Functional genetics on congenital intestinal motility disorders

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Chapter 1.

General Introduction

1. The gastrointestinal tract

The gastrointestinal (GI) tract is composed of the oral cavity, pharynx, esophagus, stomach, small intestine, large intestine, rectum, anal canal, and anus (Figure 1). It is a complex organ that performs a range of functions that are crucial for life, including digestion, absorption, secretion, propulsive movements (peristalsis), segmentation, mixing, excretion and immune response (Luongo et al., 2009; Cheng et al., 2010). To regulate these functions, there is an active coordination/communication between the Enteric nervous system (ENS), smooth muscle and Interstitial cells of Cajal (ICC), as they are essential components of the GI neuromusculature (Goldstein et al., 2016). Dysfunction of any of these components is known to affect intestinal function, leading to the development of intestinal motility disorders. In the next subsections a detailed description of each component of the GI neuromusculature will be given, as well as of how they interact with each other.

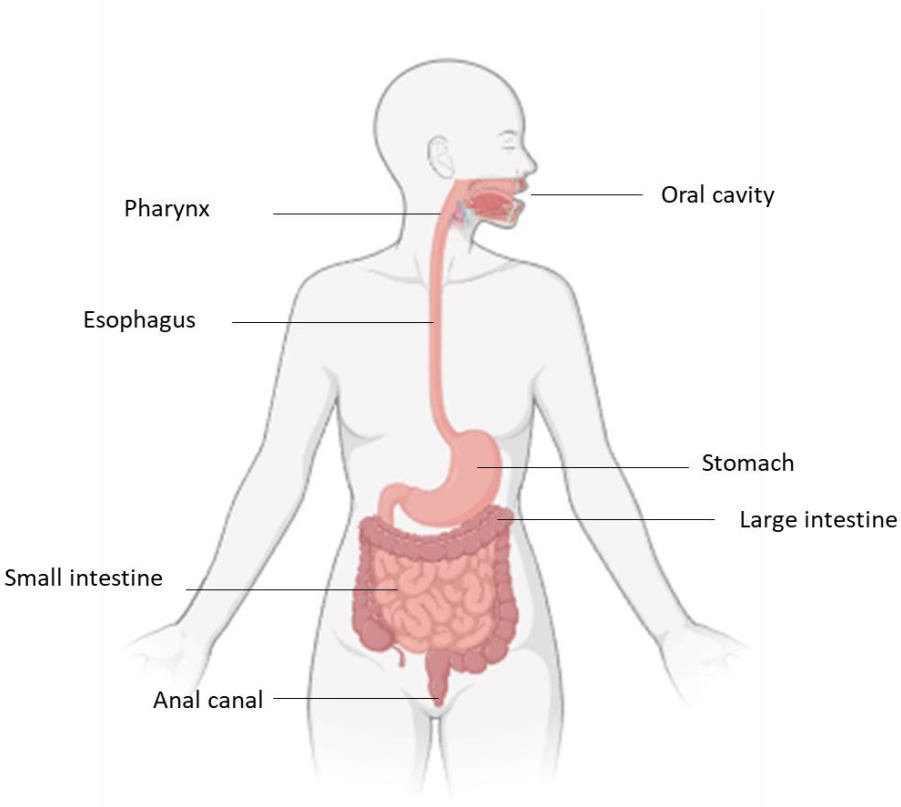

Figure 1. Human gastrointestinal (GI) tract. The GI tract consists of major organs including oral cavity, pharynx, esophagus, stomach, small intestine, large intestine and anal canal. Food and liquids travel through these organs when they are swallowed, digested, absorbed, and leave the body as fecal matter.

1a. Enteric Nervous System

The ENS (Figure 2) consists of interconnected ganglia, composed by enteric neurons and glial cells. These ganglia are located in two concentric rings throughout the GI tract. The outer ring is the myenteric (Auerbach's) plexus, located between the circular and longitudinal muscle layers, and the inner ring is the submucosal (Meissner's) plexus. In mammals, the ENS is formed by precursor cells derived primarily from vagal neural crest cells (NCCs), which arise from the neural tube. Studies in murine embryos showed that vagal NCCs invade the foregut at embryonic day 9 (E9) and are subsequently called enteric neural crest cells (ENCCs). These cells migrate in a rostro-caudal direction to colonize the entire length of the developing gut (Figure 3). At E10.5-11.5, extrinsic innervation extends from the ventral portion of the neural tube into the gut. Invasion of the gut by extrinsic nerve fibers is accompanied by Schwann cell precursors (SCPs), a multipotent pool of cells that give rise to various cell types such as melanocytes, neuroendocrine cells, Schwann cells and peripheral enteric neurons (Kastriti et al., 2017). SCPs migrate along these nerves and settle within emerging extrinsic motor axons and sensory nerves (Kameneva et al., 2021). At E13.5, the completion of the rostro-caudal migration of ENCCs occurs and coincides with the entrance of sacral NCC precursors to the hindgut, which migrate into a caudo-rostral direction (Hutchins et al., 2018). Both vagal and sacral precursors further undergo neurogenesis and gliogenesis, and their differentiation into mature neurons and glia occurs well after birth into adulthood (Goldstein et al., 2013).

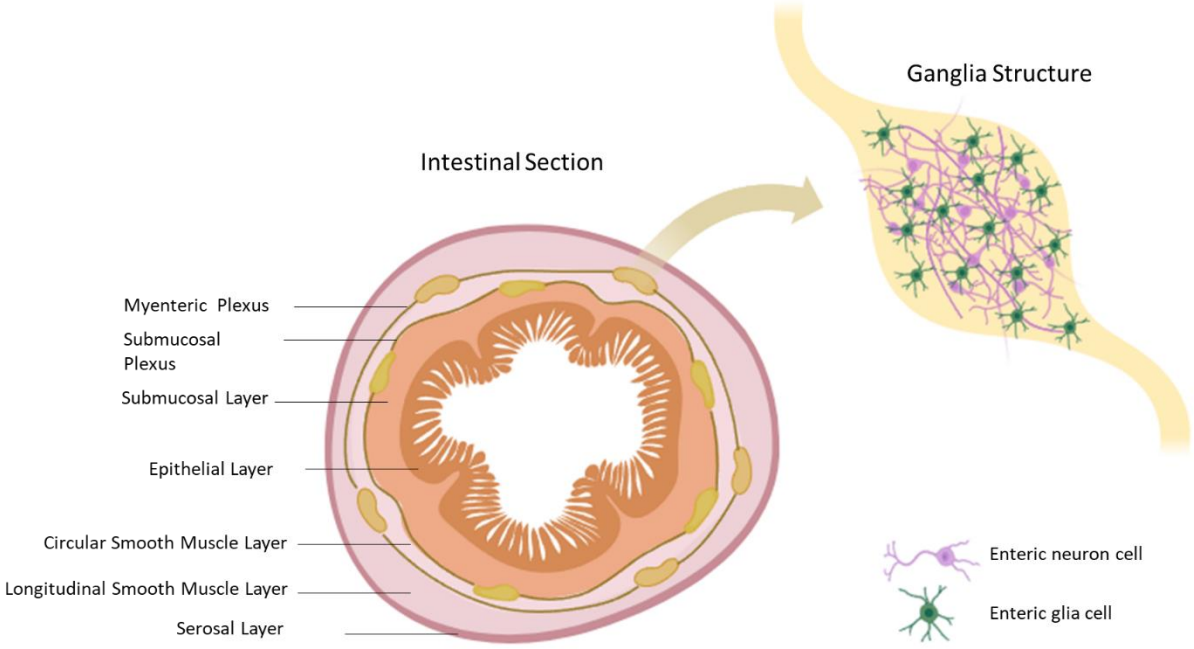

Figure 2. Schematic transverse cross section of human intestine. The structure of intestinal wall consists of several layers (from inner to outer); epithelial layer, submucosal layer, circular smooth muscle layer, longitudinal smooth muscle layer, and serosal layer. The enteric nervous system is presented as an interconnected ganglia which forms submucosal plexus (Meissner's plexus) and myenteric plexus (Auerbach's plexus). The ganglia structure consists of enteric neuron cells and glial cells.

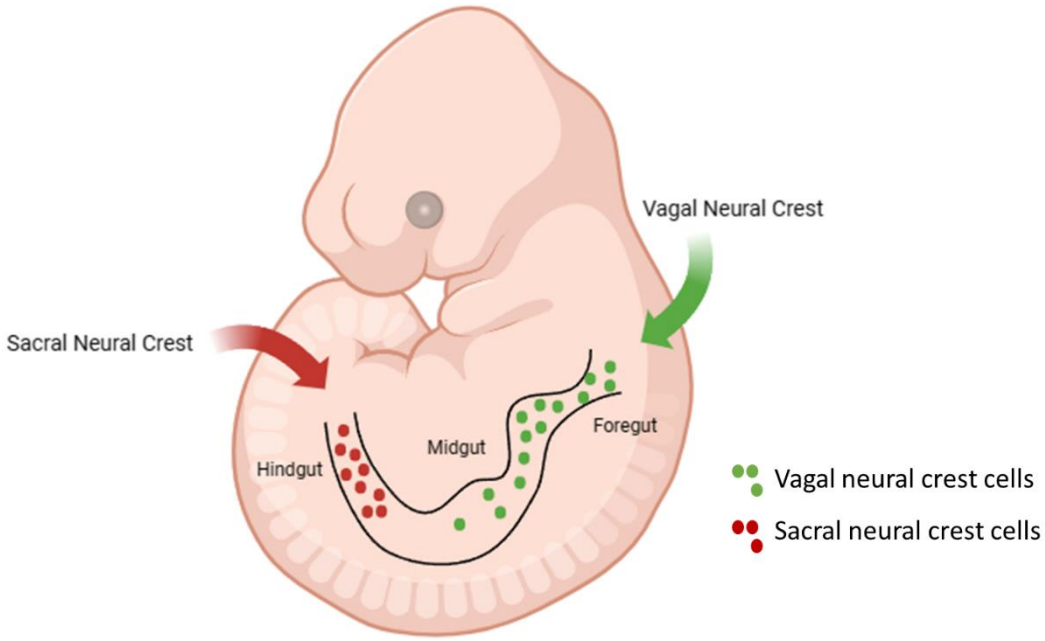

Figure 3. Schematic of enteric nervous system development in a mouse embryo. Migration of vagal neural crest cells into the embryonic gut tube and contribution of sacral neural crest cells in the hindgut.

To form a structurally and functionally mature ENS, ENCCs perform several key processes including cell proliferation to generate sufficient precursors to populate the entire gut, cell survival, directed migration, precise patterning into concentric plexuses and ganglion formation, and accurate differentiation into neurons and glia (including their subtypes). Several signaling pathways, transcription factors, and molecules have been identified to regulate the ENS development. One of the most important pathways is the Rearranged during Transfection (RET) signaling pathway which has multiple effects on key developmental processes. RET is a tyrosine kinase transmembrane receptor expressed by ENCCs, that is activated by the glial-derived neurotrophic factor (GDNF), which is expressed by the gut mesenchyme (Durbec et al., 1996). GDNF binds to a receptor complex comprised

of RET and its co-receptor, GDNF family receptor alpha 1 (GFRA1), leading to phosphorylation of RET and activation of several downstream pathways, including the RAS/mitogen-activated protein kinase (MAPK), the Jun-associated N- terminal kinase (JNK), and the phosphatidylinositol-3 kinase (PI3K) pathways (Mograbi et al., 2001). The PI3K signaling is especially crucial, as it mediates GDNF-induced ENCC proliferation (Liu et al., 2011), survival (Mograbi et al., 2001), and migration (Guo et al., 2006). RET expression in pre-enteric ENCCs is also induced by Retinoic Acid (RA) (Shepherd et al., 2004; Simkin et al., 2013), which maintains PI3K activity in migrating ENCCs (Shepherd et al., 2004). A study in murine embryos also showed that RA signaling enhances proliferation of subsets of ENS precursors in a time-dependent fashion, and increases neuronal differentiation (Sato and Heuckeroth, 2008). Two negative regulators of the GDNF-RET signaling pathway include the Sprouty RTK Signaling Antagonist 2 (SPRY-2) (Taketomi et al., 2005) and Kinesin Family Member 26A (KIF26A) (Zhou et al., 2009). Loss of these genes lead to unregulated RET signaling which resulted in decreased survival and impaired proliferation of enteric neuronal progenitor cells (Taketomi et al., 2005; Zhou et al., 2009). Endothelin receptor B (EDNRB) signaling is another important pathway in ENS development. EDNRB is a G protein-coupled receptor expressed by ENCCs (Druckenbrod et al., 2008). Its ligand, endothelin-3 (ET3) is a 21 amino acids peptide expressed in the gut mesenchyme. EDNRB signaling promotes proliferation of ENCCs (Barlow et al., 2003; Nagy and Goldstein, 2006) and inhibits their differentiation into neurons (Nagy and Goldstein, 2006), thus maintaining ENCCs in an uncommitted and proliferative state. Several transcription factors and molecules are also known to contribute to ENS development. SOX10, an HMG box-containing transcription factor (Zhu et al., 2004) and paired-like homeobox 2b gene (PHOX2B) (Pattyn et al., 1999) regulate RET expression. In addition, Zinc finger E-box binding homeobox 2 (ZEB2) acts together with SOX10 to regulate EDNRB expression (Stanchina et al., 2010; Watanabe et al., 2017). The mammalian achaete-scute homolog 1 (MASH1; ASCL1) is a basic helix-loop-helix DNA binding protein expressed by ENCCs upon arrival in the foregut (Guillemot and Joyner, 1993). The heart and neural crest derivative expressed 2 (HAND2), another basic helix-loop-helix transcription factor, is expressed by ENCCs once they colonize the intestine. Its expression is required for later stages of neurogenesis (D'Autréaux et al., 2007) and neurotransmitters specification (Lei and Howard, 2011). Both ASCL1 and HAND2 play a role in enteric neuronal subtype specification (D'Autréaux et al., 2007; Okamura and Saga, 2008; Roach et al., 2013). Moreover, other molecules such as the bone morphogenetic proteins (BMPs), particularly BMP2 and BMP4, have pleiotropic effects during ENS development. BMPs appear to have an important role in determining the ratio of neurons to glia in the gut (Chalazonitis et al., 2004, 2008, 2011), and they promote ganglionogenesis, the aggregation of ganglion cells to form clusters, by a mechanism that involves modification of the neural cell adhesion molecule (NCAM) (Fu et al., 2006; Li et al., 2011).

The gut mesenchyme also produces many extracellular matrix (ECM) proteins that have been implicated in ENS development. ECM molecules such as glycoproteins (e.g., laminins, fibronectin, tenascins), collagens, and proteoglycans, form a complex and dynamic

molecular scaffold that provides both, a physical surface for ENCC migration and important signals that regulate multiple aspects of ENS development (Nagy et al., 2016, 2018; Nishida et al., 2018). Precisely patterned ECMs modulate ENCC polarity, migration, proliferation, differentiation, aggregation into ganglia, and patterning into plexuses. Other important molecules, Netrins, known for their role in axon guidance, are secreted by the intestinal epithelium. Netrins promote the centripetal migration of neurons from the myenteric to the submucosal region (Seaman et al., 2001; Jiang et al., 2003). Hedgehog (HH) proteins, including sonic hedgehog (SHH) and Indian hedgehog (IHH) are diffusible morphogens produced by the gut epithelium from the earliest stages of intestinal organogenesis. SHH is essential for regulating proliferation and differentiation of the mesenchyme and for establishing concentric patterning along its radial axis (Sukegawa et al., 2000). HH signaling directly regulates BMP4 expression, which inhibits smooth muscle differentiation in the mesenchyme underlying the gut epithelium, regulates ENCC migration, and helps define the radial position of the forming submucosal plexus (Sukegawa et al., 2000; Chalazonitis et al., 2008). Those aforementioned signaling pathways, transcription factors, and molecules play an important role not only during development, but also to maintain a functional ENS during adulthood.

The functional mature ENS communicates with almost all intestinal cells, including muscle, epithelial cells, ICCs, platelet-derived growth factor receptor (PDGFR)α+ cells, blood vessels, and immune cells to facilitate intestinal motility (Schneider et al., 2019). In addition, enteric neurons release neurotransmitters from varicosities along neurites to regulate smooth muscle and ICC activity to support this function (Kurahashi et al., 2011; Sanders et al., 2012, 2016a; Klein et al., 2013).

1b. Smooth muscle

The GI tract is enveloped by concentric and orthogonally aligned layers of smooth muscle, normally referred to as visceral smooth muscle. Visceral smooth muscle cells (SMCs) originate from the mesoderm (McLin et al., 2009), but their development remains poorly characterized. SHH and BMP4 are two morphogens involved in SMCs development and differentiation. In mammals, Shh is expressed in the gut inducing differentiation of mesodermal precursors into visceral smooth muscle (Apelqvist et al., 1997; Ramalho-santos et al., 2000). Furthermore, Hedgehog signaling acts through Bmp to delineate the position of the inner muscle layer (circular smooth muscle layer), whereas localized Bmp inhibition is critical for formation of the outer layer (longitudinal smooth muscle layer). These two muscle layers form at different developmental stages, and during this process muscle cells are exposed to unique mechanical stimuli that direct their alignments. Differential growth within the early gut tube generates residual strain that orients the first layer circumferentially. Subsequently, spontaneous contractions of this layer align with the second layer longitudinally (Huycke et al., 2019). Once formed, the intestinal smooth

muscle provides contractile force for intestinal motility, by establishing electrical and mechanical junctions between cells that facilitate coordination of contraction (Somlyo and Somlyo, 2003; Eddinger and Meer, 2007).

In humans, the enteric gamma actin (ACTG2) is the main actin isoform expressed by visceral SMCs, being detected for the first time at 9 weeks of gestation (Halim et al., 2016). ACTG2, together with other proteins such as, Myosin heavy chain 11 (MYH11) (Dong et al., 2019), Myosin light chain kinase (MYLK) (Halim et al., 2017a), and Myosin light chain 9 (MYL9) (Moreno et al., 2018), are essential components of the contractile apparatus of visceral muscle cells. Other structural proteins such as Leiomodin 1 (LMOD1), a nucleator protein required for the formation of actin filaments in SMCs (Halim et al., 2017b), and Filamin-A (FLNA), an actin-crosslinking protein that anchors membrane proteins to the cytoskeleton (Robertson, 2005), help maintaining the architectural configuration of the smooth muscle layers. Together, these proteins are needed for actin polymerization, and to regulate actin–myosin and actin–actin interactions (Viti et al., 2023), required for smooth muscle contractility. To generate coordinated smooth muscle contraction, excitatory motor neurons from the myenteric plexus project their axons predominantly orally. In contrast, inhibitory motor neurons project axons distally, leading to smooth muscle relaxation. Simultaneous activation of excitatory and inhibitory motor neurons in a bowel region causes proximal bowel contraction and distal relaxation, a pattern called peristalsis, which is able to mix luminal contents or move undigested food towards the distal bowel for eventual elimination (Sanders et al., 2012).

1c. Interstitial cells of Cajal

ICCs represent the pacemakers of the GI tract and are found throughout the gut as distinct networks, or interspersed between muscle cells in various regions, from the submucosa to the subserosa. Several animal studies demonstrated that ICCs derive from the mesoderm (Lecoin et al., 1996; Young et al., 1996) and share the same progenitor cells as the primitive smooth muscle (Burns et al., 1996; Klüppel et al., 1998). These intestinal muscle cell progenitors initially show expression of the tyrosine kinase receptor c-Kit, and smooth muscle myosin cell heavy chain (SMMHC). At later developmental stages, ICCs will specifically keep c-Kit expression, but lose SMMHC (Klüppel et al., 1998). Interestingly, ICC differentiation and survival requires Kit Ligands, which can only be provided by neurons or cells derived from a smooth muscle lineage (Wu et al., 2000b, 2000a). Myenteric ICCs, located between the circular and longitudinal muscle layers; intramuscular ICCs, located within the circular muscle layer in colon and stomach; deep myenteric plexus ICCs, between the inner and outer parts of the circular muscle layer of the small intestine; submucosal ICCs, on the submucosal surface of the circular muscle layer of the colon; and subserosal ICCs, located in the subserosal layer (Foong et al., 2020).

Myenteric and submucosal ICCs generate rhythmic electrical slow waves of depolarization and hyperpolarization that propagate passively to SMCs and synchronize SMCs contraction.

This baseline electrical rhythm is present even when the ENS is absent (Blair et al., 2014). Intramuscular ICCs and deep myenteric plexus ICCs are closely associated with nerve varicosities, and thus, are considered to be the primary ICCs to receive input from the ENS (Sanders et al., 2010). It has even been hypothesized that ICCs might mediate inputs from enteric motor neurons due to the close apposition of these two cell types. (Beckett et al., 2005). Molecular studies have also demonstrated that ICCs have the receptors required for transduction of neurotransmitter signals (Ward et al., 2000; Beckett et al., 2005; Chen et al., 2007; Iino et al., 2009).

Detailed interactions among ENS, SMCs and ICCs have been described in several excellent studies (Sanders et al., 2016a; Foong et al., 2020). SMCs, ICC, and PDGFRα+ cells are well connected in a gap junction structure called the 'SIP syncytium' (Sanders et al., 2016b). The SIP syncytium receives input from excitatory and inhibitory motor neurons of ENS whose cell bodies reside within the myenteric plexus (Figure 4). Although SMCs were once considered the main targets of excitatory and inhibitory ENS motor neurons, neural input onto ICC and PDGFRα+ cells is likely also critical for mediating smooth muscle contractility. Finally, although the ENS can control many aspects of bowel function autonomously, *in vivo* ENS activity is also modulated by luminal contents (nutrients and microbes); muscularis macrophages; parasympathetic neurons; sympathetic innervation from the celiac, superior, and inferior mesenteric ganglia; and hormonal signals (e.g., adrenaline, thyroid hormone, corticotrophin-releasing hormone, oxytocin). Many of these extrinsic signals are influenced by central nervous system activity, which explains how emotional responses like anxiety and fear can alter bowel function (Furness, 2012).

