A lack of PTEN in the enteric nervous system induces ganglioneuromatosis and mimics intestinal pseudo-obstruction

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Supplemental Materials

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METHODS

Mice

Tyr::Cre, ZEG and Rosa26R mice (1-3) were used to produce Tyr::Cre/°; ZEG/°, Tyr::Cre/°; Rosa26R /°, °/°; ZEG/° and °/°; Rosa26R/° embryos. ZEG mice were generously provided by C. Lobe (Toronto, Canada).

Cultures of dissociated intestine

Tyr::Cre/°; ZEG intestines were dissected from mouse embryos at E14.5 and incubated with dispase/collagenase (0.5 mg/ml in Phosphate Buffered Saline-1xPBS; Roche) for 6 minutes at room temperature. The tissue was washed three times in 1xPBS, dissociated into single cells by pipetting and plated onto freshly prepared fibronectin-coated (20 µg/ml) slide wells in optiMEM (Invitrogen) supplemented with L-glutamine (1 mM; Invitrogen) and an antibiotic mixture (100 U/mL penicillin and 100 µg/mL streptomycin; Invitrogen). Cultures were maintained for up to 24 hours in an atmosphere of 5% CO2 until fixation.

Immunostaining of cultures of dissociated intestine

Before immunostaining cultures of dissociated intestine, cells were fixed in 4% PFA for 10 minutes at room temperature. Cells were washed three times in PBTr (= PBS/0.1% Triton X-100) for 5 minutes at room temperature and incubated in blocking solution (PBTr containing 1% BSA, 0.15% glycine) for 2 to 3 hours at room temperature. Primary TUJ1 antibody (mouse, Eurogentec/Covance, 1 µg/mL) was diluted in blocking solution and incubated with the samples overnight at 4°C. Cells were washed three times in PBTr for 5 minutes at room temperature and incubated with the secondary antibody (anti-mouse Alexa555, Invitrogen) for one hour at RT. After several washes with PBTr, slides were mounted and observed under an epifluorescence microscope.

Western blot analysis

Tissue and muscle layers were dissected as described in the main article for PCR analysis. The remaining strips of tunica muscularis were incubated in lysis buffer (50mM Tris-HCl pH8, 10mM EDTA, 1% SDS, 1mM DTT, 0.5 mM PMSF, 5 μg/mL aprotinin, 2 μg/mL leupeptin and 0.4 mM sodium orthovanadate) at 95°C for 5 minutes and homogenized by passage through a 18-gauge needle. The samples were centrifuged at 14,000 rpm; the resulting cleared total cell extracts were resolved by 10% SDS-PAGE followed by western blot analysis using primary antibodies to TUJ1 (mouse, 1/1,000 dilution, Covance), GFAP (mouse, 1/1,000 dilution, Cell Signaling) and, as a loading control, β-actin (mouse, 1/2,000, Chemicon International). Bound primary antibody was detected with horseradish peroxidase-conjugated secondary antibodies (Jackson) and SuperSignal detection (Pierce).

Histology, immunofluorescence and immunohistochemistry

Intestines were collected from mouse embryos, rinsed in cold PBS and fixed in 4% PFA for 2h at 4°C. Samples were incubated for 16 hours in 30% (w/v) sucrose in PBS at 4°C, embedded in OCT compound (Sakura Finetechnical) and cut into 7 μm-thick sections. Sections were rinsed twice in PBT (PBS/0.1% Tween 20), boiled for 20 minutes in 10 mM sodium citrate and treated with blocking solution (2% skimmed milk in PBT) for 20 minutes at RT. Sections were then incubated in PBT overnight at 4°C with anti-TUJ1 (mouse, 1/1,000 dilution, Covance), anti-cleaved caspase-3 (rabbit, 1/200 dilution, Cell signaling), anti-GFAP (mouse, 1/500 dilution, Cell Signaling), anti-PTEN (rabbit, 1/100 dilution, Cell signaling), or anti-HuC/D (mouse, 1/50, Invitrogen) antibodies. The anti-Sox10 antibody was generously given from M. Wegner (University of Erlingen, Germany) (guinea pig, 1/500 dilution, (4)), and the anti-BFABP antibody was generously given by T. Müller (Max-Delbrück Center, Germany) (rabbit, 1/5,000 dilution, (5). For immunofluorescence, Alexa-488 or 555 secondary antibodies (Invitrogen) were used to detect primary antibodies.

