

Enteroendocrine precursors differentiate independently of Wnt and form serotonin expressing adenomas in response to active β -catenin

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Wnt signaling is required for the maintenance of intestinal stem cells and self-renewal of the intestinal epithelium. Intestinal cancers are frequently associated with mutations that activate the Wnt pathway. The role of Wnt signaling on differentiation of lineage-specific precursors in the intestine is not well characterized. Here, we show that specification of enteroendocrine but not Paneth cells occurs independently of Wnt signals by conditional deletion of β -catenin in immature cells expressing the transcription factor, neurogenin 3. In addition, we determined whether neurogenin 3-expressing cells respond to abnormal Wnt signaling. Activation of the Wnt pathway by conditionally deleting exon 3 of the β -catenin gene at an early stage of enteroendocrine cell differentiation induced small-intestinal adenomas expressing serotonin, a feature not previously described in other tumors induced by Wnt in mice. In contrast, excision of exon 3 of β -catenin at a later stage of enteroendocrine differentiation did not produce tumors. These results provide direct evidence that some intestinal lineages are specified independently of the Wnt pathway and may lead to a better understanding of the spectrum of neuroendocrine differentiation frequently seen in human gastrointestinal cancer.

carcinoid | enteroendocrine cell | neurogenin 3 | Paneth cell | Wnt pathway

Activation of canonical Wnt signaling results in a series of events that stabilize β -catenin, with resultant activation of Wnt target gene transcription (1). Generalized disruption of Wnt signaling in the intestine resulted in widespread loss of crypts with the absence of cell proliferation in the small intestine, indicating an essential role for this pathway in the maintenance of intestinal stem cells and their dividing descendants. Enteroendocrine cells failed to develop in both TCF4-null and villin-Dkk1 mice, whereas goblet cells were absent in villin-Dkk1 but not TCF4^{-/-} animals (2, 3). The absence of enteroendocrine cells with generalized Wnt inactivation in the intestine may indicate that this lineage requires Wnt signals for differentiation. Alternatively, depletion of intestinal stem cells may preclude the ongoing replacement of enteroendocrine cells which turnover every 4–5 days rather than directly requiring Wnt signals for their specification.

Intestinal neoplasms, including colorectal cancer in humans, are frequently associated with excessive Wnt signaling. APC^{min} mice with a mutant APC allele develop adenomatous polyps in the intestine, as do mice expressing a mutant β -catenin allele lacking exon 3. In both cases, reduced targeting of β -catenin for degradation leads to redistribution of β -catenin to the nucleus with activation of Wnt target genes (4, 5).

Two basic helix–loop–helix (bHLH) proteins regulated by the Notch signaling pathway (6, 7), neurogenin 3 (NGN3) and NeuroD1, have an established role in the specification and differentiation of enteroendocrine cells. Mice lacking Ngn3 develop few enteroendocrine cells, indicating an essential role for their specification (8, 9). Ngn3 is transiently expressed in

immature cells to initiate enteroendocrine differentiation and induces expression of NeuroD1 (10). We previously showed that NeuroD1 is expressed in most enteroendocrine cells and induces cell-cycle arrest during later stages of enteroendocrine differentiation (11). Thus, expression of Ngn3 and NeuroD represent distinct stages in the differentiation of enteroendocrine cells from intestinal secretory progenitor cells in intestinal crypts.

Currently, it is not known whether cells committed to become enteroendocrine cells depend on Wnt signals for further differentiation or whether they retain the capacity to respond to Wnt. In the present work, we found that early precursors to enteroendocrine cells respond to abnormal Wnt signaling to develop serotonin-expressing adenomas of the small intestine, whereas later precursors do not. In addition, our results indicate that Wnt signaling is required for Paneth cells to differentiate but not for specification of enteroendocrine cells.

