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Wnt signaling during development of the gastrointestinal tract

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Abstract

Wnt signaling pathways have been demonstrated to play important roles in controlling tissue patterning and cell proliferation. In the gastrointestinal tract, mutations that lead to activation of the canonical Wnt pathway through β-catenin result in familial and sporadic colon cancers. The downstream transcription factor Tcf4 is required to maintain the proliferative stem cell compartment in the crypts of the small intestine. Activation of TCF-dependent transcription is a good correlate to neoplastic transformation. Despite its association with cancer in the colon, little is known of the role for Wnt signaling during development and patterning of the gut tube. We conducted a comprehensive expression screen for Wnt signaling components during different stages of gut development in the chick. Conserved expression patterns of these genes indicate that they likely play essential roles in gut morphogenesis. Based on the expression profiles of putative components of each pathway, we are able to postulate specific roles for the various pathways during gut development. Predictions of roles for canonical signaling in the developing gizzard, duodenum, and large intestine in chick were tested by viral misexpression of dominant-negative (DN) forms of the downstream cofactors Tcf4 and Lef1. In the chick, Tcf4 is expressed in the posterior gizzard mesoderm. Misexpression of DN-Tcf4 in the splanchnic mesoderm resulted in the failure of the gizzard epithelium to form microvilli. Lef1 is expressed in the chick duodenum and large intestine mesoderm. Viral misexpression of DN-Lef1 resulted in diminished mesoderm and overproliferation of the large intestine endoderm, leading to stenosis of the lumen. The results from these misexpression studies in the chick, together with evidence from colorectal lesions, indicate that the canonical Wnt pathway plays critical roles in balancing cell proliferation versus cell differentiation during gut development. The expression profiles of the Wnt signaling components presented in this paper should prove valuable in deciphering additional roles of the Wnt pathways during patterning of the vertebrate gut tube.

Introduction

The embryonic gut initially forms as a simple tube that undergoes regional specialization followed by morphogenesis and differentiation, creating the different organs of the digestive tract. The primitive gut tube is created through morphogenic movements that are initiated as two invaginations in the ventral walls of the gut endoderm at the rostal and caudal ends of the embryo. These invaginations, termed the *a*nterior *i*ntestinal *p*ortal (AIP) and *c*audal *i*ntestinal *p*ortal (CIP), migrate toward each other. As the AIP and CIP migrate, the endoderm behind them form tubes which extend and fuse at the umbillicus (Gruenwald, 1941). Functionally and morphologically, the gut tube is subdivided into three regions: foregut, midgut, and hindgut. The foregut and hindgut are derived from the AIP and CIP, respectively, while the midgut is composed of tissue originating from both the AIP and CIP (Carlson, 1999).

The primitive gut tube is composed of two tissues: a luminal lining of endoderm-derived epithelium surrounded by an outer layer of splanchnic mesoderm. As development progresses, the splanchnic mesoderm undergoes smooth muscle differentiation, causing the gut tube to alter its gross morphology. Meanwhile, the uniform luminal epithelium undergoes regional specification along the anterior–posterior (A-P) axis (Kedinger et al., 1988). Following this regionalization, the various organs subsequently differentiate along the A-P axis of the GI tract, allowing each organ to achieve its specialized function. For example, the lumen of the stomach is lined with a gastric columnar epithelium, which is required for secreting enzymes for the breakdown of food, while the lumen of the small intestine contains bulbous microvilli required for the absorption of nutrients.

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Formation of the primitive gut tube and its subsequent regionalization involves cross-talk between the mesodermal and epithelial layers along the radial axis of the gut (for review, see Roberts, 2000). By culturing early gut endoderm with non-gut mesoderm, it was demonstrated that the endoderm is responsible for inducing the overlying mesoderm to differentiate into gut-specific mesoderm. In these experiments, gut endoderm was able to induce smooth muscle differentiation in non-gut mesoderm, as indicated by the induction of smooth muscle markers (Kedinger et al., 1990). The secreted molecule Sonic Hedgehog (SHH) has been implicated as one of the inductive signals from the endoderm that specifies and patterns the overlying mesoderm. Overexpression experiments of *Shh* in both chick and transgenic mice lead to increased cell proliferation of gut mesoderm as well as smooth muscle specification (Apelqvist et al., 1997; Roberts et al., 1995, 1998). Thus, the endodermal signal allows the epithelial lumen to be surrounded by mesoderm specified to form visceral mesoderm. The mesoderm in turn signals back to the endoderm to specify the A-P position of the endoderm and to consequently define organ fate and subsequent function (Haffen et al., 1983, 1987; Kedinger et al., 1986, 1988). Again, this was demonstrated by grafting experiments. When grafted to stomach endoderm, small intestine mesoderm results in an epithelium which contains microvilli indicative of the small intestine (Kedinger et al., 1986). Further cross-talk between the endoderm and mesoderm direct organ-specific differences such that, as development proceeds, the layers of tissue across the radial axis form distinct patterns at different A-P levels. These differences are exemplified by the large muscles for grinding food found in the chick gizzard (or stomach), which are absent from the rest of the tract. While many of the tissue interactions which direct gut organogenesis have thus been defined, the molecular nature of the signals responsible for these inductive events is only starting to be elucidated.

The *Wnt* genes encode a large family of secreted molecules that play important roles in controlling tissue patterning, cell fate, and cell proliferation within a broad range of embryonic contexts. Among its various functions, Wnt signaling has been proposed to play a role in specifying the gastrointestinal tract (Wells and Melton, 1999). Wnt proteins signal in turn through the Frizzled family of cell surface receptors. To date, 19 members of the Wnt family have been identified in humans along with 10 members of the Frizzled family. The binding of Wnt ligands to Frizzled receptors activates several distinct intracellular signaling pathways. These pathways include the Wnt/β-catenin (or canonical) pathway, the Wnt/Ca^{2+} pathway, and the planar polarity pathway (for reviews, see Huelsken and Birchmeier, 2001; Kuhl et al., 2000a; Moon et al., 2002; Polakis, 2000).

During canonical signaling, binding of Wnt ligands to the Frizzled receptor results in stabilization of the transcription factor β-catenin. In the absence of the Wnt ligand, levels of β-catenin are suppressed by a complex of molecules which target β-catenin for phosphorylation and subsequent recognition by β-TrCP, an E3 ubiquitin ligase (Hart et al., 1999). The inhibitory complex required to phosphorylate β-catenin includes glycogen synthase kinase 3β (GSK3β), axin, and adenomatous polyposis coli (APC) (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998; Zeng et al., 1997). Activation of the canonical Wnt pathway, mediated by interaction between Frizzled and a second protein called Dishevelled, allows β-catenin to escape phosphorylation, and thus degradation (Noordermeer et al., 1994). As β-catenin levels rise, β-catenin translocates into the nucleus where it associates with members of the Tcf/Lef family of high-mobility-group (HMG) box transcription factors to transduce the Wnt signal (Behrens et al., 1996; Molenaar et al., 1996).

