Cell-autonomous retinoic acid receptor signaling has stage-specific effects on mouse enteric nervous system

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Retinoic acid (RA) signaling is essential for enteric nervous system (ENS) development, since vitamin A deficiency or mutations in RA signaling profoundly reduce bowel colonization by ENS precursors. These RA effects could occur because of RA activity within the ENS lineage or via RA activity in other cell types. To define cell-autonomous roles for retinoid signaling within the ENS lineage at distinct developmental time points, we activated a potent floxed dominant-negative RA receptor α (RarαDN) in the ENS using diverse CRE recombinase–expressing mouse lines. This strategy enabled us to block RA signaling at premigratory, migratory, and postmigratory stages for ENS precursors. We found that cell-autonomous loss of RA receptor (RAR) signaling dramatically affected ENS development. CRE activation of RarαDN expression at premigratory or migratory stages caused severe intestinal aganglionosis, but at later stages, RarαDN induced a broad range of phenotypes including hypoganglionosis, submucosal plexus loss, and abnormal neural differentiation. RNA sequencing highlighted distinct RA-regulated gene sets at different developmental stages. These studies show complicated context-dependent RA-mediated regulation of ENS development.

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Cell-autonomous retinoic acid receptor signaling has stage-specific effects on mouse enteric nervous system

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Introduction

The enteric nervous system (ENS) is a complex network of neurons and glia that resides in the bowel wall and is essential for intestinal function (1, 2). These ENS cells arise primarily from vagal enteric neural crest-derived cell (ENCDC) precursors that divide rapidly and colonize the bowel in a rostral to caudal direction from E9 to E13.5 in mice (3–6). In addition to vagal ENCDC, ENS precursors include sacral neural crest (7), mesenteric neural crest (8), sympatho-enteric precursors (9), Schwann cells (10), and perhaps bowel epithelial cells (11). As ENCDC colonize the bowel, they differentiate into about 20 neuron types, and many types of glia that form extensive networks to control most aspects of bowel function (2, 12–17). Maturation of the ENS continues during fetal development, and remodeling continues after birth (18–20). Retinoic acid (RA), the active metabolite of vitamin A, is an important morphogen with an integral role in ENCDC migration, proliferation, and differentiation (21–32). RA functions mainly as a transcriptional regulator that binds to and activates RAR/RXR heterodimers (33). RAR/RXR heterodimers bind DNA at RA response elements (RAREs) to regulate transcriptional activity. Several studies demonstrate vital roles for vitamin A and its active metabolite RA in ENS development. In rat and mouse primary cell culture, RA increases ENCDC proliferation and neuronal differentiation while decreasing neurite length (23, 27). In vivo, vitamin A–deficient mice (Rbp4–/– mice on a vitamin A–deficient diet) develop distal bowel aganglionosis (26), confirming that vitamin A is needed for bowel colonization by ENCDC. The extent of bowel aganglionosis dramatically increased when Ret heterozygosity was combined with vitamin A deficiency, suggesting potent gene-environment interactions in mice (26). Consistent with these observations, loss of the primary RA synthesis enzyme retinaldehyde dehydrogenase 2 (Raldh2) also causes severe intestinal aganglionosis in mice (29), with more minor effects of murine Raldh1 and Raldh3 mutations (25). Furthermore, maternal vitamin A deficiency or excess can cause intestinal hypoganglionosis without aganglionosis in rats and mice (22, 28, 32). While these results clearly show that RA signaling is needed for ENS development, many questions remain. First, because RAR and RXR receptors are expressed in diverse cell types (27), it is not clear whether RA acts directly on ENCDC or via effects on neighboring cells. Second, effects of RA signaling at distinct developmental stages remain elusive. Third, the RA-targeted gene network in ENCDC in vivo is not yet established. To address these questions, we employed a very potent
CRE-dependent dominant-negative RA receptor α (RaraDNEloxP/STV;αLoxP/+; RARαDN) (34) in combination with fluorescence-based lineage tracing in mice. We discovered that RA signaling regulates ENS development in a cell-autonomous manner, with distinct effects on different developmental stages. Furthermore, gene expression profiling showed stage-specific effects of blocking RAR signaling in developing ENCDC. These results suggest that vitamin A deficiency or excess could alter ENS structure and function in many ways during intrauterine and postnatal periods, contributing to human bowel motility disorders.

Results

Cell-autonomous RAR signaling in neural crest derivatives is required for craniofacial and ocular development. To characterize cell-autonomous roles for RAR signaling within the ENS lineage, we bred RaraDNαloxP/+ to Wnt1Cre−/− mice. RaraDNαloxP/+ produce RARαDN after CRE-mediated DNA recombination. Wnt1Cre express Cre recombinase in the CNS and many neural crest derivatives, including essentially all fetal ENS precursors (35–37). RaraDNαloxP/+; Wnt1Cre−/− mice are viable at E12.5, but they die by E14.5 (Table 1). E12.5 RaraDNαloxP/+; Wnt1Cre−/− have major malformations of neural crest–derived facial structures (Figure 1, A and B) with absent facial cartilage (Figure 1, C and D). Sectioning showed failure of nasomedial process fusion at the midline and a wide frontonasal region (Figure 1, E and F). In contrast, dorsal root ganglia (DRG), another crest-derived structure, appeared fairly normal even though Wnt1Cre induced recombination of an EYFP reporter in DRG (Figure 1, G–L). These data highlight distinct RAR roles in different neural crest–derived tissues during development. Our primary goal was to investigate RA signaling effects in the ENS.

Table 1. RaraDNαloxP/+; Wnt1Cre−/− embryos obtained at various ages

| Age   | Litters analyzed | Total embryos analyzed | Expected RaraDN; Wnt1Cre number | Actual RaraDN; Wnt1Cre number | χ2 statistic | P value |
|-------|-----------------|------------------------|---------------------------------|-------------------------------|--------------|---------|
| E10.5 | 5               | 41                     | 10.25                           | 10                            | 0.004        | 0.949571|
| E11.5 | 14              | 109                    | 27.25                           | 37                            | 2.098        | 0.147492|
| E12.5 | 22              | 147                    | 36.75                           | 26                            | 2.341        | 0.126009|
| E14.5 | 5               | 34                     | 8.5                             | 0                             | 11.333       | 0.0008  |

Embryo loss occurs between E12.5 and E14.5.

