2021

A NOVEL ROLE FOR NEUROTENSIN IN REGULATION OF STEM CELL FUNCTION IN THE SMALL INTESTINE

Stephanie Rock
University of Kentucky, sabr226@uky.edu
Author ORCID Identifier:
https://orcid.org/0000-0001-5700-6954
Digital Object Identifier: https://doi.org/10.13023/etd.2021.219

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Stephanie Rock, Student

Dr. Mark Evers, Major Professor

Dr. Isabel Mellon, Director of Graduate Studies

A NOVEL ROLE FOR NEUROTENSIN IN REGULATION OF STEM CELL FUNCTION IN THE SMALL INTESTINE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
Stephanie Anne Rock

Lexington, Kentucky

Director: Dr. B. Mark Evers M.D., Professor of Surgery

Lexington, Kentucky

2021

https://orcid.org/0000-0001-5700-6954

ABSTRACT OF DISSERTATION

A NOVEL ROLE FOR NEUROTENSIN IN REGULATION OF STEM CELL FUNCTION IN THE SMALL INTESTINE

Neurotensin (NT) is a nutrient-regulated gut hormone that plays important roles in lipid absorption, obesity, metabolic disorders, and normal and neoplastic growth in the intestine. In this study, we 1) elucidate the mechanisms regulating NT release from endocrine cells, 2) examine the role of NT on proliferation and stem cell function in the small intestine and 3) define the effects of NT on colorectal cancer stem cells. We report that NT release from endocrine cells is enhanced by the MAPK scaffold protein Kinase Suppressor of Ras 1 (KSR1) and the exocyst complex component 70 (Exo70). Moreover, free fatty acid stimulated release of NT is attenuated by inhibition of KSR1, MEK, ERK1/2, and Exo70, indicating these proteins as possible therapeutic targets for modulating the negative effects of NT on lipid absorption, obesity, and metabolic dysfunction.

Functionally, we show that NT plays an evolutionarily conserved role in maintenance of intestinal stem cells during nutritional stress. NT is required for the induction of WNT/β-catenin signaling and ISC-specific gene expression during fasting and promotes intestinal stem cell self-renewal. In the murine small intestine, loss of NT impairs crypt progenitor cell proliferation via downregulation of ERK1/2 signaling, cyclin D1 expression, and cell cycle related gene expression programs. Loss of NT impairs intestinal stem cell-specific gene expression and intestinal stem cell function after fasting, whereas NT overexpression prevents intestinal stem cell depletion in the midgut of *Drosophila* fed a nutrient-reduced diet.

We extend these findings to reveal a similar role for NT in regulation of stem cell function in colorectal cancer. NT enhances WNT/β-catenin signaling and cancer stem cell self-renewal in the human colon carcinoma cell line HCT116 and in intestinal tumor organoids derived from APC mutant models of colorectal cancer. Mechanistically, we show that NT induces phosphorylation of LRP6 in an ERK1/2 dependent manner in colorectal cancer cells and intestinal tumor organoids, indicating that NT promotes WNT/β-catenin signaling via activation of the ERK1/2 signaling pathway. In contrast, loss of NT impairs cancer stem cell function in tumor organoids derived from $APC^{min}/NT^{-/-}$

mice, and our preliminary findings indicate that NT-deficiency significantly increases lifespan in APC mutant mice.

Collectively, the studies within this dissertation identify NT as a positive regulator of normal and neoplastic growth within the small intestine through regulation of stem cell function and activation of the WNT/β-catenin pathway and identify potential therapeutic targets for modulating the effects of NT in the small intestine.

KEYWORDS: Neurotensin, Gut Hormone, Intestinal Stem Cell, Cancer Stem Cell, WNT/β-catenin Signaling, ERK1/2 Signaling

Stephanie Anne Rock

May 6th 2021

A NOVEL ROLE FOR NEUROTENSIN IN REGULATION OF STEM CELL FUNCTION IN THE SMALL INTESTINE

By

Stephanie Anne Rock

Dr. B Mark Evers
Director of Dissertation

Dr. Isabel Mellon
Director of Graduate Studies

05/06/2021
Date

For Josh, Anna, and Luna.

ACKNOWLEDGEMENTS

I would like to thank Dr. B. Mark Evers for his enduring support and guidance throughout my dissertation research and for encouraging me to pursue my PhD. To Dr. Tianyan Gao, I cannot express how immensely grateful I am for your mentorship and support. You welcomed me into you lab as one of your own students, and I could not have completed this work without your guidance, encouragement, and scientific prowess. I would also like to thank Dr. Jia Jianhang for his endless kindness, for generously sharing reagents, and for contributing all of the *Drosophila* data contained in this dissertation without asking that I learn how to dissect *Drosophila* intestines, though I am still intrigued by the idea. I am also thankful for Dr. Christine Brainson and Dr. Ying Liang for taking the time to critically review my proposals and provide helpful advice, reagents, and even protocols that aided me in my all research endeavors. I must also thank the whole of Markey Cancer Center, the Department of Toxicology and Cancer Biology, and the Department of Molecular and Cellular Biochemistry for their collaborative support. In addition, I thank Dr. Isabel Mellon for all she has done for me and continues to do for the DTCB graduate students, and Dr. Jessica Blackburn for taking the time to act as my outside examiner.

In addition, I must thank those within the Evers lab who offered their time, skills, and support to me through my graduate school career. I am particularly grateful to Jun Song for his many kindnesses and to Piotr Rychahou for always being willing to help me troubleshoot an experiment or consider alternative methods. In Dr. Gao's lab, I must thank Xiaopeng Xiong for always being willing to help me with difficult experiments and his endless willingness to share reagents, protocols, and useful scientific insights. To Ashely and Sumati, I am beyond grateful for your friendship and for welcoming me into Dr. Gao's lab without question. I learned so much from

each of you and have both of you to thank for sparking my interesting in the career I will begin after graduating. Your friendship significantly improved my graduate school experience, and for that I will always be grateful.

Finally, I must thank Josh, who above all deserves some kind of national award and a statue built in his honor for enduring the lowest lows of graduate school with me and for being excited for me during those few highs. To Anna and Luna, though they cannot read, they are the light of each day and have offered support they cannot know during the past many years. To the rest of my family and friends, I will always be grateful for your pride and interest in my work and for your continued support through the years. I look forward to being able to devote more time to you all in the very near future.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 Signaling Pathways Regulating Intestinal Homeostasis

1.1.1 Mechanisms of Proliferation and Differentiation

The intestinal epithelium is the most vigorously self-renewing mammalian tissue, turning over every 3-5 days (1, 2). This self-renewal process is maintained by a population of intestinal stem cells (ISCs) located at the base of intestinal crypts (3). These actively cycling ISCs are highly proliferative and metabolically active and contribute to intestinal epithelial homeostasis (4). During homeostatic self-renewal, ISCs give rise to progenitor cells that proliferate and migrate toward the villus tip, eventually differentiating into one of the mature intestinal cell types (2). These major cell types include absorptive (enterocytes) or secretory (goblet, Paneth, enteroendocrine) cell lineages that mediate the diverse functions of the small intestine, including nutrient absorption, digestion, and mucosal barrier function (2, 4, 5).

ISC proliferation and subsequent differentiation is controlled by numerous signaling pathways, including WNT/β-catenin, Notch, and bone morphogenic protein (BMP), signaling **(Fig 1.1)** (4). WNT/β-catenin signaling at the base of the intestinal crypts is required for ISC proliferation and self-renewal, while decreasing levels of WNT/β-catenin signaling toward the villus enable progenitor cell differentiation into one of the mature intestinal epithelial cell types (6-8). Proliferation of the active ISC population located at the crypt base is regulated in large part by WNT/β-catenin signaling, and these ISCs are identified by expression of the WNT-target gene leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*) (2, 7, 9). $Lgr5^+$ ISCs divide at the crypt base to give rise to progenitor cells, which undergo several more rounds of division in the transit-

amplifying region as they migrate up the crypt prior to terminal differentiation (2). As progenitor cells migrate toward the villus tip, they encounter signals that oppose WNT-activation and permit cell differentiation, such as BMP signaling (4). Other critical pathways that regulate intestinal homeostasis include EGF/MAPK signaling, which contributes to proliferation within intestinal crypts, and Notch signaling, which promotes self-renewal at the crypt base and plays a critical role in cell fate determination (4). In the mammalian small intestine, alteration of any one of these signaling pathways may perturb crypt cell proliferation or the trajectory of progenitor cell differentiation and therefore contribute to intestinal diseases or colorectal cancer (CRC) (2, 5, 10).

1.1.2 The WNT/β-catenin Pathway

The self-renewal properties of ISCS, and thus the whole of intestinal homeostasis, is critically dependent on WNT/β-catenin activation at the crypt base (6). The WNT/β-catenin pathway has numerous important physiological functions, including regulation of embryonic development, tissue homeostasis, and cell proliferation, migration, and fate determination, among other processes (6-8, 11). The WNT/β-catenin pathway is highly conserved and contributes to ISC function in *Drosophila* as well as mammals (12). The canonical WNT-pathway is dependent on the activity of β-catenin (11). WNT signaling is activated by WNT-ligands that cause nuclear translocation of β-catenin and activation of WNT-target gene expression **(Fig 1.2)** (11). In the absence of WNT-ligands, β-catenin is continually degraded by the actions of the β-catenin destruction complex, a protein complex composed of the scaffold protein Axin, the tumor suppressor *adenomatous polyposis coli* gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase

3 (GSK3) (11). CKI and GSK3 phosphorylate β-catenin, resulting in its ubiquitination and proteasomal degradation. Under these conditions, cytosolic β-catenin is continually degraded and WNT-target gene expression is repressed (11).

The WNT/β-catenin pathway is activated by the binding of WNT-ligands to the seven-pass transmembrane Frizzled (FZD) receptor and its co-receptor, low-density lipoprotein receptor related protein 6 (LRP6) or LRP5, which results in the formation of a FZD:LRP co-receptor complex (8, 11, 13). Upon formation of this complex, the scaffolding protein Dishevelled (DVL) binds to FZD, leading to phosphorylation of LRP6 on Ser1490 and recruitment of Axin to the receptor complex. Translocation of Axin to the FZD:LRP:DVL complex disrupts the β-catenin destruction complex, reducing the ability of CKI and GSK3 to phosphorylate β-catenin (11, 13, 14). As a result, non-phosphorylated (active) β-catenin accumulates and travels to the nucleus, where it interacts with TCF/LEF transcription factors and activates expression of WNT-target genes that regulate a number of proliferative and oncogenic functions (8). Activation of WNT/β-catenin signaling in the ISC niche is mediated by WNT-ligands secreted from Paneth cells and from subepithelial fibroblasts located beneath intestinal crypts (7). However, Paneth cells are dispersible for normal ISC function *in vivo*, indicating that subepithelial fibroblasts are the critical source of WNT ligands in the small intestine (4).

1.1.3 The MAPK Signaling Pathway

EGF/MAPK signaling is also implicated in directing the proliferation and differentiation of ISCs and crypt progenitor cells (2, 4). EGF, through binding to EGF receptor (EGFR), stimulates the MAPK pathway by activating the MAP kinase kinase

kinase (MAPKKK) Raf **(Fig 1.3)** (15). Activated Raf phosphorylates the MAP kinase kinases (MAPKKs) MEK1 and MEK2 (MEK1/2), which phosphorylate and activate ERK1/2 (16). Activated ERK1/2 can phosphorylate many different cytosolic substrates or travel to the nucleus, where it activates transcription of genes promoting cell cycle progression, DNA synthesis, and cell proliferation (15). Signaling of these MAPK complex proteins is potentiated by molecular scaffold proteins, including Kinase Suppressor of Ras 1 (KSR1). The scaffolding function of KSR1 serves to coordinate the spatial localization of the Raf/MEK/ERK proteins and enhances ERK1/2 activation (17). Disruption of MAPK signaling alters proliferation and WNT/β-catenin signaling in intestinal crypts, yet its role in intestinal homeostasis appears complex (18, 19). ERK1/2 are central regulators of cell cycle progression, and their activation has been previously shown necessary for proliferation of intestinal epithelial cells and development of the intestinal epithelium (15, 20, 21). However, recent work has revealed a more varied role for ERK1/2 signaling in intestinal crypts. In some models, ERK1/2 inactivation has been shown to enhance crypt cell proliferation and promote WNT/β-catenin signaling during development, suggesting complex crosstalk between these pathways under certain cellular contexts (22). Nonetheless, numerous studies identify a positive role for ERK1/2 signaling in regulation of intestinal epithelial cell growth and proliferation of crypt progenitor cells, and EGF is a necessary component of intestinal organoid culture media to sustain optimal organoid proliferation and maturation (2, 20, 23).

1.1.4 Nutritional Regulation of Intestinal Homeostasis

Dietary signals are key regulators of intestinal homeostasis (24). ISCs integrate diet-induced physiological signals to direct intestinal cell proliferation and differentiation (24). Nutrient-responsive pathways such as AMPK, PPARδ, and mTOR signaling regulate ISC function and proliferation in response to diet (3). In high-fat diet (HFD)-induced obesity mouse models, excess dietary fat intake promotes stemness and tumorigenicity of ISCs and progenitors by activating WNT/β-catenin signaling (25). However, long-term calorie restriction also enhances intestinal proliferation through modulation of various nutrient-responsive signaling pathways, including mTOR and AMPK signaling (26). In contrast, nutrient deprivation diminishes intestinal proliferation and induces intestinal atrophy, while boosting ISC function by enhancing fatty acid oxidation (FAO) (27). Under conditions of acute or prolonged absence of enteral nutrients, the intestinal epithelium is remodeled in the process of intestinal adaptation (28). Proliferation in intestinal crypts is diminished, apoptosis increases in the villi and crypts, and ISC function is altered (27-29). Molecules that enhance crypt proliferation or ISC function or in other ways mitigate the impacts of enteral nutrient deprivation may be promising therapeutic targets for the treatment of intestinal diseases characterized by altered intestinal homeostasis.

1.2 Mechanisms and Consequences of NT Release

1.2.1 NT Localization and Secretion

Neurotensin (NT) is a 13 amino acid peptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) found in the central nervous system (CNS) and gastrointestinal tract (30, 31). In the brain, NT is located in nerve terminal fibers and cells, while within

the intestine NT is localized to enteroendocrine (EE) cells (32, 33). Though EE cells have historically been categorized based on the peptide they secrete, such that NT-secreting EE cells are designated as "N-cells," many recent studies have highlighted extensive overlap between peptides secreted from a single EE cell type. For instance, activation of BMP signaling in glucagon-like peptide-1 (GLP-1) releasing "L-cells" has been shown to induce a switch in peptide secretion from GLP-1 to NT and peptide YY (PYY) (34). In the same study, high levels of BMP signaling in the villus region induced expression of Secretin (sct) in most EE cells (34, 35). Thus, multiple EE cell subtypes may be capable of releasing NT under various conditions.

Release of NT from EE cells occurs via the regulated secretory pathway (36). After proteolytic processing of the NT precurser fragment (pro-NT), NT is transported from the *trans* Golgi network to secretory vesicles, where it is stored until extracellular stimuli trigger NT release (36-38). Ingestion of dietary fats is the strongest extracellular stimulus regulating NT release (36), triggering exocytosis of NT-containing secretory vesicles (37, 38). Once released, NT regulates many physiological functions, including analgesia and neurotransmitter signaling in the CNS and cell proliferation and lipid absorption in the GI tract (31, 39-42). ERK1/2 and free fatty acids (FFAs) are known to stimulate NT secretion, yet the precise mechanisms contributing to NT exocytosis are unclear (43, 44). Functions of NT release in the small intestine include regulation of cell proliferation, FFA absorption, glucose homeostasis, and tumorigenesis (45-47). Thus, NT signaling is an attractive therapeutic target the treatment of metabolic disorders and various cancers. However, a more thorough understanding of the signaling events regulating NT release from endocrine cells is necessary to determine the full therapeutic potential of NT or NT-pathway targets.

1.2.2 NT/NTR1 Signaling

The effects of NT are mediated through three NT receptors (NTRs) **(Fig 1.4)**. The high affinity NTR1 and low affinity NTR2 are G-protein-coupled receptors, while NTR3 is a single-pass transmembrane domain identical to the intracellular sorting receptor sortilin (30). NTR1 is widely expressed in the CNS and various cancer cells (30, 39). NTR1 exerts many of the effects of NT in the CNS, including activation of dopamine and glutaminergic signaling (31, 39). In the CNS, NT is localized to presynaptic and postsynaptic vesicles and acts as a primary neurotransmitter (31, 39). NT-inhibition of dopamine D2 autoreceptors induces dopamine signaling, mimicking D2 receptor antagonists (31, 39). NTSR2 is 64% homologous to NTR1 but has a low-affinity for NT binding (48). NTSR2 is expressed predominantly in the CNS in areas related to pain perception (39). Administration of NT into the periaqueductal gray (PAG) and the rostral ventrolateral medulla (RVLM) induces analgesia, and inhibition of NTR2 but not NTR1 blocks this effect (39). NTR3/sortilin is widely expressed and not NT-specific (48, 49).

In the gastrointestinal tract, NTR1 mRNA is undetectable in most normal tissues but highly upregulated in various gastrointestinal cancer cells (50). In contrast, NTR2 is expressed in the gastric mucosa and EE cells of the small and large intestine but rarely detected in human tumors (50). NTR3 is broadly expressed in normal and cancer cells, including cells of the gastrointestinal tract, and may serve as a co-receptor to facilitate NT/NTR1 signaling (50, 51). Most studies indicate that the effects of NT are predominantly mediated by NTR1 (50). Precise localization of NTR1 expression in the small intestine is debated. Single-cell RNA-seq experiments fail to detect NTR1 expression in any intestinal epithelial cell populations (52), suggesting that other intestinal cell types, such as

macrophages, enteric neurons, or subepithelial cells may mediate the effects of NT in the small intestine. Interestingly, single-cell sequencing does detect NT in some putative ISCs (52), and in the colon, NTR1 expression is highest at the crypt base, near the ISC niche (53).

1.2.3 NT Regulation of MAPK Signaling

NT/NTR1 signaling leads to activation of signal transduction pathways involved in cell proliferation and survival, including PI3K/AKT, MAPK/ERK, GSK3β, and NFκB signaling **(Fig 1.5)** (31, 44, 54, 55). NT/NTR1 signaling activates phospholipase C (PLC) via Gαq/11 activation, leading to increased intracellular levels of the second messengers Inositol trisphosphate (IP3) and Phosphatidylinositol 4,5-bisphosphate (PIP2) and activation of protein kinase C (PKC) and intracellular calcium release (50). NTR3/sortilin activation in the brain is associated with activation of PI3K/AKT and MAPK/ERK signaling (48). NT-induced FFA absorption is mediated by inhibition of AMPK signaling in enterocytes (46). NT-induced activation of the MAPK/ERK pathway has been recorded in numerous cancer cell lines and is thought to be a primary mechanism of NT-induced cancer cell proliferation (47, 48). Upon binding to NTR1, NT activates a classic GPCR signaling cascade that activates MAPK signaling and ERK1/2 phosphorylation (56). Notably, Ras and ERK1/2 positively regulate NT gene expression, indicating a positive feedback loop stimulating NT release and ERK1/2 signaling (44, 57). Like NT, EGF signals predominantly via the MAPK pathway and is a critical ligand for maintaining intestinal proliferation within the ISC niche (22). Whether NT plays a similar role in maintenance of proliferation within the ISC niche is unknown.

1.2.4 NT Crosstalk WNT/β-catenin Signaling

Several studies have identified crosstalk between NT/NTR1 signaling and pathways involved in WNT/β-catenin signaling. In CRCs, NTR1 expression is activated by WNT/APC signaling, and NTR1 is positively correlated with nuclear β-catenin expression in gastric cancer (58, 59). NT has also previously been shown to contribute to phosphorylation of GSK3β, a negative regulator of WNT activation, via PKC and AKT signaling (60). Phosphorylation of GSK3β on Ser9 by AKT inhibits GSK3β activity, which serves primarily to phosphorylate and sequester β-catenin (14, 61, 62). Though some have suggested that AKT phosphorylates a pool of GSK3β that is not involved in regulation of β-catenin, multiple studies have demonstrated AKT-mediated activation of WNT/β-catenin signaling via GSK3β inhibition (14, 61-63). NT mediates phosphorylation of GSK3β in the human colon carcinoma cell line HCT116 (60), suggesting a possible role for NT in regulation of WNT/β-catenin downstream of GSK3β in CRCs. Consistent with this, NT enhanced epithelial-to-mesenchymal features and increased expression of WNT ligands, WNT-target genes, and phosphorylated-GSK3β in hepatocellular carcinoma cells in an NTR1-dependent manner (64). In glioblastoma cells, NT/NTR1 signaling is proposed to activate WNT/β-catenin signaling via activation of ERK1/2 (55). The kinase activity of ERK1/2 is known to phosphorylate LRP6 and facilitate activation of the WNT/β-catenin pathway (65). WNT/β-catenin signaling plays an important role in tumorigenesis (11). Disruption of WNT/β-catenin signaling is a fundamental step in the development of CRC, and high levels of WNT-target gene expression contribute to proliferation and tumor growth in numerous cancers (8, 66). Notably, $Lgr5^+$ ISCs are proposed to be a CRC cell-

of-origin, and WNT-activating mutations in ISCs are known to promote intestinal tumorigenesis (10). Therefore, defining the factors that control intestinal WNT/β-catenin signaling and $Lgr5^+$ ISC function is critical for understanding the mechanisms underlying the development of CRC.

1.2.5 Physiologic Functions of NT

NT plays an important role in FFA absorption in the small intestine and contributes to lipid metabolism and glucose homeostasis (46). Previous work from our lab has shown that NT-deficiency reduces intestinal lipid absorption, prevents the onset of HFD-induced obesity, and attenuates obesity-induced hepatic steatosis and insulin resistance (46). In humans, NT is a predictive marker for the development of obesity and its comorbidities. In human longitudinal studies, patients with high fasting plasma levels of pro-NT were over twice as likely to develop obesity later in life as those with the lowest pro-NT levels (46). Elevated plasma concentrations of pro-NT are also associated with insulin resistance, metabolic disorders, and visceral adipose tissue (VAT) inflammation in obese patients (67, 68) and are predictive of the presence and severity of non-alcoholic fatty liver disease (NAFLD) (69). Notably, both NT and HFD play an important role in the development of certain cancers, and FFAs promote NT secretion and intestinal cancer growth (70, 71). Given the role of NT on FFA absorption and HFD-induced obesity, NT-mediated lipid absorption may contribute to HFD-induced tumorigenesis.

The proliferative function of NT in the gastrointestinal tract has been demonstrated in multiple models of normal physiology and intestinal stress. NT stimulates growth of the mammalian small intestine and colon under basal conditions and in response to nutrient-

deprivation and intestinal injury (45). NT increases intestinal regeneration after small bowel resection (SBR) in rats, with more prominent effects in the proximal small intestine, and reverses mucosal hypoplasia due to feeding a liquid elemental diet (72-74). In mouse models of chronic intestinal inflammation, NT facilitates mucosal wound healing, suggesting a possible therapeutic role for NT in the treatment of inflammatory bowel disease (75). NT also has potent anti-apoptotic effects, which further contributes to its growth-stimulating functions (76). NT attenuates intestinal epithelial cell apoptosis in mouse models of obstructive jaundice and colitis (75, 77). Collectively, the combined effects of NT on proliferation and apoptosis during basal and stress conditions within the small intestine indicate that NT plays an important protective role in maintenance of intestinal epithelial cell integrity.

Numerous other intestinal growth factors or peptides have been shown to contribute to mucosal growth, protection, or adaptation in the mammalian small intestine (78). Like NT, glucagon-like peptide 2 (GLP-2), insulin-like growth factor-1 (IGF-1), PYY, and gastrin releasing peptide (GRP) stimulate intestinal adaptation following SBR (78). Ghrelin attenuates ISC damage and promotes epithelial cell proliferation following irradiation (79). The GLP-2 analog teduglutide is approved for the treatment of patients receiving total parenteral nutrition (TPN) as a result of clinical and preclinical studies demonstrating that GLP-2 promotes growth, proliferation, and maintenance of the intestinal epithelium (80-82). Interestingly, the enterotrophic effects of GLP-2 are enhanced by the presence of NT (83). However, the extent to which NT contributes to intestinal proliferation or ISC function during nutritional stress has not been fully investigated. In order to determine the full therapeutic potential of NT on intestinal

adaptation to nutrient state or gastrointestinal disorders, the mechanisms and consequences of NT release in the small intestine must be more fully characterized.

1.2.6 Role of NT in Cancer Stem Cell Function

In addition to the pro-growth effects of NT in the normal intestinal mucosa, NT is also known to have oncogenic effects in various cancer types, including gastric, pancreatic, hepatic, colorectal, and breast cancer (84). NT and NTR1 expression are upregulated in numerous cancer cells, and their high expression is associated with poor disease prognosis (58). In addition, some cancer cells secrete NT and express NTR1, leading to autocrine activation of the growth-stimulating pathways downstream of NT/NTR1 signaling (84). The effects of NT on NTR1-expressing cancer cells include increased proliferation, migration, invasion, and angiogenesis (13, 30, 31). Recent studies have implicated NT in regulation of stem-cell like properties of cancer cells, suggesting that NT may contribute to cancer stem cell (CSC) function (85, 86). CSCs are a subpopulation of cancer cells with stem-cell like properties, including self-renewal, differentiation, tumorigenicity, and resistance to cancer therapy (87). NT promotes tumorigenesis and self-renewal of hepatocellular carcinoma (HCC) stem cells, in part, via activation of the ERK1/2 signaling (85). Likewise, NT promotes CSC function in glioblastoma cells in an EGFR-dependent mechanism, whereas NTR1 knockdown reduces expression of glioblastoma CSC markers (86). In CRC cells, CSCs are defined by high WNT activity, and activation of WNT/β-catenin signaling in more differentiated tumor cells restores stem-cell like function and increases CSC clonogenicity, indicating that WNT/β-catenin is an important regulator of CSC function in CRC (88).

1.3 Goals and Hypotheses For Dissertation

The known functions of NT converge on multiple important physiological and pathological processes. NT is associated with lipid absorption, metabolic disorders, HFD-obesity, and oncogenic functions in a variety cancers, suggesting that control of NT release from EE cells may be therapeutically beneficial (42, 48, 89). Yet NT also exerts several protective functions on the intestinal mucosa, indicating that NT release and signaling must be tightly controlled to maintain homeostasis (40, 41, 73, 75). Given the proliferative actions of NT and its proposed crosstalk with multiple signaling pathways that are critical to the maintenance of stem cell function, including ERK1/2 and WNT/β-catenin, the primary goals of this dissertation were to 1) elucidate the mechanisms regulating NT release from endocrine cells, 2) to determine the role of NT on proliferation and stem cell function in the small intestine and 3) to define the effect of NT on colorectal cancer stem cells. We investigated the functional impacts of NT using *in vivo* models of nutrient-deprivation, NT-deficiency/overexpression, and colorectal cancer, and explored the mechanisms of NT release and function utilizing *in vitro* models of gut hormone secretion and *ex vivo* intestinal organoid and tumor organoid systems. Based on the current understanding of NT secretion and functions described here, this dissertation examines the following hypotheses:

1) **NT release from endocrine cells can be manipulated via regulation of the ERK1/2 signaling pathway.**
2) **NT regulates intestinal stem cell function via activation of WNT/β-catenin signaling.**

3) NT promotes cancer stem cell function in colorectal cancers through activation of WNT/β-catenin signaling.

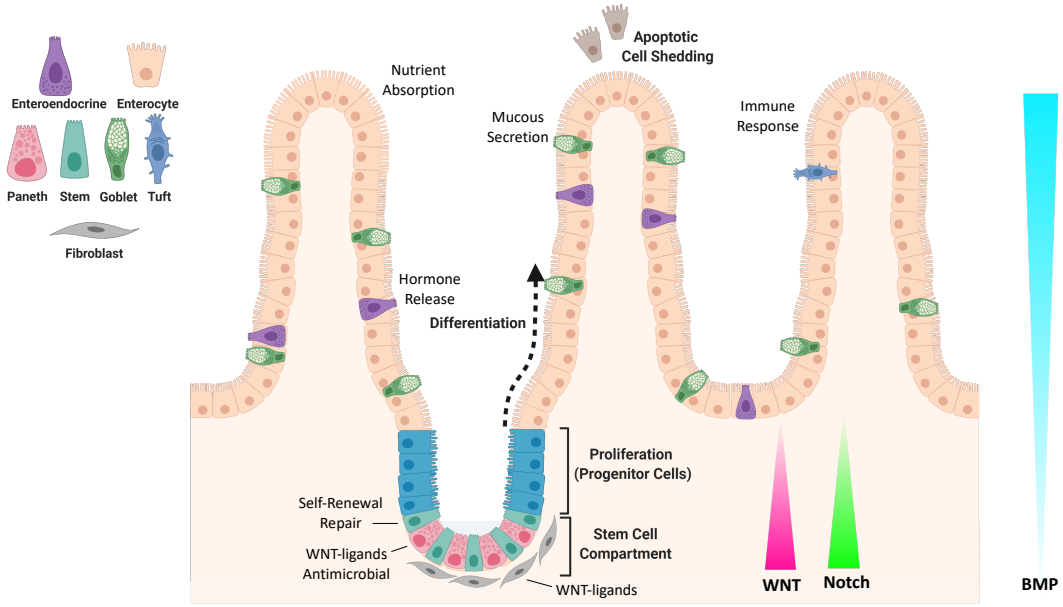


Figure 1.1: Mechanisms regulating proliferation, differentiation, and stem cell function in the small intestine.

Self-renewal of ISCs, located at the crypt base, is regulated by WNT, Notch, and MAPK signaling, whereas BMP and hedgehog signaling oppose these proliferative signals in the villus regions and enable progenitor cells to differentiate into enterocytes, enteroendocrine (EE), Paneth, goblet, or tuft cells. WNT ligands are secreted from Paneth cells and subepithelial fibroblasts and maintain high levels of WNT-activation in the ISC niche.

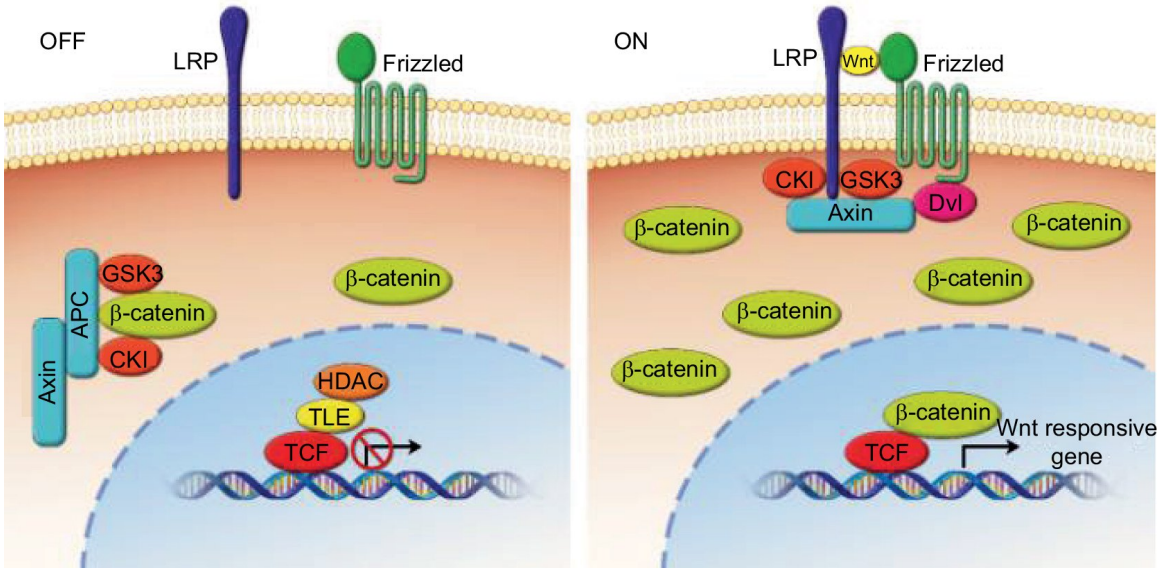


Figure 1.2: Overview of the WNT/β-catenin signaling pathway. (Left) In the absence of WNT-ligands, the β-catenin destruction complex, composed of GSK3, CKI, APC, and Axin, phosphorylates β-catenin leading to its proteasomal degradation and repression of WNT-target gene expression. Upon WNT ligand binding to Frizzled (FZD) and LRP5/6 on the plasma membrane, DVL and Axin are recruited to the plasma membrane, causing disruption of the β-catenin destruction complex and enabling β-catenin to enter accumulate, enter the nucleus, and regulate WNT-target gene expression via activation of TCF/LEF transcription factors (Adapted from Khalf et al. (90)).

