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37/67-Laminin Receptor Facilitates Neural Crest Cell Migration During Enteric Nervous System Development

Ming Fu, MD, PhD[1], **Amanda J. Barlow-Anacker, PhD**[2], **Korah P. Kuruvilla, PhD**[1], **Gary L. Bowlin, PhD**[3], **Christopher W. Seidel, PhD**[4], **Paul A. Trainor, PhD**[4,5], **Ankush Gosain, MD, PhD**[1,6,**]

[1]Division of Pediatric Surgery, Department of Surgery, University of Tennessee Health Sciences Center, Memphis, Tennessee, United States of America.

[2]Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin, United States of America.

[3]Department of Biomedical Engineering, University of Memphis, Memphis, Tennessee, United States of America.

[4]Stowers Institute for Medical Research, Kansas City, Missouri, United States of America.

[5]Department of Anatomy and Cell Biology, University of Kansas School of Medicine, Kansas City, Kansas, United States of America.

[6]Children's Foundation Research Institute, Le Bonheur Children's Hospital, Memphis, Tennessee, United States of America.

Abstract

Enteric nervous system (ENS) development is governed by interactions between neural crest cells (NCC) and the extracellular matrix (ECM). Hirschsprung disease (HSCR) results from incomplete NCC migration and failure to form an appropriate ENS. Prior studies implicate abnormal ECM in NCC migration failure. We performed a comparative microarray of the embryonic distal hindgut of wildtype and *EdnrB*$^{NCC-/-}$ mice that model HSCR and identified laminin-β1 as upregulated in *EdnrB*$^{NCC-/-}$ colon. We identified decreased expression of 37/67 kDa laminin receptor (LAMR), which binds laminin-β1, in human HSCR myenteric plexus and *EdnrB*$^{NCC-/-}$ NCC. Using a combination of in vitro gut slice cultures and ex vivo organ cultures, we determined the mechanistic role of LAMR in NCC migration. We found that enteric NCC express LAMR, which is downregulated in human and murine HSCR. Binding of LAMR by the laminin-β1 analog

[**]**Corresponding Author:** Ankush Gosain, MD, PhD, Children's Foundation Research Institute, 50 N. Dunlap Street, Room 320, Memphis, TN 38105, Phone: 901-287-6219, Fax: 901-287-4434, agosain@uthsc.edu.

Author Contributions

AJBA, MF, and AG were responsible for study concept and design. MF, AJBA, and KPK were responsible for acquisition of data. MF, AJBA, GLB, CWS, PAT, and AG were responsible for analysis and interpretation of data. MF and AG were responsible for drafting and revising of the manuscript. All authors approved the final manuscript.

Disclosures

The authors have no competing interests to declare.

Data Availability

Data from the microarray has been deposited in Minimum Information about a Microarray Experiment (MIAME)-compliant format in the National Institutes of Health Gene Expression Omnibus (GEO) repository (Accession: GSE138619).

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YIGSR promotes NCC migration. Silencing of LAMR abrogated these effects. Finally, applying YIGSR to E13.5 *EdnrB*$^{NCC-/-}$ colon explants resulted in 80–100% colonization of the hindgut. This study adds LAMR to the large list of receptors through which NCC interact with their environment during ENS development. These results should be used to inform ongoing integrative, regenerative medicine approaches to HSCR.

Keywords

Hirschsprung disease; Extracellular Matrix; Neural Crest; Development

Introduction

Hirschsprung disease (HSCR, Online Mendelian Inheritance in Man #142623) is a common cause of neonatal bowel obstruction and is potentially lethal if untreated. HSCR results from failure of neural crest cells (NCC) to migrate into and colonize the distal colon to form the enteric nervous system (ENS) which controls motility, secretion, digestion and absorption (1). The resulting aganglionic segment exhibits tonic contraction, resulting in functional bowel obstruction (2). Untreated, this leads to progressive bowel distention, the development of Hirschsprung-Associated Enterocolitis, and death (3). During development, NCC utilize receptor-ligand interactions to integrate multiple signals from their surrounding intestinal microenvironment in order to survive, proliferate, migrate and differentiate (4). More than twenty gene defects predisposing to HSCR, involving trophic factors, cell surface receptors, transcription factors and signaling molecules, have been identified (5). Many HSCR studies have noted abnormally high expression of ECM components, including laminin, collagens IV and VI, and tenascins, among others (6–9). Additionally, NCC receptors that govern interactions with the ECM (e.g. β1-integrins) have been implicated in HSCR pathogenesis (10–12). Further, studies from human HSCR patients have demonstrated that elevated levels of ECM components, in particular laminin, extend beyond the aganglionic segment and into the proximal colon (13, 14). Together, these data indicate that an altered ECM is a common finding in HSCR, regardless of specific underlying genotype.

The two most common gene defects found in HSCR are those of "*rearranged during transfection"* (*Ret*) and "*endothelin receptor B"* (*EdnrB*) (15–18). Both gene defects encode receptors on NCC that respond to ligands in the environment (glial derived neurotrophic factor (GDNF) and endothelin-3 (Et-3), respectively) and coordinately control NCC colonization of the ENS. Deletion of any of these ligands or receptors results in various degrees of NCC migration failure and aganglionosis (19–22). Interestingly, Et-3 mutant mice display increased levels of laminin in the aganglionic region (9). Laminins are heterotrimers, consisting of an α-, β- and γ-chain (23). Laminin-111 (α1, β1 and γ1), hereafter called "laminin," is the primary isoform in the developing gut. Increased laminin has been observed in both the aganglionic and ganglionated regions of HSCR patients and also when *Ret* expression is decreased (13, 24). In addition, a series of elegant studies identified a 110 kDa laminin binding protein (LBP110) on NCC that binds the laminin-α1 chain to promote NCC differentiation, and showed that NCC differentiation occurs prematurely in HSCR mice (9, 11, 25, 26). Additionally, β1-integrin receptors on NCC

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govern interactions with multiple ECM components, including the laminin-α1 chain, and mice with NCC-targeted deletion of β1-integrin show colonic aganglionosis (10, 27, 28). Finally, increased ECM laminin has been posited as an age-dependent change in the gut environment that renders it "non-permissive" to NCC migration (29). Collectively, laminin may therefore regulate interactions between NCC and the ECM, influencing their proliferation and differentiation during normal ENS development and in HSCR pathogenesis.

Laminin-β1 binds a 37/67-kDa laminin receptor (37/67LR, called 37/67-LAMR or LAMR) (30). This receptor exists in two forms, a 37-kDa laminin receptor precursor (37LRP) which undergoes homo-dimerization and fatty acid acylation, and the resultant 67LR (31, 32). Typically, both forms are present in cells, with 37LRP found primarily in the cytoplasm and nucleus, and 67LR primarily on the plasma membrane (with minimal cytoplasmic presence) where it can interact with ECM laminin (33, 34). Multiple studies in tumors have shown that increased expression of LAMR correlates with tumor invasiveness and metastasis, and inhibition of this receptor is actively being pursued as an anti-metastasis therapy (35–37). YIGSR (Tyr-Ile-Gly-Ser-Arg) is a pentapeptide that represents the site on laminin-β1 where LAMR binds (33). YIGSR has been shown in multiple cancers to bind LAMR and promote a pro-migratory, metastatic phenotype (33, 35, 37–39). In those systems, laminin/YIGSR binding to LAMR also promotes a positive feedback loop, with upregulation and cell surface expression of LAMR (40, 41). No prior studies have established the presence of LAMR on NCC or identified a functional role for YIGSR/LAMR signaling in ENS development.

Here, we have identified the presence of the 37/67 kDa laminin receptor (LAMR), which binds the laminin β1 chain, on murine NCC and NCC-derived cells and in human enteric neurons. Further, our data suggest that the NCC LAMR has a functional role in the development of HSCR aganglionosis and may be targetable as part of a multimodal therapeutic strategy to reduce or rescue colonic aganglionosis.

Methods

Animals and Tissue Collection

All animal care procedures were approved by the Animal Care and Use Committees of the University of Wisconsin-Madison (Protocol #M01394) and University of Tennessee Health Science Center (Protocols #16–021 and 16–051). Mice with NCC conditional deletion of Endothelin Receptor B ($EdnrB^{NCC-/-}$) were generated by mating animals with a floxed *EdnrB* allele ($EdnrB^{flex3/flex3}$) (Jackson Labs #003295) with mice expressing *Cre recombinase* under the control of a *Wnt-1* enhancer element, resulting in either wild-type (WT), heterozygous ($EdnrB^{NCC+/-}$) or homozygous deletion of *EdnrB* ($EdnrB^{NCC-/-}$) (42). These animals are maintained as a breeding colony both with and without the $Rosa26^{floxStop/tdTomato}$ reporter which results in expression of tdTomato in those cells where Cre recombination has taken place. Conventional *EdnrB* knockout mice were generated by insertion of bacterial beta-galactosidase (*lacZ*) into the endothelin receptor B gene locus (provided by Jim Pickel NIH/NIMH Transgenics) and are referred to as $EdnrB^{lacZ/+}$ (heterozygote) and $EdnrB^{lacZ/lacZ}$ (homozygous deletion) in this study (43). Timed breedings were performed in order to generate embryos of defined time-points, with the day

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of vaginal plugging recorded as embryonic day (E) 0.5. Animals of both genders were included throughout, and gender was considered a biological variable. Mice were housed in a specific-pathogen free environment and were allowed *ad libitum* access to standard rodent chow and water.