For immunohistochemistry, samples were either dehydrated, embedded in paraffin wax or embedded in OCT and sectioned into 5 μm-thick transverse sections. Sections were deparaffinized, rinsed in Tris-buffered saline (TBS), boiled for 20 minutes in 10mM sodium citrate and treated with 3% fresh H_2O_2 for 30 minutes. Sections were then incubated overnight at 4°C in the presence of p27 (rabbit, undiluted, Abcam) or PTEN (rabbit, 1/100 dilution, Cell signaling) antibodies. DAB (Sigma) was used to reveal bound antibodies according to the manufacturer's instructions. All sections were counter-stained with hematoxylin. The X-gal staining protocol was described in (1).

Whole-mount immunostaining was performed on E12.5 and E14.5 intestines fixed in 4% PFA for 1 hour at 4°C. Samples were incubated overnight at 4°C in blocking solution (PBS 1X, 0.1% Triton X-100, 1% BSA and 0.15% glycine), and then in blocking solution with anti-TUJ1 (mouse, 1/1,000 dilution, Covance) overnight at 4°C. Alexa-555 secondary antibody (Invitrogen) was used to detect primary antibody.

FIGURE LEGENDS

Supplemental Figure 1

Enteric neuronal cells are defloxed in Tyr::Cre mice (A) °/°; ZEG/° and Tyr::Cre/°; ZEG/° intestines were isolated from E16.5 embryos and the stomach, small intestine, cecum and colon were examined. Whole-mount intestines were fixed in PFA, mounted and observed under the epifluorescence microscope. Fluorescent cells are defloxed cells. Scale bar represents 5 _m. (B) Cultures were derived from Tyr::Cre/°; ZEG/° intestine cells, isolated from E14.5 embryos cells were fixed and immunostained for TUJ1 (red). Defloxed (=EGFP-positive) cells; (green) were TUJ1-positive. These results confirm previous findings (Puig *et al.*, 2009). Scale bar represents 25 _m.

Supplemental Figure 2

Tyr::Cre/°; PTEN F/F mice present hyperpigmentation. Macroscopic and microscopic views of WT (A, C, E, E', G, G', I) and HM (B, D, F, F', H, H', J) mice. HM tails (B), pinnae (D), paws (F, F') and olfactory bulbs (J) in p15 HM mice were more pigmented than in p15 WT

mice. Transverse sections of WT (G, G') and HM (H, H') skin show that HM hair follicles were more pigmented than WT. Scale bar (E-F' and I,J): 1 mm and scale bar (G-H'): 50 _m. °/°; PTEN F/F or °/°; PTEN F/+ are considered as WT. Tyr::Cre/°; PTEN F/F mice are HM.

Supplemental Figure 3

HM hearts and brains did not display any abnormalities. Macroscopic views of HM heart at p15 and brain at p12. Scale bar for the heart: 1 mm; scale bar for the brain: 5 mm.

Supplemental Figure 4

HM mice displayed hyperplasia of the submucosa plexus of the ENS. Transverse sections of p15 WT and HM colons were immunostained with an antibody directed against TUJ1. Note that hyperplasia was observed in the HM submucosal and mucosal ENS plexus, but not in WT (black arrowhead). Red squares correspond to the high magnification pictures on the right of the figure. Note the invasion of the nerves in the submucosal part of the intestine. Scale bar, left: 50 _m; scale bar, right: 20 _m.