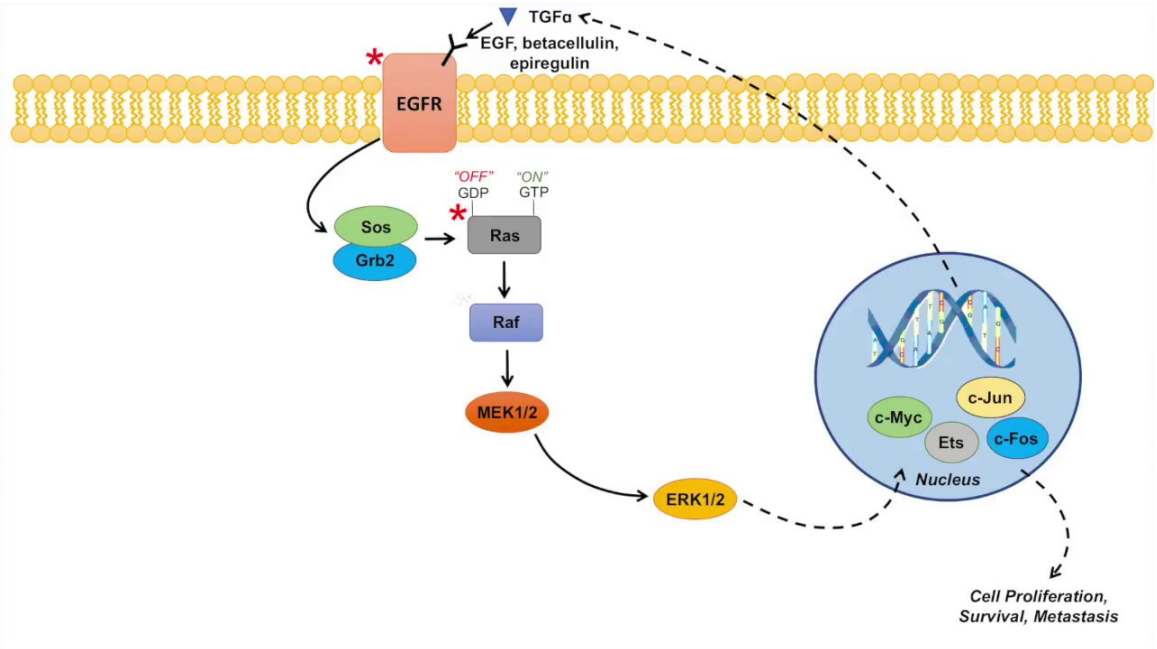


Figure 1.3: Summary of ERK/MAPK signaling. Growth factor binding to EGFR activates the MAPK signaling pathway (RAS-RAF-MEK-ERK), leading to phosphorylation and activation of ERK1/2, which regulates transcription of genes involved in cell proliferation, survival, and metastasis (Adapted from JJ Medicine).

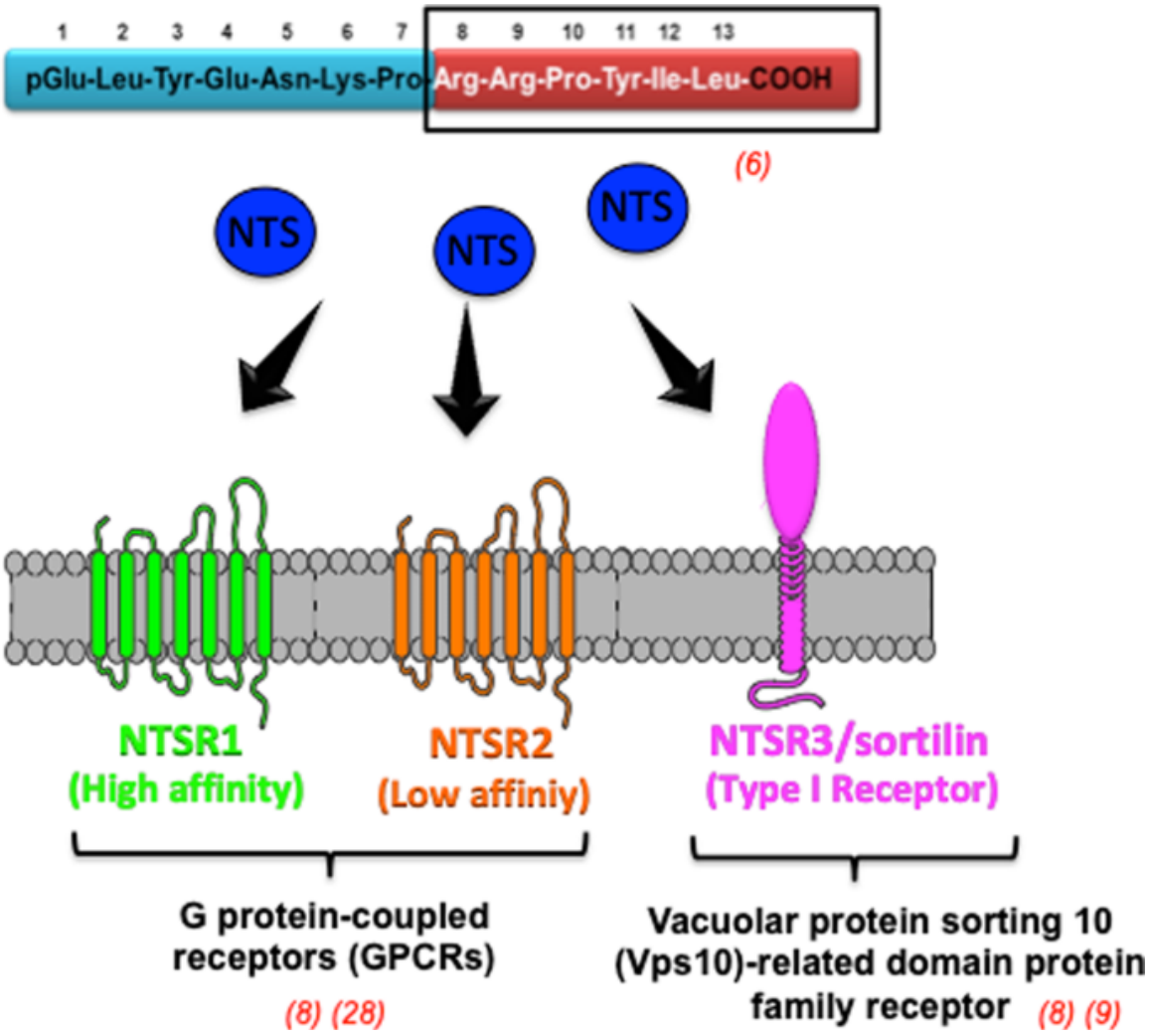


Figure 1.4: Neurotensin receptors. Neurotensin signaling is mediated by three NT receptors (NTR1, NTR1, and NTR3). NTR1 and NTR2 are GPCRs and NTR3 is type I receptor identical to sortilin. (Adapted from Christou et al. (47))

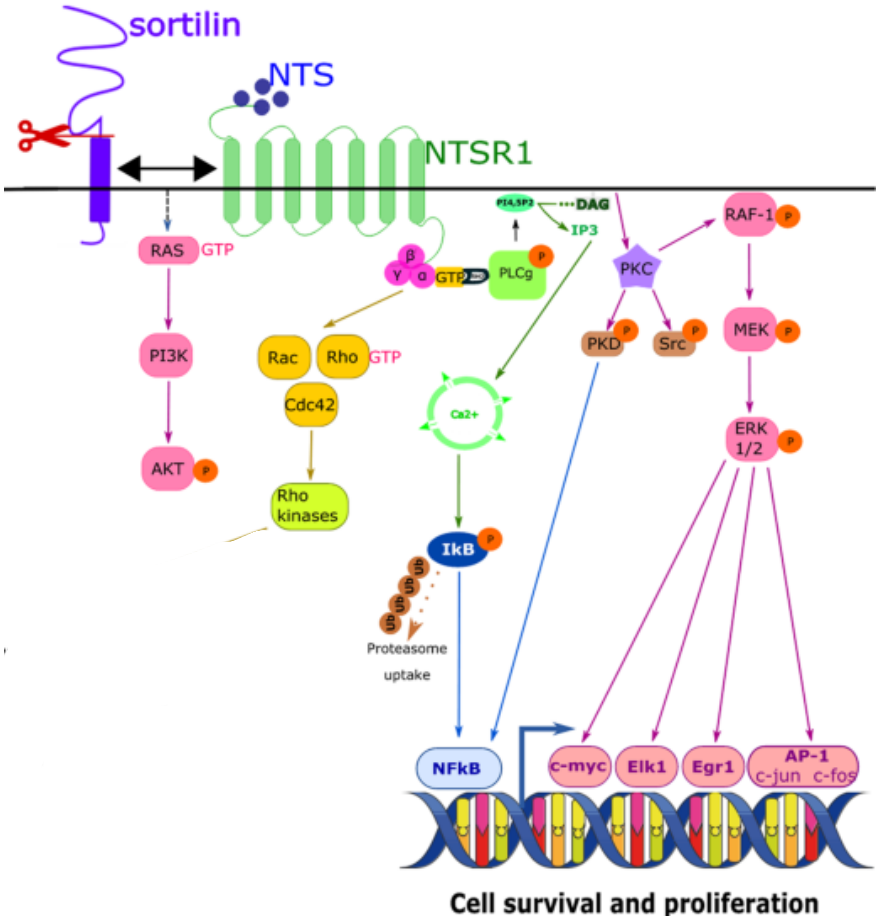


Figure 1.5: Overview of NT/NTR signaling. NT binds to NTR1 alone or in complex with NTR3 and activates PLC, PI3K/AKT, MAPK/ERK, or Rho GTPase signaling. Downstream effectors of these pathways participate in transcription of genes that regulate cell proliferation and survival. (Adapted from Christou et al. (47))

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

The following mice were used for these studies: NT wild type ($Nt^{+/+}$), knockout ($Nt^{-/-}$), Lgr5-EGFP-IRES-CreERT2 (Jackson Laboratory, strain name: B6.129P2-Lgr5tm1(cre/ERT2)Cle/J, stock number 008875), and Lgr5-EGFP-IRES-CreERT2; $Nt^{-/-}$ mice; APC^{min};$Nt^{+/+}$ and APC^{min}; $Nt^{-/-}$ mice, and APC^{min}/*KRAS* mice. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Mice were maintained on a 14 h light/10 h dark cycle and provided with food and water *ad libitum* (AL) unless otherwise indicated (3). For fasting studies, mice were fasted for 48 h beginning at 9 AM with AL access to water. Body weight was measured daily during the fasting period. Control mice were sex- and age-matched littermates fed standard chow AL. For HFD-induced obesity studies, mice were placed on a 60% HFD or 10% LFD (catalogue no. D12492 and D12450B, respectively; Research Diets, New Brunswick, NJ) at weaning for 22–24wks. Male and female mice aged 3-6 months were used for all studies.

2.2 Tissue isolation and organoid culture

Crypts were isolated from the proximal two-thirds of the small intestine as previously described (25). Briefly, the small intestine was removed, opened longitudinally, washed with PBS, and cut into 3–5mm fragments. Fragments were incubated in cold PBS containing 10mM EDTA for 30-60 min on ice at 4°C, followed by vortexing and filtration through a 70 μM cell strainer. Isolated crypts were collected for RNA and protein extraction or organoid culture. For organoid culture, crypts were embedded in growth-factor reduced Matrigel (Corning) and cultured in basal organoid media supplemented with

EGF 40 ng/ml (Peprotech), Noggin 200 ng/ml (Peprotech), and R-spondin 500 ng/m1 (Sino Biological). Basal organoid media contained Gibco, Advanced DMEM/F12 supplemented with N-acetyl-L-cysteine 1 M (Sigma-Aldrich), N2 1X (Life Technologies), B27 1X (Life Technologies), Penicillin/Streptomycin 1X (Sigma-Aldrich), HEPES 1X (Sigma-Aldrich), and Glutamax 1X (Sigma-Aldrich). Organoids were passed every 5-7 d by mechanical disruption with a 1 mL pipette and replated in fresh Matrigel.

For tumor isolation and tissue collection, mice were sacrificed and small intestine and colon removed for organoid culture and immunohistochemistry. The distal one-third of the small intestine and entire colon, excluding cecum, were fixed overnight in 10% neutral buffered formalin for immunohistochemistry. Tumors were removed from the proximal two-thirds of the small intestine for organoid culture as previously described. Briefly, tumors were rinsed in cold PBS, minced, and incubated in digestion buffer composed of DMEM-F12 supplemented with FBS, collagenase and dispase for 1h at 37°C with agitation. Following digestion, tumor fragments were allowed to settle and supernatant was collected and centrifuged for 3 min at 200g. The cell pellet was washed once with ice-cold PBS and filtered through a 40uM cell strainer. Tumor cells were embedded in Matrigel (Corning) and cultured in basal organoid media supplemented with EGF 100 ng/ml (Peprotech). Basal organoid media contained Gibco, Advanced DMEM/F12 supplemented with N-acetyl-L-cysteine 1 M (Sigma-Aldrich), N2 1X (Life Technologies), B27 1X (Life Technologies), Penicillin/Streptomycin 1X (Sigma-Aldrich), HEPES 1X (Sigma-Aldrich), and Glutamax 1X (Sigma-Aldrich). Tumor organoids were passed every 3-5 d by incubating in TrypLE Express (Invitrogen) for 5 min at 37°C, dissociating with a 1 mL pipette, and replating in fresh Matrigel.

2.3 Colony formation and viability assays

For crypt colony-formation assays, freshly isolated crypts were counted and plated in triplicate in a 48-well plate. Organoid formation was quantified 3 d after initiation of cultures and normalized to the number of crypts plated per well at day 0. For tumor organoid colony-formation assays, mature tumor organoids were incubated in TrypLE Express (Invitrogen) for 5 min at 37°C and then passed through a 23G needle to achieve a single-cell suspension. Cells were counted on a Vi Cell XR Cell Counter and 2,000 cells were plated in triplicate in a 48-well plate. Colony formation was assessed 4-7d following initiation of culture. HCT116 colony formation was assessed using the 3D CellTiter Glo Viability assay according to the manufacturer's protocol. Briefly, 7d after initiation of tumorsphere culture, 50% of the cell culture media was removed and replaced with Cell Titer Glo reagent, incubated at room temperature for 25 min with agitation, and luciferase analyzed on a Varioscan Lux plate reader.

2.4 *Drosophila* studies

Constitutive expression of NT in gut EE was achieved by cloning the gut EE cell-specific tachykinin (TK) promoter (2.0kb) into attB-UAST lacking Gal4 binding sites followed by insertion of full-length NT cDNA (91, 92). The resulting plasmid (TK-NT) was inserted at the VK5 attP locus. *esg*-Gal4-UAS-GFP/Cyo; +/TM6B and *esg*-Gal4-UAS-GFP/Cyo; TK-NT/TM6B flies were fed standard fly food (cornmeal-yeast) or low energy diet (LED) (93, 94). Briefly, flies (5d after emerging) were maintained on standard fly food for an additional 5d, split into two groups, and fed either standard fly food or LED fly food (diet composition in 100 ml media: 4 g yeast extract; 4 g sucrose; 5.2 g cornmeal; 1.5 g

agar) for a further 5d. Midguts were dissected, fixed in 4% formaldehyde in PBS for 20min, permeabilized in 0.1% Triton X-100 in PBS, and processed for either immunohistochemistry using α-GFP (Clontech) primary antibody or lipid staining using Nile Red. Fluorescence signals were acquired on an Olympus confocal microscope and images processed with Olympus FV10-ASW Ver.3.1b. 100-700 ISCs were quantified in the midgut of 8-15 flies per group and normalized to the area of midgut captured per image.

2.5 RNA-Seq, differential expression, and gene set enrichment analysis

Isolated RNA quality and quantity was assessed using the Agilent Bioanalyzer 2100 RNA Nano chip. RNA-Seq libraries were prepared using KAPA RNA Hyper + RiboErase HMR (Roche). The manufacturer's protocols were used to sequence ribosomal RNA-depleted libraries at 1x100 single-end read on an Illumina HiSeq 2500 in rapid mode, to an average depth of 32 million single-end reads per sample. The data was stored as FASTQ files. Sequencing reads were trimmed and filtered using Trimmomatic (V0.39) to remove adapters and low quality reads (95). Reads were mapped to Ensembl GRCm38 (release 100) transcripts annotation using RSEM (96). RSEM results normalization and differential expression analysis were performed using the R package edgeR (97). Significantly up/downregulated genes were determined as fold change >= 2 and q-value < 0.05. Gene set enrichment analysis was performed using GSEA software and the Hallmark gene sets in the Molecular Signature Database (MSigDB) (98)

2.6 Flow cytometry

Fresh intestinal crypts or organoids were incubated in TrypLE Express (Invitrogen) for 15 min at 37°C and then passed through a 23G needle to achieve a single-cell

suspension (25). Cells were washed in PBS, resuspended in PBS containing 1mM EDTA, 25mM HEPES, 1% FBS, and DNAse (1 μg/ml) and filtered through a 40μm cell strainer. Cells were labeled with CD11b-PE, CD31-PE, CD45-PE, CD133-PE and EPCAM-APC (BioLegend) and sorted with a BD FACS Aria II SORP cell sorter (25). Lgr5-EGFP^{+}/Ep-CAM^{+}/ CD11b^{-} CD31^{-}CD45^{-} CD133^{-} cells were quantified using FlowJo (v10) software (25). Dead cells were excluded from analysis using propidium iodide (PI) (50 μg/ml) (Abcam) (99).

2.7 Cell culture

The BON cell line was derived from a human pancreatic carcinoid tumor and characterized previously (100, 101). BON cells were cultured in Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham supplemented with 5% fetal bovine serum (FBS) in 5% CO_2 at 37°C. The QGP-1 cell line, derived from a pancreatic somatostatinoma (Japan Health Sciences Foundation, Osaka, Japan), was maintained in RPMI-1640 medium supplemented with 10% FBS (102). The human colorectal adenocarcinoma cell line HT29 and the human colon carcinoma cell line HCT116 were maintained in McCoy's 5a Medium supplemented with 10% FBS or DMEM supplemented with 10% FBS, respectively. For HCT116 tumorsphere formation, HCT116 cells were plated at 1,000 cells per well in ultra low-adhesion cell culture plates and cultured in serum-free DMEM F12.

2.8 Cell treatment, transfection, and lentiviral transduction

siRNA transfections were performed using RNAiMAX (Life Technologies, Grand Island, NY). Forty-eight h after transfection, BON and QGP-1 cells were treated with 100μM DHA in serum-free medium for 90 min. Media was collected for NT-EIA and cells

were lysed for western blot or RNA extraction. For generation of cell lines expressing KSR1-targeting shRNA, BON or QGP-1 cells were plated in 6-well plates (5×10^5 cells/well) in growth media containing purified non-targeting control (NTC) or KSR1-targeting shRNA lentivirus. Puromycin-selection was used to select for cells stably expressing KSR1 shRNA and knockdown efficiency was measured via western blot or quantitative real-time PCR (qRT-PCR). For cell lines expressing ERK2 or Exo70 shRNA, lentivirus of NTC, ERK2, or Exo70 shRNAs were co-transfected with an ectopic packaging vector into 293T cells. At 48 to 72 h post-transfection, viral supernatants were collected and filtered through a 0.45-μm Surfactant Free-Cellulose Acetate sterile syringe filter. BON cells were plated in 6-well plates (5×10^5 cells/well) and incubated with the viral supernatant for 24 h, followed by incubation with growth medium for an additional 24 h. Following stable shRNA transfection, BON cells lines were sensitized to FFA treatment and therefore treated with 30μM DHA to reduce toxicity relative to 100μM DHA treatment. Exo70 overexpression experiments were performed with 30μM DHA for consistency with Exo70 shRNA experiments.

For generation of cells overexpressing KSR1 or Exo70, DNA was isolated from MSCV-IRES-GFP, MSCV-KSR1-IRES-GFP, pEGFP-control, pECFP-N-KSR1, and pEGFP-C3-Exo70 plasmids using the Qiagen Plasmid Miniprep Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Plasmid DNA was used to transfect BON or QGP-1 cells using Lipofectamine LTX with PLUS Reagent. Cells were collected for NT-EIA at 48-72 h post-transfection and overexpression measured via western blot or qRT-PCR. ON-TARGETplus SMARTpool (KSR1) and ON-TARGETplus Non-targeting Control Pool siRNA were from GE Dharmacon (Lafayette, CO). Non-targeting control

shRNA and shRNA targeting KSR1, ERK2, and Exo70 in bacterial glycerol stock were from Sigma-Aldrich. MSCV-IRES-GFP, MSCV-KSR1-IRES-GFP, pEGFP-control and pEGFP-C3-Exo70 plasmids were from Addgene (Cambridge, MA). pECFP-N-KSR1 plasmid was from Dr. Emilia Galperin's lab (University of Kentucky).

For NT treatment of CRC cells, cells, tumorspheres, and/or tumor organoids were treated with NT (1-100nM as indicated) in the presence or absence of the NTR1 inhibitor SR48692 (5-10 μM), the MEK inhibitor PD0325901 (10 nM), or the mTOR inhibitor Rapamycin (20 nM). Adherent HCT116 and HT-29 cells were treated in serum-free DMEM/F12 or serum-free McCoy's 5a for the indicated duration. Tumor organoids and HCT116 tumorspheres were treated in basal organoid media or serum-free DMEM/F12, respectively. Inhibitor treatment was performed for 30 min prior to treatment with NT.

2.9 DHA treatment and NT enzyme immunoassay (EIA)

Cells were plated in 24-well plates at a density of $15\times10^4/cm^2$ and grown for 48 h for drug treatment and transfection. DHA treatment was performed in serum-free growth medium for 90 minutes. For treatment with PD compound, cells were pretreated with PD compound for 30 minutes in serum-free growth medium and then treated with DHA plus PD for an additional 90 minutes. Media were collected for NT secretion measurement using the NT-EIA kit from Phoenix Pharmaceuticals (Belmont, CA) as described previously (103, 104) and cells were lysed for western blotting or RNA isolation. Data obtained from NT-EIA were normalized by protein concentration from corresponding cell lysates.

2.10 *in situ* hybridization

Single molecule *in situ* hybridization was performed using Advanced Cell Diagnostics RNAscope 2.0 HD Detection Kit (ACD Bio) with mouse *Lgr5 Mm-Lgr5 (REF*

312171). Antigen retrieval was performed for 15 min and peroxidase pretreatment performed for 30 min. Stained sections were imaged using an Aperio ScanScope XT slide scanner at 20x and *Lgr5* expression quantified using HALO software (Indica Labs)

2.11 Immunohistochemistry

For histological analysis, the small intestine (including duodenum, jejunum and ileum) was divided into thirds and the proximal and distal thirds fixed overnight in 10% neutral buffered formalin, paraffin embedded, and sectioned. Antigen retrieval was performed with Tris-EDTA buffer pH 7.8 at 95°C for 64 min (standard CC1). Staining was performed on the Ventana Autostainer with Ki67 (Abcam 16667, 1:200 for 1 hour at 37°C). Alcian Blue (Sigma a-5268) staining was performed manually with 1% Alcian Blue Solution in 3% glacial acetic acid. Ventana OmniMap anti-rabbit HRP and DAB were used for visualization per manufacturer's instructions. Stained sections were imaged using an Aperio ScanScope XT slide scanner at 20x and analyzed using HALO software (Indica Labs). Ki67+ and Alcian Blue+ cells were counted in 50 crypts per section in proximal and distal small intestine from at least 5 mice per group.

2.12 Immunofluorescence

BON cells were plated on glass coverslips (#1) in 24-well plates at a density of $20\times10^4/cm^2$ and transfected with pEGFP-control and pECFP-N-KSR1 plasmid DNA using Lipofectamine LTX with PLUS Reagent. 48 h after transfection, cells were fixed in 4% paraformaldehyde/PBS, permeabilized with 0.3% TritonX-100/PBS, and blocked with 0.1% bovine serum albumin/PBS. NT antibody was from Abcam (Cambridge, UK). Cells were incubated with primary antibody for 1 hour, followed by Alexa Fluor-conjugated

secondary antibody from Invitrogen for 30 minutes. Images were observed under a Nikon confocal microscope with 40× objective.

2.13 Organoid EdU labeling

5-Ethynyl-2′-deoxyuridine (EdU)-labeling of organoids was achieved using the Click-iT EdU Cell Proliferation Kit for Imaging (Invitrogen) according to the manufacturer's instructions. EdU-positive cells were quantified using Nikon Software and expressed as Pearson's coefficient for colocalization between 4',6-Diamidino-2-Phenylindole (DAPI) and EdU. Colocalization was measured in 15-30 organoids from 3 different mice per group.

2.14 Quantitative real-time PCR

Total RNA was isolated from cells, crypts, or organoids using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from equal concentrations of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using a TaqMan Gene Expression Master Mix (ThermoFisher) and TaqMan probes for *Ksr1*, *Tcf7, c-Myc, Lgr5, Ascl2, Axin2, Olfm4, Nt*, *Actb, and GAPDH* (ThermoFisher) according to manufacturer's protocol. Relative mRNA expression was calculated using the comparative ΔΔCt method.

2.15 Protein preparation and western blotting

Protein preparation and western blotting were performed as previously described (103, 105). Briefly, cells, crypts, or organoids were lysed with lysis buffer (Cell Signaling Technology) containing 1mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of protein were resolved on 4-12% NuPAGE BisTris gels (Invitrogen, Carlsbad, CA) and

electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used for western blotting: KSR1 (LifeSpace BioSciences), GFP (Clontech), Flag (Sigma-Aldrich), pERK (CST #9101), ERK (CST #9102), GSK3β (CST #12456), pGSK3β (CST #9322), LRP6 (CST #3395), pLRP6 (CST #2568), AKT (#4691), pAKT (#4058), active-β-catenin (CST #8814), total β-catenin (CST #8480), and β-actin (Sigma Aldrich A5316). Membranes were incubated with primary antibodies overnight at 4°C followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Membranes were developed with the iBright™ FL1500 Imaging System (Invitrogen). Band intensity was measured using iBright Analysis Software (Thermo Fisher) using β-actin or total protein as loading controls.

2.16 Statistical analysis

All experiments were performed at least three independent times and all sample number (n) indicates biological replicates. For colony formation assays, 3-4 wells of organoids from 5-6 mice per group were analyzed. NT secretion values were normalized to protein concentration of corresponding lysates. All quantitative data on qRT-PCR for genes, western blot and immunohistochemistry for proteins, organoid labeling, and fluorescence levels for ISC quantification were summarized using bar graphs with means and standard deviations. Comparisons across experimental factors, which includes combination of genotype and diet, were analyzed using one-way analysis of variance or repeated measures linear mixed model to account of repeat samples within mice. Adjustment for multiple pairwise comparisons was performed within the model using the Holm's p-value stepdown method. Assessment of model assumptions were performed and data were log-transformed as necessary for optimal fit of the model.

Statistical analyses were performed using the SAS system 9.4 (Cary, NC). Bioinformatics methods for differential analysis were performed using the R package as described above.

CHAPTER 3:

KINASE SUPPRESSOR OF RAS 1 AND EXOCYST COMPLEX COMPONENT 70 PROMOTE FATTY ACID-STIMULATED NEUROTENSIN SECRETION THROUGH ERK1/2 SIGNALING IN HUMAN NEUROENDOCRINE CELLS[1]

3.1 Abstract

Neurotensin is a peptide hormone released from enteroendocrine cells in the small intestine in response to fat ingestion. Although the mechanisms regulating neurotensin secretion are still incompletely understood, our recent findings implicate a role for extracellular signal–regulated kinase 1 and 2 as positive regulators of free fatty acid-stimulated neurotensin secretion. Previous studies have shown that kinase suppressor of Ras 1 acts as a molecular scaffold of the Raf/MEK/extracellular signal–regulated kinase 1 and 2 kinase cascade and regulates intensity and duration of extracellular signal–regulated kinase 1 and 2 signaling. Here, we demonstrate that inhibition of kinase suppressor of Ras 1 attenuates neurotensin secretion and extracellular signal–regulated kinase 1 and 2 signaling in human endocrine cells. Conversely, we show that overexpression of kinase suppressor of Ras 1 enhances neurotensin secretion and extracellular signal–regulated kinase 1 and 2 signaling. We also show that inhibition of extracellular signal–regulated kinase 2 and exocyst complex component 70, a mediator of secretory vesicle exocytosis, potently inhibits basal and docosahexaenoic acid-stimulated neurotensin secretion, whereas overexpression of exocyst complex component 70 enhances basal and docosahexaenoic acid-stimulated neurotensin secretion. Together, our findings

---

[1] This research was originally published in PLoS One. Rock, S., Li, X., Song, J., Townsend, C. M., Jr, Weiss, H. L., Rychahou, P., Gao, T., Li, J., & Evers, B. M. (2019). Kinase suppressor of Ras 1 and Exo70 promote fatty acid-stimulated neurotensin secretion through ERK1/2 signaling. *PloS one*, *14*(3), e0211134. https://doi.org/10.1371/journal.pone.0211134

demonstrate a role for kinase suppressor of Ras 1 as a positive regulator of neurotensin secretion from human endocrine cells and indicate that this effect is mediated by the extracellular signal–regulated kinase 1 and 2 signaling pathway. Moreover, we reveal a novel role for exocyst complex component 70 in regulation of neurotensin vesicle exocytosis through its interaction with the extracellular signal–regulated kinase 1 and 2 signaling pathway.

3.2 Introduction

Obesity in the United States has reached epidemic levels, with approximately 70% of the population overweight and over 30% of overweight individuals classified as obese (1, 2). Cancers associated with overweight and obesity account for approximately 40% of all diagnosed cancers in the United States (1). Excess adiposity is thought to contribute to the development of cancer through the release of free fatty acids (FFAs) by adipose tissue, which have been shown to promote tumorigenesis by serving as metabolic fuel for highly proliferating cells (3-6). FFAs have also been demonstrated to regulate expression and secretion of hormones and neuropeptides, including the gastrointestinal neuropeptide neurotensin (NT), which is implicated in the promotion of obesity and the development of several types of cancer (7-12).

NT is released in the small intestine in response to fat ingestion and has been implicated in the development of metabolic disorders and many types of cancer, including breast, pancreatic, lung, prostate, and colorectal cancer (9, 10, 13-16). NT facilitates FFA absorption in the intestine and contributes to lipid metabolism and glucose homeostasis (12). In mice, NT deficiency reduces intestinal fat absorption and is protective against high-fat diet-induced obesity, hepatic steatosis, and insulin resistance (12). In humans, elevated

plasma concentrations of pro-NT (a stable NT precursor fragment produced in equimolar amounts relative to NT) are associated with insulin resistance, obesity-associated metabolic disorders, and visceral adipose tissue (VAT) inflammation in obese patients (13, 17). Moreover, high plasma pro-NT levels are predictive of the presence and severity of non-alcoholic fatty liver disease (NAFLD) (18). Interestingly, both NT secretion and cancer growth are stimulated by unsaturated FAs (7, 19). Pre-clinical and epidemiologic studies indicate that high intake of unsaturated fatty acids increases cancer risk and promotes cancer progression (5, 7, 19-22). We have recently shown that NT secretion from enteroendocrine (N) cells is enhanced by common unsaturated dietary FAs, including oleic acid, palmitoleic acid, and docosahexaenoic acid (DHA) (11). Given its intersecting role in metabolic disorders and tumorigenesis, NT may be a potential therapeutic target for metabolic diseases and cancer.