Murine Tissue Harvest and Microarray

RNA was isolated from the terminal colon of E14 wild type (WT), *EdnrB*$^{NCC-/-}$, and *EdnrB*$^{lacZ/lacZ}$ embryos and used to probe Affymetrix GeneChip mouse genome microarrays [Fig.S1]. A nine-way independent pair-wise comparison between WT and *EdnrB*-mutant tissue was performed. Log_2 mean values equivalent to a gross fold change of ±1.5 used as a threshold to identify differentially expressed genes.

Semi-quantitative PCR

Semi-quantitative PCR was then performed to validate the differential gene expression. The terminal colon was dissected from E14 WT, *EdnrB*$^{lacZ/lacZ}$ and *EdnrB*$^{NCC-/-}$ guts and total RNA was extracted using TRIzol Reagent (Life Technologies, Grand Island, NY). PCR was performed using Taq DNA polymerase (Life Technologies, Grand Island, NY) with primers specific for Laminin-β1 (forward primer 5'GAGTTCAGCTATGGCTGCGC3', reverse primer 5'GTCACAGATGATGTCATCCAC3') and β-actin (forward primer 5'CATGTACGTAGCCATCCAGGCT3', reverse primer 5'TCTCTTTGATGTCACGCACGA3'). Samples were analyzed in triplicate from n=3 terminal colons for each genotype.

Human tissue sections and immunohistochemistry

Following surgery for Hirschsprung disease, the entire length of bowel that is resected is sent for pathology analysis. This segment of bowel includes a length of at least 5 cm of ganglionated bowel proximal to the aganglionic region. With Institutional Review Board approval (16–04806-XP), patients who received surgery for rectosigmoid Hirschsprung disease at Le Bonheur Children's Hospital were identified and tissue blocks obtained for new analyses. Comparisons were made to age-matched, region-matched, non-Hirschsprung disease patients who underwent colorectal surgery during the same time period. Paraffin-embedded tissue blocks were obtained from pathology and the proximal margin of resected bowel sectioned. Sections were cut to 8μm thickness and mounted on positively charged slides. The slides were deparaffinized in xylene and rehydrated in 100% ethanol (twice for 2 min), as well as 70%, 50%, and 30% ethanol for 1 min each, followed by distilled water for 2 min. For antigen retrieval, the sections were placed in a 10mM citrate buffer in the microwave at 100% power for 6 min followed by 80% power for 6 min, and then cooled down to room temperature for 45 minutes followed by PBS wash. The sections were then blocked in PBST (Phosphate Buffered Saline with 0.2% Triton X-100) and 4% normal Donkey serum for 30 min at room temperature. Then, the first primary antibody (pan-neuronal marker HuC/D, 1:10000, Human IgG, a gift from Dr. Vanda A. Lennon, Mayo Clinic), diluted with PBST and 4% normal Donkey serum, was applied and incubated in a humidity chamber overnight at 4° C. Slides were then washed with PBS. Additional primary antibodies were used to identify LAMR expression: Rabbit 37/67 kDa Laminin receptor

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(1:200; Millipore) and Rabbit 67 kDa Laminin receptor (1:200; Abcam). Sections were washed with PBS and incubated with appropriate secondary antibodies (Life Technologies).

Embryonic whole intestine or colonic explant culture and immunohistochemistry

The whole intestine or colon, as appropriate, was taken from E12 or E12.5 mice and pinned down to 2.5% agarose with Minutien Pins (θ=0.20mm stainless steel; FST 26002–20). DMEM was supplemented with 10% fetal calf serum and penicillin 10 IU/mL and streptomycin 10 μg/mL (Gibco 15140–122), along with YIGSR (5μg/mL; Sigma, T7154) or Laminin-111 (10 μg/mL, Santa Cruz sc-29012). Explants were cultured for 24 hours before proceeding to fixation (4% PFA, 30 minutes, 25°C). The embryonic intestines were permeabilized and blocked with 4% donkey serum and 0.2% of Triton X-100 in 1xTBS (100mM Tris, 150mM NaCl) 40 minutes at 25°C, followed by incubation in 4°C for 18 hours with the following primary antibodies: Rabbit Tuj-1 (1:1000; Covance), Goat Sox10 (1:250; Santa Cruz Biotechnology Inc.), and Rabbit 37/67 kDa Laminin receptor (1:200; Millipore). Secondary antibodies (Life technologies) were applied and incubated at room temperature for 1 hour.

Slice culture and immunohistochemistry

Slice cultures allow efficient testing of multiple treatment conditions without a requirement for large numbers of animals. Additionally, large numbers of NCC can be induced to migrate out of slice cultures for analysis. Slice sections (300–400 μm length) were taken from E12.5 midgut starting 400 μm proximal to the cecum. Sectioned slices were directly placed on fibronectin-coated dishes (250 μg/mL fibronectin, Sigma; required for attachment of slice sections to the culture dish), except for the pre-treatment midgut specimens, which were incubated with GRGDSP (1 mg/mL) (44), an inhibitor of β1-integrin, at 37°C for one hour before sectioning and placement on fibronectin-coated dishes. GDNF (100 ng/mL; Gibco) with or without YIGSR (5 μg/mL) was added to Opti-MEM (Gibco), glutamine (2mM, Sigma), penicillin (10 IU/mL) and streptomycin (10 μg/mL) four hours after plating. Cultures were removed from the incubator after 20 hours of culture and fixed with 4% PFA for 20 minutes at 25°C. The slices were permeabilized and blocked with 4% donkey serum and 0.2% of Triton X-100 in 1xTBS (100mM Tris, 150mM NaCl) for 30 minutes at 25°C, followed by incubation at 4°C for 18 hours with the following primary antibodies: Rabbit Tuj-1 (1:1000; Covance), Goat Ret (1:200; Neuromics), Rabbit 37/67 kDa Laminin receptor (1:200; Millipore), 67 kDa Laminin receptor (1:200; Abcam), and Phalloidin with Alexa 488 (1:100; Cell Signaling).

Myenteric plexus whole-mount preparation

Mice, post-natal days (P) 11–21, were euthanized by isoflurane and cervical dislocation. The whole gastrointestinal tract was removed and flushed with ice-cold PBS, opened longitudinally, and flattened with pins on Sylgard dishes with mucosa side down. The smooth muscle, together with myenteric plexus, was peeled off using fine-pointed forceps under a dissection microscope. Samples were kept at −20°C in 50% glycerol until staining and analysis. The myenteric plexus sheets were permeabilized and blocked with 4% donkey serum and 0.2% of Triton X-100 in 1xTBS for 30 minutes at 25°C, followed by incubation with primary antibodies at 4°C for 18 hours with the following primary antibodies: HuC/D

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(1:10000; a gift from Dr. Vanda A. Lennon, Mayo Clinic), Rabbit 37/67 kDa Laminin receptor (1:200; Millipore), 67 kDa Laminin receptor (1:200; Abcam), followed by secondary antibody incubation: Donkey anti-Human Cy3 , 1:1000; Donkey anti-Rabbit, 1:1000 (Life technologies) at 25°C for 1 hour.

Neurosphere culture and lentiviral shRNA

RPSA siRNA (Applied Biological Material, #iV039047) and scrambled siRNA (Applied Biological Material, #LVP015-G) lentiviruses with a titer >10^7 IU/mL were prepared. E12 whole intestine was digested by collagenase (0.2 mg/mL) and dispase (0.2mg/mL) for 15 minutes at 37°C. The intestines were triturated, spun and re-suspended in NCC medium [DMEM with chicken embryo extract (10%; US Biological Life Sciences), N2 (1%; Invitrogen); B27 (2%; Invitrogen); β-mercaptoethanol (50 μM; Sigma-Aldrich), all-trans Retinoic Acid (35 ng/mL; Sigma-Aldrich), bFGF (20ng/mL; R&D Systems), EGF (20ng/mL; R&D Systems), Penicillin 10 IU/mL; Streptomycin 10 μg/mL with 1% FCS and Leukemia Inhibitory Factor (100 U/mL; Millipore ESG 1106)] on day zero. 10^5 cells were seeded in a 24-well dish. Scrambled and RPSA siRNA lentivirus (MOI 10) were added to the cultured cells with 300 μl of NCC medium. The cells were then cultured in the incubator with 5% CO_2 at 37°C until small neurospheres formed (typically 3–5 days). The neurospheres were collected, centrifuged and re-plated in ultra-low attachment 96-well (round bottom) dishes (Corning 7007) with NCC medium. Larger neurospheres (θ≈400 μm) formed after the cells were centrifuged and cultured for another two days.

Cell migration assay for lentiviral-infected cells

A 1.5mg/mL collagen gel was made by adding 0.25 volumes of 5X DMEM to 1 volume of 4mg/mL rat collagen gel (Advanced BioMatrix). 2.68 volumes of NCC medium were added to make the final collagen gel concentration 1.5mg/mL. Neurospheres were transferred from the low attachment dish to 150 μl of collagen gel in an 8-well chamber slide (Nalge Nunc International). GDNF (200 ng/mL) with or without YIGSR (10 μg/mL) was added to the well. Images were captured after 20 hours and 48 hours of culture. The culture chambers were removed from the incubator after 48 hours of culture for immunohistochemistry. Antibody to Ret (Goat IgG, 1:200) was applied and cultured at 4°C for 18 hours followed by washing and secondary antibody incubation with Donkey anti-Goat (Alexa 594, 1:1000) at 25°C for one hour.