Supplemental Figure 5

TUJ1 is more abundant in the HM colon than in the WT colon. p15 pups were generated from crosses of Tyr::Cre/°; PTENF/+ and °/°; PTENF/F mice. Results shown were obtained from two WT, one HT and two HM pups. Protein extracts from the tunica muscularis were analyzed by western blot. TUJ1 was detected as a single band at 50 kDa and actin as a single band at 42 kDa.

Supplemental Figure 6

Tyr::Cre/°; PTEN F/F mice present hyperplasia of the enteric glial cells. (A) Transverse sections of WT and HM p15 colons were immunostained for GFAP, and double-immunostained for GFAP and PTEN. Merged immunofluorescence is shown for WT and HM. Note that GFAP-positive cells produce PTEN in WT colon, but do not contain any PTEN protein in the HM colon. Scale bar for immunohistochemistry: 50 _m; scale bar for immunofluorescence: 25 _m. (B) Protein lysates from tunica muscularis isolated from the colon of two WT, one HT and two HM were analyzed by western blot with antibodies directed against GFAP and actin. Note that GFAP protein levels are increased in HM colons.

Supplemental Figure 7

p27 expression is downregulated in HM enteric neuronal cells. Transverse sections of WT and HM colons at p15 were immunostained with antibody directed against p27. The dotted lines enclose the ENS. Scale bar: 50 _m.

Supplemental Figure 8

Tyr::Cre/°; PTEN F/F enteric neuronal cells do not produce PTEN during development. Transverse sections of HM colons at E14.5, E15.5 and E17.5 were double immunostained with antibodies directed against TUJ1 (red) and PTEN (green). Merged immunofluorescence is shown. Scale bar: 20 μm.

Supplemental Figure 9

Hypertrophy occurs after hyperproliferation in HM colon. The surfaces of enteric neuronal cells from WT (black) and HM (white) colons at E18.5 were estimated using ImageJ software from transverse sections immunostained with TUJ1 antibody and counterstained with DAPI. More than one hundred cells were counted from three different embryos of each genotype derived from independent litters. Scale bar: 5 _m

Supplemental Figure 10

WT and HM enteric neuronal cells do not die by apoptosis during development. Transverse sections of WT and HM colons at E15.5 and E17.5 were double immunostained with antibodies directed against TUJ1 (red) and caspase-3 (green). Merged immunofluorescence is shown. Scale bar: 20 _m.

Supplemental Figure 11

Enteric neuronal crest cell migration during development is normal in the HM colon. Intestines were dissected from E12.5 and E14.5 WT and HM embryos and whole-mount immunostained with an antibody directed directed against TUJ1. Scale bar: 250 _m.

Supplemental Figure 12

Hyperplasia of enteric neuronal crest cells and enteric glial cells in HM colon. Transverse sections of WT and HM colons at E18.5 were double-immunostained with antibodies directed against Sox10 (green) and BFABP (red). Merged immunofluorescence is shown. Scale bar: 25 _m.

Supplemental Figure 13

PTEN is produced in floxed and defloxed cells in Tyr::Cre/°; Rosa26R/+ embryos at E10.25. Transverse sections obtained after Xgal staining of Tyr::Cre/°; Rosa26R/+ E10.25 embryos were immunostained with an antibody directed against PTEN. PTEN was detected in floxed (blue and red) and defloxed cells (red). Immunostainings carried out with secondary antibody, but not PTEN primary antibody, served as a negative control. Note that floxed cells

in PTEN immunostained sections show a more intense blue staining than the negative control. ab = antibody. Scale bar, left: 200 _m; Scale bar, right: 25 _m.

Supplemental Figure 14

The proportion of HuC/D-positive and Ki-67-positive cells is greater in HM colon than in WT colon. Transverse sections of WT and HM colons at E16.5 are double-immunostained with antibodies directed against Ki-67 (green) and HuC/D (red). Note that WT enteric neuronal cells may produce Ki-67 in HuC/D-positive cells and note that HM enteric neuronal cells producing HuC/D may not produce Ki-67 at this stage of development. Merged immunofluorescence is shown. Scale bar: 5 _m.