NT is secreted from N cells via the regulated secretory pathway (23). After proteolytic processing of the NT precursor (pro-NT), active NT peptide is transported from the trans Golgi network and stored in secretory vesicles until NT release is triggered by extracellular stimuli (23-25). NT-containing secretory vesicles are then transported to the plasma membrane and released to the cell exterior via exocytosis (24, 25). While the most potent extracellular stimulus regulating NT release from the small intestine is fat ingestion, the molecular mechanisms regulating FA-stimulated NT secretion are still incompletely understood (23). We have previously demonstrated that NT gene expression is enhanced by Ras signaling and that extracellular signal–regulated kinase 1 and 2 (ERK1/2) positively regulate NT gene expression and FA-stimulated NT peptide secretion (11, 12, 26).

ERK1/2 are mitogen activated protein kinases (MAPKs) that play a central role in the regulation of cell proliferation, differentiation, and survival (27). ERK1/2 signaling is activated upon ligand binding to membrane receptors and subsequent activation of the small GTPase Ras, which phosphorylates and activates the MAP kinase kinase kinase (MAPKKK) Raf (28). Activated Raf phosphorylates the MAP kinase kinases (MAPKKs) MEK1 and MEK2 (MEK1/2), which phosphorylate and activate ERK1/2 (28). Activated ERK1/2 phosphorylates a large number of cytosolic substrates to regulate diverse cellular functions and can also be translocated to the nucleus where it activates transcription factors regulating gene expression (27-29). Deregulation of Ras/MAPK signaling contributes to approximately one-third of human cancers (30, 31)

Coordination of the Raf/MEK/ERK protein complex and subsequent ERK1/2 phosphorylation is regulated by the scaffold protein kinase suppressor of Ras 1 (KSR1) (32-34). KSR1 coordinates formation of the Raf/MEK/ERK signaling complex, increasing specificity of MEK phosphorylation by Ras and ERK1/2 phosphorylation by MEK (35-37). Though a recent study presents contrasting evidence suggesting KSR1 allosterically regulates the interaction between Raf/MEK/ERK, the stimulatory effect of KSR1 on ERK1/2 remains well-substantiated (38). KSR1 is required for Ras-induced transformation of mouse embryonic fibroblasts (MEFs) and increases proliferative potential of mammary epithelial cells when expressed at optimal levels (34). KSR1-deficient mice exhibit reduced Ras-dependent mammary tumor formation (39, 40). In the absence of KSR1, high molecular weight complexes containing KSR1, ERK, and MEK are abolished, and KSR1 knockout mice exhibit reduced ERK signaling (40). Furthermore, reintroduction of KSR1 into KSR1-deficient mice rescues ERK signaling (34). However, KSR1 is not required for

ERK signaling or normal embryonic development. KSR1-deficient mice develop normally, despite attenuated ERK signaling and slightly reduced T-cell activation (35, 40). Because KSR1 promotes Ras and ERK activation and yet is dispensable for normal development, its potential as a therapeutic target for Ras-driven cancers is being widely investigated (41). Given the role of NT downstream of Ras and ERK1/2 signaling, we reasoned that KSR1 may also regulate NT secretion.

In this study, we examine the role of KSR1 in NT secretion and ERK1/2 signaling in the human endocrine cell lines BON and QGP-1. We demonstrate that inhibition of KSR1 reduces, while its overexpression enhances NT secretion and ERK1/2 signaling. We also show that Exo70, a component of the exocyst complex and direct substrate of ERK2, positively regulates NT secretion. These findings describe a role for KSR1 as a positive regulator of NT secretion through activation of the ERK1/2 signaling pathway and suggest that the interaction between ERK2 and Exo70 contributes to NT secretion in human endocrine cells.

3.3 Results

3.3.1 *KSR1 inhibition reduces NT secretion*

KSR1 is expressed in intestinal epithelial cells, where it is protective against cytokine-induced injury (106, 107). NT is secreted from specialized enteroendocrine (N) cells of the small intestine and is stimulated by ingestion of dietary fats (30, 31, 33, 108). To determine whether KSR1 is expressed in human endocrine cells and characterize its localization relative to NT, we used the BON cell line, which synthesizes and secretes NT in a manner equivalent to that of N cells in the small bowel. BON cells were transfected with CFP-tagged KSR1 plasmid DNA to enable detection of KSR1with fluorescent

confocal microscopy at 488nm and subsequently labeled with fluorescent anti-NT antibody (100). Consistent with previous evidence, we show that NT is localized to secretory vesicles in human endocrine cells, while KSR1 exhibits diffuse cytosolic staining **(Fig 3.1)**. Interestingly, distribution of KSR1 and NT is largely mutually exclusive, where KSR1 expressing cells do not contain NT-containing secretory vesicles and cells retaining large amounts of NT do not express high levels of KSR1. This observation suggests that KSR1 expression promotes release of NT vesicles from N cells.

We have previously demonstrated that ERK1/2 plays a stimulatory role in NT secretion (43, 44). The function of KSR1 as a scaffold of the Raf/MEK/ERK complex has been reported to enhance ERK1/2 signaling (109-111). To determine whether KSR1 is involved in NT secretion, we transfected BON cells with small interfering RNA (siRNA) against KSR1. Forty-eight h after transfection, cells were treated for 90 min with docosahexaenoic acid (DHA), a long-chain unsaturated FA that we have previously shown stimulates NT secretion, thereby mimicking the effects of fat-ingestion in the small bowel. KSR1 knockdown was confirmed via qRT-PCR and NT secretion was measured using NT-EIA. Consistently, inhibition of KSR1 attenuates DHA-stimulated NT secretion **(Fig 3.2A)**.

To verify these results, we next established BON cell lines stably expressing KSR1-targeting shRNA. shRNA-mediated knockdown of KSR1 reduces basal and DHA-stimulated NT secretion from BON cells **(Fig 3.2B)**. To further confirm these findings, we established KSR1 knockdown using siRNA in another human endocrine cell line, QGP-1. QGP-1 cells, derived from a pancreatic somatostatinoma, produce and secrete high levels of NT in response to FA-stimulation, including DHA (43). Consistent with the effect of

KSR1 on NT secretion in BON cells, inhibition of KSR1 in QGP-1 cells reduces basal and DHA-stimulated NT-secretion **(Fig 3.2C)**. Together, these data demonstrate that KSR1 regulates NT release from human endocrine cells.

3.3.2 *KSR1 inhibition reduces ERK1/2 phosphorylation*

The scaffolding function of KSR1 on the Raf/MEK/ERK complex has been demonstrated to coordinate and enhance ERK1/2 activation (17, 18, 44, 112). KSR1-deficient mice exhibit attenuated ERK1/2 signaling and a reduction in MEK- and ERK-containing high molecular weight complexes (113). To determine whether the effect of KSR1 on NT secretion is mediated by the ERK1/2 signaling pathway, BON and QGP-1 cells transfected with KSR1-targeting siRNA were treated with DHA to stimulate NT secretion and lysed for western blotting. Consistently, inhibition of KSR1 decreased ERK1/2 phosphorylation (p-ERK) **(Fig 3.3)**. These findings are consistent with studies demonstrating that KSR1 positively regulates ERK1/2 signaling and indicate that the effect of KSR1 on NT secretion in human endocrine cells is mediated by its interaction with the Raf/MEK/ERK signaling complex.

3.3.3 *KSR1 overexpression stimulates NT secretion and ERK activity.*

As a scaffold protein, KSR1 can produce both inhibitory and stimulatory effects on ERK1/2 signaling when overexpressed (109). At optimal expression levels, KSR1 facilitates maximal coordination of Raf/MEK/ERK and enhances ERK1/2 signaling, while at levels above or below this threshold, ERK1/2 signaling is inhibited. As such, the effects of KSR1 overexpression are tissue specific and based on endogenous levels of KSR1 expression (109, 112, 113). To determine the effects of KSR1 overexpression on ERK1/2

signaling in human endocrine cells, we transfected BON cells with KSR1-overexpressing plasmids and performed western blot to assess ERK1/2 activation. Overexpression of KSR1 enhances ERK1/2 phosphorylation, suggesting that endogenous KSR1 expression in BON cells is below the threshold for coordinating maximal Raf/MEK/ERK signaling **(Fig 3.4A)**. To determine whether the effect of KSR1 overexpression on ERK1/2 signaling corresponds to an increase in FA-stimulated NT secretion, we overexpressed KSR1 in BON and QGP-1 cells and performed NT-EIA. Consistent with the observed increase in ERK1/2 signaling, DHA-stimulated NT secretion is enhanced by KSR1 overexpression **(Fig 3.4B-C)**. These data establish KSR1 as a positive regulator of FA-mediated NT secretion in endocrine cells through activation of the Raf/MEK/ERK signaling pathway.

3.3.4 Exo70 positively regulates NT secretion

Upon extracellular stimulation of NT release, NT-containing vesicles are transported to the plasma membrane for exocytosis and extracellular release (37, 38). Tethering of secretory vesicles to the plasma membrane prior to secretion is mediated by the exocyst, an octameric protein complex comprised of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (114, 115). Exo70 is a direct substrate of ERK2, which phosphorylates Exo70 at Ser250 and thereby modulates the processes mediating vesicle exocytosis (116). Whether Exo70 regulates exocytosis of NT secretory vesicles is unknown. To determine whether Exo70 is involved in ERK-mediated NT secretion, we generated BON cell lines stably expressing ERK2 shRNA. Consistent with previous reports, ERK2 inhibition reduces Exo70 expression in BON cells **(Fig 3.5A)** (116). NT secretion is also significantly attenuated by ERK2 knockdown **(Fig 3.5B)**. To verify these results, we pre-treated BON cells with the MEK inhibitor PD 98059 to inhibit

phosphorylation of ERK1/2. MEK inhibition abolished ERK1/2 phosphorylation in basal and DHA-stimulated BON cells **(Fig 3.5C)** and inhibited DHA-stimulated NT secretion **(Fig 3.5D)**.

To further confirm the role of Exo70 in NT secretion, we generated BON cell lines stably expressing Exo70 shRNA and measured constitutive and FA-stimulated NT secretion. Inhibition of Exo70 potently inhibits NT secretion from both untreated and DHA-stimulated BON cells **(Fig 3.6A)**. Conversely, overexpression of Exo70 enhances both basal and DHA-stimulated NT secretion **(Fig 3.6B)**. Moreover, western blot analysis demonstrates that ERK1/2 phosphorylation is attenuated by Exo70 inhibition **(Fig 3.6C)**. Collectively, these data implicate Exo70 as a positive regulator of NT secretion and suggest that the interaction between ERK1/2 and the exocyst complex may mediate exocytosis of NT vesicles from enteroendocrine cells.

3.4 Discussion

FAs and NT play stimulatory and intersecting roles in the development of obesity and cancer. Our group recently demonstrated that stimulation of NT secretion by the unsaturated FA, DHA, is mediated by ERK1/2 signaling (43, 44). Here, we examine the role of KSR1 on DHA-stimulated NT secretion. We show that KSR1 promotes DHA-stimulated NT secretion through the ERK1/2 signaling pathway. We also show that Exo70 inhibition reduces, while its overexpression enhances NT secretion and ERK1/2 phosphorylation. Collectively, the data presented in our current study implicate KSR1 and Exo70 as positive regulators of NT secretion that are integrated through the ERK1/2 signaling pathway.

KSR1 serves as a scaffold of the Raf/MEK/ERK complex, coordinating the spatial interactions between RAF, MEK, and ERK and enhancing specificity of their sequential phosphorylation (111, 113). These interactions make KSR1 a potent regulator of ERK1/2 signaling (109-112). We show that KSR1 inhibition reduces DHA-stimulated NT secretion and correspondingly inhibits ERK1/2 phosphorylation in human endocrine cells. In contrast, overexpression of KSR1 enhances NT secretion, and ERK1/2 phosphorylation. Given the role of KSR1 as a positive regulator of ERK1/2 signaling and our previous studies showing that ERK1/2 enhances NT mRNA expression and peptide secretion, these data suggest that KSR1 positively regulates NT secretion through the Raf/MEK/ERK signaling cascade (43, 44). KSR1 has been similarly shown to regulate secretion of insulin and inflammatory cytokines, though whether its role in secretion is predominantly positive or negative is unclear (117, 118). KSR1-deficient mice have higher levels of basal insulin release relative to wild-type mice, suggesting that KSR1 negatively regulates insulin secretion (118). In T-lymphocytes and splenocytes from KSR1-deficient mice, interferon gamma (IFN-γ) secretion is enhanced relative to wild-type cells, suggesting a negative role for KSR1 in IFN-γ secretion (117). However, KSR1-deficient T cells exhibit reduced interleukin 17A (IL17A) secretion, suggesting that KSR1 enhances IL17A secretion (117).

These contrasting findings may be attributable to the role of KSR1 as a scaffold protein, which can play negative or positive roles in signal transduction depending on their expression level (109, 119-121). Scaffold proteins facilitate maximal signaling when expressed in approximate stoichiometry with their ligands (119). Scaffold inhibition reduces signaling due to depletion of the scaffold, yet scaffold overexpression can also inhibit signaling due to sequestration of the ligands, unless the ligands themselves are also

overexpressed (119). However, in cells endogenously expressing high levels of scaffold, inhibition may bring the scaffold and its ligands to near equivalent amounts, thereby increasing signaling (119). The conflicting evidence regarding the role of KSR1 in secretory processes may be attributable to variation in endogenous expression levels of KSR1 and its ligands between cell types.

In mouse embryonic fibroblasts (MEFs), expression of KSR1 at 14 times endogenous levels produces maximal ERK1/2 signaling, while levels exceeding this reduce ERK1/2 signaling to levels comparable to that of KSR1-deficient cells (112). In contrast, ectopic expression of KSR1 in 293T cells inhibits insulin- and phorbol myristate acetate (PMA)-stimulated ERK1/2 signaling (122). We show that overexpression of KSR1 enhances ERK1/2 phosphorylation and DHA-stimulated NT secretion in neuroendocrine cells. These data suggest that, like MEFs, endogenous KSR1 expression in neuroendocrine cells is below that of the threshold for maximal ERK activation. Alternatively, components of the Raf/MEK/ERK complex may also be overexpressed in response to the downstream effects of ectopic KSR1 expression, thereby restoring stoichiometry of the complex. NT has been shown to activate ERK signaling through Ras activation (54). Stimulation of NT by overexpression of KSR1 may therefore feedback positively on ERK1/2, optimizing signaling of the Raf/MEK/ERK complex.

As a secreted peptide, NT is subject to regulation by exocytotic processes, including vesicle trafficking, docking, and fusion with the plasma membrane (37, 38). Exo70, a component of the exocyst complex that regulates exocytosis, is localized to lipid rafts on the plasma membrane and facilitates docking of secretory vesicles to the membrane prior to secretion (114, 115). Exo70 is a direct substrate of ERK2, which phosphorylates Exo70

at Ser250 upon EGF stimulation (68). We have recently demonstrated that DHA-stimulated NT secretion is mediated by an ERK1/2-dependent mechanism in neuroendocrine cells and C57/BL6 mice (43). In our current study, we show that ERK2 knockdown reduces expression of total Exo70, though no antibodies are currently available for detection of phosphorylated Exo70. Ablation of ERK1/2 phosphorylation with the MEK inhibitor PD 98059 also abrogated Exo70 expression and NT-secretion. These findings support other studies demonstrating that ERK2 positively regulates Exo70 and promotes its interaction with other components of the exocyst complex (116). Activation of Exo70 by ERK1/2 signaling also mediates vesicular trafficking and matrix metalloproteinase (MMP) secretion in MDA-MB-231 human breast cancer cells (116). Our results corroborate these findings and provide further evidence to support the role of ERK1/2 signaling as a regulator of Exo70-mediated secretory processes.

We also find that Exo70 knockdown potently inhibits both basal and DHA-stimulated NT secretion. In contrast, Exo70 overexpression enhances basal and DHA-stimulated NT secretion, suggesting a positive role for Exo70 in regulating NT secretion. Consistent with our findings, Lopez et. al (123) showed that Exo70 regulates insulin secretion. Insulin granules associate with Exo70 at the plasma membrane of β-cells, suggesting that Exo70 facilitates docking of insulin granules with the membrane (123). Exo70 is also required for proper docking of Glut4-containing vesicles with the plasma membrane during insulin-stimulated Glut4 secretion (124). Additionally, we show that inhibition of Exo70 attenuates ERK1/2 phosphorylation, suggesting a role for Exo70 in feedback activation of ERK1/2 signaling. Notably, Exo70 mRNA and protein is overexpressed in colon cancer, in which both ERK1/2 and NT are also heavily implicated

(125). The exocyst complex has also been implicated in the epithelial-mesenchymal transition (EMT) in mammary epithelial cells (125, 126). Studies aimed at further delineating the interaction between Exo70 and ERK1/2 may shed light on their role in processes regulating both secretion and tumorigenesis.

In summary, the present studies demonstrate for the first time that KSR1 positively regulates FA-stimulated NT secretion and that this effect is mediated by the stimulatory effect of KSR1 on ERK1/2 signaling. Moreover, we reveal a novel role for Exo70 in the regulation of NT secretion **(Fig 3.6D)**. We also describe a role for ERK1/2 in NT secretion independent of its previously described role on NT mRNA and protein expression by demonstrating its stimulatory effect on the secretory processes mediating NT release. Collectively, these data indicate that KSR1 modulation may have therapeutic potential for diseases driven by NT gene expression and peptide secretion, such as obesity-associated metabolic disorders and Ras-driven cancers.

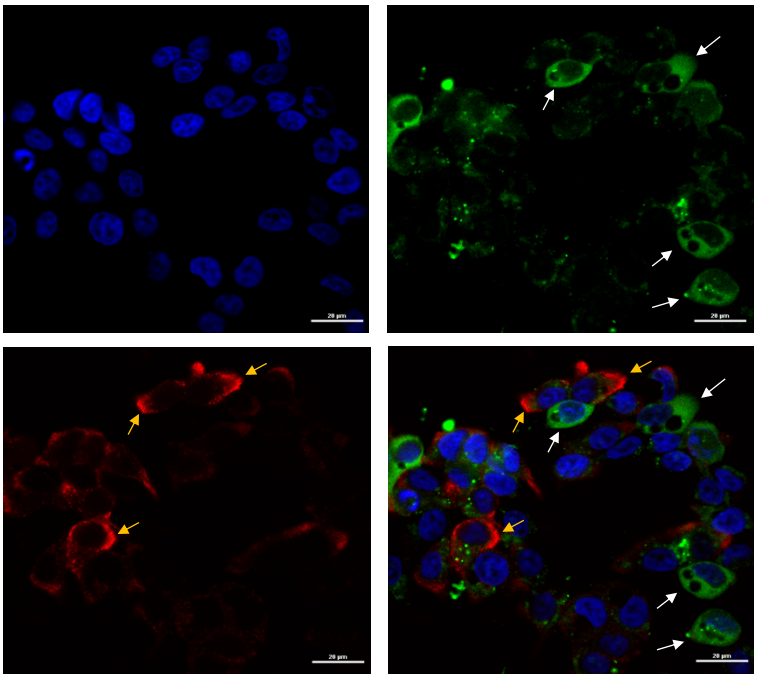


Fig 3.1. KSR1 expression stimulates NT release from BON cells. BON cells were transfected with pECFP-N-KSR1 (green) and labeled with immunofluorescent anti-NT antibody (red) and observed via confocal microscopy with 40x objective. White arrows indicate KSR1-positive cells, yellow arrows indicate NT labeling. Scale bar indicates 20μm.

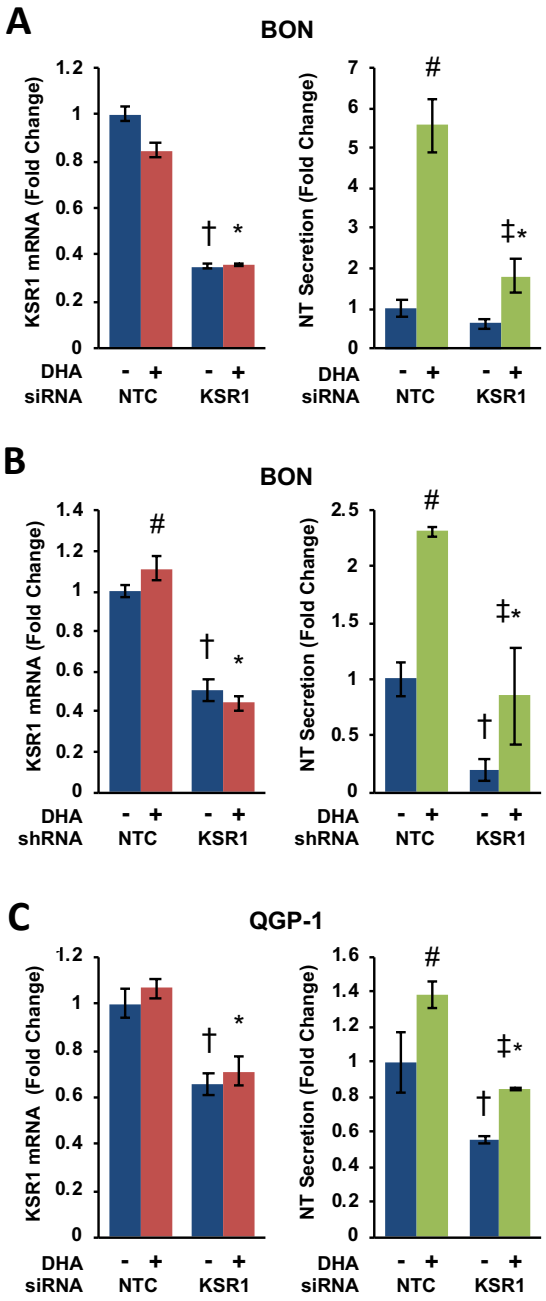


Fig 3.2. KSR1 inhibition attenuates FA-stimulated NT secretion (A) BON cells were transfected with KSR1-targeting siRNA or KSR1-targeting shRNA (B) and treated with or without DHA for 90 min. Total RNA was isolated and qRT-PCR performed targeting KSR1 with GAPDH as the internal control (left) (†$p<0.05$ vs. untreated NTC; *$p<0.05$ vs.

NTC plus DHA). Media were collected and NT-EIA performed (right) (†$p<0.05$ vs. untreated NTC; *$p<0.05$ vs. NTC plus DHA; ‡$p<0.05$ vs. untreated KSR1-siRNA/shRNA groups. (C) QGP-1 cells were transfected with KSR1-targeting siRNA and treated with or without DHA for 90 min. Total RNA was isolated and qRT-PCR performed targeting KSR1 with GAPDH as internal control (left) (†$p<0.05$ vs. untreated NTC; *$p<0.05$ vs. NTC plus DHA). Media were collected and NT-EIA performed (right) (†$p<0.05$ vs. untreated NTC; *$p<0.05$ vs. NTC plus DHA; ‡$p<0.05$ vs. untreated KSR1-siRNA group). All data represent mean +/- SD. Data are representative of three independent experiments.

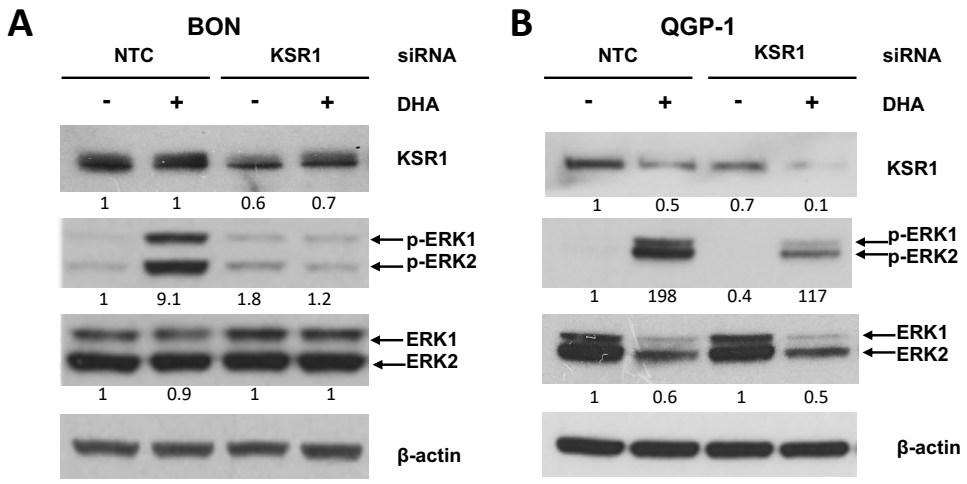


Fig 3.3. Knockdown of KSR1 attenuates ERK1/2 signaling. (A) BON and (B) QGP-1 cells were transfected with KSR1-targeting siRNA and treated with or without 100μM DHA for 90 min. Cells were lysed and western blotting analysis performed. Band intensity is indicated below the corresponding band and expressed as fold-change relative to NTC. Each blot is representative of three independent experiments.

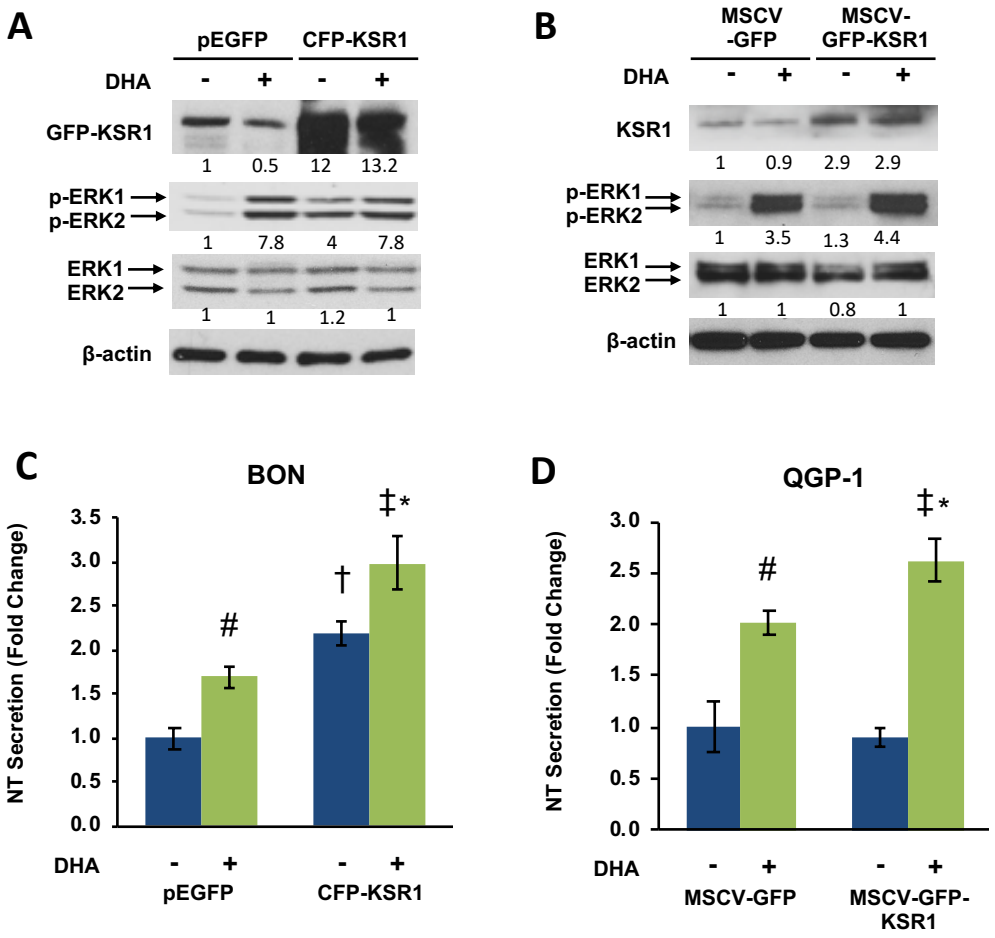


Fig 3.4. Overexpression of KSR1 enhances NT secretion and ERK activation. (A) BON cells were transfected with pEGFP-control and pECFP-N-KSR1 plasmid DNA and QGP-1 cells were transfected with MSCV-IRES-GFP and MSCV-KSR1-IRES-GFP plasmid DNA (B) and treated with or without 100μM DHA for 90 min. Cells were lysed and western blotting analysis performed. Band intensity is indicated below the corresponding band and expressed as fold-change relative to NTC. Each blot is representative of two independent experiments. (C) BON cells were transfected with pEGFP-control and pECFP-N-KSR1 plasmid DNA and QGP-1 cells were transfected with MSCV-IRES-GFP and MSCV-KSR1-IRES-GFP plasmid DNA (D) and treated with or without 100μM DHA for 90 min. Media were collected and NT-EIA performed. Panel (C):

†p<0.05 vs. untreated pEGFP; *p<0.05 vs. pEGFP plus DHA; ‡p<0.05 vs. untreated CFP-KSR1; Panel (D): †p<0.05 vs. untreated MSCV-GFP; *p<0.05 vs. MSCV-GFP plus DHA; ‡p<0.05 vs. untreated MSCV-GFP-KSR1. Data represent mean +/- SD and are representative of three independent experiments.

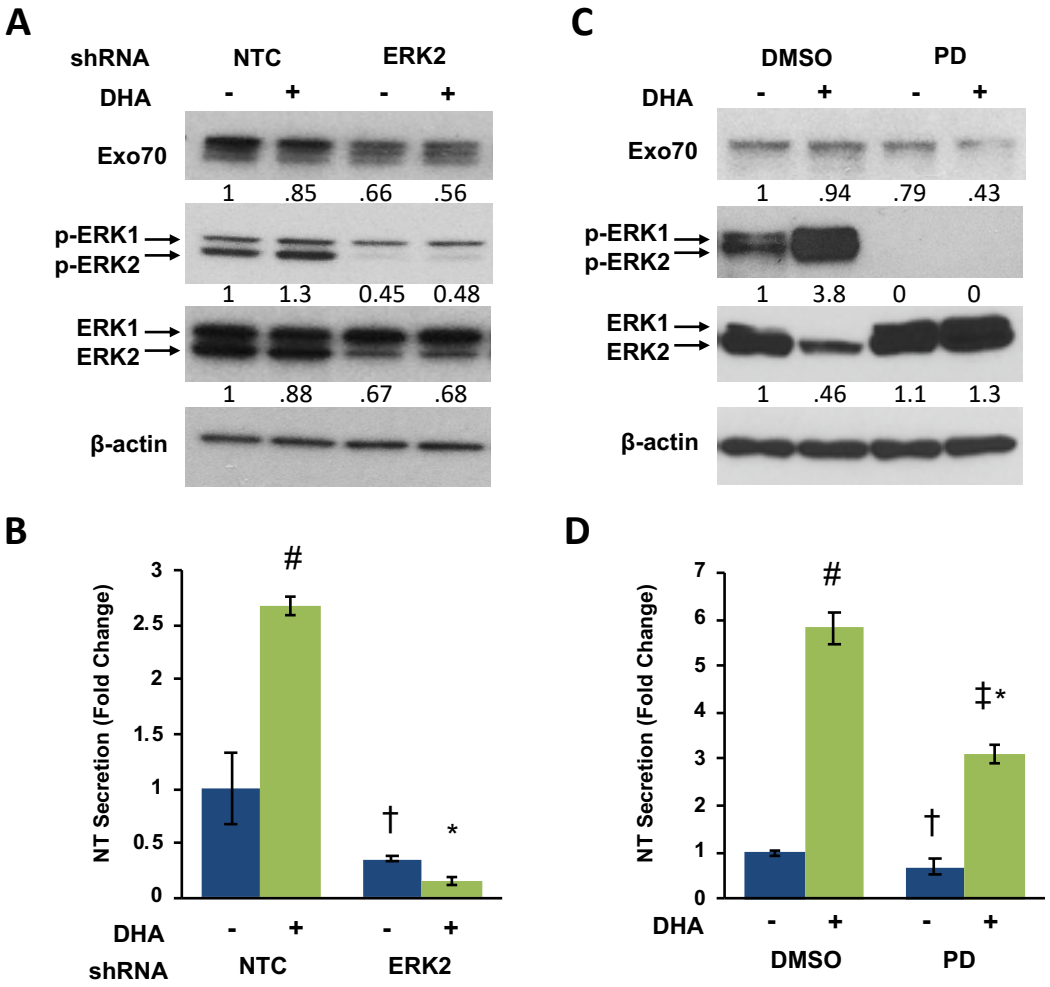


Fig 3.5. ERK1/2 inhibition attenuates Exo70 expression. (A) BON cells stably expressing NTC or ERK2 shRNAs were treated with or without 30μM DHA for 90 min. Cells were lysed and western blotting analysis performed. Band intensity is indicated below the corresponding band and expressed as fold-change relative to NTC. (B) Media were collected and NT-EIA performed (†$p<0.05$ vs. untreated NTC; *$p<0.05$ vs. NTC plus DHA; data represent mean +/- SD). (C) BON cells pre-treated with DMSO or PD 0325901 for 30 minutes were treated with or without 100μM DHA for 90 min. Cells were lysed and western blotting analysis performed. Band intensity is indicated below the corresponding band and expressed as fold-change relative to NTC. (D) Media were collected and NT-EIA

performed (†$p<0.05$ vs. DMSO without DHA; *$p<0.05$ vs. DMSO plus DHA; ‡$p<0.05$ vs. PD without DHA; data represent mean +/- SD). All data are representative of three independent experiments.

