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Hand2 is necessary for terminal differentiation of enteric neurons from crest-derived precursors but not for their migration into the gut or for formation of glia

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Hand genes encode basic helix-loop-helix transcription factors that are expressed in the developing gut, where their function is unknown. We now report that enteric *Hand2* expression is limited to crest-derived cells, whereas *Hand1* expression is restricted to muscle and interstitial cells of Cajal. Hand2 is developmentally regulated and is intranuclear in precursors but cytoplasmic in neurons. Neurons develop in explants from wild-type but not *Hand2*$^{-/-}$ bowel, although, in both, crest-derived cells are present and glia arise. Similarly, small interfering RNA (siRNA) silencing of *Hand2* in enteric crest-derived cells prevents neuronal development. Terminally differentiated enteric neurons do not develop after conditional inactivation of *Hand2* in migrating crest-derived cells; nevertheless, conditional *Hand2* inactivation does not prevent precursors from expressing early neural markers. We suggest that enteric neuronal development occurs in stages and that *Hand2* expression is required for terminal differentiation but not for precursors to enter the neuronal lineage.

KEY WORDS: Autonomic nervous system, bHLH transcription factor, Conditional knockout, Enteric nervous system, Gene knockout, Gut development, Mouse

INTRODUCTION

Basic helix-loop-helix (bHLH) transcription factors play important roles in lineage determination and differentiation (Massari and Murre, 2000). The Hand genes *Hand1* (*eHand*) and *Hand2* (*dHand*) are members of the twist family of bHLH transcription factors (Firulli, 2003). Hand genes are expressed in the developing heart, in lateral mesoderm and in subsets of neural crest-derived cells (Cserjesi et al., 1995). Hand genes are best-known for their roles in cardiac (Firulli et al., 1998; Srivastava et al., 1995; Srivastava et al., 1997), vascular (Dai et al., 2004; Yamagishi et al., 2000) and limb development (Cai and Jabs, 2005). They also play crucial roles in the development of sympathetic neurons, where they are necessary for catecholamine expression (Howard, 2005; Lucas et al., 2006). Partial redundancy might occur between *Hand1* and *Hand2* functions in heart (McFadden et al., 2005) and sympathetic (Howard et al., 1999) ganglia.

Although *Hand2* expression is linked to the specification of the noradrenergic phenotype of sympathetic neurons (Howard, 2005), *Hand2* is also expressed in parasympathetic ganglia (Morikawa et al., 2005) and gut (Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995), which do not contain noradrenergic neurons (Brookes and Costa, 2006; Furness, 2000; Lomax and Furness, 2000). The function of Hand genes in the autonomic nervous system is thus not limited to noradrenergic phenotypic expression. Because *Hand2* expression in postnatal day (P)19 embryonic carcinoma cells causes the expression of peripheral neural markers, such as peripherin 1, and its expression occurs before neurons arise (Morikawa et al., 2005), crest-derived precursors may require *Hand2* to become neurons rather than only for the specification of their neurotransmitter-defined identity (Hendershot et al., 2007; Howard, 2005).

Hand genes are expressed in developing mouse (Cserjesi et al., 1995; Hendershot et al., 2007; Hollenberg et al., 1995) and chick (Wu and Howard, 2002) gut. In situ hybridization has suggested that *Hand2* is expressed in the presumptive enteric nervous system (ENS) in mice (Dai et al., 2004) and chicks (Wu and Howard, 2002). We now report that *Hand2*, but not *Hand1*, is necessary for crest-derived cells to become enteric neurons. We found that crest-derived cells selectively express *Hand2*, whereas mesodermal derivatives express *Hand1*, in mouse gut. Additionally, our results show that crest-derived precursors from *Hand2*$^{-/-}$ mouse gut survive and give rise to glia in vitro but fail to develop as neurons. *Hand2* expression was also found to be developmentally regulated, but was found to continue at a low level in mature neurons. We also show that Hand2 is intranuclear during differentiation, but cytoplasm-restricted in mature neurons. Although the conditional knockout of *Hand2* in neural crest cells did not prevent the limited expression of early neural markers, it did block the terminal differentiation of enteric neurons.

MATERIALS AND METHODS

Animals

Adult mice (CD-1; Charles River Laboratories) and rats (Sprague-Dawley, Charles River, Waltham MA) were killed by CO_2 inhalation. *Hand2*$^{-/-}$ embryos were obtained by interbreeding *Hand2*$^{+/-}$ mice (Srivastava et al., 1997). Mice were considered wild type only when verified to be *Hand2*$^{+/+}$ and not *Hand2*$^{+/-}$. The generation and characterization of transgenic mice bearing a *lacZ* reporter gene encoding β-galactosidase under the control of the *Hand1* promoter have previously been described (Dai et al., 2004). To generate mice carrying 'floxed' *Hand2* alleles, the targeting construct of the *Hand2* gene included loxP sites placed 5′ of the start of transcription and within the first intron. Recombination between the loxP sites deletes the first intron, which includes the bHLH domain and most of the coding region. A manuscript describing the generation of this mouse line will be presented elsewhere (Morikawa et al., 2007). Mice lacking *Hand2* in crest-derived cells (Wnt1-Cre-H2$^{\Delta}$) were generated by crossing males carrying

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Table 1. Antibodies and labels

Primary antibodies

Antigen	Antibodies	Dilution	Source	Secondary antibodies (numbers correspond to secondary antibodies, below)
β3-tubulin	Rabbit monoclonal	1/1000	Covance, Richmont, CA	2 or 4 (ABC kit, DAB)
β-galactosidase	Rabbit polyclonal	1/500	5′-3′, Boulder, CO	3
B-FABP	Rabbit polyclonal	1/500	Gift from T. Müller, Max Delbruck Center for Molecular Medicine, AG Medical Genetics, Berlin, Germany	1 or 2
Dbh	Rabbit polyclonal	1/500	Chemicon, Temecula, CA	4 (ABC kit, DAB)
Dbh	Sheep polyclonal	1/500	Chemicon	9
GAP-43	Mouse monoclonal	1/5000	Gift from D. Schreyer, Stanford University, Palo Alto, CA	5
HuC/D	Mouse monoclonal biotinylated	1/10	Invitrogen-Molecular Probes, Carlsbad, CA	(Steptavidin Alexa-Fluor-488), (Alexa-Fluor-594) or (ABC kit, DAB)
Kit	Goat polyclonal (C14)	1/1000	Santa Cruz Biotechnology,	8 (Steptavidin Alexa-Fluor-594)
MAP2	Chicken	1/1000	Chemicon	10
nNOS	Rabbit polyclonal	1/500	Santa Cruz Biotechnology,	3
$p75^{NTR}$	Rabbit polyclonal	1/1500	Gift from M. Chao, Skirball Institute, New York University, NY	1, 2 or 4 (Streptavidin Alexa-Fluor-350)
PGP9.5	Rabbit polyclonal	1/1000	Biogenesis, Poole, UK	2 or 4 (ABC kit, DAB)
Phox2b	Rabbit polyclonal	1/1000	Gift from J.-F. Brunet, CNRS, École normale supérieure, Paris, France	3 or 4 (ABC kit, DAB)
Ret	Goat polyclonal	1/200	R&D Systems, Berkeley, CA	8 (ABC kit, DAB)
Sox10	Goat polyclonal	1/10	Gift from D. Anderson, California Institute of Technology, Howard Hughes Medical Institute, Pasadena, CA	7 or 8 (ABC kit, DAB)
Th	Sheep polyclonal	1/500	Chemicon	9

Secondary antibodies

Antibodies antigen	Antibodies	Dilution	Source	Label
1- Rabbit	Goat	1/600	Molecular probes, Eugene, OR	Alexa-Fluor-488
2- Rabbit	Goat	1/600	Molecular probes	Alexa-Fluor-594
3- Rabbit	Donkey	1/300	Molecular probes	Alexa-Fluor-594
4- Rabbit	Donkey	1/200	Jackson ImmunoResearch, West Grove, PA	Biotin
5- Mouse	Goat	1/600	Molecular probe	Alexa-Fluor-488
6- Goat	Donkey	1/300	Molecular probes	Alexa-Fluor-594
7- Goat	Donkey	1/300	Jackson ImmunoResearch	FITC
8- Goat	Donkey	1/200	Jackson ImmunoResearch	Biotin
9- Sheep	Donkey	1/300	Molecular probes	Alexa-Fluor-488
10- Chicken	Goat	1/400	Molecular probes	Alexa-Fluor-488

Other labels

Name	Dilution	Source
Streptavidin Alexa-Fluor-488, -594 or -350	1/300	Molecular probes
Vectastain ABC kit		Vector Laboratories, UK
DAB		Vector Laboratories

the *Wnt1-Cre* transgene and heterozygous for the null allele of *Hand2* (Srivastava et al., 1997) with females homozygous for the 'floxed' *Hand2* allele. Mutant embryos were identified by PCR genotyping of extraembryonic membranes. Mice with the *Wnt1-Cre* transgenes (Danielian et al., 1998) were contributed by Henry M. Sukov (University of Southern California, Los Angeles, CA; permission from Andrew MacMahon, University of Illinois, Urbana, IL).

Isolation of crest-derived cells

To obtain enteric crest-derived cells, fetal mouse or rat gut was dissociated with collagenase. The resulting cellular suspension was cultured or purified by immunoselection with rabbit polyclonal antibodies to the common neurotrophin receptor $p75^{NTR}$ (gift from Moses Chao, New York University, NY) as described previously (Chalazonitis et al., 1994; Chalazonitis et al., 1997).