ENS development requires cell autonomous RAR signaling. At E12.5 in Wnt1Cre−/− (control) mice, ENCDC had colonized the esophagus, stomach, small intestine, and half of the colon, as seen by TuJ1 (neuron specific β3-tubulin) antibody staining (Figure 2A). In contrast, E12.5 RaraDNαloxP/+; Wnt1Cre−/− mice only had TuJ1+ cells in the esophagus and stomach (Figure 2B). Recognizing that the absence of TuJ1 staining might reflect impaired neuronal differentiation in RaraDNαloxP/+; Wnt1Cre−/− mice or could reflect absent ENS, we bred to R26R-TdTomato reporter mice so that cells undergoing CRE-mediated DNA recombination are unambiguously marked. While TdTomato+ ENS and HuC/D+ (neuronal RNA binding protein) neurons were readily detected in stomach, small bowel, and proximal colon of control animals (Figure 2, C–E and I), the RaraDNαloxP/+; Wnt1Cre−/−; R26R-TdTomato+ mice did not have TdTomato+ or HuC/D+ cells in the small bowel or colon and had fewer ENS cells in stomach than controls (Figure 2, F–H and J). These analyses confirmed that blocking cell-autonomous RAR signaling in the Wnt1Cre lineage completely prevented these ENS precursors from colonizing the small bowel and colon.

Blocking RAR signaling causes defects in ENCDC migration and differentiation by E10.5. To determine if RaraDN expression within neural crest–derived ENS precursors acts at earlier developmental stages, we examined E10.5 whole embryo via 3DISCO tissue clearing (38) and confocal microscopy (Figure 3, A and B). TuJ1 and SOX10 antibody staining showed many ENCDC in the esophagus and stomach in control mice (Figure 3, C–F) but very few stained ENCDC in the proximal bowel of RaraDNαloxP/+; Wnt1Cre−/− mice (Figure 3, G–J), consistent with a defect in early stages of bowel colonization. More specifically, while there were some SOX10+ ENCDC near the vagus in mutant mice (Figure 3, G–J), the control mice had many SOX10+ ENCDC that had migrated far beyond the vagus and into the stomach (Figure 3, C–F). Furthermore, in control mice, many ENCDC were TuJ1 immunoreactive, suggesting early neuronal differentiation (Figure 3, C and E), but there were almost no TuJ1+ cells in the esophagus or stomach of RaraDNαloxP/+; Wnt1Cre−/− mice (Figure 3, G and I). Collectively, these data suggest that cell-autonomous RAR signaling is needed for ENCDC to populate the bowel and differentiate into neurons.

Cell autonomous RAR signaling is required for RET and PHOX2B expression in ENCDC. The defect in bowel colonization by ENCDC of RaraDNαloxP/+; Wnt1Cre−/− mice closely resembles the phenotype in Ret and Phox2b null mice.
Because RA was previously reported to induce Ret expression in quail ENCDC migrating from the neural tube to bowel (24), we stained E12.5 bowel from RarαDNLoxP/+; Wnt1Cre+; R26R-EYFP+ and controls (Wnt1Cre+; R26R-EYFP+) with RET antibody. A total of 100% of stomach EYFP+ ENCDC expressed RET in controls as expected (Figure 4, D–F). Curiously, while 1 RarαDNLoxP/+; Wnt1Cre+; EYFP+ mouse had many RET+EYFP+ ENCDC in the stomach (42%), the other mice evaluated had very few RET+EYFP+ ENCDC (0%, 0%, 0.4%, and 4.9%) (Figure 4, G–J). RARαDN also dramatically reduced the percentage of lineage-marked ENCDC that express PHOX2B in the stomach or esophagus of RarαDNLoxP/+; Wnt1Cre+; TdTomato mice (Figure 5, A–F and H). EdU incorporation into lineage marked ENCDC in E12.5 stomach of RarαDNLoxP/+; Wnt1Cre+; TdTomato mice, however, was not statistically different from controls (Figure 5, A–F and G); therefore, it is not clear that reduced proliferation within stomach ENCDC fully accounts for the phenotype. Collectively, these data suggest that RAR signaling is needed in the Wnt1Cre ENS lineage for Ret and Phox2b expression, further suggesting that loss of either RET or PHOX2B could cause this type of extensive intestinal aganglionosis (39, 40).

**Loss of RAR signaling causes defective vagal nerve development.** In parallel with the loss of ENCDC in fetal stomach, E11.5 RarαDNLoxP/+; Wnt1Cre+ mice had smaller vagus nerves than control littermates (Figure 6, A–C). The reduction in vagal nerve fibers in the stomach appears to be non–cell autonomous because TuJ1+ vagal nerve fibers are TdTomato-negative in Wnt1Cre+; TdTomato+ and RarαDN; Wnt1Cre+; TdTomato+ mice (Figure 6D, Supplemental Figure 1, and Supplemental Videos 1 and 2; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.145854DS1). Enlarged images also demonstrate many TdTomato+ cells migrating along the TuJ1+ vagal nerve fibers in Wnt1Cre+; TdTomato+ and RarαDN; Wnt1Cre+; TdTomato+ mice (Supplemental Figure 1, E, F, K, and L). Furthermore, while ENCDC migrate far beyond the vagus in control animals, TuJ1+ and SOX10-labeled ENCDC remained close to vagal fibers in E11.5 RarαDNLoxP/+; Wnt1Cre+ mice (Figure 3, D and H, and Figure 6, A and B). These observations suggest synergistic interactions between growing vagal nerve fibers and migrating ENCDC that colonize the bowel to form ENS.