Results and Discussion

To determine whether enteroendocrine progenitor cells respond to abnormal Wnt signaling, we conditionally deleted exon 3 of a mutant β -catenin allele in *Catnb*^{Flox(ex3)/+} mice (4) expressing Cre under control of the Ngn3 gene (12) to generate a stabilized β -catenin protein. *Ngn3-Cre:Catnb*^{Flox(ex3)/+} mice were born with the expected frequency. After 4 weeks of age, we observed increasing numbers of adenomatous polyps throughout the small intestine (Fig. 1A) but not in other Ngn3-expressing gastrointestinal organs, including colon, stomach, and pancreas, consistent with earlier work suggesting that the small intestine is the predominant organ in the GI tract targeted by exon 3 deletions of β -catenin where mice harboring up to 3,000 adenomas fail to develop colonic tumors (4, 5). Polyps appeared to originate from aberrant crypts (Fig. 1B and C) and in some cases displayed high-grade dysplasia with nuclear pseudostratification, increased mitotic figures, and mucosal ulceration consistent with intraepithelial carcinoma (Fig. 1D). At 20 weeks of age, we observed 246 ± 40 polyps per mouse (Fig. 3A). Most *Ngn3-Cre:Catnb*^{Flox(ex3)/+} mice developed a single or occasionally two bulky invasive adenocarcinomas with tumor glands invading beyond the muscularis mucosa into the submucosal space to the muscularis propria (Fig. 1E–G) and died without distant metastasis by 6–9 months of age (Fig. 3B). The very low frequency of invasive neoplasia is consistent with earlier observations follow-

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Abbreviation: NE, neuroendocrine.

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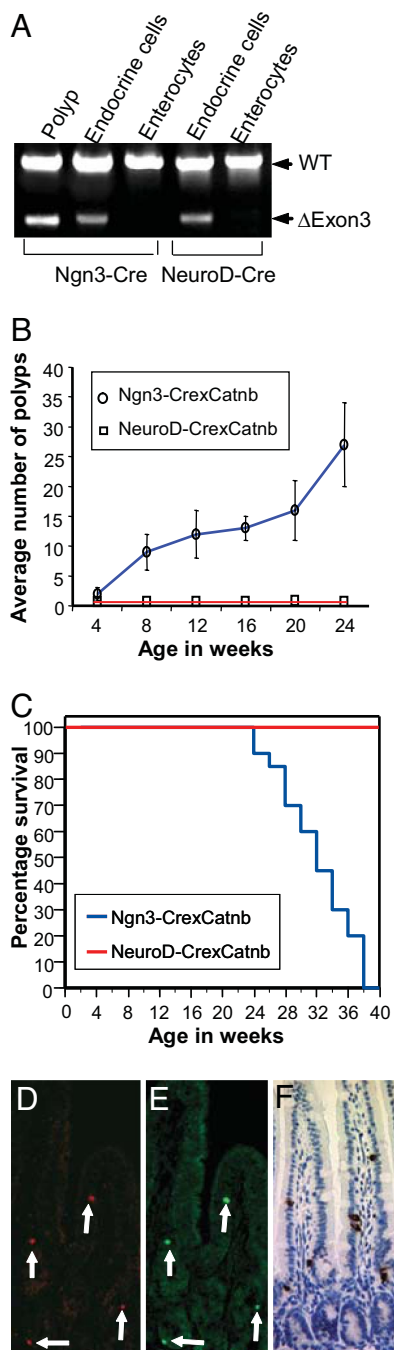