The canonical Wnt pathway has been found to be essential for a number of developmental processes, including establishment of the dorsoventral body axis (Parr and McMahon, 1994; Wodarz and Nusse, 1998). A number of Wnts and Fz's have

Table 1
Chick probes with a description of their fragments

Chick probes	Gut exp	Fragments	Reference
Lef1	+	~1 kb	Kengaku et al., 1998
LefX	−	full-length cDNA	C. Hartmann, unpublished
Tcf4	+	510 bp of coding	Hartmann and Tabin, 2000
Wnt1	+	complete cDNA + poly(A) (~2.2 kb)	Fung et al., 1985
Wnt2	+	~400 bp	D. Smith, unpublished
Wnt2b	+	1 kb of 3′ cDNA	Jasoni et al., 1999
Wnt3a	+	~400 bp	Hollyday et al., 1995
Wnt4	−	starts at aa 200 to stop codon	Yoshioka et al., 1994
Wnt5a	+	380 bp genomic	Gavin et al., 1990
Wnt5b	+	~400 bp fragment	C. Hartmann, unpublished
Wnt7a	+		T. Brown
Wnt7b	+	380 bp PCR genomic fragment	Gavin et al., 1990
Wnt8b	+	PCR fragment	A. McMahon
Wnt8c	+	1.7 kb full-length cDNA	
Wnt10a	+	~400 bp fragment	D. Smith, unpublished
Wnt11	+	1827 bp ORF	Tanda et al., 1995
Wnt14	−	550 bp (aa 77-263)	Hartmann and Tabin, 2001
Frzb1	+	~640 bp fragment	H. McBride/Fraser Lab
Crescent	+	full-length cDNA	H. McBride/Fraser Lab
sFRP1	+	full-length cDNA	H. McBride/Fraser Lab
sFRP2	+	224 bp	H. McBride/Fraser Lab
Fz1	+	1.6 kb partial cDNA	Stark et al., 2000
Fz2	+	1.6 kb start codon to aa 535	Stark et al., 2000
Fz4	+	3.7 kb	Stark et al., 2000
Fz7	+	full-length cDNA	Stark et al., 2000
Fz8	+	1.1 kb partial cDNA	Stark et al., 2000
Fz9	+	1.6 kb	Stark et al., 2000
Fz10	−	1 kb	Stark et al., 2000

Note. Expression of these genes during gut development is indicated by the plus and minus signs.

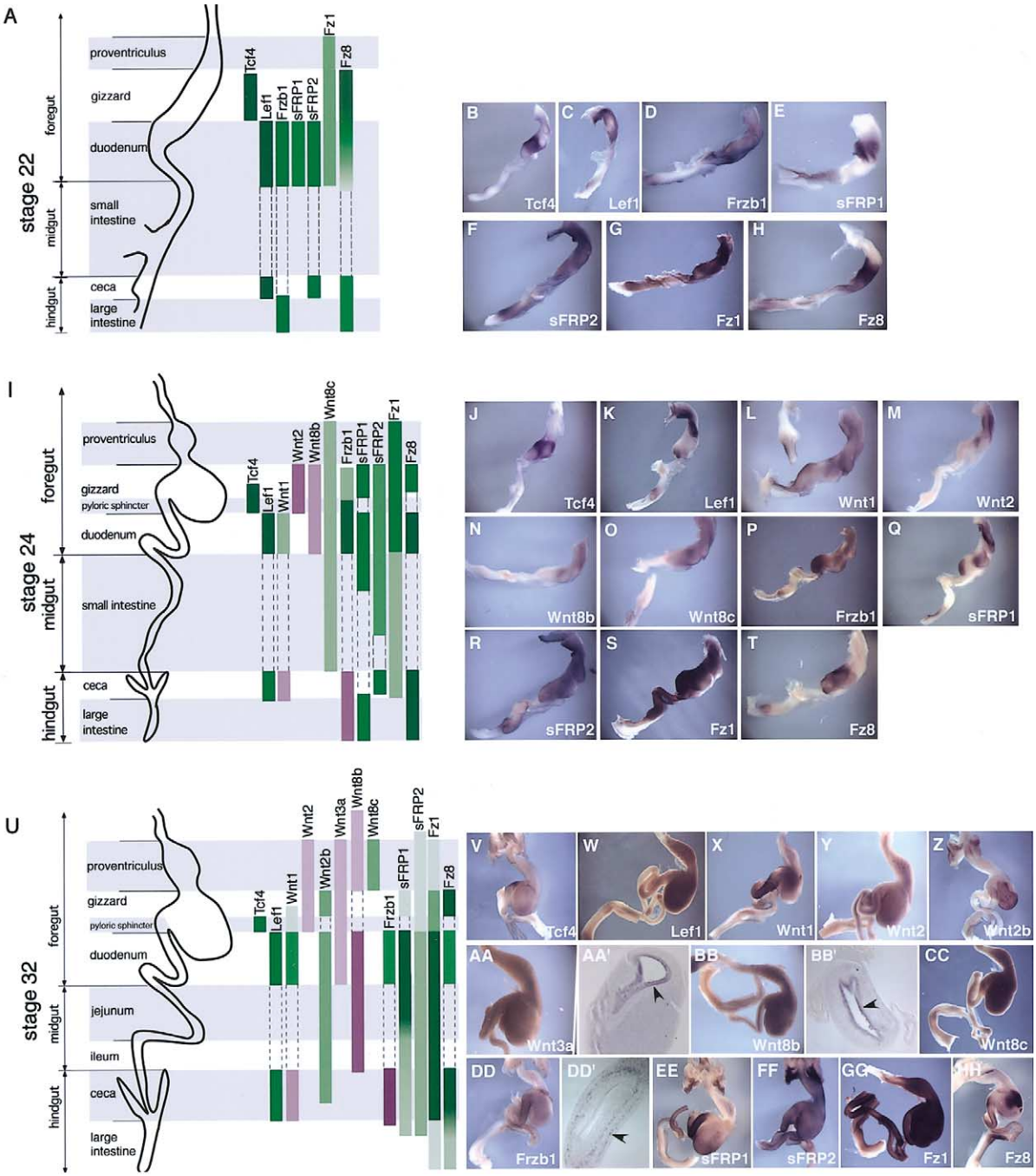

Fig. 1. Expression profiles of genes from the Wnt/β-catenin pathway during gut development in chick. The figure illustrates the expression profiles by whole-mount in situ of components of the canonical Wnt pathway during three stages of gut development in chick: patterning at stage 22 (A–H), morphogenesis at stage 24 (I–T), and differentiation at stage 32 (U–HH). Sections (AA′), (BB′), and (DD′) illustrate in situ hybridization for probes *Wnt3a*, *Wnt8b*, and *Frzb1*, respectively. Black arrowheads highlight expression of *Wnt3a* in the gizzard endoderm (AA′), *Wnt8b* expression in the small intestine endoderm, and *Frzb1* expression in the duodenum mesoderm (DD′). (A), (I), and (U) represent schematic drawings of the developing gut at stages 22, 24, and 32, respectively. The guts are oriented from anterior (top) to posterior (bottom), starting with the proventriculus and stomach down through the ceca and large intestine. Alongside each schematic is a bar depicting the expression domain of each gene along the anterior–posterior axis of the gut. Green bars identify expression in the gut mesoderm, while purple bars refer to expression in the gut endoderm. Attempts have also been made to quantify relative levels of signal, with the shades of color corresponding to relative intensities of signal from light (low signal) to dark (strong signal). However, it should be noted that the relative intensities of signals illustrated in (A), (I), and (U) are slightly deceptive as RNA probes in whole mount can give strong or weak signals, depending on the gene fragment used.

been classified into the canonical pathway by their ability to induce axis duplication in early *Xenopus* embryos and to transform epithelial cells. Wnts from this class include Wnt1, 2b, 3a, 8, 8b, and 8c (Du et al., 1995). Their putative paired receptors include Fz1, 3, 5, 7, 8, and 10 (Tanaka et al., 1998; Terasaki et al., 2002; Umbhauer et al., 2000).