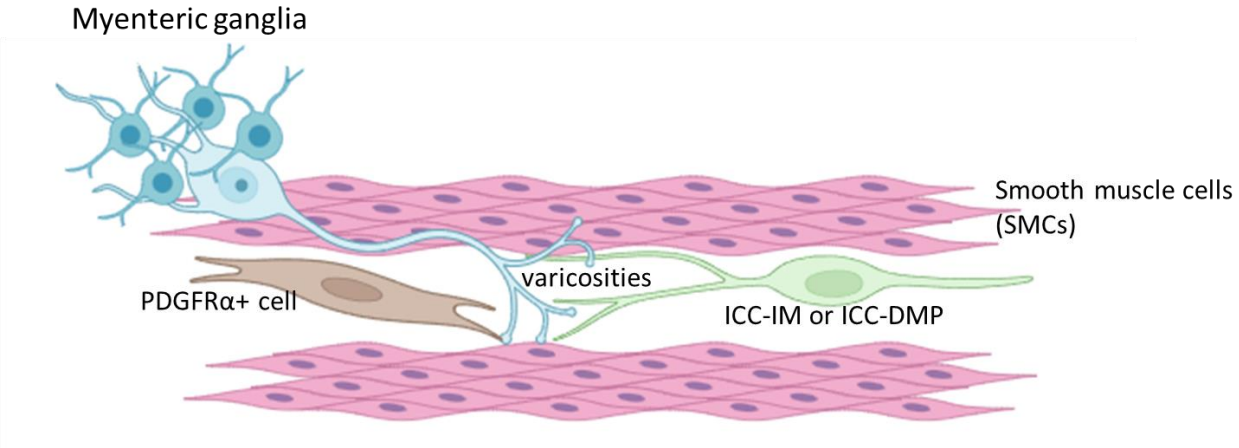

Figure 4. Schematic of SIP syncytium which is composed of SMCs, ICC, and PDGFRα+ cells that receive input from ENS varicosities.

2. Congenital intestinal motility disorders

Congenital intestinal motility disorders are characterized by abnormal function of the GI tract, as a result of defective smooth muscle cells, ICCs, and/or absence of an ENS. Pediatric intestinal pseudo-obstruction (PIPO) is a form of congenital intestinal dysmotility, characterized by a severe impairment of GI propulsion, due to partial or complete intestinal obstruction in the absence of any lesion restricting or occluding the intestinal lumen (Di Nardo et al., 2017a). PIPO represents the most severe form of intestinal dysmotility with potential lethal consequences (Goldstein et al., 2016). It is a rare condition in which the majority of cases occur sporadically. To date, only a few epidemiological studies reported the incidence and prevalence of PIPO. Data from the American Pseudo-obstruction and Hirschsprung Society (now part of the International Foundation for Functional Gastrointestinal Disorders) suggest that less than 100 infants are born every year in the USA with primary PIPO (Thapar et al., 2018). In 2014, a nationwide Japanese survey reported an estimated pediatric prevalence of 3.7 in 1 million individuals under 15 years of age. More than half of the children (57%) diagnosed with PIPO developed symptoms in the neonatal period (<1 month old), 19% within infancy (1–12 months old), 15% in childhood (1–7 years of age), and 9% in school age or later (7–15 years of age) (Muto et al., 2014).

Diagnostic criteria for PIPO were proposed by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition in 2018 with a goal to improve diagnosis, management and treatment, while decreasing morbidity and mortality in patients. A stepwise diagnostic approach for suspected PIPO should consider the vulnerability of neonatal or infant patients. In principle, diagnostic approaches should exclude mechanical occlusion of the GI tract, confirm impaired GI motility, and rule out treatable causes. Histopathology studies on muscle and ganglia also contribute to diagnosis and can be used to inform management, for example, the use of parenteral nutrition in intestinal myopathies and prokinetics in intestinal neuropathies (Thapar et al., 2018). However, the role of histopathology in the diagnosis of PIPO is still controversial, as there is no single pathognomonic finding for the disease. Only some signs, such as aganglionosis, ganglioneuromatosis, neuropathies with inclusions and mitochondrial disorders (Giordano et al., 2009), muscularis propria malformations (Kapur et al., 2010), and severe degenerative (vacuolar) myopathies (Knowles et al., 2010), have a high diagnostic specificity.

The cause of PIPO can be divided into primary, secondary and idiopathic. Primary disorders leading to PIPO are defined as abnormalities within the development, degeneration, or inflammation of the enteric neuromusculature. These disorders include sporadic or familial forms of myopathy, neuropathy, mesenchymopathy, mitochondrial diseases, or neuropathy associated with multiple endocrine neoplasia type II. Primary PIPO has a strong genetic nature and many causative genes have been identified due to recent advances in genetic testing (Gamboa and Sood, 2019). However, for the majority of cases, the genetic etiology of the disease is unknown. Secondary PIPO includes a variety of conditions (multiple systemic illnesses, infections, toxins, autonomic nervous system disorders) which have a later effect in the ENS, SMCs and ICCs. Finally, idiopathic PIPO is defined when primary and secondary causes for the disease cannot be identified. Based on where the abnormalities are found within the GI tract, PIPO can be classified

as a neuropathy, myopathy, or mesenchymopathy. In this thesis, primary neuropathic and myopathic PIPO were our main focus, and thus, are further described in the next subsections.

2a. Primary neuropathic PIPO

Neuropathic PIPO typically arises due to loss of enteric neurons or due to inflammation within the enteric ganglia and/or neuronal connections (Knowles et al., 2010). Mutations in several genes have been described to cause primary neuropathic PIPO (Supplementary Table 1). Hirschsprung disease (HSCR) is the most common example of primary neuropathic PIPO. The incidence of HSCR is estimated to be 1/5000 live births. However, this number varies significantly among ethnic groups (1.0, 1.5, 2.1, and 2.8 per 10 000 live births in Hispanics, Caucasian-Americans, African Americans, and Asians, respectively (Granström et al., 2016; Taghavi et al., 2019). There is also a sex bias in which HSCR affects males more frequently than females, with a ratio ranging from 2.8:1 in European population to 4.0:1 in UK and Ireland population (Best et al., 2014; Bradnock et al., 2017). However, this pattern does not hold true in syndromic HSCR (defined when HSCR is associated with other congenital anomalies), or in long-segment and total colonic aganglionosis (Montalva et al., 2023; Xiao et al., 2023). HSCR has a strong genetic background with more than 30 genes identified (Karim et al., 2021; Tang et al., 2023). HSCR is a congenital malformation of the hindgut characterized by the absence of parasympathetic intrinsic ganglion cells in the submucosal and myenteric plexuses (Amiel et al., 2008). It is regarded as the consequence of premature arrest of the cranio-caudal migration of vagal NCCs in the hindgut, between the fifth and twelfth week of gestation, and is therefore classified as a neurocristopathy. While the internal anal sphincter is the constant inferior limit, patients could be classified as short-segment HSCR (S-HSCR: 72% of cases) when the aganglionic segment does not extend beyond the upper sigmoid, long-segment HSCR (L-HSCR: 15% of cases) when aganglionosis extends proximal to the sigmoid and total colonic aganglionosis (TCA, 8% of cases) in which aganglionosis extend <5cm beyond ileum. There are also some very rare cases in which aganglionosis extends into the small intestine, > 5cm beyond ileum (5% of cases), and total intestinal HSCR where ganglionated intestine is found in < 20cm beyond the ligament of Treitz (1% of cases) (Karim et al., 2021; Montalva et al., 2023).

2b. Primary Myopathic PIPO

In primary myopathic PIPO, abnormalities within the intestinal smooth muscle were found to be associated with the distribution and morphology of SMCs. Similar to enteric neuropathies, enteric myopathies typically present in infants and children and are congenital due to the presence of a genetic mutation (Supplementary Table 2). Visceral

myopathy (VSCM) is the most common form of myopathic PIPO. In patients affected by this disorder, not only the intestine, but also other visceral organs are affected, such as the bladder and the uterus. VSCM leads to inefficient movement of air and nutrients through the bowel, impairs bladder emptying, and affects normal uterine contraction and relaxation, particularly during pregnancy. The onset of symptoms and severity exist as a spectrum in patients with VSCM, even in a family member with the same disease-causing mutation (Wangler et al., 2014; Matera et al., 2016). For example, children suffering severe disease from infancy can have parents that are only mildly affected in a familial VSCM case. This suggests that second-site genetic modifiers or non-genetic factors (e.g., diet and gut microbes) are likely to impact disease manifestations (Viti et al., 2023b). Megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS) is the most severe form of myopathic PIPO and has the most debilitating defects, including dysfunctional bowel, intrauterine colonic growth defect (microcolon), and bladder-emptying defects which require catheterization. MMIHS has a high fatality outcome and patients often die early in childhood (Matera et al., 2016). Congenital Short Bowel Syndrome (CSBS), another visceral myopathic condition, has also a high mortality rate within the first few months after birth, although some long-term survivors of CSBS have been reported. CSBS is characterized by a shortened small intestine (approximately 50 cm, while normal length at birth is around 275 cm) and intestinal malrotation (Ordonez et al., 2006). Visceral myopathies like MMIHS and myopathic PIPO are most commonly caused by mutations in genes involved in smooth muscle contraction (Hashmi et al., 2023).

3. Treatment options for congenital intestinal motility disorders

Available treatments for congenital intestinal motility disorders such as PIPO, include three aspects: nutritional, pharmacological and surgical therapy. Nutritional therapy is required for hydration and nutrition, as around 80% of patients require parenteral nutrition to maintain normal growth and development. Specialized feeds (e.g, hydrolysed protein feeds) and feeding routes (e.g, jejunal) are also used to promote enteral feed tolerance. Pharmacological therapy for PIPO is still debatable, as there is no evidence of benefits based on controlled trials (Di Nardo et al., 2017b, 2017a). Surgical procedures such as venting ostomies, are commonly used to decompress and reduce pseudo-obstructive events (Thapar et al., 2018). They are often a last resort, but in the majority of cases, they are life-saving. In HSCR, surgery to remove the aganglionic part of the bowel is currently the only available option (Smith et al., 2020; Jensen and Frischer, 2022). The underlying principle is to connect the normal ganglionic bowel to the anus, and to release the tonic contraction of the internal anal sphincter. Since the initial protocol described by Swenson in 1948, a series of operative approaches, such as the Soave and Duhamel procedures, have been developed (Soave, 1964; Swenson, 2002). Laparoscopic and trans-anal pull-through techniques have also been more recently proposed for HSCR patients (Van De Ven et al., 2013). Details of surgery techniques in HSCR management and post-operative treatments are well reviewed in Montalva et al (Montalva et al., 2023). In MMIHS, treatment using prokinetic drugs and GI hormones have been generally unsuccessful, and surgery interventions such as gastrostomy, jejunostomy, ileostomy, cecostomy, segmental resection of jejunum and ileum,

lysis of adhesions and internal sphincter myectomy, have been used for nutrition delivery (Lee et al., 2010; Gosemann and Puri, 2011). Despite advances in surgical procedures and their clear benefits, intestinal functional outcome may vary and life-long GI complications often occur in PIPO patients (Conway et al., 2007; Laughlin et al., 2012)**.** Therefore, other therapeutic options to re-establish optimal intestinal functions are still needed.

4. Aims and outline of this thesis

This thesis focuses on basic and translational research of primary PIPO caused by defects in ENS development (neuropathic PIPO) and smooth muscle function (myopathic PIPO). It brings new insights into the etiology of these diseases by the identification of new causing genes, and contributes to the understanding of the underlying cellular and molecular changes involved in PIPO pathogenesis.

In **Chapter 2,** a heterozygous deletion in the transcription factor AP-2β (*TFAP2B*) was found in a neuropathic PIPO patient. *TFAP2B* has never been linked to intestinal dysmotility**.** Therefore, *in vitro* and *in vivo* functional studies were performed to investigate the role of this gene in intestinal development. **Chapter 3** focuses on the role of Filamin A (FLNA) in intestinal development and its implication in X-linked myopathic PIPO. In **Chapter 4**, we focused on HSCR, the most common form of neuropathic PIPO. Despite the fact that more than 30 genes have been implicated in its pathogenesis, the genetic cause for more than half of the cases is still unknown. Studying epigenetic modifications, such as DNA methylation, occurring especially in ENS cells derived from HSCR patients, could lead to the identification of potential new HSCR genes. **Chapter 5**, describes the generation of hiPSC derived enteric neuronal progenitors as a disease model to understand differentiation of ENCCs towards an ENS, as well as proliferation and migrations of these cells in healthy controls vs HSCR patients. In this chapter, we also discuss the possibility of using these cells for therapy. Finally, **Chapter 6** summarizes and discusses how the work described in this thesis improves our understanding of the genetic and cellular mechanisms required for intestinal development, and how they can lead to disease. Knowledge of these processes can potentially provide insights and clues for new treatment avenues for primary PIPO.

References

Amiel, J., Sproat-Emison, E., Garcia-Barcelo, M., Lantieri, F., Burzynski, G., Borrego, S., et al. (2008). Hirschsprung disease, associated syndromes and genetics: A review. *J Med Genet* 45, 1–14. doi: 10.1136/jmg.2007.053959

Apelqvist, Å., Ahlgren, U., and Edlund, H. (1997). Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Current Biology* 7, 801–804. doi: 10.1016/S0960-9822(06)00340-X

Barlow, A., De Graaff, E., and Pachnis, V. (2003). Enteric Nervous System Progenitors Are Coordinately Controlled by the G Protein-Coupled Receptor EDNRB and the Receptor Tyrosine Kinase RET. *Neuron* 40, 905–916. doi: 10.1016/S0896-6273(03)00730-X

Beckett, E. A. H., Takeda, Y., Yanase, H., Sanders, K. M., and Ward, S. M. (2005). Synaptic specializations exist between enteric motor nerves and interstitial cells of cajal in the murine stomach. *Journal of Comparative Neurology* 493, 193–206. doi: 10.1002/cne.20746

Best, K. E., Addor, M. C., Arriola, L., Balku, E., Barisic, I., Bianchi, F., et al. (2014). Hirschsprung's disease prevalence in Europe: A register based study. *Birth Defects Res A Clin Mol Teratol* 100, 695–702. doi: 10.1002/bdra.23269

Blair, P. J., Rhee, P. L., Sanders, K. M., and Ward, S. M. (2014). The significance of interstitial cells in neurogastroenterology. *J Neurogastroenterol Motil* 20, 294–317. doi: 10.5056/jnm14060

Bradnock, T. J., Knight, M., Kenny, S., Nair, M., and Walker, G. M. (2017). Hirschsprung's disease in the UK and Ireland: Incidence and anomalies. *Arch Dis Child* 102, 722–727. doi: 10.1136/archdischild-2016-311872

Burns, A. J., Lomax, A. E. J., Torihashi, S., Sanders, K. M., and Ward, S. M. (1996). Interstitial cells of Cajal mediate inhibitory neurotransmission in the stomach. *Proc Natl Acad Sci U S A* 93, 12008–12013. doi: 10.1073/pnas.93.21.12008

Chalazonitis, A., D'Autréaux, F., Guha, U., Pham, T. D., Faure, C., Chen, J. J., et al. (2004). Bone Morphogenetic Protein-2 and -4 Limit the Number of Enteric Neurons but Promote Development of a TrkC-Expressing Neurotrophin-3-Dependent Subset. *Journal of Neuroscience* 24, 4266–4282. doi: 10.1523/JNEUROSCI.3688-03.2004

Chalazonitis, A., D'Autréaux, F., Pham, T. D., Kessler, J. A., and Gershon, M. D. (2011). Bone morphogenetic proteins regulate enteric gliogenesis by modulating ErbB3 signaling. *Dev Biol* 350, 64–79. doi: 10.1016/j.ydbio.2010.11.017

Chalazonitis, A., Pham, T. D., Li, Z., Roman, D., Guha, U., Gomes, W., et al. (2008). Bone morphogenese protein regulation of enteric neuronal phenotypic diversity: Relationship to timing of cell cycle exit. *Journal of Comparative Neurology* 509, 474–492. doi: 10.1002/cne.21770

Chen, H., Redelman, D., Ro, S., Ward, S. M., Ördög, T., and Sanders, K. M. (2007). Selective labeling and isolation of functional classes of interstitial cells of Cajal of human and murine small intestine. *Am J Physiol Cell Physiol* 292, 497–507. doi: 10.1152/ajpcell.00147.2006

Cheng, L. K., O'Grady, G., Du, P., Egbuji, J. U., Windsor, J. A., and Pullan, A. J. (2010). Gastrointestinal system. *Wiley Interdiscip Rev Syst Biol Med* 2, 65–79. doi: 10.1002/wsbm.19

Conway, S. J., Craigie, R. J., Cooper, L. H., Turner, K., Turnock, R. R., Lamont, G. L., et al. (2007). Early adult outcome of the Duhamel procedure for left-sided Hirschsprung disease-a prospective serial assessment study. *J Pediatr Surg* 42, 1429–1432. doi: 10.1016/j.jpedsurg.2007.03.046

D'Autréaux, F., Morikawa, Y., Cserjesi, P., and Gershon, M. D. (2007). Hand2 is necessary for terminal differentiation of enteric neurons from crest-derived precursors but not for their migration into the gut or for formation of glia. *Development* 134, 2237–2249. doi: 10.1242/dev.003814

Di Nardo, G., Di Lorenzo, C., Lauro, A., Stanghellini, V., Thapar, N., Karunaratne, T. B., et al. (2017a). Chronic intestinal pseudo-obstruction in children and adults: diagnosis and therapeutic options. *Neurogastroenterology and Motility* 29. doi: 10.1111/nmo.12945

Di Nardo, G., Karunaratne, T. B., Frediani, S., and De Giorgio, R. (2017b). Chronic intestinal pseudo-obstruction: Progress in management? *Neurogastroenterology and Motility* 29. doi: 10.1111/nmo.13231

Dong, W., Baldwin, C., Choi, J., Milunsky, J. M., Zhang, J., Bilguvar, K., et al. (2019). Identification of a dominant MYH11 causal variant in chronic intestinal pseudo-obstruction: Results of whole-exome sequencing. *Clin Genet* 96, 473–477. doi: 10.1111/cge.13617

Druckenbrod, N. R., Powers, P. A., Bartley, C. R., Walker, J. W., and Epstein, M. L. (2008). Targeting of endothelin receptor-B to the neural crest. *Genesis* 46, 396–400. doi: 10.1002/dvg.20415

Durbec, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., et al. (1996). GDNF signalling through the Ret receptor tyrosine kinase. *Nature* 381, 789–793. doi: 10.1038/381789a0

Eddinger, T. J., and Meer, D. P. (2007). Myosin II isoforms in smooth muscle: Heterogeneity and function. *Am J Physiol Cell Physiol* 293. doi: 10.1152/ajpcell.00131.2007

Foong, D., Zhou, J., Zarrouk, A., Ho, V., and O'Connor, M. D. (2020). Understanding the biology of human interstitial cells of cajal in gastrointestinal motility. *Int J Mol Sci* 21, 1–18. doi: 10.3390/ijms21124540

Fu, M., Vohra, B. P. S., Wind, D., and Heuckeroth, R. O. (2006). BMP signaling regulates murine enteric nervous system precursor migration, neurite fasciculation, and patterning via altered Ncam1 polysialic acid addition. *Dev Biol* 299, 137–150. doi: 10.1016/j.ydbio.2006.07.016

Furness, J. B. (2012). The enteric nervous system and neurogastroenterology. *Nat Rev Gastroenterol Hepatol* 9, 286–294. doi: 10.1038/nrgastro.2012.32

Gamboa, H. E., and Sood, M. (2019). Pediatric Intestinal Pseudo-obstruction in the Era of Genetic Sequencing. *Curr Gastroenterol Rep* 21. doi: 10.1007/s11894-019-0737-y

Giordano, C., Powell, H., Leopizzi, M., De Curtis, M., Travaglini, C., Sebastiani, M., et al. (2009). Fatal congenital myopathy and gastrointestinal pseudo-obstruction due to POLG1 mutations. *Neurology* 72, 1103–1105. doi: 10.1212/01.wnl.0000345002.47396.e1

Goldstein, A. M., Hofstra, R. M. W., and Burns, A. J. (2013). Building a brain in the gut: Development of the enteric nervous system. *Clin Genet* 83, 307–316. doi: 10.1111/cge.12054

Goldstein, A. M., Thapar, N., Karunaratne, T. B., and De Giorgio, R. (2016). Clinical aspects of neurointestinal disease: Pathophysiology, diagnosis, and treatment. *Dev Biol* 417, 217–228. doi: 10.1016/j.ydbio.2016.03.032

Gosemann, J. H., and Puri, P. (2011). Megacystis microcolon intestinal hypoperistalsis syndrome: Systematic review of outcome. *Pediatr Surg Int* 27, 1041–1046. doi: 10.1007/s00383-011-2954-9

Granström, A. L., Svenningsson, A., Hagel, E., Oddsberg, J., Nordenskjöld, A., and Wester, T. (2016). Maternal risk factors and perinatal characteristics for hirschsprung disease. *Pediatrics* 138, 1–8. doi: 10.1542/peds.2015-4608

Guillemot, F., and Joyner, A. L. (1993). Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. *Mech Dev* 42, 171–185. doi: 10.1016/0925-4773(93)90006-J

Guo, F., Debidda, M., Yang, L., Williams, D. A., and Zheng, Y. (2006). Genetic deletion of Rac1 GTPase reveals its critical role in actin stress fiber formation and focal adhesion complex assembly. *Journal of Biological Chemistry* 281, 18652–18659. doi: 10.1074/jbc.M603508200

Halim, D., Brosens, E., Muller, F., Wangler, M. F., Beaudet, A. L., Lupski, J. R., et al. (2017a). Loss-of-Function Variants in MYLK Cause Recessive Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *Am J Hum Genet* 101, 123–129. doi: 10.1016/j.ajhg.2017.05.011

Halim, D., Hofstra, R. M. W., Signorile, L., Verdijk, R. M., Van Der Werf, C. S., Sribudiani, Y., et al. (2016). ACTG2 variants impair actin polymerization in sporadic Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *Hum Mol Genet* 25, 571–583. doi: 10.1093/hmg/ddv497

Halim, D., Wilson, M. P., Oliver, D., Brosens, E., Verheij, J. B. G. M., Han, Y., et al. (2017b). Loss of LMOD1 impairs smooth muscle cytocontractility and causes megacystis microcolon intestinal hypoperistalsis syndrome in humans and mice. *Proc Natl Acad Sci U S A* 114, E2739–E2747. doi: 10.1073/pnas.1620507114

Hashmi, S. K., Ceron, R. H., and Heuckeroth, R. O. (2023). Visceral myopathy : clinical syndromes , genetics , pathophysiology , and fall of the cytoskeleton. *Am J Physiol Gastrointest Liver Physiol*. 919–935. doi: 10.1152/ajpgi.00066.2021

Hutchins, E. J., Kunttas, E., Piacentino, M. L., Howard, A. G. A., Bronner, M. E., and Uribe, R. A. (2018). Migration and diversification of the vagal neural crest. *Dev Biol* 444, S98–S109. doi: 10.1016/j.ydbio.2018.07.004

