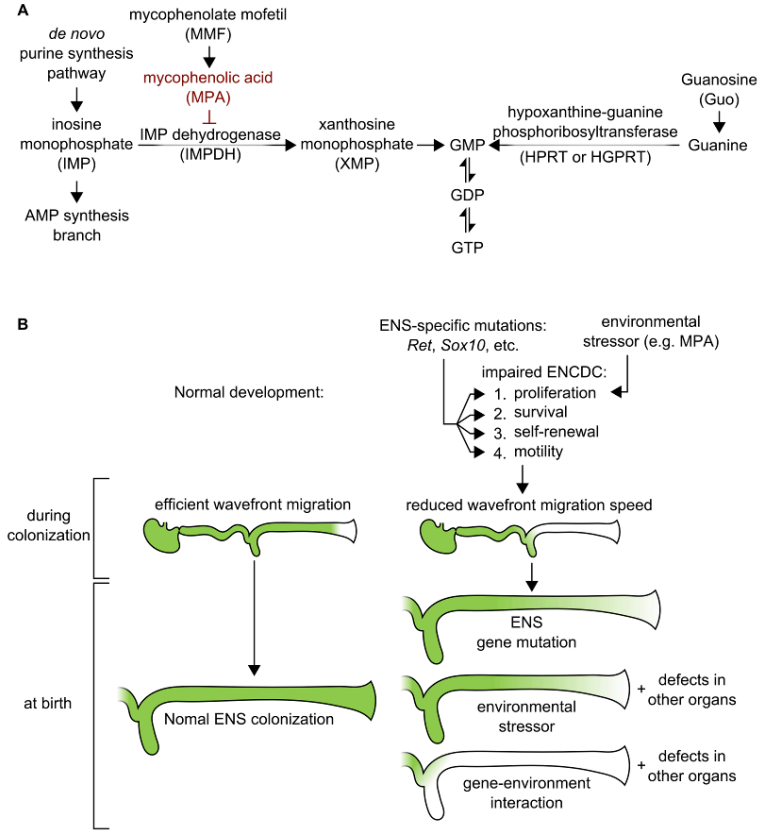
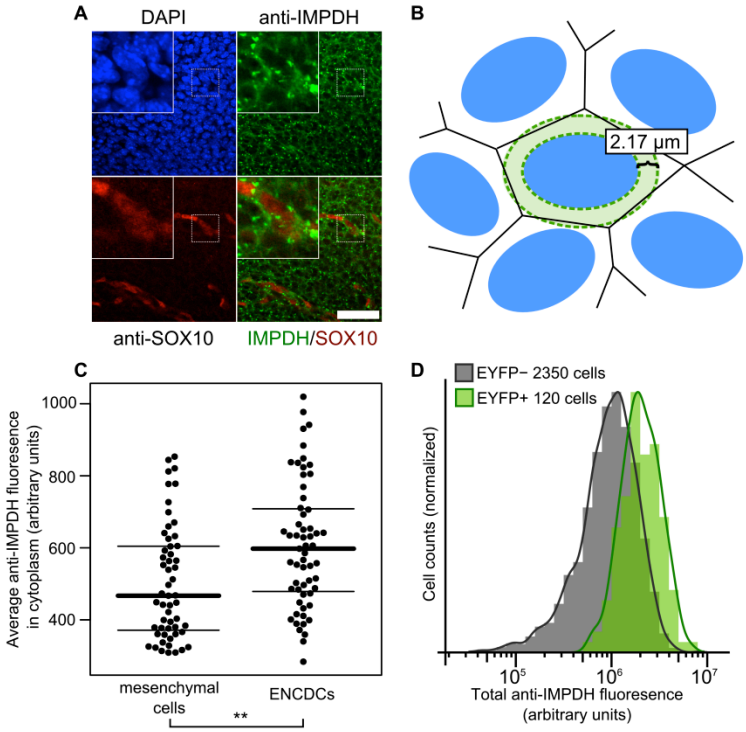
Supplemental Figures