An association between the GI tract and the canonical Wnt pathway first came from the observation that components of this pathway are often mutated in familial and sporadic colon cancers (Polakis, 2000). Mutations that result in tumor formation are those that lead to stabilization of β-catenin and hence constitutive activation of the pathway. These include mutations in axin that eliminate β-catenin binding sites and inactivation of APC, which affects β-catenin regulation (most likely axin binding sites). Similarly, mutations in β-catenin have been found in cancer cell lines that disrupt the phosphorylation sites required for its targeted degradation by β-TrCP (Hart et al., 1999).

Several downstream components of β-catenin signaling have also been found mutated in colorectal cancers, while others are ectopically activated in colon cancer. Although Lef1 is a transcriptional mediator of canonical Wnt signaling in many tissues, Lef1 is not normally expressed in the colon, and indeed mice lacking the Lef1 gene do not display any gut abnormalities (van Genderen et al., 1994). However, Lef1 has been found ectopically expressed in human colon carcinomas (Hovanes et al., 2001). In contrast, mice deficient in the related gene *Tcf1*, which is a downstream target of Tcf4/β-catenin in the gut, have a 15% rate of adenomatous intestinal polyp development in knock-out mice (Roose et al., 1999).

Although several components of canonical signaling have been shown to act as tumor suppressors in the gut, little is known about the role of this pathway during gut development. An exception to this is the downstream transcription factor Tcf4, which is expressed in the human colon (Korinek et al., 1998a). The *Tcf4* knock-out mouse displays a single gut abnormality: failure to maintain the proliferating stem cell compartment in the crypts of the small intestine (Korinek et al., 1998a; van de Westering et al., 2002). Thus, if canonical Wnt signaling plays a role in other aspects of gut development, it must work through distinct or redundant Tcf/Lef family members. The *Tcf4* loss-of-function phenotype shows that the normal role of activating Tcf4 (through the stabilization of β-catenin) is to induce proliferation of undifferentiated cells. Since polyps form from the out-pocketing of intestinal epithelium at the crypt-villus border, activation of TCF-dependent transcription is a good correlate to neoplastic transformation.

As with canonical Wnt signaling through β-catenin, the planar cell polarity and Wnt/Ca^{2+} pathways signal through the Frizzled receptors. The planar polarity pathway branches from the canonical pathway at the level of Dishevelled, activating Rho kinase and the Jun-N-terminal kinase (JNK) (Li et al., 1999; Mlodzik, 2000; Moriguchi et al., 1999; Wallingford et al., 2000). Activation of the planar polarity pathway directs cytoskeletal organization and epithelial cell polarization (Winter et al., 2001). The developmental processes regulated by the Wnt/planar polarity pathway in vertebrates include the convergent extension movements of the mesoderm during gastrulation. Among the Wnt ligands and receptors implicated in activating JNK during convergent extension are Wnt11 and Fz7 (Djiane et al., 2000; Heisenberg et al., 2000; Marlow et al., 2002). The Wnt/Ca^{2+} pathway has also been implicated in convergent extension movements as well as ventralization of the *Xenopus* embryo (Kuhl et al., 2000b; Moon et al., 1993). Stimulation of the Wnt/Ca^{2+} pathway results in the release of intracellular Ca^{2+} through activation of phospholipase C, protein kinase C, and calmodulin-dependent kinase II (Slusarski et al., 1997b). Both Wnt5a and Fz2 induce intracellular Ca^{2+} release and this process is most likely mediated via G-proteins (Slusarski et al., 1997a, b; Wallingford et al., 2001).

It is noteworthy that Fz7, which mediates convergent-extension movements via the Wnt/planar polarity pathway, has also been implicated in mediating canonical Wnt signaling (as noted above). Similarly, Wnt5a, which mainly triggers activation of the Wnt/Ca^{2+} pathway (see above), can activate canonical Wnt signaling in the presence of Fz5 (He et al., 1997). These examples highlight the fact that, while the Wnt ligands and Fz receptors can be categorized according to the pathways that they principally stimulate, these groupings cannot be viewed as absolute and are to some extent context-dependent. Nonetheless, knowledge of the signaling pathways used by these ligands and receptors in other developmental contexts can give insight into the pathways most likely utilized in the gut.

In addition to factors regulating the activation of Wnt pathways, inhibition of Wnt signaling also contributes to patterning of the vertebrate body plan (Leyns et al., 1997; Wang et al., 1997a; Xu et al., 1998). The various Wnt antagonists, such as the *s*ecreted *F*rizzled-*r*elated *p*roteins (sFRP's), interact directly with Wnt ligands to block their activity. Since these antagonists, to varying extents, discriminate between different Wnt ligands (which themselves display specificity in activating downstream pathways, as described above), there is some specificity to the Wnt signaling pathways that the antagonists affect. For example, the antagonists Frzb1 and sFRP2 can both inhibit Wnt1 (Wang et al., 1997b). However, while Frzb1 can additionally inhibit Wnt8, sFRP2 cannot, although sFRP2 can inhibit Wnt4. This specificity exists despite the fact that Wnt's 1, 4, and 8 are all ligands known to activate signaling through the canonical Wnt pathway (Lee et al., 2000). Thus, understanding the roles of Wnt antagonists as well as the different classes of Wnt ligands and Fz receptors will be important in understanding the roles of these pathways during gut development.

Over the past few years, reports of knock-out mice lacking various signaling components, together with misexpression studies carried out in other vertebrates, have elucidated roles for the different Wnt pathways during vertebrate development (Korinek et al., 1998a; Wang et al., 2001). Of those, only a few examples have shed light on the roles of Wnt signaling during patterning of the gut. As mentioned above, disruption of *Tcf4* by homologous recombination results in depletion of the epithelial stem cell compartment

contained in the crypts of the small intestine (Korinek et al., 1998a). Maintenance of the stem cell compartment may be mediated by Cdx1, a downstream target of Tcf4 expressed in the crypts of the intestinal epithelium (Lickert et al., 2000). Further evidence for the role of Tcf4 in endoderm specification comes from the induction of gut markers in animal cap explants of early *Xenopus* embryos upon injection of *Tcf4* (Lee et al., 1999). Within the Wnt/Ca^{2+} pathway, mice homozygous mutant for *Fz4* lack muscle differentiation in the esophagus leading to esophageal distension (Wang et al., 2001). Thus, despite the large size of the Wnt family and their known involvement in many other aspects of vertebrate embryogenesis, surprisingly few links have been made between the Wnt pathways and their roles during gut development.

To begin to understand roles for the various Wnt pathways during gut development, we undertook a comprehensive expression screen of Wnt's, Fz's, Wnt antagonists, and their downstream transcriptional cofactors during different stages of gut development. This paper describes the expression profiles for putative components of each pathway, and based on these data, attempts to identify possible signaling centers where the various pathways may play key roles during gut development. In the case of signaling through β-catenin, we took advantage of the avian-specific retroviral system to misexpress downstream targets to define a role for the canonical pathway within the developing gizzard and large intestine.

