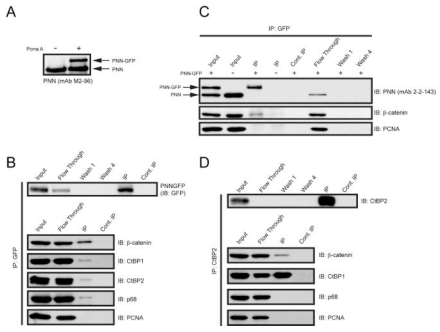

Fig. 7.

Association between PNN and β-catenin. (A) Ponasterone A (4μM) treatment effectively induces robust exogenous PNN expression in EcR293-PNNGFP cells. (B) Co-immunoprecipitation assays reveal that PNN forms a complex with β-catenin in EcR293-PNNGFP cells. While CtBP1, CtBP2, and p68 previously known to interact with PNN are successfully immunoprecipitated with PNN, non-PNN interacting PCNA is not detected in PNN immunoprecipitates. (C) PNN's association with β-catenin is also observed in OE33 esophageal adenocarcinoma cells. PCNA serves as a negative interaction control. (D) CtBP2 is also in a complex with β-catenin. Co-immunoprecipitation of endogenous CtBP2 shows the presence of β-catenin. While large amount of CtBP1 is co-immunoprecipitated, p68 and PCNA are not detectable in CtBP2 immunoprecipitates. IP: immunoprecipitation; IB: immunoblotting; Input: 4% of total sample.

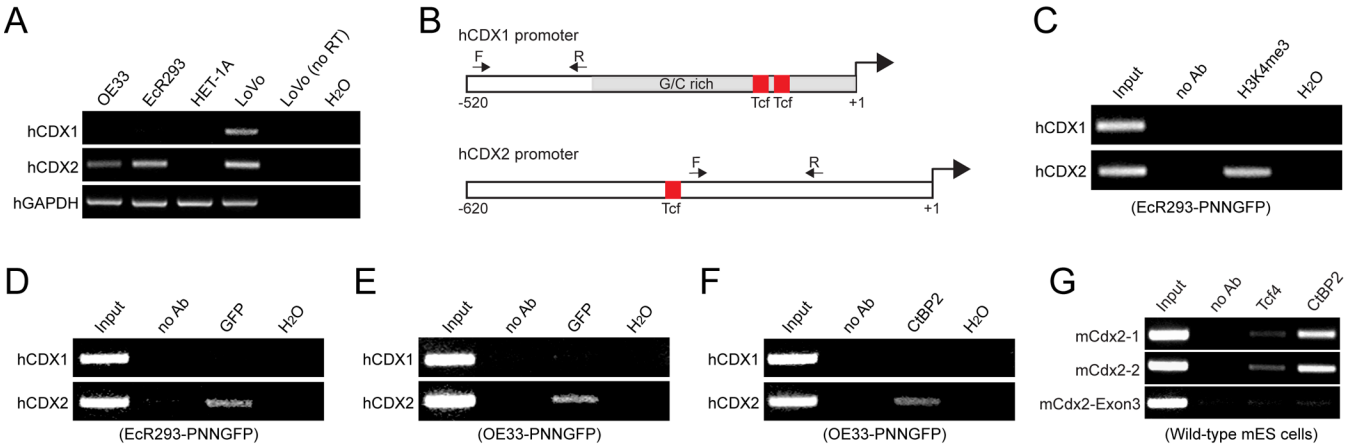

Fig. 8.

Specific recruitment of PNN to the active proximal promoter of *CDX2*. (A) Semiquantitative RT-PCR analysis shows that OE33 and EcR293 cells are expressing *CDX2*, but not *CDX1*. HET-1A (Human esophageal epithelial cell line) and LoVo (human colon adenocarcinoma cell line) cells serve as negative and positive controls, respectively. (B) A schematic illustration shows the location of primers used for ChIP assays on the promoter area of human *CDX* genes. Numbers are relative to the transcription start site (+1). Tcf binding elements are shown as red blocks. (C) ChIP assay with antibody against H3K4me3 shows active chromatin status of *CDX2* promoter in EcR293-PNNGFP cells. (D, E) ChIP analyses demonstrate specific recruitment of PNN to active *CDX2* promoter, but not to the inactive *CDX1* promoter area in EcR293 (D) and OE33 (E) cells. (F, G) CtBP2 is also recruited to *CDX2* promoter in OE33 (F) and mouse ES cells (G) as evidenced by ChIP assay with anti-CtBP2 antibody. As expected, Tcf4 is also shown to be recruited to *Cdx2* promoter in ES cells.