Fig. 3. Absence of neoplasia in *NeuroD-Cre:Catnb^{lox(ex3)/+}* mice. (A) Genotyping for excision of exon 3 of the β -catenin gene by amplification of DNA from chromogranin A-stained enteroendocrine cells or enterocytes isolated by laser microdissection from the small intestine of *Ngn3-Cre:Catnb^{lox(ex3)/+}* and *NeuroD-Cre:Catnb^{lox(ex3)/+}* mice. Arrows denote the positions of the wild-type and exon 3 deleted alleles. DNA was amplified 45–54 cycles. (B) *Ngn3-Cre:Catnb^{lox(ex3)/+}* mice (circles) accumulate neoplasms with increasing age, whereas *NeuroD-Cre:Catnb^{lox(ex3)/+}* mice (squares) fail to develop polyps at any age. For each time point, polyps were counted in three to four animals and results were expressed as the number of polyps \pm SEM per cm of intestinal length. (C) Survival curves for *Ngn3-Cre:Catnb^{lox(ex3)/+}* (blue) and *NeuroD-Cre:Catnb^{lox(ex3)/+}* (red) mice showing the percentage of mice remaining alive at 2-week intervals. The analysis included 20 animals for each genotype. Most *Ngn3-Cre:Catnb^{lox(ex3)/+}* mice died by the age of 9 months in contrast to *NeuroD-Cre:Catnb^{lox(ex3)/+}* mice. (D and E) Colocalization of Cre (C) and NeuroD (D) by double immunofluorescence in the small intestine of *NeuroD-Cre* mice. (F) *NeuroD-Cre:Catnb^{lox(ex3)/+}* mouse display normal intestinal morphology and normal enteroendocrine cells immunostained for chromogranin A.

NE markers and may be related to “mixed exocrine-endocrine carcinoma” (MEEC), which contain significant endocrine and nonendocrine components (16).

Genotyping of normal enteroendocrine cells in *Ngn3-Cre:Catnb^{lox(ex3)/+}* mice revealed recombination at exon 3 of β -catenin, suggesting that excision of exon 3 preceded polyp formation (Fig. 3A). The delayed appearance of polyps suggests that development of neoplasia may require ongoing exposure to excessive Wnt signaling. The absence of tumors in mice with biallelic deletion of APC in the intestine also suggests the importance of sustained abnormal Wnt signaling for tumorigenesis. Despite replacement of the intestinal epithelium with proliferating crypt cells, these mice fail to develop neoplasms, presumably because of their short lifespan (17).

To determine whether enteroendocrine precursors retain the ability to respond to abnormal Wnt signals as they differentiate, we generated mice expressing Cre recombinase under control of 115 kb of 5' sequence and 78 kb of 3' sequence of the *NeuroD* gene, a bHLH transcription factor induced by *Ngn3* in enteroendocrine cells. Thus, the onset of *NeuroD* expression represents a later stage in the differentiation of enteroendocrine cells than expression of *Ngn3*. Expression of the Cre transgene appeared identical to the endogenous *NeuroD* gene because it was restricted to *NeuroD*⁺ cells in the small intestine with all *NeuroD*⁺ cells showing expression of Cre (Fig. 3C and D). We crossed the *NeuroD-Cre* mice with *Catnb^{lox(ex3)/+}* mice to initiate excision of the third exon of the β -catenin gene with the onset of *NeuroD* expression, later in differentiation compared with the *Ngn3-Cre:Catnb^{lox(ex3)/+}* mice.

NeuroD-Cre:Catnb^{lox(ex3)/+} mice remained healthy and free of polyps with normal intestinal morphology and enteroendocrine cells at 1 year of age (Fig. 3A, B, and E). Amplification of the β -catenin gene from enteroendocrine cells isolated from *NeuroD-Cre:Catnb^{lox(ex3)/+}* mice by laser microdissection showed excision of exon 3 from the floxed β -catenin allele but not the wild-type allele as expected. Excision occurred specifically in enteroendocrine cells but not in enterocytes, indicating that the failure to develop intestinal neoplasms did not result from the absence of recombination in enteroendocrine cells (Fig. 3A). We have previously shown that expression of *NeuroD* is important for differentiating enteroendocrine cells to undergo cell-cycle arrest (11). Thus, initiation of *NeuroD* expression may represent a stage when differentiating enteroendocrine cells permanently exit the cell cycle and lose their response to Wnt signals.