Supplemental Figure 1



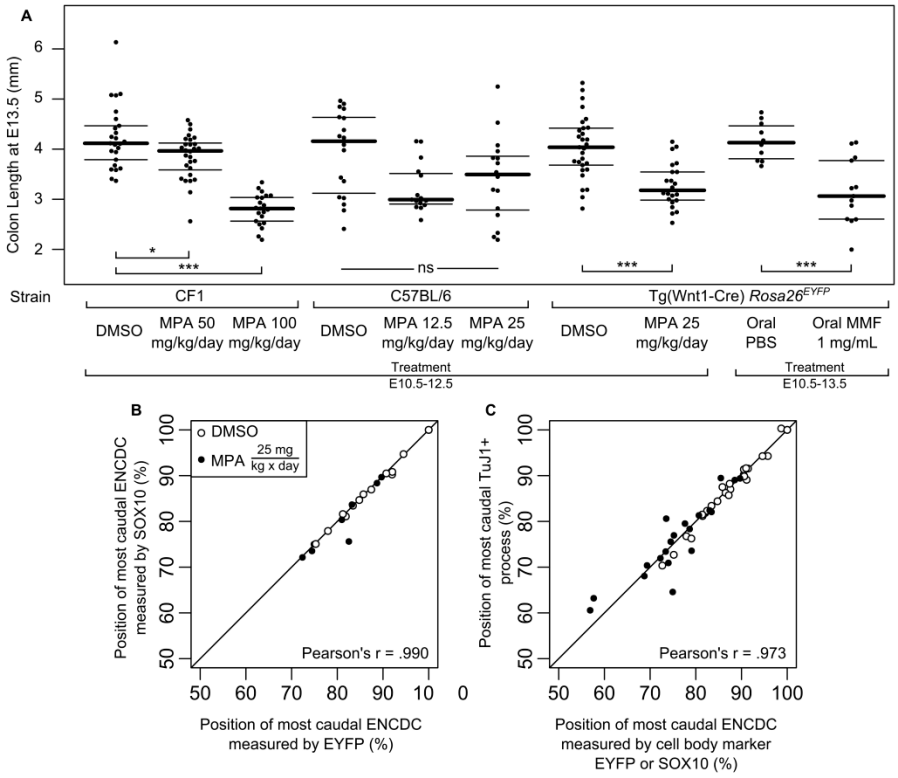
The *de novo* and salvage purine synthesis pathways are shown converging on GMP synthesis (**A**). Enzymes and intermediates not involved in MPA action and/or guanosine salvage are omitted for simplicity. A model (**B**) for the individual and synergistic effects of MPA (or other similar antiproliferative insults) and HSCR-predisposing mutations on the developing ENS. Normally, the ENCDCs that migrate into the developing bowel complete their colonization of the terminal colon with relatively little time remaining before the bowel microenvironment becomes more resistant to colonization. Either defects in classical ENS development genes or prolonged exposure to antimetabolites (e.g. MPA) can result in partially penetrant aganglionosis because of this developmental window. ENCDCs may be individually more sensitive to MPA than other bowel cells and or simply cannot migrate effectively if proliferation of all cells slows. MPA interacts with genes both affecting proliferation (*Ret*) and other aspects of ENS development (*Sox10*) suggesting that multiplicative effects on disease penetrance and severity can result from two hits in converging but largely separate signaling pathways. Transient environmental insults that are removed as the ENCDC migration window closes can result in phenotypes ranging from complete recovery to lethal aganglionosis dependent on genotype.