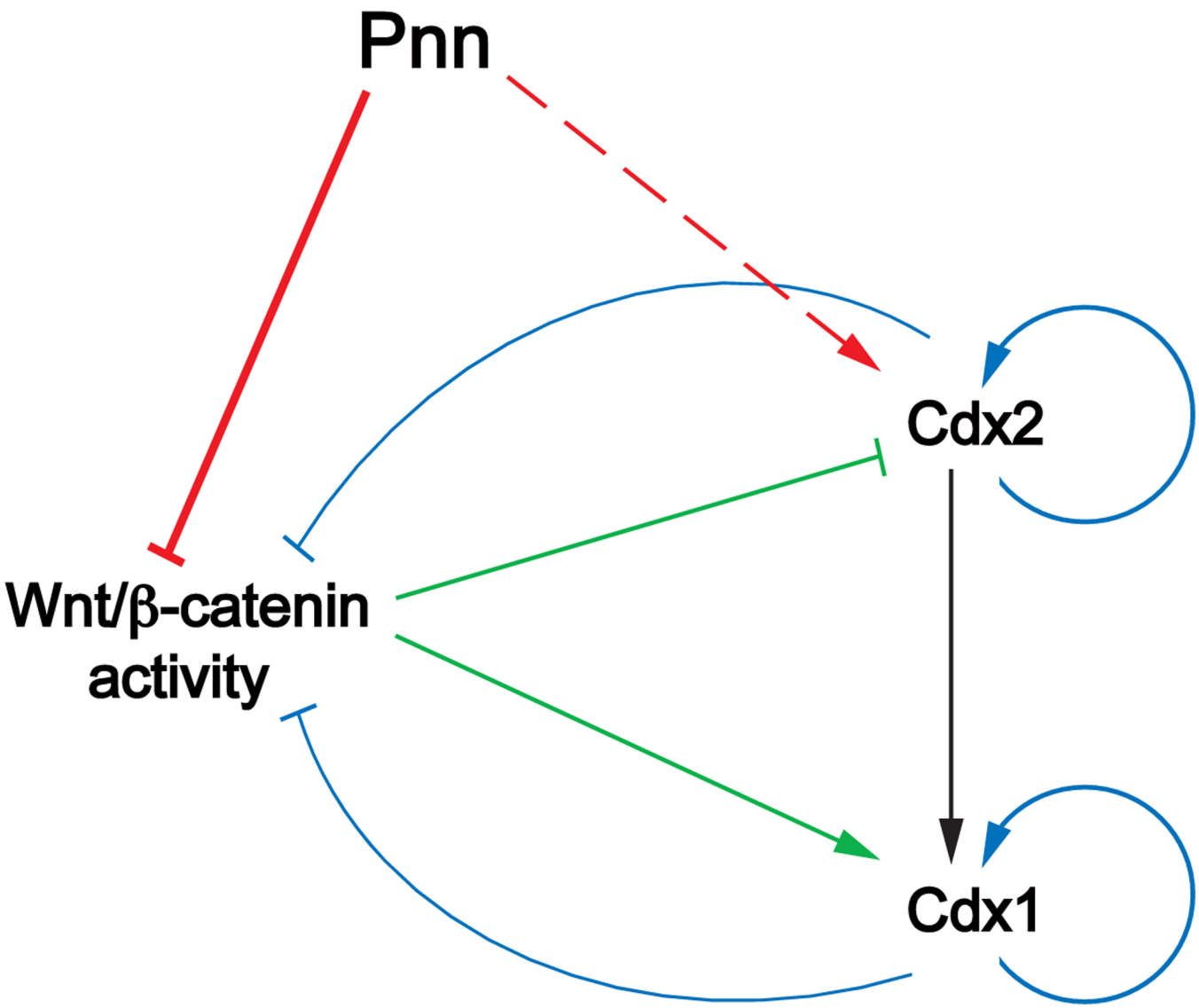

Fig. 9.

A model for functional interaction between Pnn, Wnt/β-catenin signaling, and Cdx proteins. Pnn may modulate Wnt/β-catenin activity through its interaction with β-catenin and, in turn, influence *Cdx2* expression. Besides, Pnn might be directly involved in *Cdx2* regulation at its promoter. It is very tempting to contemplate that Pnn may coordinate these two possible roles in an interdependent manner to ensure tight regulation of *Cdx2* expression during mouse small intestinal development. Autoregulatory mechanism and inhibitory role of Cdx proteins on Wnt/β-catenin activity are shown in blue. The impact of Wnt/β-catenin activity on *Cdx* genes is shown in green.