Cell and organotypic tissue culture

Suspensions of dissociated or immunoselected cells were plated (2×10^5 cells/ml) on laminin-coated charged glass chamber slides (NUNC, Denmark). The maintenance medium consisted of Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with L-glutamine (2 mM; Invitrogen), neurotrophin 3 (1.4 nM), glial cell-line-derived neurotrophic factor (0.3 nM), epithelial growth factor (1.7 nM), and basic fibroblast growth factor (0.2 nM). Cells were plated in the maintenance medium enriched with 10% fetal bovine serum (FBS) and 20% horse serum (plating medium), and were transferred to the maintenance medium with B-27 Supplement (Invitrogen) 1 day later. Explanted foregut was cultured in a three-dimensional collagen gel as previously described (Natarajan et al., 1999; Tessier-Lavigne et al., 1988). Foregut explants were cultured in the plating medium for 2 days, then transferred to maintenance medium supplemented with 2% FBS. Media were changed at 2-day intervals.

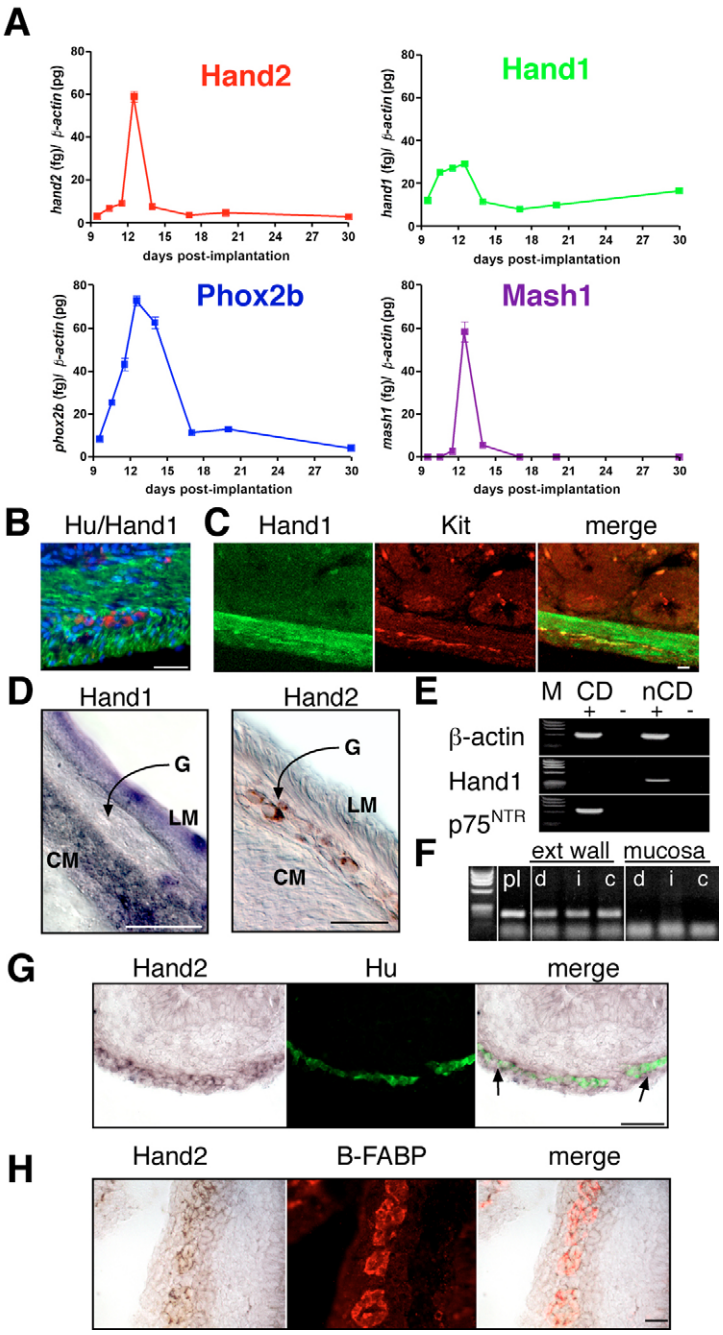

Fig. 1. Hand genes are expressed and developmentally regulated in the gut. (**A**) Transcripts encoding Hand2 (red), Hand1 (green), Phox2b (blue) and Mash1 (purple) quantified relative to β-actin and plotted as a function of age. (**B**,**C**) β-galactosidase expressed under the control of the *Hand1* promoter in transgenic mice to locate sites of *Hand1* expression. β-galactosidase immunoreactivity visualized by confocal microscopy (green). Hu (B; red) or Kit (C; red) immunoreactivities co-localized with that of β-galactosidase to identify, respectively, neurons and interstitial cells of Cajal. (**D**) In situ hybridization reveals *Hand1* transcripts in muscle layers, but not ganglia, of adult mouse intestine, whereas transcripts encoding Hand2 are located in neurons within ganglia, but not smooth muscle. (**E**) Transcripts encoding Hand1, but not those encoding $p75^{NTR}$, are detected, by reverse transcriptase (RT)-PCR, in a preparation from which crest-derived (CD) cells have been removed by immunoselection (nCD). Transcripts encoding Hand1 cannot be detected in a $p75^{NTR}$-rich immunoselected preparation of crest-derived cells. (**F**) Transcripts encoding Hand2 are detected by RT-PCR in mucosa-free preparations of bowel wall (ext wall) but not in isolated mucosa from adult mouse duodenum (d), ileum-jejunum (i) and colon (c). (**G**,**H**) Transcripts encoding Hand2 were found in Hu+ (G; green) and B-FABP+ (H; red) cells. Arrows in G show *Hand2* transcripts in cells that are not Hu^+. d, duodenum; c, colon; CD, crest-derived; CM, circular muscle; G, ganglion; i, ileum-jejunum; LM, longitudinal muscle; M, size markers; nCD, non-crest-derived; pl, plasmid carrying DNA encoding Hand2 (positive control). Scale bars: 50 μm.

Explants of $Hand2^{-/-}$ gut were transfected with a pcDNA3.1 construct containing the coding sequence of *Hand2* with an in frame histidine tag in a transfection mixture (50 μl; plasmid DNA 0.2 μg, 0.5 μl lipofectamine 2000) that was microinjected directly into the explants.

Silencing Hand2 expression with siRNA

The entire bowel was isolated from embryonic day (E)10 wild-type fetuses and cultured as a free-floating organ in opti-MEM (Invitrogen) supplemented with L-glutamine (2 mM) (Natarajan et al., 1999). The cultures were transfected twice with small interfering (si)RNA, once at 2 hours after explantation and the second time at 2 days after explantation. A commercial kit (Silencer siRNA construction; Ambion, Austin, TX), used according to the manufacturer's directions, was employed to prepare siRNA directed against *Hand2*. A control siRNA was directed against *Gapdh*. Three different Cy3-labeled siRNAs (Silencer siRNA labeling kit; Ambion) were made to target *Hand2* mRNA sequences (siRNA1: 5′-AAGATCAAGACACTGCGCCTG-3′; siRNA2: 5′-AAGGCGGAGATCAAGAAGACC-3′; and siRNA3: 5′-AAGAAGACCGACGTGAAAGAG-3′) and pooled. Transfection was carried out, using transfection reagent (Siport; Ambion). The transfection mixture (75 μl; siRNA mixture 60 pM, 1.5 μl transfection reagent) was added directly to the culture medium (300 μl total volume) in four well plates (NUNC). Lentivirus-based vectors (pFIV-H1/U6-copGFP; System Biosciences, Mountain View, CA) were engineered to express siRNA1, siRNA2 or siRNA3 (see above; $siRNA^{Hand2}$/GFP), or a control siRNA ($siRNA^{scrambled}$/GFP) with a scrambled target sequence (siScrambled: 5′-AAGTAAAGCCAATCGCCGCGT-3′), and were then transfected into cultured immunoselected or dissociated intestinal cells using lipofectamine 2000 (Invitrogen). The transfection mixture (50 μl; plasmid DNA 0.2 μg, 0.5 μl lipofectamine 2000) was added directly to the culture medium (200 μl total volume) in eight-well chamber slides (NUNC). The ability of $siRNA^{Hand2}$/GFP transfection to silence *Hand2* was evaluated in stably