**Loss of RAR signaling in SOX10 lineage of ENCDC causes aganglionosis.** To evaluate cell-autonomous effects of RAR beyond E12.5 when RarαDNLoxP/+; Wnt1Cre+ die, we generated RarαDNLoxP/+; SOX10Cre+ mice. These mice express Cre from Sox10 regulatory elements, beginning at E8.5 when ENCDC migrate through the paraxial mesoderm to foregut (41). To confirm CRE activation in the ENS lineage, we examined SOX10Cre; R26R-TdTomato...
mice at E12.5 and found essentially complete overlap of the TdTomato lineage marker and RET antibody staining in the bowel (Supplemental Figure 2). Unlike controls (Figure 7A) and like RaraαDNLoxP/+; Wnt1Cre+, the E12.5 RaraαDNLoxP/+; SOX10Cre+ had TuJ1+ ENCDC predominantly confined to the stomach at E12.5 (Figure 7, B–E), with a few sparsely distributed TuJ1+ ENCDC in the proximal small intestine (Figure 7D). By E15.5, RaraαDNLoxP/+; SOX10Cre+ had obvious eye and craniofacial defects but remained viable (Figure 7F). The E15.5 ENS network was dense in the esophagus and stomach but was sparse in the proximal small intestine (Figure 7, G, J, L, and N) compared with controls (Figure 7, I, K, and M). Distal small intestine (Figure 7P) and colon were aganglionic with extrinsic nerve fibers in the distal colon (Figure 7, H and R) of mutant mice in contrast to controls (Figure 7, O and Q). Neurons in the small bowel were often associated with extrinsic nerve fibers and separated from more proximal ENS (Figure 7, S–V). These data are consistent with the hypothesis that RAR signaling is required as ENCDC migrate from the neural tube to bowel and that it is not needed by ENS precursors before E8.5 in the neural tube.

Blocking RAR signaling in the TyrCre lineage reduces enteric neurons and alters ENS patterning. We next examined the ENS in mice that express Cre in ENCDC from the tyrosinase promoter (RaraαDNLoxP/+. TyrCre+) starting at E10.5 (41, 42) when ENCDC normally reach the midgut (3). In these mice, the extent of bowel colonization by
ENCDC was similar to controls at E13.5 (Figure 8, A and E). Stomach ENS appeared normal in RarαDNLoxP/+; TyrCre+ mice (Figure 8, B and F), but the small bowel and colon were hypoganglionic relative to controls (Figure 8, C, D, G, and H). ENS organization was also abnormal in the small bowel and colon of mutants at E13.5. Instead of the fine network of cells seen in controls, RarαDNLoxP/+; TyrCre+ ENS had thick chains of cells (Figure 8, A, C–E, G, and H). At birth, RarαDNLoxP/+; TyrCre+ mice appeared healthy but did not feed (absent milk spot, Figure 8I) and consistently died within 12 hours (n = 6). To avoid analysis of dying mice, we examined E18.5 ENS. Although the RarαDNLoxP/+; TyrCre+; R26R-TdTomato bowel looked grossly normal (Figure 8J), lineage-marked TdTomato+ ENS cells were less dense in the mutant bowel throughout the small intestine and colon (Figure 8K). HuC/D IHC confirmed that enteric neurons were sparse (Figure 8, L, and N–P) and that all neurons in RarαDNLoxP/+; TyrCre+; R26R-TdTomato ENS were TdTomato+ (Figure 8, L and M). To determine if blocking RAR impacted neuron subtype ratios, we counted NOS1 and HuC/D double-labeled cells and discovered that more HuC/D+ neurons were NOS1+ in RarαDNLoxP/+; TyrCre+ ENS (Figure 8, Q–S) compared with controls. Collectively, these results show that cell-autonomous RAR signaling is important after E10.5 for ENS patterning, to increase neuron number, and for neuron subtype specification.

Inactivation of RAR signaling in the RET lineage causes distal bowel hypoganglionosis. Sparse ENS in the preceding models might reflect inadequate RET, making it difficult to identify other roles for RAR signaling in ENCDC. Our prior studies suggest that RA is not needed to sustain Ret expression in ENCDC that already express Ret (26, 27). We therefore decided to use mice expressing CRE-ERT2 from the Ret locus to activate RarαDN expression in ENCDC that already express Ret. For these experiments, we also needed a CRE-dependent fluorescent reporter (EYFP) to track CRE activity. Because Ret is on mouse chromosome 6 near the ROSA26 locus that drives expression of RarαDN, as well as most fluorescent reporters, we bred RarαDNLoxP/+ mice to RET-CreERT2-EYFPlox/loxnes mice to generate RarαDNLoxP/+; RETCreERT2-EYFPlox/loxnes and RarαDNLoxP/+ controls. The
RARαDNLoxP/+; RETCreERT2-EYFP tandem have undergone recombination so RETCreERT2 and a CRE-activated EYFP–Channelrhodopsin-2 (ChR2) protein are on the same chromosome. E13.5 control RARαDNLoxP/+ lacking CreERT2 had the bowel fully colonized by ENS precursors after E10.5 tamoxifen treatment (Figure 9, A, B, D, F, and H). E10.5 tamoxifen-treated RARαDNLoxP/+; RETCreERT2-EYFP tandem also had normally patterned ENS in the esophagus and small bowel (Figure 9, A, C, E, and G) but had hypoganglionic ENS in the colon, with thick chains of ENS cells (Figure 9, C and I). We confirmed high levels of CRE-activation in the ENS after tamoxifen treatment and almost no CRE activation in the absence of tamoxifen in these RETCreERT2-EYFP tandem mice (Supplemental Figure 3). Because tamoxifen effects may take 12–18 hours, we also tamoxifen treated at E8.5 but found similar phenotypes (Figure 9, J–M). RET and PHOX2B were readily detected in EYFP+ ENCDC of E13.5 RARαDNLoxP/+; RETCreERT2-EYFP tandem mice, consistent with our prior studies (26, 27). These data suggest that RAR is needed to activate RET and PHOX2B expression in the absence of tamoxifen in these RETCreERT2-EYFP tandem mice (Figure 4, D–I, and Figure 5, A–F and H) but not to maintain expression once regulatory elements are activated (Figure 9, N–Q). The unusual patterning in the distal colon of tamoxifen-treated E13.5 RARαDNLoxP/+; RETCreERT2-EYFP tandem mice suggests that RAR regulates additional ENS patterning genes in ENCDC.