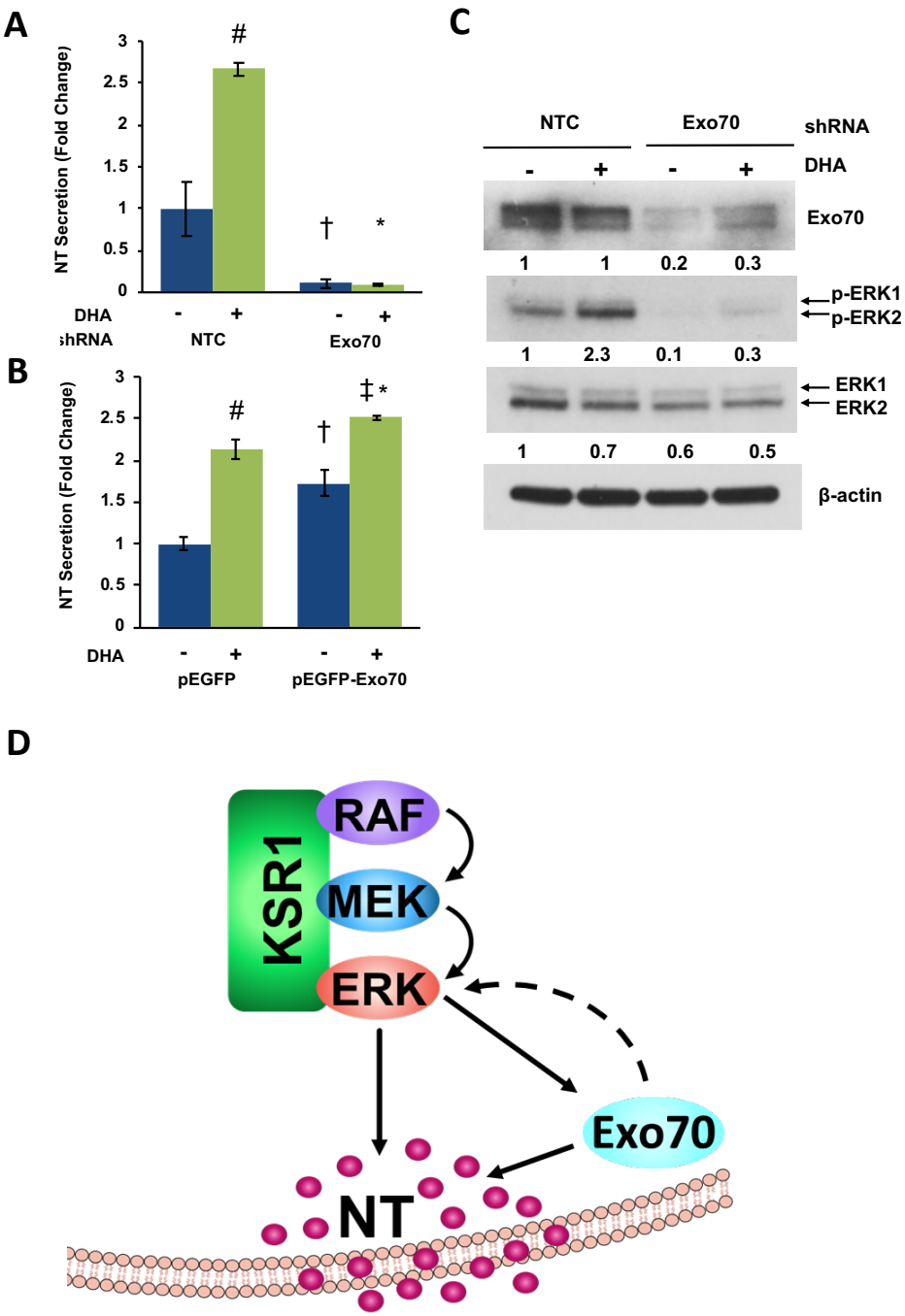


Fig 3.6. Exo70 positively regulates NT secretion. (a) BON cells stably expressing NTC or Exo70 shRNAs were treated with or without 30μM DHA for 90 min. Media were collected and NT-EIA performed (†$p<0.05$ vs. untreated NTC; *$p<0.05$ vs. NTC plus

DHA; data represent mean +/- SD). **(b)** BON cells were transfected with pEGFP-control or pEGFP-C3-Exo70 plasmid DNA and treated with or without 30μM DHA for 90 min. Media were collected and NT-EIA performed (†$p<0.05$ vs. untreated pEGFP; *$p<0.05$ vs. pEGFP plus DHA; ‡$p<0.05$ vs. untreated pEGFP-Exo70; data represent mean +/- SD). **(c)** BON cells stably expressing NTC or Exo70 shRNAs were treated with or without 30μM DHA for 90 min. Cells were lysed and western blotting analysis performed. Band intensity is indicated below the corresponding band and expressed as fold-change relative to NTC. All data are representative of three independent experiments. **(d)** Proposed model of KSR1/ERK/Exo70 signaling in the control of NT secretion.

CHAPTER 4:
NEUROTENSIN PROMOTES STEM CELL FUNCTION IN THE SMALL INTESTINE AND COLORECTAL CANCER CELLS

4.1 Abstract

Actively cycling intestinal stem cells (ISCs), expressing the WNT target gene *Lgr5,* are highly proliferative and metabolically active, and are sensitive to dietary alterations and nutrient availability. Neurotensin (NT), a gut peptide localized predominantly to the small bowel and released by fat ingestion, stimulates the growth of intestinal mucosa under basal conditions and with nutrient deprivation, suggesting a possible role for NT on ISC function. Here we demonstrate that NT regulates proliferation and stem cell function in the small intestine and that these effects are highly dependent on nutrient state. Under nutrient-rich conditions, NT stimulates ERK1/2 signaling and the expression of genes that promote cell cycle progression, including cyclin D1, leading to maintenance of crypt cell proliferation. Under conditions of nutrient depletion, NT stimulates WNT/β-catenin signaling and promotes an ISC gene signature, leading to enhanced ISC function. Importantly, we show that NT is required for the induction of WNT/β-catenin signaling and ISC-specific gene expression during nutrient depletion. Functionally, loss of NT reduces crypt cell proliferation and impairs ISC function and *Lgr5* expression in the intestine during fasting. Conversely, the expression of NT in the midgut enteroendocrine cells of *Drosophila* prevents loss of ISCs during nutrient deprivation. Collectively, our findings establish an evolutionarily-conserved role for NT in ISC maintenance during nutritional stress.

4.2 Introduction

The intestinal mucosa is in a continuous state of proliferation with complete epithelial cell turnover occurring every 4-5 days (2, 127). This rapid self-renewal process is maintained by intestinal stem cells (ISCs) that reside at the base of intestinal crypts (4). ISCs give rise to progenitor cells that rapidly proliferate within the crypt and terminally differentiate as they progress up the crypt-villus axis. Progenitor cells differentiate into either absorptive (enterocytes) or secretory (goblet, Paneth, enteroendocrine [EE]) cells that carry out the primary functions of the intestinal epithelium, including nutrient absorption, digestion, and protection from microbial infections (2, 4, 5). The process of proliferation and differentiation within the small intestine is highly sensitive to nutrient state. Nutrient deprivation causes atrophy of the intestinal mucosa, reduces crypt cell proliferation, and enhances crypt and villus cell apoptosis (28). In contrast, ISC function is enhanced by short-term fasting, and ISC numbers are increased by long-term calorie restriction (26, 27). Proliferation and stem cell function in the small intestine are controlled by signaling pathways that are tightly coupled to metabolic status, including mitogen-activated protein kinase (MAPK) and WNT/β-catenin signaling (4, 128-131).

WNT/β-catenin signaling is an evolutionarily-conserved pathway that plays a critical role in intestinal homeostasis (12). WNT/β-catenin signaling is activated by translocation of β-catenin to the nucleus, where it activates expression of WNT-target genes that regulate cell proliferation, embryonic development, and tissue homeostasis (6, 11). Reduction of WNT/β-catenin signaling in the intestine impairs crypt cell proliferation and ISC function, whereas hyperactivation of the WNT/β-catenin pathway contributes to neoplasia (6, 8). WNT ligands secreted from subepithelial mesenchymal cells and Paneth

cells maintain high levels of WNT/β-catenin signaling at the base of intestinal crypts to support rapid proliferation and ISC self-renewal (7, 132-134). In the mammalian small intestine, active ISCs positioned at the base of crypts are specifically labeled by expression of the WNT target gene *Lgr5* (3, 7). Likewise, the *Drosophila* midgut epithelium is replenished by a population of ISCs regulated, in part, by expression of the *Drosophila* WNT homolog, *wingless* (*wg*) (12, 135). The WNT/β-catenin pathway is sensitive to nutrient state and metabolic status. Excess dietary fat intake activates WNT/□-catenin signaling in intestinal crypts, leading to enhanced stemness and tumorigenicity of ISCs and progenitor cells (25). Free-fatty acids (FFAs) increase β-catenin target gene expression in intestinal organoids, and high glucose levels are required for WNT-stimulated nuclear translocation of β-catenin in enteroendocrine (EE) cells and various tumor-derived cell lines (25, 136).

Nutrient state is a also a critical regulator of intestinal hormone release. The peptide hormone neurotensin (NT) is localized to EE cells predominantly in the distal small intestine (33, 35, 137). NT, released primarily by ingestion of dietary fats, facilitates intestinal mucosal proliferation, FFA absorption, lipid metabolism, and glucose homeostasis (36, 46, 137-139). The well-characterized physiologic effects of NT are primarily endocrine-mediated; however, NT is also known to act in a paracrine and autocrine fashion in some cell types, such as certain cancers and intestinal cells (39, 140, 141). NT signaling in the small intestine is predominantly mediated by the G-protein coupled NT receptor 1 (NTR1), which leads to the activation of growth stimulating pathways including PI3K/AKT and MAPK signaling (30, 51, 60, 142-145). Exogenous NT stimulates growth of the normal intestinal mucosa, contributes to adaptation following

intestinal injury, and has an anti-apoptotic role in intestinal epithelial cells and hepatocytes (40, 45, 72, 74, 76, 146). NT has been implicated in regulation of the WNT/β-catenin pathway in glioblastoma and hepatocellular carcinoma and is a downstream target of the WNT pathway in neuroendocrine tumor cells (55, 64, 147). In colorectal cancer (CRC) cells, NT mediates phosphorylation and inhibition of GSK3β, a repressor of WNT/β-catenin signaling (60, 147). Whether the intestinotrophic effect of NT is mediated through the regulation of ISCs is not known. Here we demonstrate that NT differentially regulates proliferation and stem cell function in the small intestine based on nutrient status. Under nutrient-rich conditions, NT promotes crypt cell proliferation via ERK1/2 signaling and positive regulation of the cell cycle, whereas during nutritional stress, NT promotes WNT/β-catenin signaling and contributes to ISC maintenance in mice and *Drosophila*. Collectively, our findings demonstrate that NT contributes to the maintenance of intestinal homeostasis through regulation of ERK1/2 and WNT/β-catenin signaling and identify an evolutionarily conserved role for NT in the maintenance of ISCs during nutritional stress.

4.3 Results

4.3.1 NT-deficiency impairs intestinal crypt cell proliferation.

We first investigated the impact of NT on the intestinal epithelium using Lgr5-EGFP, NT wild type ($Nt^{+/+}$) and Lgr5-EGFP, NT knockout ($Nt^{-/-}$) mice. NT activates the MAPK family members ERK1 and ERK2 (ERK1/2) in multiple cancer cell lines, including CRC (142, 143). The ERK1/2 pathway is a master regulator of the cell cycle, and MAPK/ERK signaling mediates ISC and crypt cell proliferation in the small intestine (4, 15). To determine whether NT contributes to ERK1/2 signaling or stemness in the small intestine, we cultured organoids from $Nt^{+/+}$and $Nt^{-/-}$ mice to examine ERK1/2 activation

and ISC number. NT deficiency did not alter the appearance of intestinal organoids **(Fig. S4.1A)**; however, the absence of NT reduced phosphorylated ERK1/2 (p-ERK1/2) expression by approximately 50% **(Fig. S4.1B)**, which corresponded to an approximate 50% decrease in expression of the ISC markers *Lgr5* and *Olfm4* compared to $Nt^{+/+}$organoids **(Fig. S4.1C-D)**. Fluorescence-activated cell sorting (FACS) analysis revealed that NT deficiency reduced the percentage of Lgr5-GFP^{+} ISCs in intestinal organoids by approximately 50% relative to intestinal organoids from $Nt^{+/+}$ mice **(Fig. S4.1E)**, indicating that NT plays a role in maintenance of ISC number. However, the number of Lgr5-GFP^{+} ISCs in freshly isolated crypts was unaltered by the absence of NT **(Fig. S4.1F)**, suggesting that other factors in the ISC niche likely compensate for the loss of NT *in vivo*.

The reduction of ERK1/2 signaling and ISC number in $Nt^{-/-}$ organoids prompted us to ask whether NT contributes to these functions *in vivo* under non-homeostatic conditions. Under conditions of nutritional stress, including acute fasting or long term calorie restriction, crypt proliferation is impaired and ISC function is altered (5). As an intestinotrophic hormone released in response to nutrient ingestion, we hypothesized that NT may contribute to intestinal adaptation depending upon the nutrient state (i.e, fed or fasted). To investigate the role of NT in the small intestine during nutritional stress, $Nt^{+/+}$ and $Nt^{-/-}$ mice were fed standard chow *ad libitum* (AL) or fasted for 48 h. We confirmed loss of NT by evaluating *Nt* mRNA from intestinal crypts **(Fig. S4.2A)**. Both $Nt^{+/+}$ and $Nt^{-/-}$ mice lost approximately 20% of their body weight compared with AL-fed mice; there was no difference in body weight between fasted $Nt^{+/+}$ and $Nt^{-/-}$ mice **(Fig. S4.2B)**.

NT has been shown to promote ERK1/2 signaling in various CRC cell lines *in vitro*, yet whether NT regulates ERK1/2 signaling in the small intestine *in vivo* is not known. In organoids from $Nt^{-/-}$ mice, pERK1/2 expression was downregulated relative to $Nt^{+/+}$ organoids **(Fig. S4.1B)**, indicating that NT promotes ERK1/2 signaling in intestinal cells *ex vivo* . To determine whether NT regulates the ERK1/2 pathway *in vivo*, we measured p-ERK1/2 in crypts isolated from AL and fasted $Nt^{+/+}$ and $Nt^{-/-}$ mice. Consistent with our findings in $Nt^{-/-}$ organoids, absence of NT reduced p-ERK1/2 expression in the crypts by approximately 50% relative to AL-fed controls, although p-ERK1/2 expression was not altered in the crypts of fasted $Nt^{-/-}$ mice **(Fig. 4.1A)**. In agreement with this data, expression of the ERK1/2 target gene, cyclin D1, was reduced by approximately 50% in crypts from $Nt^{-/-}$ mice relative to AL-fed controls **(Fig. 4.1B)**. Cyclin D1 expression was reduced to a similar extent by fasting alone but was not further reduced by the absence of NT. To quantify gene expression changes associated with NT during nutrient stress, we performed a bulk RNAseq analysis using crypts isolated from AL-fed and fasted $Nt^{+/+}$ and $Nt^{-/-}$ mice. Because ERK1/2 and cyclin D1 are critical regulators of cell cycle progression and proliferation, we performed a gene set enrichment analysis (GSEA) to better delineate the role of NT on pathways regulating the cell cycle and cell proliferation. GSEA revealed that absence of NT reduced expression of genes related to cell cycle progression, mitosis, and DNA synthesis compared to AL-fed and fasted $Nt^{+/+}$ mice **(Fig. 4.1C-D)**, thus providing further evidence that NT positively regulates crypt cell proliferation.

To validate these results *in vivo,* we quantified expression of Marker of Proliferation protein Ki67 (Ki67) (148) in the proximal and distal small intestine. Consistent with previous studies (6, 48), fasting reduced the number of $Ki67^{+}$ cells in the

crypts by approximately 50% compared to AL-fed mice in both the proximal and distal small intestine **(Fig. 4.1E and Fig S4.2C-D)**. Moreover, absence of NT significantly reduced the number of $Ki67^+$ cells in the distal crypts under both AL-fed and fasted conditions relative to $Nt^{+/+}$ mice **(Fig. 4.1E)**. However, NT deficiency did not impact the frequency of $Ki67^+$ cells in the proximal small intestine **(Fig S4.2C-D)**, suggesting that the effect of endogenous NT is greatest in the distal small intestine, where NT expression is highest (149). The reduction in proliferating distal crypt cells in $Nt^{-/-}$ mice corresponded to increased epithelial cell differentiation measured via Alcian blue staining of goblet cells. $Nt^{-/-}$ approximately doubled the percentage of goblet cells in the distal crypts of AL-fed and fasted mice compared to $Nt^{+/+}$ mice **(Fig 4.1F)**, whereas no significant changes were observed in goblet cell frequency in the proximal small intestine **(Fig. S4.2E-F).** Together, these data demonstrate that NT contributes to cell proliferation and differentiation in the distal small intestine independent of nutrient status. These effects are likely mediated by NT regulation of ERK1/2 signaling.

4.3.2 Fasting activates WNT/β-catenin signaling in a NT-dependent manner.

Crosstalk between NT/NTR1 and the WNT/β-catenin pathway has been demonstrated in multiple cancer cell lines (55, 64, 147), yet whether NT regulates WNT/β-catenin signaling in the small intestine is unknown. To determine whether NT-mediated effects on intestinal proliferation are associated with alterations in the WNT/β-catenin pathway, we examined expression of the TCF/LEF transcription factor *Tcf7*, a main effector of WNT/β-catenin signaling (150), in crypts from AL-fed and fasted $Nt^{+/+}$ and $Nt^{-/-}$ mice. Strikingly, despite reducing crypt cell proliferation, fasting increased *Tcf7* mRNA expression by approximately 80% in $Nt^{+/+}$ crypts relative to AL-fed controls **(Fig. 4.2A)**.

Although the absence of NT had no impact on *Tcf7* mRNA in the crypts of AL-fed mice, loss of NT completely abrogated the induction of *Tcf7* mRNA during fasting. We observed a similar expression of the WNT target gene *c-myc*, which was increased by approximately 80% in fasted $Nt^{+/+}$ crypts but was not increased in the crypts of fasted $Nt^{-/-}$ mice **(Fig. 4.2B)**. In agreement with WNT/β-catenin activation, fasting increased expression of active-β-catenin in the crypts from $Nt^{+/+}$ mice by greater than 50% compared to crypts from AL-fed $Nt^{+/+}$ mice but did not increase active-β-catenin in the crypts from $Nt^{-/-}$ mice **(Fig. 4.2C)**, suggesting that NT positively regulates WNT/β-catenin signaling during periods of fasting.

To elucidate the mechanism by which NT activates WNT/β-catenin during fasting, we next examined pathways downstream of NT signaling that may converge on the WNT/β-catenin pathway. In CRCs, NT signaling promotes phosphorylation of GSK3β, a negative regulator of WNT/β-catenin signaling (60). GSK3β phosphorylates β-catenin on *Ser*33, Ser37, and Thr41, leading to ubiquitination and proteasomal degradation of β-catenin (13). Phosphorylation of GSK3β on Ser9 by AKT inhibits GSK3β activity, thereby stabilizing β-catenin and enabling its nuclear translocation and activation of WNT signaling **(Fig. 4.2D)** (14, 61, 62). To determine whether NT promotes WNT/β-catenin signaling through regulation of GSK3β, we examined phosphorylation of GSK3β on Ser9 in crypts isolated from AL-fed and fasted $Nt^{+/+}$ and $Nt^{-/-}$ mice. The absence of NT reduced p-GSK3β expression relative to crypts harvested from $Nt^{+/+}$ mice under both AL-fed and fasted conditions **(Fig. 4.2E)**. Though fasting alone did not alter the ratio of phosphor- and total GSK3β, total GSK3β expression was significantly reduced by fasting **(Fig. S4.2G-H)**, suggesting GSK3β depletion as a potential mechanism for WNT/β-catenin activation

during fasting. Consistent with p-GSK3β expression, *Nt*$^{-/-}$ reduced p-AKT expression by approximately 50% in the crypts from AL-fed mice **(Fig. 4.2E)**. Fasting alone reduced p-AKT to a similar extent; however, no additional decrease of p-AKT was observed in the crypts of fasted *Nt*$^{-/-}$ mice. These data demonstrate that NT contributes to the induction of WNT/β-catenin signaling in the small intestine during fasting. Moreover, our findings suggest that this induction may occur through NT regulation of the AKT/GSK3β pathway.

4.3.3 Exogenous NT activates WNT/β-catenin signaling in the small intestine.

We next determined whether exogenous NT activates WNT/β-catenin and AKT/GSK3β signaling in the small intestine. qRT-PCR analysis revealed that intestinal organoids do not express NTR1 **(Fig. S2I)**, therefore we utilized an *ex vivo* full-thickness intestine model for exogenous NT treatment. Full-thickness small intestine was isolated from *Nt*$^{+/+}$ mice and treated with NT (100nM or 1μM) in PBS for 15 or 30 min at 37°C. Western blot analysis demonstrated increased AKT phosphorylation after a 15 min incubation with either 100nM or 1μM NT **(Fig 4.3A)**. After 30 min exposure, p-AKT expression remained elevated relative to controls but was decreased relative to the 15 min timepoint, indicating that short-term treatment with NT potently activates AKT signaling in the small intestine. Consistent with AKT activation, we found that 15 min exposure to 100nM NT increased p-GSK3β and p-LRP6, a marker of WNT/β-catenin activation, by approximately 10- and 2.5-fold, respectively **(Fig 4.3A, 4.3C)**. In agreement with our initial findings that NT depletion reduces ERK1/2 signaling in crypts, exogenous NT increased phosphorylation of ERK1/2 by approximately 6-fold **(Fig 4.3A, 4.3C)**.

To further confirm that activation of these pathways was mediated by NT/NTR1 signaling, we treated full-thickness small intestine with 100nM NT for 15 min with or without 20 min pre-treatment with the NTR1 inhibitor SR48692 (151). NT consistently increased phosphorylation of AKT, GSK3β, LRP6, and ERK1/2, whereas pre-treatment with SR48692 prevented NT-mediated phosphorylation of these proteins **(Fig 4.3B, 4.3C)**. Collectively, these data demonstrate that NT/NTR1 signaling activates WNT/β-catenin, AKT/GSK3β, and ERK1/2 signaling in the small intestine.

4.3.4 NT promotes an ISC gene expression program during fasting.

WNT/β-catenin signaling is critical for the proliferation and maintenance of ISCs, which are characterized by their high expression of WNT target genes, including *Lgr5*, *Ascl2*, *Sox9*, *Msi-1,* and *EphB3 (3, 4, 152-154)*. Consistent with activation of WNT/β-catenin signaling in the crypts of fasted $Nt^{+/+}$ mice, RNAseq analysis showed that fasting increased expression of ISC-specific genes in the crypts of $Nt^{+/+}$ mice relative to AL-fed controls, and that the absence of NT blunted the increase of these ISC markers during fasting **(Fig 4.4A)**. qRT-PCR was used to validate expression of *Lgr5* and *Ascl2,* which are highly restricted to active ISCs (4)*,* and confirmed that fasting induced expression of these ISC markers in $Nt^{+/+}$ mice but not in $Nt^{-/-}$ mice **(Fig 4.4B)**, indicating that NT is required for the induction of an ISC gene signature during fasting (154). We next analyzed the frequency of $Lgr5^{+}$ cells in the crypts using FACS. In contrast to the pattern of *Lgr5* mRNA during fasting, neither fasting nor absence of NT altered the total number of Lgr5-GFP^{+} ISCs **(Fig S4.2J)**. To more precisely delineate the localization of *Lgr5* mRNA expression *in vivo*, we performed single-molecule *in situ* hybridization in the distal small intestine, where we observed that the effects of NT are most pronounced. In agreement with RNAseq

and qRT-PCR analyses, fasting increased *Lgr5* mRNA copies per crypt cell by 2-fold relative to AL-fed controls, but did not significantly increase *Lgr5* expression in the crypts of $Nt^{-/-}$ mice **(Fig 4.4C-D)**. Thus, rather than increasing the number of $Lgr5^{+}$ ISCs, fasting increased the expression of *Lgr5* in the established population of ISCs and progenitor cells in an NT-dependent manner.

4.3.5 NT increases ISC function during nutrient deprivation.

Fasting has been previously shown to augment ISC function without altering ISC frequency (27, 29). To determine whether the NT-dependent increase in *Lgr5* mRNA expression during fasting is associated with changes in ISC function, crypts were isolated from AL-fed and fasted $Nt^{+/+}$ and $Nt^{-/-}$ mice for *ex vivo* colony formation assays. Consistent with others (27), we found a greater than two-fold increase in organoid forming efficiency in fasted $Nt^{+/+}$ crypts relative to the crypts from AL-fed $Nt^{+/+}$ mice **(Fig 4.4E).** However, organoid formation was significantly reduced in the crypts from fasted $Nt^{-/-}$ mice compared to $Nt^{+/+}$ mice. Furthermore, analysis of EdU incorporation, an indicator of cell proliferation (155), in crypt-derived organoids demonstrated a significant reduction in EdU uptake in fasted $Nt^{-/-}$ mice relative to fasted $Nt^{+/+}$ mice **(Fig 4.4F-G).** We next examined whether NT augments fatty acid oxidation (FAO), which promotes ISC function during fasting via activation of PPARδ signaling and enhanced function of Cpt1a, the rate-limiting enzyme in FAO (27). Interestingly, FAO and PPARδ gene sets were enriched in $Nt^{-/-}$ mice relative to $Nt^{+/+}$ mice under both AL-fed and fasted conditions **(Fig. S4.2K),** suggesting a role for NT in negative regulation of these pathways. However, no significant changes in the PPARδ target genes *Cpt1a* or *Hmgcs2* were observed between fasted $Nt^{+/+}$ and fasted $Nt^{-/-}$ mice **(Fig S4.2L)**, suggesting that the effects of NT on ISC function are not mediated by

alterations in FAO. Instead, our findings indicate that NT increases ISC function during fasting via upregulation of WNT-target genes (i.e. *Lgr5*) in ISCs and progenitor cells.

Regulation of the intestinal epithelium is highly conserved, such that similar signaling pathways and cellular processes regulate intestinal homeostasis in the mammalian and *Drosophila* intestine (135, 156). The *Drosophila* midgut is composed of ISCs that differentiate into the same major lineages as those in the mammalian small intestine, and ISC proliferation is regulated by expression of the *Drosophila* WNT homolog *wg* (12, 135). Therefore, the *Drosophila* ISC reporter line *esg*-Gal4-GFP, in which GFP is fused to the *Drosophila* ISC marker *esgargot*, is a powerful model for studying ISC function (157). We previously identified CG9918 (Pyrokinin 1 receptor, PK1-R) as the endogenous NTR-like receptor in *Drosophila* (46). NT expression in the *Drosophila* midgut mimics the effects of NT on intestinal lipid absorption and AMPK activation in the mammalian small intestine, suggesting that the effects of NT on the intestinal epithelium are highly conserved (46). To further delineate the role of NT on ISC function, we expressed human full-length NT cDNA in midgut EE cells using the EE cell-specific tachykinin (TK) promoter and examined ISCs labeled by *esg*-Gal4-GFP (*esg*-GFP) **(Fig 4.4H)** (92, 158). *esg*-GFP$^+$ TK-NT and *esg*-Gal4-GFP control flies were maintained for 5 d on a standard diet (SD) or a nutrient-reduced low-energy diet (LED) (93, 94). Consistent with our previous findings, NT overexpression increased the accumulation of lipid droplets in *Drosophila* midgut measured by Nile Red staining **(Fig 4.4H, center panels)** (46). In flies fed SD, NT expression caused a slight but significant increase in *esg*-GFP$^+$ ISCs relative to control flies **(Fig 4.4I)**. In flies fed LED, the frequency of *esg*-GFP$^+$ ISCs was reduced by over 50% compared to flies fed SD. However, expression of NT in flies fed

LED restored *esg*-GFP^{+} ISCs numbers to that of control flies fed SD **(Fig 4.4I)**. These data indicate that NT promotes ISC frequency in *Drosophila* midgut and attenuates ISC depletion during nutrient-deprivation. Collectively, our findings demonstrate that NT contributes to the maintenance of ISCs in both mice and *Drosophila* during nutrient-depletion, thus indicating an evolutionarily-conserved function of NT on ISCs.

4.3.6 NT does not contribute to HFD-induced proliferation or ISC function

Work from our lab previously demonstrated that NT contributes to HFD-induced obesity by increasing intestinal lipid absorption (46). Beyaz et al. demonstrated an important role for HFD and FFAs in stimulating ISC function and stemness of progenitor cells (25). The importance of nutrient state in regulation of ISCs by NT led us to ask whether NT-mediated intestinal lipid absorption potentiates HFD-induced ISC function. $NT^{+/+}$ and $NT^{-/-}$ were fed HFD or low-fat diet (LFD) control chow and crypts isolated for qRT-PCR analysis of the ISC markers *Lgr5* and *Olfm4*. Consistent with Beyaz et al., expression of *Olfm4* mRNA was significantly increased in mice fed HFD relative to mice fed LFD but was not altered by $NT^{-/-}$ under either dietary condition **(Fig 4.5A)** (25). Though we didn't observe significant differences in *Lgr5* mRNA expression across groups, we observed a trend similar to that of *Olf4m4* mRNA, with increased expression in mice fed HFD relative to mice fed LFD. In colony formation assays, $NT^{-/-}$ did not alter organoid forming efficiency of crypts fed LFD or HFD **(Fig 4.5B)**, indicating that NT does not contribute to HFD-induced ISC function.

Crypt cell proliferation is also enhanced in mice fed HFD relative to mice fed a control diet (25). Having established NT as an important regulator of crypt cell

proliferation in mice fed AL, we tested the effect of *NT*$^{-/-}$ on HFD-induced crypt cell proliferation. Consistent with Beyaz et al., HFD significantly increased the number of Ki67+ cells per crypt in the proximal and distal small intestine **(Fig 4.6A-D)** (25). However, *NT*$^{-/-}$ did not reduce crypt cell proliferation under either dietary condition. Collectively, these data indicate that the effects of NT on intestinal lipid absorption do not contribute to HFD-induced ISC function or crypt cell proliferation. Taken together with the results of our fasting study, these data suggest that NT serves a protective role on ISCs that is critical to maintenance of ISC numbers and function during conditions of nutrient stress. Under nutrient-rich conditions, which serve to enhance ISC function, protection of ISCs is unnecessary and NT function on ISCs is dispensable.

4.3.7 NT promotes WNT/β-catenin signaling and CSC function in CRC cells

The role of NT on WNT/β-catenin signaling and ISC function prompted to ask whether NT regulates stem cell function in CRC cells. Numerous studies have demonstrated the role of NT/NTR1 signaling in promoting cancer cell growth, proliferation, invasion, and tumor progression (47, 48). NTR1 is highly overexpressed in CRCs and intestinal tumors relative to normal intestinal tissues, and high levels of NTR1 expression are associated with poor prognosis in CRC (50). Like crypt epithelial cells and ISCs, proliferation and stemness of cancer cells are regulated, in part, by the ERK1/2 and WNT/β-catenin pathways, and within heterogenous CRC cell populations, high WNT-activity identifies the CSC population (88, 159). Though NT has been implicated in crosstalk with the WNT/β-catenin pathway in other cancers (55), whether NT promotes WNT/β-catenin signaling or stem cell function in CRC is unknown.