Huycke, T. R., Miller, B. M., Gill, H. K., Nerurkar, N. L., Sprinzak, D., Mahadevan, L., et al. (2019). Genetic and Mechanical Regulation of Intestinal Smooth Muscle Development. *Cell* 179, 90-105.e21. doi: 10.1016/j.cell.2019.08.041

Iino, S., Horiguchi, K., Nojyo, Y., Ward, S. M., and Sanders, K. M. (2009). Interstitial cells of Cajal contain signalling molecules for transduction of nitrergic stimulation in guinea pig caecum. *Neurogastroenterology and Motility* 21. doi: 10.1111/j.1365-2982.2008.01236.x

Jensen, A. R., and Frischer, J. S. (2022). Surgical history of Hirschsprung disease. *Semin Pediatr Surg* 31, 151174. doi: 10.1016/j.sempedsurg.2022.151174

Jiang, Y., Liu, M. tsai, and Gershon, M. D. (2003). Netrins and DCC in the guidance of migrating neural crest-derived cells in the developing bowel and pancreas. *Dev Biol* 258, 364–384. doi: 10.1016/S0012-1606(03)00136-2

Kameneva, P., Kastriti, M. E., and Adameyko, I. (2021). Neuronal lineages derived from the nerve-associated Schwann cell precursors. *Cellular and Molecular Life Sciences* 78, 513–529. doi: 10.1007/s00018-020-03609-5

Kapur, R. P., Robertson, S. P., Hannibal, M. C., Finn, L. S., Morgan, T., Van Kogelenberg, M., et al. (2010). Diffuse abnormal layering of small intestinal smooth muscle is present in patients with FLNA mutations and X-linked intestinal pseudo-obstruction. *American Journal of Surgical Pathology* 34, 1528–1543. doi: 10.1097/PAS.0b013e3181f0ae47

Karim, A., Tang, C. S. M., and Tam, P. K. H. (2021). The Emerging Genetic Landscape of Hirschsprung Disease and Its Potential Clinical Applications. *Front Pediatr* 9, 1–14. doi: 10.3389/fped.2021.638093

Kastriti, M. E., and Adameyko, I. (2017). Specification, plasticity and evolutionary origin of peripheral glial cells. *Curr Opin Neurobiol* 47, 196–202. doi: 10.1016/j.conb.2017.11.004

Klein, S., Seidler, B., Kettenberger, A., Sibaev, A., Rohn, M., Feil, R., et al. (2013). Interstitial cells of Cajal integrate excitatory and inhibitory neurotransmission with intestinal slow-wave activity. *Nat Commun* 4. doi: 10.1038/ncomms2626

Klüppel, M., Huizinga, J. D., Malysz, J., and Bernstein, A. (1998). Developmental origin and Kit-dependent development of the interstitial cells of Cajal in the mammalian small intestine. *Developmental Dynamics* 211, 60–71. doi: 10.1002/(SICI)1097-0177(199801)211:1<60::AID-AJA6>3.0.CO;2-5

Knowles, C. H., De Giorgio, R., Kapur, R. P., Bruder, E., Farrugia, G., Geboes, K., et al. (2010). The London Classification of gastrointestinal neuromuscular pathology: Report on behalf of the gastro 2009 international working group. *Gut* 59, 882–887. doi: 10.1136/gut.2009.200444

Kurahashi, M., Zheng, H., Dwyer, L., Ward, S. M., Don Koh, S., and Sanders, K. M. (2011). A functional role for the “fibroblast-like cells” in gastrointestinal smooth muscles. *Journal of Physiology* 589, 697–710. doi: 10.1113/jphysiol.2010.201129

Laughlin, D. M., Friedmacher, F., and Puri, P. (2012). Total colonic aganglionosis: A systematic review and meta-analysis of long-term clinical outcome. *Pediatr Surg Int* 28, 773–779. doi: 10.1007/s00383-012-3117-3

Lecoin, L., Gabella, G., and Le Douarin, N. (1996). Origin of the c-kit-positive interstitial cells in the avian bowel. *Development* 122, 725–733. doi: 10.1242/dev.122.3.725

Lee, G., Chambers, S. M., Tomishima, M. J., and Studer, L. (2010). Derivation of neural crest cells from human pluripotent stem cells. *Nat Protoc* 5, 688–701. doi: 10.1038/nprot.2010.35

Lei, J., and Howard, M. J. (2011). Targeted deletion of Hand2 in enteric neural precursor cells affects its functions in neurogenesis, neurotransmitter specification and gangliogenesis, causing functional aganglionosis. *Development* 138, 4789–4800. doi: 10.1242/dev.060053

Li, Z., Chalazonitis, A., Huang, Y. Y., Mann, J. J., Margolis, K. G., Yang, Q. M., et al. (2011). Essential roles of enteric neuronal serotonin in gastrointestinal motility and the development/survival of enteric dopaminergic neurons. *Journal of Neuroscience* 31, 8998–9009. doi: 10.1523/JNEUROSCI.6684-10.2011

Liu, P., Cheng, H., Roberts, T. M., and Zhao, J. J. (2011). targeting the phosphoinositide 3-kinase (Pi. *Cancer* 8, 627–644. doi: 10.1038/nrd2926.Targeting

Luongo, D., D'Arienzo, R., Bergamo, P., Maurano, F., and Rossi, M. (2009). Immunomodulation of gut-associated lymphoid tissue: Current perspectives. *Int Rev Immunol* 28, 446–464. doi: 10.3109/08830180903236486

Matera, I., Rusmini, M., Guo, Y., Lerone, M., Li, J., Zhang, J., et al. (2016). Variants of the ACTG2 gene correlate with degree of severity and presence of megacystis in chronic intestinal pseudo-obstruction. *European Journal of Human Genetics* 24, 1211–1215. doi: 10.1038/ejhg.2015.275

McLin, V. A., Henning, S. J., and Jamrich, M. (2009). The Role of the Visceral Mesoderm in the Development of the Gastrointestinal Tract. *Gastroenterology* 136, 2074–2091. doi: 10.1053/j.gastro.2009.03.001

Mograbi, B., Bocciardi, R., Bourget, I., Busca, R., Rochet, N., Farahi-Far, D., et al. (2001). Glial cell line-derived neurotrophic factor-stimulated phosphatidylinositol 3-Kinase and Akt activities exert opposing effects on the ERK pathway: Importance for the rescue of neuroectodermic cells. *Journal of Biological Chemistry* 276, 45307–45319. doi: 10.1074/jbc.M101220200

Montalva, L., Cheng, L. S., Kapur, R., Langer, J. C., Berrebi, D., Kyrklund, K., et al. (2023). Hirschsprung disease. *Nat Rev Dis Primers* 9. doi: 10.1038/s41572-023-00465-y

Moreno, C. A., Sobreira, N., Pugh, E., Zhang, P., Steel, G., Torres, F. R., et al. (2018). Homozygous deletion in MYL9 expands the molecular basis of megacystis-microcolon-intestinal hypoperistalsis syndrome. *European Journal of Human Genetics* 26, 669–675. doi: 10.1038/s41431-017-0055-5

Muto, M., Matsufuji, H., Tomomasa, T., Nakajima, A., Kawahara, H., Ida, S., et al. (2014). Pediatric chronic intestinal pseudo-obstruction is a rare, serious, and intractable disease: A report of a nationwide survey in Japan. *J Pediatr Surg* 49, 1799–1803. doi: 10.1016/j.jpedsurg.2014.09.025

Nagy, N., Barad, C., Graham, H. K., Hotta, R., Cheng, L. S., Fejszak, N., et al. (2016). Sonic hedgehog controls enteric nervous system development by patterning the extracellular matrix. *Development (Cambridge)* 143, 264–275. doi: 10.1242/dev.128132

Nagy, N., Barad, C., Hotta, R., Bhave, S., Arciero, E., Dora, D., et al. (2018). Collagen 18 and agrin are secreted by neural crest cells to remodel their microenvironment and regulate their migration during enteric nervous system development. *Development (Cambridge)* 145. doi: 10.1242/dev.160317

Nagy, N., and Goldstein, A. M. (2006). Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. *Dev Biol* 293, 203–217. doi: 10.1016/j.ydbio.2006.01.032

Nishida, S., Yoshizaki, H., Yasui, Y., Kuwahara, T., Kiyokawa, E., and Kohno, M. (2018). Collagen VI suppresses fibronectin-induced enteric neural crest cell migration by downregulation of focal adhesion proteins. *Biochem Biophys Res Commun* 495, 1461–1467. doi: 10.1016/j.bbrc.2017.11.184

Okamura, Y., and Saga, Y. (2008). Notch signaling is required for the maintenance of enteric neural crest progenitors. *Development* 135, 3555–3565. doi: 10.1242/dev.022319

Ordonez, P., Sondheimer, J. M., Fidanza, S., Wilkening, G., & Hoffenberg, E. J. (2006). Long-term outcome of a patient with congenital short bowel syndrome. *Journal of Pediatric Gastroenterology and Nutrition*, *42*(5), 576–580. https://doi.org/10.1097/01.mpg.0000189360.84169.da

Pattyn, A., Morin, X., Cremer, H., Goridis, C., and Brunet, J. F. (1999). The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature* 399, 366–370. doi: 10.1038/20700

Ramalho-santos, M., Melton, D. A., and Mcmahon, A. P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 127, 2763–2772. doi: 10.1007/bf03270579

Roach, G., Heath Wallace, R., Cameron, A., Emrah Ozel, R., Hongay, C. F., Baral, R., et al. (2013). Loss of ascl1a prevents secretory cell differentiation within the zebrafish intestinal epithelium resulting in a loss of distal intestinal motility. *Dev Biol* 376, 171–186. doi: 10.1016/j.ydbio.2013.01.013

Robertson, S. P. (2005). Filamin A: Phenotypic diversity. *Curr Opin Genet Dev* 15, 301–307. doi: 10.1016/j.gde.2005.04.001

Sanders, K. M., Hwang, S. J., and Ward, S. M. (2010). Neuroeffector apparatus in gastrointestinal smooth muscle organs. *Journal of Physiology* 588, 4621–4639. doi: 10.1113/jphysiol.2010.196030

Sanders, K. M., Kito, Y., Hwang, S. J., and Ward, S. M. (2016a). Regulation of gastrointestinal smooth muscle function by interstitial cells. *Physiology* 31, 316–326. doi: 10.1152/physiol.00006.2016

Sanders, K. M., Kito, Y., Hwang, S. J., and Ward, S. M. (2016b). Regulation of gastrointestinal smooth muscle function by interstitial cells. *Physiology* 31, 316–326. doi: 10.1152/physiol.00006.2016

Sanders, K. M., Koh, S. D., Ro, S., and Ward, S. M. (2012). Regulation of gastrointestinal motility—insights from smooth muscle biology. *Nat Rev Gastroenterol Hepatol* 9, 633–645. doi: 10.1038/nrgastro.2012.168.

Sato, Y., and Heuckeroth, R. O. (2008). Retinoic acid regulates murine enteric nervous system precursor proliferation, enhances neuronal precursor differentiation, and reduces neurite growth in vitro. *Dev Biol* 320, 185–198. doi: 10.1016/j.ydbio.2008.05.524

Schneider, S., Wright, C. M., and Heuckeroth, R. O. (2019). Unexpected Roles for the Second Brain: Enteric Nervous System as Master Regulator of Bowel Function. *Annu Rev Physiol* 81, 235–259. doi: 10.1146/annurev-physiol-021317-121515

Seaman, C., Anderson, R., Emery, B., and Cooper, H. M. (2001). Localization of the netrin guidance receptor, DCC, in the developing peripheral and enteric nervous systems. *Mech Dev* 103, 173–175. doi: 10.1016/S0925-4773(01)00350-1

Shepherd, I. T., Pietsch, J., Elworthy, S., Kelsh, R. N., and Raible, D. W. (2004). Roles for GFRα1 receptors in zebrafish enteric nervous system development. *Development* 131, 241–249. doi: 10.1242/dev.00912

Simkin, J. E., Zhang, D., Rollo, B. N., and Newgreen, D. F. (2013). Retinoic Acid Upregulates Ret and Induces Chain Migration and Population Expansion in Vagal Neural Crest Cells to Colonise the Embryonic Gut. *PLoS One* 8. doi: 10.1371/journal.pone.0064077

Smith, C., Ambartsumyan, L., and Kapur, R. P. (2020). Surgery, Surgical Pathology, and Postoperative Management of Patients With Hirschsprung Disease. *Pediatric and Developmental Pathology* 23, 23–39. doi: 10.1177/1093526619889436

Soave, F. (1964). Hirschsprung's disease: A new surgical technique. *Arch Dis Child* 39, 116–124. doi: 10.1136/adc.39.204.116

Somlyo, A. P., and Somlyo, A. V. (2003). Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: Modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 83, 1325–1358. doi: 10.1152/physrev.00023.2003

Stanchina, L., Van de Putte, T., Goossens, M., Huylebroeck, D., and Bondurand, N. (2010). Genetic interaction between Sox10 and Zfhx1b during enteric nervous system development. *Dev Biol* 341, 416–428. doi: 10.1016/j.ydbio.2010.02.036

Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., and Iba, H. (2000). <Sukegawa 2000.pdf>. 1980, 1971–1980.

Swenson, O. (2002). Hirschsprung ' s Disease : A Review. 109.

Taghavi, K., Goddard, L., Evans, S. M., Hobson, A., Beasley, S. W., Sankaran, S., et al. (2019). Ethnic variations in the childhood prevalence of Hirschsprung disease in New Zealand. *ANZ J Surg* 89, 1246–1249. doi: 10.1111/ans.14857

Taketomi, T., Yoshiga, D., Taniguchi, K., Kobayashi, T., Nonami, A., Kato, R., et al. (2005). Loss of mammalian Sprouty2 leads to enteric neuronal hyperplasia and esophageal achalasia. *Nat Neurosci* 8, 855–857. doi: 10.1038/nn1485

Tang, C. S. man, Karim, A., Zhong, Y., Chung, P. H. yu, and Tam, P. K. hang (2023). Genetics of Hirschsprung's disease. *Pediatr Surg Int* 39, 1–7. doi: 10.1007/s00383-022-05358-x

Thapar, N., Saliakellis, E., Benninga, M. A., Borrelli, O., Curry, J., Faure, C., et al. (2018). Paediatric Intestinal Pseudo-obstruction: Evidence and Consensus-based Recommendations from an ESPGHAN-Led Expert Group. *J Pediatr Gastroenterol Nutr* 66, 991–1019. doi: 10.1097/MPG.0000000000001982

Van De Ven, T. J., Sloots, C. E. J., Wijnen, M. H. W. A., Rassouli, R., Van Rooij, I., Wijnen, R. M., et al. (2013). Transanal endorectal pull-through for classic segment Hirschsprung's disease: With or without laparoscopic mobilization of the rectosigmoid? *J Pediatr Surg* 48, 1914–1918. doi: 10.1016/j.jpedsurg.2013.04.025

Viti, F., De Giorgio, R., Ceccherini, I., Ahluwalia, A., Alves, M. M., Baldo, C., et al. (2023a). Multi-disciplinary Insights from the First European Forum on Visceral Myopathy 2022 Meeting. *Dig Dis Sci* 68, 3857–3871. doi: 10.1007/s10620-023-08066-1

Viti, F., De Giorgio, R., Ceccherini, I., Ahluwalia, A., Alves, M. M., Baldo, C., et al. (2023b). Multi-disciplinary Insights from the First European Forum on Visceral Myopathy 2022 Meeting. *Dig Dis Sci* 68, 3857–3871. doi: 10.1007/s10620-023-08066-1

Wangler, M. F., Gonzaga-Jauregui, C., Gambin, T., Penney, S., Moss, T., Chopra, A., et al. (2014). Heterozygous De Novo and Inherited Mutations in the Smooth Muscle Actin (ACTG2) Gene Underlie Megacystis-Microcolon-Intestinal Hypoperistalsis Syndrome. *PLoS Genet* 10. doi: 10.1371/journal.pgen.1004258

Ward, S. M., Beckett, E. A. H., Wang, X. Y., Baker, F., Khoyi, M., and Sanders, K. M. (2000). Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *Journal of Neuroscience* 20, 1393–1403. doi: 10.1523/jneurosci.20-04-01393.2000

Watanabe, Y., Stanchina, L., Lecerf, L., Gacem, N., Conidi, A., Baral, V., et al. (2017). Differentiation of Mouse Enteric Nervous System Progenitor Cells Is Controlled by Endothelin 3 and Requires Regulation of Ednrb by SOX10 and ZEB2. *Gastroenterology* 152, 1139-1150.e4. doi: 10.1053/j.gastro.2016.12.034

Wu, J. J., Rothman, T. P., and Gershon, M. D. (2000a). Development of the interstitial cell of Cajal: Origin, kit dependence and neuronal and nonneuronal sources of kit ligand. *J Neurosci Res* 59, 384–401. doi: 10.1002/(SICI)1097-4547(20000201)59:3<384::AID-JNR13>3.0.CO;2-4

Wu, M., Hemesath, T. J., Takemoto, C. M., Horstmann, M. A., Wells, A. G., Price, E. R., et al. (2000b). c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi. *Genes Dev* 14, 301–312. doi: 10.1101/gad.14.3.301

Xiao, J., Hao, L. W., Wang, J., Yu, X. S., You, J. Y., Li, Z. J., et al. (2023). Comprehensive characterization of the genetic landscape of familial Hirschsprung's disease. *World Journal of Pediatrics* 19, 644–651. doi: 10.1007/s12519-023-00686-x

Young, H. M., Ciampoli, D., Southwell, B. R., and Newgreen, D. F. (1996). Origin of interstitial cells of Cajal in the mouse intestine. *Dev Biol* 180, 97–107. doi: 10.1006/dbio.1996.0287

Zhou, R., Niwa, S., Homma, N., Takei, Y., and Hirokawa, N. (2009). KIF26A Is an Unconventional Kinesin and Regulates GDNF-Ret Signaling in Enteric Neuronal Development. *Cell* 139, 802–813. doi: 10.1016/j.cell.2009.10.023

Zhu, L., Lee, H. O., Jordan, C. R. S., Cantrell, V. A., Southard-Smith, E. M., and Shin, M. K. (2004). Spatiotemporal regulation of endothelin receptor-B by SOX10 in neural crest-derived enteric neuron precursors. *Nat Genet* 36, 732–737. doi: 10.1038/ng1371

Supplementary Table1. Genetic causes of primary neuropathic PIPO

Gene	Disorder	Function	Inheritance	Phenotype	Age of onset
Sox10 (Herbarth et al., 1998; Sánchez-Mejías et al., 2010)	Type IV Waardenburg syndrome	A transcription factor involved in broad range of developmental process especially in neural crest cells and derivatives development	Autosomal dominant, sporadic	Peripheral neuropathy with hypomyelination, sensorineural deafness and pseudoobstruction, intestinal aganglionosis (HSCR)	Neonatal period
RET proto-oncogene (Receptor tyrosine kinase) (Garcia-Barceló et al., 2004; Fadista et al., 2018)	Isolated HSCR, MEN2A, MEN2B	Expressed in the neural crest cells of the enteric ganglia and encodes a member of the receptor tyrosine kinase family of transmembrane receptors	Autosomal dominant with incomplete penetrant	Haploinsufficiency associated with intestinal aganglionosis (HSCR), Gain in function mutation associated with intestinal ganglioneuromas leading to increased cell number in the myenteric plexus and dysmotility	Infancy to 3rd decade of life
GDNF (Borghini et al., 2002; Ruiz-Ferrer et al., 2011)	Isolated HSCR, accompanied by RET variant in some patients	A RET ligand act as a major survival factor for many types of neurons	Autosomal dominant, sporadic	Haploinsufficiency is associated with short, long and total colonic aganglionosis	Neonatal period
EDNRB (Tang et al., 2018a, 2018b)	Type IV Waardenburg syndrome/ Isolated HSCR	G-protein-coupled heptahelical receptors that transduce signals through the endothelins	Autosomal recessive/ Autosomal Dominant	Peripheral neuropathy with hypomyelination, sensorineural deafness and pseudo-obstruction/ Haploinsufficiency is associated with intestinal aganglionosis (HSCR)	Neonatal period
EDN3 (Endothelin 3) (Tilghman et al., 2019)	Type IV Waardenburg syndrome/ Isolated HSCR	Endothelin protein interaction with EDNRB is essential for development of neural crest derived cell lineages	Autosomal recessive/ Autosomal Dominant Autosomal recessive	Peripheral neuropathy with hypomyelination, sensorineural deafness and pseudo-obstruction /Haploinsufficiency is associated with intestinal aganglionosis (HSCR)	Neonatal period
IHH (Indian Hedgehog Signaling Molecule) (Sribudiani et al., 2018)	Isolated HSCR	Hedgehog family protein are essential secreted signalling including growth, patterning and morphogenesis	Autosomal Dominant	intestinal aganglionosis (HSCR)	Neonatal period
NRTN (Neurturin) (Ruiz-Ferrer et al., 2011)	Isolated HSCR	A RET ligand act as a major survival factor for many types of neurons	Autosomal dominant, sporadic	Short segment intestinal aganglionosis (HSCR)	Neonatal period
ARTN (Artemin) (Ruiz-Ferrer et al., 2011)	Isolated HSCR	A RET ligand act as a major survival factor for many types of neurons	Autosomal dominant, sporadic and familial	Short to long segment intestinal aganglionosis (HSCR)	Neonatal period
PSPN (Persephin) (Ruiz-Ferrer et al., 2011)	Isolated HSCR	A RET ligand act as a major survival factor for many types of neurons	Autosomal dominant, sporadic	Short segment intestinal aganglionosis (HSCR)	Neonatal period
GFRA1 (GDNF Family Receptor Alpha 1) (Tang et al., 2018b; Tilghman et al., 2019)	Isolated HSCR	A receptor for GDNF and NTRN and mediates RET activation, plays role in neuron survival and differentiation	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period