Western blot analysis

For Western blot analysis of LAMR expression, the myenteric plexus, together with the smooth muscle, was isolated. ProteoExtract Subcellular Proteome Extraction Kit (Millipore) was used to perform subcellular protein extraction. The membrane and organelle fraction (fraction #2 based on manufacturer recommendations) was subjected to immunoblotting using antibodies to 37/67 kDa and 67 kDa laminin receptor. For Western blot analysis of LAMR expression after slice culture with GDNF and GDNF with YIGSR, cells that migrated out of the slices were used for protein extraction. To accomplish this, slice cultures were performed in 6-well fibronectin-coated culture dishes. After 24 hours of culture, the slices were removed, leaving only the cells that had migrated out of the slice. TRI reagent

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(Sigma) was used as lysis buffer. For Western blot analysis of siRNA efficiency, the lentiviral infected cells were used.

Cells were incubated with lysis buffer on ice for 10 minutes, homogenized by passing through a 20G PrecisionGlide needle (B-D Biosciences) 20 times, and centrifuged for 10 min at 20,000g at 4°C. Supernatants were collected, and protein concentration determined by protein assay (Bio-Rad). 50 μg of protein was used for gel electrophoresis using Mini-Protean TGX gels (Bio-Rad). Protein was transferred electrophoretically from gels to Nitrocellulose membranes (Bio-Rad). The membranes were incubated with appropriate antibodies. In brief, membranes were incubated with blocking solution (5% nonfat dry milk powder in PBS, 0.1% Tween 20 in PBS) at 25°C for 1 hour on a shaker. They were incubated overnight at 4°C with anti-37/67 kDa Laminin receptor (1:500) and anti-β-actin (1:1000) or anti-GAPDH (1:1000). Membranes were washed thoroughly and incubated for 1 hour with HRP conjugated anti-rabbit antibody for 1 hour at 25°C on a shaker. Membranes were washed and incubated with SuperSignal West Pico PLUS Chemiluminescent substrate (ThermoScientific) for 1 minute. The reagent was drained and the membranes were imaged.

Statistical analysis

Data are presented as the mean values ± standard error of the mean (SEM). Parametric (Unpaired Student's t-tests, one-way ANOVA and Tukey's post-hoc test) and non-parametric (Mann-Whitney test, Kruskal-Wallis test followed by Dunn's multiple comparisons test) were performed as appropriate. *p*-values <0.05 were considered significant. Statistical analysis was performed with GraphPad Prism software.

Results

Laminin-β1 is upregulated in *Endothelin Receptor B* mutant models of HSCR

To begin exploring the molecular mechanisms governing NCC migration failure in *EdnrB* mutant mice, we performed comparative microarrays of the embryonic day (E) 14 distal colon in two HSCR mouse models: $EdnrB^{lacZ/lacZ}$ (conventional knock-out) and $EdnrB^{NCC-/-}$ (NCC-conditional deletion of *EdnrB*) [Fig.S1]. Of the top genes upregulated more than 1.5-fold in the HSCR models, *Lamb1*, which encodes the β1 chain of laminin-111, the primary laminin isoform found in the developing colon, was upregulated more than 2.5-fold in both *EdnrB* mutant models [Supplementary Table 1]. qPCR verified upregulation of *Lamb1* transcripts in $EdnrB^{NCC-/-}$ [Fig.S1].

37/67-LAMR, the receptor for laminin-β1, is downregulated in murine and human HSCR

Laminin-β1 binds a 37/67-kDa laminin receptor (37/67LR, called 37/67-LAMR or LAMR) (30). We identified the presence of LAMR on post-natal (P7–11) NCC-derived neurons in the colonic myenteric plexus, with down-regulation in $EdnrB^{NCC-/-}$ [Fig.1]. Localization of LAMR appeared altered, with decreased cell surface expression of 67LR in $EdnrB^{NCC-/-}$ [Fig.1A] and expression of 37/67-LAMR appeared restricted to the nucleus [Fig.1B]. This observation was supported by Western Blot analysis of the sub-cellular membrane fraction of neural crest cells [Fig.1C]. Examination of post-natal $EdnrB^{NCC-/-}$ small intestine also

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demonstrated decreased LAMR expression and altered localization compared to WT [Fig.S2]. Of note, postnatally LAMR was not expressed in NCC-derived glial cells [Fig.S3].

To determine relevance to human HSCR, paraffin-embedded tissue blocks from patients that had undergone bowel resection for HSCR versus control patients (undergoing surgery for non-ENS related indications) were obtained and the proximal margin of resected bowel sectioned and analyzed (n=12 HSCR patients, n=15 controls). Immunohistochemistry confirmed that 67LR [Fig.2A] and 37/67-LAMR [Fig.2B] are expressed in HuC/D(+) enteric neurons, with decreased expression in the colon of HSCR affected individuals [Fig.2C], similar to the $EdnrB^{NCC-/-}$ mouse model.

To understand the time course of these alterations, and if they may play a role during development, we examined the prenatal bowel in the murine model. We first noted protein expression of 37/67-LAMR in WT mice in the migratory wavefront of NCC between E10.5 and E12, which coincides with NCC entry and migration into the colon [Fig.S4]. Importantly, comparison of WT and $EdnrB^{NCC-/-}$ prenatal samples demonstrated decreased expression of 37/67-LAMR in migratory NCC in the HSCR model [Fig.3]. These data suggested that LAMR receptor alterations may contribute to NCC-ECM interaction during ENS development.

NCC migration is inhibited by laminin-111 and enhanced by the laminin-β1 analog YIGSR

Prior studies have demonstrated that NCC binding of the laminin-α1 chain shifts NCC from a migratory to differentiation phenotype and that NCC differentiation occurs prematurely in HSCR mice which exhibit elevated levels of laminin in the hindgut (9, 11, 25, 26). To understand if Laminin-β1 and LAMR are involved in NCC migration, we utilized an embryonic organ culture model [Fig.4]. Explanted E12.5 colons were treated with exogenous laminin-111 or YIGSR (a pentapeptide analog that represents the portion of laminin-β1 that binds LAMR). Laminin-111 reduced NCC migration while YIGSR yielded an increase in migration [Fig.4B]. This suggests that targeting of distinct NCC laminin receptors may have differential effects on NCC migratory phenotype.

YIGSR upregulates NCC LAMR expression and enhances NCC migration

Blocking of laminin-β1 binding to LAMR reduces cancer cell invasion and metastasis, indicating that laminin-β1 and LAMR are involved in cell migration (35, 37). Further, YIGSR has been shown in multiple cancers to bind LAMR and promote a pro-migratory, metastatic phenotype (33, 35, 37–39). In those systems, laminin-β1/YIGSR binding to LAMR also promoted a positive feedback loop, with upregulation and cell surface expression of LAMR (40, 41). We hypothesized that YIGSR would exert a similar effect on NCC. E12.5 mouse midgut slices were cultured on fibronectin-coated dishes to investigate NCC migration in response to GDNF. Migration distance from the edge of the midgut slice and the furthest migratory Ret(+) NCC was determined. In the presence of YIGSR, migration distance was increased by approximately 30% as compared to samples treated with GDNF alone [Fig.5A,B]. Consistent with the existing literature, we observed a requirement of GDNF for NCC migration, and no migration of NCC was observed in preparations using YIGSR alone [Fig.S5].

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Closer examination of the Ret(+) NCC at the leading edge of migration revealed re-localization of 67LR fluorescence signals to the lamellipodia [Fig.5C], where they co-localized with phalloidin [Fig.5D]. 67LR expression more precisely overlapped with the actin-bind protein vinculin [Fig.5E], suggesting a potential role for LAMR in actin cytoskeleton dynamics. Protein immunoblot on these cells indicated enhanced expression of LAMR after culture with YIGSR [Fig.5F], suggesting a positive feedback loop on LAMR expression upon receptor binding.

67LR and β1-integrin may cooperate in enhancing NCC migration

In bone marrow-derived mesenchymal stem cells, β1-integrin binding promotes directional migration through focal adhesion turnover and actin dynamics in lamellipodia (45). In migratory embryonic stem cells, laminin binds both α6β1 integrin and 67LR to induce migration through FAK/paxillin and cAMP/Epac1/Rap1 pathways (46). Additionally, β1-integrin binding of the laminin-α1 chain of laminin is critical for NCC migration into the hindgut (47). We therefore hypothesized that 67LR and β1-integrin may cooperate in NCC migration. To test this, Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP), an inhibitor of β1-integrin, was employed as pre-treatment to block the effect of β1-integrin binding to laminin in slice cultures. We focused on the Ret(+) cells at the leading edge of migration [Fig.6A]. Embryonic midgut slice cultures were pre-treated with GRGDSP for one hour prior to adding GDNF, with or without YIGSR. Quantification demonstrated that YIGSR enhanced GDNF-induced NCC migration distance while the β1-integrin inhibitor blocked NCC migration [Fig.6B]. Interestingly, YIGSR was able to overcome GRGDSP-mediated inhibition of NCC migration [Fig.6B], suggesting that downstream pathways triggered by LAMR may override those triggered by β1-integrin.