Neoplasms in *Ngn3-Cre:Catnb^{lox(ex3)/+}* mice showed nuclear and cytoplasmic β -catenin staining indicative of active Wnt signaling, versus the predominant localization at the cell membrane in adherens junctions of normal mice (Fig. 4A and B). In normal mice, expression of the Wnt target gene, *c-Myc*, was confined to cells in the lower crypt (Fig. 4C). Dysplastic epithelial cells invading the lamina propria of polyps showed strong nuclear staining for *c-Myc*, consistent with excessive Wnt signaling (Fig. 4D). We examined polyps for increased expression of two additional Wnt target genes, EphrinB3 and CD44, by semiquantitative RT-PCR. Polyps showed increased expression of both of these genes and of *c-myc* compared with adjacent normal epithelium (Fig. 4G).

We also detected nuclear *c-Myc* staining in a fraction of enteroendocrine cells in normal appearing intestine of *Ngn3-Cre:Catnb^{lox(ex3)/+}* mice (Fig. 4E) but not in *NeuroD-Cre:Catnb^{lox(ex3)/+}* mice (Fig. 4F). Thus, excision of exon 3 of the β -catenin gene at this later stage of differentiation with the onset of *NeuroD* expression did not result in activation of the Wnt target gene, *c-Myc*. The failure to activate Wnt target genes in response to nuclear β -catenin has been described for mature T lymphocytes. Introduction of stabilized β -catenin into mature T lymphocytes did not result in Wnt target gene expression despite the formation of β -catenin–LEF1 complexes in the nucleus, in

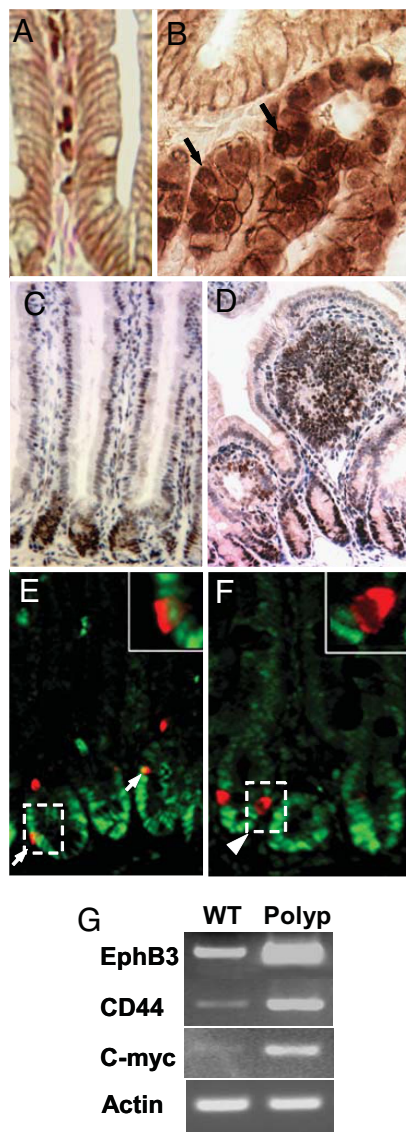


Fig. 4. Abnormal Wnt signaling in the intestine of *Ngn3-Cre:Catnb^{Flox(ex3)}+* mice. (A and B) Intracellular localization of β -catenin. (A) Immunostaining for β -catenin at the cell membrane in a normal mouse. (B) Nuclear and cytoplasmic β -catenin immunostaining in a large adenoma. (C) Immunostaining for c-Myc in normal mice is restricted to crypts. (D) Polyp with widespread c-Myc staining. (E) Double immunofluorescent staining for c-Myc (green) and chromogranin A (red) shows some enteroendocrine cells in normal intestinal tissue of *Ngn3-Cre:Catnb^{Flox(ex3)}+* mice that express c-Myc. Higher magnification (Inset) shows a single cell with red cytoplasmic staining for chromogranin A, colocalized with green nuclear c-Myc staining. (F) Absence of c-Myc staining in enteroendocrine cells of *NeuroD-Cre:Catnb^{Flox(ex3)}+* mice. (G) Semiquantitative RT-PCR analysis comparing expression of Wnt target genes, c-myc, CD44, and EphrinB3, in polyps versus normal epithelium.

contrast to activation of Wnt target genes in the Jurkat T cell line (18). The underlying mechanism accounting for this differential response to β -catenin remains to be elucidated.