Materials and methods

Expression analysis

Fertilized White Leghorn chick eggs were obtained from SPAFAS (Norwich, CT). Expression analysis of Wnt signaling molecules in the chick was performed on three different stages representing a range of events during gut development (stages 22, 24, and 32; according to Hamburger Hamilton) (Hamburger and Hamilton, 1951). Embryos were fixed in 4% paraformaldehyde/PBS, pH 7.4, for 5–12 h at 4°C. Embryos destined for whole-mount in situ hybridization were dehydrated to 100% methanol and stored at −20°C until used. For section in situ hybridization, embryos were embedded in paraffin (Allen, 1994).

Whole-mount in situ hybridization was performed by using digoxigenin-labeled riboprobes as described by Riddle et al., (1993) and Burke et al. (1995). All staining patterns were confirmed by sectioning whole mounts or by in situ hybridization on sections (Murtaugh et al., 1999). Probes in this study were generously provided by a large group of investigators (see Table 1 for references).

Retroviral misexpression

For misexpression, we utilized two varieties of the replication-competent retroviral vector, RCASBP(A) and RCASBP(B). Constructs carrying N-terminal deleted (dominant negative) forms of Tcf4 and Lef1 were made and viruses were generated as previously described (G. Kardon and B. Harfe, unpublished observations) (Hughes et al., 1987; Logan and Tabin, 1998; van de Westering et al., 2002). Retrovirus was injected into the intraceolomic cavity at HH stage 12, enabling viral particles to infect the splanchnic mesoderm. Viruses containing A and B coat proteins were simultaneously injected in order to increase the number of viral infections, resulting in stronger phenotypes than single viruses alone. Embryos were allowed to develop and harvested at HH stage 32. Guts were dissected from harvested embryos and fixed with 4% paraformaldehyde/PBS, pH 7.4, for 12 h at 4°C prior to processing for histologic analysis.

Histologic and immunohistochemistry analysis

Infected guts were embedded in paraffin and cut into 8-micron sections. Adjacent sections were collected on sequential slides for direct comparison of areas of viral infection with gut histology (visualized by H & E staining; Allen, 1994). Regions of viral infection were detected with the 3C2 antibody against the *gag* viral protein and visualized by staining with DAB.

Results

Characterization of Wnt signaling molecules during gut development

In order to develop a comprehensive view of Wnt signaling during normal gut development, we examined the expression of a large number of genes in this pathway, including Wnt ligands, Fz receptors, Wnt antagonists, and transcriptional cofactors. Expression analysis of Wnt signaling molecules was performed at three different stages representing a range of developmental events during formation of the GI tract. The earliest stage examined, stage 22, is just prior to fusion of the AIP and CIP at the midgut. At this stage, the gut is a simple straight tube with a slight thickening defining the stomach and the horns of the future ceca. By stage 24, the walls of the midgut have fused and the general morphology of the gut has begun to take shape; with the characteristic out-pouching of the stomach, the slight elongation of the small intestine, and the branching of the ceca. During these earlier stages, the gut is composed of a columnar epithelium surrounded by undifferentiated mesenchyme. At stage 32, the different organs of the digestive tract can be distinguished by the histologic differentiation of the endoderm and different muscle groups in the overlying

mesoderm. Thus, by choosing these stages, we hoped to get a comprehensive view of the roles for Wnt signaling during early patterning prior to significant morphogenesis (stage 22), during morphogenesis of the organs (stage 24), and following differentiation of gut organs with distinct functions (stage 32).

To examine the expression profiles of Wnt signaling molecules during normal gut development, in situ hybridization was initially performed on embryos in whole mount. However, while whole mounts with digoxygenin-labeled probes can be valuable in evaluating general expression domains, relative expression levels and exact boundaries of expression domains are often ambiguous. This is especially true for the gut, where specific endoderm expression is often obscured by extensive pooling in the gut lumen. Thus, in an attempt to eliminate discrepancies between probes and provide more accurate comparison of expression boundaries, all results from whole-mount in situ analysis were confirmed by sectioning whole-mount embryos or by performing in situ hybridization on sectioned embryos.

Our initial overview of gene expression revealed that the majority of Wnt signaling molecules tested were expressed during various stages of gut development (Table 1). Of the 16 Wnt ligands analyzed, only 4 were not expressed during early or late stages of gut development: *Wnt3, 4, 7b*, and *14*. In addition, all Wnt antagonists examined were found widely expressed, as were all Fz's with the exception of *Fz10*, which was not expressed in the developing gut. Of the 3 downstream targets of canonical signaling tested (*Lef1*, *LefX*, and *Tcf4*), only *LefX* was not expressed in the gut. The large number of Wnts and their signaling components found expressed during different stages of gut development suggests a broad range of roles for Wnt signaling during formation and patterning of the GI tract. Because of the large number of Wnt pathway genes found expressed during gut development, there are likely to be some redundancies in their functions. However, since Wnt proteins are known to signal through several different transduction pathways, the expression data also indicate that the various Wnt signals are likely to play multiple distinct roles during gut development. To allow patterns of gene expression reflective of these roles to emerge from the complex set of data, we have organized the data by signaling pathways. Although, as discussed above, some of the genes appear to act in more than one pathway and the categorization of these molecules is still incomplete, organizing the data in this way using the information currently available has allowed us to discern a number of interesting patterns.

Expression profile of canonical pathway molecules

The canonical Wnt pathway signals through β-catenin and is the best understood of the Wnt pathways on the molecular level (Moon et al., 2002). Many molecules involved in canonical signaling were expressed at stage 22 during early formation of the gut tube (Fig. 1A–H). Although no canonical Wnt ligands were detected early at stage 22, two receptors (*Fz1* and *Fz8*) and the downstream transcriptional cofactors *Tcf4* and *Lef1* were all found expressed in the mesoderm (Fig. 1G and H, and B and C). Already at this early stage, regions of potential signaling can be identified based on expression profiles of signaling components. *Tcf4*, *Fz1*, and *Fz8* are expressed in the gizzard, while the region that gives rise to the duodenum expresses *Lef1*, *Fz1*, and *Fz8*. The appearance of the Wnt antagonists *Frzb1*, *sFRP1*, and *sFRP2* in the presumptive duodenum suggests that one or more as yet unidentified Wnt ligands are expressed in this region (Fig. 1D–F). It also seems likely that the initial expression of *Tcf4* and *Lef1* is regulated independently of Wnt signaling at these early stages, as no canonical Wnt ligands appear to be expressed at this time.

Canonical signaling molecules also appear to be expressed early in the ceca and large intestine (Fig. 1A). Here, we can begin to see different family members of the same canonical signaling system expressed at different sites. Positive regulators of canonical signaling *Lef1* and *Fz8* are expressed in the ceca as is the Wnt antagonist *sFRP2*, while *Tcf4*, *Frzb1*, and *sFRP1* are absent. *Fz8* is also expressed in the large intestine as is the antagonist *Frzb1* but not *sFRP1* or *sFRP2*. This differential regulation of the receptors *Fz1* and *Fz8* and the downstream molecules *Tcf4* and *Lef1* could indicate that they play different roles in patterning the early gut tube, perhaps activating distinct sets of target genes in response to Wnt signaling in different regions.