ECE1 (Endothelin Converting Enzyme 1) (Robert et al., 1999)	HSCR with cardiac, craniofacial, and autonomic disorders	ECE1 involved in the proteolytic processing of big endothelin 1, 2, and 3	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
PHOX2B (Paired Like Homeobox 2B) (Fernández et al., 2013)	Haddad syndrome (Congenital Central Hypoventilation Syndrome with HSCR) Neuroblastoma with HSCR HSCR with dysmorphic facial features	A transcription factor involved in the development of several noradren- ergic neuron populations	Autosomal dominant, sporadic and familial	Short to long intestinal aganglionosis (HSCR)	Neonatal period
ZEB2 (Zinc Finger E-Box Binding Homeobox 2) (Garavelli and Mainardi, 2007)	Mowat-Wilson syndrome	A DNA binding transcriptional repressor which interacts with activated SMADs, play role in proliferation of ENCCs	Autosomal dominant, sporadic and familial	intestinal aganglionosis (HSCR), intellectual disability, genital anomalies (particularly hypospa- dias in males), congenital heart disease (CHD), agenesis of the corpus callosum (ACC) and eye defects	Neonatal period
KIFBP (Kinesin Famili Binding Protein) (Brooks et al., 2005)	Goldberg–Shprintzen syndrome	Regulating transport of the mitochondria	Autosomal dominant, sporadic and familial	intestinal aganglionosis (HSCR), mi- crocephaly, mental retardation, facial dysmorphism	Neonatal period
NRG1 (Neurugulin 1) (Garcia-Barcelo et al., 2009)	Isolated HSCR	A member of the epidermal growth factor family of receptor tyrosine kinase protein ligands, involved in activation of proliferation, survival, and differentiation neuron	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
ERBB2 (ErbB-2 tyrosine kinase) or HER2 (Human Epidermal Growth Factor 2) (Tang et al., 2018b)	Isolated HSCR	A member of the epidermal growth factor family of receptor tyrosine kinase protein ligands, involved in activation of proliferation, survival, and differentiation neuron	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
SEMA3C/D (Semaphorin 3 C/D) (Tilghman et al., 2019)	Isolated HSCR	A glycoprotein belongs to the semaphoring class 3 family of neuronal guidance cues	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
GLI1 (GLI Family Zinc Finger 1) (Ngan et al., 2011)	Isolated HSCR	An effector of Hh pathway, Regulate stem cell proliferation	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
GLI2 (GLI Family Zinc Finger 2) (Ngan et al., 2011)	Isolated HSCR	An effector of Hh pathway, , Regulate stem cell proliferation	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
GLI3 (GLI Family Zinc Finger 3) (Ngan et al., 2011; Sribudiani et al., 2018)	Isolated HSCR	An effector of Hh pathway, , Regulate stem cell proliferation	Autosomal dominant, sporadic and familial	intestinal aganglionosis (HSCR)	Neonatal period
L1CAM (L1 Cell Adhesion Molecule)	X-linked hydrocephalus with HSCR	Encodes an axonal glycoprotein belonging to the immunoglobulin supergene family. L1CAM is a cell	X-linked	intestinal aganglionosis (HSCR)	Neonatal period

(Takenouchi et al., 2012)		adhesion molecule that plays role in neuronal migration and differentiation			
ARID1B (AT-Rich Interaction Domain 1B) (Takenouchi et al., 2016)	Isolated HSCR	A component of SNI/SNF chromatin remodelling complex, play role in cell cycle activation	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR), congenital cataracts	Neonatal period
ITGB4 (Intergrin Subunit Beta 4)(Tang et al., 2018a)	Isolated HSCR	A receptor for the Laminins. ITBG4 mediates cell-matrix or cell-cell adhesion, and transduced signals that regulate gene expression and cell growth.	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
PTK2 (Protein Tyrosine Kinase 2) (Tang et al., 2018a)	Isolated HSCR	PTK2 plays important early step in cell growth and intracellular signal transduction pathways triggered in response to certain neural peptides or to cell interactions with the extracellular matrix.	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
DENND3 (DENN Domain Containing 3) (Gui et al., 2017)	Isolated HSCR	A protein coding gene related to pathways such as vesicle mediated transport and Rab regulation of trafficking	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
NCLN (Nicalin) (Gui et al., 2017)	Isolated HSCR	A protein complex that antagonizes Nodal signalling which in vertebrates is involved in induction of the mesoderm and endoderm	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
NUP98 (Nucleoporin 96 and 98 Precursor) (Gui et al., 2017)	Isolated HSCR	NUP98 is involved in transcriptional regulation of the HSCR genes SEMA3A, DSCAM, NRG1, and the NRG1 receptor ERBB4 in human neural progenitor cells	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
TBATA (Thymus, Brain, And Testes Associated) (Gui et al., 2017)	Isolated HSCR	TBATA is expressed in early differentiating neurons.	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
VCL (Vinculin) (Lai et al., 2017)	Isolated HSCR	Involved in anchoring F-actin to the membrane	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
BACE2 (Beta Secretase 2) (Tang et al., 2018b)	Isolated HSCR	An integral membrane glycoprotein that functions as an aspartic protease. BACE2 is crucial for ENCCs survival	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
ACSS2 (Acyl-CoA Synthetase Short Chain Family Member 2) (Tilghman et al., 2019)	Isolated HSCR	A cytosolic enzyme that catalyzes the activation of acetate for use in lipid synthesis and energy generation	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
ENO3 (Enolase 3) (Tilghman et al., 2019)	Isolated HSCR	ENO3 and its family play a role in skeletal muscle development and regeneration	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
SH3PXD2A (SH3 and PX Domains 2A) (Tilghman et al., 2019)	Isolated HSCR	Involved in osteoclast fusion and superoxide metabolic process.	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period
UBR4 (Ubiquitin Protein Ligase E3 Component N-Recognin 4) (Tilghman et al., 2019)	Isolated HSCR	UBR4 appears to be a cytoskeletal component in the cytoplasm and part of the chromatin scaffold in the nucleus.	Autosomal dominant, sporadic	intestinal aganglionosis (HSCR)	Neonatal period

RAD21 (RAD21 Cohesin Complex Component) (Bonora et al., 2015)	Mungan syndrome	Part of a cohesion complex that controls pairing and unpairing in cell replication. Plays an important role in epithelial and neuronal survival and ABOP regulation in the gastrointestinal tract	Autosomal recessive	Pseudo-obstruction, megaduodenum, long segment Barrett's esophagus and cardiac abnormalities	1st–2nd decade of life
SGOL1 (Shugosin-like 1) (Chetaille et al., 2014)	Chronic atrial and intestinal dysrhythmia (CAID)	Component of the cohesion pathway	Autosomal recessive	Accelerated cell cycle progression and enhanced activation of TGF-B signaling leading to changes in both the enteric nervous system and smooth muscle	1st to 4th decade of life

Supplementary Table 2. Genetic causes of myopathic PIPO

Gene	Disorder	Function	Inheritance	Phenotype	Age of onset
FLNA (filamin A) (Gargiulo et al., 2007; Kapur et al., 2010; Van Der Werf et al., 2013)	Chronic idiopathic intestinal pseudo-obstruction (CIIPX), Congenital Short Bowel Syndrome (CSBS)	Encodes large cytoskeletal proteins	X-linked recessive	Abnormal filamin A leads to cytoskeletal abnormalities and potentially disrupts enteric-neuron structure and function. Seizures and progressive abdominal distension and obstruction	Neonatal period
ACTG2 (enteric smooth muscle actin–gamma 2)(Halim et al., 2016; Milunsky et al., 2017)	Familial visceral myopathy; megacystis-microcolon-intestinal hypoperistalsis syndrome	Encodes enteric smooth muscle Actin	Autosomal dominant, sporadic	Altered ACTG2 protein in the muscularis propria leads to impaired contractility	Neonatal, 3rd decade in life
MYH11 (myosin heavy	Megacystis-microcolon-intestinal	Encodes myosin light chain	Autosomal recessive	Abnormal MYH11 in smooth muscle myosin leads to impaired contractility	Neonatal-3rd decade in life

chain 11) (Dong et al., 2019)	hypoperistalsis syndrome)				
MYLK (myosin light chain kinase)(Halim et al., 2017a)	Megacystis-microcolon-intestinal hypoperistalsis syndrome	Encodes a kinase required for myosin activation and subsequent interaction with actin filaments	Autosomal recessive	Abnormal MYLK leads to impaired smooth muscle cell contraction	Neonatal-3rd decade in life
LMOD1 (leiomodin 1)(Halim et al., 2017b)	Megacystis-microcolon-intestinal hypoperistalsis syndrome	Encodes visceral smooth muscle cells	Sporadic	Abnormal LMOD1 leads to impaired intestinal smooth muscle contractility	Neonatal-3rd decade in life
MYL9 (myosin regulatory light chain 9) (Moreno et al., 2018)	Megacystis-microcolon-intestinal hypoperistalsis syndrome	Encodes a regulatory myosin light chain	Autosomal recessive	Abnormal MYL9 leads to impaired intestinal smooth muscle contractility	Neonatal-3rd decade in life

Supplementary Table References

Bonora, E., Bianco, F., Cordeddu, L., Bamshad, M., Francescatto, L., Dowless, D., et al. (2015). Mutations in RAD21 disrupt regulation of apob in patients with chronic intestinal pseudo-obstruction. *Gastroenterology* 148, 771-782.e11. doi:10.1053/j.gastro.2014.12.034.

Borghini, S., Bocciardi, R., Bonardi, G., Matera, I., Santamaria, G., Ravazzolo, R., et al. (2002). Hirschsprung associated GDNF mutations do not prevent RET activation. *Eur. J. Hum. Genet.* 10, 183–187. doi:10.1038/sj.ejhg.5200785.

Brooks, A. S., Bertoli-Avella, A. M., Burzynski, G. M., Breedveld, G. J., Osinga, J., Boven, L. G., et al. (2005). Homozygous nonsense mutations in KIAA1279 are associated with malformations of the central and enteric nervous systems. *Am. J. Hum. Genet.* 77, 120–126. doi:10.1086/431244.

Chetaille, P., Preuss, C., Burkhard, S., Côté, J. M., Houde, C., Castilloux, J., et al. (2014). Mutations in SGOL1 cause a novel cohesinopathy affecting heart and gut rhythm. *Nat. Genet.* 46, 1245–1248. doi:10.1038/ng.3113.

Dong, W., Baldwin, C., Choi, J., Milunsky, J. M., Zhang, J., Bilguvar, K., et al. (2019). Identification of a dominant MYH11 causal variant in chronic intestinal pseudo-obstruction: Results of whole-exome sequencing. *Clin. Genet.* 96, 473–477. doi:10.1111/cge.13617.

Fadista, J., Lund, M., Skotte, L., Geller, F., Nandakumar, P., Chatterjee, S., et al. (2018). Genome-wide association study of Hirschsprung disease detects a novel low-frequency variant at the RET locus. *Eur. J. Hum. Genet.* 26, 561–569. doi:10.1038/s41431-017-0053-7.

Fernández, R. M., Mathieu, Y., Luzón-Toro, B., Núñez-Torres, R., González-Meneses, A., Antiñolo, G., et al. (2013). Contributions of PHOX2B in the Pathogenesis

of Hirschsprung Disease. *PLoS One* 8. doi:10.1371/journal.pone.0054043.

Garavelli, L., and Mainardi, P. C. (2007). Mowat-Wilson syndrome. *Orphanet J. Rare Dis.* 2, 1–12. doi:10.1186/1750-1172-2-42.

Garcia-Barcelo, M. M., Tang, C. S. M., Ngan, E. S. W., Lui, V. C. H., Chen, Y., So, M. T., et al. (2009). Genome-wide association study identifies NRG1 as a susceptibility locus for Hirschsprung's disease. *Proc. Natl. Acad. Sci. U. S. A.* 106, 2694–2699. doi:10.1073/pnas.0809630105.

Garcia-Barceló, M., Sham, M. H., Lee, W. S., Lui, V. C. H., Chen, B. L. S., Wong, K. K. Y., et al. (2004). Highly Recurrent RET Mutations and Novel Mutations in Genes of the Receptor Tyrosine Kinase and Endothelin Receptor B Pathways in Chinese Patients with Sporadic Hirschsprung Disease. *Clin. Chem.* 50, 93–100. doi:10.1373/clinchem.2003.022061.

Gargiulo, A., Auricchio, R., Barone, M. V., Cotugno, G., Reardon, W., Milla, P. J., et al. (2007). Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. *Am. J. Hum. Genet.* 80, 751–758. doi:10.1086/513321.

Gui, H., Schriemer, D., Cheng, W. W., Chauhan, R. K., Antiňolo, G., Berrios, C., et al. (2017). Whole exome sequencing coupled with unbiased functional analysis reveals new Hirschsprung disease genes. *Genome Biol.* 18, 1–13. doi:10.1186/s13059-017-1174-6.

Halim, D., Brosens, E., Muller, F., Wangler, M. F., Beaudet, A. L., Lupski, J. R., et al. (2017a). Loss-of-Function Variants in MYLK Cause Recessive Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *Am. J. Hum. Genet.* 101, 123–129. doi:10.1016/j.ajhg.2017.05.011.

Halim, D., Hofstra, R. M. W., Signorile, L., Verdijk, R. M., Van Der Werf, C. S., Sribudiani, Y., et al. (2016). ACTG2 variants impair actin polymerization in sporadic Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *Hum. Mol. Genet.* 25, 571–583. doi:10.1093/hmg/ddv497.

Halim, D., Wilson, M. P., Oliver, D., Brosens, E., Verheij, J. B. G. M., Han, Y., et al. (2017b). Loss of LMOD1 impairs smooth muscle cytocontractility and causes megacystis microcolon intestinal hypoperistalsis syndrome in humans and mice. *Proc. Natl. Acad. Sci. U. S. A.* 114, E2739–E2747. doi:10.1073/pnas.1620507114.

Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., et al. (1998). Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. *Proc. Natl. Acad. Sci. U. S. A.* 95, 5161–5165. doi:10.1073/pnas.95.9.5161.

Kapur, R. P., Robertson, S. P., Hannibal, M. C., Finn, L. S., Morgan, T., Van Kogelenberg, M., et al. (2010). Diffuse abnormal layering of small intestinal smooth muscle is present in patients with FLNA mutations and X-linked intestinal pseudo-obstruction. *Am. J. Surg. Pathol.* 34, 1528–1543. doi:10.1097/PAS.0b013e3181f0ae47.

Lai, F. P. L., Lau, S. T., Wong, J. K. L., Gui, H., Wang, R. X., Zhou, T., et al. (2017). Correction of Hirschsprung-Associated Mutations in Human Induced Pluripotent Stem Cells Via Clustered Regularly Interspaced Short Palindromic Repeats/Cas9, Restores Neural Crest Cell Function. *Gastroenterology* 153, 139-153.e8. doi:10.1053/j.gastro.2017.03.014.

Milunsky, A., Baldwin, C., Zhang, X., Primack, D., Curnow, A., and Milunsky, J. (2017). Diagnosis of Chronic Intestinal Pseudo-obstruction and Megacystis by Sequencing the ACTG2 Gene. *J. Pediatr. Gastroenterol. Nutr.* 65, 384–387. doi:10.1097/MPG.0000000000001608.

Moreno, C. A., Sobreira, N., Pugh, E., Zhang, P., Steel, G., Torres, F. R., et al. (2018). Homozygous deletion in MYL9 expands the molecular basis of megacystis-microcolon-intestinal hypoperistalsis syndrome. *Eur. J. Hum. Genet.* 26, 669–675. doi:10.1038/s41431-017-0055-5.

Ngan, E. S. W., Garcia-Barceló, M. M., Yip, B. H. K., Poon, H. C., Lau, S. T., Kwok, C. K. M., et al. (2011). Hedgehog/notch-induced premature gliogenesis represents a new disease mechanism for Hirschsprung disease in mice and humans. *J. Clin. Invest.* 121, 3467–3478. doi:10.1172/JCI43737.

Robert, H., Valdenaire, O., Arch, E., Osinga, J., Kroes, H., Loffler, B.-M., et al. (1999). A loss-of-function mutation in the Endothelin-Converting Enzyme 1 (ECE1) associated with Hirschsprung disease, cardiac defect, and autonomic dysfunction. *Am. J. Hum. Genet.* 64, 304–308. doi:10.2959/logo.2002.13.2.109.

Ruiz-Ferrer, M., Torroglosa, A., Luzón-Toro, B., Fernández, R. M., Antiñolo, G., Mulligan, L. M., et al. (2011). Novel mutations at RET ligand genes preventing receptor activation are associated to Hirschsprung's disease. *J. Mol. Med.* 89, 471–480. doi:10.1007/s00109-010-0714-2.

Sánchez-Mejías, A., Watanabe, Y., Fernández, R. M., López-Alonso, M., Antiñolo, G., Bondurand, N., et al. (2010). Involvement of SOX10 in the pathogenesis of Hirschsprung disease: Report of a truncating mutation in an isolated patient. *J. Mol. Med.* 88, 507–514. doi:10.1007/s00109-010-0592-7.

Sribudiani, Y., Chauhan, R. K., Alves, M. M., Petrova, L., Brosens, E., Harrison, C., et al. (2018). Identification of Variants in RET and IHH Pathway Members in a Large Family With History of Hirschsprung Disease. *Gastroenterology* 155, 118-129.e6. doi:10.1053/j.gastro.2018.03.034.

Takenouchi, T., Nakazawa, M., Kanemura, Y., Shimozato, S., Yamasaki, M., Takahashi, T., et al. (2012). Hydrocephalus with Hirschsprung disease: Severe end of X-linked hydrocephalus spectrum. *Am. J. Med. Genet. Part A* 158 A, 812–815. doi:10.1002/ajmg.a.35245.

Takenouchi, T., Yoshihashi, H., Sakaguchi, Y., Uehara, T., Honda, M., Takahashi, T., et al. (2016). Hirschsprung disease as a yet undescribed phenotype in a patient with ARID1B mutation. *Am. J. Med. Genet. Part A* 170, 3249–3252. doi:10.1002/ajmg.a.37861.

Tang, C. S. M., Zhuang, X., Lam, W. Y., Ngan, E. S. W., Hsu, J. S., Michelle, Y. U., et al. (2018a). Uncovering the genetic lesions underlying the most severe form of Hirschsprung disease by whole-genome sequencing. *Eur. J. Hum. Genet.* 26, 818–826. doi:10.1038/s41431-018-0129-z.

Tang, C. S. man, Li, P., Lai, F. P. L., Fu, A. X., Lau, S. T., So, M. T., et al. (2018b). Identification of Genes Associated With Hirschsprung Disease, Based on Whole-Genome Sequence Analysis, and Potential Effects on Enteric Nervous System Development. *Gastroenterology* 155, 1908-1922.e5. doi:10.1053/j.gastro.2018.09.012.

Tilghman, J. M., Ling, A. Y., Turner, T. N., Sosa, M. X., Krumm, N., Chatterjee, S., et al. (2019). Molecular Genetic Anatomy and Risk Profile of Hirschsprung's Disease. *N. Engl. J. Med.* 380, 1421–1432. doi:10.1056/nejmoa1706594.

Van Der Werf, C. S., Sribudiani, Y., Verheij, J. B. G. M., Carroll, M., O'Loughlin, E., Chen, C. H., et al. (2013). Congenital short bowel syndrome as the presenting symptom in male patients with FLNA mutations. *Genet. Med.* 15, 310–313. doi:10.1038/gim.2012.123.

Chapter 2

TFAP2B haploinsufficiency impacts gastrointestinal function and leads to Pediatric Intestinal Pseudo-obstruction

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ABSTRACT

Pediatric Intestinal Pseudo-obstruction (PIPO) is a congenital enteric disorder characterized by severe gastrointestinal (GI) dysmotility, without mechanical obstruction. Although several genes have been described to cause this disease, most patients do not receive a genetic diagnosis. Here, we aim to identify the genetic cause of PIPO in a patient diagnosed with severe intestinal dysmotility shortly after birth. Whole exome sequencing (WES) was performed in the patient and unaffected parents, in a diagnostic setting. After identification of the potential disease-causing variant, its functional consequences were determined in vitro and in vivo. For this, expression constructs with and without the causing variant, were overexpressed in HEK293 cells. To investigate the role of the candidate gene in GI development and function, a zebrafish model was generated where its expression was disrupted using CRISPR/Cas9 editing. WES analysis identified a de novo heterozygous deletion in TFAP2B (NM_003221.4:c.602-5_606delTCTAGTTCCA), classified as a variant of unknown significance. In vitro studies showed that this deletion affects RNA splicing and results in loss of exon 4, leading to the appearance of a premature stop codon and absence of TFAP2B protein. Disruption of tfap2b in zebrafish led to decreased enteric neuronal numbers and delayed transit time. However, no defects in neuronal differentiation were detected. tfap2b crispants also showed decreased levels of ednrbb mRNA, a downstream target of tfap2b. We showed that TFAP2B haploinsufficiency leads to reduced neuronal numbers and GI dysmotility, suggesting for the first time that this gene is involved in PIPO pathogenesis.

Keywords: Chronic intestinal pseudo-obstruction; enteric nervous system; intestinal motility; crispant; ednrbb

INTRODUCTION

Chronic intestinal pseudo-obstruction (CIPO) is characterized by severe intestinal dysmotility imitates mechanical obstruction, and mainly affects small bowel and colon (Di Nardo et al., 2017). CIPO represents a spectrum of heterogenous disorders with multiple pathological mechanisms, affecting the structure and/or function of the intestinal neuromuscular and interstitial cells of Cajal (Downes et al., 2018; Knowles et al., 2010). CIPO has been recognized in adult and children, where its etiology, onset, clinical features, and natural history harbor a fundamental difference. Therefore, when diagnosed in children, CIPO is considered a separate entity and referred to as pediatric intestinal pseudo-obstruction (PIPO) (Thapar et al., 2018). Overlapping histopathological features with other gastrointestinal neuromuscular disorders, lack of molecular biomarkers and no single diagnostic test which is pathognomonic for CIPO/PIPO, hinder this disorder to be early diagnosed (Downes et al., 2018). Consequently, most cases remain undiagnosed until advanced stages of disease. In addition, only few epidemiological studies reporting CIPO prevalence in the pediatric population have been documented (Muto et al., 2014), thus the prevalence of PIPO worldwide is difficult to estimate. From a genetic perspective, the majority of PIPO cases are sporadic with a de novo dominant pattern of inheritance (Gamboa & Sood, 2019). Mutations in FLNA following an X-linked pattern of inheritance (Gargiulo et al., 2007; Jenkins et al., 2018; Van Der Werf et al., 2013) and autosomal dominant mutations in ACTG2 (Halim et al., 2016; Milunsky et al., 2017; Ravenscroft et al., 2018) have been identified in PIPO patients. In addition, mutations in TYMP (Nishino et al., 1999), CLMP (Van Der Werf et al., 2012), POLG1 (Giordano et al., 2009; Van Goethem et al., 2003), SGOL1 (Chetaille et al., 2014) and RAD21 (Bonora et al., 2015), which follow an autosomal recessive inheritance pattern, have also been linked to this disease. Despite the identification of several genes, for the majority of PIPO patients, the underlying genetic cause and molecular mechanisms remain unknown.