Ret lineage loss of RAR signaling profoundly reduced submucosal neuron density and altered cell identity. RARαDNLoxP/+; RETCreERT2-EYFP tandem treated with tamoxifen at E10.5 (Figure 10) were born at a normal Mendelian ratio, grew normally, and had a normal-appearing bowel (Figure 10B), permitting analysis of adult ENS. We therefore stained the bowel of 2-month-old mice with HuC/D and TuJ1 antibodies (Figure 10, C–N). Quantitative analysis demonstrated a 50%–65% reduction in myenteric neurons in small bowel and colon and a 90% loss of submucosal neurons in RARαDN-expressing mutant mice (Figure 10, O–T). In contrast to neurons, adult enteric glia marked by SOX10 antibody appeared similar in abundance in tamoxifen-treated RARαDNLoxP/+; RETCreERT2-EYFP tandem and control mice (Supplemental Figure 4A). Interestingly, E10.5 tamoxifen treatment also led to a dramatic increase in NOS1+ submucosal neurons in RARαDNLoxP/+; RETCreERT2-EYFP tandem small bowel (Supplemental Figure 4B), and a mild increase in NOS1+ myenteric neurons. These studies confirm distinct age-dependent effects of cell-autonomous RAR signaling in the ENS.

Transcriptional profiling shows that RARαDN differentially impacts gene expression in E11.5 stomach and E13.5 colon ENCDC. Because RAR regulates transcription, we performed pooled-cell RNA sequencing (RNA-seq) to characterize RARαDN-induced changes in ENCDC gene expression. For RARαDNLoxP/+; Wnt1Cre; R26R-TdTomato mice, we analyzed E11.5 stomach ENCDC because RARαDN eliminated ENCDC beyond the stomach.
For $Rar\alpha{\loxp/+}; RET\text{CreERT2-EYFP}\text{loxP}$, we analyzed E13.5 colon ENCDC after tamoxifen treatment at E10.5 because RARα-expressing colon ENCDC had patterning defects. Controls expressed fluorescent protein in ENCDC but lacked $Rar\alpha$. Flow sorting cleanly separated fluorescent cells from other bowel cell types (Figure 11, A and B). Multidimensional scaling (MDS) showed separate groups of control versus $Rar\alpha$-expressing ENCDC (Figure 11, C and D). Among approximately 23,000 transcripts at each age, we found 1413 genes differentially expressed between control and $Rar\alpha\text{Loxp/+}; Wnt1\text{Cre+}$ ENCDC (FDR < 0.05) at E11.5 (Supplemental Table 1) and 1140 genes differentially expressed between control and $Rar\alpha\text{Loxp/+}; \text{RETCreERT2-EYFP}\text{loxP}$ (FDR < 0.05) at E13.5 (Supplemental Table 2), as represented in volcano plots (Figure 11, E and F). The top 20 upregulated and downregulated genes at E11.5 (Supplemental Figure 5A) and E13.5 (Supplemental Figure 5B) and main dysregulated pathways (Figure 12A for E11.5 and Figure 12B for E13.5) suggest that many distinct biological processes are influenced by RAR signaling.

One initially surprising feature at E13.5 was that many hemoglobin genes were less abundant in flow-sorted cells from $Rar\alpha\text{Loxp/+}; \text{RETCreERT2-EYFP}\text{loxP}$ compared with controls. We suspect that this occurred because $Ret$ is expressed in the hematopoietic stem cell (HSC) lineage, where RAR supports erythropoiesis (43, 44) and hemoglobin genes are normally expressed at high levels. Consistent with this hypothesis, some $\text{RETCreERT2-EYFP}\text{loxP}$-lineage cells in the colon were stained with TER119 (erythroid lineage) and CD31 (endothelial, platelet, and leucocyte lineage) antibodies (Supplemental Figure 6). To define a gene set clearly linked to the ENS, we
compared differentially expressed genes in RaraDNα; RETCreERT2-EYFP; Wnt1Cre mice. In total, 594 genes were differentially regulated versus age-matched control cells in both mouse lines (Supplemental Figure 7 and Supplemental Table 3). Of these, a core set of 115 genes were regulated in the same direction by RARαDNα in ENCDC at E11.5 and E13.5 (Supplemental Tables 4 and 5). Gene enrichment pathway analysis of the aforementioned 2 sets of genes showed many pathways related to neuron development (Supplemental Figure 7). To validate RNA-seq data, we selected 2 differentially expressed genes of ENCDC at E11.5 (Stmn2 and Pax3) and performed quantitative PCR (qPCR), which confirmed differences predicted by RNA-seq (Supplemental Figure 8). The RNA-seq data for Ret and Phox2B in E11.5 stomach also correlate well with our IHC for RET and PHOX2B protein (Figure 4, Figure 5, and Supplemental Figure 9).

**Discussion**

RA regulates activity of the RAR/RXR transcription factor family to alter gene expression and influence many aspects of development (33). Prior studies using constitutive KO mice, RAR antagonist, stem cells, or vitamin A depletion in mice and rats show critical roles for RA in the developing ENS (21–32). For example, loss of the RA biosynthetic enzyme RALDH2 causes extensive bowel aganglionosis in Raldh2−/− mice.
While Raldh1−/− and Raldh3−/− mice have milder ENS defects (25), the Raldh2−/− phenotype probably occurs because RA is needed in vagal paraxial mesoderm starting at E9 in mice (or E1.5 in avians) to induce Ret expression in ENCDC as these cells migrate from neural tube to bowel (24). In contrast, when RAR signaling was blocked by the chemical pan-RAR antagonist BMS493 at E11.5 in organ culture (26) or at E12.5 in dissociated cell culture (27), RET expression in ENCDC appeared unaffected by RAR inhibition. Nonetheless, RAR inhibition at these later stages impaired ENCDC proliferation, bowel colonization, nerve growth, neuronal differentiation, and ENS patterning (23, 26, 27). Consistent with these observations, nutritional deficiency in the RA precursor vitamin A causes distal bowel aganglionosis in mice, mimicking human Hirschsprung disease (HSCR) (26), and gene variants in people with HSCR may alter RA signaling (45, 46). These studies suggest that RA signaling could impact ENS development in many ways, depending on the timing and severity of the RA signaling defect.