With the basic patterning plan of the gut established by stage 24, we begin to detect transcripts for Wnt ligands *Wnt1, 2, 8b*, and *8c* (Fig. 1I). The Wnt ligands are expressed in regions similar to those of the early Fz receptors: in the gizzard (*Wnt2* and *8b*; Fig. 1M and N), the duodenum (*Wnt1, 8b*, and *8c*; Fig. 1L, N, and O), and the ceca (*Wnt1*, Fig. 1L). It should be noted that the Wnt ligands are expressed in both tissue layers of the gut, i.e., in the mesoderm (*Wnt1* in the duodenum, and *Wnt8c*) as well as the gut endoderm (*Wnt1* in the ceca, *Wnt2* and *Wnt8b*) (depicted in Fig. 1I). Comparing expression profiles at stage 22 and stage 24, we observe that some expression domains expand during this time, while others become more restricted. For example, *Fz8* is expressed throughout the gizzard and anterior duodenum at stage 22, but becomes excluded from the region that will give rise to the pyloric sphincter (the region directly connecting the stomach to the duodenum) by stage 24 (compare Fig. 1H with T). In contrast, the expression of the Wnt antagonist *sFRP2* expands from the duodenum at stage 22 to the gizzard and much of the small intestine by stage 24 (compare Fig. 1F with R). Another interesting observation was the shift in expression of *Frzb1* from the large intestine mesoderm in stage 22 to the endoderm by stage 24 (Fig. 1D and P, and 1A and I).

By stage 32 in the chick, the different organs along the GI tract have been established and are distinguishable on the histologic level, with the epithelium becoming specialized for specific organ functions along the A-P axis. Along with

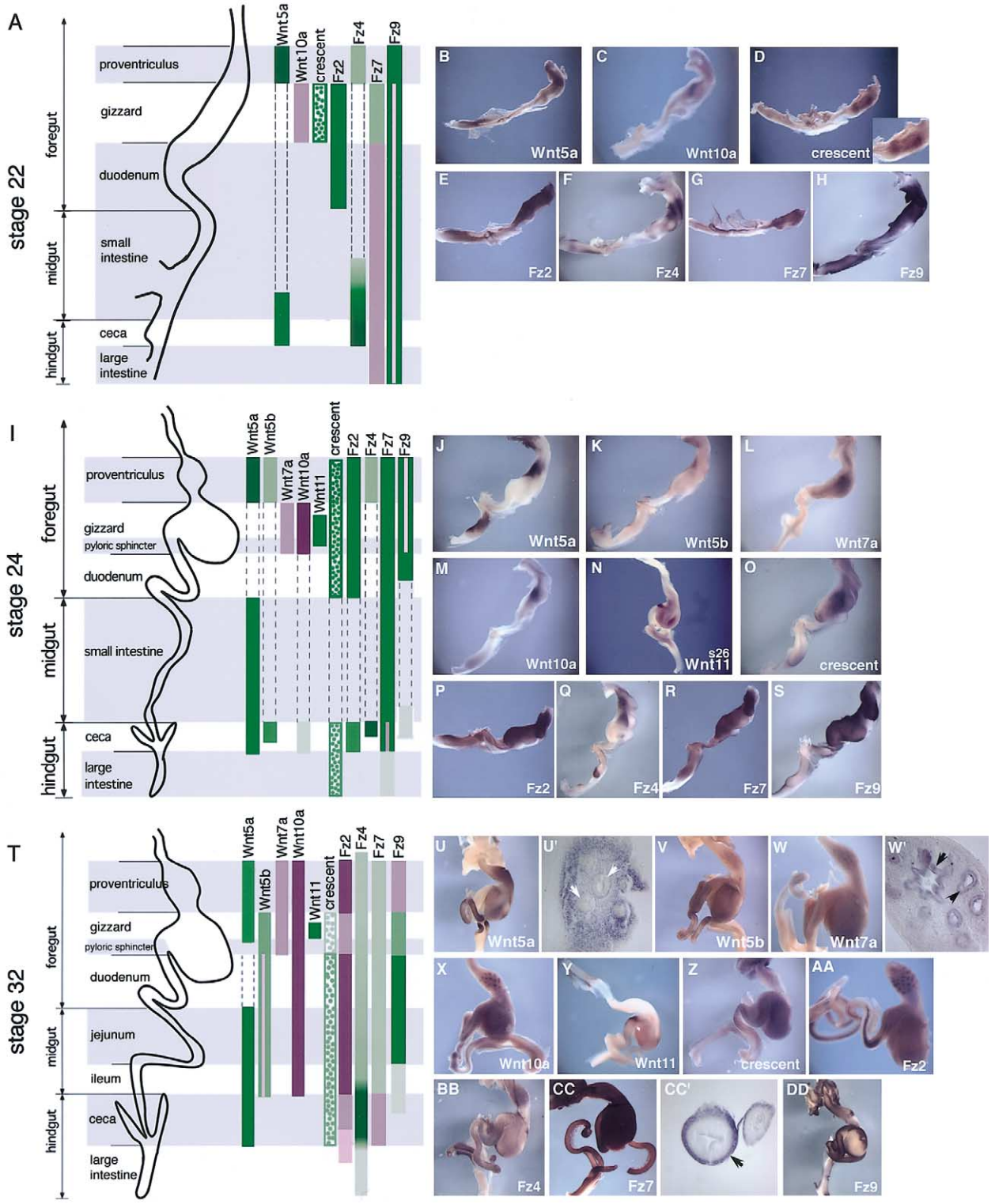

Fig. 2. Expression profiles of genes from the noncanonical Wnt pathways during gut development in chick. The expression profiles of genes in the noncanonical Wnt pathways (including the Wnt/Ca^{2+} and planar polarity pathways) are illustrated as described in Fig. 1. Depicted in this figure are expression profiles for stage 22 (A–H), stage 24 (I–S), and stage 32 (T–DD), with the exception of (N), which is at stage 26. Sections (U′), (W′), and (CC′) illustrate in situ hybridization for probes *Wnt5a*, *Wnt7a*, and *Fz7*, respectively. (U′) *Wnt5a* expression throughout the mesoderm of the proventriculus, with white arrows highlighting the absence of *Wnt5a* from the endoderm. Black arrows indicate the expression of *Wnt7a* in the proventriculus endoderm (W′) and *Fz7* expression in the small intestine mesoderm (CC′). Like Fig. 1, vertical bars spanning relative regions along the A-P axis of the gut illustrate gene expression domains. Relative levels of gene expression are depicted by the different shades of green (mesoderm) and purple (endoderm).

the molecules observed earlier in development, expression of *Wnt2b* and *Wnt3a* is also observed by stage 32 (Fig. 1Z, AA). The late onset of expression of *Wnt2b* and *3a* suggests that they may play a role in maintaining the integrity of the endoderm of the foregut. Again, by stage 32, some molecules become more restricted in their expression domain (*Tcf4, Frzb1, Wnt8c*), while other molecules are expressed in a more expanded domain (*Wnt1, sFRP1,* and *sFRP2*) (Fig. 1U).