Supplemental Figure 2



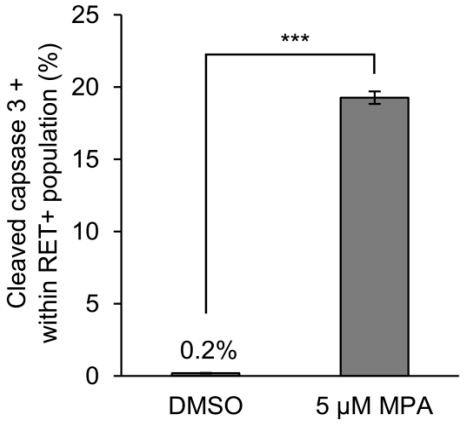
IMPDH is ubiquitously expressed in the bowel at the time that ENCDCs colonize the colon. Since we anticipated that IMPDH levels might vary by position within the bowel and ENS, we first used whole mount IMPDH immunohistochemistry to visualize and quantify the expression in situ. (**A**) IMPDH reactivity was diffusely cytoplasmic with rod and ring aggregates. Since ENCDCs appeared to have more intense cytoplasmic immunoreactivity for IMPDH, we measured the average fluorescence signal intensity in 2.17 micron thick rings (**B**) (represented by the shaded green region) around mesenchymal and ENCDC (SOX10-positive) nuclei (blue ovals). This strategy was designed to minimize overlap with the cytoplasm of neighboring cells (black lines). (**C**) Intensity values. Each dot is mean fluorescence intensity from a single cell. Measurements are from optical sections of three separate E11.5 colons from the CF1 strain. Thick line = median; thin lines = 25th and 75th percentile. Values have extensive overlap, but ENCDCs have a statistically significant increase in average IMPDH fluorescence intensity. ** = $P < 0.01$, rank-sum test. Since whole bowel staining did not reveal any obvious differences in anti-IMPDH reactivity between regions of the bowel, we quantified (**D**) total cellular anti-IMPDH reactivity in cells dissociated from six E12.5 Tg(Wnt1-Cre); *Rosa26*^{EYFP} bowels that were cultured for 3 hours on fibronectin-coated glass at a density of 3.1×10^4 cells/cm^2 before fixation and staining. Total-cellular anti-IMPDH fluorescence for each cell was quantified with ImageJ and is shown for EYFP-negative and EYFP-positive populations as histograms.

Supplemental Figure 3



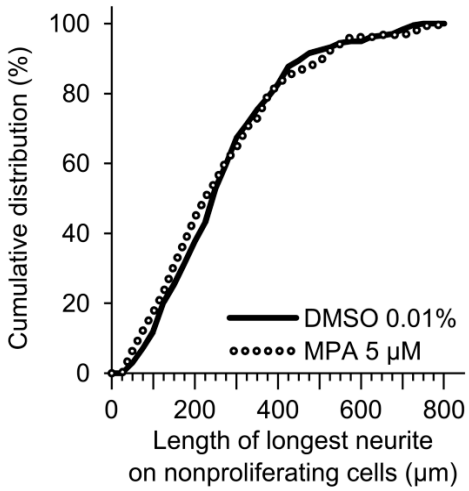
MPA/MMF treatment from E10.5-E13.5 reduced colon lengths at E13.5. (**A**) Higher doses of MPA significantly reduced the length of CF1-strain colons at E13.5 ($P < .001$, Kruskal-Wallis test) while lower doses of MPA and oral MMF significantly reduced the length of Wnt1-Cre *Rosa26*^{EYFP}-strain colons (t-tests). Lower doses of MPA did not significantly affect bowel length in C57Bl/6 fetuses, though variability was high (Kruskal-Wallis test). Thick line = median; thin lines = 25th and 75th percentile. *** = $P < 0.001$, * = $P < .05$. (**B,C**) Plots of ENS colonization extent assessed with double-stained E13.5 Wnt1-Cre *Rosa26*^{EYFP}-strain colons. (**B**) Cellular markers such as neural-crest lineage marker EYFP or SOX10 have near-perfect concordance for ENS colonization extent measurements. The most caudal TuJ1+ process also reliably indicates colonization extent, albeit with some random error. MPA treatment does not appear to have any systematic effect on the extension of neuronal processes up to ENCDCs at the wavefront.