Generalized disruption of Wnt signaling in the intestine interferes with specification of all three secretory lineages (2, 3). The failure to develop secretory lineages in mice with the complete loss of Wnt signaling may result from depletion of stem cells rather than a lineage-specific requirement for this pathway. We conditionally deleted the β -catenin gene from *Ngn3*-expressing cells to determine whether Wnt signaling has a direct effect on the differentiation of enteroendocrine cells as opposed to its known role in

stem-cell maintenance. We crossed *Ngn3-Cre* transgenic mice to animals with one null allele and one conditional β -catenin allele with loxP sites flanking exons 2–6 (19) into a R26R background to allow us to identify cells that expressed Cre on the basis of β -gal activity arising from the ROSA26 gene. The small intestine of *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice appeared normal, revealing normal crypt-villus architecture with proliferating cells confined to the crypt. The number of β -gal-expressing enteroendocrine cells in *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice was not significantly different from *Ngn3-Cre:R26R* mice, indicating that Wnt signaling was not required for their specification or terminal differentiation (Fig. 5A).

We previously showed that ≈ 5 –10% of small-intestinal goblet cells, and 45% of Paneth cells arose from cells expressing *Ngn3* by recombination based lineage analysis (12). To examine whether canonical Wnt signaling was required for specification of nonendocrine cells that arise from *Ngn3*⁺ cells, we examined the effect of deleting β -catenin in *Ngn3*⁺ cells in the small intestine of *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice on the number of β -gal expressing Paneth cells and goblet cells. The fraction of goblet cells expressing β -gal in *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice was identical to *Ngn3-Cre:R26R* mice, suggesting that this subpopulation of goblet cells did not depend on Wnt signaling for differentiation (Fig. 5B). However, we observed an 80% reduction in the fraction of Paneth cells expressing β -gal in *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice (Fig. 5C), suggesting that a major fraction of this lineage may depend on Wnt for specification. Recombination based lineage analysis also revealed that a very small fraction of pluripotent cells in the intestine expressed *Ngn3*, resulting in β -gal expression in all epithelial cells of single crypt-villus units at a frequency of $<1\%$. We observed a 74% reduction in the frequency of crypt-villus units showing generalized β -gal expression in *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice, consistent with the established role of the canonical Wnt pathway in the maintenance of crypt stem cells (Fig. 5D).

The importance of Wnt signaling for Paneth cell specification is unresolved at present. Paneth cells in cryptless regions of the intestine of villin-Dkk1 mice were dramatically reduced in number. The loss of Paneth cells may have resulted from the relative absence of crypt stem cells rather than from a direct lineage-specific requirement (2, 3). Activation of the Wnt pathway by conditional APC deletion under control of the villin gene resulted in crypt expansion with increased numbers of Paneth cells, reduced goblet cell numbers, and a slight reduction in enteroendocrine cells, suggesting that Wnt signaling favors Paneth-cell differentiation at the expense of other secretory lineages (20).

The sparing of the enteroendocrine and goblet lineages after conditional deletion of β -catenin in the present work indicates that the loss of Paneth cells did not result from stem-cell depletion. Disruption of Wnt signaling by conditional deletion of *Frizzled5* (*Fz5*) in keratin 19-expressing cells resulted in mispositioning of Paneth cells in the intestine, leading to the suggestion that Wnt signaling was required for maturation of Paneth cells but not for their specification (21, 22). However, multiple *Frizzled* family proteins, including *Frizzled 5*, *Frizzled 6*, and *Frizzled 7*, were identified at the crypt base overlapping the location of Paneth cells and their precursors (23). Thus, deletion of *Frizzled 5* may only result in a partial loss of Wnt signaling and is consistent with mispositioning of Paneth cells in parts of the intestine of villin-Dkk1 mice with normal appearing crypts. We never observed mispositioning of Paneth cells to villi in *Ngn3-Cre:R26R:Catnb^{Flox/-}* mice, suggesting that Paneth cells were not specified with complete disruption of the canonical Wnt pathway. Thus, specification, differentiation, and maturation of Paneth cells may require different components of the Wnt pathway.