Expression profiles of Wnt/Ca^{2+} pathway molecules

The Wnt/Ca^{2+} pathway signals through CamKII to release intracellular Ca^{2+} and regulate convergent extension movements and ventralization of the early Xenopus embryo (Kuhl et al., 2000b; Moon et al., 1993). Of the Wnt signaling molecules analyzed in this expression screen, three have been identified as signaling through CamKII: Wnt5a, Fz2, and Fz4 (Slusarski et al., 1997a, b; Wang et al., 2001). These molecules are all expressed early at stage 22 of gut development (Fig. 2A). *Wnt5a* is expressed in the future region of the proventriculus and hindgut mesoderm (Fig. 2B). *Fz4* has an expression profile similar to *Wnt5a,* with expression at the proventriculus and hindgut mesoderm (Fig. 2F). In contrast, *Fz2* is expressed in the gizzard and duodenum mesoderm (Fig. 2E). Expression of these molecules persists and remains relatively constant until late stages of gut development (Fig. 2I). At stage 32, *Fz4* expression expands at low levels throughout the gut mesoderm with higher levels at the ceca (Fig. 2BB). During digit formation, Wnt5a has been proposed to signal through Fz4 (Chimal-Monroy et al., 2002). The parallel expression patterns of *Wnt5a* and *Fz4,* especially at stages 22 and 24 in the gut, suggest that perhaps Fz4 transduces the Wnt5a signal during gut development as well.

Expression profiles of planar polarity signaling molecules

The planar polarity pathway signals through Rho kinase and JNK to regulate cytoskeletal organization and epithelial cell polarization, and to effect changes in gene expression (Winter et al., 2001). Wnt11, Wnt5b, and Fz7 have all been shown to signal through the planar polarity pathway (Djiane et al., 2000; Heisenberg et al., 2000; Pandur et al., 2002; Tada and Smith, 2000; Winklbauer et al., 2001). Transcripts for *Wnt11* and *5b* are not detectable in the gut until stage 24, after morphogenesis of gut organs has begun. At stage 24, *Wnt5b* is expressed in the mesoderm of the proventriculus and ceca (Fig. 2I and K). By stage 32, this pattern has shifted to expression in the gizzard and small intestine mesoderm and the small intestine endoderm, suggesting different roles for Wnt5b during early morphogenesis and within the mature differentiated gut (Fig. 2T and V). *Wnt11* expression is detectable at stage 26 in a restricted region at the posterior edge of the gizzard adjacent to the duodenum, and is maintained at later stages (Fig. 2N and Y).

Fz7 appears to have a very dynamic expression profile during gut development. At stage 22, *Fz7* is expressed in the endoderm of the small and large intestines, while also being expressed in the gizzard mesoderm (Fig. 2A and G). By stage 24, expression in the endoderm is restricted to the ceca, while in the mesoderm, *Fz7* expression has expanded beyond the gizzard to the proventriculus, intestines, and ceca (Fig. 2I and R). The expression profile of *Fz7* suggests diverse functions for Fz7 in the gut, which include a possible role in regulating planar polarity of the ceca epithelium, mesoderm–endoderm signaling, and possibly maintaining the integrity of the foregut and midgut mesoderm. Such a dynamic expression profile also suggests that multiple Wnt's may signal through Fz7.

Expression profiles of unclassified signaling molecules

Several Wnt signaling molecules analyzed in this expression screen have not been attributed to any specific noncanonical signaling pathway. These include *Wnt7a, Wnt10a, crescent,* and *Fz9* (Fig. 2L, M, O, S, W, X, Z, DD). The expression of *crescent* was distinct in that instead of being uniformly expressed in the mesoderm, the transcripts were distributed in a punctate manner (Fig. 2D, and inset). This punctate expression appeared in different layers of the mesoderm and thus did not appear to define a particular cell type (section in situ not shown). Once these molecules have been placed in specific signaling pathways, knowledge of their expression profiles will allow their functions during gut development to be more quickly defined.

Roles for canonical signaling during gut development

Based on the expression profiles of the downstream transcription factors *Tcf4* and *Lef1,* we predicted that canonical Wnt signaling could be taking place in the regions of the gizzard, duodenum, and ceca. To test this hypothesis, we misexpressed N-terminal deleted forms of the transcriptional cofactors *Lef1* and *Tcf4,* which lack β-catenin binding sites and thus act as dominant negatives in the developing splanchnic mesoderm.

The *Lef1* transcript is expressed from early through late stages in the developing duodenum and ceca mesoderm (Fig. 1A, I, and U). Misexpression of the dominant-negative form of *Lef1* led to phenotypes in regions that corresponded to its normal domains of expression. In the duodenum, overexpression of the dominant-negative *Lef1* in the gut mesoderm resulted in a smaller duodenum (Fig. 3A–C). This was the result of a decrease in the size of the mesodermal segment of the gut when compared with wild type tissue. Despite normal differentiation of the epithelium, the lumen of *DN-Lef1*-infected duodenum appeared disorganized, most likely due to the constraints of a diminished area of surrounding mesoderm. At the ileal–cecal junction, misexpression of *DN-Lef1* had the effect of inducing proliferation of the endoderm, leading to stenosis of the lumen

(Fig. 3D–F). Stenosis due to endoderm proliferation was also observed in the large intestine at the level of the ceca, but not in the horns of the ceca itself. Importantly, since *DN-Lef1* infection was limited to the splanchnic mesoderm and its derivatives (Fig. 3F), overproliferation of the gut endoderm following infection of the viral construct appeared to be due to a secondary signal induced by Lef1, which regulates proliferation of the gut endoderm. This differed from Lef1's direct effect in the duodenum, where the infected mesoderm was smaller than wild type, either due to a failure of the mesoderm to proliferate or induction of an apoptotic program (Fig. 3C). Thus, Lef1 appears to have dual roles during gut development: directly regulating cell proliferation of the mesoderm in the duodenum, and indirectly regulating cell proliferation via a secondary signal in the endoderm of the large intestine.

Tcf4 expression spans a small region of the posterior gizzard throughout gut development (Fig. 1A, I, and U). When infected with virus expressing the dominant-negative form of *Tcf4*, the gizzard epithelium failed to form microvilli (Fig. 3G–I). Regulation of epithelial differentiation by Tcf4 was specific to the gizzard, as the adjacent epithelium of the pyloric sphincter contained normal microvilli in the presence of viral misexpression in the surrounding mesoderm (Fig. 3J and K). As was the case with Lef1 in the ceca, the specification of gizzard epithelium appeared to be regulated by Tcf4 through a secondary signal from the mesoderm to the endoderm.

Discussion

In this study, we performed a comprehensive analysis of the expression of Wnt signaling molecules during gut development. This expression profile screen was performed over three distinct stages of gut development: patterning (stage 22), morphogenesis (stage 24), and differentiation (stage 32). The components of the Wnt pathway included in this study consisted of the Wnt ligands, Fz receptors, Wnt antagonists, and several downstream effectors. Prior reports have demonstrated roles for specific Wnt signaling molecules during gut development (Korinek et al., 1998a; Lee et al., 1999; Tanaka et al., 1998; van de Westering et al., 2002; Wang et al., 2001). However, the full level of involvement of Wnt genes during gut development may be underestimated in loss-of-function studies due to redundancies between signaling molecules. Thus, we have attempted here to shed light on the additional roles of Wnt genes during gut development by comprehensively examining the expression of Wnt signaling components and organizing the data by putative signaling pathways.