Supplemental Figure 15

Transversal section of the patient (CIPO#3) presenting ganglioneuromatosis with no signs of MEN2B or NF-1, which do not produce PTEN. c m = cirucular muscle, l m = longitudinal muscle, myen p = myenteric plexus, s = serosa, subm = submucosa.

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Analysis of offspring genotypes obtained from mating Tyr::Cre/°; $PTEN^{F/+}$ with °/°; $PTEN^{F/F}$. °/°; $PTEN^{F/F}$ and °/°; $PTEN^{F/+}$ mice are considered as wild type (WT), Tyr::Cre/°; $PTEN^{F/+}$ as heterozygous (HT) and Tyr::Cre/°; $PTEN^{F/F}$ as homozygous (HM). Classical Mendelian segregation was observed. Theo: Theoretical, Nb Obs: number observed.

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Main characteristics of the nine human subjects studied; two were normal and seven had CIPO. F = female, M = male, CIPO = chronic intestinal pseudo obstruction, GNM = ganglioneuromatosis.

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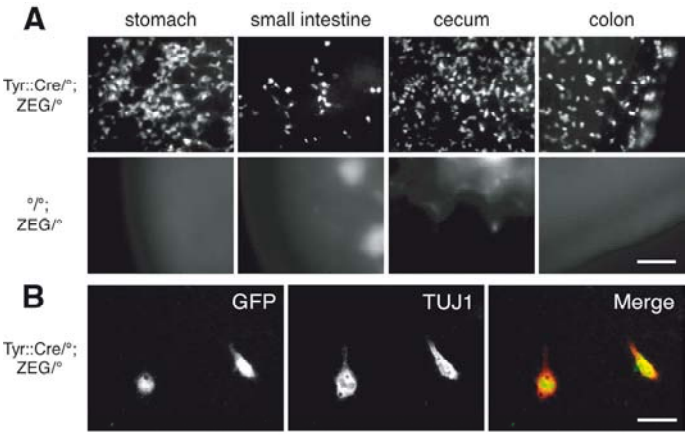

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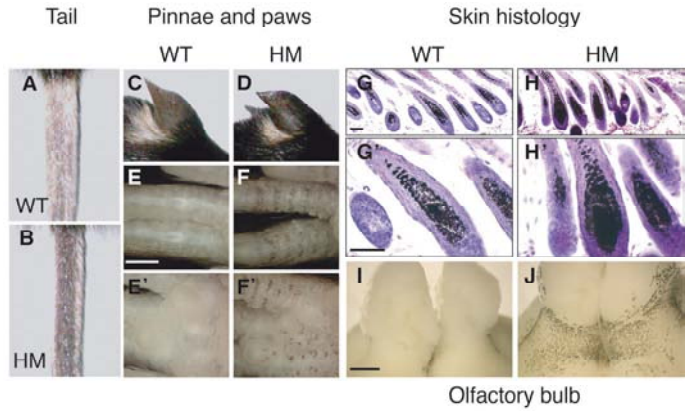

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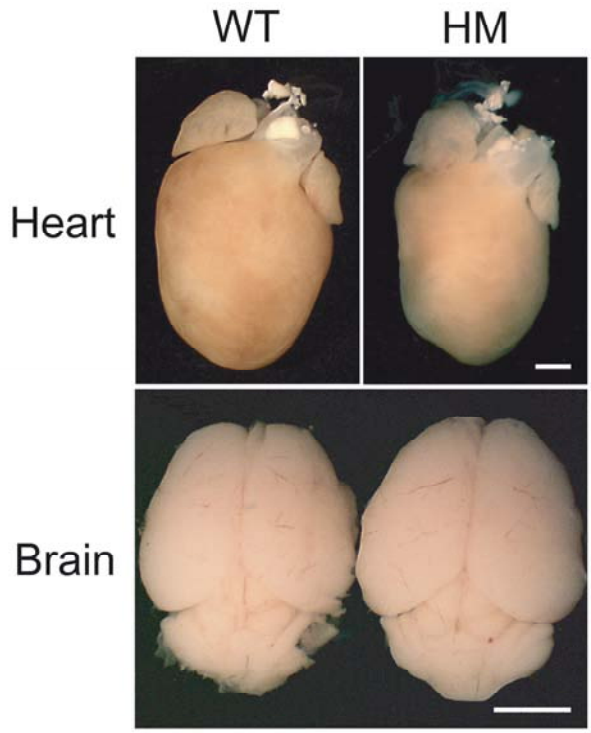