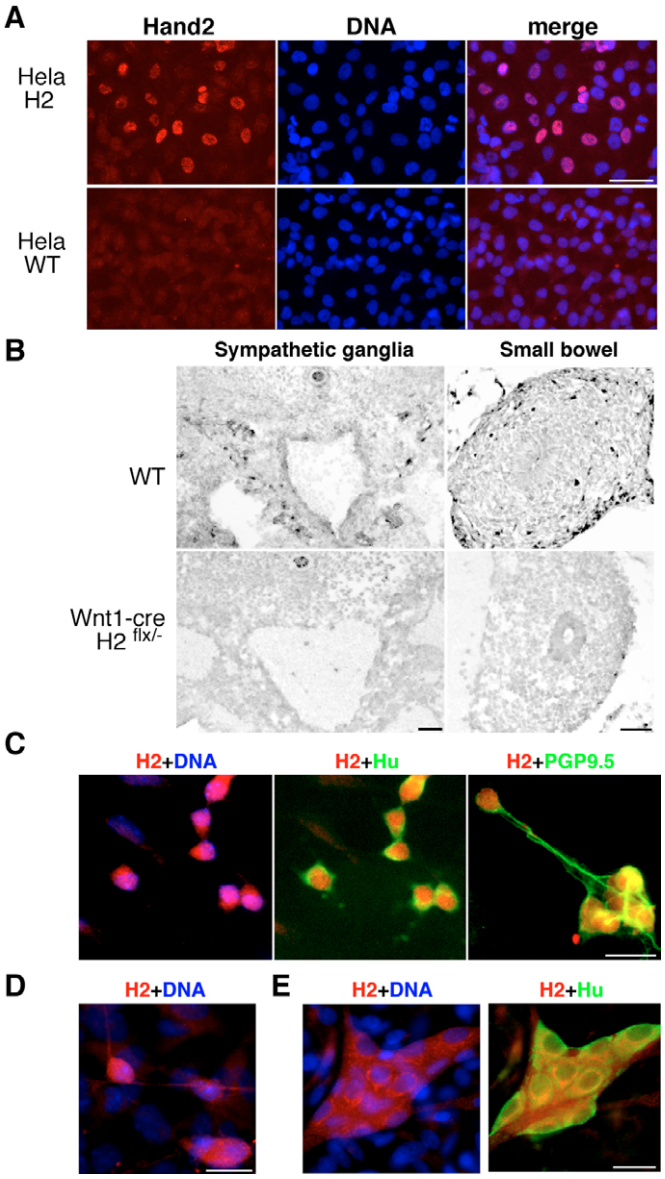

Fig. 2. Hand2 immunoreactivity is intranuclear in developing enteric neurons but cytoplasmic when they mature. (**A**) Polyclonal antibodies raised against a 15-amino acid N-terminal sequence of Hand2 react with transfected *Hand2*-expressing (Hela H2) but not non-transfected Hela [Hela wild type (WT)] cells. (**B**) At E12, Hand2 antibodies react with crest-derived cells in the gut and prevertebral sympathetic ganglia of wild-type (WT) but not Wnt1-Cre-H2$^{\Delta}$ fetuses. (**C**) Fetal gut dissociated at E11.5 and cultured overnight; the nuclei of Hu+ and PGP9.5+ neurons are Hand2+ (H2; red). (**D**) Fetal gut dissociated at E14 and cultured similarly to those in C; nuclei and cytoplasm are both Hand2+ (red). DNA is blue. (**E**) A dissected laminar preparation of adult bowel containing a submucosal ganglion viewed as a whole mount. Hand2 immunoreactivity (red) is restricted to the cytoplasm of Hu+ (green) neurons. DNA (blue) stained with bisbenzimide. Scale bars: 50 μm in A,B; and 25 μm in C-E.

transfected *Hand2*-expressing HeLa cells. The control level of *Hand2* expression was taken as that found in cells exposed to siRNA^{scrambled}/GFP. Each of the three different siRNA^{Hand2}/GFP constructs were found to silence *Hand2* expression; one of these constructs (siRNA3), which reduced *Hand2* expression by 77% (P<0.001), was selected for subsequent experiments. Information on methods used to select the target sequences can be found at http://www.rockefeller.edu/labheads/tuschl/sirna.html (revised May 2006).

Reverse-transcriptase PCR and real-time PCR

Extraction of RNA and preparation of cDNA have previously been described (Chalazonitis et al., 2004). All PCR products were sequenced and found to match the appropriate sequences in the GenBank. Transcripts encoding *Hand2* (sense: 5′-TACCAGCTACATCGCCTACCT-3′; antisense: 5′-TCACTGCTTGAGCTCCAGGG-3′), *Hand1* (sense: 5′-TTGAAGGC-TCGACTCAAGGT-3′; antisense: 5′-AGCGACAAGAAGGAAAACCA-3′), *Phox2b* (sense: 5′-CCTCAATTCCTCTGCCTACG-3′; antisense: 5′-AGTTTGTATGGAACTGCGGC-3′), *Mash1* (*Ascl1*; sense: 5′-GACT-TGAACTCTATGGCGG-3′; antisense: 5′-AGATGCAGGATCTGCT-3′), and *β-actin* (sense: 5′-TGTTTGAGACCTTCAACAC-3′; antisense: 5′-CAGTAATCTCCTTCTGCATCC-3′) were quantified in fetal and mature mouse gut by using real-time PCR (SYBR Green I; LightCycler, Roche Molecular Biochemicals, Indianapolis, IN). Three independent experiments were carried out and a standard curve was obtained for each gene product. Transcripts encoding rat *Hand1* (sense: 5′-CCAACATGAACCTCGT-3′; antisense: 5′-CCTGAACCTTTTCGCC-3′), rat *p75^{NTR}* (sense: 5′-GAGGGCACATACTCAGACGAAGCC-3′; antisense: 5′-GTCTATATGTTCAGGCTGGTAA-3′) and rat *β-actin* (see above) were amplified by using reverse transcriptase (RT)-PCR.

In situ hybridization and immunocytochemistry

Details of the procedures used have been presented previously (Li et al., 2006). Briefly, tissues were fixed with 4% formaldehyde (from paraformaldehyde) in 0.2 M phosphate buffer at pH 7.4. Fetuses or dissected bowel were fixed overnight at 4°C, whereas cultures were fixed for 30 minutes at ambient temperature. Fixed preparations were dehydrated, cleared and paraffin sectioned or cryoprotected (30% sucrose; 4°C), embedded in Neg50 (Richard Allan Scientist, Kalamazoo, MI), frozen (liquid N_2), and cryosectioned. Sections were collected on Superfrost slides (Fisher Scientific, UK). Digoxygenin (Dig)-labeled full-length cRNA probes encoding mouse Hand2 (1.2 kb) and Hand1 (1.7 kb) were synthesized and used for in situ hybridization. Pre-hybridization and hybridization were carried out for 2 hours at 70°C and for 18-22 hours at 70°C, respectively.

For immunocytochemical detection of markers, sections were washed with phosphate-buffered saline with Tween (PBST) and, if horseradish peroxidase (HRP) was to be used to visualize antigens, they were exposed for 20 minutes to 0.3% H_2O_2 to quench endogenous peroxidase activity. Primary and secondary antibodies (Table 1) were applied as described previously (Li et al., 2006). Sections were blocked overnight with monovalent goat Fab fragments against mouse IgG (Jackson Laboratories, West Grove, PA) before applying primary antibodies of mouse origin. DNA in sections was stained with bisbenzimide to enable cell density to be determined. Coverslips were mounted in 50% glycerol in 0.5 M bicarbonate buffer (pH 8.6).

Cell-death assay

Activated caspase-3 (caspase 3) was detected immunocytochemically (Cell Signaling, Danvers, MA). The TUNEL method was employed (‘In situ cell death detection kit’, Roche) according to the manufacturer’s directions.

Quantitative imaging

Immunocytochemically labeled cells or those expressing GFP were counted when cells could individually be discerned. Counts were normalized to the total number of cells, which was determined by counting bisbenzamide-stained nuclei. Alternatively, the density of labeled cells was ascertained by computer-assisted morphometry (Openlab software; Improvision, Lexington, MA). Images were acquired using a cooled CCD camera (Retiga; Q Imaging) installed on a Leica DMRXA2 microscope. Where indicated in the text, confocal images were obtained with a Zeiss LSM 510 NLO Multiphoton Confocal Microscope.

Statistical analyses

Student’s t-test was used to compare sample means. Equality of variances was analyzed with an F test and Welch’s correction was employed when variances of populations was significantly different.