The absence of goblet, Paneth, and enteroendocrine cells in mice lacking the bHLH protein MATH1 may indicate a common progenitor cell for the three secretory lineages (24). However,

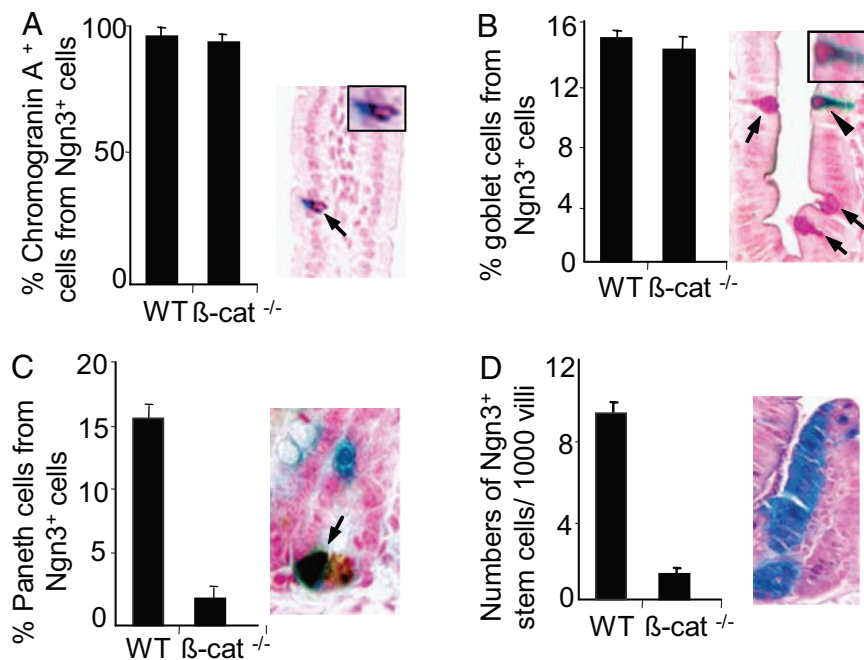


Fig. 5. Specification of enteroendocrine and goblet cells but not Paneth cells occurs independently of Wnt signaling. (A–D) Effects of conditionally deleting β -catenin in intestinal epithelial lineages arising from Ngn3^+ cells in a R26R background. The numbers of enteroendocrine, goblet, Paneth, and pluripotent cells arising from Ngn3^+ cells stained for β -gal activity (blue) using X-gal histochemistry were counted. At least 500 cells were counted for each lineage. (A) Conditional deletion of the β -catenin gene in endocrine precursor cells expressing Ngn3 has no effect on enteroendocrine cell differentiation. Mice show normal numbers of chromogranin A-stained enteroendocrine cells (Left) and with normal distribution (arrow; Right). Higher magnification (Inset) shows a single cell with cytoplasmic staining for β -gal (blue) and chromogranin A (brown). (B) Number of β -gal-expressing cells showing PAS staining for mucin (red) was unaffected by the deletion of β -catenin (Right). Shown are examples of goblet cells double positive for PAS and β -gal (arrowhead) or PAS alone (arrows). Higher magnification (Inset) shows a single cell with cytoplasmic staining for PAS and β -gal. (C) Deletion of β -catenin resulted in an 85% reduction in the number of Paneth cells expressing β -gal activity (Right). Shown are examples of X-gal-positive (blue) and lysozyme-positive (brown) Paneth cells. (D) Descendants of pluripotent cells expressing Ngn3 appear as entire crypt-villus units that stain for β -gal activity (Right) and were reduced by 75% in number when β -catenin was deleted under control of the Ngn3 gene. Results were expressed as the percentage of goblet and Paneth cells expressing β -gal activity (B and C) or as the frequency of crypt-villus units per 1,000 showing expression of β -gal in all epithelial cells.