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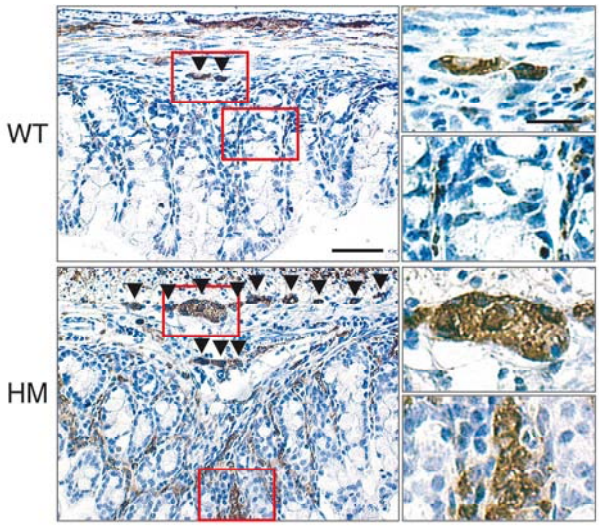

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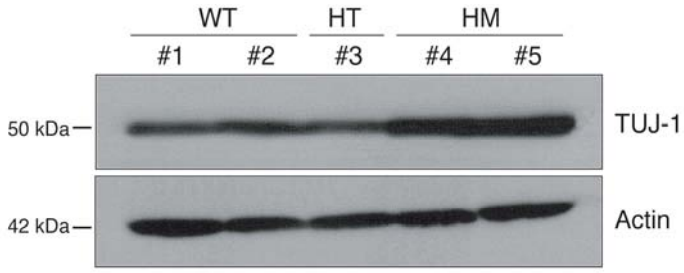

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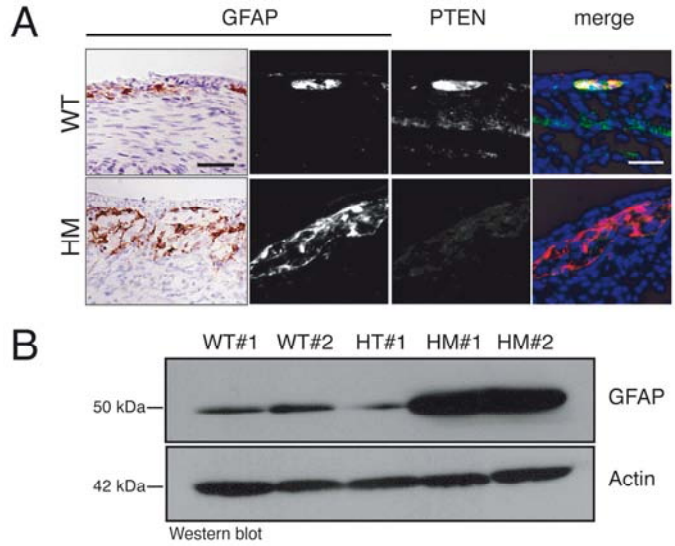