Supplemental Figure 4



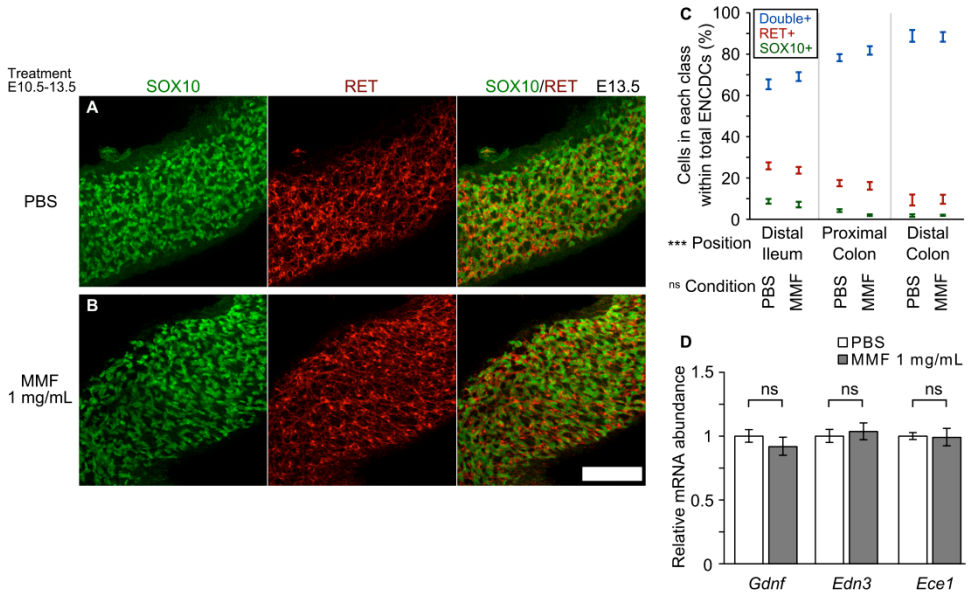
Cleaved caspase 3 staining of 24-hour explant cultures reveals that MPA induces apoptosis in cultured ENCDCs. *** = $P < 0.001$, t-test.

Supplemental Figure 5



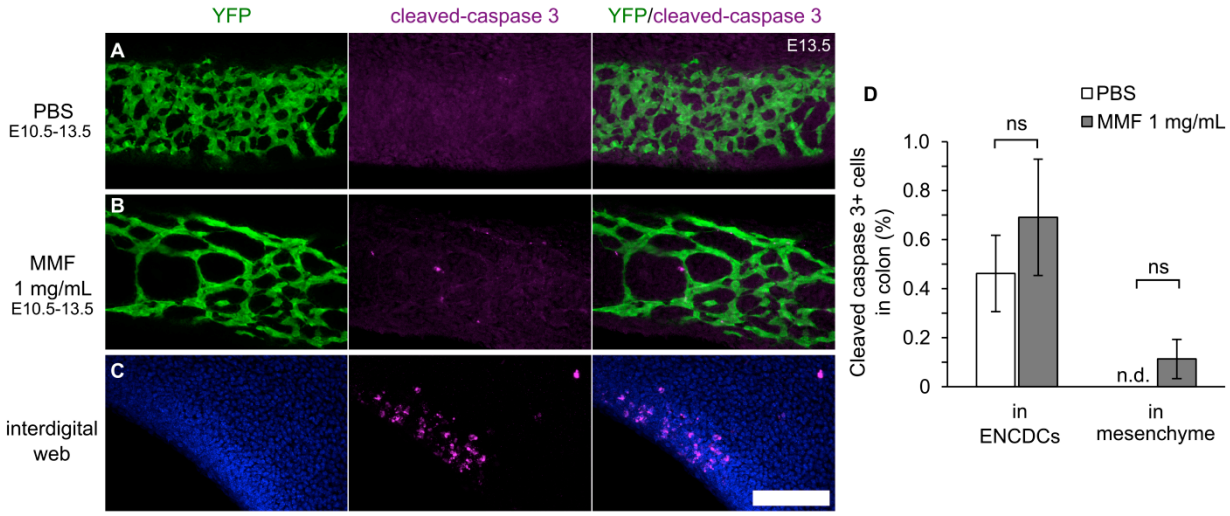
MPA did not alter neurite growth in post-mitotic neurons. Data show the length of the longest neurite on each TuJ1+ cell that did not incorporate BrdU over the course of 48 hours in culture with GDNF, (n>150 cells/group).