Here, we report for the first time, a PIPO patient carrying a de novo heterozygous deletion in the Transcription Factor Activating Protein-2 Beta (TFAP2B) gene (NM_003221.4:c.602-5_606delTCTAGTTCCA). TFAP2B encodes for a member of the AP-2 transcription factors family, which in mammals include TFAP2A, TFAP2B, TFAP2C, TFAP2D and TFAP2E (Hilger-Eversheim et al., 2000). TFAP2B is highly expressed in migrating neural crest cells (NCCs) during embryonic development of vertebrates (Hong et al., 2011; Schmidt et al., 2011; Seok et al., 2008). Since NCCs give rise to various tissues including peripheral neurons and glia, cardiovascular tissue, melanocytes, craniofacial bone and teeth, cartilage, and connective tissue (Prasad et al., 2010), variants in TFAP2B have been associated with different disorders, often classified as neurocristopathies. Char syndrome (OMIM#169100), characterized by patent ductus arteriosus (PDA), facial dysmorphism and hand anomalies, was the first known congenital genetic disorder

linked to TFAP2B. This syndrome is caused by heterozygous missense variants in the gene, suspected to result in a dominant negative effect (Satoda et al., 2000; Zhao et al., 2001; Edward et al., 2019). The same type of variants have also been reported in patients with severe dental anomalies and incomplete penetrance of PDA (Tanasubsinn et al., 2017), as well as in a pediatric Char patient diagnosed with central diabetes insipidus, scoliosis and hearing loss (Edward et al., 2019). Moreover, TFAP2B loss of function variants have been linked to Char syndrome with variable expressivity of PDA (Mani et al., 2005), familial and non-familial isolated PDA (Chen et al., 2011; Ji et al., 2014; Khetyar et al., 2008; Xiong et al., 2013) and syndromic craniosynostosis (Timberlake et al., 2019). Genome wide association studies and animal models have also shown an involvement of TFAP2B in metabolic syndromes and obesity risk (Kraja et al., 2014), as well as retinal (Jin et al., 2015) and renal development (Moser et al., 2003). However, this gene has never been involved in GI complaints, despite being recently described as one of the regulators of the Endothelin Receptor Type B (EDNRB) expression (Ling & Sauka-Spengler, 2019), a known Hirschsprung disease (HSCR) gene involved in GI development and function (Bondurand et al., 2018).

MATERIAL AND METHODS

Patient characteristics

The first child of non-consanguineous Dutch parents, a boy, presented shortly after birth with biliary vomiting. One day post-partum, an abdominal X-ray showed distended bowel loops without air in the rectum. A contrast study was performed and showed slow passage from stomach to duodenum. A laparotomy also showed distended small bowel loops, which gradually tapered to a small lumen. Full-thickness biopsies were taken on different sites of the intestine, which showed normal ganglion cells, ruling out HSCR. Since this procedure, spontaneous passage of feces was hardly seen. At a few months of age, an antro-duodenal manometry study was performed overnight, showing abnormal small bowel motor activity, suggestive of a neuropathic phenotype. Unfortunately, colonic manometry was aborted due to a small caliber colon and risk of bleeding and perforation. A venting gastrotomy was also performed to discharge gastric fluid. Currently, the patient still alive, but total parenteral nutrition is given to support feeding. Informed consent was obtained from the parents for diagnostic genetic analysis.

DNA isolation, whole exome sequencing analysis and variant interpretation

Peripheral blood from patient and parents were taken and used for DNA isolation using the Chemagic DNA Blood 4k Kit (PerkinElmer, Waltham, MA, USA). Three micrograms of double-stranded DNA were fragmented (Covaris, Woburn, MA, USA) and exonic sequences were captured using the Agilent SureSelect Clinical Research Exome V2 (Agilent, Santa Clara, CA, USA).

Paired-end sequencing was performed on a HiSeq 4000 platform (150bp paired end) and an average coverage of at least 50X. Reads were mapped against the human reference genome GRCh37/hg19 with the Burrows-Wheeler Aligner (Li & Durbin, 2009), and variants were called using the Genome Analysis toolkit (Broad Institute, Cambridge, MA, USA). Alissa Interpret software (Agilent, Santa Clara, CA, USA) was used to filter and prioritize variants. Variants were classified according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants (Richards et al., 2015).

Sanger sequencing of TFAP2B

For validation of the de novo deletion identified in TFAP2B, Sanger sequencing was performed as previously described (Sribudiani et al., 2018), using the following primers: 5' CCTGGTTCCCAGCACAGTCC 3' (forward) and 5' CATTCAGGGGGCGACAGC 3' (reverse). Touchdown 650C-550C PCR program was used to amplify target genomic regions. PCR products underwent ExoSAP treatmentBDT reaction, and were sequenced on both strands using the BigDye v3.1 kit (Life Technologies, Carlsbad, CA, USA). Electropherograms were visualized with Chromas Lite v 2.1 (www.technelysium.com.au).

TFAP2B minigene for exon trapping assay

An in vitro splicing assay was carried out using a pSPL3 exon-trapping vector (Addgene, Watertown, MA, USA). Briefly, the pSPL3 vector contains a small artificial gene composed of a SV40 promoter and exon SD6-intron-exon SA2 sequence with functional splice donor and acceptor sites. A late polyadenylation signal is also present. Genomic DNA fragment from control and patient that comprises entire TFAP2B exon 4 plus additional 233 basepairs (bp) (5') and 309 bp (3') of the flanking intronic region was amplified by PCR, with primers containing additional XhoI (forward) and BamHI (reverse) restriction sites. The following primers: 5' CATATACTCGAGCCTGGTTCCCAGCACAGTCC 3' (forward) and 5' TATCGTGGATCCCATTCAGGGGGGCGACAGC 3' (reverse) were used for this purpose. After PCR amplification, products were purified and subjected to restriction enzyme digestion. They were subsequently inserted into the pSPL3 vector to create a minigene construct. The minigene constructs were Sanger sequenced to confirm the presence of the wild type and mutant DNA fragments.

Expression vectors

pCMV-Myc-tagged AP2 beta (TFAP2B) was purchased from Origene (OriGene, Rockville, Maryland, USA). The following TFAP2B variants: deletion of whole exon 4, c.706 C>T, c.898 C>T

and c.1144 C>T were generated by site directed mutagenesis according to the Q5 Site Directed Mutagenesis manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Primers used in site directed mutagenesis experiment were listed in Supplementary Materials Table 1. Following mutagenesis, the entire TFAP2B insert was evaluated by Sanger sequencing, using the following primers: 5' GCATGGGTGACAGCCTCTCG 3' (forward) and 5' GGTCACTCGGGTCTGTGTC 3' (reverse).

Cell Culture and transfection

Human Embryonic Kidney cells (HEK293) were cultured in DMEM (Lonza, Basel, Switzerland), supplemented with 10% fetal calf serum (Sigma-Aldrich, Burlington, MA, USA) and 1% penicillin/streptomycin (Gibco-Life Technologies, Renfrewshire, UK). Cells were maintained at 37oC and 5% CO2. For transient transfection, 500,000 cells were seeded in 6 well-plates. Twenty-four hours later, cells were transfected with the wild type and mutant constructs, using GeneJuice Transfection Reagent (MilliporeSigma, Burlington, MA, USA), according to the manufacturer's instructions. Experiments were performed in duplicate.

RNA isolation, cDNA preparation and qRT-PCR

Total RNA was harvested 48 hours post-transfection using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was quantified using the NanoDrop (Thermo Fisher Scientific, Waltham, MA USA) and 1 ug RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. Gene expression levels of TFAP2B were measured by quantitative real time (qRT)-PCR using iTaq universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The following primers: 5' TATGAGGACCGGCACGATG 3' (forward) and 5' GTAGGGCGGCTGGAAGTC 3' (reverse) were used for amplifying the TFAP2B transcript. GAPDH (5' CGACCTTCACCTTCCCCAT 3' (forward) and 5' TAAAAGCAGCCCTGGTGACC 3' (reverse))and β-Actin (5' AACCGCGAGAAGATGACCC 3' (forward primer) and 5' GCCAGAGGCGTACAGGGATAG 3' (reverse primer)) were used as housekeeping genes. Two independent experiments were performed for statistical analysis.

Cell lysates and Western Blot analysis

Protein lysates were collected 48 hours post-transfection. Cells were washed with PBS and incubated with a lysis buffer containing 150mM NaCl, 20mMTris, 1% Triton X, 1x protease inhibitors cOmpleteTM (Roche, Basel, Switzerland), for 30 minutes on ice. Lysates were stored at -80oC. Protein quantification was performed using the Pierce BCA kit (Thermo Fisher Scientific, Waltham, MA, USA) and 40ug of protein was loaded into a criterion TGX precast gel (Bio-Rad,

Hercules, CA, USA). The following primary antibodies were used: Myc 1:3000 (Cell Signaling Technology, Danvers, MA, USA), β-Actin 1: 1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GFP 1:2000 (AbCam, Cambridge, UK). Secondary antibodies used were IRDye 800CW Goat anti mouse (Li-Cor, Lincoln, NE, USA) and IRDye 680RD Goat anti-Rabbit (Li-Cor, Lincoln, NE, USA).

Zebrafish larvae maintenance

Zebrafish Tg (phox2bb:GFP) embryos and larvae (Nechiporuk et al., 2007) were kept at 28oC on a 14-10 hour light/dark cycle in 1 M HEPES buffered (pH 7.2) E3 medium (34.8 g NaCl, 1.6 g KCL, 5.8 g CaCl2.2H2O, 9.78 g MgCl2). For quantification of enteric neurons, E3+ 0,003% 1-phenyl 2-thiourea (PTU) was added to the medium at 1 day post fertilization (dpf) to prevent pigmentation. Animal experiments were approved by the Animal Experimentation Committee of the Erasmus MC, Rotterdam (No. AVD1010020209425).

Zebrafish crispant tfap2b generation

tfap2b specific guide RNA (gRNA) was designed to target the beginning of exon 4 (5' CGTCAACGAGGTTTTCTGCT 3', https://www.idtdna.com/). The synthesized gRNA (1 µl) was mixed with 4 ng of Cas9-nuclease and the final volume was adjusted to 6 µL with 300 mM KCl. Approximately, 1 nL of the mix was injected into a one-cell stage fertilized zebrafish oocytes. Genomic DNA surrounding the gRNA target site was Sanger sequenced and efficiency of insertions/deletions (InDels) was determined using the online tool TIDE (shinyapps.datacurators.ni/tide/), as previously described (Brinkman et al., 2014; Kuil et al., 2019). For neuronal quantification and intestinal transit assay, gRNA efficiency was evaluated for each individual larva. For gene transcript expression analysis, the gRNA efficiency was determined from a pool of 25 larvae. We refer to the generation of larvae that are directly injected with gRNA/Cas9 protein complexes as crispants (F0). F0 fish were maintained until adulthood to generate stable line mutants.

Generation of tfap2b+/- mutant zebrafish stable line

Fin clips of founder (F0) fish were used for DNA isolation using a mixture containing Tris-HCl (pH9.0), KCl, Triton X-100, and protease K (Sigma Aldrich, St. Louis, Missouri, USA). After incubation at 550C for one hour, protease K was inactivated at 980C for 10 min. The digested fin mx was used directly as a template for a PCR reaction using a standard PCR touchdown program (650C-550C) and the following primers: 5' TCCACGCACAGTTCCAGTTCC 3' (forward) and 5' ACCCCACCAAGCAGAGACGC 3'(reverse) were used to amplify the genomic target. Sanger sequencing was used to confirm the presence of InDels in tfap2b. Sanger reads were analyzed

using Chromas Lite v 2.1 (www.technelysium.com.au). F0 were crossed out with wild-type fish to generate F1 fish. F1 fish with a heterozygous deletion of 7 bp in exon 4 of tfap2b (ENSDART00000174808.2: c.629-635delTTTTCTG, frame shift) were maintained (Supplementary Figure 1a&1b). For the experiments, F1 fish were crossed, the offspring was used to determine the number of enteric neurons and differentiated enteric neurons, as well as for the intestinal transit time experiments.

Quantification of enteric neuronal numbers in zebrafish

Five dpf F2 zebrafish larvae (phox2bb:GFP) treated with PTU were used to determine the number of enteric neurons in the gut. For imaging, larvae were anaesthetized with 0.016% Tricaine (MS-222) and placed on a 1.8% agarose coated Petridish, to be observed under the fluorescent microscope (Leica M165FC). Fish containing the transgene were selected using the GFP signal and images were taken. The enteric neurons were counted using Image J software (National Institute of Health, Maryland, USA). All larvae were genotyped at the end of the experiment. The number of enteric neurons were also analysed in F0 crispants, cas9 injected and uninjected embryos.

Zebrafish intestinal transit time assay

To increase feeding efficiency, 5dpf F2 larvae were fed for one day with dry food pellet. At 7 dpf, larvae (control group n= 30, crispant group= 29) were fed with a fluorescence pellet generated by mixing the dry food pellet with FluoSpheres carboxylate (Invitrogen, Waltham, MA, USA). After 2 hours, fish with pellet located in zone 1 of the gut (0 hour) were selected and placed in a new Petridish, at 280C. After 16 hours, fish were visualized again with the fluorescent microscope (Leica M165FC) to determine location of the pellet in the gut. All larvae were genotyped at the end of the experiment.

Zebrafish RNA isolation and expression analysis

Zebrafish F0 crispant and uninjected larvae were collected at six different time points during development 8 hours post fertilization (hpf), 1 dpf, 2 dpf, 3 dpf, 4 dpf, 5 dpf, snapped frozen and stored at -80oC (n=50 per time point). RNA from these larvae was isolated using Trizol reagent (Ambion, Austin, TX, USA) and 500 ng of total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacture's protocol. elfa (5' TTGAGAAGAAAATCGGTGGTGCTG 3' (forward primer) and 5' GGAACGGTGTGATTGAGGGAAATTC 3' (reverse primer) and β-actin (5' CGAGCAGGAGATGGGAACC 3' (forward primer) and 5' CAACGGAAACGCTCATTGC 3' (reverse

primer)) were used as housekeeping genes. Comparison of the expression levels of crispants and wild-type embryos was performed for each analyzed developmental stage.Two independent technical replications were performed for the statistical analysis.

Quantification of the proportion of differentiated enteric neurons in zebrafish

To determine the proportion of terminally differentiated enteric neurons, 5 dpf F2 zebrafish larvae (tfap2b+/-, phox2bb:GFP) treated with PTU were immunostained with antiHuC/HuD, a mature neuronal marker. Larvae were incubated on ice for 30 minutes before being fixed in 4% PFA and washed in 1x phosphate buffer solution/ 0.25% Triton X-100 for 1 hour, at room temperature. Whole mount antibody staining was performed according to previous reports (Uyttebroek et al., 2010). Anti HuC/HuD (1:100, A-21271, Invitrogen, Waltham, Massachusetts, USA) was used as primary antibody and Cy3 Mouse IgG (1:500, Thermo Fisher Scientific, Waltham, Massachusetts, USA) as secondary antibody. Larvae were imaged under the confocal microscope (Leica SP5 AOBS, Leica Camera, Wetzlar, Germany). The number of phox2bb:GFP+ and HuC/HuD+ cells were counted using Fiji Image J software. The proportion of differentiated enteric neuron was determined by calculating the ratio number of HuC/HuD+ cells to phox2bb:GFP+ cells. All larvae were genotyped at the end of the experiment.

Statistical analysis

RNA expression results of HEK293-transfected cells and zebrafish were presented as fold change expressions. Potential differences between two groups were evaluated using an unpaired T-test (http://graphpad.com/). The number of enteric neurons present in the zebrafish gut is presented as the number of neurons per 100µm and the enteric neuronal differentiation is presented as ratio of HuC/HuD+ :phox2bb:GFP+ . Potential difference between groups was tested using an unpaired T-test from the GraphPad package. Differences in zone location for the intestinal transit time assay were tested using the Proportion's Test (https://www.medcalc.org/calc/comparison_of_proportions.php).

RESULTS

Identification of a novel TFAP2B deletion in a PIPO patient

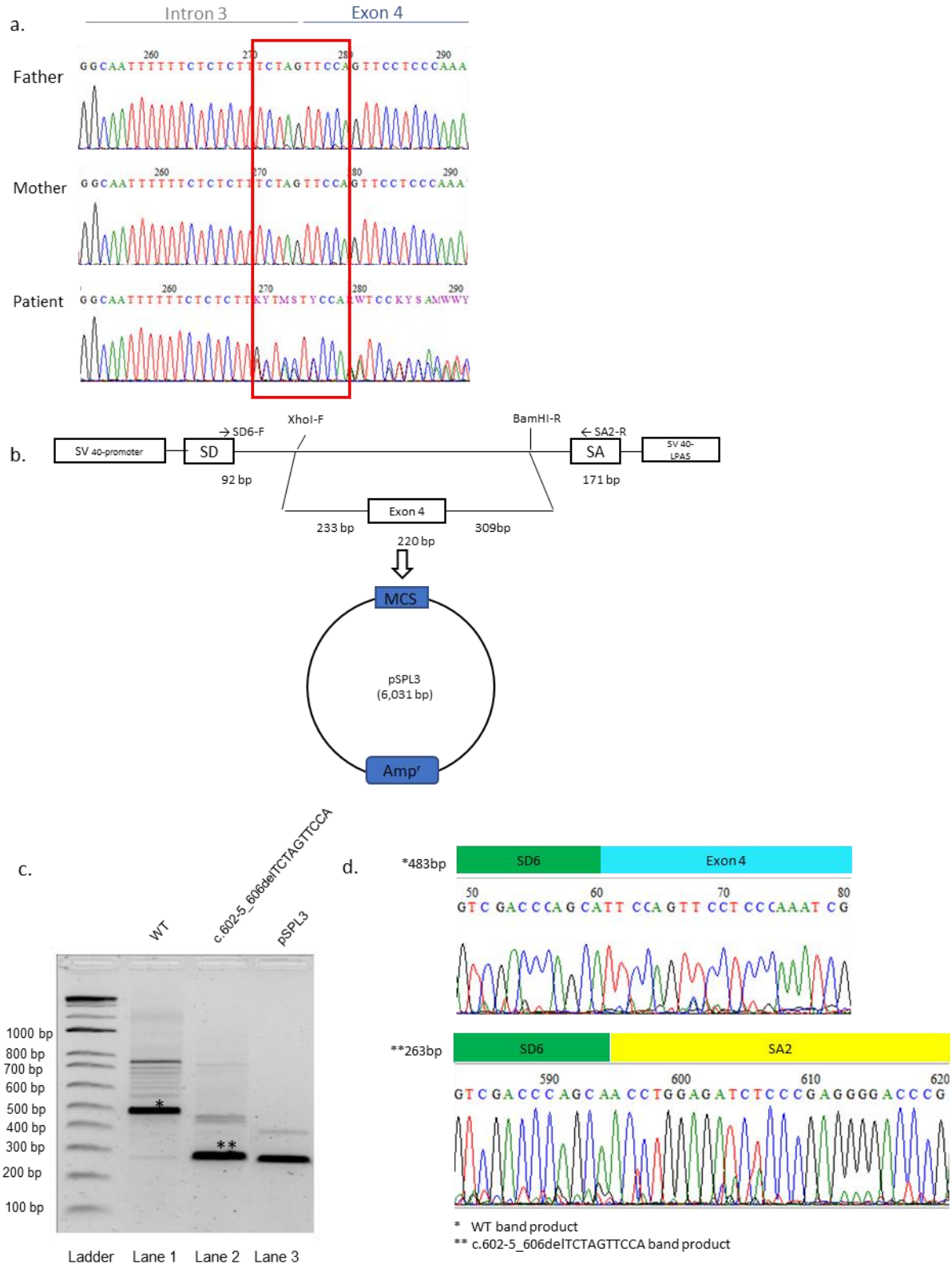
Whole exome sequencing (WES) was performed on genomic DNA isolated from proband and both parents. Data analysis identified a de novo heterozygous deletion in TFAP2B (NM_003221.4:c.602-5_606delTCTAGTTCCA, frame shift) which was located at the intron 3-exon 4 boundary (Figure 1a). This deletion was predicted to affect a canonical splice site and was

therefore, considered ‘potentially deleterious’. However, since this was the first time a variant in this gene was identified in a PIPO patient, it was classified as a variant of unknown significance (VUS). This deletion variant was registered in ClinVar database with accession number SCV002507296. This exact deletion is absent from GnomAD cohort (control cohort), but three loss of function variants in TFAP2B (c.82-1254C>A, MAF=0.000006590; and c.602-2_602delTAG, MAF=0.00001315, in European population; c.419C>A, MAF=0.000006570, in African-American population) have been documented (GnomADv3.1.2). One of these variants (c.602-2_602delTAG) affects the same region deleted in our PIPO patient.

TFAP2B deletion leads to exon skipping and the appearance of a premature stop codon

To test pathogenicity of the TFAP2B deletion, we first evaluated the potential effect of the c.602-5_606delTCTAGTTCCA variant, on splicing. Minigenes (Figure 1b) that carried the wild-type and mutant sequences were transfected into HEK293 cells. Reverse transcriptase (RT)-PCR analysis was performed on total RNA isolated from transfected cells and detected a 483bp band in cells transfected with the wild-type plasmid (Figure 1c). Direct sequencing of this band revealed that it corresponded to exon 4, as expected. In contrast, the mutant minigene showed a shorter fragment which lacked exon 4 (Figure 1d). This deleted exon 4 is located in transactivation domain of TFAP2 family protein (http://us.expasy.org/sprot/), together with other variants described in syndromic craniosysnostosis (Timberlake et al., 2019) and syndromic PDA (Mani et al., 2005) depicted in Figure 1e. Skipping of exon 4 leads to a premature stop codon at position c.863, and therefore we considered this deletion to be pathogenic (Figure 1f).