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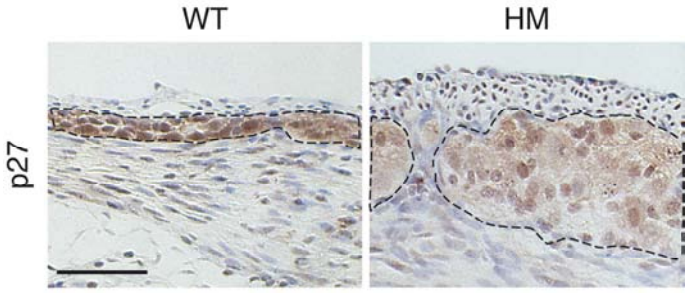

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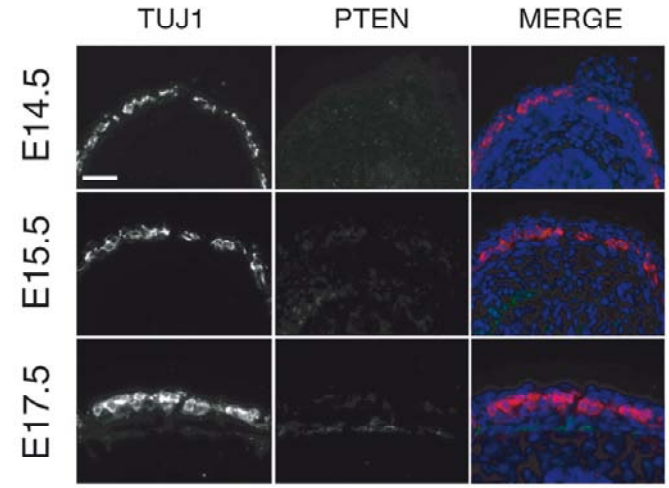

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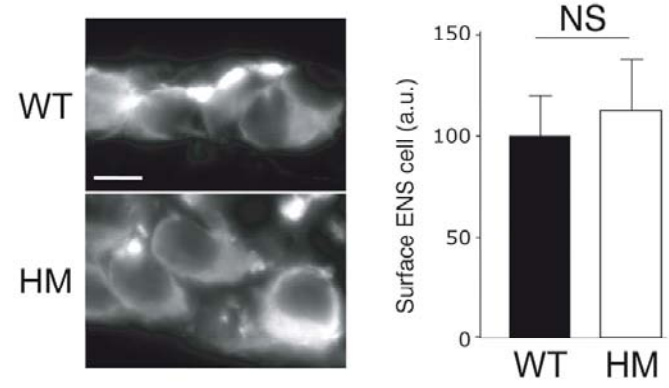

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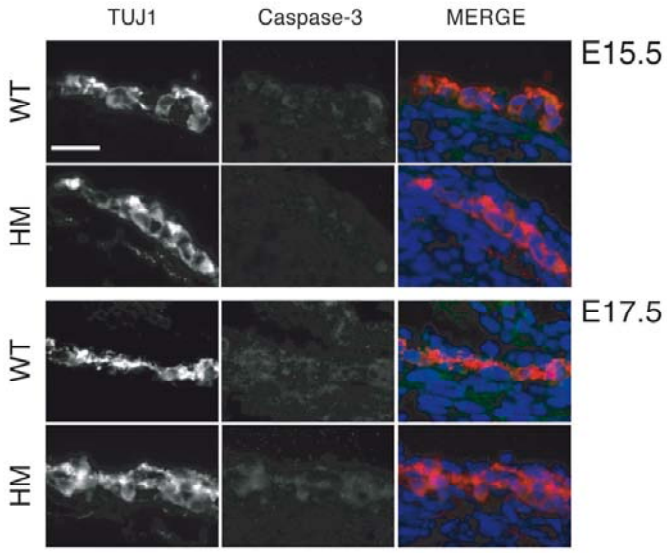