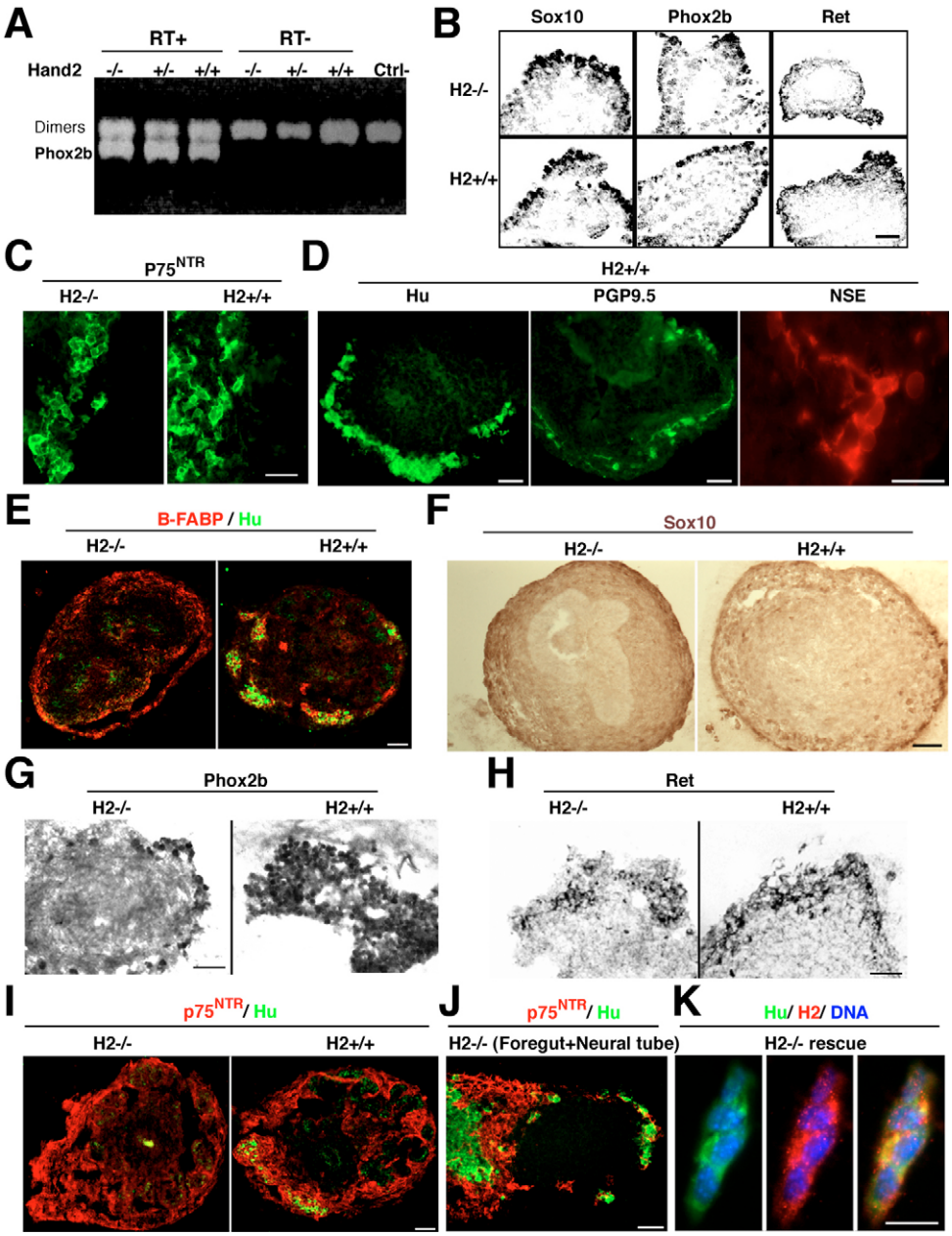

Fig. 3. Development of neurons, but not glia, in explants of fetal gut is Hand2-dependent. (**A**) Transcripts encoding Phox2b detected by reverse transcriptase (RT)-PCR in fetal mouse gut at E9.5 in wild-type (+/+), *Hand2*$^{+/-}$ and *Hand2*$^{-/-}$ mice. (**B**) Sox10+, Phox2b+ and Ret+ cells are detected at E9.5 in the outer mesenchyme of wild-type (H2+/+) and *Hand2*$^{-/-}$ (H2–/–) gut. (**C**) p75^{NTR}+ cells are found in longitudinal sections cut through the foregut of wild-type and *Hand2*$^{-/-}$ mice. (**D**) Hu+, PGP9.5+ and NSE+ neurons develop in cultures of wild-type gut explanted at E9.5. (**E**) B-FABP+ (red), as well as Hu+ (green), neurons develop in cultures of wild-type gut explanted at E9.5. B-FABP+ glia, but no Hu+ neurons, develop in cultures of *Hand2*$^{-/-}$ gut explanted at E9.5. (**F-H**) Sox10 (F), Phox2b (G) and Ret (H) immunoreactivities are found in cultured explants of E9.5 gut from *Hand2*$^{-/-}$ and wild-type mice. (**I**) p75^{NTR}+ precursors (red) are present in explants of E9.5 gut from wild-type and *Hand2*$^{-/-}$ mice; however, Hu+ neurons (green) develop in vitro only in the explants of wild-type, and not *Hand2*$^{-/-}$, gut. (**J**) Co-culture of E9.5 *Hand2*$^{-/-}$ gut with somites containing primordial dorsal root ganglia. Hu+ neurons (green) develop. (**K**) Differentiation of neurons occurs when Hand2$^{-/-}$ explants are rescued by transfection with *Hand2*; neurons co-express Hu (green) and Hand2 (red) immunoreactivity. Ctrl, control. Scale bars: 50 μm in B-J; 10 μm in K.

RESULTS

Developmental regulation of Hand gene expression

The abundance of transcripts encoding Hand1 and Hand2 was quantified by real-time reverse-transcriptase (RT)-PCR in fetal mouse gut from the age of embryonic day (E)9.5 to adult. Expression of *Hand1* and *Hand2* was compared to that of *Phox2b*, which is required early in ontogeny by all enteric crest-derived cells (Pattyn et al., 1999), and to that of *Mash1* (also known as Ascl1 – Mouse Genome Informatics), which is required later by a subset of neural precursors (Blaugrund et al., 1996). *Hand2* expression was developmentally regulated in the mouse gut (Fig. 1A, red curve). Transcripts encoding *Hand2* were detected at E9.5, when the first émigrés from the crest begin to colonize the bowel (Rothman et al., 1984; Young et al., 1999). *Hand2* expression was later upregulated and peaked at E12.5. By E14, *Hand2* expression declined to a lower level, which was maintained throughout adult life. *Hand1* expression was detected by E9.5 but, unlike that of *Hand2*, was bimodal (Fig. 1A, green curve), peaking at E12.5, declining by E14, and being upregulated again during adult life. *Phox2b* expression was upregulated before that of *Hand2* (Fig. 1A, blue curve). Although transcripts encoding *Hand2*, *Hand1* and *Phox2b* were detectable at all ages tested, transcripts encoding *Mash1* (Fig. 1A, purple curve) were not detected prior to E11 or after E17.

Hand1 and *Hand2* are expressed by enteric cells of different lineages

The cells that express *Hand1* and *Hand2* in the developing mouse gut have not previously been identified. Knock-in mice (P14) expressing β-galactosidase under the control of the *Hand1* promoter (Morikawa and Cserjesi, 2004) were analyzed to identify *Hand1*-expressing cells. β-galactosidase was detected in

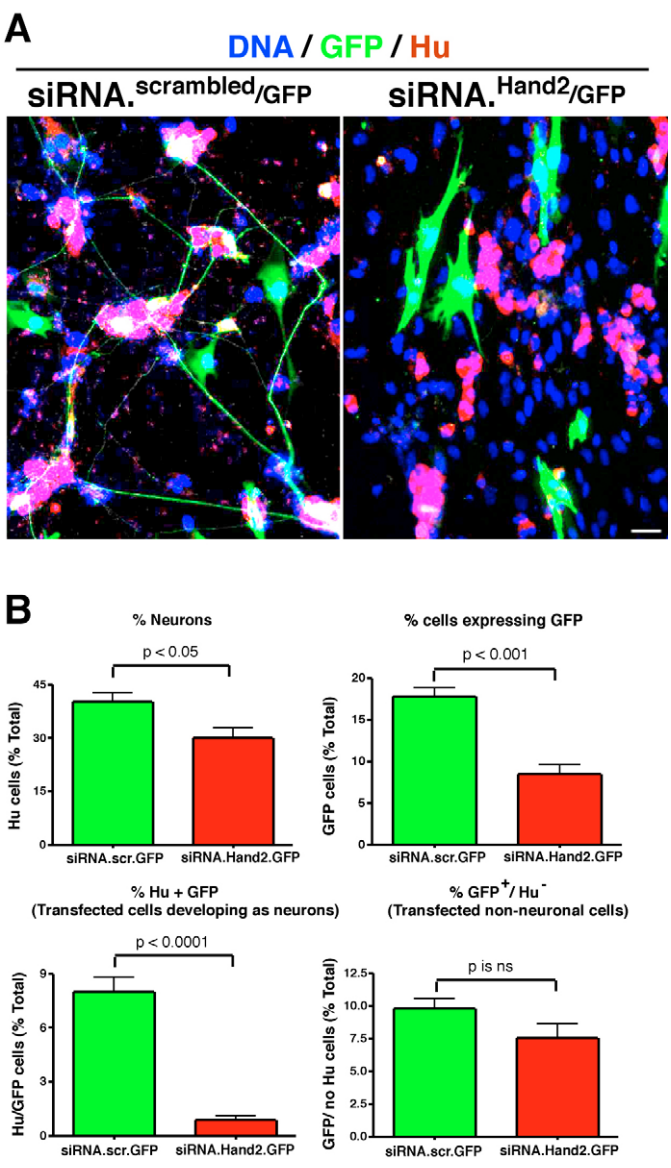

Fig. 4. Silencing of *Hand2* prevents the in vitro development of enteric neurons. (**A**) Crest-derived cells immunoselected from E13 mouse gut, cultured and transfected with siRNA^{Hand2}/GFP or siRNA^{scrambled}/GFP. siRNA^{Hand2}/GFP interfered with the development of Hu+ neurons (pink). Notice the GFP-fluorescent axons (green) of the Hu+ neurons in cultures transfected with siRNA^{scrambled}/GFP (A; left), which are absent in those transfected with siRNA^{Hand2}/GFP (A; right). (**B**) Quantitative results. Overall density both of Hu+ neurons and transfected cells developing as neurons (doubly labeled with GFP and Hu) in cultures transfected with siRNA^{Hand2}/GFP are lower than those seen in cultures transfected with siRNA^{scrambled}/GFP. siRNA^{Hand2}/GFP does not differ from siRNA^{scrambled}/GFP in transfection efficiency measured on non-neuronal cells. Scale bar: 50 μm.

smooth muscle cells in the stomach, and in the small and large intestines, but not in ganglia (Fig. 1B). Interstitial cells of Cajal (ICCs), identified by coincident immunostaining with antibodies to Kit, also contained β-galactosidase (Fig. 1C). This localization was confirmed by the reciprocal in situ localization of transcripts encoding Hand1 in muscle layers, but not in neural crest derivatives, and encoding Hand2 in neural crest derivatives, but not in smooth muscle (Fig. 1D). Further confirmation was obtained by separating crest and non-crest-derived cells from dissociated preparations of rat fetal bowel by positive and negative immunoselection with antibodies to p75^{NTR}. Transcripts encoding Hand1 were not detected in preparations of immunoselected crest-derived cells, but were abundant in preparations of non-crest-derived cells (Fig. 1E).

In situ hybridization was used to locate *Hand2*-expressing cells in the developing bowel wall (E12-E14). These transcripts were detected only in cells located in the outer gut mesenchyme, in a distribution corresponding to that of crest-derived cells (data not shown). No mucosal expression of *Hand2* was apparent. Because *Hand2* expression has been reported in chick colonic mucosa (Wu and Howard, 2002), mucosal expression of *Hand2* was investigated further. RNA was extracted separately from the mucosa and from the ganglion-containing layer. Transcripts encoding Hand2 were detected, by RT-PCR, in the ganglion-containing bowel wall but not in the mucosa (Fig. 1F). Different lineages of cells in the developing gut thus express *Hand2* and *Hand1*.