Most of the leading cells migrating out of the slice cultures demonstrated smaller lamellipodia in the setting of GRGDSP treatment alone [Fig.6A, white arrows], confirming that β1-integrin binding promotes cytoskeleton re-arrangement in NCC. Treatment with YIGSR resulted in a larger area of 67LR(+) expression colocalized with phalloidin(+) staining, both when YIGSR was used alone and when used in combination with GRGDSP [Fig.6D]. This suggests that YIGSR enhances migration through cytoskeleton re-arrangement and that the 67LR is involved in this process. Importantly, YIGSR treatment increased 67LR expression area [Fig.6D], but was not sufficient to completely overcome GRGDSP-mediated inhibition of lamellipodia formation [Fig.6C]. Together, rescue of migration distance and partial rescue of 67LR re-distribution and colocalization in lamellipodia suggests the existence of a regulatory signaling pathway that is independent of β1-integrin.

Silencing LAMR decreases NCC migration

To test the specificity of YIGSR acting through LAMR to impact NCC migration, we used lentiviral shRNA against LAMR (siRPSA) to treat neurospheres derived from E12.5 embryonic gut [Fig.7]. Neurospheres were employed rather than midgut slices because of the prolonged culture period required for lentiviral infection. The lentivirus-infected NCC were identified as double-positive for GFP and Ret-immunoreactivity [Fig.7D inset; Fig.S6]. The activity of lentiviral siRNA against LAMR was confirmed by protein immunoblot with

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antibody to 37/67-LAMR [Fig.7B] and immunohistochemistry after culture [Fig.S7]. Neurospheres were cultured with GDNF alone or GDNF with YIGSR, and observed after 20 hours [Fig.7A] and 48 hours [Fig.7C]. Following culture for 48 hours, immunohistochemical staining was performed with Ret to identify the GFP(+) NCC and TuJ1 to demonstrate neuronal cells with neurites. GFP(+) cells that had migrated greater than 400 μm out of the neurospheres at 48 hours were quantified [Fig.7C,D]. With siControl, GDNF and GDNF/YIGSR-treated cultures demonstrated increased numbers of GFP(+) cells migrating to the edge of and beyond the established neurite network [Fig.7C]. In the siRPSA condition, fewer GFP(+)/Ret(+) cells had migrated over 400 μm. GFP(−) cells still responded to GDNF (with/without YIGSR), indicating that silencing LAMR abrogates the beneficial effect of YIGSR on NCC migration. When quantified, the number of cells that migrated out of the neurospheres after 48 hours was reduced by 60% when cultured with siRPSA versus siRNA control [Fig.7E]. This data further supports a role for laminin-β1 binding to LAMR in promoting GDNF-induced NCC migration.

YIGSR treatment rescues NCC migration and colonization in *EdnrB*$^{NCC-/-}$ hindgut *ex vivo*

Based on our mechanistic findings and prior literature demonstrating increased laminin in the distal hindgut of HSCR models, we hypothesized that YIGSR would enhance NCC migration and colonization in *EdnrB*$^{NCC-/-}$ animals. We employed *ex vivo* organ cultures of embryonic guts from E13.5 *EdnrB*$^{NCC-/-}$ and *EdnrB*$^{NCC+/+}$ animals [Fig.8A]. NCC colonization was limited to the proximal colon in more than 90% of *EdnrB*$^{NCC-/-}$ mice when treated with PBS control. However, treatment with YIGSR resulted in markedly increased colonization, reaching the distal colon in 80% of *EdnrB*$^{NCC-/-}$ mice, suggesting that YIGSR may be able to rescue NCC migration and colonization in *EdnrB*$^{NCC-/-}$ mice [Fig.8B, C].

Discussion

We have identified the presence of the 37/67 kDa laminin receptor (LAMR), which binds the laminin β1 chain, on NCC and our data suggest that the NCC LAMR may have a role in the development of HSCR aganglionosis. We identified the presence of this receptor on post-natal NCC-derived neurons in the colon, with down-regulation in the *EdnrB*$^{NCC-/-}$ animal model of HSCR and human HSCR specimens. Additionally, postnatal localization of 37/67-LAMR in the colon is altered, with decreased cell surface expression in *EdnrB*$^{NCC-/-}$. *EdnrB*$^{NCC-/-}$ small intestine also demonstrated decreased 37/67-LAMR expression and altered localization, suggesting that these receptor alterations may contribute to NCC-ECM interaction during development. Further, we found that *in vitro* treatment with YIGSR enhanced NCC migration through a mechanism involving LAMR and likely involving actin cytoskeleton dynamics in the lamellipodia. Finally, we have demonstrated the first pharmacologic approach to rescue NCC migration in HSCR intact gut: *ex vivo* treatment of embryonic *EdnrB*$^{NCC-/-}$ colon with laminin-β1 analog (YIGSR) resulted in near-complete colonization of the distal colon. Our study adds new information to the large list of receptors that NCC use to interact with their environment during normal and aberrant ENS development.

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YIGSR has been shown in multiple cancers to bind LAMR and promote a pro-migratory, metastatic phenotype, thus inhibiting LAMR is being investigated as an anti-metastasis target (33, 35, 37–39). Ret/GDNF and EdnrB/Et-3 are required for migration of NCC (19–22). Intracellular signaling through these receptors occurs primarily through mitogen-activated protein kinase (MAPK) and phosphotidylinositol-3-kinase (PI3K) pathways(15). Specifically, GDNF/Ret binding promotes a pro-migration effect mediated primarily through a PI3K pathway (4, 48). Consistent with these known mechanisms, application of PI3K inhibitor (LY294002) to inhibit GDNF-Ret signaling in slice cultures *in vitro* blocked NCC migration, an effect that could not be overcome by YIGSR (data not shown), supporting the critical role of Ret signaling for NCC migration. ET-3/EdnrB binding promotes NCC migration through PKA-dependent activation of PI3K (4). Ret-mediated NCC survival and proliferation are Akt-dependent, while both differentiation and proliferation occur through MAPK pathways (primarily ERK). However, *EdnrB* also prevents premature differentiation of NCC through MAPK/ERK and LAMR signals through this pathway in tumor models. NCC receptor-ECM ligand interactions (e.g. via β1-integrin and LBP110) occur through the same pathways and others (e.g. focal adhesion kinase (FAK)) (10–12). However, studies of cancer metastasis indicate that LAMR binding primarily activates MAPK pathways, resulting in cellular proliferation and migration (33). In the current study, we identified potential crosstalk between LAMR and β1-integrin through effects on the actin cytoskeleton in lamellipodia of migration cells, which is likely related to FAK signaling, but may also involve other intracellular signaling pathways (MAPK, PI3K, Akt) as well as physical changes to the cytoskeleton. In fact, inhibition of PI3K signaling abrogated any beneficial effect of YIGSR on migration, confirming that this pathway is critical for NCC migration (49). The precise mechanisms for potential LAMR interaction with components of the actin cytoskeleton and focal adhesion formation/turnover remain to be determined and will require detailed study utilizing relevant genetic models.

One seemingly contradictory finding in our current study is that of elevated laminin expression combined with decreased expression of LAMR in the *EdnrB*$^{NCC-/-}$ colon. The finding of increased laminin expression has been previously established and replicates human HSCR findings from >25 years ago (13, 14). However, the finding of decreased expression of the receptor, through which YIGSR enhances NCC migration, requires further consideration. First, it makes conceptual sense that there is reciprocal expression of the ligand and receptor pair; when laminin is increased there is decreased expression of the receptor. Our data also show that YIGSR promotes NCC migration and ENS colonization. Additionally, our data indicate that NCC migration is suppressed by exogenous laminin but addition of YIGSR can overcome this inhibition. Taken together, we postulate that YIGSR may selectively enhance LAMR-mediated pro-migratory effects in NCC, without activating other pathways downstream of laminin isoform binding to receptors on NCC (e.g. NCC differentiation via binding LBP110). Our working hypothesis is that LAMR binding results in a positive feedback loop with increased LAMR expression and preferentially promotes NCC migration through a PI3K-dependent pathway. Studies to determine how these receptors are activated and regulated *in vivo* will be required to reconcile seemingly contradictory results obtained when examining the function of single receptor types *in vitro*.

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While current diagnosis and treatment of HSCR focuses on the aganglionic segment, postsurgical patients often experience long-term morbidity, including motility disorders, constipation, and enterocolitis (5, 50). This suggests that the ganglionated portion of the remaining colon post-surgery may not be normally-innervated and may not sustain normal bowel function (51). This has been demonstrated in both mice (51) and humans (52), where decreased neuronal density and an imbalance of neurotransmitter phenotypes are observed. Ideally, non-surgical therapy for HSCR will result in complete colonization of the GI tract with a full complement of neurotransmitters, thereby providing normal ENS structure and function. However, multiple authors have suggested that reducing the length of aganglionosis, which would thereby reduce the extent of surgery required, would beneficially impact patient outcomes (5). Our results indicate that *ex vivo* treatment of E13.5 $EdnrB^{NCC-/-}$ colonic explants rescues NCC migration, resulting in colonization that is statistically equivalent to controls. However, it is unknown if YIGSR-mediated colonization results in a functioning ENS. While the ENS has many functions along the GI tract (motility, secretion, digestion, absorption), the primary functions of the distal colon and rectum (the typical aganglionic regions in HSCR) are the storage and emptying of feces (motility) (53). Expert consensus has identified that demonstrating amelioration of the HSCR motility defect in mouse models is necessary before translation into humans (54). Additionally, there is currently no widely-available pre-natal diagnostic modality for non-familial/syndromic HSCR (5), although pre-natal MRI is emerging as a possibility (55). Therefore, therapeutic approaches to HSCR must consider post-natal therapy to have translational benefit to patients (56). Studies to understand the ability of YIGSR to impact late-gestation and/or post-natal NCC migration and the function of the ENS in YIGSR-treated $EdnrB^{NCC-/-}$ hindgut are ongoing in our laboratory.