To examine the role of NT on WNT/β-catenin signaling in CRC cells, we used the human colorectal adenocarcinoma cell line HT29, the human colon carcinoma cell line HCT116, and the patient-derived CRC cell line PT130. Cells were treated with 100nM NT for 15 min and WNT/β-catenin activation analyzed via western blot. Consistent with our findings demonstrating that NT promotes WNT/β-catenin signaling in the normal small intestine, NT treatment increased expression of active-β-catenin and p-LRP6 in all CRC cell lines, indicating activation of the WNT/β-catenin pathway **(Fig 4.7A)**. To validate these results, we utilized an *ex-vivo* tumor organoid model derived from mouse models of CRC. Mutations in APC are a leading cause of CRC in humans, which is exacerbated by accumulation of additional mutations in oncogenic proteins such as K-ras and p-53 (160). To mimic the effects of CRC in a mouse model, we utilized APC^{min} and APC^{min};$K\text{-}ras^{mt}$ mice (161, 162).

Tumor organoids were cultured from the small intestinal tumors of APC^{min};$Nt^{+/+}$ mice and treated with 100nM NT for 48 h. Treatment with NT increased expression p-LRP6 and the WNT/β-catenin target c-Myc **(Fig 4.7B)**. We further confirmed these findings by examining expression of WNT target genes in APC^{min};$Nt^{+/+}$ after 48 h treatment with NT. Consistent with activation of WNT/β-catenin signaling, NT treatment increased expression of the WNT target genes *Lgr5*, *Axin2*, and c-*Myc* by approximately two-fold relative to untreated tumor organoids **(Fig 4.7C)**. Moreover, we observed similar levels of upregulation of WNT target gene expression in tumor organoids derived from APC^{min};$K\text{-}ras^{mt}$;$Nt^{+/+}$ mice. To confirm that this was mediated by NT/NTR1 signaling, we treated APC^{min};$K\text{-}ras^{mt}$;$Nt^{+/+}$ tumor organoids with NT in the presence or absence of the NTR1 inhibitor SR48692 (5 μM) **(Fig 4.7D)**. Treatment with the NTR1 inhibitor alone or

in combination with NT reduced expression of *Lgr5*, *Axin2*, and c-*Myc* mRNA relative to controls. Collectively, these data demonstrate that NT promotes WNT/β-catenin signaling in CRC cells and small intestinal tumor organoids in an NTR1-dependent manner.

Constitutive activation of WNT/β-catenin in CRC cells contributes to aberrant cell proliferation and promotes stemness of CSCs (66, 88). Stemness of CRC cells is measured via colony-formation assays that examine the ability of single tumor cells to form tumor organoid colonies, which requires an undifferentiated cancer stem cell phenotype (163). To determine whether NT-mediated activation of WNT/β-catenin signaling in CRC cells was associated with increased clonogenicity of CSCs, we performed colony formation assays on APC^{min};$Nt^{+/+}$ tumor organoids and HCT116 tumorspheres treated with NT. Consistent with upregulation of WNT target gene expression, NT treatment enhanced colony formation of single APC^{min};$Nt^{+/+}$ tumor organoid cells by approximately two-fold and caused dose-dependent increases in HCT116 tumorsphere colony formation **(Fig 4.8A-B)**. Together, these data indicate that NT/NTR1 signaling activates WNT/β-catenin signaling and enhances stem cell function in CRC cells.

4.3.8 NT induces LRP6 phosphorylation via ERK1/2 in CRCs and tumor organoids.

We next sought to define the mechanism by which NT may regulate WNT/β-catenin signaling in CRC cells. We demonstrated that NT positively regulates ERK1/2 signaling in the normal small intestine, and NT has previously been shown to promote ERK1/2 phosphorylation in various CRC cell lines. In certain contexts, ERK1/2 phosphorylates LRP6 on Ser1490, leading to activation of WNT/β-catenin signaling (65). We confirmed the role of NT on ERK1/2 signaling in our CRC models by treating HT29,

HCT116, PT130 and *APC*^{min};*Nt*$^{+/+}$ tumor organoids with NT. ERK1/2 phosphorylation was upregulated by treatment with 100nM NT in HT29, HCT116 and *APC*^{min};*Nt*$^{+/+}$ tumor organoids **(Fig 4.9A)**. NT treatment also increased p-ERK1/2 expression in PT130 cells, but to a lesser extent than the other CRC cell lines even at the highest dose, which may reflect lower levels of NTR1 expression **(Fig 4.9B)**. We also observed increased mTORC1 activation in HT29, HCT116, and *APC*^{min};*Nt*$^{+/+}$ tumor organoids following NT treatment, suggesting that the stimulatory effect of NT on CRC cell growth may be mediated in part by the protein synthesis processes regulated by the mTOR signaling pathway **(Fig 4.9C-F)** (164).

We next asked whether NT promotes phosphorylation of LRP6 via activation of ERK1/2. HT29 and HCT116 cell were treated with 100nM NT for 15 min with or without 20 min pre-treatment with the MEK inhibitor PD0325901 (10 nM) to block NT-mediated phosphorylation of ERK1/2. Treatment with NT produced consistent upregulation of p-ERK1/2 and p-LRP6 expression in both cell lines, whereas MEK inhibition blocked NT-mediated phosphorylation of ERK1/2 and LRP6 **(Fig 4.10)**. Notably, MEK inhibition completely ablated p-LRP6 expression in HT29 cells even in the absence of NT, suggesting that ERK1/2 signaling may be a critical mediator of WNT-activation in HT29 cells. Collectively, these data demonstrate that NT mediates phosphorylation of LRP6 on Ser1490 in CRCs via activation of ERK1/2 signaling. As phosphorylation of LRP6 on Ser1490 is sufficient for WNT/β-catenin activation, and we observe enhanced active β-catenin and WNT-target gene expression following NT treatment, these findings suggest that NT promotes WNT/β-catenin signaling via ERK1/2 mediated phosphorylation of LRP6.

4.3.9 Loss of NT reduces WNT/β-catenin signaling and CSC function in intestinal tumor organoids.

Our findings in HCT116, HT29, and PT130 cells and *APC*^{min} NT$^{+/+}$ tumor organoids indicate that exogenous NT activates WNT/β-catenin signaling and enhances CSC function. To determine the impact of NT-deficiency on CSC function, we utilized *APC*^{min} *NT*$^{+/+}$ and *APC*^{min} *NT*$^{-/-}$ mouse models. Tumor organoids cultured from single intestinal tumor cells from *APC*^{min} *NT*$^{+/+}$ mice were significantly larger than those cultured from *APC*^{min} *NT*$^{-/-}$ mice, indicating reduced proliferation as a result of NT-deficiency **(Fig 4.11A)**. We performed colony formation assays to examine the effect of NT on CSC clonogenicity and found that colony forming efficiency and viability of tumor organoids was reduced by approximately 60% and 80%, respectively, by NT-deficiency **(Fig 4.11B-C)**. Consistent with the positive role of NT on WNT/β-catenin activation, loss of NT reduced expression of the WNT-target gene *Axin2* by approximately 50%, suggesting that absence of NT reduces WNT/β-catenin signaling in intestinal tumor cells **(Fig 4.11D)**. In addition, our preliminary analysis of survival of *APC*^{min} *NT*$^{+/+}$ and *APC*^{min} *NT*$^{-/-}$ mice shows that loss of NT significantly increases overall survival in APC mutant mice **(Fig 4.12)**. Collectively, these data suggest that NT contributes to stem cell function in CRC cells and APC mutant mice via positive regulation of WNT/β-catenin signaling.

4.4 Discussion

Our study identifies a novel and evolutionarily-conserved role for NT, acting through NTR1, in the nutrient-dependent regulation of ISC function. We find that NT contributes to ISC maintenance in mice and *Drosophila* during nutritional stress and

activates AKT/ GSK3β and WNT/β-catenin signaling in the small intestine. In addition, we demonstrate that NT positively regulates WNT/β-catenin signaling and stem-like traits in CRC cells and show that NT-deficiency reduces CSC function in CRC tumor organoids and increases survival in *APC*^{min} mice. Previous studies show that NT contributes to stem cell maintenance in hepatocellular carcinoma (85) and promotes stem-like traits in glioblastoma cells (86), suggesting that NT regulates self-renewal and stem cell function in multiple cellular contexts. Consistent with the role of NT on ISCs, glucagon-like peptide 2 (GLP-2) has been reported to exert similar protective effects on ISCs during intestinal stress (79, 82). The GLP-2 analog teduglutide promotes ISC expansion after intestinal resection, protects ISCs from radiation damage, and facilitates ISC repair during graft-versus-host disease in mice and humans (165-167). Notably, GLP-2 prevents mucosal atrophy caused by total parenteral nutrition (TPN) through activation of AKT/ GSK3β and WNT/β-catenin signaling, suggesting that GLP-2 and NT exert their trophic effects through a common mechanism (168). In a manner similar to that observed with GLP-2, NT prevents mucosal hypoplasia caused by enteral feeding and facilitates mucosal repair following chronic intestinal inflammation or small-bowel resection (72, 73, 75). In addition, the enterotrophic effects of GLP-2 are enhanced by the presence of NT (83). The analogous mechanism and functions of NT described here suggest that NT may provide therapeutic benefits similar to those observed with GLP-2.

To confirm the positive role of NT on ISC function in mice, we performed a complementary study using *Drosophila,* another powerful model of ISC physiology. We have previously shown that constitutive NT expression in EE cells of the *Drosophila* midgut increases lipid droplet accumulation and impairs AMPK signaling in a manner

analogous to that observed in the mammalian small intestine (46), therefore establishing *Drosophila* as another useful model for studying NT function. Corroborating the observation that NT contributes to ISC maintenance in the mammalian small intestine, NT expression increases ISC number in the midgut of *Drosophila* fed a standard diet and prevents ISC loss during nutrient-depletion. Other intestinal hormones have demonstrated similar nutrient-regulated effects on ISC proliferation in *Drosophila* (92). Deletion of EE cells in *Drosophila* midgut prevents nutrient-stimulated expression of the *Drosophila* insulin-like peptide 3 (*Dilp3*) and impairs ISC proliferation, whereas increasing EE cell number promotes *Dilp3* expression and ISC division, suggesting that EE cells are important nutrient-sensing modulators of ISC homeostasis (92). Our findings lend further support to the idea that EE cells, through gut hormone release, support ISC function in *Drosophila,* and indicate that the role of NT on ISCs is highly conserved.

Our data suggest that NT plays a protective role on ISCs, which is consistent with previous studies documenting the protective effects of exogenous NT in the small intestine. NT administration reverses hypoplasia of the small intestinal mucosa in rats challenged with a liquid elemental diet and promotes mucosal regeneration following small-bowel resection (72, 73). Interestingly, several studies report a more pronounced effect for exogenous NT on growth in the proximal small intestine relative to the distal small intestine (41, 73). Our findings indicate that endogenous NT has a more prominent effect in the distal crypts, which is likely due to the primarily distal localization of NT expression in the small intestine. This highlights potential differences in the function of exogenous and endogenous NT in regulation of intestinal function along the proximal-to-distal axis of the small intestine and suggests that endogenous NT release from distal EE cells regulates the

local ISC niche in a paracrine manner. Like NT, many other gut hormones are secreted from EE cells that are localized to discrete regions of the intestine (169). Given the important local effects of NT in the distal small intestine, it is interesting to speculate that other gut hormones may exert similar paracrine functions that influence the local microenvironment in which they are released. In addition to its trophic function, NT has also demonstrated anti-apoptotic effects in gastrointestinal tissues (76). NT reduces intestinal crypt cell apoptosis caused by obstructive jaundice and reduces caspase-3 expression in mouse models of colitis (77, 170). The collective pro-proliferative and anti-apoptotic actions of NT suggest it plays a protective role on epithelial cells under various stress conditions. We advance these findings to show that the protective function of NT in the small intestine extends to ISCs challenged by nutrient-depletion.

Our data, utilizing mice deficient in NT, corroborate findings in a human model of nutritional stress that show *Lgr5* mRNA and WNT target gene expression are upregulated in the crypts of human ileum after prolonged absence of enteral nutrients, despite a reduction in total ISC numbers, suggesting that *Lgr5* expression is enhanced in ISCs during nutrient depletion in order to prime them for rapid proliferation upon the reintroduction of nutrients (171). In light of our findings that NT contributes to the induction of *Lgr5* expression in fasted crypts, NT may have therapeutic benefit to patients receiving parenteral nutrition. Our findings also support that of other studies demonstrating enhanced ISC function during fasting with no changes in ISC frequency (27, 29). Upregulation of FAO via PPARδ signaling and *Cpt1a* expression was shown to be critical for enhanced ISC function after a 24h fast (27). In agreement with this, we find induction PPARδ signaling and FAO target genes, including *Hmgcs2* and *Cpt1a*, in crypts from fasted mice

relative to AL fed mice. However, we find that loss of NT impairs fasting-induced ISC function without altering expression of *Cpt1a*, suggesting that the effect of NT on ISC function during nutrient deprivation is independent of FAO or PPARδ signaling. Instead, we find that fasting promotes WNT/β-catenin signaling in intestinal crypts in a NT-dependent manner. Mechanistically, NT increased *Lgr5* mRNA expression in ISCs and progenitor cells and conferred increased ISC function during fasting. As PPARδ is a β-catenin-target gene, it is plausible that the increase in PPARδ signaling during fasting observed by us and others is a product of WNT/β-catenin activation (172).

NT-mediated activation of the WNT/β-catenin pathway corresponded to enhanced phosphorylation of AKT and GSK3β. The role of NT on AKT/GSK3β signaling has been demonstrated previously in CRCs, in which NT promotes phosphorylation of AKT and GSK3β and enhances cell proliferation (60). Consistent with this, p-AKT and p-GSK3β expression were enhanced by exogenous NT and attenuated by the absence of NT. Phosphorylation of GSK3β by AKT is inhibitory, resulting in accumulation of active β-catenin and enhanced WNT/β-catenin signaling (14, 61, 62). Consistent with this model, we found that NT upregulates expression of active β-catenin and WNT target genes during fasting. Though we did not detect NT-dependent differences in AKT activity in fasted crypts, our findings corroborate previous work demonstrating nearly undetectable levels of p-AKT in crypts after a 48h fast (29). We speculate that the observed level of AKT activity during fasting is the minimum required for crypt maintenance and is therefore not further inhibited by loss of NT. Interestingly, loss of NT markedly reduces p-AKT and p-GSK3β expression in the crypts of AL-fed without altering WNT pathway activation. We postulate that under nutrient-rich conditions, in which the intestinal crypts are undergoing rapid

proliferation, the effect of NT on these growth stimulating pathways serves mainly to contribute to crypt cell proliferation. In light of our observation that fasting reduces total GSK3β expression, the pool of GSK3β phosphorylated downstream of NT may become more critical in regulation of WNT/β-catenin signaling during nutrient-deprivation.

This study also conclusively establishes NT as a positive regulator of ERK1/2 signaling in the small intestine. Under nutrient-rich conditions, exogenous NT activated, whereas NT-deficiency attenuated ERK1/2 phosphorylation in the small intestine. ERK1/2 controls the G1 to S-phase and G2 to M-phase transitions of the cell cycle and regulates cyclin D1 transcription during G1/S phase, and activation of ERK1/2 is required for S-phase entry and proliferation of intestinal epithelial cells (20, 21, 173-175). Consistent with the role of ERK1/2 as a central regulator of cell cycle progression and cyclin D1, NT-deficiency significantly reduced cyclin D1 protein expression and impaired progenitor cell proliferation in the distal crypts. Attenuation of crypt cell proliferation in *Nt*$^{-/-}$ mice was associated with increased crypt cell differentiation, evident by the increase in cells stained by Alcian blue, a marker of differentiated mucin-secreting goblet cells (176). Cyclin-CDK complexes regulate the switch from active cell proliferation to terminal differentiation, and cyclin D1 expression is inversely correlated with differentiation of goblet cells in multiple mouse models (177-179). Thus, attenuation of cyclin D1 expression in *Nt*$^{-/-}$ mice may impair crypt progenitor cell proliferation in favor of terminal differentiation. Together, these data establish NT as a positive regulator of crypt cell proliferation, ERK1/2 signaling, and cyclin D1 expression, and suggest that the trophic effects of NT in the small intestine are mediated, in part, by positive regulation of the cell cycle.

The role of NT on ISC function and activation of ERK1/2 and WNT/β-catenin signaling led us to interrogate the role of NT on these pathways in CRC. Consistent with previous work in glioblastoma cells suggesting that NT may promote WNT/β-catenin signaling via ERK1/2 activation (55), we found that NT promoted LRP6 phosphorylation and WNT activation in an ERK1/2 dependent manner. ERK1/2 kinase activity is known phosphorylate LRP6 on Ser1490, leading to activation of the WNT pathway (65). In support of this model, treatment with NT enhanced, whereas treatment with NT in combination with MEK inhibition blocked phosphorylation of ERK1/2 and LRP6, indicating that NT promotes LRP6 phosphorylation via ERK1/2 activation. Consistent with increased WNT activation, NT treatment increased the expression of WNT target genes in an NTR1-dependent manner and enhanced CSC function in HCT116 cells and *APC*^{min} *NT*$^{+/+}$ tumor organoids. In support of our findings, NT/NTR1 signaling has been shown to promote WNT signaling in hepatocellular carcinoma and promote CSC-like traits in hepatocellular carcinoma and glioblastoma cells (64, 85, 86). Our data extend these findings to demonstrate a similar role for NT on stem cell function in CRC. Of important clinical relevance, our preliminary findings indicate that NT-deficiency markedly reduces CSC clonogenicity in *APC*^{min} tumor organoids, reduces WNT target gene expression, and significantly increases survival in mouse models of CRC. Collectively, these findings demonstrate that NT/NTR1 signaling activates WNT/β-catenin signaling via LRP6 and ERK1/2 phosphorylation and establish NT as an important regulator of stem cell function in CRC.

In conclusion, our study defines a novel function for NT in maintenance of stem cell function in the normal small intestine and in CRC cells. We demonstrate that, through

activation of ERK1/2 and WNT/β-catenin signaling in intestinal crypts, NT differentially regulates progenitor cell proliferation and ISC function based on nutrient availability (**Figure 4 J**). Moreover, we find that NT exerts these effects primarily in the distal small intestine, and therefore increase our understanding of the paracrine effects of NT, which we show include regulation of the local ISC niche in response to nutritional cues. We further show that, in CRC cells, NT promotes WNT/β-catenin signaling via ERK/LRP6 signaling and enhances CSC function. These findings provide new insight into the local effects of gut hormone function in the small intestine and suggest that gut hormones play an important role in their local microenvironment in a paracrine manner. Collectively, we reveal a novel role for NT on stem cell maintenance during nutritional stress and in CRC, suggesting that NT serves as a critical link between nutrient-sensing pathways, intestinal homeostasis, and tumorigenesis.

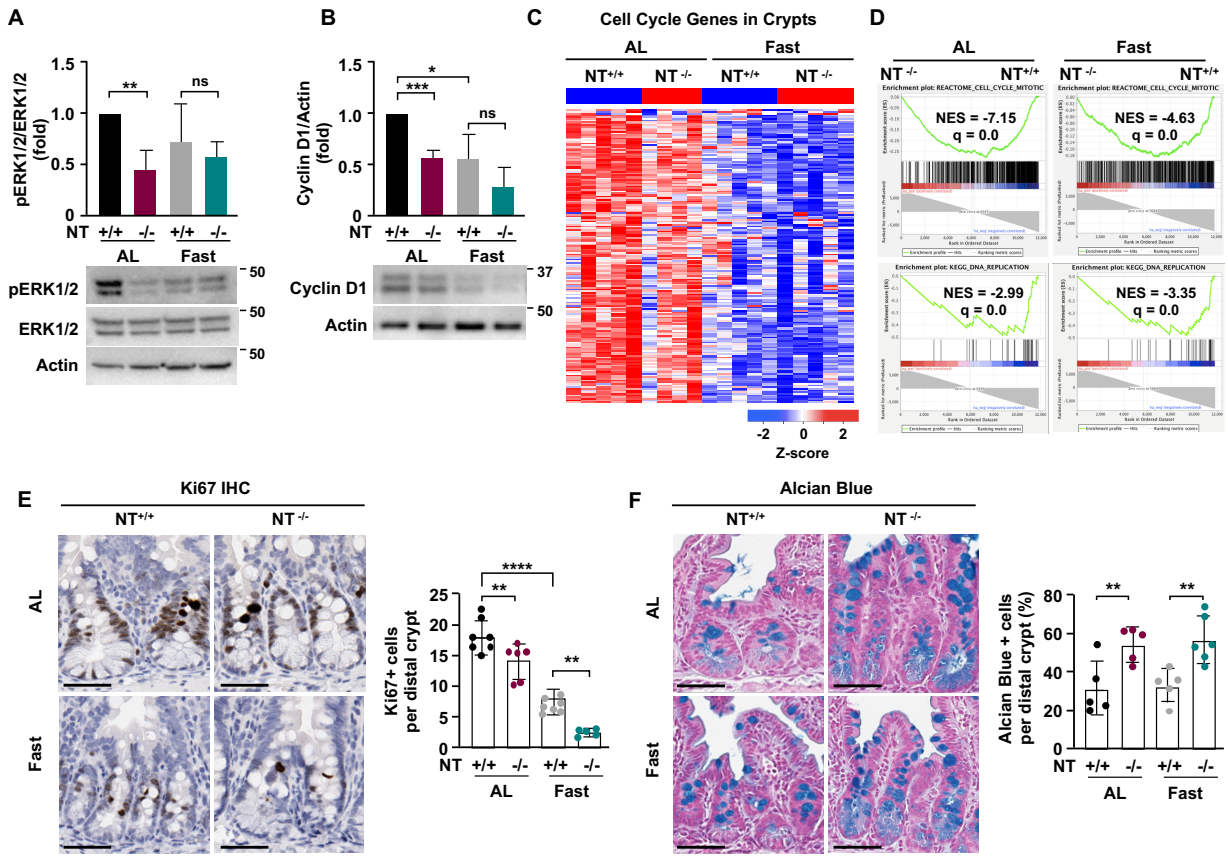


Figure 4.1. Loss of NT impairs crypt cell proliferation and promotes differentiation.

(A) Representative western blot of phospho- and total-ERK1/2 in intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either ad libitum (AL) or fasted for 48 h. (*Top*) Phospho-ERK1/2 expression was quantified relative to total ERK1/2 expression in n=5 mice per group. (B) Representative western blot of cyclin D1 expression in intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. (*Top*) Cyclin D1 expression was quantified relative to Actin in n=5 mice per group. (C) Heatplot of RNA-seq analysis showing expression of genes from the Cell Cycle gene set in crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. n= 5 mice per group for $Nt^{+/+}$ AL, $Nt^{+/+}$ Fast, and $Nt^{-/-}$ Fast groups, n=4 for $Nt^{-/-}$ AL group. (D) Enrichment plots generated by GSEA for Mitosis and DNA Replication gene sets based on RNAseq data from crypts isolated

from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. NES, normalized enrichment score relative to $Nt^{+/+}$ groups. q, False-discovery rate (FDR)-adjusted p-value. (E) Representative Ki67 staining in distal intestinal crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. *(Right)* Quantification of the average number of Ki67+ cells per crypt over 50 crypts per section in n=6-7 mice per group. (F) Representative Alcian Blue staining in distal intestinal crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. *(Right)* Quantification of the percentage of Alcian Blue+ cells per crypt over 50 crypts per section in n= 5-6 mice per group. Data shown are means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant. (Scale bars indicate 50μM).

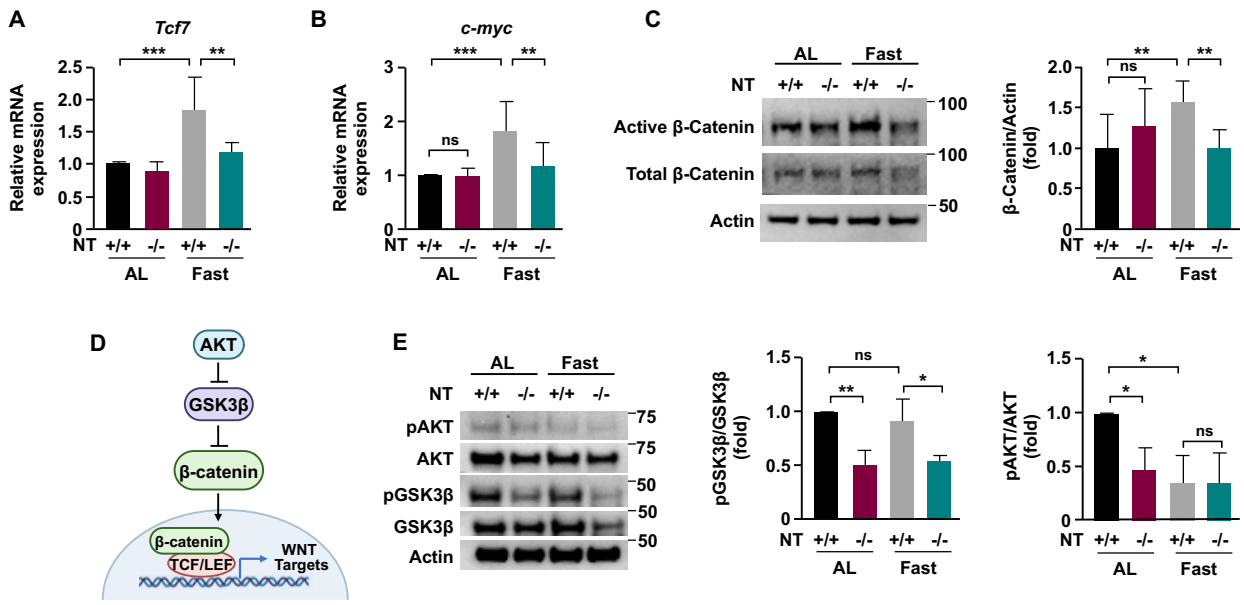


Figure 4.2. NT is required for induction of WNT/β-catenin signaling in intestinal crypts during fasting.

(A) qRT-PCR analysis of *Tcf7* mRNA expression in RNA from intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either ad libitum (AL) or fasted for 48 h. n= 5 mice per group. (B) qRT-PCR analysis of *c-Myc* mRNA expression in RNA from intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. n=5 mice per group. (C) Representative western blot of active- and total- β-catenin expression in intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. (*Right*) Active-β-catenin expression was quantified relative to Actin expression in n=6-8 mice per group. (D) Schematic of AKT/GSK3β mediated activation of the WNT/β-catenin pathway. AKT phosphorylates and inhibits GSK3β, leading to β-catenin stabilization and nuclear translocation. (E) Representative western blot of phospho- and total-AKT and phospho- and total-GSK3β expression in intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. (*Right*) Phospho-AKT and phospho-GSK3β expression were quantified relative to total protein expression in n=5 mice per group. Data shown are

means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

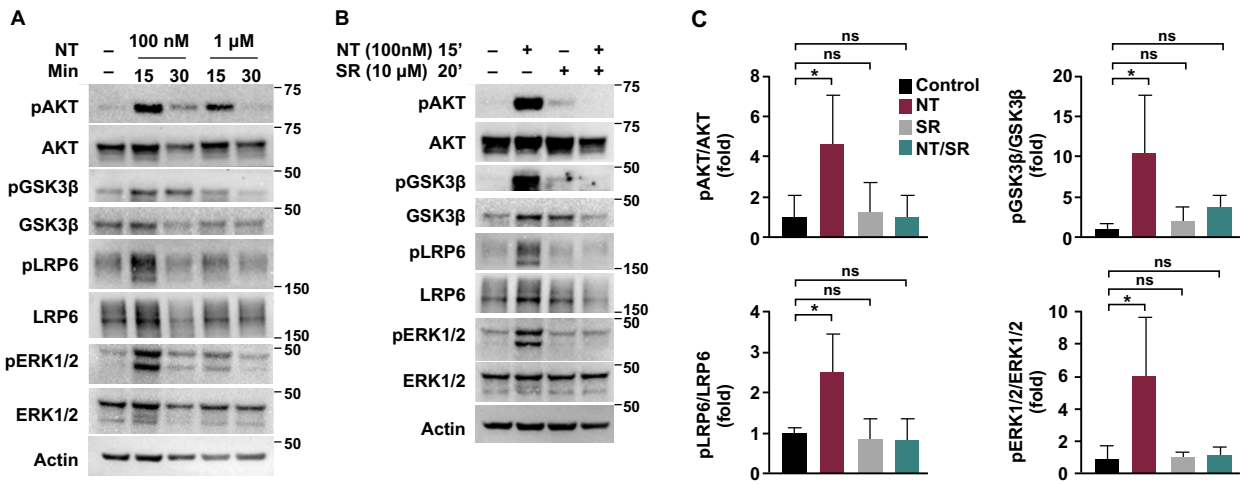


Figure 4.3. Exogenous NT activates WNT/β-catenin signaling in the small intestine.

(A) Representative western blot analysis of proteins in the WNT/β-Catenin and AKT/GSK3β signaling pathways in full-thickness small intestine treated with NT (100nM) for 15 min, SR48692 (10µM) for 20 min, or pre-treated with SR48692 (10µM) for 20 min and treated with NT (100nM) for 15 min. (B) Representative western blot of phospho and total forms of AKT, GSK3β, LRP6, ERK1/2 in in full-thickness small intestine treated *ex vivo* with 100nM or 1µM NT for 15 or 30 min. (C) Phosphorylated AKT, GSK3β, LRP6, and ERK1/2 expression was quantified relative to total protein expression in n=3-4 mice per group. Data shown are means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

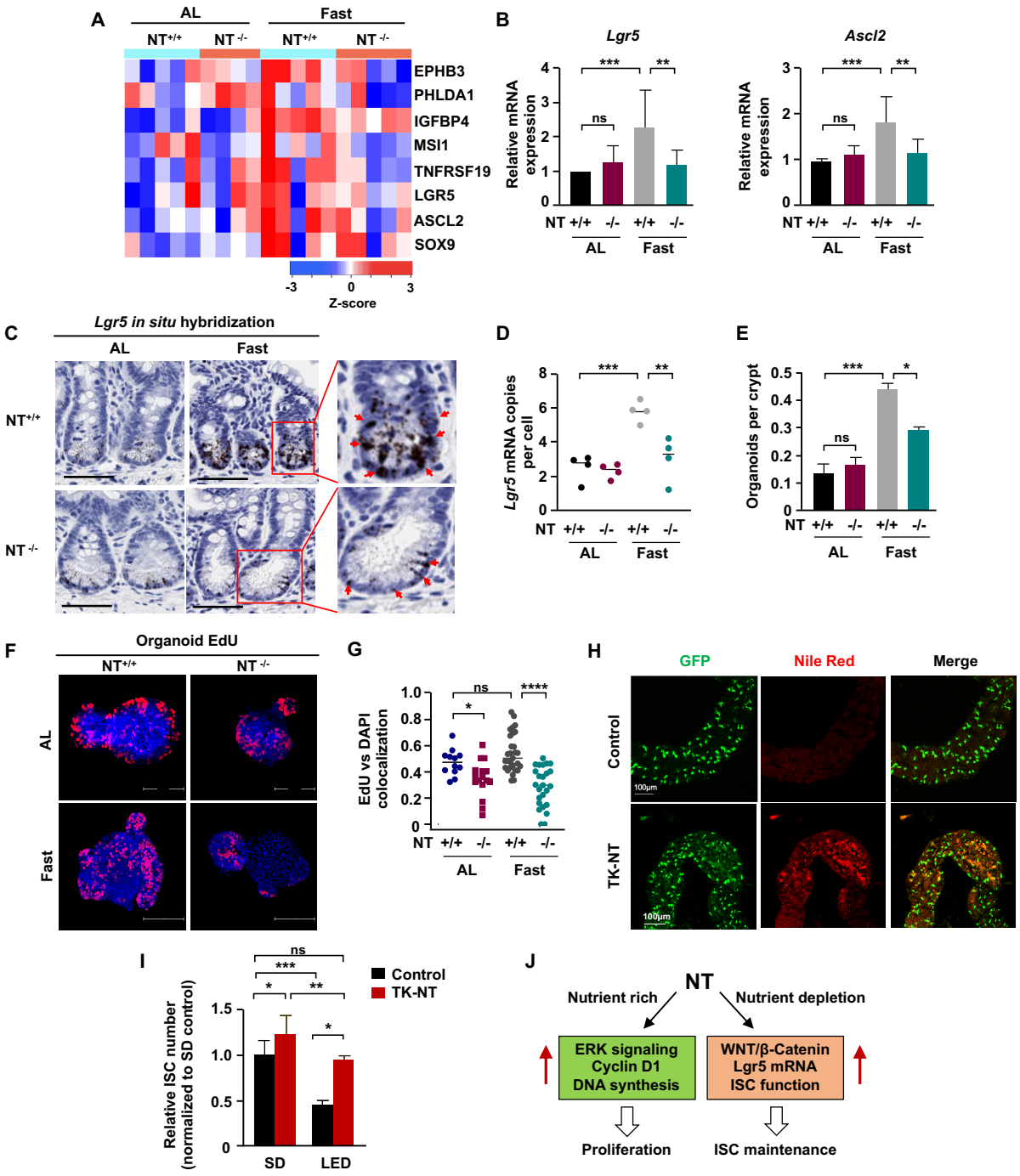


Figure 4.4. NT contributes to ISC maintenance during nutrient-stress.