the dependence of Paneth cell but not goblet or enteroendocrine cell differentiation on Wnt signaling suggests that differentiation of intestinal secretory lineages may involve a series of secretory progenitor cells that arise from cells expressing MATH1. A recent study describing increased numbers of enteroendocrine cells, reduced numbers of goblet cells, and absent Paneth cells in *Gfi-1*-null mice (25) is also consistent with additional secretory precursors segregating from MATH1-dependent cells.

The response to Wnt signaling and developmental requirement for these signals may be highly dependent on their timing for different cell lineages. Our observations provide direct evidence that enteroendocrine cells are specified independently of the Wnt pathway, whereas Paneth cells are not. In contrast to the Wnt, Notch signaling is one pathway that appears to be critical for enteroendocrine lineage specification through its effects on bHLH proteins (6, 7). In addition, the ability to activate Wnt target genes via the canonical Wnt pathway may be restricted to immature cells in the intestine and may be lost as cells differentiate. The features in the intestinal tumors reported here have not been previously described in mice with abnormal Wnt signaling and may provide further insight toward understanding the spectrum of neuroendocrine differentiation in human intestinal tumors.

Materials and Methods

Transgenic Mice. To conditionally express a stabilized β -catenin allele in Ngn3 - or *NeuroD*-expressing cells, *Ngn3-Cre* or *NeuroD-Cre* mice were crossed with the *Catnb^{Flox(ex3)/+}* line, which has loxP sites flanking the third exon. Genotyping for the Cre allele

and *Catnb^{Flox(ex3)/+}* allele were performed as described previously. The offspring inherited a *Catnb^{Flox(ex3)/+}* allele, and either an *Ngn3-Cre* or *NeuroD-Cre* allele were analyzed for phenotypes.

Mice carrying an allele of β -catenin gene with loxP sites flanking gene sequence from exon 2 through exon 6 were obtained from The Jackson Laboratory (*Catnb^{m2kem}/J*) as homozygotes. Mice with one β -catenin gene null allele were generated by crossing *Catnb^{m2kem}/J* with *EIIa-Cre* mice, a general deleter strain from The Jackson Laboratory (*B6.FVB-Tg (EIIa-Cre)C5379Lmgd/J*). To conditionally delete β -catenin in Ngn3 -expressing cells, a mating scheme was devised that only one conditional allele needs to undergo recombination to create tissue-specific null for β -catenin gene as described in ref. 19. The knockout animals were further crossed to homozygous R26R mice (*B6.129S4-Gt 26 Sor^{tmSor}*) from The Jackson Laboratory (26) to generate mice with both β -catenin alleles deleted in Ngn3^+ cells in a R26R background. Use of vertebrate animals was approved by the Institutional Animal Care and Use Committee at Tufts–New England Medical Center.

Histology and Immunohistochemistry. Tissue samples were fixed in 4% paraformaldehyde and processed for paraffin sections. Slides were incubated with the following antibodies: mouse anti- β -catenin at 1:50 (Transduction Laboratories), rabbit anti-c-Myc at 1:100 (Santa Cruz Biotechnology), rabbit anti-chromogranin A at 1:1,000 (ImmunoStar), rabbit anti-serotonin at 1:10,000 (ImmunoStar), rabbit anti-secretin at 1:500 (W. Chey, University of Rochester, Rochester, NY), rabbit anti-cholecystokinin at 1:8,000 (Chemicon), rabbit anti-somatostatin at 1:3,000 (R. Lechan, Tufts University), rabbit anti-Ki67 at 1:1,000 (NeoMarkers), rabbit anti-PGP9.5 at