Therapeutic approaches to rescue ENS development in HSCR are being actively pursued by many research groups (56–58). The current results do not suggest that modulation of NCC interaction with laminin alone will be sufficient to rescue human ENS development in HSCR. Indeed, this study adds to the growing literature implicating NCC interactions with the ECM as a major contributing factor in the pathogenesis of HSCR and as potential targets for therapy. Prior studies have demonstrated temporal changes in the hindgut ECM that restrict NCC migration beyond E14.5, hypothesizing that this may be secondary to laminin accumulation (29). Other ECM components that have been implicated as involved in ENS development include collagens, tenascins, fibronectin and others (7, 47, 59–62). Additionally, there is growing literature that NCC both produce and break down ECM components during migration and ENS development (60, 62). Additionally, alterations in NCC function and the ECM milieu are likely more complex in human HSCR patients, very few of whom map to the reductionist, single-gene defects studied in most animal models of HSCR (5). Together, our results and those from other studies should be used to inform ongoing integrative, regenerative medicine approaches to HSCR, including stem cell therapies and bioengineered intestinal replacements (57, 58, 63–69).

Supplementary Material

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Abbreviations

37-LRP	37 kDa laminin receptor precursor
37/67-LAMR	37/67 kDa laminin receptor
67-LR	67 kDa laminin receptor
EdnrB	Endothelin receptor B
E	embryonic
ENS	enteric nervous system
ET-3	Endothelin-3
GDNF	Glial derived neurotrophic factor
HSCR	Hirschsprung disease
LAMR	laminin receptor (refers to 37, 37/67 or 67)
LBP110	110 kDa laminin binding protein
NCC	Neural crest cells
NFES	Near-field electrospinning
NSC47924	Small molecule inhibitor of LAMR
P	post-natal
Ret	Rearranged during transfection
SEM	standard error of the mean
YIGSR	LAMR binding pentapeptide

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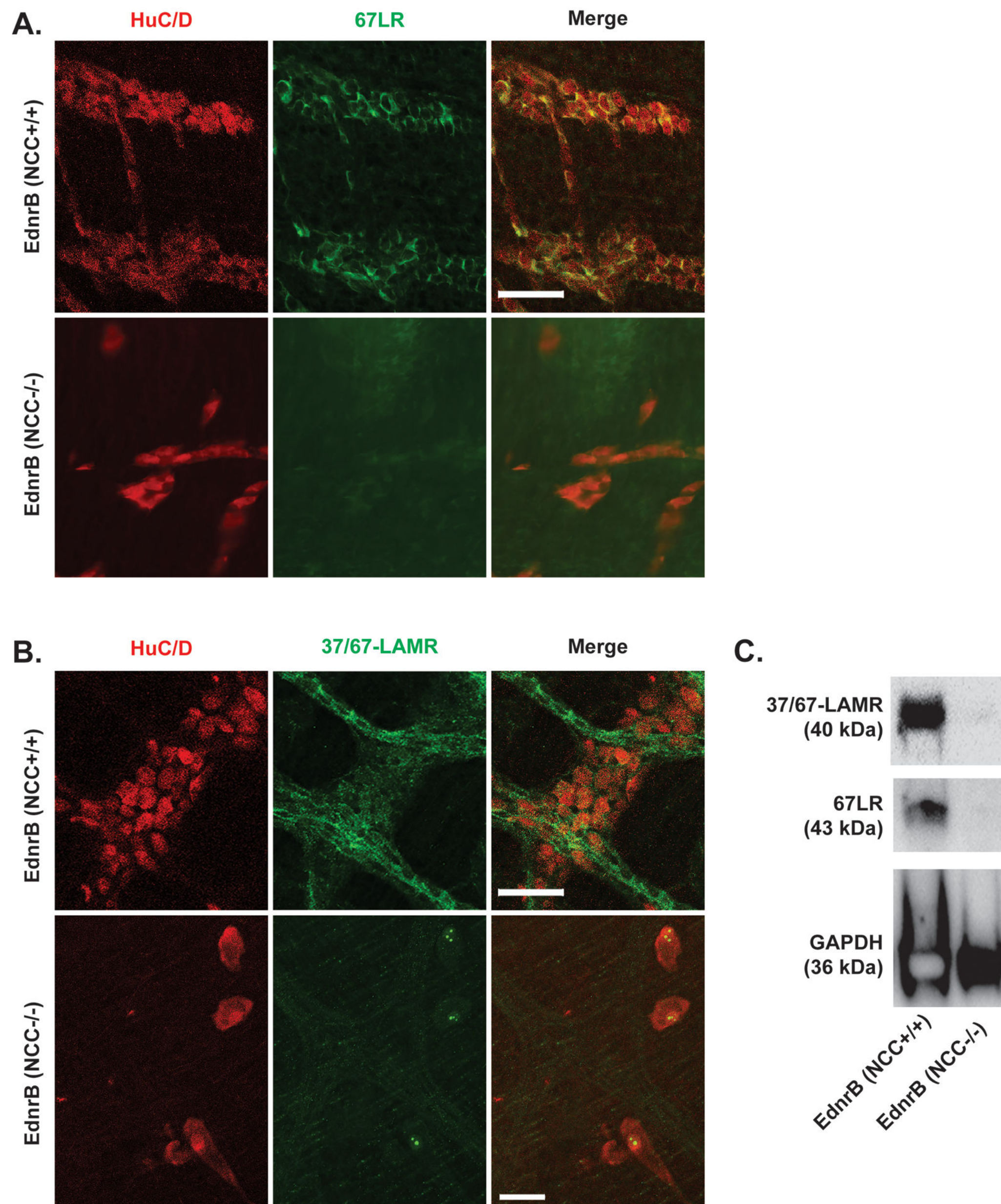

Figure 1. 37/67 kDa Laminin receptor (37/67-LAMR) and 67kDa Laminin receptor (67LR) are expressed in *EdnrB*$^{NCC+/+}$ myenteric plexuses, with reduced expression in *EdnrB*$^{NCC-/-}$ mice.
Whole-mount myenteric plexus from (A) *EdnrB*$^{NCC+/+}$ colon and *EdnrB*$^{NCC-/-}$ colon stained with antibody to HuC/D (red, pan-neuronal marker), 67LR (green, on the membrane of neurons). Scale bar: 50 μm. Whole-mount myenteric plexus from (B) *EdnrB*$^{NCC+/+}$ and *EdnrB*$^{NCC-/-}$ mice stained with antibody to HuC/D (red, all neurons), 37/67-LAMR (green, on the cytoplasm and nuclei). Localization is altered (intracellular). Images in both A and B for *EdnrB*$^{NCC-/-}$ colon are taken from the region of the neural crest cell migration wavefront. Scale bar: 50 μm in upper row and 20 μm in lower row. (C) Membrane protein fraction (see Methods) taken from *EdnrB*$^{NCC+/+}$ and *EdnrB*$^{NCC-/-}$ myenteric plexus, blotted with antibody against 37/67-LAMR and 67LR. GAPDH was used as a control. Images and WB are representative from n=3 animals per group.

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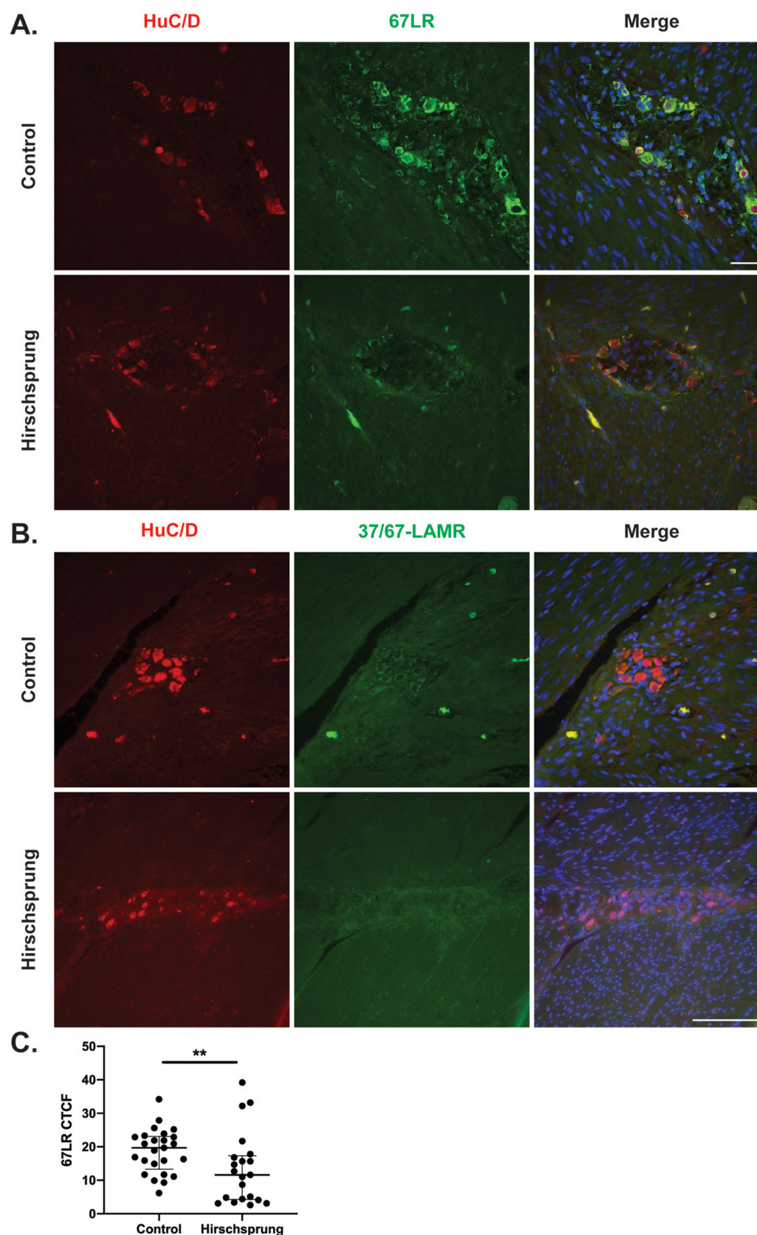

Figure 2. 37/67-LAMR and 67LR are expressed in human myenteric neurons, with reduced expression in Hirschsprung disease colon.