Hand2 is expressed in the gut by cells in neuronal and glial lineages

Double labeling was employed with antibodies to the neuronal markers HuC and HuD (collectively referred to here as Hu), and a riboprobe to detect transcripts encoding Hand2 (Fig. 1G). At E14, all Hu-immunoreactive (Hu+) cells contained transcripts encoding Hand2. *Hand2* is thus expressed at E14 by all cells developing as neurons; however, the presence of *Hand2*-expressing cells that lack Hu (Fig. 1G, arrows) is consistent with the possibility that *Hand2* is also expressed by glia. The immunoreactivity of the early glial marker brain-specific fatty acid-binding protein (B-FABP, also known as Fabp7 – Mouse Genome Informatics) was thus demonstrated simultaneously with transcripts encoding Hand2 (Fig. 1H). A subset of *Hand2*-expressing cells was B-FABP+. Transcripts encoding *Hand2*, therefore, are found in cells developing as neurons and glia. The pattern and timing of *Hand2* expression are consistent with its involvement in the development of enteric neurons and/or glia.

The intracellular distribution of Hand2 changes during neuronal development

The persistence of *Hand2* expression in mature enteric neurons raises the possibility that *Hand2* acquires another function following the differentiation of neurons and/or glia. Such a change in function might be associated with a change in its intracellular compartmentation. Hand2 protein was thus located immunocytochemically by using a rabbit polyclonal antibody raised against a synthetic peptide corresponding to the sequence VKEEKRKKELNEILK, which is found in the amino terminal domain of Hand2 (Dai and Cserjesi, 2002). The antibodies showed Hand2 protein in the nuclei of transfected *Hand2*-expressing HELA cells, but it did not react with parental HELA cells (Fig. 2A). More importantly, the antibodies immunostained cells in the presumptive ENS and sympathetic ganglia of wild-type mice, but did not do so in those of Wnt1-Cre-H2$^{\Delta}$ animals (Fig. 2B).

Enteric neurons, differentiating in vitro from cultures of dissociated gut at E11.5 or E14, were Hand2+ within 24 hours of plating. Only nuclei were Hand2+ at 24 hours in cells dissociated at E11.5 (Fig. 2C); however, by 6 days after plating, both nuclei and cytoplasm were Hand2+. In contrast to neurons developing from E11.5 gut, the cytoplasm as well as the nuclei of neurons in cultures of bowel

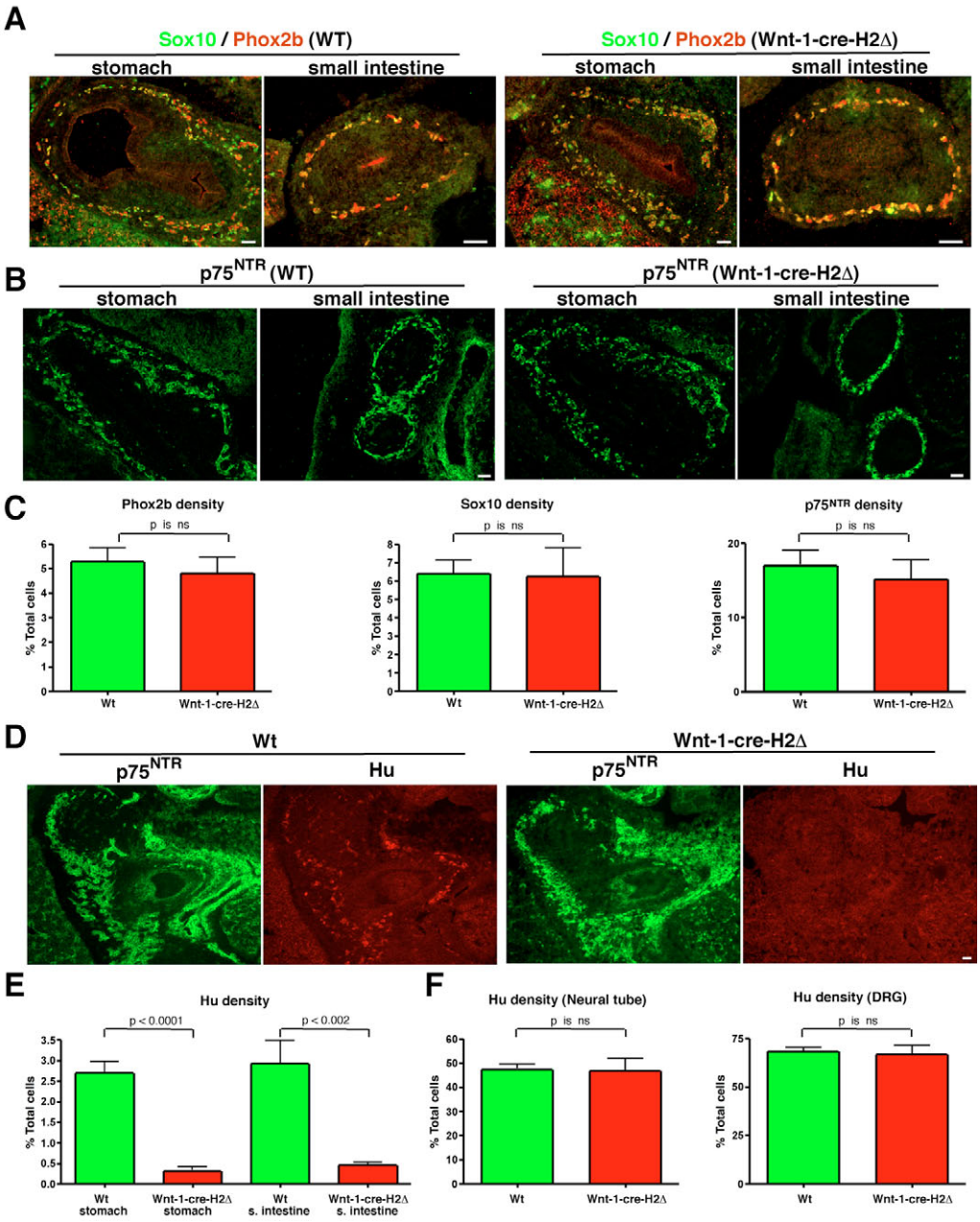

Fig. 5. *Hand2* deletion does not prevent the colonization of the bowel by enteric nervous system precursor cells in vivo but interferes with their development as neurons. (**A-C**) Sox10+ (green, A), Phox2b+ (red, A) and $p75^{NTR}$+ (green, B) cells are present and similarly distributed in the stomachs and small intestines of E12 wild-type (WT) and Wnt-1-CreH2$^{\Delta}$ mice; (C) quantified data. (**D**) $p75^{NTR}$+ (green) and Hu+ (red) cells in the stomachs of Wnt-1-CreH2$^{\Delta}$ mice (right) and wild-type littermates (left). $p75^{NTR}$ is present, but Hu is virtually absent, in Wnt-1-CreH2$^{\Delta}$ mice. (**E**) The densities of Hu+ cells at E12 in the stomachs and small intestines of Wnt1-CreH2$^{\Delta}$ are lower than those in the stomachs and small intestines of wild-type mice. (**F**) The densities of Hu+ cells in the neural tubes and dorsal root ganglion (DRG) of E12 Wnt-1-CreH2$^{\Delta}$ mice and wild-type mice are similar. ns, not significant. Scale bars: 50 μm.

dissociated at E14 was already Hand2+ by 24 hours post-dissociation (Fig. 2D). Hand2 immunoreactivity was restricted to the cytoplasm in essentially all enteric neurons of adult mice, in situ, in whole mounts of laminar preparations of the bowel wall, and in culture (Fig. 2E). These observations are consistent with the idea that Hand2 is intranuclear and thus able to influence transcription during differentiation; however, after differentiation is completed, cytoplasmic sequestration may inactivate Hand2 as a transcription factor.

Colonization of the gut by crest-derived cells is Hand2-independent

The effects of *Hand2* deletion were studied to test the hypothesis that Hand2 plays a role in ENS development. Unfortunately, *Hand2*$^{-/-}$ fetuses die at E10.5 from cardiac and vascular abnormalities (Firulli et al., 1998; Srivastava et al., 1995), and enteric neurons cannot be recognized prior to E10.5-E12 (Rothman and Gershon, 1982; Young et al., 2003). ENS precursors, however, enter the foregut at E9-E9.5, and give rise to neurons and glia in cultured explants (Rothman et al., 1984). Transcripts encoding the crest marker *Phox2B* are detectable by RT-PCR in bowel of *Hand2*$^{-/-}$ mice at E9.5 (Fig. 3A), suggesting that crest-derived cells do enter this gut. To confirm their presence, the immunoreactivities of four crest markers – Phox2B (Pattyn et al., 1999), $p75^{NTR}$ (Anderson et al., 2006; Baetge et al., 1990), Ret (Durbec et al., 1996; Pachnis et al., 1993; Schuchardt et al., 1994) and Sox10 (Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998) – were compared in sections of bowel cut from E9.5 *Hand2*$^{-/-}$ and wild-type littermates. Phox2B, Ret, Sox10 (Fig. 3B) and $p75^{NTR}$ (Fig. 3C) immunoreactivities were each found, distributed similarly in the outer gut mesenchyme, and comparable in abundance in *Hand2*$^{-/-}$ and wild-type animals. These observations suggest that the *Hand2*$^{-/-}$ gut is normally colonized by crest-derived precursors and that *Hand2* expression is not required for the initial expression of Phox2B, Ret, Sox10 or $p75^{NTR}$.