Expression profiles of Wnt signaling molecules coincide with morphological boundaries

The expression screen revealed that, even before morphogenesis has begun, Wnt signaling molecules are expressed in very distinct domains along the anterior–posterior axis of the gut tube, corresponding with the boundaries of the future organs of the digestive tract. For molecules in the canonical pathway, their expression profiles define domains that give rise to the future gizzard, duodenum, ceca, and large intestine (for example, *Tcf4*, *Lef1*, *Fz1*, and *Fz8*; see Fig. 1). Conversely, the expression profiles of noncanonical pathway members define regions that will become the proventriculus, posterior small intestine, and ceca (for example, *Wnt5a*, *Fz2*, *Fz4*, and *Fz7*; Fig. 2). Thus it appears that the canonical and noncanonical pathways may define distinct domains along the A-P axis of the gut tube, as well as having different functions in regions of the gut where their expression overlaps (such as in the ceca).

Expression profiles predict regions of canonical Wnt signaling

Grouping the expression profiles of Wnt signaling molecules by signaling pathways enabled us to make some predictions on the possible roles of different Wnt pathways during gut development. Wnt signaling through the canonical pathway has an early role in patterning the gut tube. *Tcf4*, *Fz1*, and *Fz8* are expressed early in the gizzard, while *Lef1*, *Fz1*, and *Fz8* are expressed in the duodenum and large intestine (Fig. 1B, C, G, and H). Interestingly, no transcript for a Wnt ligand was detected at stage 22. This could be because the transcript levels were too low to be detected by whole mount. Alternatively, the ligand *Wnt5a*, which has been demonstrated to signal through both canonical and noncanonical pathways, is expressed in the proventriculus at stage 22 and may be responsible for initial induction of *Tcf4* expression in the adjacent gizzard (Fig. 2B). Transcripts for three different Wnt antagonists were detected in the duodenum (Fig. 1D–1F). Without a detectable Wnt ligand expressed in this region, it is difficult to account for the need for these three antagonists, but this does stress the importance of the tight regulation of canonical signaling in the region of the duodenum.

The expression profiles of canonical signaling components also suggest a coordinated role both in defining morphological boundaries along the A-P axis of the gut tube and in cross-talk between the endoderm and mesoderm. For example, at stage 24, the ligands *Wnt2* and *Wnt8b* are expressed in the gizzard and duodenum endoderm, while their potential receptors *Fz1* and *Fz8* are in the adjacent mesoderm (Fig. 1I). This type of expression profile suggests a role for Wnt2 and Wnt8b in communicating with the adjacent mesoderm. Other components of the pathway are expressed in different tissues layers at different A-P positions along the gut tube. The *Wnt1* transcript was detected in both the duodenum mesoderm and the ceca endoderm, indicating separate roles for the same molecule in different regions and tissues of the gut (Fig. 1I, L, U, and X). *Frzb1* is expressed early in the duodenum and large intestine mesoderm, but by stage 24, it expands to the mesoderm of

the gizzard and duodenum and to the endoderm of the large intestine (Fig. 1A, D, I, P, U, DD). The complexity of the spatial and temporal regulation of these genes will complicate further functional studies.

One way in which to simplify analyses of this kind is to examine the downstream transcription cofactors of the Wnt pathways. In the chick, *Tcf4* and *Lef1* expression are tightly restricted to specific positions (the posterior gizzard and the duodenum and ceca, respectively) (Fig. 1B and C). There are several possible explanations for the restricted expression of *Tcf4* and *Lef1*, and the absence of other tested transcription factors of the canonical pathway we examined, in spite of the fact that upstream components of the pathway are expressed throughout the entire gut tube. The restricted expression of downstream transcription factors *Tcf4* and *Lef1* could imply that, despite the broad expression of Wnt's and Fz's throughout the gut, the Wnt antagonists act to dramatically restrict their action. Although the Wnt antagonists overlap in their expression profiles, a single antagonist, *sFRP1*, is absent from the posterior gizzard in the same region that the *Tcf4* transcript is found (Fig. 1). Alternatively, it is possible that other yet unidentified downstream components may be expressed in different regions of the gut. Finally, it is possible that a Wnt-independent pathway may regulate the initial expression of *Tcf4* and *Lef1* in the gut, and that the extent of their domains serves to regulate the locations where ligands are able to act.

Tcf4 and Lef1 regulate cell proliferation and differentiation in the developing chick gut

Studies in mutant mice and human colorectal lesions have demonstrated a role for the canonical pathway in maintaining cell proliferation at the expense of cell differentiation in the gut epithelium. Mice homozygous mutant for *Tcf4* fail to maintain a proliferative stem cell compartment in the crypts of the small intestine and instead undergo differentiation programs (Korinek et al., 1998a; van de Westering et al., 2002). Although *Lef1* is not expressed in the normal human colon, *Lef1* is ectopically expressed in colon carcinomas (Hovanes et al., 2001). Experiments in colorectal cancer cells (CRC) have demonstrated that dominant-negative forms of TCF family members fail to bind β-catenin and act as inhibitors of endogenous β-catenin/TCF complexes in the nucleus. The effect is cell cycle arrest in the proliferative zones of the crypts and differentiation (van de Westering et al., 2002). Thus, analyses from both knockout mice and CRC cells support a role for TCF family members in balancing cell proliferation with cell differentiation.

To discern a role for the canonical pathway in the developing chick gut, we first examined the expression profiles of the downstream transcription factors. Interestingly, the expression profiles of *Tcf4* and *Lef1* in the chick gut differ from their patterns in the mouse. In the developing mouse gut, *Tcf4* is expressed in the epithelium of the midgut and becomes restricted to the intervillus crypts later in development (Korinek et al., 1998a,b; Lee et al., 1999). Unlike *Tcf4, Lef1* is absent from the developing mouse gut (Hovanes et al., 2001; Oosterwegel et al., 1993). In contrast, in the chick, *Tcf4* is expressed in the posterior stomach mesoderm, while *Lef1* is expressed in the mesoderm of the intestines at the level of the duodenum and ceca.

In chick, the expression profiles of *Tcf4* and *Lef1* predict a role for canonical signaling in the developing gizzard, duodenum, and ceca. Predictions of function based on expression patterns were tested in the chick by performing viral misexpression experiments of dominant-negative forms of the downstream cofactors *Tcf4* and *Lef1*. Viral infection of *DN-Tcf4* and *DN-Lef1* in the sphanchnic mesoderm at HH stage 12 allowed us to elucidate the roles of these genes during early patterning of the developing chick gut. In the duodenum, *DN-Lef1*-expressing cells of the mesoderm failed to proliferate, which led to diminished intestine size (compare Fig. 3A with B). In the ceca, *DN-Lef1* expression had a secondary effect on the adjacent epithelium, inducing cell proliferation leading to stenosis of the lumen (Fig. 3D–F). Viral misexpression of *DN-Tcf4* in the gizzard mesoderm interrupted epithelial differentiation, resulting in the failure to form microvilli (Fig. 3G and H). Thus, Tcf4 and Lef1 appear to be essential in balancing cell proliferation versus cell differentiation events in the developing gut.