Paraffin sections taken from age-matched, region-matched, non-Hirschsprung disease patients undergoing colon resection and Hirschsprung disease colon, taken from the region immediately proximal to the aganglionic segment, stained with antibody to HuC/D (red, all neurons), (A) 67LR and (B) 37/67-LAMR. (C) Quantification of IHC through corrected cell total fluorescence (CTCF), demonstrating decreased 67LR in human Hirschsprung colon. **$p<0.01$ by Mann-Whitney test. Scale bar: A=50 μm; B=100 μm. N=21–25 per group.

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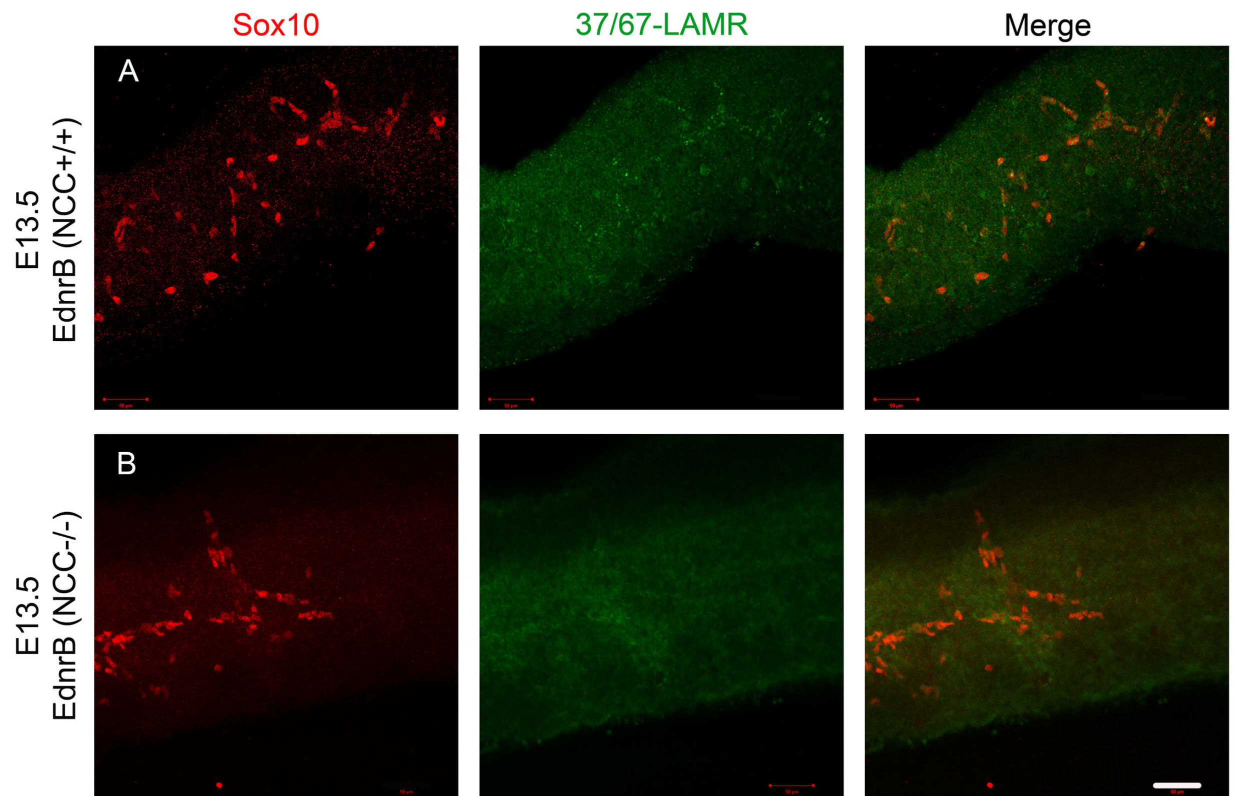

Figure 3. 37/67-LAMR expression is decreased in embryonic *EdnrB*$^{NCC-/-}$ mouse colon.
EdnrB$^{NCC+/+}$ and *EdnrB*$^{NCC-/-}$ mouse colon samples from E13.5, stained with antibodies to Sox10 (red, neural crest progenitors) and 37/67-LAMR (green). Expression of 37/67-LAMR is decreased in *EdnrB*$^{NCC-/-}$. Scale Bar: 50 μm.

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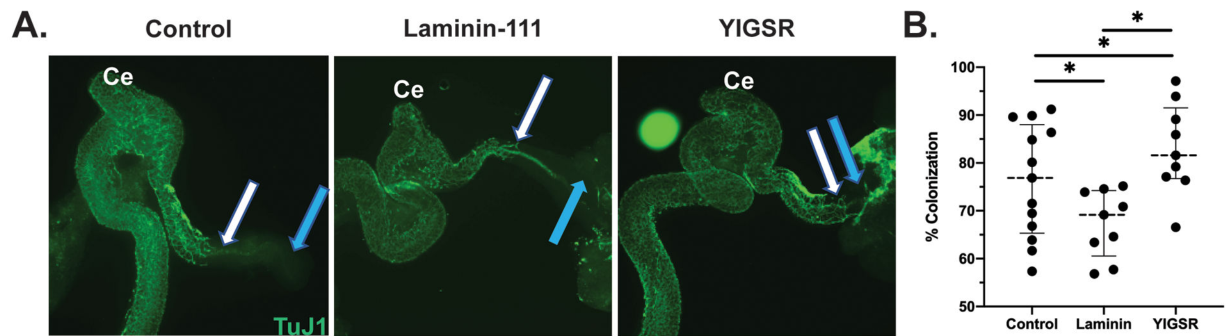

Figure 4. NCC migration in E12.5 murine colon is inhibited by laminin-111 and enhanced by the laminin-β1 analog YIGSR.

E12.5 WT mouse gut explants were cultured for 24 hours (A) under control conditions (untreated), with Laminin-111, or with YIGSR. Whole-mount immunohistochemistry for TuJ1 showed neuronal cells and neurites within the developing ENS. White arrows in distal colon indicate the leading edge of cells in migration wavefront. Blue arrows indicate the end of the distal colon. "Ce" is the tip of cecum. The measure from Ce to the blue arrow represents the whole length of colon. The measure from Ce to the white arrow represents the NCC-colonized segment. (B) Quantitative analysis shows laminin-111 treatment reduced the extent of NCC migration while YIGSR treatment resulted in an increase in migration. *$p<0.05$ by Kruskal-Wallis test followed by Dunn's multiple comparisons test. Control: n=15; Laminin-111: n=10; YIGSR: n=10.