Supplemental Figure 6



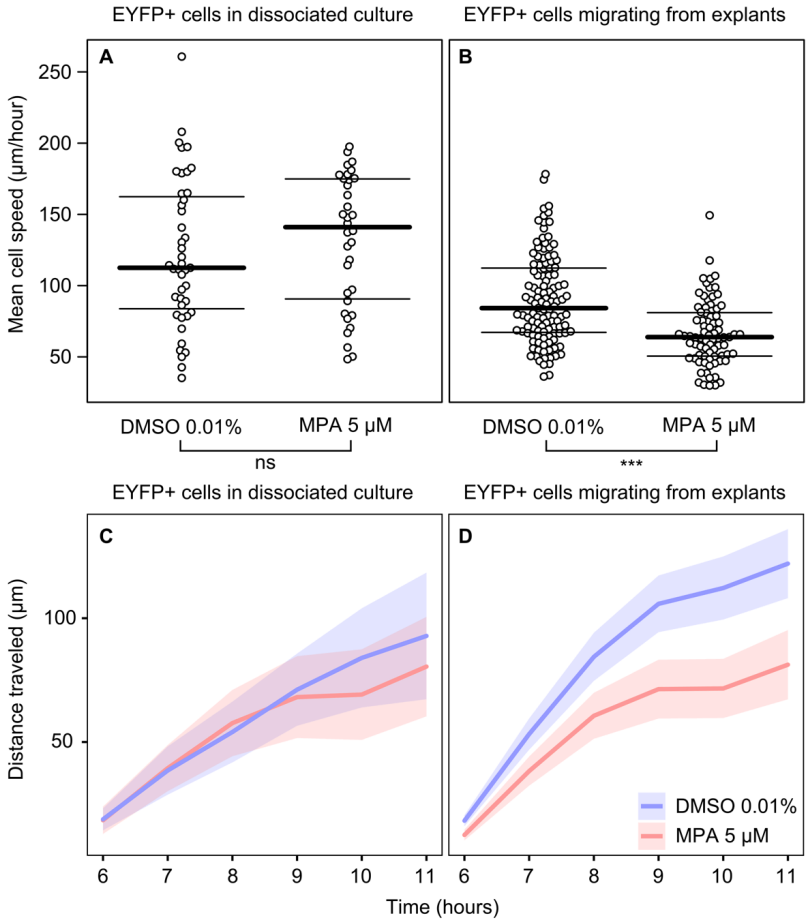
MMF treatment at doses that significantly reduced ENCDC proliferation and bowel colonization in vivo did not cause premature differentiation at E13.5. 8 μm-thick maximum-intensity projections of SOX10 (pseudocolored green) and RET (pseudocolored red) distal small intestine (**A**,**B**, scale bar = 100 μm) and quantification of each cell population within total ENCDCs (**C**) demonstrate the expected reduction in the percentage of SOX10, RET double positive progenitor cells within the ENCDC population as we move from distal (e.g. more recently colonized) to proximal. Concomitantly, the RET+, SOX10− and SOX10+, RET− single positive populations increase in proportion as the double-positive proportion falls. Since enteric neurons already exist at this time and are known to lose SOX10 and retain RET expression, the RET single-positive population is very likely a neuronal lineage. Increased differentiation would manifest as a reduction in the proportion of double-positive cells. MMF treatment did not significantly change the percentage of SOX10+, RET+ progenitor cells within the ENCDC population (two-way ANOVA, interaction term was not significant). (**D**) qRT-PCR analysis of mRNAs encoding mesenchyme derived factors important for ENS colonization. RNA was isolated from E13.5 bowels after treatment from E10.5-E13.5 with PBS (5 embryos) or MMF (4 embryos). *Gapdh* is used as an internal reference gene for relative quantitation (t-tests). *** = $P < 0.001$, ns = not significant.