Figure 1.


a.
Intron 3
Exon 4
Father
Mother
Patient
b.
SV 40-promoter
SD
→ SD6-F
XhoI-F
BamHI-R
← SA2-R
SA
SV 40-LPAS
92 bp
171 bp
Exon 4
233 bp
309bp
220 bp
MCS
pSPL3
(6,031 bp)
Amp^r
c.
WT
c.602-5_606delTCTAGTTCCA
pSPL3
1000 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
Ladder
Lane 1
Lane 2
Lane 3
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SD6
Exon 4
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** c.602-5_606delTCTAGTTCCA band product

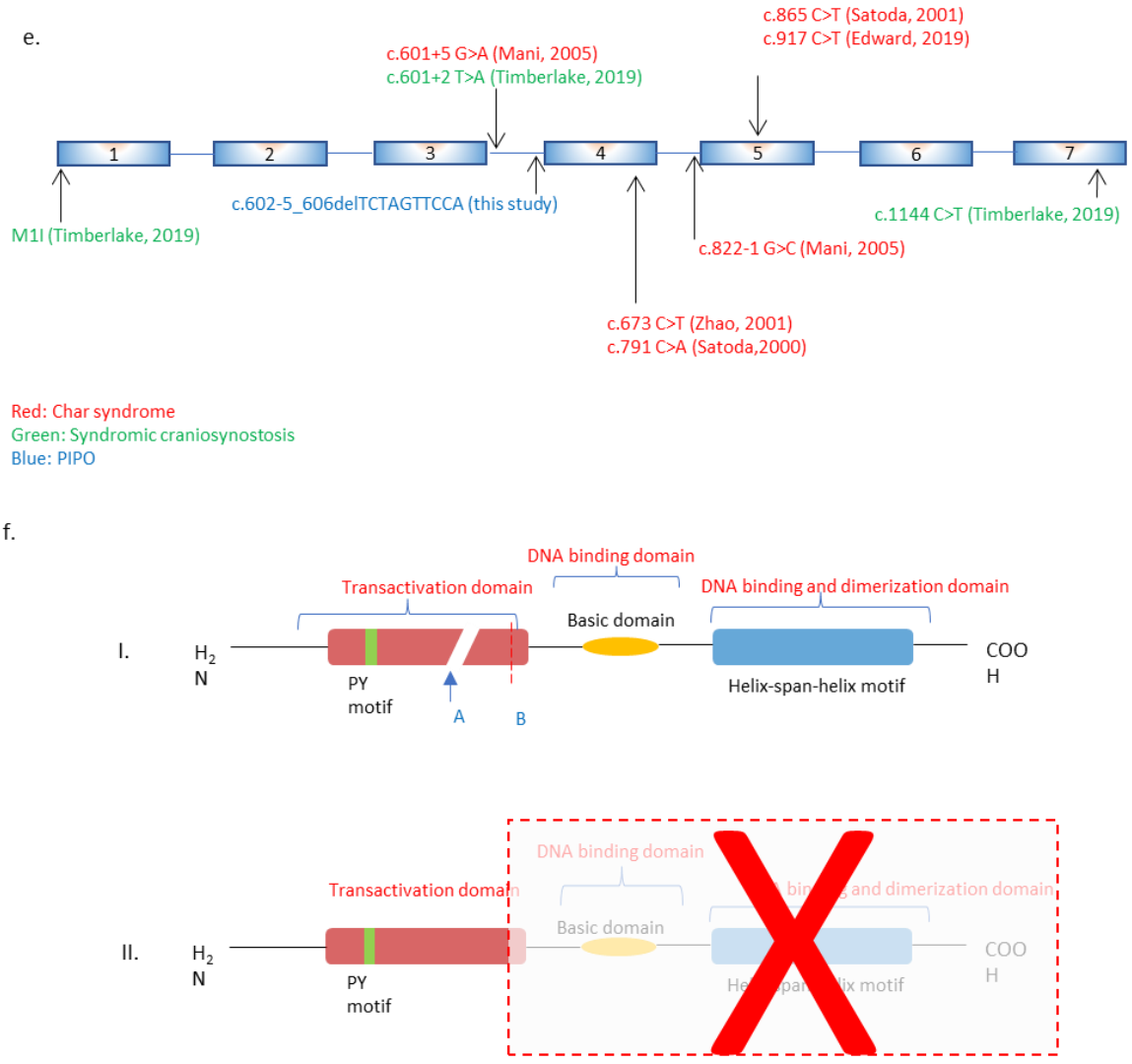


Figure 1. Exon trapping-splice site assay shows that the TFAP2B deletion (c.602-5_606delTCTAGTTCCA) identified in the PIPO patient is deleterious. a) The heterozygous 10 base pair (bp) deletion

(NM_003221.4: c.602-5_606delTCTAGTTCCA, frame shift) harbors the last 5 bp of intron 3 and 5 bp of adjacent exon 4, identified in the PIPO patient (red box). This deletion was not inherited from the father or the mother. b) Schematic representation of the minigene construct for the exon trapping splice assay. pSPL3 vector composed of a SV40 promoter and exon SD6-intron-exon SA2, was used to create a mini gene expression construct containing Exon 4 and its flanking region (233 bp of intron 3 and 309 bp of intron 4) from control and patient DNA. Xhol and BamHI restriction enzyme sites were used. c) RT-PCR of the minigene expression assay shows a 483bp product (*), consisting of SD6 (92bp) +exon4 (220bp)+SA2 (171bp) for cells transfected with the wild-type (WT) construct. Cells transfected with the mutant construct (c.602-5_606delTCTAGTTCCA) show a 263bp product (**), consisting of SD6 (92bp)+SA2 (171 bp). Lane 1, 2, 3 represent WT, c.602-5_606delTCTAGTTCCA, and empty pSPL3, respectively. d) Sanger sequencing of the RT-PCR product band obtained from the WT and mutant constructs, show that exon 4 is spliced out in the presence of the deletion described in this study. e) Schematic representation of TFAP2B with published variants in Char syndrome and syndromic craniosynostosis depicted in red and green, respectively. The variant identified in this study in the PIPO patient is depicted in blue. f) Schematic representation of TFAP2B protein monomer (I). TFAP2B contains 460 amino acids including transactivation domain and DNA binding domain including a dimerization domain (adapted from SwissProt, ID:Q92481). Splicing out exon 4 (A) due to c.602-5_606delTCTAGTTCCA variant located in transactivation domain and resulted in early stop codon at c.863 position in basic domain (B). This rearrangement results in loss of the dimerization domain (II).

Deletion of exon 4 leads to reduced expression levels of TFAP2B in vitro

To study the impact of the exon 4 deletion identified, we determined the expression levels of TFAP2B in HEK293 cells, by overexpressing a construct containing the wild-type TFAP2B and the exon 4 deletion, tagged C-terminally with a Myc-tag. For comparison, we also included two other TFAP2B constructs overexpressing missense variants known to cause Char syndrome (c.706 C>T and c.898 C>T) and one construct containing a nonsense variant identified in craniosynostosis (c.1144 C>T). qRT-PCR analysis of TFAP2B showed a slight increase in mRNA levels for the variants associated with craniosynostosis and CIPO, while missense variants associated to Char syndrome showed a slight decrease of transcript levels when compared to the wildtype (Figure 2a). However, neither of these changes was statistically significant. Subsequent protein analysis showed absence of TFAP2B protein for the constructs containing the deletion identified in the PIPO patient, as well as the nonsense variant identified in craniosynostosis. For both the Char syndrome variants, protein levels of TFAP2B were still detectable and comparable to the wild type (Figure 2b), which is in line with the suspected dominant negative effect previously reported for missense variants associated with this syndrome (Zhao et al., 2011).

Figure 2.

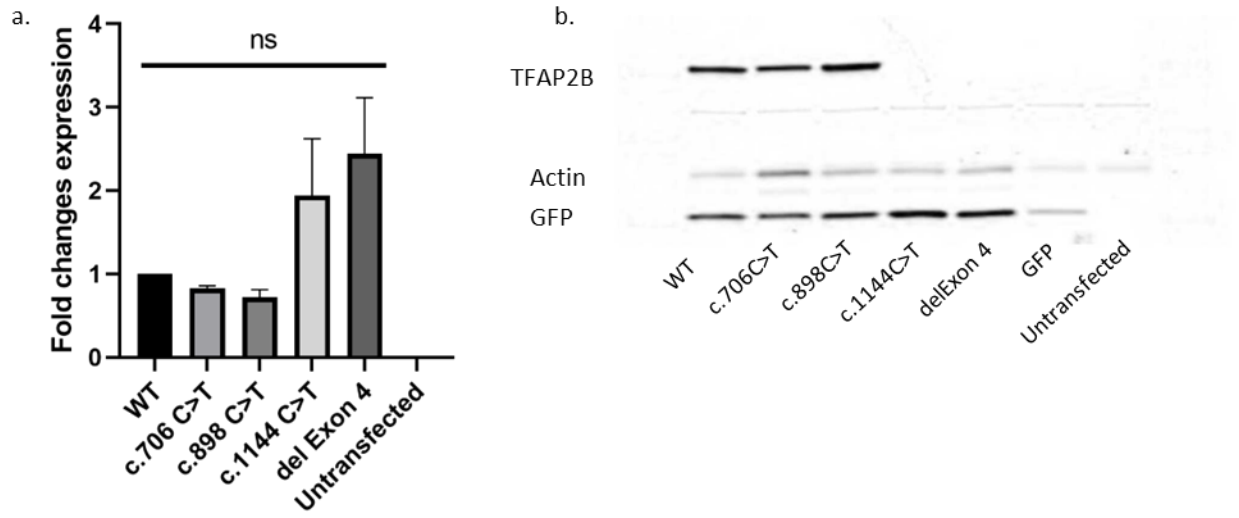


Figure 2. TFAP2B transcript and protein expression analysis. a). qRT-PCR analysis of TFAP2B transcript levels of WT, c.706 C>T, c.898 C>T, c.1144 C>T, and exon 4 deletion expression constructs show no statistically significant difference among the samples normalized to GAPDH and b-Actin. b) Western blot analysis of all tested variants shows absence of protein products for the syndromic craniosynostosis variant (c.1144 C>T) and PIPO variant (del Exon 4). Protein products are still detectable for the Char syndrome variants (c.706 C>T and c.898 C>T). A Myc-tag antibody was used to visualize TFAP2B protein levels.

Loss of tfap2b leads to decrease enteric neuronal numbers and delayed intestinal transit time in a zebrafish model

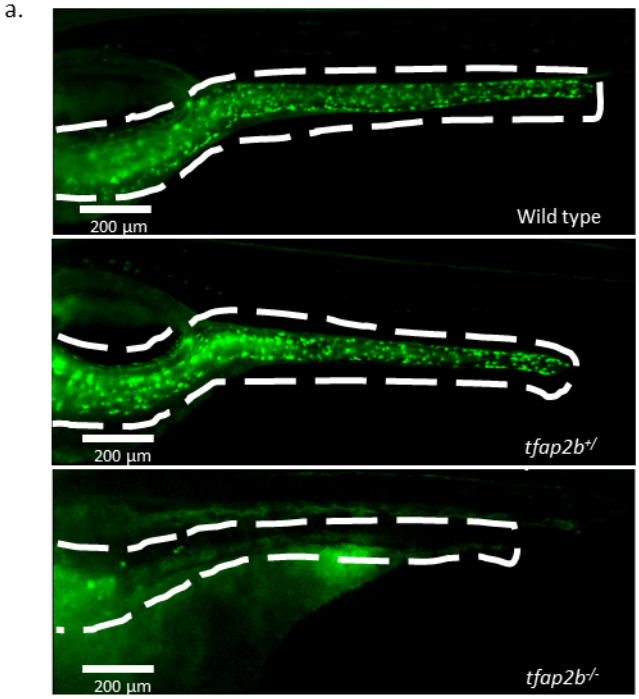
To determine if TFAP2B is involved in the development and function of the GI tract, we disrupted this gene in zebrafish using CRISPR/Cas9 editing. Zebrafish Tfap2b shares 88.71% sequence identity with the human protein (Supplementary Figure 1a). Based on the literatures, tfap2b is only expressed in neural crest-derived cells in early embryonic development (Bradford et al., 2022) and in differentiating distal nephron segment at the 28 somite-stage (Chambers et al., 2019). At 5 dpf, tfap2b seems to be only expressed in the optic tectum, retinal inner layer, and spinal cord, with no expression expected in the intestine. To disrupt expression of tfap2b, the Cas9 protein/gRNA complexes were injected into fertilized zebrafish oocytes at one-cell stage. The gRNA was specifically designed to target the beginning of exon 4 of tfap2b. Efficiency of

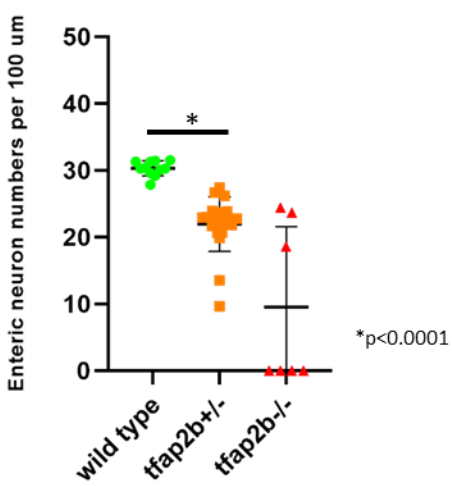
targeting was evaluated by the generation of InDels and was determined by Sanger sequencing of genomic DNA from individual 5dpf larvae (n=48). The TIDE program was used to check sequence decomposition, showing that on average 73.46% (SD±11.288) of the alleles harbored InDels (i.e mutagenesis efficiency).

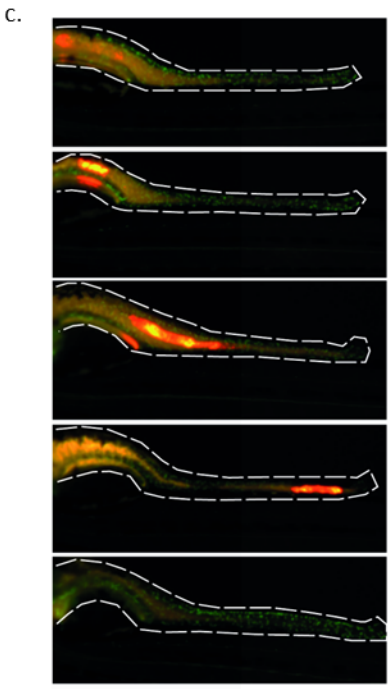
By counting the number of enteric neurons in 5dpf F0 fish, we observed a reduction in neuronal numbers per 100µm (mean= 24.431, SD± 3.594) in crispants, when compared to uninjected or cas9-injected fish (mean= 30.144, SD±1.473; mean= 30.369, SD± 1.864, respectively, $p<0.0001$, Student's T test; Supplementary Figure 2a). In addition, the length of the GI tract was evaluated in crispants and wild-type fish, by measuring the ratio of total body length to mouth-distal intestinal length. However, no significant difference was found at 5dpf (Supplementary Figure 2b). These results were consistently observed in F2 tfap2b+/- stable mutant fish in neuronal numbers per 100 µm (mean= 21.961, SD± 4.087) when compared to wild type (mean= 30.296, SD± 1.129, $p<0.0001$, Student's T test) as depicted in Figure 3a&3b. Interestingly, we observed that the majority of tfap2b-/- showed total intestinal aganglionosis (4 out of 7).

To evaluate the effect of reduced enteric neuronal numbers on intestinal transit time, we performed an intestinal transit time assay in F2 fish. We divided the intestine in 4 zones (Figure 3c) and observed that while the majority of wild-type fish showed an empty gut 16h after feeding (75%), only 38.4% of tfap2b+/- showed the same ($p <0.0001$, Proportion test). The majority of tfap2b+/- fish showed retention of the fluorescent pellet in proximal zones 2,3 and 4, suggesting a delay in intestinal transit in the Tfap2b haploinsufficiency. All the tfap2b-/- fish showed retention of pellet in zone 2 (Figure 3d). Furthermore, we evaluated the length of the GI tract in wild type, tfap2b+/-, and tfap2b-/- by measuring the ratio of total body length to mouth-distal intestinal length. We found no difference between the groups (Figure 3e).

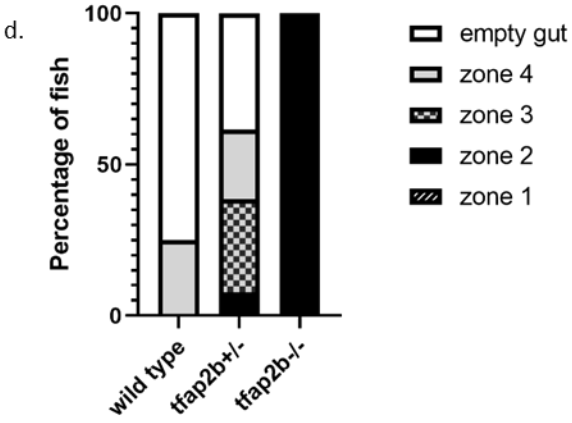
Figure 3.







a: Zone 1
b: Zone 2
c: Zone 3
d: Zone 4
Red fluorescent: pellet
Green signals: enteric neurons



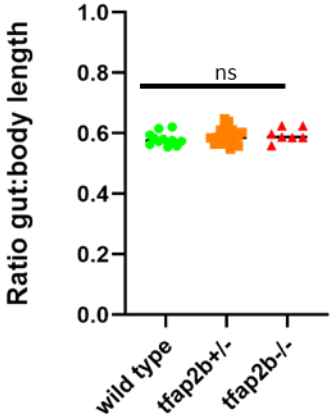


Figure 3. Decreased enteric neuronal numbers and delayed intestinal transit time in tfap2b+/- zebrafish. a). Image of the zebrafish wild type, tfap2b+/- and tfap2-/- intestine. Enteric neurons are shown in green and a white dash line delineates the intestine. b). A significant reduced number of enteric neurons (*p<0.0001, unpaired t-test) was found in the tfap2b+/- fish (n=20) when compared to the wild type fish (n=11). Neurons were counted and presented per 100µm. Four out of 7 tfap2b-/- fish showed total aganglionosis. c) Intestinal transit time was determined by the presence of a fluorescent pellet (red fluorescent) in the intestine 16 hours (16h) after food intake (final measurement). To help evaluation, the zebrafish intestine was divided in 4 zones, proximal to distal, and empty. Only fish with fluorescent pellet in zone 1 at time 0 (initial measurement), were selected for this study. d). A significant delay in intestinal transit time was observed in tfap2b+/- fish, as 75% of wild type fish had an empty intestine 16h after food intake, in comparison with only 38.4% of tfap2b+/- fish (p<0.0001, Proportion test). All tfap2b-/- fish had pellet retained in zone 2.

Loss of Tfap2b leads to a reduction of ednrbb transcript levels

TFAP2B was recently suggested to be one of transcription factors involved in the regulation of EDNRB expression (Ling & Sauka-Spengler, 2019). Since EDNRB is known to cause HSCR and is therefore, involved in GI development and function (Bondurand et al., 2018), we evaluated expression levels of this gene in our crispant tfap2b zebrafish model. The following primers: 5' GATCACTGAGGGAAAAGCTGG 3' (forward) and 5' AGTTCGTTTGGATCAGTGTGC 3' (reverse) were used to capture tfap2b transcript. Two ednrb orthologs are present in zebrafish, ednrba and ednrbb. However, only ednrbb seems to have an effect on GI development, as knocking down this gene in zebrafish showed less enteric neurons (Tilghman et al., 2019). Therefore, we decided to specifically determine the levels of ednrbb (5' CCGGTGCGAATCAAAGACG 3' (forward primer) and 5' ACTGCCGATCACAATGTTGG 3' (reverse primer) in our crispant fish at different time points of development (8hpf to 5 dpf). As expected, a decrease in tfap2b transcript levels was detected at each time point analyzed starting from 1 dpf, when compared to wild type fish (p<0.05, unpaired t-test; Figure 4a). A significant reduction in ednrbb transcript levels was also detected in crispants, but only at 4 and 5 dpf (p<0.05, unpaired t-test; Figure 4b).

Figure 4.

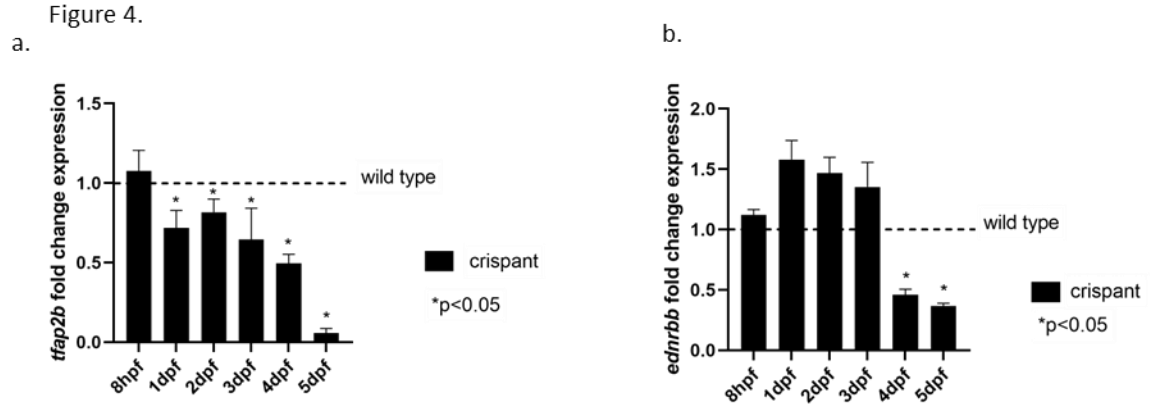


Figure 4. Expression levels of tfap2b and ednrbb during zebrafish development. a). Expression levels of tfap2b in crispant fish at 8 hpf, 1 dpf, 2 dpf, 3 dpf, 4 dpf, and 5 dpf show a significant decrease when compared to control, starting from 1 dpf onwards ($p<0.05$, unpaired t-test). b) Expression levels of ednrbb in tfap2b crispants show a significant decrease when compared to wild type fish, at 4 and 5 dpf ($p<0.05$, unpaired t-test).

Enteric neuronal differentiation is not affected in tfap2b+/- fish

Since EDNRB is involved in enteric neuronal differentiation, we evaluated if this process was affected in our tfap2b+/- fish. For this, a HuC/HuD immunostaining was performed to identify terminally differentiated enteric neurons, and HuC/HuD/phox2bb:GFP ratio was determined. Interestingly, we found no difference in the proportion of differentiated enteric neurons of tfap2b+/- (mean= 0.970, SD± 0.020, n=7) when compared to the wild type (mean= 0.967, SD± 0.006, n=5) (**Figure 5a and 5b**).

Figure 5.