Prior strategies did not define the cell types influenced by RA signaling. To determine how cell-autonomous RAR signaling affects the ENS lineage, we used a potent CRE-inducible dominant-negative RAR transgene (RARαT403) that blocks all 3 RAR receptors (i.e., RarαDNLoxP/+ mice) (47). By breeding to several different CRE-expressing mouse lines, we induced RARαT403 (RARαDN) expression selectively within ENCDC at specific times during development. Our RaraDNLoxP/+; Wnt1Cre+ studies show that cell-autonomous RAR signaling within ENCDC is required to activate Ret expression early in development, consistent with data from zebrafish (21) and avian models (24). RAR signaling is also needed to activate Ret in the ureteric bud, suggesting that similar cell-autonomous mechanisms may control Ret expression in the kidney and developing ENS (34, 48). In contrast, when CRE expression is driven by Ret regulatory elements, RaraDN

Figure 7. RaraDNLoxP/+; Sox10Cre mice had extensive distal bowel aganglionosis. (A–E) E12.5 control or RaraDNLoxP/+; Sox10Cre whole bowel was stained with TuJ1 antibody (red) and DAPI (blue). Insets highlight mutant stomach (C), proximal small intestine (D), and distal small intestine (E). Sparse TuJ1+ nerve cell bodies were seen in the proximal small bowel (D) but not in more distal small bowel (E) of RaraDNLoxP/+; Sox10Cre mice. Scale bars: 500 μm (A and B) and 100 μm (C–E). (F) Images of either RaraDNLoxP/+ (control) or RaraDNLoxP/+; Sox10Cre E15.5 embryos. Note the defective eye and craniofacial development of the mutant embryos. (G and H) E15.5 RaraDNLoxP/+; Sox10Cre bowel were stained with TuJ1 (green) and counter-stained with DAPI. Note the TuJ1+ network at proximal small intestine (G) and distal colon (H). Scale bars: 100 μm. (I–R) RaraDNLoxP/+ (control) or RaraDNLoxP/+; Sox10Cre bowel were stained with HuC/D (red) and TuJ1 (green) antibodies. Representative images of esophagus (I and J), stomach (K and L), proximal small intestine (M and N), distal small intestine (O and P), and end of distal colon (Q and R). Scale bar: 50 μm. (S) Proximal small intestine of E15.5 RaraDNLoxP/+; Sox10Cre bowel. Regions outlined by boxes indicate which areas are enlarged in (T–V). Note extrinsic nerve fibers (T and U). Scale bars: 50 μm (S) and 50 μm (T–V). n = 3, RaraDNLoxP/+ (control); n = 3, RaraDNLoxP/+; Sox10Cre.
Figure 8. TyrCre-driven RarαDN reduces enteric neuron number and causes abnormal ENS patterning. (A–H) E13.5 RarαDN<sup>loxP/+; TyrCre<sup>+</sup> or TyrCre<sup>+</sup> (control) bowels stained with TuJ1 antibody (red) and DAPI. Scale bars: 1 mm (A and E). Boxes in A and E indicate regions of stomach (B and F), midgut (C and G), and distal colon (D and H) that are magnified in adjacent images. Scale bars: 50 μm (B–H). (I) RarαDN<sup>loxP/+; TyrCre<sup>+</sup> appear similar to control (TyrCre<sup>+</sup>) at birth but did not feed and lack a milk spot in stomach (n = 6). (J) RarαDN<sup>loxP/+; TyrCre<sup>+</sup> distal bowel appeared grossly similar to control (TyrCre<sup>+</sup>) at P0 (n = 10). (K) TdTomato<sup>+</sup> images of proximal small intestine (PSI) and colon of either RarαDN<sup>loxP/+; TyrCre<sup>+</sup> or TyrCre<sup>+</sup> (control) E18.5 pups. Scale bar: 1 mm. (L) Whole-mount IHC of E18.5 RarαDN<sup>loxP/+; TyrCre<sup>+</sup> or TyrCre<sup>+</sup> (control) bowels using HuC/D (red) and NOS1 (green) antibodies and via TdTomato (blue) fluorescence. Scale bar: 100 μm. (M) All TdTomato<sup>+</sup> cells in the small bowel and colon were HuC/D<sup>+</sup> in E18.5 RarαDN<sup>loxP/+; TyrCre<sup>+</sup> and TyrCre<sup>+</sup> (control) mice. (N–S) Total HuC/D<sup>+</sup> neurons were reduced in PSI, distal small intestine (DSI) and colon of RarαDN<sup>loxP/+; TyrCre<sup>+</sup> mice (N–P), but the percentage of HuC/D<sup>+</sup> neurons expressing NOS1 was increased in mutants (Q–S), n = 3, TyrCre<sup>+</sup> (control); n = 3, RarαDN<sup>loxP/+; TyrCre<sup>+</sup>. Two-tailed unpaired Student’s t tests were used for statistics.
induction did not lead to loss of RET protein in ENCDC, suggesting that RA is needed to turn on Ret but not to maintain Ret expression, consistent with our prior BMS493 data (26, 27). We were surprised to discover that PHOX2B protein was also undetectable in ENCDC of RarαDNLoxP/+; Wnt1Cre+ mice. Loss of PHOX2B could, by itself, explain the loss of RET in RarαDNLoxP/+; Wnt1Cre+ ENCDC (40), but we are unable to find evidence that PHOX2B regulates RET in the kidney, suggesting that Ret is regulated by RA via additional PHOX2B-independent mechanisms, as supported by prior studies (30, 45, 49).