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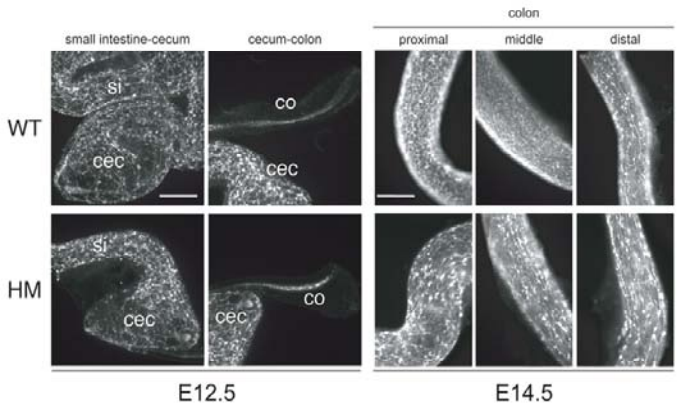

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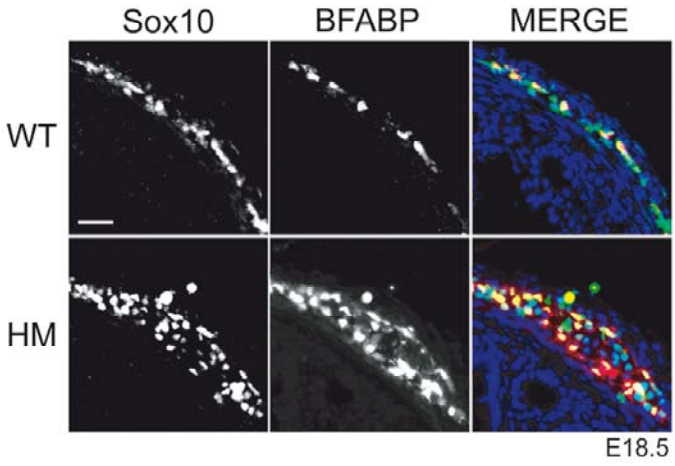

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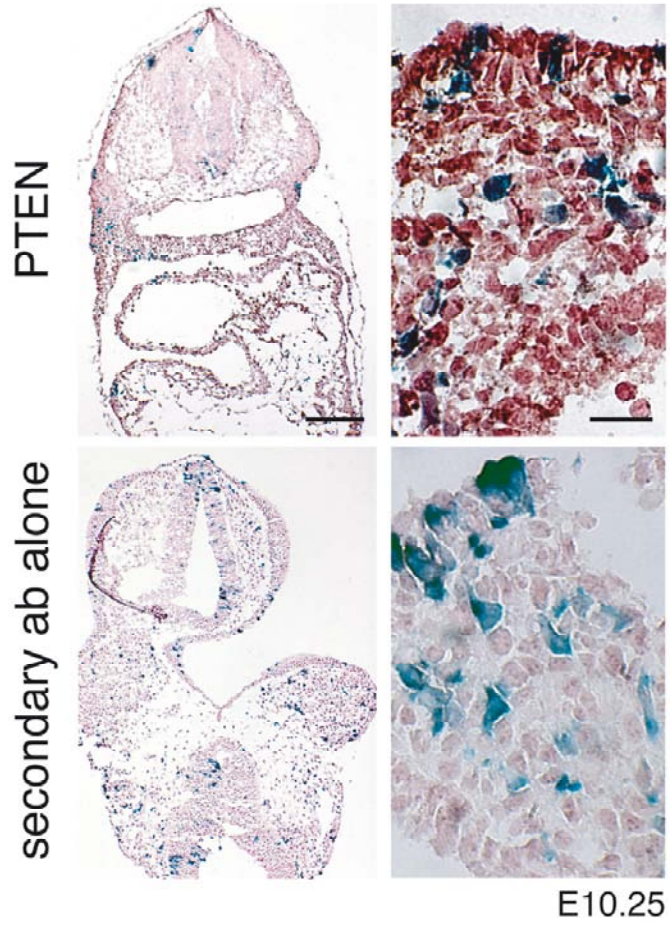