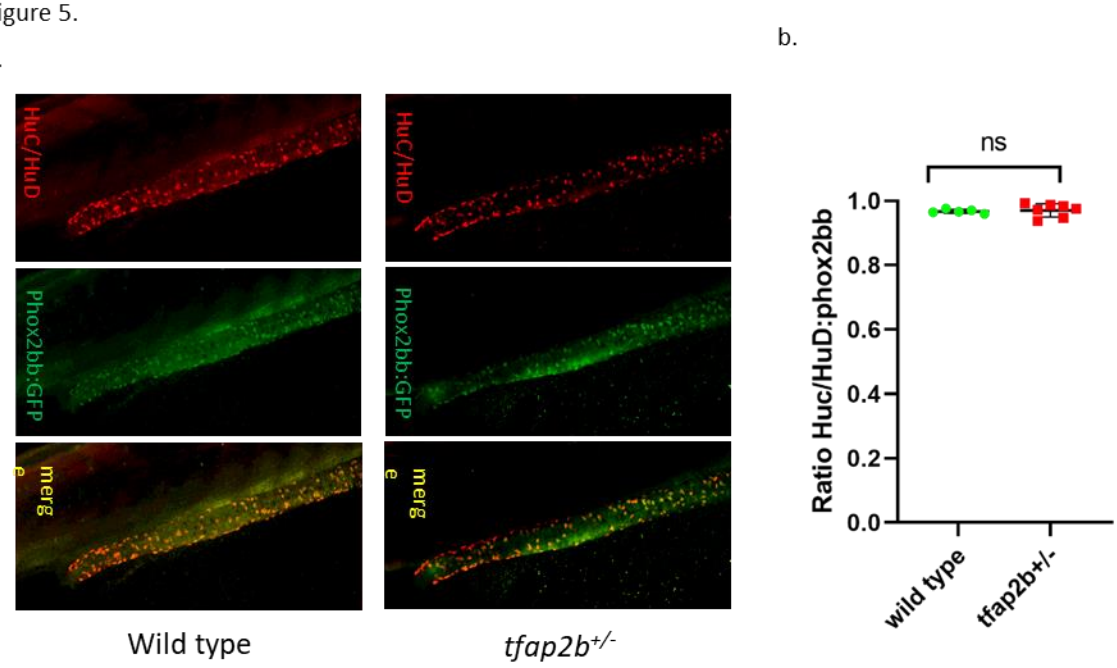


Figure 5. Differentiated enteric neurons in tfap2b+/- fish. a). Confocal microscope images of HuC/HuD and phox2bb:GFP positive cells in wild-type and tfap2b+/- F2 fish. b). No significant difference in the ratio of HuC/HuD and phox2bb:GFP positive cells in wild type and tfap2b+/- F2 fish was found ($p>0.05$, unpaired t-test).

DISCUSSION

In this manuscript, we show that TFAP2B loss of function is associated to a congenital GI disorder, by identifying a de novo heterozygous deletion in this gene (c.602-5_606delTCTAGTTCCA), in a PIPO patient. Our in vitro studies showed that this deletion leads to exon 4 skipping and to the appearance of an early stop codon at position c.863. Consequently, a reduction in TFAP2B expression levels was observed. Although it is tempting to speculate that this reduction possibly occurred through the process of nonsense mediated decay, as we used the C-terminal Myc-tag to detect expression of TFAP2B, we cannot exclude the possibility that exon 1-3 are still intact. Considering that the transactivation domain of TFAP2B is located in this region, it might well be that a small TFAP2B protein is expressed. However, since this small protein will miss the dimerization domain (Figure 1f), it is unlikely to be functional. Our zebrafish model confirmed the involvement of TFAP2B in GI development and function, as tfap2b crispant and tfap2b+/- F2 fish showed a reduction of enteric neurons and delayed intestinal transit time, with no change in total body to mouth-distal intestine length ratio when compared to wild-type. Therefore, we classified this de novo deletion variant as pathogenic based on American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants.

Interestingly, three loss of function variants in TFAP2B have been reported in control cohorts in GnomAD. However, since GI complaints might not be recorded in medical charts and can be easily missed due to variation in symptoms severity, we cannot definitively exclude that these controls have no clinical GI features. Such phenomenon is not uncommon in GI diseases, as mildest symptoms can be found in family members carrying the same pathogenic variant as the affected proband (Sribudiani et al., 2018).

TFAP2B together with TFAP2A, TFAP2C, TFAP2D and TFAP2E compose the AP-2 transcription factors family, known to form homo- or hetero-dimers with themselves, to function and bind specific DNA sequences as their targets (Hilger-Eversheim et al., 2000). These transcription factors are thought to stimulate proliferation and suppress terminal differentiation of specific cell types during embryonic development (Eckert et al., 2005), and were recently shown to be core regulators in orchestrating delineation of vagal neural crest cells, in an avian model (Ling & Sauka-Spengler, 2019). One of the genes found to be indirectly controlled by the TFAP2 family is EDNRB, a known gene required for enteric nervous system (ENS) development. In fact, mutations in EDNRB have been found in patients with HSCR, the most common enteric neuropathy characterized by lack of enteric neurons in the distal colon (Bondurand et al., 2018). Due to this link, we decided to evaluate transcript levels of ednrbb in our crispant tfap2b fish and observed a significant reduction of this gene, at 4 and 5dpf. Physiologically, at these developmental time points, colonization of the zebrafish gut by enteric neuronal progenitors is completed and neuronal differentiation is prominent (Howard et al., 2021; Kuil et al., 2021). Therefore, it was tempting to hypothesize that neuronal differentiation would be affected in the absence of TFAP2B, due to reduction of ednrbb levels. Our results showed that this was not the case (Figure 5b), as no difference in the ratio of HuC/D/phox2bb neurons was detected. EDN3/EDNRB signaling has also been reported to control proliferation of the enteric neural progenitors (Barlow et al., 2003; Nagy & Goldstein, 2006). Considering that our tfap2b-/- stable line show a HSCR phenotype, we suspect that a reduction in the number of progenitors is responsible for the reduction in enteric neurons identified.

Although this is the first time TFAP2B is linked to GI development, this is not the first time that this gene is involved in a congenital genetic disorder. Char syndrome and syndromic craniosynostosis are both caused by pathogenic mutations in this gene. However, while the majority of Char syndrome patients carry an autosomal dominant heterozygous missense variant in TFAP2B, nonsense, splice site, and missense variants have been identified in syndromic craniosynostosis (Figure 1e). These findings indicate a phenotypic variability resulting from TFAP2B defects, suggesting that variants in this gene lead to pleiotropic effects that vary in severity. Such a phenomenon is not new, as variants in other genes are known to result in a spectrum of genetic disorders. Heterozygous Inactive Lysine Methyltransferase 2E (KMT2E) variants result in a spectrum of neurodevelopmental disorders, epilepsy, and functional GI disturbances (O'Donnell-Luria et al., 2019). Furthermore, variants in Coiled-Coil and C2 Domain Containing 2A (CC2D2A) have been recognized to cause a group of genetic disorders classified as

ciliopathies. These include Meckel syndrome type 6, Joubert syndrome type 9, Bardet-Biedl syndrome and nephronophthisis (Lewis et al., 2019). Since we have now linked TFAP2B variants to PIPO, it will be interesting to evaluate if GI complaints are also present when assessing other obvious malformations, such as the ones described for Char syndrome and craniosynostosis. To date, there are no reports describing co-occurrence of Char syndrome and PIPO or craniosynostosis and PIPO, but this might be because GI symptoms were missed or considered to be secondary. Therefore, although it is too early to present TFAP2B as a new CIPO gene since only one patient has been identified, our results suggest that a better characterization of Char syndrome and syndromic craniosynostosis patients is needed, to improve counselling and define optimal treatment strategies.

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Contribution to the Field Statement

Pediatric intestinal pseudo-obstruction (PIPO) is a specific form of Chronic intestinal pseudo-obstruction diagnosed in children. PIPO is a congenital gastrointestinal (GI) dysmotility which involves multiple pathology mechanisms affecting the structure and/or function of the intestinal neuromuscular and interstitial cells of Cajals. Therefore, clinical manifestations can be highly pleiomorphic, depending on location and the extent of the affected GI segment. Although several genes have been described to be the cause for this disorder, the majority of PIPO patients do not receive a genetic diagnosis, and this study aims to identify the genetic cause of PIPO. We identified a de novo heterozygous deletion in TFAP2B (NM_003221.4:c.602-5_606delTCTAGTTCCA) in a PIPO patient and showed that haploinsufficiency of this gene led to reduced enteric neuronal numbers and intestinal motility delays. To date, this is the first PIPO patient described with a pathogenic variant in TFAP2B. Furthermore, this is the first time that TFAP2B is directly implicated in intestinal development and function. In this manuscript, we present TFAP2B as a novel genetic cause for PIPO, bringing new insights to disease pathogenesis.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contributions: Conceptualization: A.Z., L.K., B.M.dG., E.B., M.M.A.; Data Curation: A.Z., B.M.dG., J.W., N.K., A.B., B.dK., M.vS.; Formal Analysis: A.Z., L.K., B.M.dG., M.vS., E.B., M.M.A.; Funding acquisition: R.M.W.H., E.B., M.M.A.; Investigation: A.Z., L.K., B.M.dG.; Methodology: A.Z., L.K., B.M.dG., M.M.A.; Resources: L.K., B.M.dG., J.W., A.B., M.vS., B.dK.; Software: A.Z.; Visualization: A.Z., B.M.dG.; Writing Original Draft: AZ, MMA; Writing-review & editing: A.Z., L.K., B.M.dG., J.W., A.B., M.vS., B.dK., R.W., V.M., E.B., M.M.A.; Supervision: V.M., E.B., M.M.A.

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References

Barlow, A., De Graaff, E., & Pachnis, V. (2003). Enteric Nervous System Progenitors Are Coordinately Controlled by the G Protein-Coupled Receptor EDNRB and the Receptor Tyrosine Kinase RET. *Neuron*, *40*(5), 905–916. https://doi.org/10.1016/S0896-6273(03)00730-X

Bondurand, N., Dufour, S., & Pingault, V. (2018). News from the endothelin-3/EDNRB signaling pathway: Role during enteric nervous system development and involvement in neural crest-associated disorders. *Developmental Biology*, *444*(August), S156–S169. https://doi.org/10.1016/j.ydbio.2018.08.014

Bonora, E., Bianco, F., Cordeddu, L., Bamshad, M., Francescatto, L., Dowless, D., Stanghellini, V., Cogliandro, R. F., Lindberg, G., Mungan, Z., Cefle, K., Ozcelik, T., Palanduz, S., Ozturk, S., Gedikbasi, A., Gori, A., Pippucci, T., Graziano, C., Volta, U., … De Giorgio, R. (2015). Mutations in RAD21 disrupt regulation of apob in patients with chronic intestinal pseudo-obstruction. *Gastroenterology*, *148*(4), 771-782.e11. https://doi.org/10.1053/j.gastro.2014.12.034

Bradford, Y. M., Van Slyke, C. E., Ruzicka, L., Singer, A., Eagle, A., Fashena, D., Howe, D. G., Frazer, K., Martin, R., Paddock, H., Pich, C., Ramachandran, S., & Westerfield, M. (2022). Zebrafish information network, the knowledgebase for Danio rerio research . *Genetics*, *220*(4). https://doi.org/10.1093/genetics/iyac016

Chambers, B. E., Gerlach, G. F., Clark, E. G., Chen, K. H., Levesque, A. E., Leshchiner, I., Goessling, W., & Wingert, R. A. (2019). Tfap2a is a novel gatekeeper of nephron differentiation during kidney development. *Development (Cambridge)*, *146*(13). https://doi.org/10.1242/dev.172387

Chen, Y. W., Zhao, W., Zhang, Z. F., Fu, Q., Shen, J., Zhang, Z., Ji, W., Wang, J., & Li, F. (2011). Familial nonsyndromic patent ductus arteriosus caused by mutations in TFAP2B. *Pediatric Cardiology*, *32*(7), 958–965. https://doi.org/10.1007/s00246-011-0024-7

Chetaille, P., Preuss, C., Burkhard, S., Côté, J. M., Houde, C., Castilloux, J., Piché, J., Gosset, N., Leclerc, S., Wünnemann, F., Thibeault, M., Gagnon, C., Galli, A., Tuck, E., Hickson, G. R., Amine, N. El, Boufaied, I., Lemyre, E., De Santa Barbara, P., … Andelfinger, G. (2014). Mutations in SGOL1 cause a novel cohesinopathy affecting heart and gut rhythm. *Nature Genetics*, *46*(11), 1245–1248. https://doi.org/10.1038/ng.3113

Di Nardo, G., Karunaratne, T. B., Frediani, S., & De Giorgio, R. (2017). Chronic intestinal pseudo-obstruction: Progress in management? *Neurogastroenterology and Motility*, *29*(12). https://doi.org/10.1111/nmo.13231

Downes, T. J., Cheruvu, M. S., Karunaratne, T. B., De Giorgio, R., & Farmer, A. D. (2018). Pathophysiology, Diagnosis, and Management of Chronic Intestinal Pseudo-Obstruction. *Journal of Clinical Gastroenterology*, *52*(6), 477–489. https://doi.org/10.1097/MCG.0000000000001047

Eckert, D., Buhl, S., Weber, S., Jäger, R., & Schorle, H. (2005). The AP-2 family of transcription factors. *Genome Biology*, *6*(13). https://doi.org/10.1186/gb-2005-6-13-246

Edward, H. L., D'Gama, A. M., Wojcik, M. H., Brownstein, C. A., Kenna, M. A., Grant, P. E., Majzoub, J. A., & Agrawal, P. B. (2019). A novel missense mutation in TFAP2B associated with Char syndrome and central diabetes insipidus. *American Journal of Medical Genetics, Part A*, *179*(7), 1299–1303. https://doi.org/10.1002/ajmg.a.61150

Gamboa, H. E., & Sood, M. (2019). Pediatric Intestinal Pseudo-obstruction in the Era of Genetic Sequencing. *Current Gastroenterology Reports*, *21*(12). https://doi.org/10.1007/s11894-019-0737-y

Gargiulo, A., Auricchio, R., Barone, M. V., Cotugno, G., Reardon, W., Milla, P. J., Ballabio, A., Ciccodicola, A., & Auricchio, A. (2007). Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. *American Journal of Human Genetics*, *80*(4), 751–758. https://doi.org/10.1086/513321

Giordano, C., Powell, H., Leopizzi, M., De Curtis, M., Travaglini, C., Sebastiani, M., Gallo, P., Taylor, R. W., & D'Amati, G. (2009). Fatal congenital myopathy and gastrointestinal pseudo-obstruction due to POLG1 mutations. *Neurology*, *72*(12), 1103–1105. https://doi.org/10.1212/01.wnl.0000345002.47396.e1

Halim, D., Hofstra, R. M. W., Signorile, L., Verdijk, R. M., Van Der Werf, C. S., Sribudiani, Y., Brouwer, R. W. W., van IJcken, W. F. J., Dahl, N., Verheij, J. B. G. M., Baumann, C., Kerner, J., Van Bever, Y., Galjart, N., Wijnen, R. M. H., Tibboel, D., Burns, A. J., Muller, F., Brooks, A. S., & Alves, M. M. (2016). ACTG2 variants impair actin polymerization in sporadic Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *Human Molecular Genetics*, *25*(3), 571–583. https://doi.org/10.1093/hmg/ddv497

Hilger-Eversheim, K., Moser, M., Schorle, H., & Buettner, R. (2000). Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell-cycle control. *Gene*, *260*(1–2), 1–12. https://doi.org/10.1016/S0378-1119(00)00454-6

Hong, S. J., Huh, Y. H., Leung, A., Choi, H. J., Ding, Y., Kang, U. J., Yoo, S. H., Buettner, R., & Kim, K. S. (2011). Transcription factor AP-2β regulates the neurotransmitter phenotype and maturation of chromaffin cells. *Molecular and Cellular Neuroscience*, *46*(1), 245–251. https://doi.org/10.1016/j.mcn.2010.09.007

Howard, A. G. A., Baker, P. A., Ibarra-García-padilla, R., Moore, J. A., Rivas, L. J., Tallman, J. J., Singleton, E. W., Westheimer, J. L., Corteguera, J. A., & Uribe, R. A. (2021). An atlas of neural crest lineages along the posterior developing zebrafish at single-cell resolution. *ELife*, *10*, 1–31. https://doi.org/10.7554/eLife.60005

Jenkins, Z. A., Macharg, A., Chang, C. Y., van Kogelenberg, M., Morgan, T., Frentz, S., Wei, W., Pilch, J., Hannibal, M., Foulds, N., McGillivray, G., Leventer, R. J., García-Miñaúr, S., Sugito, S., Nightingale, S., Markie, D. M., Dudding, T., Kapur, R. P., & Robertson, S. P. (2018). Differential regulation of two FLNA transcripts explains some of the phenotypic heterogeneity in the loss-of-function filaminopathies. *Human Mutation*, *39*(1), 103–113. https://doi.org/10.1002/humu.23355

Ji, W., Benson, M. A., Bhattacharya, S., Chen, Y., Hu, J., & Li, F. (2014). Characterization of transcription factor AP-2 beta mutations involved in familial isolated patent ductus arteriosus suggests haploinsufficiency. *Journal of Surgical Research*, *188*(2), 466–472. https://doi.org/10.1016/j.jss.2014.01.015

Jin, K., Jiang, H., Xiao, D., Zou, M., Zhu, J., & Xiang, M. (2015). Tfap2a and 2b act downstream of Ptf1a to promote amacrine cell differentiation during retinogenesis. *Molecular Brain*, *8*(1), 1–14. https://doi.org/10.1186/s13041-015-0118-x

Khetyar, M., Syrris, P., Tinworth, L., Abushaban, L., & Carter, N. (2008). Novel TFAP2B mutation in nonsyndromic patent ductus arteriosus. *Genetic Testing*, *12*(3), 457–459. https://doi.org/10.1089/gte.2008.0015

Knowles, C. H., De Giorgio, R., Kapur, R. P., Bruder, E., Farrugia, G., Geboes, K., Lindberg, G., Martin, J. E., Meier-Ruge, W. A., Milla, P. J., Smith, V. V., Vandervinden, J. M., Veress, B., & Wedel, T. (2010). The London Classification of gastrointestinal neuromuscular pathology: Report on behalf of the gastro 2009 international working group. *Gut*, *59*(7), 882–887. https://doi.org/10.1136/gut.2009.200444

Kraja, A. T., Chasman, D. I., North, K. E., Reiner, A. P., Yanek, L. R., Kilpeläinen, T. O., Smith, J. A., Dehghan, A., Dupuis, J., Johnson, A. D., Feitosa, M. F., Tekola-Ayele, F., Chu, A. Y., Nolte, I. M., Dastani, Z., Morris, A., Pendergrass, S. A., Sun, Y. V., Ritchie, M. D., … Borecki, I. B. (2014). Pleiotropic genes for metabolic syndrome and inflammation. *Molecular Genetics and Metabolism*, *112*(4), 317–338. https://doi.org/10.1016/j.ymgme.2014.04.007

Kuil, L. E., Chauhan, R. K., Cheng, W. W., Hofstra, R. M. W., & Alves, M. M. (2021). Zebrafish: A Model Organism for Studying Enteric Nervous System Development and Disease. *Frontiers in Cell and Developmental Biology*, *8*(January), 1–15. https://doi.org/10.3389/fcell.2020.629073

Lewis, W. R., Bales, K. L., Revell, D. Z., Croyle, M. J., Engle, S. E., Song, C. J., Malarkey, E. B., Uytingco, C. R., Shan, D., Antonellis, P. J., Nagy, T. R., Kesterson, R. A., Mrug, M. M., Martens, J. R., Berbari, N. F., Gross, A. K., & Yoder, B. K. (2019). Mks6 mutations reveal tissue-and cell type-specific roles for the cilia transition zone. *FASEB Journal*, *33*(1), 1440–1455. https://doi.org/10.1096/fj.201801149R

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, *25*(14), 1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Ling, I. T. C., & Sauka-Spengler, T. (2019). Early chromatin shaping predetermines multipotent vagal neural crest into neural, neuronal and mesenchymal lineages. *Nature Cell Biology*, *21*(12), 1504–1517. https://doi.org/10.1038/s41556-019-0428-9

Mani, A., Radhakrishnan, J., Farhi, A., Carew, K. S., Warnes, C. A., Nelson-Williams, C., Day, R. W., Pober, B., State, M. W., & Lifton, R. P. (2005). Syndromic patent ductus arteriosus: Evidence for haploinsufficient TFAP2B mutations and identification of a linked sleep disorder. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(8), 2975–2979. https://doi.org/10.1073/pnas.0409852102

Milunsky, A., Baldwin, C., Zhang, X., Primack, D., Curnow, A., & Milunsky, J. (2017). Diagnosis of Chronic Intestinal Pseudo-obstruction and Megacystis by Sequencing the ACTG2 Gene. *Journal of Pediatric Gastroenterology and Nutrition*, *65*(4), 384–387. https://doi.org/10.1097/MPG.0000000000001608

Moser, M., Dahmen, S., Kluge, R., Gröne, H., Dahmen, J., Kunz, D., Schorle, H., & Buettner, R. (2003). Terminal renal failure in mice lacking transcription factor AP-2β. *Laboratory Investigation*, *83*(4), 571–578. https://doi.org/10.1097/01.LAB.0000064703.92382.50

Muto, M., Matsufuji, H., Tomomasa, T., Nakajima, A., Kawahara, H., Ida, S., Ushijima, K., Kubota, A., Mushiake, S., & Taguchi, T. (2014). Pediatric chronic intestinal pseudo-obstruction is a rare, serious, and intractable disease: A report of a nationwide survey in Japan. *Journal of Pediatric Surgery*, *49*(12), 1799–1803. https://doi.org/10.1016/j.jpedsurg.2014.09.025

Nagy, N., & Goldstein, A. M. (2006). Endothelin-3 regulates neural crest cell proliferation and differentiation in the hindgut enteric nervous system. *Developmental Biology*, *293*(1), 203–217. https://doi.org/10.1016/j.ydbio.2006.01.032

Nechiporuk, A., Linbo, T., Poss, K. D., & Raible, D. W. (2007). Specification of epibranchial placodes in zebrafish. *Development*, *134*(3), 611–623. https://doi.org/10.1242/dev.02749

Nishino, I., Spinazzola, A., & Hirano, M. (1999). Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. *Science*, *283*(5402), 689–692. https://doi.org/10.1126/science.283.5402.689

O'Donnell-Luria, A. H., Pais, L. S., Faundes, V., Wood, J. C., Sveden, A., Luria, V., Abou Jamra, R., Accogli, A., Amburgey, K., Anderlid, B. M., Azzarello-Burri, S., Basinger, A. A., Bianchini, C., Bird, L. M., Buchert, R., Carre, W., Ceulemans, S., Charles, P., Cox, H., … Rodan, L. H. (2019). Heterozygous Variants in KMT2E Cause a Spectrum of Neurodevelopmental Disorders and Epilepsy. *American Journal of Human Genetics*, *104*(6), 1210–1222. https://doi.org/10.1016/j.ajhg.2019.03.021