Although the outcomes of loss of Tcf4 activity in the chick (misexpression of *DN-Tcf4*) and mouse (*Tcf4* knockout experiments) appear similar with both leading to endodermal differentiation defects, there are important differences in the way the phenotypes arise in the two species. In the mouse, loss of *Tcf4* function in the gut resulted in G_1 arrest of crypt cells and the induction of differentiation markers. In the chick, the misexpression of *DN-Tcf4* in the mesoderm led to the failure of microvilli to form in the adjacent endoderm, likely through a secondary secreted signal. *Tcf4* is also expressed at low levels in the chick endoderm, but the activity there would be unaffected in our mesoderm infections with the *DN-Tcf4* virus. A thick keratin-like substance normally found coating the microvilli of the gizzard was found to be secreted into the lumen of *DN-Tcf4*-infected gizzards, suggesting that some epithelial differentiation had occurred (Fig. 3G and J), in spite of the failure to form villi in the gizzard. Further studies with molecular markers will elucidate the degree of normal epidermal differentiation in the region. Significantly, however, the effect of *Tcf4* on the formation of microvilli was specific to the gizzard, as the adjacent epithelium of the pyloric sphincter contained normal microvilli even in the presence of viral misexpression (Fig. 3J and K). A potential caveat in our interpretation concerns the specificity of our dominant-negative constructs. The dominant-negative form of *Tcf4* could, in principle, block the activity of all TCF family members and, hence, misexpression of *DN-Tcf4* could potentially interfere with the activity of other cofactors ex-

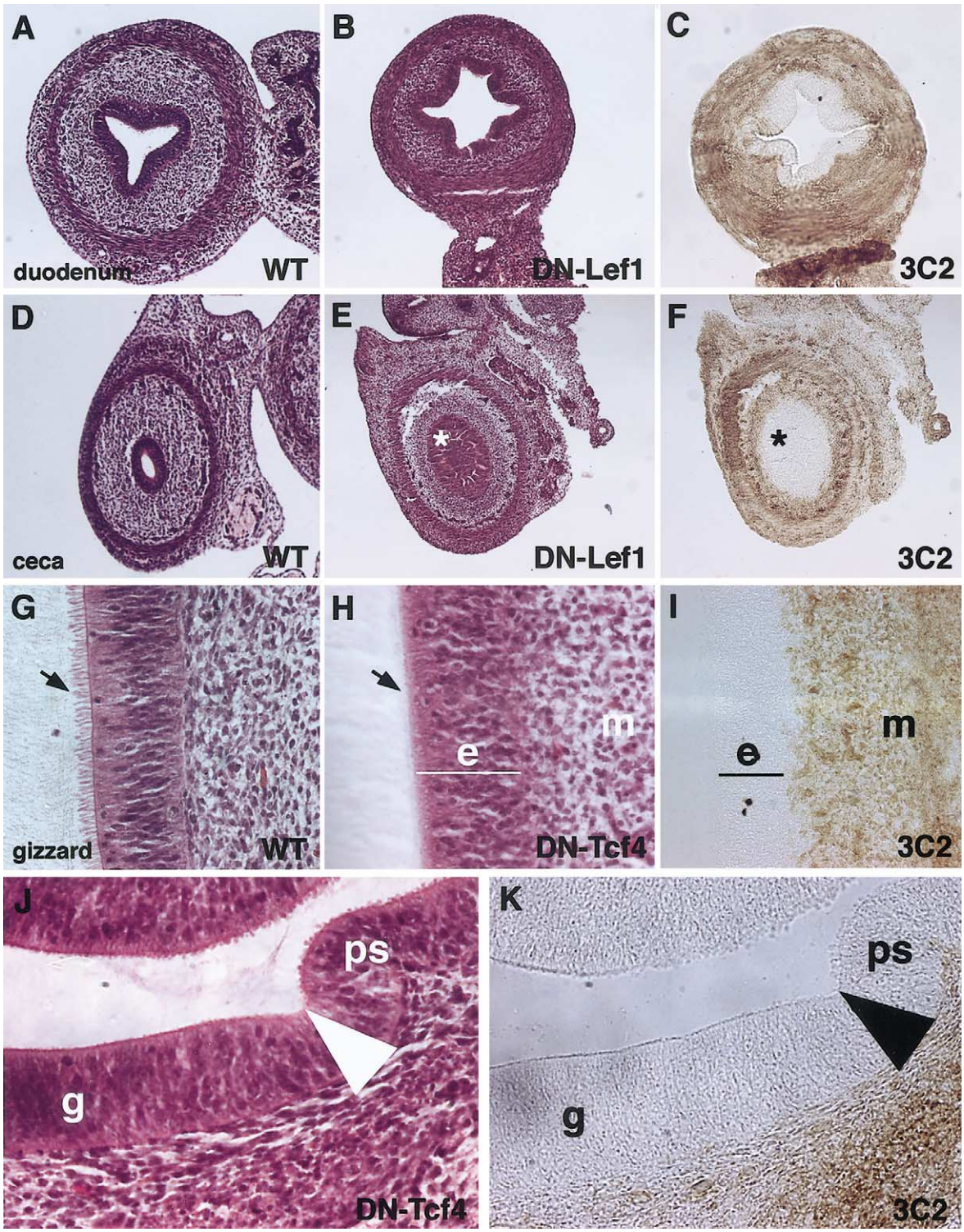

Fig. 3. Misexpression phenotypes of dominant-negative forms of *Lef1* and *Tcf4* in the chick gut. Sections (8 μm) through gut tissue at the level of the duodenum (A–C), ceca (D–F), and gizzard (G–K). Sections (A, B, D, E, G, H, and J) are stained with hematoxylin and eosin, while sections (C, F, I, and K) are stained with the 3C2 antibody against the viral *gag* protein to detect regions of viral infection. Viral misexpression of the *DN-Lef1* construct resulted in a decrease in the size of the mesoderm by cross-section (B) when compared with wild type (A). (C) Detection of viral particles by the 3C2 antibody in the adjacent section to (B) reveals that infection occurred throughout the gut mesoderm but not in the inner endodermal layer. (D, E) Infection of *DN-Lef1* in the ceca leads to stenosis of the lumen (indicated by asterisk in E). (F) In the adjacent section to (E), viral infection is observed throughout the mesoderm (as indicated by 3C2 staining), but is absent from the overproliferated epithelium (asterisk). (G–K) Viral misexpression of the *DN-Tcf4* construct leads to failure of the gizzard epithelium to properly differentiate and form microvilli [compare wild type microvilli (arrow in G) to infected tissue (arrow in H)]. (I) Adjacent section to (H) stained with 3C2 antibody illustrating viral infection throughout the mesoderm (m) but absent from the endoderm (e). The magnification in (G) and (H) is 63×, and is 40× in (I). (J, K) Division of the region spanning the gizzard (g) and pyloric sphincter (ps) is indicated by arrowheads. Despite infection throughout the gizzard and pyloric mesoderm (K), only the gizzard epithelium fails to differentiate, while the pyloric microvilli appear normal.

pressed in the mesoderm. We can eliminate this possibility, however, since misexpression of *DN-Lef1* does not result in loss of microvilli in the gizzard endoderm.

In conclusion, we undertook a comprehensive expression screen of Wnt signaling components in an attempt to identify possible signaling centers for Wnt pathways during gut development. In the case of canonical signaling through β-catenin, the downstream cofactors Tcf4 and Lef1 regulate cell proliferation and cell differentiation during development of the chick gut. Our data implicate specific roles for Tcf4 and Lef1 in the chick gut mesoderm. The breadth of this expression profile screen provides the foundation for future functional studies in elucidating roles for the different Wnt pathways during vertebrate gut development.

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