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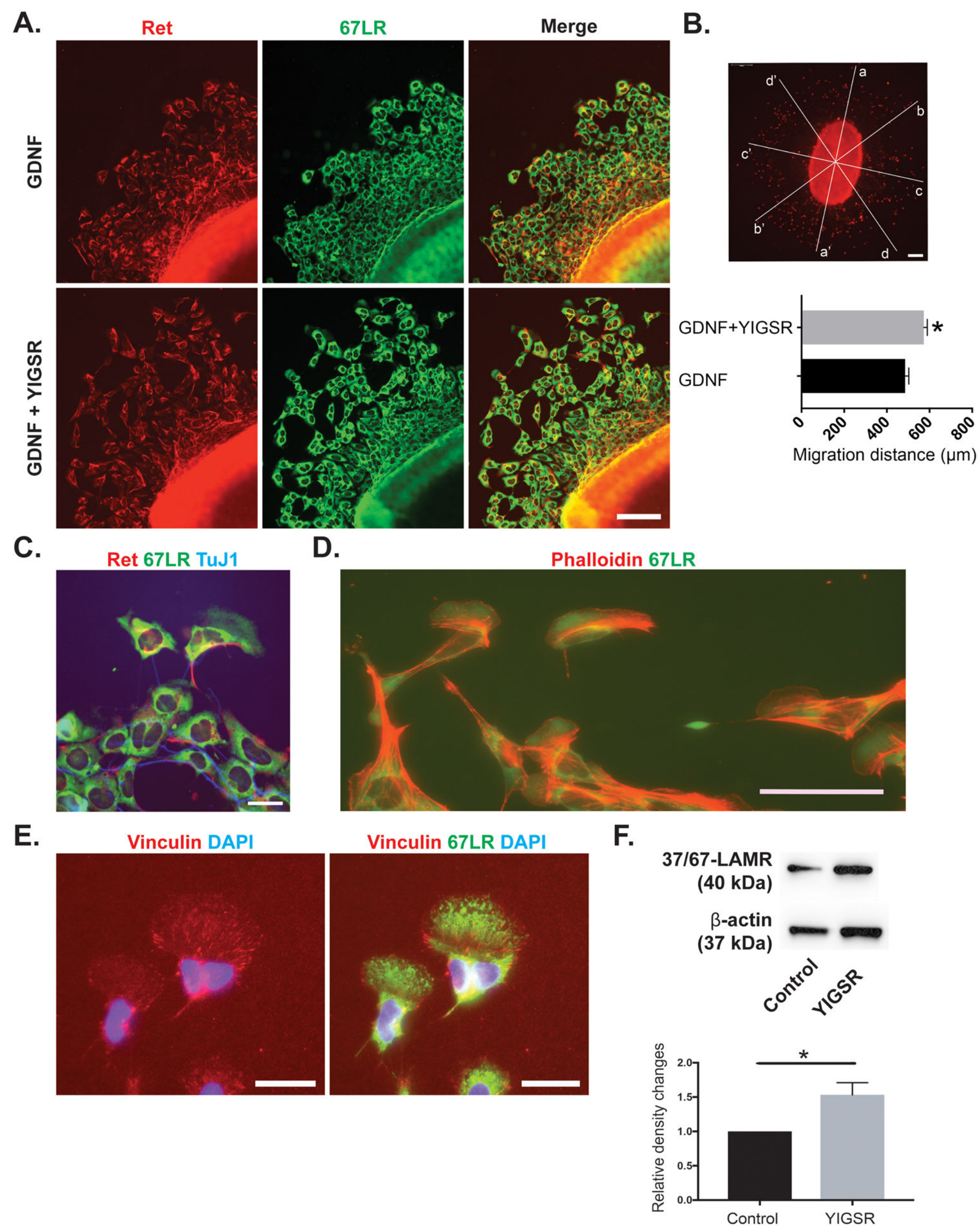

Figure 5. YIGSR localizes 67LR to the actin-binding region in lamellipodia, upregulates NCC LAMR expression and enhances NCC migration.

E12.5 mice embryonic gut slice explants were cultured on fibronectin coated dishes. Migration distance was measured after 20 hours of culture with (A) GDNF or GDNF with YIGSR (red, all Ret positive neural crest cells; green, 67LR). (B) Eight radiating lines were drawn in standard positions extending from the edge of the explant. The point of the most distant Ret+ cell along these lines was measured. The mean migration distance of Ret+ cells from the edge of gut slice confirmed increased migration with treatment of GDNF plus YIGSR. (C) The leading migrating cells were stained by antibody to Ret (red), 67LR (green) and TuJ1 (blue). (D) Cell morphology was characterized with antibody to Phalloidin (red) and 67LR (green) or with (E) antibody to Vinculin (red), 67LR (green) and DAPI (blue). In

both D and E, 67LR was seen to localize to the same region as actin binding proteins in the lamellipodia. (F) Increased expression of LAMR after culture with GDNF and YIGSR was confirmed by protein immunoblot using antibody to 37/67-LAMR. Scale bar: A-B, 100 μm; C: 25 μm; D: 50 μm, E: 250 μm. Images and WB are representative from n=6 separate experiments; n>300 cells were analyzed. *$p<0.05$ by Mann-Whitney test.

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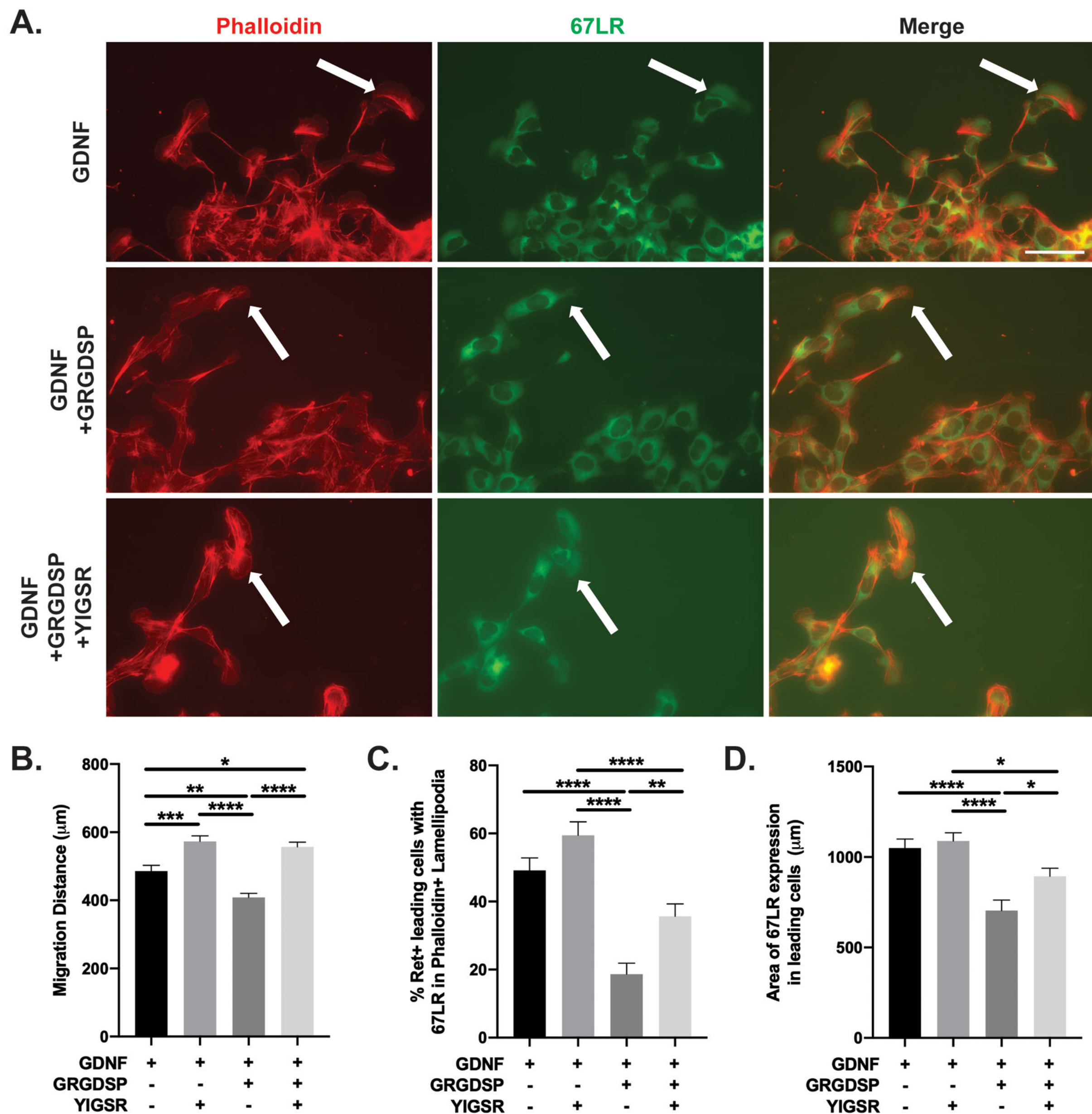

Figure 6. LAMR may act by stabilizing actin and promoting lamellipodia formation in migrating cells.

(A) E12.5 slice explants were cultured to allow NCC to migrate out in response to GDNF (red, phalloidin-labeled protrusion; green, 67LR located in the actin-binding region). E12.5 embryonic guts were cultured with GDNF alone (A, top row), GRGDSP (an inhibitor of β1-integrin) for 1 hour, followed by GDNF (A, middle row), or GRGDSP followed by GDNF plus YIGSR (A, bottom row). Neural crest cell migration distance was measured as in Fig.5B. Phalloidin-labeled protrusions (arrows) regressed after pre-treatment with GRGDSP (middle row vs. top row). (B) Cell migration distance was reduced with GRGDSP pre-treatment. YIGSR treatment restored migration distance back to a similar level as with GDNF plus YIGSR. (C) The percentage of Ret+ leading cells with 67LR in Phalloidin+ protrusions versus total Ret+ leading cells indicated that YIGSR is able to partially rescue

the inhibitory effect of GRGDSP in lamellipodia formation. (D) This was confirmed by measuring the area of 67LR expression in lamellipodia of the leading migratory cells. Scale bar: 50 μm; n>400 cells were analyzed in each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by ANOVA and Tukey's post-hoc test.