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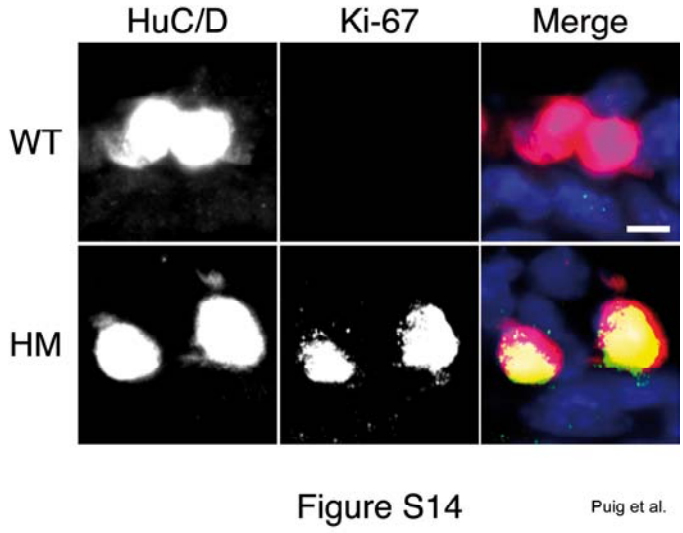

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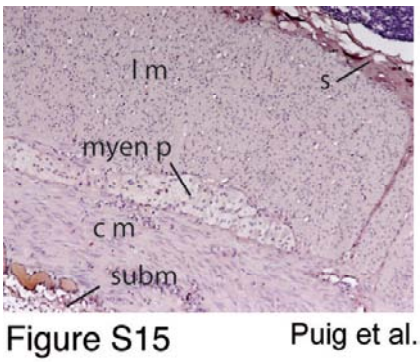

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Transversal section of the patient (CIPO#3) presenting ganglioneuromatosis with no signs of MEN2B or NF-1, which do not produce PTEN. c m = cirucular muscle, l m = longitudinal muscle, myen p = myenteric plexus, s = serosa, subm = submucosa.

Tyr::Cre/°; PTEN$^{F/+}$ x °/°; PTEN$^{F/F}$		Theo %	Nb Obs	% Obs
WT=	°/°; PTEN$^{F/+}$	25	28	22.4
WT=	°/°; PTEN$^{F/F}$	25	20	20.0
HT=	Tyr::Cre/°; PTEN$^{F/+}$	25	38	30.4
HM=	Tyr::Cre/°; PTEN$^{F/F}$	25	34	27.2

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Analysis of offspring genotypes obtained from mating Tyr::Cre/°; PTEN$^{F/+}$ with °/°; PTEN$^{F/F}$. °/°; PTEN$^{F/F}$ and °/°; PTEN$^{F/+}$ mice are considered as wild type (WT), Tyr::Cre/°; PTEN$^{F/+}$ as heterozygous (HT) and Tyr::Cre/°; PTEN$^{F/F}$ as homozygous (HM). Classical Mendelian segregation was observed. Theo: Theoretical, Nb Obs: number observed.

Case	Age	Gender	Clinical findings	Sample type	diagnostic
#1	10 months	F	Normal	Full-thickness biopsy colon	Normal
#2	2 years	M	Normal	Colectomy	Normal
#3	3 years	F	CIPO	Colectomy	Diffuse GNM
#4	2 years	M	CIPO	Full-thickness biopsy colon	Diffuse GNM
#5	5 years	M	CIPO	Full-thickness biopsy colon	Diffuse GNM
#6	3 years	M	CIPO	Colectomy	Diffuse GNM
#7	6 years	M	CIPO	Full-thickness biopsy colon	Diffuse GNM
#8	20 months	M	CIPO	Colectomy	Diffuse GNM
#9	2 years	F	CIPO	Colectomy	Diffuse GNM

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Supplemental Table 2

Main characteristics of the nine human subjects studied; two were normal and seven had CIPO. F = female, M = male, CIPO = chronic intestinal pseudo obstruction, GNM = ganglioneuromatosis.

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