Supplemental Figure 7



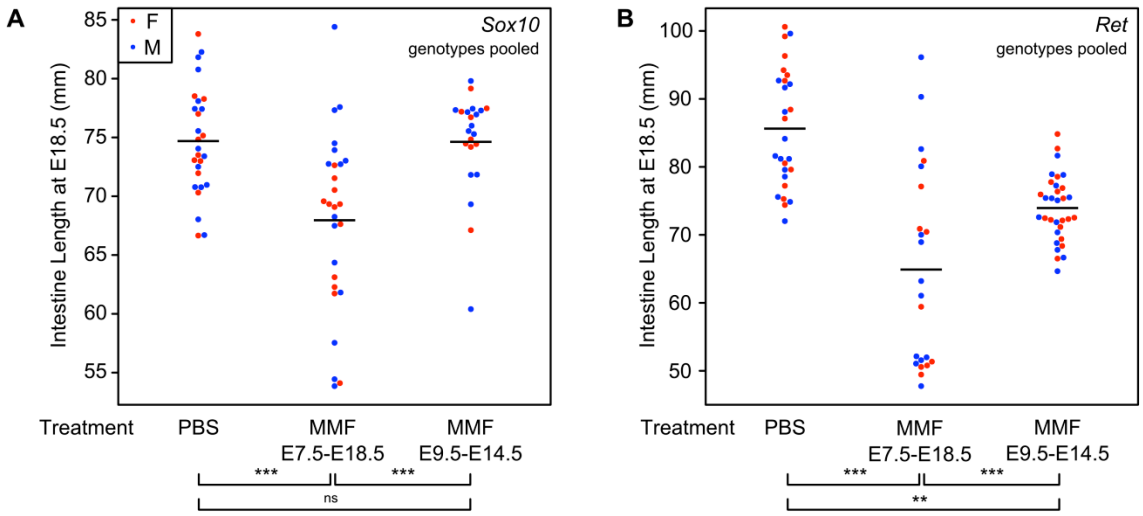
MMF treatment at doses that significantly reduced ENCDC proliferation and bowel colonization in vivo did not cause detectable canonical caspase-mediated ENCDC apoptosis. Cleaved-caspase 3 reactivity was readily detected in the interdigital web of E13.5 mice (**C**) but was infrequently detected in ENCDCs or non-neural crest mesenchymal cells with or without MMF treatment (**A-B,D**) ns = not significant, t-test). Bowels shown in (**A-B**) are the same as in Figure 3E-F.

Supplemental Figure 8



Time lapse imaging of isolated ENCDC and bowel explant cultures reveals that (**A**, **C**) MPA does not alter the speeds or migration distance of individual ENCDCs when they are cultured at low density (Kolmogorov-Smirnov test), but does reduce (**B**, **D**) the speed and migration of ENCDCs migrating from bowel explants (t-test on log-transformed speeds). Isolated ENCDCs were prepared from the same region of Wnt1-Cre *Rosa26*^{EYFP} bowel as were the explants (small intestine) and were plated at low density (3125 cells/cm^2) to minimize cell-cell collisions. Migrating EYFP+ cells were randomly selected for tracking at 8 hours after GDNF addition and tracked for the duration of culture that they remained in the field of view, alive, and non-dividing. Measurements from the middle third (approximately hours 6-11) of 16-hour time-courses were chosen for analysis because most chosen cells stayed in field for this interval. ns = not significant, *** = P <.05. Thick lines in **A-B** = median. Thin lines in **A-B**= 25th and 75th percentile. Thick lines and shaded region in **C-D** = Mean and 95% confidence interval.

Supplemental Figure 9



MMF treatment during gestation reduces intestine length at E18.5 in (**A**) *Sox10* and (**B**) *Ret* interaction experiments. Data are shown with all genotypes pooled since *Ret* or *Sox10* genotype did not affect intestine length. Black lines = mean, *** = $P < 0.001$, ns = not significant, statistical test: Welch ANOVA.

Supplemental Tables

Supplemental Table 1:

Complete list of screened compounds and results are provided as a separate spreadsheet.

Supplemental Table 2: Incidence of neural tube and heart defects in the offspring of MMF treated dams