(A) Heatplot of RNA-seq analysis showing expression of ISC-specific WNT-target genes in crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either ad libitum (AL) or fasted for 48 h. n= 5 mice per group for $Nt^{+/+}$ AL, $Nt^{+/+}$ Fast, and $Nt^{-/-}$ Fast groups, n=4 for $Nt^{-/-}$ AL group.

(B) qRT-PCR analysis of *Lgr5* and *Ascl2* mRNA expression in RNA from intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. n= 5-8 mice per group. (C) Representative images of *Lgr5 in situ* hybridization in distal crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. Scale bars indicate 50 μM. (Inset) Arrows indicate *Lgr5*+ cells. (D) Quantification of *Lgr5* mRNA copies per cell determined by *in situ* hybridization. n=4 mice per group. (E) Organoid-forming efficiency of crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. n= 5-6 mice per group. (F) Representative EdU staining and quantification of EdU positive cells (G) in primary organoids cultured from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. Scale bars indicate 100 μM. EdU positive cells were quantified using Pearson's coefficient of colocalization between EdU and DAPI in 20-30 organoids from 3-4 mice per group.(H) Representative *esg*-GAL4-GFP and Nile Red staining in the midgut of +/+ (control) and NT-expressing (TK-NT) drosophila. (I) Quantification of *esg*-GAL4-GFP+ ISCs in the midgut of +/+ (control) and NT-expressing (TK-NT) drosophila fed a standard diet (SD) or low-energy diet (LED). Data represent the number of ISCs in 6-15 flies per group. (J) Summary of the nutrient-state dependent function of NT on crypt cell proliferation and ISC maintenance. Data shown are means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

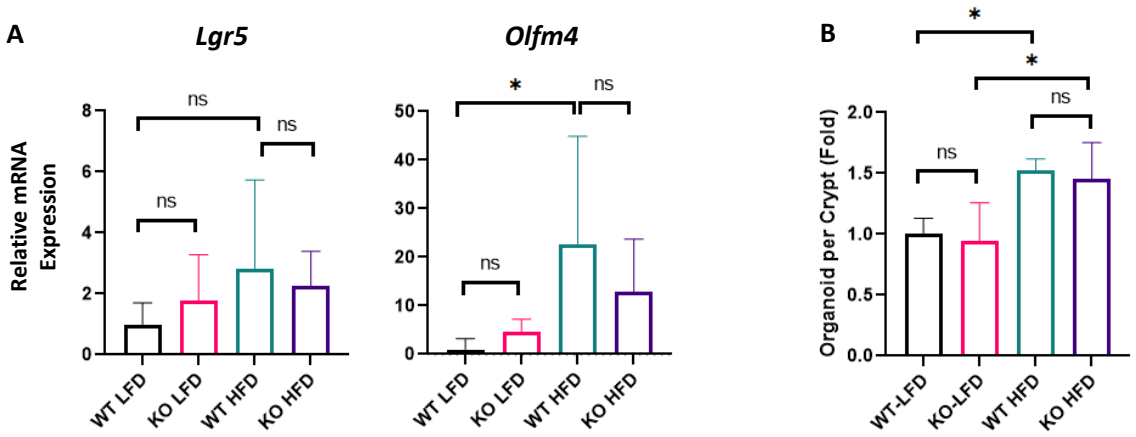
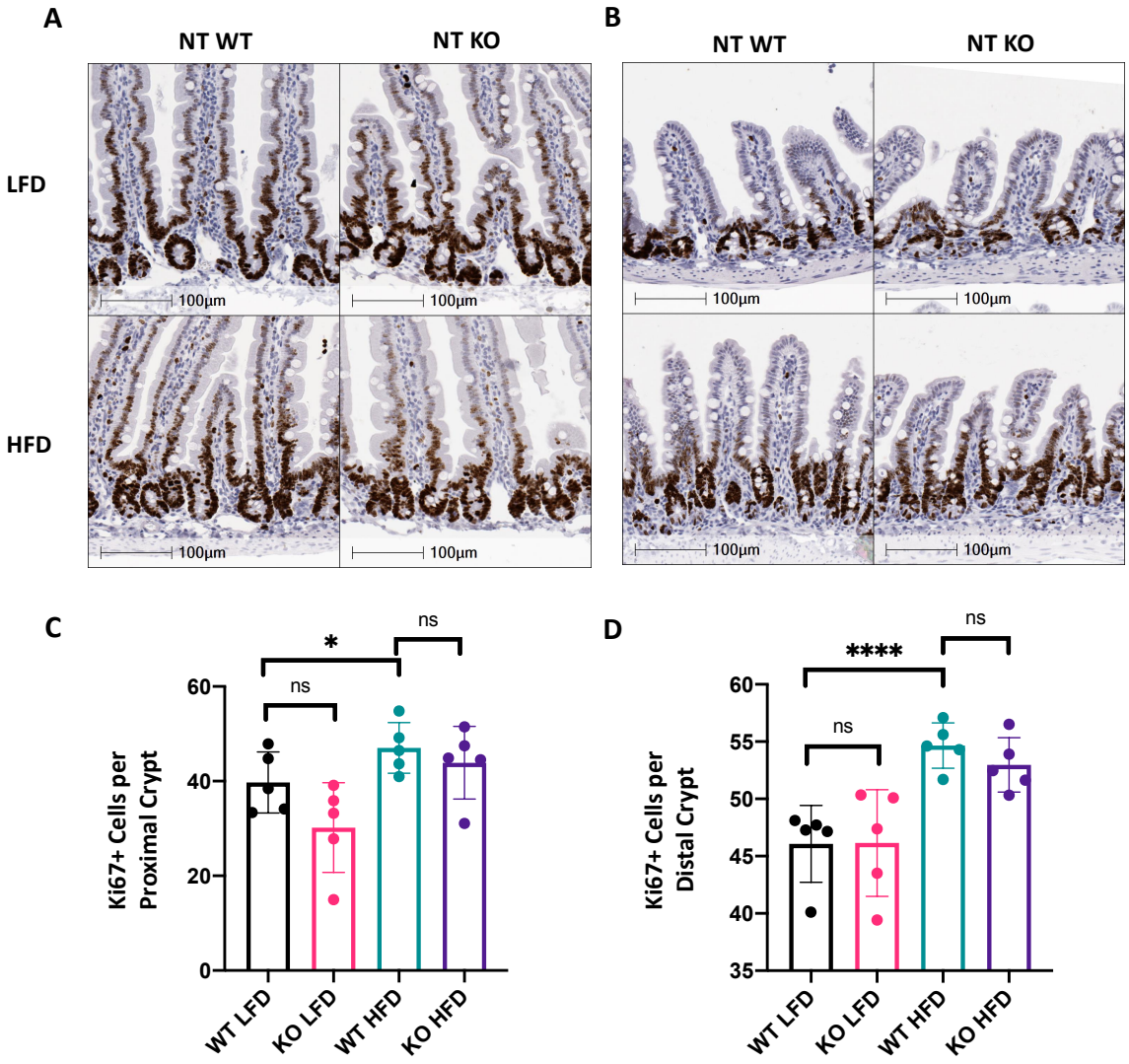


Figure 4.5 NT does not alter HFD-induced ISC function. (A) Expression of *Lgr5* and (right) *Olfm4* mRNA in crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed LFD or HFD. (B) Colony forming efficiency of primary crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed LFD or HFD. n= 4-5 mice per group. Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.



4.6. NT does not alter HFD-induced crypt cell proliferation. (A) Representative Ki67 staining in proximal and (B) distal small intestine. (C) Quantification of Ki67+ cells in the proximal and (D) distal small intestine. n= 4-5 mice per group. Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

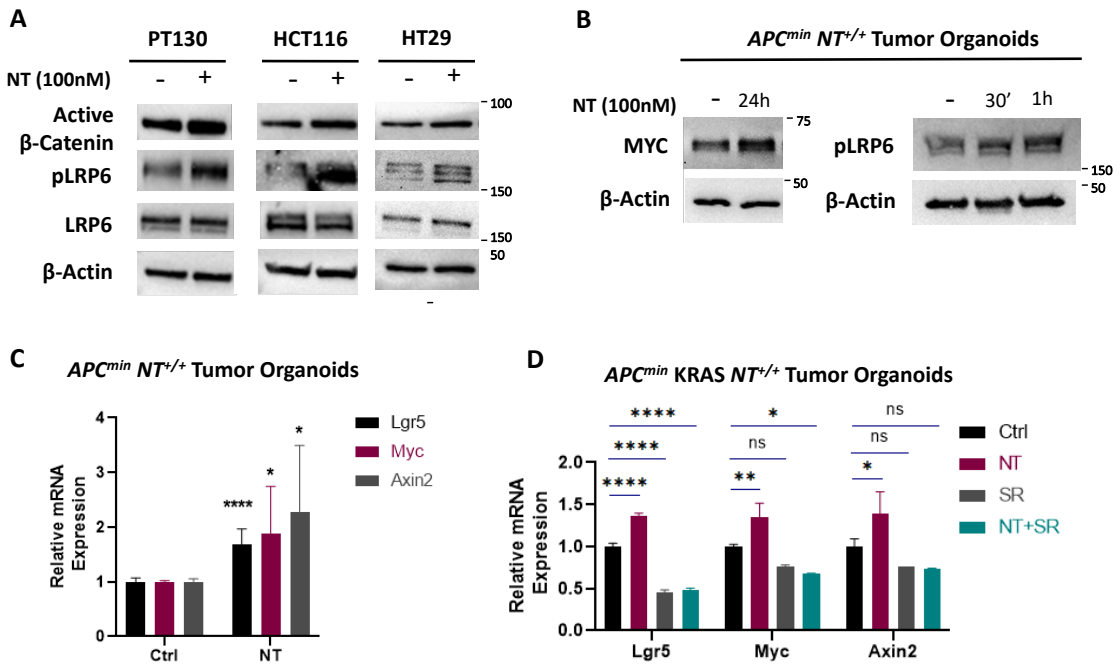


Figure 4.7. NT Activates WNT/β-catenin signaling in colorectal cancer cells and *APC^{min}* tumor organoids. (A) Representative western blot of active β-catenin and phospho- and total-LRP6 in indicated CRC cell lines treated with or without NT for 15 min. (B) Representative western blot of c-myc protein and phospho-LRP6 expression in *APC^{min}* tumor organoids treated with or without NT for the indicated times. (C) qRT-PCR analysis of *Lgr5, Myc, and Axin2* in *APC^{min}* NT$^{+/+}$ tumor organoids treated with or with NT (100nM) for 48h. *(D)* qRT-PCR analysis of *Lgr5, Myc, and Axin2* in *APC^{min} KRAS* NT$^{+/+}$ tumor organoids treated with NT (100nM) for 48h with or without pretreatment with the NTR1 inhibitor SR48692 (5 μM) for 20 min. Data are representative of 2-4 independent experiments and are shown as means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

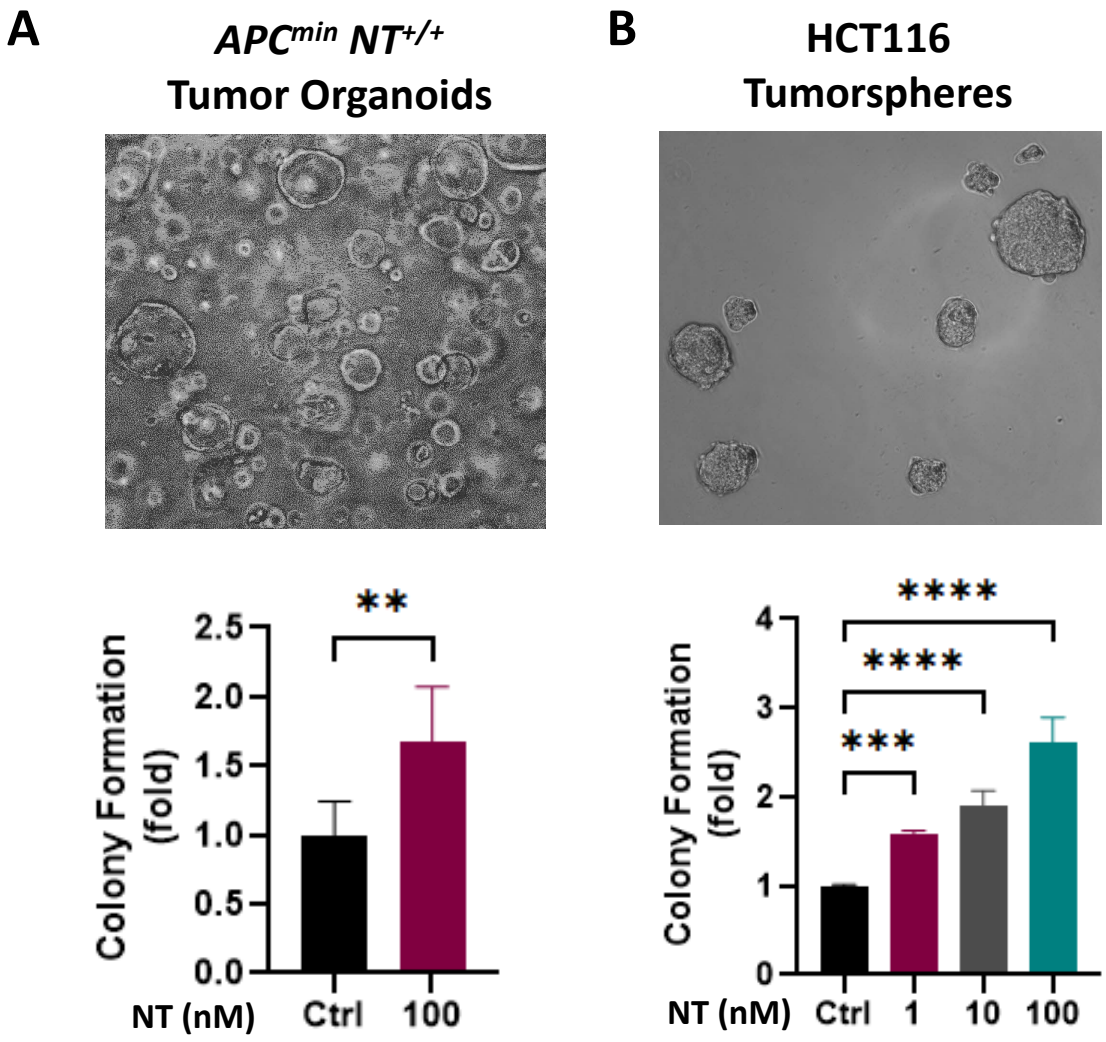


Figure 4.8. NT promotes CSC function in CRCs and intestinal tumor organoids. (A) Representative image of *APC*^{min} NT$^{+/+}$ tumor organoids (top) and colony forming efficiency (bottom) in *APC*^{min} NT$^{+/+}$ tumor organoids treated with NT (100nM) for 72 h. (B) Representative image of HCT116 tumorspheres (top) and colony forming efficiency (bottom) in HCT116 tumorspheres treated with NT at the indicated doses for 72 h. Data are representative of 2 independent experiments and are shown as means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

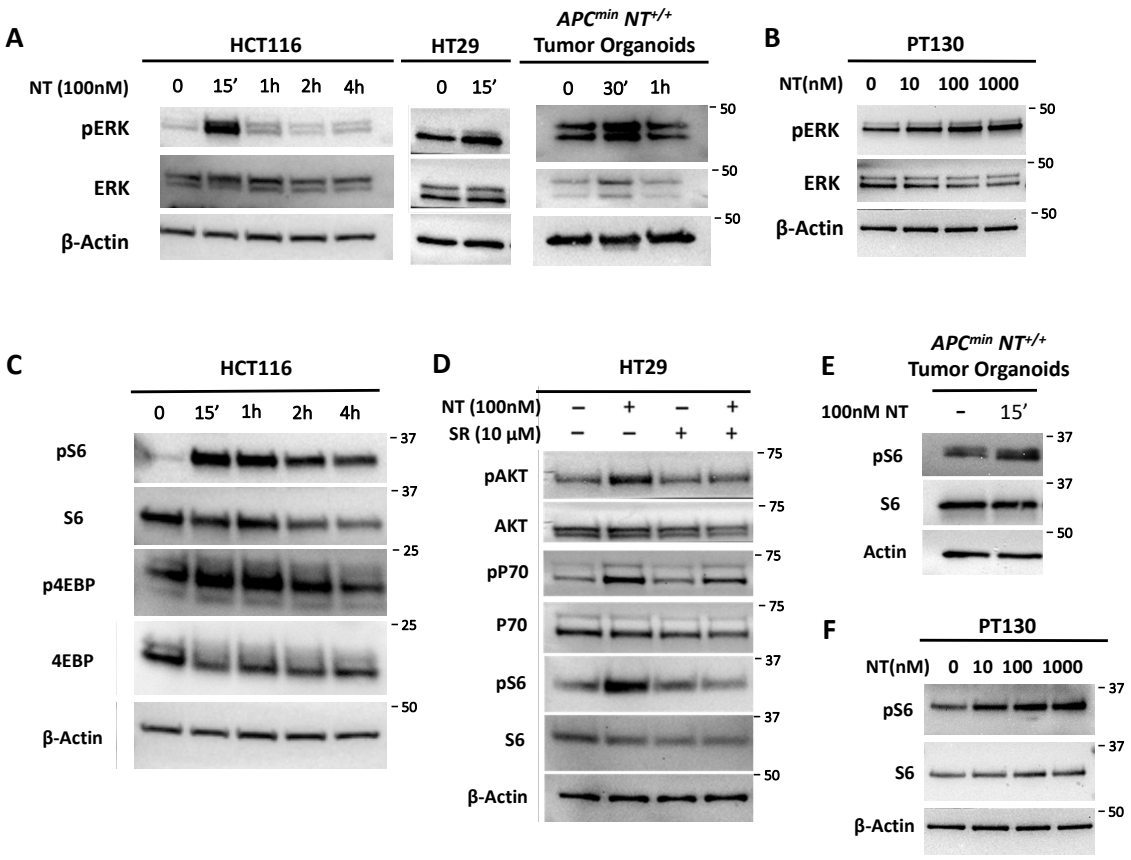


Figure 4.9. NT activates ERK1/2 and mTORC1 signaling in CRCs and intestinal tumor organoids. (A) ERK1/2 phosphorylation in HCT116, HT29, and *APC*^{min} NT$^{+/+}$ tumor organoids treated with 100nM NT for the indicated times. (B) ERK1/2 phosphorylation in PT130 cells treated with NT at the indicated concentrations for 15 min. (C) mTORC1 signaling activation in HCT116 cells treated with 100nM NT for the indicated times. (D) mTORC1 signaling in HT29 cells treated with 100nM NT for 15 min and/or SR48692 (10 μM) pre-treatment for 20 min. (E) S6 activation in *APC*^{min} NT$^{+/+}$ tumor organoids treated with 100nM NT for 15 min. (F) S6 activation in PT130 cells treated with 100nM NT at the indicated concentrations for 15 min. Data are representative of 2-3 independent experiments.

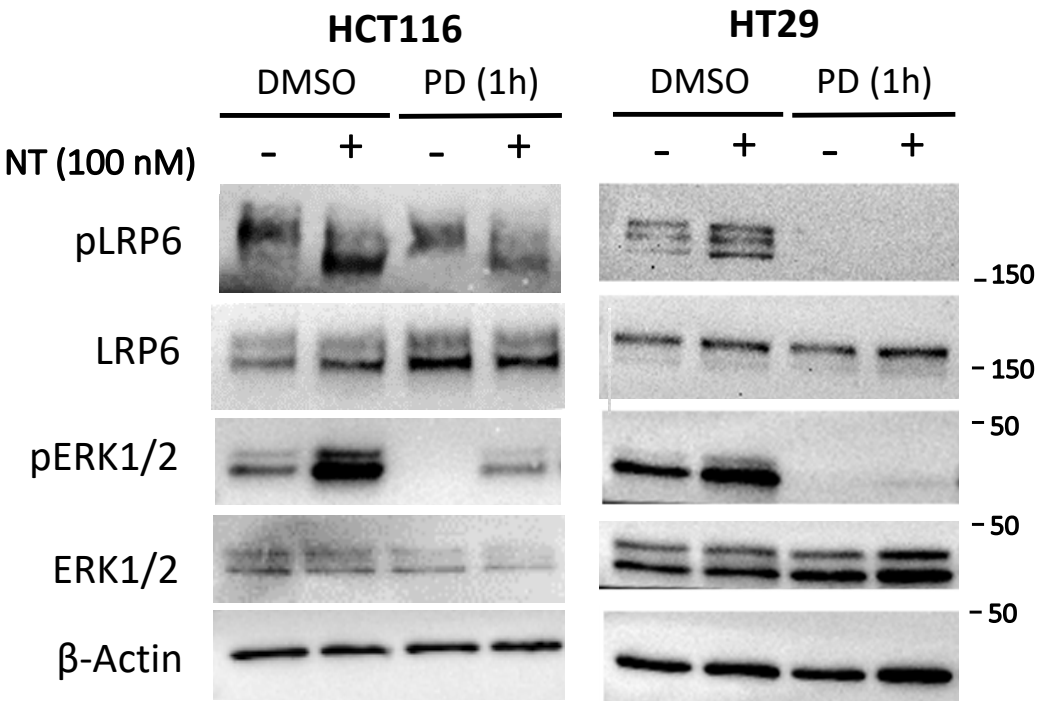


Figure 4.10. NT-mediated phosphorylation of LRP6 requires ERK1/2 activation. ERK1/2 and LRP6 phosphorylation in HCT116 and HT29 cells treated with NT (100nM) and/or PD0325901 (10 nM) for 15 min. Data are representative of 2-3 independent experiments.

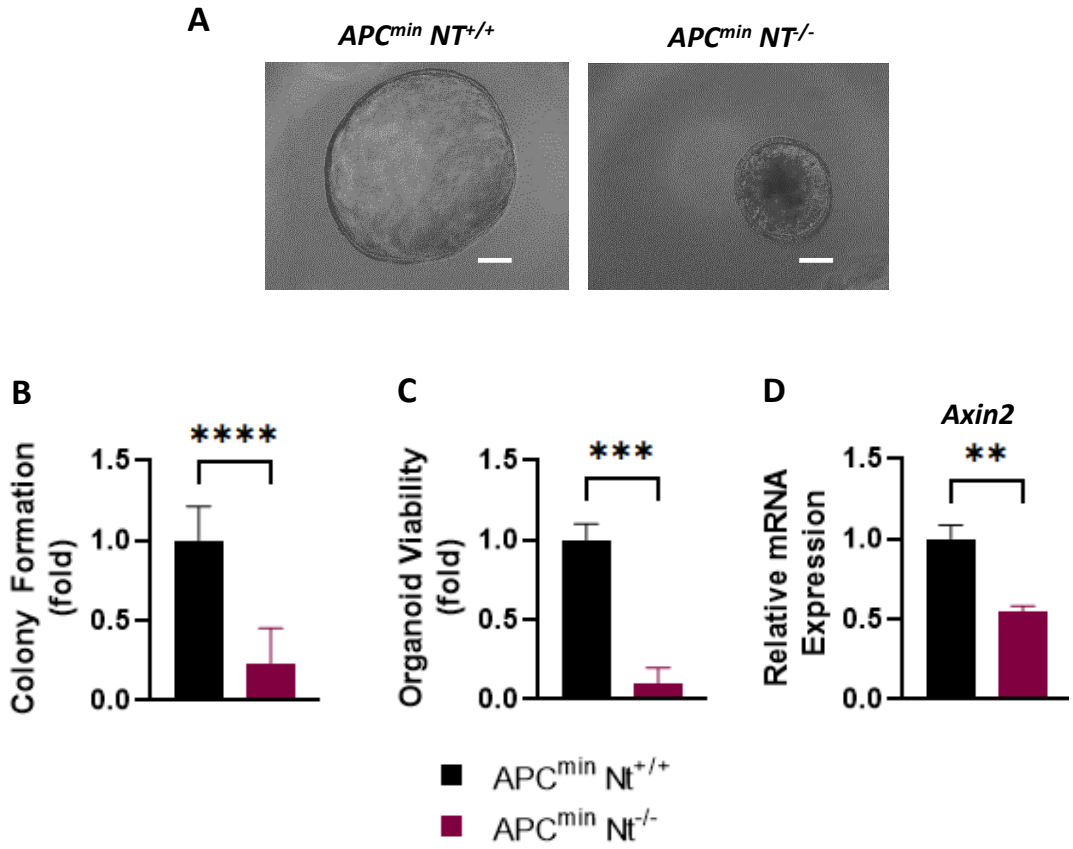


Figure 4.11. NT-deficiency reduces CSC function and WNT-target gene expression in *APC^{min}* tumor organoids. (A) Representative images of tumor organoids cultured from *APC^{min}* NT$^{+/+}$ and *APC^{min}* NT$^{-/-}$ tumors. (B) Colony formation and (C) viability of tumor organoids cultured from *APC^{min}* NT$^{+/+}$ and *APC^{min}* NT$^{-/-}$ tumors. (D) mRNA expression of the WNT-target gene Axin2 in *APC^{min}* NT$^{+/+}$ and *APC^{min}* NT$^{-/-}$ tumor organoids. Data are representative of 2 independent experiments and are shown as means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant.

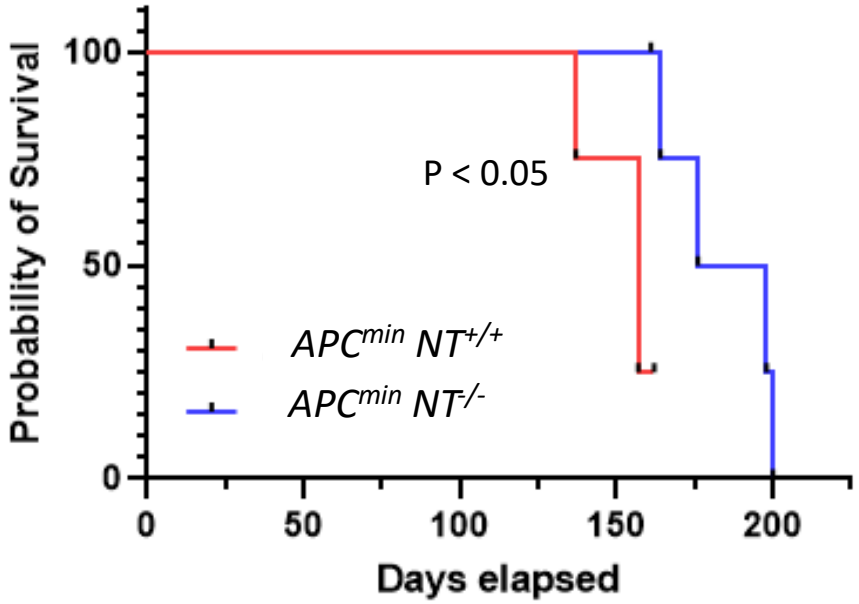
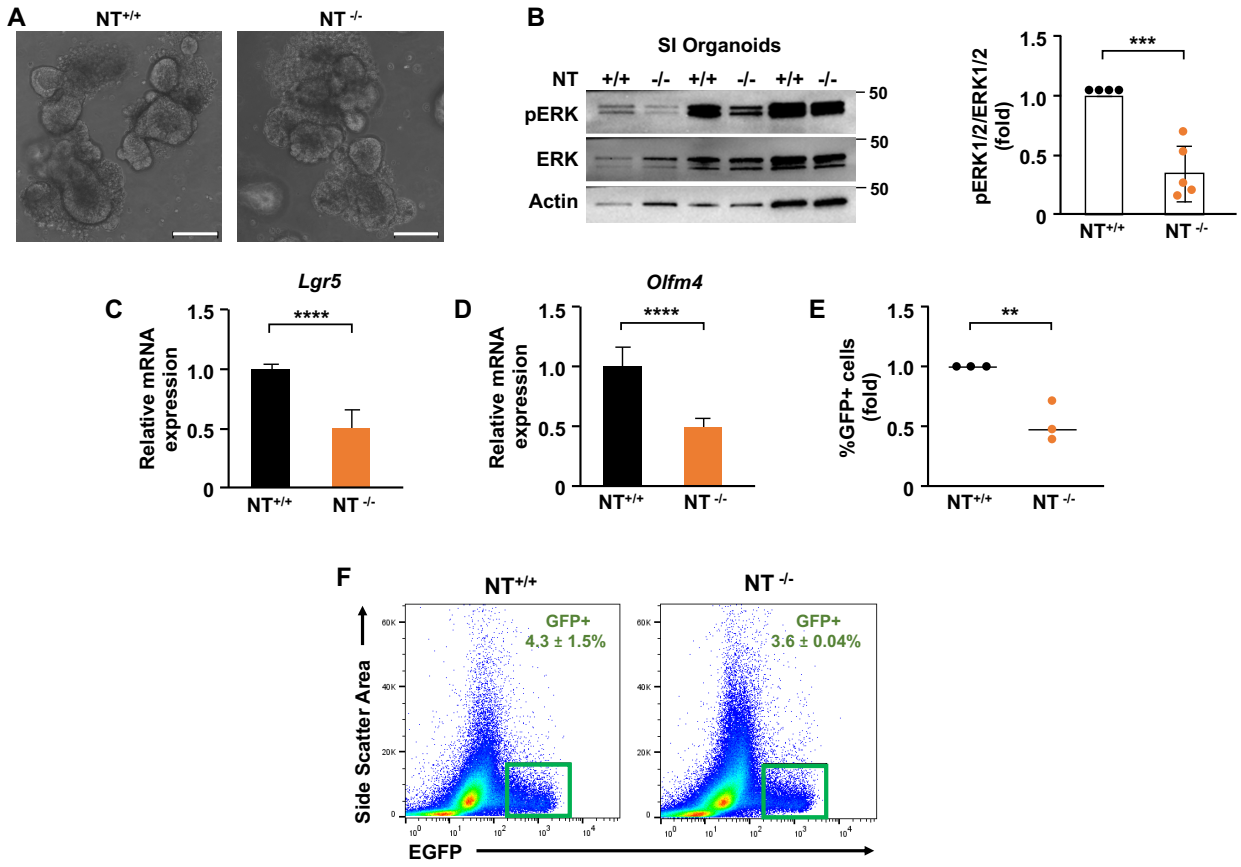
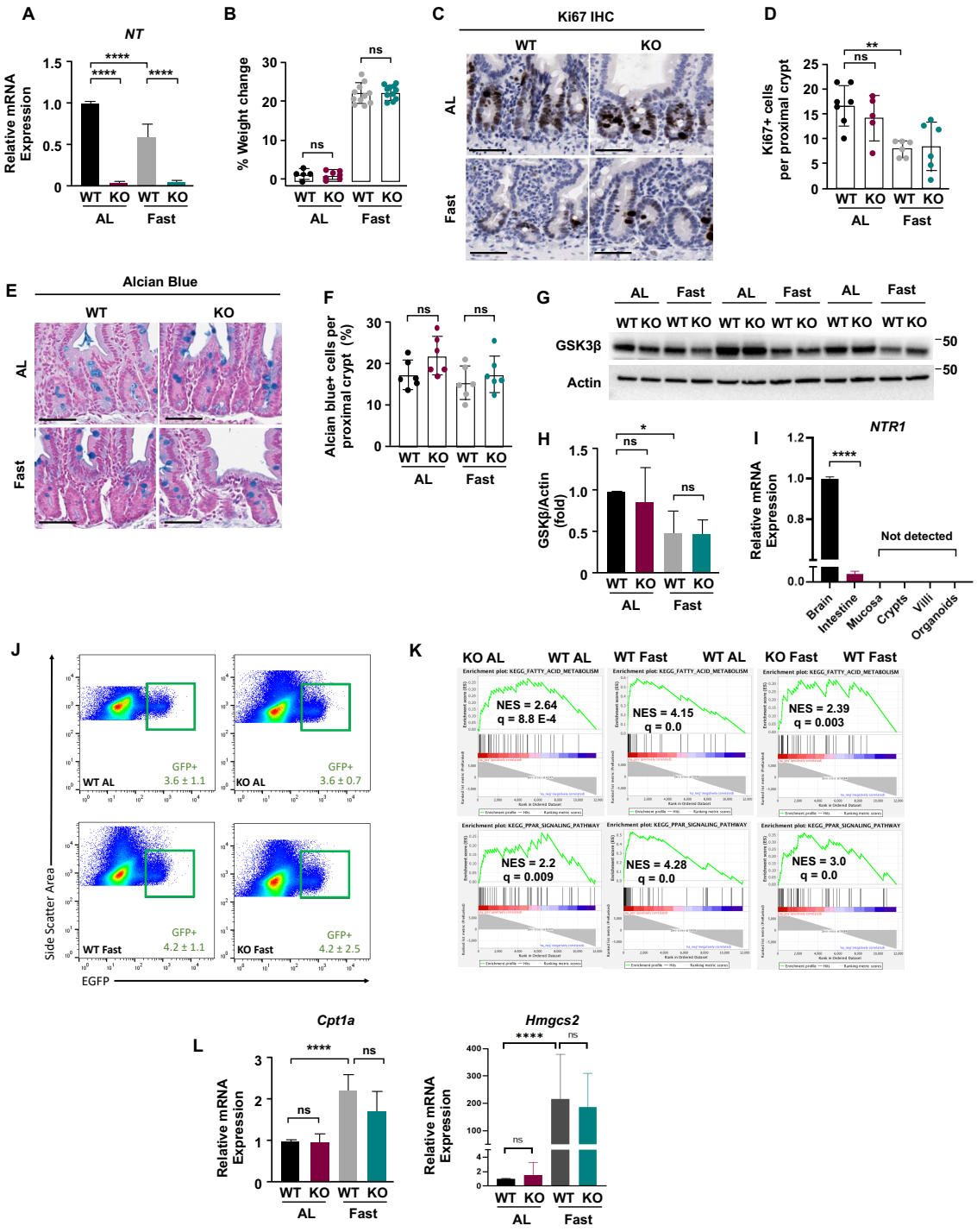


Figure 4.12. NT-deficiency significantly increases the lifespan of *APC^{min}* mice. Kaplan-Meier curve shows the survival distribution of two cohorts of mice: *APC^{min}* NT$^{+/+}$ and *APC^{min}* NT$^{-/-}$. Numbers of mice in the two cohorts are: *APC^{min}* NT$^{+/+}$ (n=4) and *APC^{min}* NT$^{-/-}$(n=5). Statistical significance (determined by Log Rank test) is given for comparisons between *APC^{min}* NT$^{+/+}$ and *APC^{min}* NT$^{-/-}$ ($p<0.05$).