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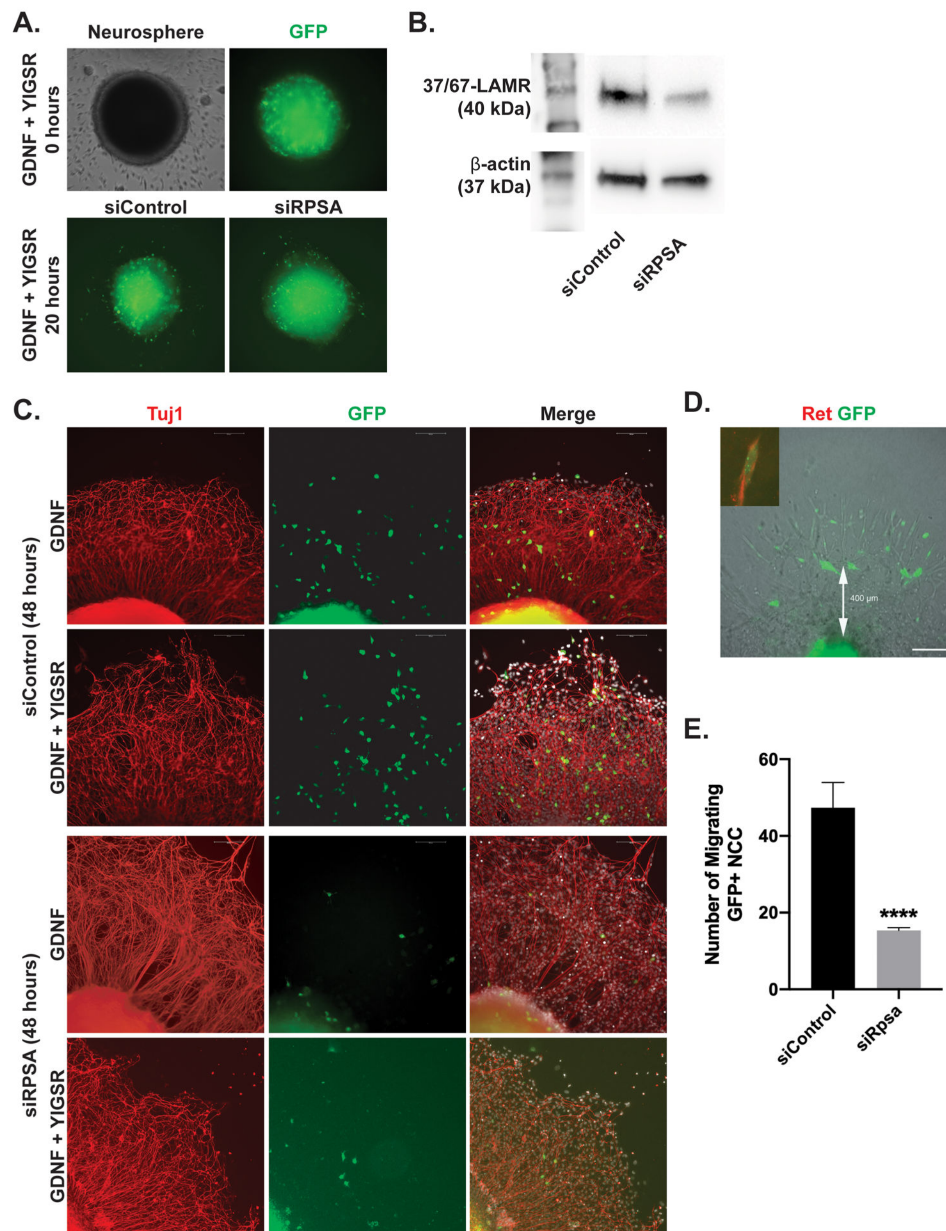

Figure 7. Silencing LAMR decreases NCC migration.

Lentivirus against 37/67-LAMR (siRPSA) or control lentivirus (siControl) was used to infect neurospheres generated from E12.5 embryonic gut. (A) Lentiviral-infected cells express GFP. At 20 hours of treatment with GDNF and YIGSR, GFP(+) cells can be seen migrating out of neurospheres. (B) Protein immunoblot using antibody to 37/67-LAMR demonstrated that siRNA silenced 50% of 37/67-LAMR expression. (C) After 48 hours of culture, staining with TuJ1 (red) demonstrated neurites, GFP (green) indicated lentivirus infected cells, and DAPI was used to visualize all cells. In both the siControl and siRPSA conditions, YIGSR-treated cultures demonstrated increased numbers of cells migrating to the edge of and beyond the established neurite network. In the siRPSA condition, these cells were not green, indicating that silencing LAMR abrogates the beneficial effect of YIGSR on

NCC migration. (D) GFP+ cells that had migrated past a boundary 400 μm from the edge of the neurosphere (white double arrow) were analyzed. Ret immunohistochemistry (inset, red, Ret+ neural crest cells, green: GFP+ lentiviral-infected cells) was used to confirm that migratory GFP+ cells were NCC. (E) Quantification of GFP+ NCC that migrated at the 48-hour timepoint indicates that silencing 37/67-LAMR markedly decreased NCC migration. n=3 separate experiments. ****$p<0.0001$ by Mann-Whitney test.

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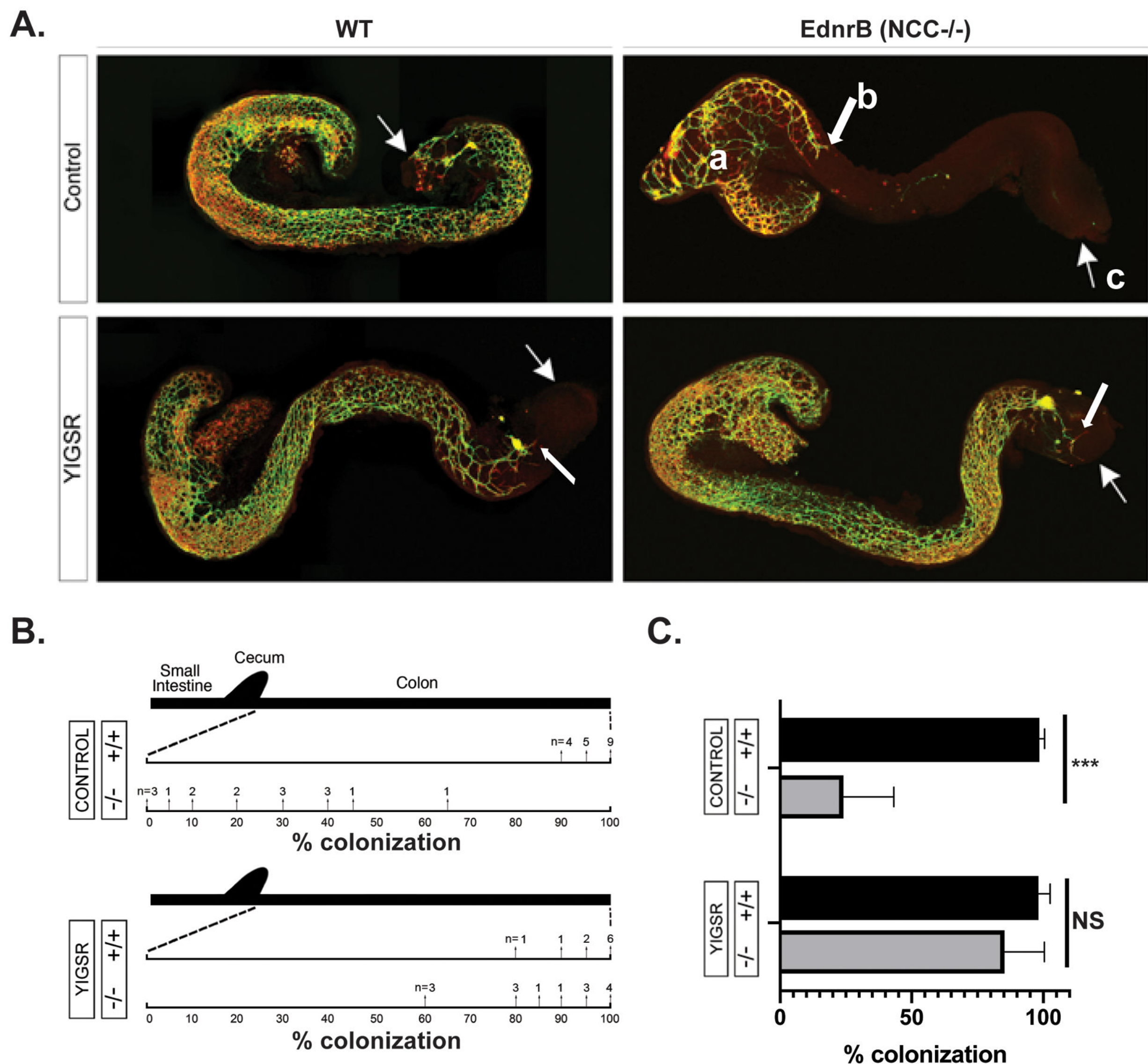

Figure 8. YIGSR rescues NCC migration in *EdnrB*$^{NCC-/-}$ colon.

(A) E13.5 colon explants were treated with PBS (control) or YIGSR. % colonization was calculated: "a" marks cecum, "b" marks extent of migration, "c" marks end of colon. % Colonization = 100 * ab/ac. Large arrows indicate distal extent of colonization; small arrows indicate end of colon. YIGSR treatment resulted in increased colonization of the hindgut in *EdnrB*$^{NCC-/-}$. (NCC are tdTomato+ red and HuC/D is green) (B) Length of colonization is indicated on a schematic of the colon, with the number of individual colons showing specific % colonization indicated. NCC colonized 90–100% of distal colon in *EdnrB*$^{NCC+/+}$ colon at this age, while the furthest neural crest colonization only reached to 60% of *EdnrB*$^{NCC-/-}$ colon cultured with control. Twelve out of fifteen *EdnrB*$^{NCC-/-}$ colons achieved 80–100% colonization when cultured with YIGSR. (C) Quantification: YIGSR treatment results in complete or near-complete colonization of the hindgut in *EdnrB*$^{NCC-/-}$. WT n=28; *EdnrB*$^{NCC-/-}$ n=31. ***$p<0.001$ by Mann-Whitney test.