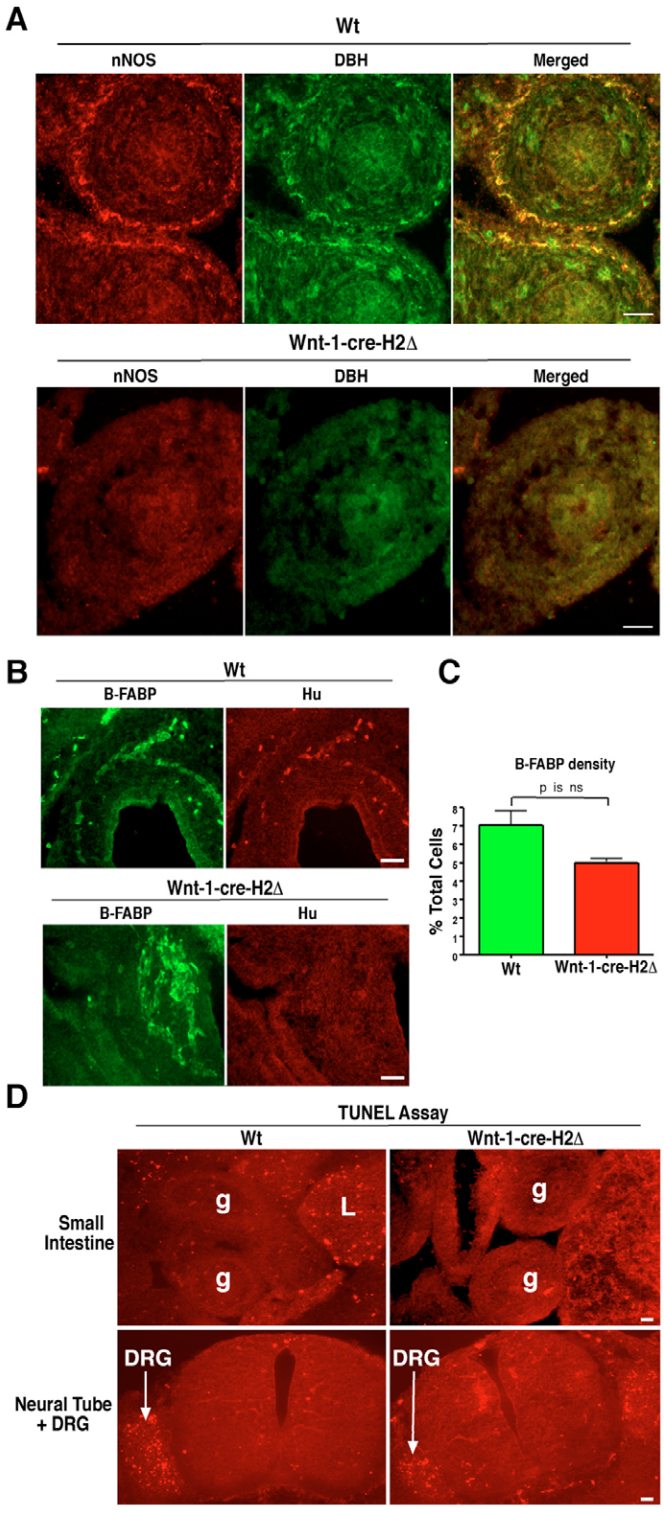

Fig. 6. Terminal differentiation of enteric neurons, but not glia, is abnormal in Wnt-1-CreH2$^{\Delta}$ mice. (**A**) nNOS+ (red) and Dbh+ (green) neurons are found in the presumptive enteric nervous system (ENS) of E12 wild-type (Wt) mice but not in Wnt-1-CreH2$^{\Delta}$ bowel. (**B**) Hu+ (red) neurons and B-FABP+ (green) glia are present in the stomachs of wild-type mice at E12. Wnt1-CreH2$^{\Delta}$ stomachs contain B-FABP+ glia but almost no Hu+ neurons. (**C**) The densities of B-FABP+ cells in Wnt1-CreH2$^{\Delta}$ and wild-type mice are similar. (**D**) The number of gut cells revealed by the TUNEL technique to be undergoing apoptosis is similar in Wnt1-CreH2$^{\Delta}$ and wild-type mice. The dorsal root ganglion (DRG) and neural tube were studied as positive controls. DRG, dorsal root ganglion; g, gut; L, liver; ns, not significant. Scale bars: 50 μm.

Neurogenesis, but not gliogenesis, fails in explants of *Hand2*$^{-/-}$ gut

Because crest-derived precursors are present in the foregut of *Hand2*$^{-/-}$ fetuses, it was possible to explant the bowel before the death of the animals, culture the explants, and evaluate the development of neurons and glia in vitro. Foregut was explanted from wild-type and *Hand2*$^{-/-}$ mice at E9.5 and cultured for 6-10 days in three-dimensional collagen gels (Natarajan et al., 1999). Markers used to demonstrate the development, if any, of neurons were Hu (Fairman et al., 1995; Phillips et al., 2004), PGP9.5 (also known as Uchl1 – Mouse Genome Informatics) (Sidebotham et al., 2001; Wilkinson et al., 1989) and neuron-specific enolase (NSE; also known as Eno2 – Mouse Genome Informatics) (Bishop et al., 1985; Hearn et al., 1999). Antibodies to B-FABP were employed to demonstrate glia (Simon et al., 1993; Young et al., 2003). Hu+, PGP9.5+ and NSE+ neurons developed reproducibly in cultures from wild-type mice (n=20) (Fig. 3D). By contrast, cultures obtained from *Hand2*$^{-/-}$ mice (n=9) never contained Hu+ cells (Fig. 3E,I), although they did contain B-FABP+ cells, which were comparable in form and abundance to B-FABP+ cells in cultures from wild-type mice (Fig. 3E). Despite the absence of Hu+ neurons in cultures of *Hand2*$^{-/-}$ bowel, these cultures contained many Sox10+ (Fig. 3F), Phox2b+ (Fig. 3G), Ret+ (Fig. 3H) and p75^{NTR}+ (Fig. 3I) cells; therefore, the knockout of *Hand2* evidently prevented neither the colonization of the bowel by émigrés from the neural crest, nor their survival in vitro.

Because no Hu+ neurons were found, despite the presence of cells expressing *Sox10*, *Phox2b*, *Ret* and *p75^{NTR}*, in cultures of *Hand2*$^{-/-}$ gut, control experiments were carried out to determine whether the microenvironment of the *Hand2*$^{-/-}$ bowel supports in vitro neurogenesis. Explants of E9.5 *Hand2*$^{-/-}$ gut were either co-cultured with somites 1-20 from the same *Hand2*$^{-/-}$ fetuses (Fig. 3J) or transfected with a construct encoding Hand2 (Fig. 3K). Somites contain precursors of dorsal root ganglion (DRG) neurons, which do not express *Hand2*. Hu+, as well as p75^{NTR}+, cells were present in the DRG-gut co-cultures (Fig. 3J), and co-expression of Hu and Hand2 immunoreactivities was found in the gut following rescue by transfection with the construct encoding Hand2 (Fig. 3K). Following transfection, all Hu+ cells were also p75^{NTR}+, showing that they were, as expected, crest-derived. Thus, crest-derived cells are able to give rise to neurons within the microenvironment of a *Hand2*$^{-/-}$ gut if they are Hand2-independent (DRG) or induced to express *Hand2* (rescue).

Silencing of Hand2 expression prevents neuronal expression in cultures of enteric crest-derived cells

To verify independently that *Hand2* expression is necessary for the development of neurons, *Hand2* expression was silenced with small interfering RNA (siRNA). Experiments were carried out with crest-derived cells, immunoselected with antibodies to p75^{NTR}, from E13 bowel (Fig. 4A,B). The percentage of Hu+ neurons developing in cultures transfected with siRNA^{Hand2}/GFP was significantly less

than that developing in control cultures transfected with siRNA^{scrambled}/GFP (Fig. 4B, top left). More strikingly, the percentage of GFP+ cells (Fig. 4B, top right) and, even more, the percentage of GFP+/Hu+ doubly labeled cells were selectively reduced by transfection with siRNA^{Hand2}/GFP (Fig. 4B, bottom left). By contrast, the numbers of GFP+ cells that did not co-express Hu were similar in cultures transfected with siRNA^{Hand2}/GFP and siRNA^{scrambled}/GFP (Fig. 4B, bottom right), suggesting that the transfection efficiency of siRNA^{Hand2}/GFP and siRNA^{scrambled}/GFP is comparable. Silencing of *Hand2* expression, therefore, appears to interfere with the development and/or survival of neurons. Comparable results were obtained when siRNA^{Hand2} was used to transfect organotypic cultures of gut explanted at E10 or cultures of cells dissociated from fetal bowel at E14 (data not shown). Again, siRNA^{Hand2}-transfected crest-derived cells failed to give rise to neurons, but neurons did arise from crest-derived cells transfected with control siRNA. Together, these data suggest that interference with *Hand2* expression inhibits enteric neurogenesis and does so no matter whether *Hand2* is silenced prior to the in situ appearance of neurons (E10) or after neuronal differentiation has already begun (E13-E14).