There were many other interesting observations. First, the thick chains of enteric neurons seen in the distal bowel of RarαDNLoxP/+; RETCreERT2-EYFP tandem mice closely resemble the colon ENS patterning defect we observed in BMS493-treated fetal gut organ cultures (26). This suggests that the normal dispersion of enteric neurons into small colon ganglia is RA dependent. Second, in fetal RarαDNLoxP/+; SOX10Cre+ mice, we saw some clustered small bowel enteric neurons near extrinsic nerve fibers but far from the more proximal ENS cells. These clusters closely resemble Schwann cell–derived ENS described by Uesaka et al., but the ENCDC we observed were restricted to small regions of fetal bowel (10). Alternatively, these cells might originate from the “mesenteric neural crest cells” recently described by Yu et al. that they hypothesize contribute to human skip segment HSCR (8). Third, vagus nerves at E11.5 in RarαDNLoxP/+; Wnt1Cre+ mice occupied a smaller area of the stomach compared with control animals. Since vagus nerve fibers were not TdTomato labeled in Wnt1Cre; R26R-TdTomato mice, this suggests reciprocal interactions between migrating Wnt1Cre lineage ENC-DC and growing vagal fibers. Fourth, in addition to approximately 80% reduction in total neuron number in the ENS of RarαDNLoxP/+; TyrCre+ mice, there was a striking increase in the percentage of enteric neurons that express NOS1 (nitric oxide synthase). We confirmed that these NOS1+ neurons had expressed CRE using an R26R-TdTomato reporter. This suggests that NOS1+ neuron differentiation is less dependent on RAR signaling than other neuron subtypes or that RAR turns off Nos1 expression in some enteric neuron subtypes. Finally, we found an almost complete loss of submucosal neurons in RarαDNLoxP/+; RETCreERT2-EYFP tandem mice treated

**Figure 9. Inactivation of RAR signaling in Ret lineage at E8.5 or E10.5.** (A) RaraDN<sup>cre+/-</sup>; RETCreERT2-EYFP<sup>CreERT2</sup> have CreERT2 knocked into the Ret locus and ChR2-Lox-Stop-Lox-EYFP in the nearby Rosa26 locus on the same chromosome. Tamoxifen activates CreERT2 to induce expression of RaraDN and EYFP. (B and C) E13.5 whole bowel from control (RaraDN<sup>cre+/-</sup>) that lacks RETCreERT2-EYFP<sup>CreERT2</sup> (B) RaraDN<sup>cre+/-</sup>; RETCreERT2-EYFP<sup>CreERT2</sup> (C) after E10.5 tamoxifen treatment. ENS was visualized using TuJ1 (red) and GFP (green) antibodies. (D-I) Boxes indicate regions of esophagus (D and E), small bowel (F and G), and distal colon (H and I) that are magnified in adjacent images. Scale bars: 1000 μm (B and C) and 500 μm (D-I). (J and K) E13.5 bowels after E8.5 tamoxifen treatment. Scale bar: 1000 μm. (L and M) Regions of magnified images from RaraDN<sup>cre+/-</sup>; RETCreERT2-EYFP<sup>CreERT2</sup> small bowel (L) or distal colon (M). Scale bar: 100 μm. (N and O) RET (red) and GFP (green) antibody stained E13.5 colon after E10.5 tamoxifen treatment. (P and Q) PHOX2B (green), GFP (red), and DAPI (blue) stained E13.5 colon after E10.5 tamoxifen treatment. Scale bars: 100 μm. n = 3 in each group.
with tamoxifen at E10.5, suggesting a critical role for RA signaling in radial migration of ENCDC to form submucosal plexus. The normal postnatal growth of these tamoxifen-treated RaroDN mice and normal appearance of the adult bowel suggests that a loss of approximately 90% of submucosal neurons is well tolerated, at least in mice. This is interesting, in part, because we know little about mechanisms controlling radial migration of ENS precursors to the submucosal plexus, with prior studies implicating only GDNF and netrin/DCC signaling in this process (50, 51). These observations highlight the remarkable range of ENS abnormalities that may occur when RAR signaling is inadequate and are consistent with the observation that low serum vitamin A is associated with increased constipation in children with autism (52).

One concern is that we did not evaluate ENS biology in every possible control group, so we cannot exclude some effects of tamoxifen, Cre alleles, fluorescent reporters, or the RaroDN^{cre/+} allele in isolation. For example, a recent study clearly shows that Wnt1Cre^{+/-} increases the severity of the Ednrb^{-/-} ENS phenotype (8). We also were underpowered to examine the effect of sex on ENS phenotypes, an issue that could be important for some of the milder phenotypes we examined.

One advantage of our strategy is that CRE-dependent expression of RARαDN and a fluorescent reporter in the same cells facilitated flow sorting and RNA-seq to identify RAR targets. Our analyses identified thousands of differentially expressed genes in RARαDN-expressing versus control ENCDC at E11.5 and E13.5

Figure 10. Postnatal ENS phenotype when RAR signaling is inactivated in Ret lineage starting from E10.5. (A) Tamoxifen was given to pregnant mice at E10.5 to activate the RaroDN transgene, and bowel was harvested at about 2 months of age. (B) RaroDN^{cre/+} (control) vs. RaroDN^{cre/+}: RETCreERT2-EYFP^{tandem} (mutant) bowels appear similar. (C–N) Myenteric and submucosal plexus in RaroDN^{cre/+} (control) or RaroDN^{cre/+}: RETCreERT2-EYFP^{tandem} (mutant) animals were visualized in whole mounts using HuC/D (green) and TuJ1 (red) antibodies plus DAPI (blue). Scale bar: 100 μm. (O–T) Quantification of HuC/D+ neurons shows about 50% fewer myenteric and about 90% fewer submucosal neurons in RaroDN: RETCreERT2-EYFP^{tandem} (mutant) animals compared with RaroDN^{cre/+} (control) (n = 3 in each group). Two-tailed unpaired Student’s t tests were used for statistics.
with 115 genes regulated in the same way by RARαDN at each age. Interpreting these data is complicated, since RARαDN induces changes in cell type ratios, in addition to changes in gene expression in individual cells. Furthermore, it is not clear if the induced changes occur because of direct effects on RAREs or if they reflect more global effects on the differentiation state of the sequenced cells. For example, loss of PHOX2B in E11.5 ENCDC should change the expression of many genes, independent of effects on RAR activity.