Supplemental Figure 4.1.

(A) Representative images of organoids from *Nt*$^{+/+}$ and *Nt*$^{-/-}$ crypts. (B) Western blot analysis of phospho- and total-ERK1/2 in organoids from *Nt*$^{+/+}$ and *Nt*$^{-/-}$ crypts. Organoids from n=3 mice per group are shown. Densitometry analysis was performed on western blots of organoids from n=5 mice per group. (C) *Lgr5* and (D) *Olfm4* mRNA expression in organoids cultured from *Nt*$^{+/+}$ and *Nt*$^{-/-}$crypts. N=5 mice per group. (E) Lgr5-GFP+ ISC frequency in organoids cultured from *Nt*$^{+/+}$ and *Nt*$^{-/-}$ crypts. N=3 mice per group. (F) Lgr5-GFP+ ISC frequency in freshly isolated crypts from *Nt*$^{+/+}$ and *Nt*$^{-/-}$ mice fed ad libitum. N=3-4 mice per group. Data shown are means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not statistically significant. Scale bars indicate 100 µM.



Supplemental Figure 4.2.

(A)*Nt* mRNA expression in crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed AL or fasted for 48 h. n=7-8 mice per group. (B) Body weight of $Nt^{+/+}$ and $Nt^{-/-}$ mice fed AL or fasted for

48h. n= 5-11 mice per group. (C) Representative Ki67 staining in proximal intestinal crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. (D) Quantification of the average number of Ki67+ cells per crypt over 50 crypts per section in n=5-7 mice per group. (E) Representative Alcian Blue staining in proximal intestinal crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. (F) Quantification of the percentage of Alcian Blue+ cells per crypt over 50 crypts per section in n= 6 mice per group. (G) Representative western blot analysis of total GSK3β in crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. n=3 mice per group are shown. (H) Densitometry analysis was performed to quantify total GSK3β expression relative to Actin in n=5 mice per group. (I) *NTR1* mRNA expression in indicated tissues. (J) Lgr5-GFP+ ISC frequency in freshly isolated crypts from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either AL or fasted for 48 h. N=4 mice per group. (K) Enrichment plots generated by GSEA for Fatty Acid Metabolism and PPARδ signaling gene sets based on RNAseq data from crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed AL or fasted for 48 h. NES, normalized enrichment score relative to $Nt^{+/+}$ groups. q, False-discovery rate (FDR)-adjusted p-value. (L) qRT-PCR analysis of *Cpt1a* and *Hmgcs2* mRNA expression in RNA from intestinal crypts isolated from $Nt^{+/+}$ and $Nt^{-/-}$ mice fed either ad libitum (AL) or fasted for 48 h. n= 5-7 mice per group. Data shown are means ± standard deviation (SD). Significant differences are shown with asterisks. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; , not statistically significant. Scale bars indicate 50 μM).

CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS

Neurotensin is a trophic hormone that promotes proliferation of the intestinal mucosa and several types of cancer cells (31, 48, 89). In this study, we investigated 1) the mechanisms regulating NT release from endocrine cells and methods for modulating NT secretion, 2) the role of NT on proliferation and stem cell function in the normal small intestine and 3) the impact of NT on stem cell function in CRC. These studies provide greater mechanistic and functional insight into the processes controlling NT secretion and its physiological and pathological effects in the intestine.

NT is released from EE cells, a rare population of differentiated epithelial cells in the small intestine that secrete gut peptides (35). Multiple sub-populations of EE cells exist that predominantly secrete only specific gut peptides, with the NT-specific EE cell traditionally referred to as N-cells (34, 86). Thus, isolating and culturing the NT-secreting cells for mechanistic studies is complicated by the rarity of the cell population in addition to the low fidelity of normal epithelial cell cultures (180). To overcome these limitations, we utilized two endocrine tumor cells lines that release NT in a manner analogous to that of N-cells (181, 182). Our findings demonstrate that FFA-stimulated NT release from endocrine cells is positively regulated by the RAF-MEK-ERK scaffold protein KSR1, and that NT release can be controlled via modulation of KSR1 expression or the activity of MEK or ERK in the MAPK signaling pathway. Furthermore, we identify a novel role for the exocyst complex component Exo70 in regulation of NT release and ERK1/2 signaling. Given the dichotomous role of NT, understanding the mechanisms controlling NT release will provide greater insight into how NT may be used therapeutically. The role of NT on intestinal lipid absorption and metabolic dysfunction makes NT a particularly attractive

target for the treatment of obesity associated metabolic disorders. Our findings identify a novel role for KSR1 and Exo70 in regulation of NT expression and secretion, suggesting that these proteins may have therapeutic potential in the treatment of NT-driven metabolic disorders.

A major goal of this study was to further our understanding of the trophic effects of NT in the small intestine. Most recent studies investigating the proliferative role of NT have been carried out in cancer cells (48). Though early *in vivo* studies using rat models of intestinal resection or nutrient deprivation identified the trophic function of NT in the intestine, the relative difficulty of culturing normal intestinal epithelial cells *in vitro* has limited investigations into the molecular mechanisms regulating NT-induced growth in the intestine (40, 41, 74, 180, 183). The establishment of ISC-derived organoid models provides a powerful method for studying normal intestinal physiology *in vitro,* and methods for effective isolation of whole intestinal crypts enables rapid analysis of *in vivo* signaling relative to histological analysis (184). Using these state-of-the-art methods, we were able to gain greater insight into the functions of NT in the normal small intestine under physiological conditions.

We demonstrated that NT plays an important role in regulation of the cell cycle, crypt progenitor cell proliferation, and positive regulation of the ERK1/2 signaling pathway, providing greater insight into the mechanisms underlying the trophic effects of NT in the small intestine. In addition, this study is the first to identify a role for NT in regulation of ISC function. Our findings demonstrate that the functional consequences of NT in the small intestine are dependent on nutrient status, implicating NT as an important link between nutrient-sensing pathways and intestinal homeostasis. While NT appears to

predominantly regulate cell proliferation via ERK1/2 and cyclin D1 expression during nutrient-rich conditions, nutrient depletion switches the effects of NT from maintenance of cell proliferation to maintenance of ISC integrity. Under stress conditions, in which too few nutrients are available for normal levels of cell proliferation, NT increases WNT/β-catenin signaling and promotes an ISC gene expression signature, leading to enhanced ISC function. Moreover, we find that the function of NT on ISCs is conserved in *Drosophila* during nutrient-depletion, suggesting that NT plays an important role in preservation of ISC integrity during intestinal stress in order to prevent irreparable crypt loss and mucosal damage. This is consistent with the similar effects of GLP-2, which maintains crypt cell proliferation during TPN via activation of WNT/β-catenin signaling (168). Like GLP-2, NT is an attractive therapeutic target for promoting mucosal cell growth during periods of nutrient-depletion, inflammation, or mechanical damage (72, 73, 75). Our findings provide greater insight into the mechanisms by which NT promotes mucosal growth and identifies a novel role for NT in ISC function, thus expanding the potential clinical applications of NT.

The effects of NT on ISC function led us to interrogate the role of NT on CSC function. While our findings suggest that NT confers enhanced CSC function in multiple CRC cell lines, many additional questions remain regarding the full extent of NT on tumor initiation and CSC function. The next steps in fully characterizing the contribution of NT in CRC stem cell function and tumor growth is a comprehensive study of the effects of NT deficiency in mouse models of CRC. Our APC^{min} tumor organoid model has yielded promising preliminary data suggesting that NT promotes tumor stem cell clonogenicity, and our survival analysis thus far indicates that loss of NT significantly increases survival

in APC mutant mice. However, the extent to which NT regulates tumor number, size, or progression remains to be seen. Mechanistically, these studies show that the effects of NT on ISC function and CSC function are mediated by positive regulation of WNT/β-catenin signaling. Thus, as in many other systems, WNT activation downstream of NT/NTR1 signaling has positive and negative benefits depending on cell type. The role of NT on WNT/β-catenin signaling and ISC function during nutritional stress indicates that NT has a beneficial role on ISC maintenance and crypt integrity under conditions of low nutrient availability. In contrast, in highly proliferative CRC cells, NT-mediated WNT activation exacerbates the effects of aberrant cell proliferation and tumor cell clonogenicity. Thus, like WNT/β-catenin signaling, NT signaling should be tightly controlled to maintain normal ISC physiology without contributing to aberrant cell growth and tumor progression. Given the role of NT in promoting HFD-induced obesity (42) and the known stimulatory effects of HFD on WNT/β-catenin signaling and intestinal tumorigenesis (25), it is interesting to speculate that NT may promote HFD-induced CRC. An exciting future experiment to address this could examine the effect of feeding a HFD in our APC^{min} $NT^{+/+}$ and APC^{min} $NT^{-/-}$ mouse models to determine whether $NT^{-/-}$ is protective against HFD-induced tumorigenesis.

Regulation of ERK1/2 signaling is a persistent theme in our studies of NT release and function. Previous work from our lab showed that NT gene expression and secretion is enhanced by ERK1/2 signaling (44), and the present study shows that NT/NTR1 signaling activates ERK1/2 signaling in intestinal crypts and tumor organoids, leading to enhanced proliferation and CSC function, respectively. These findings suggest that NT and ERK1/2 may be involved in a positive feedback loop that sustains NT gene expression and

secretion and enhances the mitogenic effects of ERK1/2-dependent functions. In intestinal epithelial cells, this may serve as a positive signal indicating a nutrient-rich environment suitable for rapid cell proliferation, wherein nutrient-intake activates ERK1/2 in EE cells leading to NT release, which then binds to NTR1 and activates ERK1/2 signaling in crypt epithelial cells, promoting cell proliferation. However, in cancer cells that release NT and also express NTR1, the autocrine effects of NT may activate a positive feedback loop that constitutively activates ERK1/2 signaling and leads to sustained cell proliferation and enhanced tumor growth and disease progression. Thus, KSR1 and Exo70 may be useful therapeutic targets for controlling NT release and excessive ERK1/2 activation in cancer cells. Future studies that examine whether KSR1 or Exo70 manipulation alter the effects of NT on CRC cells or ISCs will be needed to determine their full therapeutic potential.

Notably, the effects of endogenous NT on proliferation and ISC function were greatest in the distal small intestine, where NT expression and secretion is highest (33). That NT regulates proliferation and ISC function primarily in the crypts closest to the site of NT release suggests that these functions occur in a paracrine manner. A major question still remaining to be addressed is whether NT regulates ISC function directly or indirectly. NTR1 expression is largely undetectable in normal intestinal epithelial cells (30, 52), yet we find that NT elicits robust effects on cell proliferation, ISC function, and ERK1/2, and WNT/β-catenin signaling. To address the question of NTR1 localization, we examined NTR1 mRNA expression and showed that NTR1 is not expressed in intestinal crypts, villi, or mucosal scrapings, but we did detect NTR1 expression in full-thickness small intestine, which includes the mucosa and submucosa. Notably, subepithelial fibroblasts and the mesenchymal cells located beneath the crypt layer have recently been identified as an

important source WNT ligands and other ISC niche signals that support ISC function (134). It is interesting to speculate that NT may mediate its effects on WNT/β-catenin signaling and ISC function by regulating WNT ligand secretion from subepithelial fibroblasts. However, future studies are needed to conclusively identify NTR1-expressing cells in the small intestine. Isolation and culture of subepithelial fibroblasts from the small intestine may represent a critical experiment for determining whether NTR1 is expressed in this cell population and, if so, whether NT impacts secretion of ISC niche factors.

In conclusion, this study identifies a novel role for NT as a regulator of stem cell function and WNT/β-catenin signaling in the normal small intestine and in CRC. Moreover, we identify KSR1, a molecular scaffold of the RAS/RAF/ERK pathway, and Exo70, a mediator of exocytosis, as potential therapeutic targets for modifying NT release **(Fig 5.1)**. Collectively, these studies reveal a new function of NT as a regulator of ISC and CSC function and identify clinically-relevant targets for regulating NT-mediated effects in the normal intestine and in intestinal tumors.

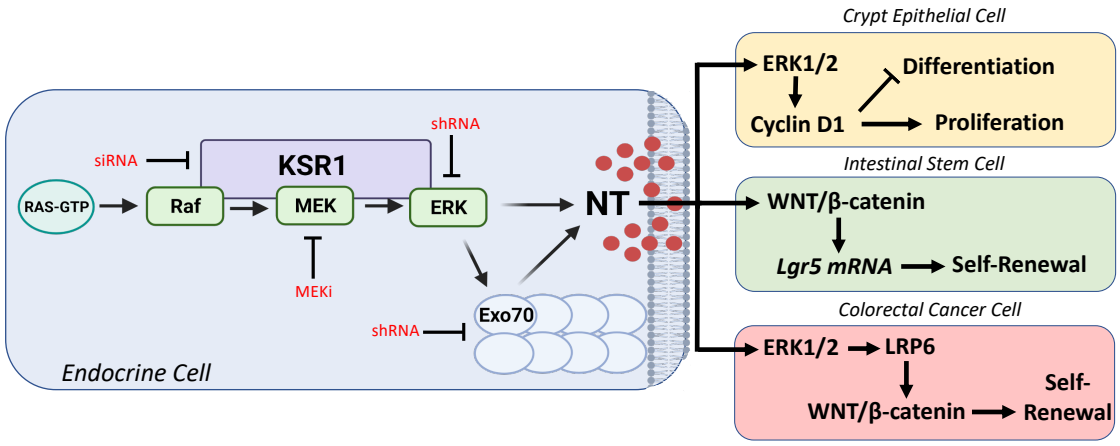


Figure 5.1. Mechanisms regulating NT release and function in the small intestine and CRC cells. Release of NT from endocrine cells is stimulated by KSR1 and Exo70 and attenuated by inhibition of KSR1, MEK, ERK1/2, or Exo70. Functions of NT described in this study include regulation of proliferation and differentiation in crypt epithelial cells via activation of ERK1/2 and cyclin D1, stimulation of ISC self-renewal during nutrient-deprivation via activation of WNT/β-catenin and *Lgr5* mRNA expression in ISCs, and enhanced WNT/β-catenin signaling and CSC self-renewal in CRC cells through phosphorylation of LRP6 by ERK1/2.

REFERENCES

1. Clevers H. Searching for adult stem cells in the intestine. EMBO Mol Med. 2009;1(5):255-9.
2. van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol. 2009;71:241-60.
3. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;449(7165):1003-7.
4. Baulies A, Angelis N, Li VSW. Hallmarks of intestinal stem cells. Development. 2020;147(15).
5. Beumer J, Clevers H. Regulation and plasticity of intestinal stem cells during homeostasis and regeneration. Development. 2016;143(20):3639-49.
6. Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev. 2003;17(14):1709-13.
7. Farin HF, Van Es JH, Clevers H. Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. Gastroenterology. 2012;143(6):1518-29 e7.
8. Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature. 2005;434(7035):843-50.
9. Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. Am J Anat. 1974;141(4):537-61.
10. Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature. 2009;457(7229):608-11.
11. Logan CY, Nusse R. The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 2004;20:781-810.
12. Perochon J, Carroll LR, Cordero JB. Wnt Signalling in Intestinal Stem Cells: Lessons from Mice and Flies. Genes (Basel). 2018;9(3).
13. Gao C, Xiao G, Hu J. Regulation of Wnt/beta-catenin signaling by posttranslational modifications. Cell Biosci. 2014;4(1):13.
14. Tejeda-Munoz N, Robles-Flores M. Glycogen synthase kinase 3 in Wnt signaling pathway and cancer. IUBMB Life. 2015;67(12):914-22.
15. Meloche S, Pouyssegur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene. 2007;26(22):3227-39.
16. Wortzel I, Seger R. The ERK Cascade: Distinct Functions within Various Subcellular Organelles. Genes Cancer. 2011;2(3):195-209.
17. Roy F, Laberge G, Douziech M, Ferland-McCollough D, Therrien M. KSR is a scaffold required for activation of the ERK/MAPK module. Genes Dev. 2002;16(4):427-38.
18. Kolch W. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nature reviews Molecular cell biology. 2005;6(11):827-37.
19. Kabiri Z, Greicius G, Zaribafzadeh H, Hemmerich A, Counter CM, Virshup DM. Wnt signaling suppresses MAPK-driven proliferation of intestinal stem cells. J Clin Invest. 2018;128(9):3806-12.

20. Boucher MJ, Jean D, Vezina A, Rivard N. Dual role of MEK/ERK signaling in senescence and transformation of intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol. 2004;286(5):G736-46.
21. Chambard JC, Lefloch R, Pouyssegur J, Lenormand P. ERK implication in cell cycle regulation. Biochim Biophys Acta. 2007;1773(8):1299-310.
22. Wei G, Gao N, Chen J, Fan L, Zeng Z, Gao G, et al. Erk and MAPK signaling is essential for intestinal development through Wnt pathway modulation. Development. 2020;147(17).
23. Fesler Z, Mitova E, Brubaker PL. GLP-2, EGF, and the Intestinal Epithelial IGF-1 Receptor Interactions in the Regulation of Crypt Cell Proliferation. Endocrinology. 2020;161(4).
24. Mana MD, Kuo EY, Yilmaz OH. Dietary Regulation of Adult Stem Cells. Curr Stem Cell Rep. 2017;3(1):1-8.
25. Beyaz S, Mana MD, Roper J, Kedrin D, Saadatpour A, Hong SJ, et al. High-fat diet enhances stemness and tumorigenicity of intestinal progenitors. Nature. 2016;531(7592):53-8.
26. Igarashi M, Guarente L. mTORC1 and SIRT1 Cooperate to Foster Expansion of Gut Adult Stem Cells during Calorie Restriction. Cell. 2016;166(2):436-50.
27. Mihaylova MM, Cheng CW, Cao AQ, Tripathi S, Mana MD, Bauer-Rowe KE, et al. Fasting Activates Fatty Acid Oxidation to Enhance Intestinal Stem Cell Function during Homeostasis and Aging. Cell Stem Cell. 2018;22(5):769-78 e4.
28. Shaw D, Gohil K, Basson MD. Intestinal mucosal atrophy and adaptation. World J Gastroenterol. 2012;18(44):6357-75.
29. Richmond CA, Shah MS, Deary LT, Trotier DC, Thomas H, Ambruzs DM, et al. Dormant Intestinal Stem Cells Are Regulated by PTEN and Nutritional Status. Cell reports. 2015;13(11):2403-11.
30. Vincent JP, Mazella J, Kitabgi P. Neurotensin and neurotensin receptors. Trends in pharmacological sciences. 1999;20(7):302-9.
31. Mustain WC, Rychahou PG, Evers BM. The role of neurotensin in physiologic and pathologic processes. Curr Opin Endocrinol Diabetes Obes. 2011;18(1):75-82.
32. Uhl GR, Goodman RR, Snyder SH. Neurotensin-containing cell bodies, fibers and nerve terminals in the brain stem of the rat: immunohistochemical mapping. Brain Res. 1979;167(1):77-91.
33. Evers BM, Ehrenfried JA, Wang X, Townsend CM, Jr., Thompson JC. Temporal-specific and spatial-specific patterns of neurotensin gene expression in the small bowel. Am J Physiol. 1994;267(5 Pt 1):G875-82.
34. Beumer J, Artegiani B, Post Y, Reimann F, Gribble F, Nguyen TN, et al. Enteroendocrine cells switch hormone expression along the crypt-to-villus BMP signalling gradient. Nat Cell Biol. 2018;20(8):909-16.
35. Polak JM, Sullivan SN, Bloom SR, Buchan AM, Facer P, Brown MR, et al. Specific localisation of neurotensin to the N cell in human intestine by radioimmunoassay and immunocytochemistry. Nature. 1977;270(5633):183-4.
36. Drewe J, Mihailovic S, D'Amato M, Beglinger C. Regulation of fat-stimulated neurotensin secretion in healthy subjects. The Journal of clinical endocrinology and metabolism. 2008;93(5):1964-70.

37. Michael DJ, Cai H, Xiong W, Ouyang J, Chow RH. Mechanisms of peptide hormone secretion. Trends Endocrinol Metab. 2006;17(10):408-15.
38. Park JJ, Loh YP. How peptide hormone vesicles are transported to the secretion site for exocytosis. Mol Endocrinol. 2008;22(12):2583-95.
39. Boules M, Li Z, Smith K, Fredrickson P, Richelson E. Diverse roles of neurotensin agonists in the central nervous system. Front Endocrinol (Lausanne). 2013;4:36.
40. Evers BM, Izukura M, Chung DH, Parekh D, Yoshinaga K, Greeley GH, Jr., et al. Neurotensin stimulates growth of colonic mucosa in young and aged rats. Gastroenterology. 1992;103(1):86-91.
41. Wood JG, Hoang HD, Bussjaeger LJ, Solomon TE. Neurotensin stimulates growth of small intestine in rats. Am J Physiol. 1988;255(6 Pt 1):G813-7.
42. Li J, Song J, Zaytseva YY, Liu Y, Rychahou P, Jiang K, et al. An obligatory role for neurotensin in high fat diet-induced obesity. Nature. In Revision.
43. Jing Li JS, Xian Li, Stephanie B. Rock, Heather F. Sinner, Heidi L. Weiss, Todd Weiss, Courtney M. Townsend Jr., Tuanyan Gao, B. Mark Evers. FFAR4 is involved in regulation of neurotensin release from neuroendocrine cells and male C57BL/6 mice. Endocrinology 2018.
44. Li J, Liu J, Song J, Wang X, Weiss HL, Townsend CM, Jr., et al. mTORC1 inhibition increases neurotensin secretion and gene expression through activation of the MEK/ERK/c-Jun pathway in the human endocrine cell line BON. Am J Physiol Cell Physiol. 2011;301(1):C213-26.
45. Evers BM. Neurotensin and growth of normal and neoplastic tissues. Peptides. 2006;27(10):2424-33.
46. Li J, Song J, Zaytseva YY, Liu Y, Rychahou P, Jiang K, et al. An obligatory role for neurotensin in high-fat-diet-induced obesity. Nature. 2016;533(7603):411-5.
47. Christou N, Blondy S, David V, Verdier M, Lalloue F, Jauberteau MO, et al. Neurotensin pathway in digestive cancers and clinical applications: an overview. Cell Death Dis. 2020;11(12):1027.
48. Nikolaou S, Qiu S, Fiorentino F, Simillis C, Rasheed S, Tekkis P, et al. The role of Neurotensin and its receptors in non-gastrointestinal cancers: a review. Cell Commun Signal. 2020;18(1):68.
49. Kim JT, Napier DL, Weiss HL, Lee EY, Townsend CM, Jr., Evers BM. Neurotensin Receptor 3/Sortilin Contributes to Tumorigenesis of Neuroendocrine Tumors Through Augmentation of Cell Adhesion and Migration. Neoplasia. 2018;20(2):175-81.
50. Wu Z, Martinez-Fong D, Tredaniel J, Forgez P. Neurotensin and its high affinity receptor 1 as a potential pharmacological target in cancer therapy. Front Endocrinol (Lausanne). 2012;3:184.
51. Martin S, Navarro V, Vincent JP, Mazella J. Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line. Gastroenterology. 2002;123(4):1135-43.
52. Haber AL, Biton M, Rogel N, Herbst RH, Shekhar K, Smillie C, et al. A single-cell survey of the small intestinal epithelium. Nature. 2017;551(7680):333-9.
53. Gui X, Guzman G, Dobner PR, Kadkol SS. Increased neurotensin receptor-1 expression during progression of colonic adenocarcinoma. Peptides. 2008;29(9):1609-15.

54. Zhao D, Keates AC, Kuhnt-Moore S, Moyer MP, Kelly CP, Pothoulakis C. Signal transduction pathways mediating neurotensin-stimulated interleukin-8 expression in human colonocytes. J Biol Chem. 2001;276(48):44464-71.
55. Xiao H, Zeng Y, Wang Q, Wei S, Zhu X. A Novel Positive Feedback Loop Between NTSR1 and Wnt/beta-Catenin Contributes to Tumor Growth of Glioblastoma. Cell Physiol Biochem. 2017;43(5):2133-42.
56. Besserer-Offroy E, Brouillette RL, Lavenus S, Froehlich U, Brumwell A, Murza A, et al. The signaling signature of the neurotensin type 1 receptor with endogenous ligands. European journal of pharmacology. 2017;805:1-13.
57. Evers BM, Zhou Z, Celano P, Li J. The neurotensin gene is a downstream target for Ras activation. J Clin Invest. 1995;95(6):2822-30.
58. Souaze F, Viardot-Foucault V, Roullet N, Toy-Miou-Leong M, Gompel A, Bruyneel E, et al. Neurotensin receptor 1 gene activation by the Tcf/beta-catenin pathway is an early event in human colonic adenomas. Carcinogenesis. 2006;27(4):708-16.
59. Zhou Z, Xie J, Cai Y, Yang S, Chen Y, Wu H. The significance of NTR1 expression and its correlation with beta-catenin and EGFR in gastric cancer. Diagn Pathol. 2015;10:128.
60. Wang Q, Zhou Y, Evers BM. Neurotensin phosphorylates GSK-3alpha/beta through the activation of PKC in human colon cancer cells. Neoplasia. 2006;8(9):781-7.
61. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 1995;378(6559):785-9.
62. Fukumoto S, Hsieh CM, Maemura K, Layne MD, Yet SF, Lee KH, et al. Akt participation in the Wnt signaling pathway through Dishevelled. J Biol Chem. 2001;276(20):17479-83.
63. Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. Pharmacology & therapeutics. 2015;148:114-31.
64. Ye Y, Long X, Zhang L, Chen J, Liu P, Li H, et al. NTS/NTR1 co-expression enhances epithelial-to-mesenchymal transition and promotes tumor metastasis by activating the Wnt/beta-catenin signaling pathway in hepatocellular carcinoma. Oncotarget. 2016;7(43):70303-22.
65. Cervenka I, Wolf J, Masek J, Krejci P, Wilcox WR, Kozubik A, et al. Mitogen-activated protein kinases promote WNT/beta-catenin signaling via phosphorylation of LRP6. Mol Cell Biol. 2011;31(1):179-89.
66. Schatoff EM, Leach BI, Dow LE. Wnt Signaling and Colorectal Cancer. Curr Colorectal Cancer Rep. 2017;13(2):101-10.
67. Barchetta I, Cimini FA, Capoccia D, Bertoccini L, Ceccarelli V, Chiappetta C, et al. Neurotensin Is a Lipid-Induced Gastrointestinal Peptide Associated with Visceral Adipose Tissue Inflammation in Obesity. Nutrients. 2018;10(4).
68. Melander O, Maisel AS, Almgren P, Manjer J, Belting M, Hedblad B, et al. Plasma proneurotensin and incidence of diabetes, cardiovascular disease, breast cancer, and mortality. JAMA : the journal of the American Medical Association. 2012;308(14):1469-75.
69. Barchetta I, Cimini FA, Leonetti F, Capoccia D, Di Cristofano C, Silecchia G, et al. Increased Plasma Proneurotensin Levels Identify NAFLD in Adults With and Without

Type 2 Diabetes. The Journal of clinical endocrinology and metabolism. 2018;103(6):2253-60.
70. Bhathena SJ. Relationship between fatty acids and the endocrine and neuroendocrine system. Nutr Neurosci. 2006;9(1-2):1-10.
71. Bartsch H, Nair J, Owen RW. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. Carcinogenesis. 1999;20(12):2209-18.
72. Evers BM, Izukura M, Townsend CM, Jr., Uchida T, Thompson JC. Neurotensin prevents intestinal mucosal hypoplasia in rats fed an elemental diet. Dig Dis Sci. 1992;37(3):426-31.
73. Izukura M, Evers BM, Parekh D, Yoshinaga K, Uchida T, Townsend CM, Jr., et al. Neurotensin augments intestinal regeneration after small bowel resection in rats. Ann Surg. 1992;215(5):520-6; discussion 6-7.
74. Chung DH, Evers BM, Shimoda I, Townsend CM, Jr., Rajaraman S, Thompson JC. Effect of neurotensin on gut mucosal growth in rats with jejunal and ileal Thiry-Vella fistulas. Gastroenterology. 1992;103(4):1254-9.
75. Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, et al. Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol. 2005;288(4):G621-9.
76. Devader C, Beraud-Dufour S, Coppola T, Mazella J. The anti-apoptotic role of neurotensin. Cells. 2013;2(1):124-35.
77. Assimakopoulos SF, Scopa CD, Zervoudakis G, Mylonas PG, Georgiou C, Nikolopoulou V, et al. Bombesin and neurotensin reduce endotoxemia, intestinal oxidative stress, and apoptosis in experimental obstructive jaundice. Ann Surg. 2005;241(1):159-67.
78. Martin GR, Beck PL, Sigalet DL. Gut hormones, and short bowel syndrome: the enigmatic role of glucagon-like peptide-2 in the regulation of intestinal adaptation. World J Gastroenterol. 2006;12(26):4117-29.
79. Kwak SY, Shim S, Park S, Kim H, Lee SJ, Kim MJ, et al. Ghrelin reverts intestinal stem cell loss associated with radiation-induced enteropathy by activating Notch signaling. Phytomedicine. 2021;81:153424.
80. Tsai CH, Hill M, Drucker DJ. Biological determinants of intestinotrophic properties of GLP-2 in vivo. Am J Physiol. 1997;272(3 Pt 1):G662-8.
81. Drucker DJ. The Discovery of GLP-2 and Development of Teduglutide for Short Bowel Syndrome. ACS Pharmacol Transl Sci. 2019;2(2):134-42.
82. Tsai CH, Hill M, Asa SL, Brubaker PL, Drucker DJ. Intestinal growth-promoting properties of glucagon-like peptide-2 in mice. Am J Physiol. 1997;273(1 Pt 1):E77-84.
83. Litvak DA, Evers BM, Hellmich MR, Townsend CM, Jr. Enterotrophic effects of glucagon-like peptide 2 are enhanced by neurotensin. J Gastrointest Surg. 1999;3(4):432-39; discussion 9-40.
84. Carraway RE, Plona AM. Involvement of neurotensin in cancer growth: evidence, mechanisms and development of diagnostic tools. Peptides. 2006;27(10):2445-60.
85. Tang KH, Ma S, Lee TK, Chan YP, Kwan PS, Tong CM, et al. CD133(+) liver tumor-initiating cells promote tumor angiogenesis, growth, and self-renewal through neurotensin/interleukin-8/CXCL1 signaling. Hepatology. 2012;55(3):807-20.