Conditional knockout of *Hand2* in the neural crest selectively blocks the terminal differentiation of enteric neurons

The in vitro data described above are consistent with the hypothesis that *Hand2* expression is required for the development of enteric neurons but not glia. To test this hypothesis in vivo, experiments were carried out with Wnt1-Cre-H2$^{\Delta}$ mice, in which the knockout of *Hand2* is restricted to crest-derived cells. This restriction would not be expected to prevent the development of cardiac abnormalities because *Hand2* is expressed in the cardiac crest; nevertheless, the restriction of the knockout of *Hand2* to crest-derived cells would be anticipated to mitigate the resulting defect because the restricted knockout would not interfere with *Hand2* expression by cardiomyocytes. Because the Wnt1-promoted expression of Cre occurs in premigratory crest cells, the postmigratory crest-derived cells in the sympathetic ganglia and bowel of Wnt1-Cre-H2$^{\Delta}$ mice do not express *Hand2* and thus lack Hand2 immunoreactivity (see above; Fig. 2B). Wnt1-Cre-H2$^{\Delta}$ mice hemorrhage in the cardiac outflow tract and die at E12.5; nevertheless, fetuses survive long enough to permit enteric neurogenesis to be analyzed. Wnt1-Cre-H2$^{\Delta}$ mice were compared with wild-type littermates at E12, when neurons are morphologically recognizable in the wild-type mouse gut (Rothman and Gershon, 1982; Young et al., 2003). Sox10+, Phox2b+ (Fig. 5A) and p75^{NTR}+ cells (Fig. 5B) were each present in the gut of Wnt1-Cre-H2$^{\Delta}$ mice and were distributed identically to that observed in their wild-type littermates; moreover, their densities in Wnt1-Cre-H2$^{\Delta}$ and wild-type bowel also did not differ significantly (Fig. 5C). These observations confirm that deletion of *Hand2* does not interfere with the colonization of the gut by crest-derived cells. By striking contrast, Hu+ cells were greatly diminished in the Wnt1-Cre-H2$^{\Delta}$ stomach and small intestine (Fig. 5D,E); moreover, cells expressing MAP2 (also known as Mtap2 – Mouse Genome Informatics; not illustrated) and type-specific neuronal markers, such as Dbh and nNOS (Nos1 – Mouse Genome Informatics), were virtually absent in the stomach and small intestine of Wnt1-Cre-H2$^{\Delta}$ mice (Fig. 6A). Despite this reduction in enteric Hu+ cells in the Wnt1-Cre-H2$^{\Delta}$ gut, there were no changes from wild type in the Hu+ cells of DRG or prevertebral sympathetic ganglia, or in the spinal cord of Wnt1-Cre-H2$^{\Delta}$ fetuses (Fig. 5F). Very few tyrosine hydroxylase (TH)+ or Dbh+ neurons, however, were observed in paravertebral and prevertebral sympathetic neurons of Wnt1-Cre-H2$^{\Delta}$ mice (not illustrated). These observations confirm that the knockout of *Hand2* interferes specifically with the terminal differentiation of enteric neurons.

In contrast to Hu+, Dbh+ and nNOS+ neurons, B-FABP+ glia were present in Wnt1-Cre-H2$^{\Delta}$ bowel and were distributed in a manner that could not be distinguished from that in wild-type animals (Fig. 6B). The density of B-FABP+ glia, furthermore, did not differ significantly between Wnt1-Cre-H2$^{\Delta}$ and wild-type mice (Fig. 6C). It is noteworthy that the failure of neuronal differentiation in the gut in the absence of *Hand2* expression did not lead to a shift of crest-derived precursors towards the glial lineage. The TUNEL procedure and activated caspase-3 (caspase 3) immunostaining were thus employed to test the hypothesis that the knockout of *Hand2* causes the death of crest-derived precursors that would otherwise have developed as neurons. This hypothesis was not confirmed because the abundance of cells in the gut demonstrated by TUNEL (Fig. 6D) or activated caspase-3 immunoreactivity (not illustrated), which were very low, did not detectably differ in wild-type and Wnt1-Cre-H2$^{\Delta}$ mice. In contrast to gut, where apoptosis is uncommon during development (Gianino et al., 2003), many TUNEL- and activated caspase-3-demonstrable cells were found in the E12 DRG of wild-type (Ernfors, 2001) and Wnt1-Cre-H2$^{\Delta}$ animals (Fig. 6D).

Because no evidence suggested that, in the absence of *Hand2* expression, crest-derived enteric precursors shift development towards the glial lineage or die, experiments were carried out to test the hypothesis that these cells begin to develop as neurons but fail to terminally differentiate. Neuronal markers, other than Hu, which are expressed by still-proliferating neuronal precursor cells were thus investigated, including β3-tubulin (Fig. 7A,B), GAP-43 (Gap43; Fig. 7C,D) and PGP9.5 (Fig. 7E,F). The densities of cells expressing each of these markers were decreased in the Wnt1-Cre-H2$^{\Delta}$ bowel, although not to the same extent as was Hu (Fig. 5E). In general, the decreases in cells expressing β3-tubulin (Fig. 7B), GAP-43 (Fig. 7D) and PGP9.5 (Fig. 7F) were more severe in the primordial stomach than in the small intestine. The marker least affected by the knockout of *Hand2* was PGP9.5; therefore, subsets of cells were found in Wnt1-Cre-H2$^{\Delta}$, but not wild-type, mice that were PGP9.5+ but not GAP-43+ (Fig. 7G). Another test of the hypothesis that cells begin to develop as neurons despite the knockout of *Hand2* was to examine the coincidence of Sox10 and Phox2b immunoreactivities. These transcription factors are co-expressed in uncommitted precursor cells, but enteric neurons maintain expression of Phox2b while downregulating Sox10; glia downregulate Phox2b while maintaining Sox10. Many strongly Phox2b+ cells, which co-expressed little or no Sox10, were observed in the gut of both wild-type and Wnt1-Cre-H2$^{\Delta}$ mice (Fig. 7H), suggesting that the inactivation of *Hand2* does not prevent precursors from entering the neuronal lineage.

DISCUSSION

The current experiments were carried out to test the hypotheses that enteric expression of *Hand2* is restricted to crest-derived cells and necessary for their terminal differentiation as neurons. Observations support these hypotheses. Expression of *Hand2*, in contrast to that of *Hand1*, is specific for crest-derived cells and, as these cells mature, becomes coincident with neural markers. *Hand2* expression is developmentally regulated and the peak of its expression coincides with peak enteric neurogenesis (Pham et al., 1991; Young et al., 2003). Hand2 immunoreactivity is intranuclear in early neuronal precursors; however, in mature

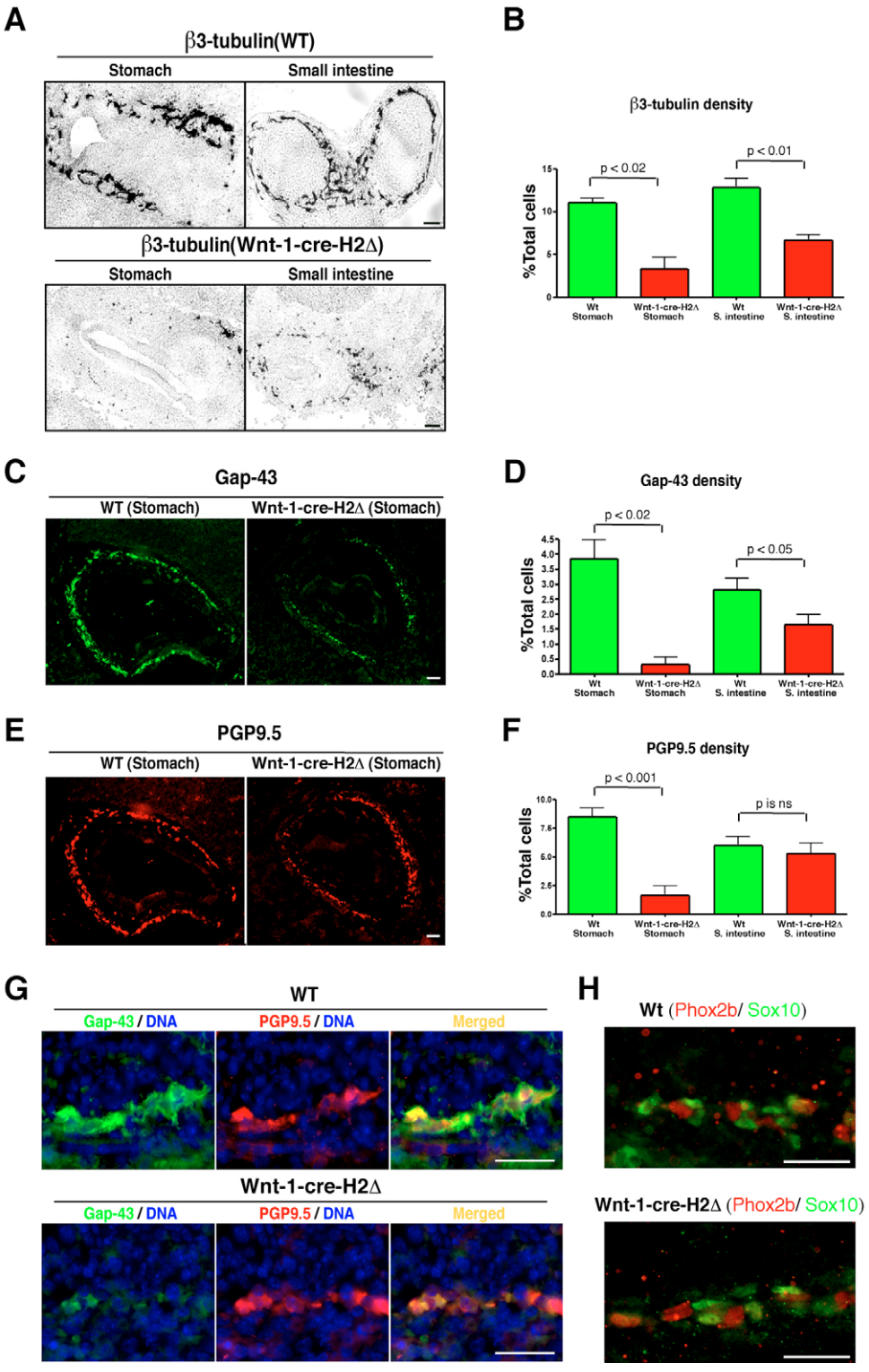

Fig. 7. Hand2 is not required for precursor cells to begin neuronal differentiation. (**A-F**) β3-tubulin+, GAP-43+ and PGP9.5+ neuronal precursors are present, but at a lower density, in the Wnt-1-CreH2$^{\Delta}$ gut than in wild type. (A) β3-tubulin immunoreactivity; (B) β3-tubulin cell density; (C) GAP-43 immunoreactivity; (D) GAP-43-cell density; (E) PGP9.5 immunoreactivity; (F) PGP9.5 cell density. (**G**) GAP-43 (green) and PGP9.5 (red) immunoreactivities are fully coincident in wild-type (WT) mice (top) but, in Wnt-1-CreH2$^{\Delta}$ animals, many PGP9.5+ cells lack GAP-43 (bottom). (**H**) Neural precursors lose Sox10 (green) immunoreactivity while retaining that of Phox2b (red) in both wild-type fetuses (top) and Wnt-1-CreH2$^{\Delta}$ mice (bottom). Scale bars: 50 μm.