As one strategy to further define the role of RAR signaling, we narrowed our lists to include only differentially expressed genes (FDR < 0.2) where expression changed in the same direction at E11.5 and E13.5 for RARαDN+ cells (Supplemental Tables 4 and 5). At E11.5, there were 55 genes expressed at higher levels in WT ENCDC than in RARαDN+ ENCDC. Thirty-five of these genes had easily identified functions in neurons. Thirteen genes at E11.5 were more abundant in RARαDN+ ENCDC than in WT ENCDC. This list included Rest, an epigenetic master negative regulator of neurogenesis (53, 54), and Bmp4, a gene with complex roles in ENS patterning (55–62). At E13.5, our list included 44 genes more abundant in WT than in RARαDN+ cells. Twenty-nine of these genes had easily identified roles in neurons. Genes more abundant in RARαDN+ than in WT cells at E13.5 included Rest, Sox10, and Ets1, consistent with a role for RAR in ENS neurogenesis. The SOX10 transcription factor is essential for ENS development (63), at least in part because SOX10 activates RET expression (64, 65). However, as multipotent ENCDC differentiate into enteric neurons, SOX10 expression is lost, while enteric glia continue to express SOX10 through adulthood (3). ETS1 enhances Sox10 expression and is essential to make radial glia in Xenopus (66). Consistent with these mRNA findings, SOX10+ cells were abundant in myenteric and submucosal plexus of adult Raraa-DN-expressing mice, even though Raraa-DNLoxP/+; RETCreERT2-EYFPlox/lox had an approximately 90% reduction in submucosal neurons and the enteric glia are

Figure 11. RNA-seq shows that many genes are dysregulated after Raraa-DN expression in the ENS. (A) ENCDC were isolated from E11.5 stomach of Raraa-DNLoxP/+; Wnt1Cre; R26R-TdTomato and Wnt1Cre; R26R-TdTomato (control) mice based on TdTomato fluorescence (n = 4 each). (B) ENCDC were isolated from E13.5 colon of Raraa-DNLoxP/+; RETCreERT2-EYFPlox/lox or RETCreERT2-EYFPlox/lox (control) mice based on EYFP fluorescence after tamoxifen treatment at E10.5 (n = 4 each, but 1 Raraa-DNLoxP/+; RETCreERT2-EYFPlox/lox failed quality control, leaving n = 3 for this genotype). (C and D) Multidimensional scaling analysis (MDS) of RNA-seq data. (E and F) Volcano plots show differentially expressed genes.
derived from RET- ENCDC (67). In addition, many Wnt ligands (Wnt1, Wnt2, Wnt4, and Wnt10a) were highly differentially expressed in E11.5 ENCDC of RarαDNLoxP/+; Wnt1Cre versus control. Since Wnt signaling plays important roles in many aspects of neural development (68–70), RAR might alter Wnt signaling to regulate ENCDC development. Collectively, these data suggest that cell-autonomous RAR signaling directs neurogenesis in the ENS from multipotent ENCDC and that cell-autonomous RAR signaling has distinct effects at many stages of ENS development. Our observations further suggest that maternal retinoid status during pregnancy and postnatal vitamin A deficiency or excess could have long-term effects on ENS structure and function. This may be important because vitamin A deficiency is a common problem in many regions of the world (71).

Methods

Mouse strains. RarαDNLoxP-STOP-LoxP/+ (previously described in ref. 34) were rederived from sperm into C57BL/6J background by the CHOP transgenic core. H2az2(Tg[Wnt1cre]11Rth) (Research Resource Identifiers [RRID]: 2386570_JAX:003829; referred to as Wnt1Cre), TyrCre (RRID: MGI:358524_JAX:029788), SOX10Cre (MGI:3586900_JAX:025807), Rosa26R-tdTomato (Gt[ROSA]26Sortm1[tdTomato]Hze, MGI: 2449038, RRID:IMSR_EM:09668), and Gt[ROSA]26Sortm9(CAGtdTomato)Hze (RRID:IMSR_JAX:007909; referred to as R26R-TdTomato) were from The Jackson Laboratory and maintained in C57BL/6J background. RETCreERT2/EYFPmice, a gift from Wenqin Luo (University of Pennsylvania), were generated by breeding RETCreERT2 (RRID: MGI:4437245) to mice with ChR2-Lox-Stop-EYFP in the Rosa26 locus (72). Mouse Ret and Rosa26 loci are close together (about 5 million bp), but RETCreERT2-EYFPmice recombined so that they are on the same chromosome. Ret<sup>TGM/TGM</sup> (Ret<sup>-/-</sup> allele Ret<sup>tm1en</sup>) on C57BL/6J were previously described (73). A complete list

![Figure 12. Pathway analysis for genes dysregulated by RARαDN.](image)
of mouse strains appears in Supplemental Table 6. Genotyping was performed using previously published and potentially novel primers (Supplemental Table 7).

**Mouse husbandry.** Mice had a 12-hour light-dark cycle with free access to food and water. Additional details based on ARRIVE guidelines (74) are in Supplemental Table 8. For timed pregnant mating, RaraDN female mice were bred with Cre-expressing male mice in the late afternoon. Successful mating was confirmed by vaginal plug the next morning, which was designated E0.5.

**EdU incorporation assay.** EdU analyses were performed using a Click-iT EdU imaging kit (Thermo Fisher Scientific, C10337). Timed pregnant animals (E12.5) were injected (i.p.) with EdU (12.5 μg/gm body weight). Tissues were dissected 4 hours after EdU and fixed (4% paraformaldehyde [PFA], 30 minutes at room temperature) before staining, using manufacturer’s protocol.

**Whole mouse embryo imaging using 3DISCO clearing.** 3DISCO imaging was performed as described (38) with modification. Briefly, E10.5 embryos from timed-pregnant females were washed in PBS (pH 7.4), fixed (4% PFA, 1 hour, room temperature), and then incubated in SOX10 (1:200, Novus Biologicals, AF2864) and TuJ1 (1:2000 dilution, Covance, PRB-435P, RRID:AB_2313773) antibodies in 1× PBS with 0.5% Triton X-100 (PBST) and 5% normal donkey serum (NDS) (4°C, constant rocking, 72 hours). After washing 4 times (PBS, room temperature, 15 minutes per wash), embryos were incubated in secondary antibodies and Hoechst 33342 for nuclear staining (2 μg/mL, Invitrogen, catalog H3570) in PBST with 5% NDS (4°C, overnight) and then washed 4 times (PBS, room temperature, 15 minutes per wash) and subjected to 3DISCO clearing using a series of tetrahydrofuran (MilliporeSigma, 401757) incubations (room temperature, 50%, 70%, and then 80% in double-distilled water, 60 minutes each, with rocking), followed by incubation in 100% tetrahydrofuran (3 × 20 minutes, room temperature, rocking). Embryos were then incubated overnight in dibenzyl ether either (MilliporeSigma, 108014-1KG) at room temperature and mounted in dibenzyl ether before confocal imaging.