86. Zhou J, Yi L, Ouyang Q, Xu L, Cui H, Xu M. Neurotensin signaling regulates stem-like traits of glioblastoma stem cells through activation of IL-8/CXCR1/STAT3 pathway. Cellular signalling. 2014;26(12):2896-902.
87. Atashzar MR, Baharlou R, Karami J, Abdollahi H, Rezaei R, Pourramezan F, et al. Cancer stem cells: A review from origin to therapeutic implications. J Cell Physiol. 2020;235(2):790-803.
88. Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol. 2010;12(5):468-76.
89. Qiu S, Pellino G, Fiorentino F, Rasheed S, Darzi A, Tekkis P, et al. A Review of the Role of Neurotensin and Its Receptors in Colorectal Cancer. Gastroenterol Res Pract. 2017;2017:6456257.
90. Khalaf AM, Fuentes D, Morshid AI, Burke MR, Kaseb AO, Hassan M, et al. Role of Wnt/beta-catenin signaling in hepatocellular carcinoma, pathogenesis, and clinical significance. J Hepatocell Carcinoma. 2018;5:61-73.
91. Song W, Veenstra JA, Perrimon N. Control of lipid metabolism by tachykinin in Drosophila. Cell reports. 2014;9(1):40-7.
92. Amcheslavsky A, Song W, Li Q, Nie Y, Bragatto I, Ferrandon D, et al. Enteroendocrine cells support intestinal stem-cell-mediated homeostasis in Drosophila. Cell reports. 2014;9(1):32-9.
93. Min KJ, Flatt T, Kulaots I, Tatar M. Counting calories in Drosophila diet restriction. Exp Gerontol. 2007;42(3):247-51.
94. Skorupa DA, Dervisefendic A, Zwiener J, Pletcher SD. Dietary composition specifies consumption, obesity, and lifespan in Drosophila melanogaster. Aging Cell. 2008;7(4):478-90.
95. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
96. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323.
97. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40.
98. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A. 2005;102(43):15545-50.
99. Crowley LC, Scott AP, Marfell BJ, Boughaba JA, Chojnowski G, Waterhouse NJ. Measuring Cell Death by Propidium Iodide Uptake and Flow Cytometry. Cold Spring Harb Protoc. 2016;2016(7).
100. Evers BM, Townsend CM, Jr., Upp JR, Allen E, Hurlbut SC, Kim SW, et al. Establishment and characterization of a human carcinoid in nude mice and effect of various agents on tumor growth. Gastroenterology. 1991;101(2):303-11.
101. Parekh D, Ishizuka J, Townsend CM, Jr., Haber B, Beauchamp RD, Karp G, et al. Characterization of a human pancreatic carcinoid in vitro: morphology, amine and peptide storage, and secretion. Pancreas. 1994;9(1):83-90.

102. Doihara H, Nozawa K, Kojima R, Kawabata-Shoda E, Yokoyama T, Ito H. QGP-1 cells release 5-HT via TRPA1 activation; a model of human enterochromaffin cells. Mol Cell Biochem. 2009;331(1-2):239-45.
103. Li J, O'Connor KL, Cheng X, Mei FC, Uchida T, Townsend CM, Jr., et al. Cyclic adenosine 5'-monophosphate-stimulated neurotensin secretion is mediated through Rap1 downstream of both Epac and protein kinase A signaling pathways. Mol Endocrinol. 2007;21(1):159-71.
104. Li J, Chen LA, Townsend CM, Jr., Evers BM. PKD1, PKD2, and their substrate Kidins220 regulate neurotensin secretion in the BON human endocrine cell line. J Biol Chem. 2008;283(5):2614-21.
105. Li J, Hellmich MR, Greeley GH, Jr., Townsend CM, Jr., Evers BM. Phorbol ester-mediated neurotensin secretion is dependent on the PKC-alpha and -delta isoforms. Am J Physiol Gastrointest Liver Physiol. 2002;283(5):G1197-206.
106. Yan F, John SK, Polk DB. Kinase suppressor of Ras determines survival of intestinal epithelial cells exposed to tumor necrosis factor. Cancer Res. 2001;61(24):8668-75.
107. Yan F, John SK, Wilson G, Jones DS, Washington MK, Polk DB. Kinase suppressor of Ras-1 protects intestinal epithelium from cytokine-mediated apoptosis during inflammation. J Clin Invest. 2004;114(9):1272-80.
108. Mazella J, Beraud-Dufour S, Devader C, Massa F, Coppola T. Neurotensin and its receptors in the control of glucose homeostasis. Front Endocrinol (Lausanne). 2012;3:143.
109. Frodyma D, Neilsen B, Costanzo-Garvey D, Fisher K, Lewis R. Coordinating ERK signaling via the molecular scaffold Kinase Suppressor of Ras. F1000Res. 2017;6:1621.
110. McKay MM, Ritt DA, Morrison DK. Signaling dynamics of the KSR1 scaffold complex. Proc Natl Acad Sci U S A. 2009;106(27):11022-7.
111. Razidlo GL, Kortum RL, Haferbier JL, Lewis RE. Phosphorylation regulates KSR1 stability, ERK activation, and cell proliferation. J Biol Chem. 2004;279(46):47808-14.
112. Kortum RL, Lewis RE. The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. Mol Cell Biol. 2004;24(10):4407-16.
113. Nguyen A, Burack WR, Stock JL, Kortum R, Chaika OV, Afkarian M, et al. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol Cell Biol. 2002;22(9):3035-45.
114. Wang S, Hsu SC. The molecular mechanisms of the mammalian exocyst complex in exocytosis. Biochem Soc Trans. 2006;34(Pt 5):687-90.
115. Wu B, Guo W. The Exocyst at a Glance. J Cell Sci. 2015;128(16):2957-64.
116. Ren J, Guo W. ERK1/2 regulate exocytosis through direct phosphorylation of the exocyst component Exo70. Dev Cell. 2012;22(5):967-78.
117. Goettel JA, Scott Algood HM, Olivares-Villagomez D, Washington MK, Chaturvedi R, Wilson KT, et al. KSR1 protects from interleukin-10 deficiency-induced colitis in mice by suppressing T-lymphocyte interferon-gamma production. Gastroenterology. 2011;140(1):265-74.
118. Klutho PJ, Costanzo-Garvey DL, Lewis RE. Regulation of glucose homeostasis by KSR1 and MARK2. PLoS One. 2011;6(12):e29304.

119. Burack WR, Shaw AS. Signal transduction: hanging on a scaffold. Curr Opin Cell Biol. 2000;12(2):211-6.
120. Good MC, Zalatan JG, Lim WA. Scaffold proteins: hubs for controlling the flow of cellular information. Science. 2011;332(6030):680-6.
121. Shaw AS, Filbert EL. Scaffold proteins and immune-cell signalling. Nat Rev Immunol. 2009;9(1):47-56.
122. Joneson T, Fulton JA, Volle DJ, Chaika OV, Bar-Sagi D, Lewis RE. Kinase suppressor of Ras inhibits the activation of extracellular ligand-regulated (ERK) mitogen-activated protein (MAP) kinase by growth factors, activated Ras, and Ras effectors. J Biol Chem. 1998;273(13):7743-8.
123. Lopez JP, Turner JR, Philipson LH. Glucose-induced ERM protein activation and translocation regulates insulin secretion. Am J Physiol Endocrinol Metab. 2010;299(5):E772-85.
124. Inoue M, Chang L, Hwang J, Chiang SH, Saltiel AR. The exocyst complex is required for targeting of Glut4 to the plasma membrane by insulin. Nature. 2003;422(6932):629-33.
125. Xiao L, Zheng K, Lv X, Hou J, Xu L, Zhao Y, et al. Exo70 is an independent prognostic factor in colon cancer. Sci Rep. 2017;7(1):5039.
126. Lu H, Liu J, Liu S, Zeng J, Ding D, Carstens RP, et al. Exo70 isoform switching upon epithelial-mesenchymal transition mediates cancer cell invasion. Dev Cell. 2013;27(5):560-73.
127. Yousefi M, Li L, Lengner CJ. Hierarchy and Plasticity in the Intestinal Stem Cell Compartment. Trends Cell Biol. 2017;27(10):753-64.
128. Papa S, Choy PM, Bubici C. The ERK and JNK pathways in the regulation of metabolic reprogramming. Oncogene. 2019;38(13):2223-40.
129. Sethi JK, Vidal-Puig A. Wnt signalling and the control of cellular metabolism. The Biochemical journal. 2010;427(1):1-17.
130. Sethi JK, Vidal-Puig AJ. Wnt signalling at the crossroads of nutritional regulation. The Biochemical journal. 2008;416(2):e11-3.
131. Biteau B, Jasper H. EGF signaling regulates the proliferation of intestinal stem cells in Drosophila. Development. 2011;138(6):1045-55.
132. Degirmenci B, Valenta T, Dimitrieva S, Hausmann G, Basler K. GLI1-expressing mesenchymal cells form the essential Wnt-secreting niche for colon stem cells. Nature. 2018;558(7710):449-53.
133. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, et al. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature. 2011;469(7330):415-8.
134. Valenta T, Degirmenci B, Moor AE, Herr P, Zimmerli D, Moor MB, et al. Wnt Ligands Secreted by Subepithelial Mesenchymal Cells Are Essential for the Survival of Intestinal Stem Cells and Gut Homeostasis. Cell reports. 2016;15(5):911-8.
135. Zwick RK, Ohlstein B, Klein OD. Intestinal renewal across the animal kingdom: comparing stem cell activity in mouse and Drosophila. Am J Physiol Gastrointest Liver Physiol. 2019;316(3):G313-G22.
136. Anagnostou SH, Shepherd PR. Glucose induces an autocrine activation of the Wnt/beta-catenin pathway in macrophage cell lines. The Biochemical journal. 2008;416(2):211-8.

137. Walker JP, Fujimura M, Sakamoto T, Greeley GH, Jr., Townsend CM, Jr., Thompson JC. Importance of the ileum in neurotensin released by fat. Surgery. 1985;98(2):224-9.
138. Barber DL, Cacace AM, Raucci DT, Ganz MB. Fatty acids stereospecifically stimulate neurotensin release and increase [Ca2+]i in enteric endocrine cells. Am J Physiol. 1991;261(3 Pt 1):G497-503.
139. Li J, Song J, Li X, Rock SB, Sinner HF, Weiss HL, et al. FFAR4 Is Involved in Regulation of Neurotensin Release From Neuroendocrine Cells and Male C57BL/6 Mice. Endocrinology. 2018;159(8):2939-52.
140. Kim JT, Weiss HL, Evers BM. Diverse expression patterns and tumorigenic role of neurotensin signaling components in colorectal cancer cells. Int J Oncol. 2017;50(6):2200-6.
141. Zhao D, Pothoulakis C. Effects of NT on gastrointestinal motility and secretion, and role in intestinal inflammation. Peptides. 2006;27(10):2434-44.
142. Ehlers RA, Zhang Y, Hellmich MR, Evers BM. Neurotensin-mediated activation of MAPK pathways and AP-1 binding in the human pancreatic cancer cell line, MIA PaCa-2. Biochem Biophys Res Commun. 2000;269(3):704-8.
143. Muller KM, Tveteraas IH, Aasrum M, Odegard J, Dawood M, Dajani O, et al. Role of protein kinase C and epidermal growth factor receptor signalling in growth stimulation by neurotensin in colon carcinoma cells. BMC Cancer. 2011;11:421.
144. Ehlers RA, 2nd, Bonnor RM, Wang X, Hellmich MR, Evers BM. Signal transduction mechanisms in neurotensin-mediated cellular regulation. Surgery. 1998;124(2):239-46; discussion 46-7.
145. Bakirtzi K, Hatziapostolou M, Karagiannides I, Polytarchou C, Jaeger S, Iliopoulos D, et al. Neurotensin signaling activates microRNAs-21 and -155 and Akt, promotes tumor growth in mice, and is increased in human colon tumors. Gastroenterology. 2011;141(5):1749-61 e1.
146. Assimakopoulos SF, Alexandris IH, Scopa CD, Mylonas PG, Thomopoulos KC, Georgiou CD, et al. Effect of bombesin and neurotensin on gut barrier function in partially hepatectomized rats. World J Gastroenterol. 2005;11(43):6757-64.
147. Kim JT, Liu C, Zaytseva YY, Weiss HL, Townsend CM, Jr., Evers BM. Neurotensin, a novel target of Wnt/beta-catenin pathway, promotes growth of neuroendocrine tumor cells. Int J Cancer. 2015;136(6):1475-81.
148. Gerdes J, Schwab U, Lemke H, Stein H. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer. 1983;31(1):13-20.
149. Evers BM, Rajaraman S, Chung DH, Townsend CM, Jr., Wang X, Graves K, et al. Differential expression of the neurotensin gene in the developing rat and human gastrointestinal tract. The American journal of physiology. 1993;265(3 Pt 1):G482-90.
150. Cadigan KM, Waterman ML. TCF/LEFs and Wnt signaling in the nucleus. Cold Spring Harb Perspect Biol. 2012;4(11).
151. Gully D, Canton M, Boigegrain R, Jeanjean F, Molimard JC, Poncelet M, et al. Biochemical and pharmacological profile of a potent and selective nonpeptide antagonist of the neurotensin receptor. Proc Natl Acad Sci U S A. 1993;90(1):65-9.

152. Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, et al. Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation. 2003;71(1):28-41.
153. van der Flier LG, van Gijn ME, Hatzis P, Kujala P, Haegebarth A, Stange DE, et al. Transcription factor achaete scute-like 2 controls intestinal stem cell fate. Cell. 2009;136(5):903-12.
154. Spit M, Koo BK, Maurice MM. Tales from the crypt: intestinal niche signals in tissue renewal, plasticity and cancer. Open Biol. 2018;8(9).
155. Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc Natl Acad Sci U S A. 2008;105(7):2415-20.
156. Jiang H, Edgar BA. Intestinal stem cells in the adult Drosophila midgut. Exp Cell Res. 2011;317(19):2780-8.
157. Loza-Coll MA, Southall TD, Sandall SL, Brand AH, Jones DL. Regulation of Drosophila intestinal stem cell maintenance and differentiation by the transcription factor Escargot. EMBO J. 2014;33(24):2983-96.
158. Song W, Cheng D, Hong S, Sappe B, Hu Y, Wei N, et al. Midgut-Derived Activin Regulates Glucagon-like Action in the Fat Body and Glycemic Control. Cell Metab. 2017;25(2):386-99.
159. Zhan T, Ambrosi G, Wandmacher AM, Rauscher B, Betge J, Rindtorff N, et al. MEK inhibitors activate Wnt signalling and induce stem cell plasticity in colorectal cancer. Nat Commun. 2019;10(1):2197.
160. Hsieh JS, Lin SR, Chang MY, Chen FM, Lu CY, Huang TJ, et al. APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. Am Surg. 2005;71(4):336-43.
161. Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science. 1990;247(4940):322-4.
162. Luo F, Poulogiannis G, Ye H, Hamoudi R, Arends MJ. Synergism between K-rasVal12 and mutant Apc accelerates murine large intestinal tumourigenesis. Oncol Rep. 2011;26(1):125-33.
163. Rajendran V, Jain MV. In Vitro Tumorigenic Assay: Colony Forming Assay for Cancer Stem Cells. Methods Mol Biol. 2018;1692:89-95.
164. Cargnello M, Tcherkezian J, Roux PP. The expanding role of mTOR in cancer cell growth and proliferation. Mutagenesis. 2015;30(2):169-76.
165. Garrison AP, Dekaney CM, von Allmen DC, Lund PK, Henning SJ, Helmrath MA. Early but not late administration of glucagon-like peptide-2 following ileo-cecal resection augments putative intestinal stem cell expansion. Am J Physiol Gastrointest Liver Physiol. 2009;296(3):G643-50.
166. Norona J, Apostolova P, Schmidt D, Ihlemann R, Reischmann N, Taylor G, et al. Glucagon-like peptide 2 for intestinal stem cell and Paneth cell repair during graft-versus-host disease in mice and humans. Blood. 2020;136(12):1442-55.
167. Booth C, Booth D, Williamson S, Demchyshyn LL, Potten CS. Teduglutide ([Gly2]GLP-2) protects small intestinal stem cells from radiation damage. Cell Prolif. 2004;37(6):385-400.
168. Feng Y, Demehri FR, Xiao W, Tsai YH, Jones JC, Brindley CD, et al. Interdependency of EGF and GLP-2 Signaling in Attenuating Mucosal Atrophy in a Mouse Model of Parenteral Nutrition. Cell Mol Gastroenterol Hepatol. 2017;3(3):447-68.

169. Svendsen B, Pedersen J, Albrechtsen NJ, Hartmann B, Torang S, Rehfeld JF, et al. An analysis of cosecretion and coexpression of gut hormones from male rat proximal and distal small intestine. Endocrinology. 2015;156(3):847-57.
170. Akcan A, Muhtaroglu S, Akgun H, Akyildiz H, Kucuk C, Sozuer E, et al. Ameliorative effects of bombesin and neurotensin on trinitrobenzene sulphonic acid-induced colitis, oxidative damage and apoptosis in rats. World J Gastroenterol. 2008;14(8):1222-30.
171. Wieck MM, Schlieve CR, Thornton ME, Fowler KL, Isani M, Grant CN, et al. Prolonged Absence of Mechanoluminal Stimulation in Human Intestine Alters the Transcriptome and Intestinal Stem Cell Niche. Cell Mol Gastroenterol Hepatol. 2017;3(3):367-88 e1.
172. He TC, Chan TA, Vogelstein B, Kinzler KW. PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell. 1999;99(3):335-45.
173. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem. 1996;271(34):20608-16.
174. Daksis JI, Lu RY, Facchini LM, Marhin WW, Penn LJ. Myc induces cyclin D1 expression in the absence of de novo protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene. 1994;9(12):3635-45.
175. Zeiser R. Trametinib. Recent Results Cancer Res. 2014;201:241-8.
176. Greco V, Lauro G, Fabbrini A, Torsoli A. Histochemistry of the colonic epithelial mucins in normal subjects and in patients with ulcerative colitis. A qualitative and histophotometric investigation. Gut. 1967;8(5):491-6.
177. Ruijtenberg S, van den Heuvel S. Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. Cell Cycle. 2016;15(2):196-212.
178. Hulit J, Wang C, Li Z, Albanese C, Rao M, Di Vizio D, et al. Cyclin D1 genetic heterozygosity regulates colonic epithelial cell differentiation and tumor number in ApcMin mice. Mol Cell Biol. 2004;24(17):7598-611.
179. Li C, Zhou Y, Rychahou P, Weiss HL, Lee EY, Perry CL, et al. SIRT2 Contributes to the Regulation of Intestinal Cell Proliferation and Differentiation. Cell Mol Gastroenterol Hepatol. 2020;10(1):43-57.
180. Chopra DP, Dombkowski AA, Stemmer PM, Parker GC. Intestinal epithelial cells in vitro. Stem Cells Dev. 2010;19(1):131-42.
181. Carraway RE, Mitra SP, Evers BM, Townsend CM, Jr. BON cells display the intestinal pattern of neurotensin/neuromedin N precursor processing. Regul Pept. 1994;53(1):17-29.
182. Tateishi K, Funakoshi A, Kitayama N, Matsuoka Y. Secretion of neurotensin from a human pancreatic islet cell carcinoma cell line (QGP-1N). Regul Pept. 1993;49(2):119-23.
183. Evers BM, Izukura M, Rajaraman S, Parekh D, Thakore K, Yoshinaga K, et al. Effect of aging on neurotensin-stimulated growth of rat small intestine. Am J Physiol. 1994;267(2 Pt 1):G180-6.
184. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van den Brink S, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. Gastroenterology. 2011;141(5):1762-72.

VITA

Stephanie A. Rock
Doctoral Candidate

Education:

2017-2021	Ph.D., Department of Toxicology and Cancer Biology, College of Medicine, University of Kentucky
2013-2016	M.S., Department of Biology, College of Arts and Sciences, University of Kentucky
2007-2011	B.S., Department of Biology, Western Kentucky University

Professional Experience:

2019-2020	Adjunct Instructor, BIO 150: Principles of Biology I, Department of Natural Sciences, Bluegrass Community and Technical College
2016-2017	Research Analyst, Markey Cancer Center, University of Kentucky
2015-2016	Science Writer, AA'S Inc.
2013-2015	Teaching Assistant, Department of Biology, University of Kentucky
2011	National Park Ranger, National Park Service, Mammoth Cave National Park

Academic Awards and Honors:

2021	Poster of Distinction, Digestive Disease Week
2020	Women in Medicine and Science Rising Star Award Nominee
2019	American Gastroenterological Association Early Career Scientist Award
2019	American Gastroenterological Association Student Abstract Award
2019	Markey Cancer Center Trainee Travel Award, University of Kentucky, College of Medicine
2019	Office of Biomedical Education Travel Award, University of Kentucky, College of Medicine
2018	College of Medicine Fellowship for Excellence in Graduate Research, University of Kentucky
2017	STEM Woman of the Week, I Am a Woman in STEM Organization, University of Kentucky
2015	Travel and Conference Fellowship Award, Okinawa Institute of Science and Technology
2013-2014	Graduate School Travel Award, Department of Biology University of Kentucky
2013-2014	Gertrude Flora Ribble Travel Award, University of Kentucky
2011	Honor's Program Graduate, Western Kentucky University
2011	President's Scholar, Western Kentucky University
2011	Magna Cum Laude, Western Kentucky University
2007-2011	Dean's List, Western Kentucky University

Grant Funding:

2018-2020 T32 DK007778, National Institute of Health, "Pharmacology and Nutritional Sciences: Multidisciplinary Approaches for Metabolic Disease" Department of Pharmacology and Nutritional Sciences, University of Kentucky

Publications:

Stephanie Rock, Heidi L. Weiss, Chi Wang, Jianhang Jia, Tianyan Gao, B. Mark Evers. Neurotensin facilitates intestinal regeneration and colorectal cancer stem cell function. (Manuscript in preparation).

Jing Li, Xian Li, Jun Song, Baoxiang Yan, **Stephanie Rock**, Jianhang Jia, Jinpeng Liu, Chi Wang, Todd Weiss, Heidi L. Weiss, Tianyan Gao, Ashfaqul Alam, B. Mark Evers. Absence of neurotensin attenuates intestinal dysbiosis and inflammation by maintaining Mmp7/α-defensin axis in diet-induced obese mice. FASEB Journal. 2020 Jun;34(6):8596-8610. PMID: 32359121

Stephanie Rock, Xian Li, Jun Song, Courtney M. Townsend, Jr., Heidi L. Weiss, Piotr Rychahou, Tianyan Gao, Jing Li, and B. Mark Evers. Kinase suppressor of Ras 1 and Exo70 promote fatty acid- stimulated neurotensin secretion through ERK1/2 signaling. PLOS ONE. 2019 Mar 27;14(3):e0211134. PMID: 30917119

Jing Li, Jun Song, Xian Li, **Stephanie Rock**, Heather F. Sinner, Heidi L. Weiss, Courtney M. Townsend, Jr., Tianyan Gao, B. Mark Evers. FFAR4 is involved in regulation of neurotensin release from neuroendocrine cells and male C57BL/6 mice. Endocrinology. 2018 Aug 1;159(8):2939-2952. PMID: 29796668

Paige E. Herman, Angelos Papatheodorou, **Stephanie Bryant**, Courtney K. M. Waterbury, Joseph R. Herdy, Anthony A. Arcese, Joseph D. Buxbaum, Jeramiah J. Smith, Jennifer R. Morgan & Ona Bloom. Highly conserved molecular pathways, including Wnt signaling, promote functional recovery from spinal cord injury in lampreys. Sci Rep. 2018 Jan 15;8(1):742. PMID: 29335507

Stephanie Bryant, Joseph Herdy, Chris Amemiya, Jeramiah J. Smith. Characterization of Somatically- Eliminated Genes During Development of the Sea Lamprey (*Petromyzon marinus*) Mol Biol Evol. (IF: 14.8) 2016 Sep;33(9):2337-44 PMID: 27288344

Abstracts:

Stephanie Rock, Yuanyuan Wu, Xiaopeng Xiong, Jing Li, Heidi L. Weiss, Chi Wang, Tianyan Gao, B. Mark Evers. Neurotensin promotes stem cell function in the small intestine and in intestinal tumor organoids. Digestive Disease Meeting (Virtual), 2021

Stephanie Rock, Baoxiang Yan, Xiaopeng Xiong, Tianyan Gao, B. Mark Evers. Neurotensin promotes WNT/beta-catenin signaling and colorectal cancer stem cell function. American Association of Cancer Research, Virtual Annual Meeting II. 2020

Stephanie Rock, Jun Song, Baoxiang Yan, Xiaopeng Xiong, Jing Li, Tianyan Gao, B. Mark Evers. Defining the Role of Neurotensin on Intestinal Wnt Signaling. Department of Toxicology and Cancer Biology Annual Retreat, University of Kentucky. 2019

Stephanie Rock, Jun Song, Baoxiang Yan, Daheng He, Xiaopeng Xiong, Jing Li, Tianyan Gao, B. Mark Evers. Neurotensin Differentially Regulates WNT Signaling in Normal Intestinal Cells and Tumors. Wnt Signaling Networks in Development, Disease and Regeneration Gordon Research Conference, Mt. Snow Vermont. 2019

Stephanie Rock, Xian Li, Jun Song, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. KSR1 and Exo70 interact with ERK1/2 to promote Neurotensin secretion in human endocrine cells. American Gastroenterological Association, Digestive Disease Week, San Diego, CA. 2019

Jing Li, Jun Song, Xian Li, **Stephanie Rock**, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. Deficiency of NT-NTR1 signaling prevents the disruption of bile acid homeostasis in mice fed high-fat diet. American Gastroenterological Association, Digestive Disease Week, San Diego, CA. 2019

Xian Li, Jun Song, **Stephanie Rock**, Heidi Weiss, Courtney Townsend Jr., Jing Li, B. Mark Evers. Neurotensin (NT)-α-defensins axis mediates the influence of high fat diet (HFD) on gut microbiota. American Gastroenterological Association, Digestive Disease Week, San Diego, CA. 2019

Stephanie Rock, Xian Li, Jun Song, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. KSR1 and Exo70 interact with ERK1/2 to promote Neurotensin secretion in human endocrine cells. Markey Cancer Research Day, University of Kentucky. 2019

Stephanie Bryant, Jun Song, Jing Li, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. KSR1 positively regulates ERK1/2 signaling and Neurotensin secretion in endocrine cells. American Gastroenterological Association, Digestive Disease Week, Washington, D.C. 2018

Jing Li, Jun Song, **Stephanie A. Bryant**, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. FFAR4 agonists stimulate Neurotensin secretion in vitro and in vivo. American Gastroenterological Association, Digestive Disease Week, Washington, D.C. 2018

Jing Li, **Stephanie A. Bryant**, Jun Song, Heidi L. Weiss, Courtney Townsend Jr., B. Mark Evers. FFAR1 but not FFAR4 regulates fatty acid-stimulated neurotensin secretion through ERK and AMPK signaling. American Gastroenterological Association, Digestive Disease Week, Chicago, IL 2017

Stephanie Bryant, Jun Song, Jing Li, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. KSR1 positively regulates ERK1/2 signaling and Neurotensin secretion in endocrine cells. Markey Cancer Research Day, University of Kentucky. 2018

Stephanie Bryant, Jun Son, Jing Li, Courtney Townsend, B. Mark Evers. KSR1/2 regulates ERK activation and Neurotensin secretion. American Gastroenterological Association, Digestive Disease Week, Chicago, IL. 2017

Stephanie Bryant, Vladimir Timoshevskiy, Jeramiah J. Smith. Characterization of Somatically- Eliminated Genes in the Sea Lamprey. Plant and Animal Genome XXIII Conference, San Diego, CA. 2015

Stephanie Bryant, Jeramiah J. Smith. Genomics and Regeneration in Petromyzon marinus. Vertebrate Development and Regeneration Interest Group, University of Kentucky. 2014

Stephanie Bryant, Jeramiah J. Smith. Analysis of Pluripotency Genes in Spinal Cord Regeneration of the Sea Lamprey (Petromyzon marinus). Plant and Animal Genome XXII Conference, San Diego, CA. 2014

Stephanie Bryant, William G. Hess, Margaret M. Crowder. Effects of Climate Change on Marine Chemistry and Biodiversity. Campus Community Partnership for Sustainability Conference, Bowling Green, KY. 2009

Oral Presentations:

Stephanie Rock, Jun Song, Baoxiang Yan, Xiaopeng Xiong, Jing Li, Tianyan Gao, B. Mark Evers. Defining the Role of Neurotensin on Intestinal Stem Cell Function. Markey Cancer Center Career Enhancement Club, University of Kentucky, 2020

Stephanie Rock, Jun Song, Baoxiang Yan, Xiaopeng Xiong, Jing Li, Tianyan Gao, B. Mark Evers. Defining the Role of Neurotensin on Intestinal Stem Cell Function. Department of Toxicology and Cancer Biology Student and Staff Seminar, University of Kentucky, 2020

Stephanie Rock, Jun Song, Baoxiang Yan, Xiaopeng Xiong, Jing Li, Tianyan Gao, B. Mark Evers. Defining the Role of Neurotensin on Intestinal Wnt Signaling. Department of Toxicology and Cancer Biology Annual Retreat, University of Kentucky, 2019

Stephanie Rock, Xian Li, Jun Song, Heidi Weiss, Courtney Townsend Jr., B. Mark Evers. KSR1 and Exo70 interact with ERK1/2 to promote Neurotensin secretion in human endocrine cells. Digestive Disease Week, San Diego, CA. 2019

Xian Li, Jun Song, **Stephanie Rock**, Heidi L. Weiss, Tianyan Gao, Jing Li*, B. Mark Evers. Neurotensin (NT)-α-defensins axis mediates the influence of high fat diet (HFD) on gut microbiota. Digestive Disease Week, San Diego, CA. 2019

Jing Li, **Stephanie Bryant**, Jun Song, Heidi L. Weiss, Courtney Townsend, B. Mark Evers. FFAR1 but not FFAR4 regulates fatty acid-stimulated neurotensin secretion through ERK and AMPK signaling. Digestive Disease Week, Chicago, IL 2017

Stephanie Bryant, Joseph R. Herdy, Jeramiah J. Smith. Molecular Correlates of Programmed DNA Elimination. Presentation, Plant and Animal Genome XXIII Conference, San Diego, CA. 2015

Sara K. Wigginton, Joseph N. Edwards*, Nikki H. Roff, Heidi Steinhaus, Steven Milesko, Joseph Webb, **Stephanie Bryant**, Jenna Binion, Eric Smith, Marie Tinsley, Mary Penick, Scott Grubbs, Albert Meier. A Study of the Aquatic Macrophyte Podostemum ceratophyllum in the Green River, Kentucky. Kentucky Academy of Science Meeting, Bowling Green, KY. 2010