neurons, Hand2 is predominantly cytoplasmic. This subcellular localization is compatible with the ideas that Hand2 affects the transcription of genes involved in neuronal differentiation but, when that process is completed, Hand2 is both downregulated and sequestered in the cytoplasm. Cytoplasmic sequestration might inactivate Hand2 but, if reversible, might permit Hand2 to regain transcriptional activity when plasticity is required. That remains to be demonstrated; however, the rapid downregulation (between E12-E14; see Fig. 1A) and cytoplasmic sequestration of Hand2 suggests that it might not play the role previously postulated for it as a determinant of neurotransmitter choice (Hendershot et al., 2007; Howard, 2005), which, for neurons using neurotransmitters, such as the neurotransmitters vasoactive intestinal polypeptide (Vip) and calcitonin gene-related peptide, does not occur until after E15-E16 (Blaugrund et al., 1996; Pham et al., 1991). The

universal expression of *Hand2* by all Hu+ neurons also suggests that *Hand2* expression is not sufficient for specifying any particular neurotransmitter.

Studies of the in vitro development of crest-derived cells from *Hand2*$^{-/-}$ mice provide evidence that *Hand2* expression is necessary for enteric neuronal differentiation. *Hand2*$^{-/-}$ animals die from the cardiovascular effects of the knockout at E10.5. At E9.5, *Hand2*$^{-/-}$ and wild-type gut were comparable in their complement of cells expressing the crest markers Phox2B, p75^{NTR}, Ret and Sox10 (Anderson et al., 2006; Young et al., 2003; Young et al., 1999). Because crest-derived cells are present, neurons and glia develop in cultures of wild-type gut explanted at E9.5; however, only glia arise in similar explants from *Hand2*$^{-/-}$ mice. The conclusion that enteric neurogenesis is Hand2-dependent was further supported by the demonstration that siRNA silencing of *Hand2* expression in crest-derived cells from wild-type gut also specifically blocked neuronal differentiation. In *Hand2*-expressing HeLa cells, siRNA reduced expression by approximately 77%. It is likely, therefore, that siRNA did not totally extinguish *Hand2* expression in enteric crest-derived cells. Because neurogenesis was nevertheless abolished in siRNA-transfected cells, there is probably a threshold of *Hand2* expression below which neuronal differentiation fails.

The conditional inactivation of *Hand2* in Wnt1-Cre-H2$^{\Delta}$ mice permitted the in vivo verification of its role in enteric neurogenesis. Virtually no neurons expressing Hu, nNOS, Dbh or MAP2 develop in the Wnt1-Cre-H2$^{\Delta}$ bowel. This failure of neurons to develop raises the question of what happens to the crest-derived cells that colonize the Wnt1-Cre-H2$^{\Delta}$ gut? In contrast to neurons, glia develop, but glial abundance does not increase. *Hand2* deletion thus does not appear to cause common neural-glial progenitors to generate glia instead of neurons. TUNEL- or activated caspase-detectable apoptosis also fails to account for the progenitors that do not develop as neurons. Development of neurons from migrating crest-derived precursors has been proposed to occur in stages (Sommer et al., 1995). Sympathetic neuronal precursors, for example, become noradrenergic while still proliferating (Rothman et al., 1978). Mash1-dependent enteric neuronal precursors also proliferate, but contain neurofilament proteins, GAP-43 and peripherin 1; these cells are catecholaminergic from E10-E13 but terminally differentiate as non-catecholaminergic neurons (Baetge and Gershon, 1989; Baetge et al., 1990; Blaugrund et al., 1996). We thus looked at early markers in the E12 Wnt1-Cre-H2$^{\Delta}$ gut to determine whether neuronal precursors might be present but unable to complete development. The Wnt1-Cre-H2$^{\Delta}$ bowel contained GAP-43+, β3-tubulin+ and PGP9.5+ cells, albeit at a lower density than that found in wild-type gut. These markers are expressed by still-proliferating neuronal precursor cells (Baetge et al., 1990; Sidebotham et al., 2001; Sommer et al., 1995; Young et al., 2003). *Hand2* expression is thus not essential for enteric crest-derived cells to enter the neuronal lineage; however, it is required to enable them to complete neuronal differentiation.

The effects on sympathetic and enteric neuronal development of the conditional knockout of *Hand2* appear to differ. Whereas Hu immunoreactivity was not expressed in the primordial ENS of Wnt1-Cre-H2$^{\Delta}$ mice, it was expressed in developing sympathetic neurons of the same animals; nevertheless, Th and Dbh in sympathetic ganglia were virtually absent. Similar observations have recently been made on the role of *Hand2* in developing sympathetic neurons of zebrafish (Lucas et al., 2006). In *hands off* (also known as *hand2* – Zebrafish Information Network) mutant embryos, which lack *hand2*, crest-derived cells migrate to presumptive ganglia and express the generic neuronal marker Elavl3 (HuC). They fail, however, to express Th and Dbh and later genes, such as *gata2* and *tfap2a*. A common sympathoadrenal-enteric progenitor has been proposed (Blaugrund et al., 1996; Carnahan et al., 1991); it is thus interesting that *Hand2* deletion interferes with terminal differentiation in both lineages. The *Hand2*-independent expression of Hu orthologs in mouse and zebrafish sympathetic neurons suggests that *Hand2* may function at different stages in enteric and sympathetic differentiation.

On the basis of a study of the effects of the Wnt1-Cre-mediated conditional knockout of *Hand2* in mice, Hendershot et al. have recently postulated that *Hand2* expression is sufficient and required specifically for the generation of Th+ and Vip+ neurons, the choice of these cell-type-specific markers, and the migration of precursors to pattern the ENS (Hendershot et al., 2007). By contrast, our data indicate that Hand2 is required for precursors that have entered the neuronal lineage to become neurons. Failure of development into neurons implies that events downstream of cells becoming neurons, including the acquisition of subtype-specific neurotransmitters or markers, will be affected and not limited to specific enteric neuronal subsets. We also found that Hand2 is not necessary for the migration of neuronal and glia precursors to and within the gut, although the pattern of ganglia in which neurons cannot terminally differentiate might appear abnormal. The animals studied by Hendershot et al. survived to birth, whereas all of the Wnt1-Cre-H2$^{\Delta}$ animals that we analyzed died by E12.5. We anticipated fetal lethality in Wnt1-Cre-H2$^{\Delta}$ mice because of the excision of *Hand2* in crest-derived cells of the cardiac outflow tract and sympathetic nervous system. Norepinephrine (NE) is essential for fetal survival (Kobayashi et al., 1995; Thomas et al., 1995; Zhou et al., 1995) and *Hand2* expression is necessary for the acquisition of the noradrenergic sympathetic phenotype (Howard et al., 1999; Howard, 2005; Lucas et al., 2006; Xu et al., 2003). Indeed, we found that Th and Dbh were almost absent in sympathetic ganglia of Wnt1-Cre-H2$^{\Delta}$ mice. By contrast, *Th* expression was observed in the conditional knockout animals studied by Hendershot et al., which might account for the ability of those mice to survive to gestation (Hendershot et al., 2007). At E14, the age at which Hendershot et al. observed enteric Th, the wild-type gut is known to contain no Th+ cells (Baetge and Gershon, 1989; Blaugrund et al., 1996; Teitelman et al., 1981). Such cells do not appear until dopaminergic neurons develop from non-catecholaminergic Mash1-independent progenitors at the end of gestation (Li et al., 2004). The gut, however, receives a Th+ sympathetic innervation, which is extensive in the stomach at E14, and is made up of axons with large varicosities that can be misidentified as nerve cell bodies. Differences between our observations and those of Hendershot et al. might be explained by differences in the design or configuration of loxP-*Hand2* alleles. Hendershot et al. studied *Hand2*$^{loxP/loxP;Wnt1\text{-}Cre}$ mice, whereas the Wnt1-Cre-H2$^{\Delta}$ mice that we investigated were *Hand2*$^{loxP/null;Wnt1\text{-}Cre}$. When two floxed alleles, instead of one, are used for Cre-mediated recombination, a mosaic often results (Kwan, 2002). The presence of two floxed alleles might also delay the deletion of *Hand2* beyond the time when Hand2 is required for early enteric and sympathetic neurogenesis.

The hypothesis that *Hand2* is required for terminal differentiation of enteric neurons, but not glia, is strongly supported by the concordance we report of in vitro and in vivo observations; nevertheless, because enteric neurons of different phenotypes are born at different times of development (E8.5 to P21) (Pham et al., 1991), it remains formally possible that only early-born subsets of

enteric neurons are Hand2-dependent. The observation that late-appearing neurons develop in explants of bowel from *Hand2*$^{+/+}$, but not *Hand2*$^{-/-}$, mice when the explants were maintained for up to 10 days is consistent with the idea that the terminal differentiation of all enteric neurons requires *Hand2*. Still, it will be necessary in the future to investigate the differentiation of enteric neurons in mice that lack *Hand2* expression in crest-derived cells but survive past the age when late-born neurons are generated.

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