**IHC.** For whole-mount staining, fetal bowel was fixed as a tube. Adult bowels were opening along mesentery and pinned flat to Sylgard before fixation. Bowels were fixed with 4% PFA (40 minutes, room temperature, rocking), washed (4 × 10 minutes, PBS, room temperature), blocked (5% NDS in PBST, room temperature, 1 hour), and then incubated overnight (4°C) with primary antibodies in 5% NDS in PBST. Secondary antibody staining, unless otherwise specified, was done by incubating samples in a 1:400 dilution of the appropriate antibody (1 hour, 25°C). All the primary and secondary antibodies are listed in Supplemental Table 9.

**RNA-seq analyses.** E11.5 stomach ENCDC were isolated from RaraDN<sup>lox/lox</sup>; Wnt1Cre<sup>+</sup>; R26R-TdTomato and Wnt1Cre<sup>+</sup>; R26R-TdTomato (control) mice. E13.5 colon ENCDC were isolated from RaraDN<sup>lox/lox</sup>; RETCreERT2-EYFP<sup>lox/lox</sup> or RETCreERT2-EYFP<sup>lox/lox</sup> (control) mice after treatment with tamoxifen (oral gavage, 233 mg/kg body weight dissolved in corn oil) at E10.5. Individual E11.5 stomach and E13.5 colon were incubated with collagenase (MilliporeSigma, C0130, 0.2 mg/mL) and dispase (MilliporeSigma, 494207800, 0.2 mg/mL) in Ham’s F12 media (Thermo Fisher Scientific, 11765054) with 1% BSA (37°C, 5% CO<sub>2</sub>, 20 minutes) and then triturated by pipetting up and down 15 times using a 1000 pipette tip before passing through a 30 μm strainer (Corning, 352340). Cells with TdTomato or EYFP fluorescence were purified by flow sorting (MoFlo Astrios high speed cell sorter, 100 μm nozzle), pelleted by centrifugation (500g, 4°C, 5 minutes), washed once with PBS, and frozen immediately at −80°C until analysis. RNA isolation, library preparation, and RNA-seq analysis were performed by University of Pennsylvania Next-Generation Sequencing Core. RNA extracted using the GenElute Single Cell RNA Purification Kit (MilliporeSigma, RNB300) was analyzed on a 2100 Agilent Bioanalyzer using an RNA 6000 Pico Kit (Agilent, 5067-1513). The libraries were made using the combination of the CloneTech (now Takara), SMART (cDNA synthesis and amplification), and Illumina Nextera XT (cDNA to library) kits. Libraries were then single-end sequenced on an Illumina HiSeq 4000, using Sequencer Software Real-Time Analysis (RTA) 2.7.7 and Illumina HiSeq Control Software (HCS) HD 3.4.0.38. Reads were aligned to remove repeat sequences and ribosomal RNA reads and then processed using RNA-seq unified mapper (RUM) package (75). RUM files were visualized in the TessLA browser for down-stream analyses. MDS analysis, volcano plot, and heatmaps were made on R 4.0.2 platform using limma, edgeR, Glimma, gplots, org.Mm.eg.db, and RCcolorBrewer. We used ggrepel, ggplot2, and reshape to generate Volcano plots, and we used tidyr, gplots, and ggplot2 to generate heatmaps. Gene expression pathway analyses were performed using Ingenuity Pathway Analysis (IPA) package (Qiagen) and Metascape Gene Annotation & Analysis Resource (76). A Venn diagram was generated using the Draw Venn Diagram software of Bioinformatics & Evolutionary Genomics, VIB/UGent, Belgium. The RNA-seq raw data have been deposited in the GEO NIH data repository under the accession no. GSE165344.

**Real time PCR.** E11.5 stomach ENCDC of RaraDN<sup>lox/lox</sup>; Wnt1Cre<sup>+</sup>; R26R-TdTomato and Wnt1Cre<sup>+</sup>; R26R-TdTomato (control) mice were isolated via the same protocol as for RNA-seq analysis. RNA was purified using
PicoPure RNA isolation kit (Arcturus, KIT0202). cDNA was made by using First-strand cDNA synthesis using superscript II RT kit (Invitrogen, 18064-022) with Oligo(dT)12-18 primer (Invitrogen, 18418012). Quantitative PCR (qPCR) was performed using SYBR Green qPCR mixture (MilliporeSigma, S4438). All primers have been previously described and listed in Supplemental Table 10. Ct values were normalized to Gapdh mRNA.

Confocal microscopy. High-resolution 3-dimensional images were acquired with a Zeiss LSM 710 confocal microscope using Zeiss ZEN 2.3 SP1 FP3 (black) (Version 14.0.18.201; Zeiss) software and a 20×/0.8 air or 63×/1.4 oil DIC M27 Plan-Apochromat objective, except for whole bowel E12.5 and E13.5 imaging, which employed a 5× objective and the ZEN Tile Scan function. Image stitching employed ZEN software. Z-stacks were created with ImageJ/FIJI. Increments between each slice were 1 μm, except for scanning the whole E10.5 embryo, where increments between slices were 3 μm. Videos of Z-stacks were created with the animation function of Imaris 9.02.

Image analysis. Early embryonic, perinatal, and adult mouse bowel cell counting used > 5 randomly selected ×20 fields per animal in each region and FIJI CellCounter module (NIH). Investigators were blinded to genotype when comparing RarαDN mutant and control mice.

Statistics. All experiments include at least 3 replicates. Statistical analyses employed GraphPad Prism 7 software. Data normality analyses employed the Shapiro-Wilk test. Comparisons between groups used 2-tailed unpaired Student’s t tests. P < 0.05 is considered statistically significant. Data show mean ± SEM.

Study approval. Mouse studies were performed with approval from the Institutional Use and Care Committee at The Children’s Hospital of Philadelphia (CHOP) Research Institute (IAC 19-001041) and the Animal Studies Committee of Washington University School of Medicine in St. Louis (no. 20120130).

Author contributions
TG and ROH led this study and wrote the manuscript. TG, ECWJ, RS, JBA, and ROH designed research studies, conducted experiments, acquired data, analyzed data, and edited the manuscript.

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