Paternal Strain / Genotype	Maternal Strain / Genotype	Treatment	Fetus Genotype	N Fetuses	Exencephaly	Heart / Great Vessel Defects
C3HFe/J *Sox10*$^{+/LacZ}$	C57Bl/6	0.25X PBS E7.5-E18.5	*Sox10*$^{+/+}$	11	0 (0%)	0 (0%)
			Sox10$^{+/LacZ}$	16	0 (0%)	0 (0%)
		MMF 1 mg/mL E7.5-E18.5	*Sox10*$^{+/+}$	10	4 (40%)	1 (10%)
			Sox10$^{+/LacZ}$	17	6 (35%)	4 (23%)
		MMF 1 mg/mL E9.5-E14.5	*Sox10*$^{+/+}$	9	0 (0%)	0 (0%)
			Sox10$^{+/LacZ}$	13	0 (0%)	1 (8%)[A]
B6;129X1 *Ret*$^{9/+}$	C57Bl/6 *Ret*$^{TGM/+}$	0.25X PBS E7.5-E18.5	*Ret*$^{+/+}$	7	0 (0%)	0 (0%)
			Ret$^{9/+}$	6	0 (0%)	0 (0%)
			Ret$^{+/–}$	6	0 (0%)	0 (0%)
			Ret$^{9/–}$	8	0 (0%)	0 (0%)
		MMF 1 mg/mL E7.5-E18.5	*Ret*$^{+/+}$	5	3 (60%)	0 (0%)
			Ret$^{9/+}$	4	2 (50%)	0 (0%)
			Ret$^{+/–}$	4	2 (50%)	0 (0%)
			Ret$^{9/–}$	8	5 (63%)	3 (38%)
		MMF 1 mg/mL E9.5-E14.5	*Ret*$^{+/+}$	9	0 (0%)	0 (0%)
			Ret$^{9/+}$	4	0 (0%)	0 (0%)
			Ret$^{+/–}$	5	0 (0%)	0 (0%)
			Ret$^{9/–}$	14	0 (0%)	0 (0%)

A – Affected fetus was much smaller than littermates

Supplemental Table 3: Genotyping oligonucleotides

Gene	Allele	Direction	Sequence	Amplicon Size (bp)
Ret	common	Forward	5'CAGCGCAGGTCTCTCATCAGTACCGCAACC3'	multiple[A]
Ret	wild-type 1	Reverse	5'ACGTCGCTTTCGCCATCGCCCGTGCGCGCG3'	230
Ret	Ret^{TGM}	Reverse	5'CCCTGAGCATGATCTTCCATCACGTCGAAC3'	290
Ret	wild-type 2	Reverse	5'GCGCAGCAGCTAGCCGCAGCGACCCGGTTC3'	450
Ret	Ret^{9}	Reverse	5'CCCAGTAAGCATCCCTCGAGAAGTAGAGGC3'	320
Hprt	wild-type	Forward	5'TCTGGTTTTATATGGGTACTGGGGGATCT3'	218
Hprt	$Hprt^{b\text{-}m3}$	Forward	5'AGCATTCCTGCCCCAACAATGATTC3'	288
Hprt	common	Reverse	5'CATGCAGGCACTCACACATACAAGTAAAAA3'	multiple[B]
Sry	N/A	Forward	5'TTGTCTAGAGAGCATGGAGGGCCATGTCAA3'	273
Sry	N/A	Reverse	5'CCACTCCTCTGTGACACTTTAGCCCTCCGA3'	
EGFP/ EYFP	N/A	Forward	5'GCACGACTTCTTCAAGTCCGCCATGCC3'	265
EGFP/ EYFP	N/A	Reverse	5'GCGGATCTTGAAGTTCACCTTGATGCC3'	

A – To detect Ret^{+} and Ret^{TGM}, a PCR reaction was performed with three primers (common, wild-type 1, and *TGM*). To distinguish Ret^{+} and Ret^{9}, an analogous reaction was used (common, wild-type 2, and *Ret9*).

B – To detect Hprt genotype, two separate PCR reactions were performed for wild-type (wild-type and common) and *b-m3* (*b-m3* and common).

Supplemental Table 4: Primary antibodies

Antibody	Manufacturer / Investigator	Dilution used
rabbit anti-TuJ1	Covance (PRB-435P)	1:10000
goat anti-RET	Neuromics (GT15002)	1:200
rabbit anti-p75NTR	Promega (G323A)	1:200
chicken anti-GFP	Aves Labs (GFP-1020)	1:1000
goat anti-SOX10	Santa Cruz Biotechnology	1:200
rabbit anti-SoxE	Craig Smith, MRCI, Australia	1:4000
rabbit anti-IMPDH	Proteintech (12948-1-AP)	1:100 on bowel; 1:200 on cells
rabbit anti-cleaved Caspase 3	Cell Signaling (#9661)	1:250
Alexa 594-conjugated mouse anti-BrdU	Life Technologies (PRB–1)	1:50
biotin-XX conjugated anti-HuC/HuD	Life Technologies (A21272)	400 ng/mL