Prasad, P. D., Stanton, J. A. L., & Assinder, S. J. (2010). Expression of the actin-associated protein transgelin (SM22) is decreased in prostate cancer. *Cell and Tissue Research*, *339*(2), 337–347. https://doi.org/10.1007/s00441-009-0902-y

Ravenscroft, G., Pannell, S., O'Grady, G., Ong, R., Ee, H. C., Faiz, F., Marns, L., Goel, H., Kumarasinghe, P., Sollis, E., Sivadorai, P., Wilson, M., Magoffin, A., Nightingale, S., Freckmann, M. L., Kirk, E. P., Sachdev, R., Lemberg, D. A., Delatycki, M. B., … Laing, N. G. (2018). Variants in ACTG2 underlie a substantial number of Australasian patients with primary chronic intestinal pseudo-obstruction. *Neurogastroenterology and Motility*, *30*(9), 1–9. https://doi.org/10.1111/nmo.13371

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., & Rehm, H. L. (2015). Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, *17*(5), 405–424. https://doi.org/10.1038/gim.2015.30

Schmidt, M., Huber, L., Majdazari, A., Schütz, G., Williams, T., & Rohrer, H. (2011). The transcription factors AP-2β and AP-2α are required for survival of sympathetic progenitors and differentiated sympathetic neurons. *Developmental Biology*, *355*(1), 89–100. https://doi.org/10.1016/j.ydbio.2011.04.011

Seok, J. H., Lardaro, T., Myung, S. O., Huh, Y., Ding, Y., Un, J. K., Kirfel, J., Buettner, R., & Kim, K. S. (2008). Regulation of the noradrenaline neurotransmitter phenotype by the transcription factor AP-2β. *Journal of Biological Chemistry*, *283*(24), 16860–16867. https://doi.org/10.1074/jbc.M709106200

Sribudiani, Y., Chauhan, R. K., Alves, M. M., Petrova, L., Brosens, E., Harrison, C., Wabbersen, T., de Graaf, B. M., Rügenbrink, T., Burzynski, G., Brouwer, R. W. W., van IJcken, W. F. J., Maas, S. M., de Klein, A., Osinga, J., Eggen, B. J. L., Burns, A. J., Brooks, A. S., Shepherd, I. T., & Hofstra, R. M. W. (2018). Identification of Variants in RET and IHH Pathway Members in a Large Family With History of Hirschsprung Disease. *Gastroenterology*, *155*(1), 118-129.e6. https://doi.org/10.1053/j.gastro.2018.03.034

Tanasubsinn, N., Sittiwangkul, R., Pongprot, Y., Kawasaki, K., Ohazama, A., Sastraruji, T., Kaewgahya, M., & Kantaputra, P. N. (2017). TFAP2B mutation and dental anomalies. *Journal of Human Genetics*, *62*(8), 769–775. https://doi.org/10.1038/jhg.2017.37

Thapar, N., Saliakellis, E., Benninga, M. A., Borrelli, O., Curry, J., Faure, C., De Giorgio, R., Gupte, G., Knowles, C. H., Staiano, A., Vandenplas, Y., & Di Lorenzo, C. (2018). Paediatric Intestinal Pseudo-obstruction: Evidence and Consensus-based Recommendations from an ESPGHAN-Led Expert Group. *Journal of Pediatric Gastroenterology and Nutrition*, *66*(6), 991–1019. https://doi.org/10.1097/MPG.0000000000001982

Timberlake, A. T., Jin, S. C., Nelson-Williams, C., Wu, R., Furey, C. G., Islam, B., Haider, S., Loring, E., Galm, A., Steinbacher, D. M., Larysz, D., Staffenberg, D. A., Flores, R. L., Rodriguez, E. D., Boggon, T. J., Persing, J. A., & Lifton, R. P. (2019). Erratum: Mutations in TFAP2B and previously unim-plicated genes of the BMP, Wnt, and Hedgehog pathways in syndromic craniosynostosis (Proceedings of the National Academy of Sciences of the United States of America 116 (15116–15121) DOI: 10.1073/pnas.19020. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(34), 17130. https://doi.org/10.1073/pnas.1912893116

Van Der Werf, C. S., Sribudiani, Y., Verheij, J. B. G. M., Carroll, M., O'Loughlin, E., Chen, C. H., Brooks, A. S., Liszewski, M. K., Atkinson, J. P., & Hofstra, R. M. W. (2013). Congenital short bowel syndrome as the presenting symptom in male patients with FLNA mutations. *Genetics in Medicine*, *15*(4), 310–313. https://doi.org/10.1038/gim.2012.123

Van Der Werf, C. S., Wabbersen, T. D., Hsiao, N., Paredes, J., Etchevers, H. C., Kroisel, P. M., Tibboel, D., Babarit, C., Schreiber, R. A., Hoffenberg, E. J., Vekemans, M., Zeder, S. L., Ceccherini, I., Lyonnet, S., Ribeiro, A. S., Seruca, R., Te Meerman, G. J., Van Ijzendoorn, S. C. D., Shepherd, I. T., … Hofstra, R. M. W.

(2012). CLMP is required for intestinal development, and loss-of-function mutations cause congenital short-bowel syndrome. *Gastroenterology*, *142*(3), 453-462.e3. https://doi.org/10.1053/j.gastro.2011.11.038

Van Goethem, G., Schwartz, M., Löfgren, A., Dermaut, B., Van Broeckhoven, C., & Vissing, J. (2003). Novel POLG mutations in progressive external ophthalmoplegia mimicking mitochondrial neurogastrointestinal encephalomyopathy. *European Journal of Human Genetics*, *11*(7), 547–549. https://doi.org/10.1038/sj.ejhg.5201002

Xiong, F., Li, Q., Zhang, C., Chen, Y., Li, P., Wei, X., Li, Q., Zhou, W., Li, L., Shang, X., & Xu, X. (2013). Analyses of GATA4, NKX2.5, and TFAP2B genes in subjects from southern China with sporadic congenital heart disease. *Cardiovascular Pathology*, *22*(2), 141–145. https://doi.org/10.1016/j.carpath.2012.07.001

Zhao, F., Bosserhoff, A. K., Buettner, R., & Moser, M. (2011). A Heart-Hand syndrome gene: TFAP2B plays a critical role in the development and remodeling of mouse ductus arteriosus and limb patterning. *PLoS ONE*, *6*(7), 3–10. https://doi.org/10.1371/journal.pone.0022908

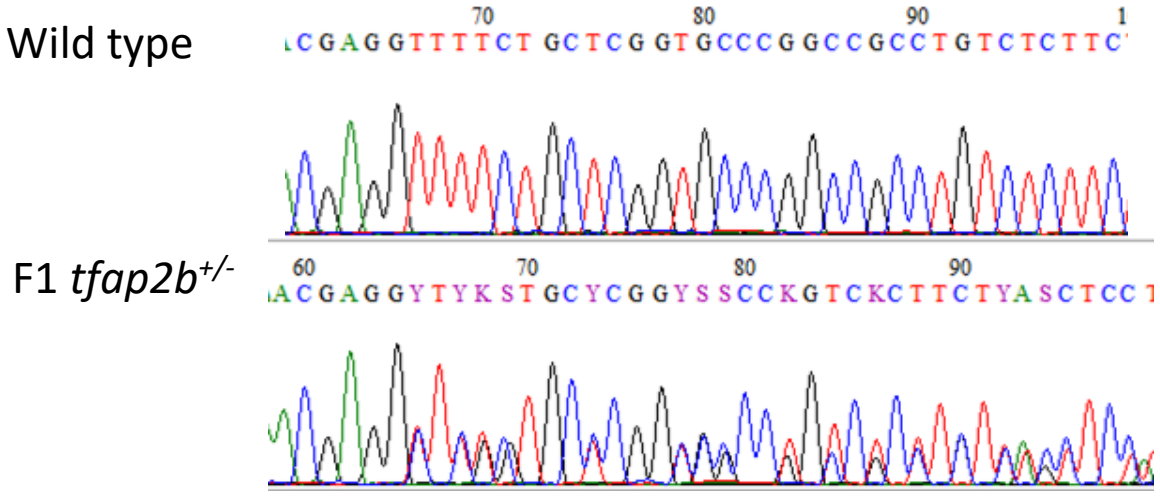
Supplementary Material

Supplementary Table 1. **List of site directed mutagenesis primers used in this study.**

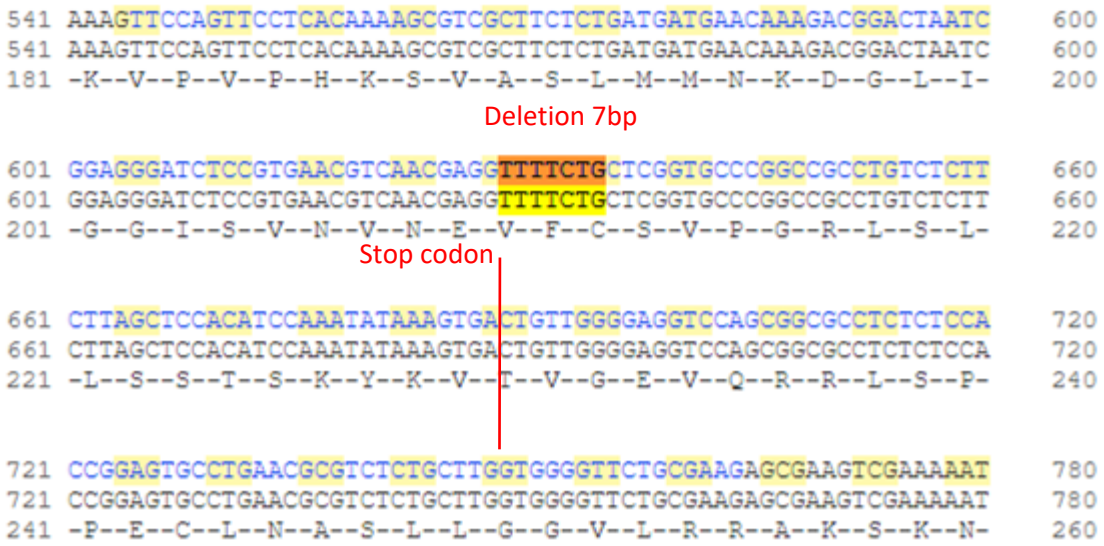
Gene	Sequence
TFAP2B-sdm-C706T-F	CGTCCCAGGCtGTTTGTCTCT
TFAP2B-sdm-C706T-R	GAGCAAAACACCTCGCCG
TFAP2B-sdm-C898T-F	CGCGGGCAGGtGCAAAGCAGC
TFAP2B-sdm-C898T-R	GGTAAATTCAAACCGATTTTTTCTAGCCTTTCTCGC
TFAP2B-sdm-delex4-F	AGCCAAATCGAAAAATGG
TFAP2B-sdm-delex4-R	CTTTTTTAATGACAGACTGG
TFAP2B-sdm-C1144T-F	AGGGAACAGCtGACCCAGCCC
TFAP2B-sdm-C1144T-R	ATCGGTGTCCGGTCCTGC

Supplementary Figure 1a.

Supplementary Figure 1b.



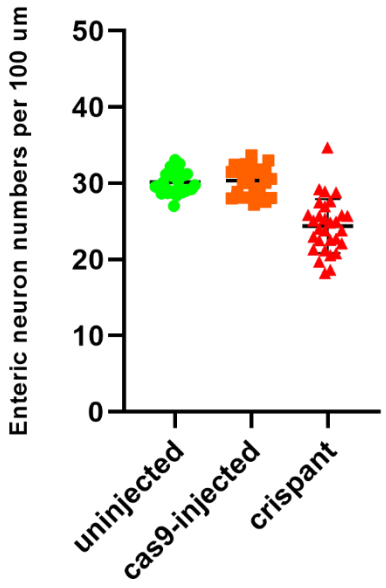
Supplementary Figure 1c.



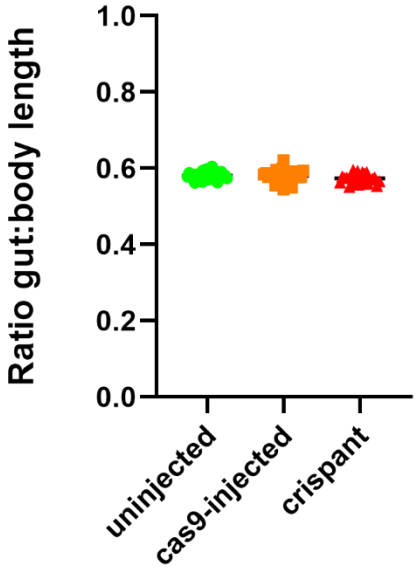
Supplementary Figure 1. De novo heterozygous Deletion of 7 basepairs (bp) in tfap2b+/- zebrafish leads to premature stop codon. a). The alignment of human TFAP2B (NM_003221.4) and zebrafish tfap2b (ENSDART00000174808.2) transcript sequence shows 88.71% homology

(BLAST Tools, ensemble.org). b). Sequencing validation of F1 zebrafish generated from crispant (F0), shows heterozygous deletion of 7 basepairs (bp) (ENSDART00000174808.2: c.629-635delTTTTCTG, frame shift). c). The c.629-635delTTTTCTG leads to a frame shift resulting in stop codon at position c.689.

Supplementary Figure 2a.



Supplementary Figure 2b.



Supplementary Figure 2. Effect of tfap2b reduction in the number of enteric neurons and in the gut vs total body length ratio. a). A significant decreased number of enteric neurons (*p<0.0001, Student's T test) was found in the crispant fish (n=30, mean= 24.431, SD± 3.594) when compared to the uninjected (n=30, mean= 30.144, SD±1.473) or cas9-injected (n=30, mean= 30.369, SD± 1.864). Neurons were counted and presented per 100µm. b). No effect of the gut and body length was detected in crispant fish when compared to uninjected or cas9-injected fish.

Chapter 3

The long Filamin-A isoform is required for intestinal development and motility: implications for Chronic Intestinal Pseudo-Obstruction

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ABSTRACT

Filamin A (FLNA) is a cytoplasmic actin binding protein, recently shown to be expressed as a long and short isoform. Mutations in *FLNA* are associated with a wide spectrum of disorders, including an X-linked form of chronic intestinal pseudo-obstruction (CIPO). However, the role of FLNA in intestinal development and function is largely unknown. In this study, we show that FLNA is expressed in the muscle layer of the small intestine from early human fetal stages. Expression of *FLNA* variants associated with CIPO, blocked expression of the long flna isoform and led to an overall reduction of RNA and protein levels. As a consequence, contractility of human intestinal smooth muscle cells was affected. Lastly, our transgenic zebrafish line showed that the *flna* long isoform is required for intestinal elongation and peristalsis. Histological analysis revealed structural and architectural changes in the intestinal smooth muscle of homozygous fish, likely triggered by the abnormal expression of intestinal smooth muscle markers. No defect in the localization or numbers of enteric neurons was observed. Taken together, our study demonstrates that the long FLNA isoform contributes to intestinal development and function.

Since loss of the long FLNA isoform does not seem to affect the enteric nervous system, it likely results in a myopathic form of CIPO, bringing new insights to disease pathogenesis.

Keywords: Filamin A, chronic intestinal pseudo-obstruction, X-linked, intestinal development, intestinal motility.

INTRODUCTION

Filamin A (FLNA) is a cytoplasmic protein with a well-characterized role. It was the first actin filament cross linking protein to be identified in non-muscle cells, and is involved in a series of events required for cell motility, migration, and maintenance of cytoskeletal integrity (Nakamura et al., 2011; Onoprishvili et al., 2008). Several binding partners are known for FLNA, including ion channels, receptors, intracellular signalling molecules and transcription factors (Nakamura et al., 2011). Therefore, it is not surprising that loss of function variants affecting the normal expression levels of FLNA are associated with various human disorders (Bernstein et al., 2011; Kyndt et al., 2007; Oegema et al., 2013; Robertson, 2005; Van Der Werf et al., 2013; Wade et al., 2020). These disorders are frequently called filaminopathies, and show a wide phenotypic variability, including abnormal neuronal migration, as well as vascular and cardiac defects, and intestinal dysmotility characteristic of chronic intestinal pseudo-obstruction (CIPO (Wade et al., 2020).

CIPO encompasses a heterogeneous group of disorders of which, Congenital Short Bowel Syndrome (CSBS), Megacystis Microcolon Intestinal Hypoperistaltic Syndrome (MMIHS), and Hirschsprung disease (HSCR) are examples of severe forms. Several genes have been identified as the cause of neuronal and myopathic forms of CIPO, and depending on the affected gene, various degrees of intestinal dysmotility have been reported (Gamboa & Sood, 2019; Thapar et al., 2018). *FLNA* is one of the genes previously described to cause an X-linked form of CIPO. Two independent studies reported the presence of two base pair deletions in this gene (NM_001110556.2: c.16-17delTC, c.65-66AC) in five male patients diagnosed with CIPO associated CSBS (Gargiulo et al., 2007; Van Der Werf et al., 2013). These deletions, located in exon two of *FLNA*, result in a frameshift with the subsequent appearance of an early stop codon a few base pairs later. Interestingly, exon two of *FLNA* has a peculiarity, the presence of two methionine's separated by 28 amino acids. Due to this feature, it has been suggested that two FLNA isoforms exist, and that in the presence of the two base pair deletions, only expression of the long isoform is affected (Gargiulo et al., 2007). This hypothesis was recently confirmed by the identification of three distinct transcription start sites in *FLNA*, while studying the pathogenicity of another *FLNA* deletion (NM_001110556.2: c.18-19delTC) identified in a CIPO patient (Jenkins et al., 2018). Two of these sites were shown to produce a protein isoform using the first methionine (ATG^{+1}), while the third one used a methionine located 81 nucleotides after the first starting site (ATG^{+82}). Moreover, tissue-specific regulation of FLNA was reported during development, with the long FLNA isoform being predominantly expressed in intestinal smooth

muscle, while the short isoform was more abundant in other tissues/organs, including the brain (Jenkins et al., 2018). Despite these findings, the role of FLNA in intestinal development is still poorly understood, making it difficult to pinpoint the pathogenic mechanisms involved in the development of CIPO.

In this study, we bring new insights into the role of FLNA, by further investigating the existence of two FLNA isoforms, and providing evidence that the long isoform is required for smooth muscle contractility, intestinal elongation, and intestinal motility.

MATERIAL AND METHODS

Immunohistochemistry in human small intestinal specimens

Paraffin-embedded human small intestinal specimens from controls were collected at weeks 17 and 22 of embryonic development, at neonatal stage and at a post-natal stage at 3 months of age, from the department of Pathology, Erasmus University Medical Center. Slides were stained with a specific antibody against FLNA (dilution 1:1000; Eurogentec, Seraing, Belgium), using a previously described protocol(Halim et al., 2016).

Expression vectors and site-directed mutagenesis

pAAV2.1-CMV-EGFP-FLNA wild-type (WT) and pAAV2.1-CMV-EGFP-FLNA mutant 2 (Mut2, c.65-66delAC) constructs were kindly provided by Prof. Alberto Auricchio from the University of Napoli. pCMV6-FLNA(Myc-DDK) was purchased from OriGene (Rockeville, Maryland, USA).

pAAV2.1-CMV-EGFP-FLNA mutant 1 (Mut1, C.16-17delTC), pCMV6-FLNA(Myc-DDK)mut1 (c.16-17delTC) and pCMV6-FLNA(Myc-DDK)mut 2 (c.65-66delAC) were generated by site-directed mutagenesis according to the the Q5 Site Directed Mutagenesis manufacturer's instructions (New England Biolabs, Ipswich, Massachusetts, USA), using the WT constructs. Primers used for site directed mutagenesis are described in Supplementary Table 1.

Cell culture and transfection

Human embryonic kidney cells (HEK293) were cultured in DMEM with high glucose content supplemented with 10% FBS and 1% penicillin/streptomycin. Human osteoblastoma cells (U2OS) were cultured as described before (Halim et al., 2016). Human intestinal smooth muscle cells (hiSMCs) were obtained from ScienceCell (Carlsbad, California, USA) and cultured according to the instructions provided. All cells were incubated at 37^0C in the presence of 5% CO2.

For transient transfection, 300,000 cells of HEK293 and U2OS, and 500,000 of hiSMCs were cultured in a 6 well plate. Twenty-four hours after, cells were transfected with 1 µg of plasmid DNA using GeneJuice (Millipore, Burlington, Massachusetts, USA) as transfection reagent, according to the manufacturer's instructions.

Cell lysis and Western Blot

Cells were lysed 48 hours after transfection using m-PER (Thermo Scientific, Waltham, Massachusetts, USA) and 1X protease inhibitors (Roche, Basel, Switzerland). Cell lysate

preparation, protein quantification and Western blot analysis were performed as described before (Halim et al., 2016). The following antibodies were used: anti-HA (Cell Signaling Technology, Danvers, Massachusetts, USA), anti-Myc (Cell Signaling Technology, Danvers, Massachusetts, USA), anti-FLNA (Abcam, Cambridge, UK), anti-GAPDH (Millipore, Burlington, Massachusetts, USA) and anti-actin (Santa Cruz Biotechnologies, Dallas, Texas, USA). Secondary antibodies used were IRDye 800CW Goat anti-mouse, IRDye 680RD Goat anti-Rabbit and IRDye 680RD Donkey anti-Goat (Li-Cor, Lincoln, Nebraska, USA).

RNA isolation, cDNA preparation, RT-PCR and qRT-PCR

Zebrafish larvae collected at different embryonic stages were lysed in Trizol. RNA isolation from zebrafish larvae and cell lines was performed with the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. 1 microgram of RNA was used for cDNA preparation with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA). For Reverse transcriptase (RT) PCR, 100 ng/µl of cDNA was used as a template. For quantitative real time (qRT) PCR, relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to actin and GAPDH. Primers used can be found in Supplementary Table 1.

Cell contractility assays

Twenty-four hours after transfection, 2X10^6 hiSMCs expressingpCMV6-FLNA(Myc-DDK)WT, pCMV6-FLNA(Myc-DDK)Mut1 and pCMV6-FLNA(Myc-DDK)Mut2 were trypsinized, mixed with collagen (Cell Biolabs, San Diego, California, USA) and plated in 24 well plates. One day later, collagen matrices were dislodged from the wells and cellular contractility was measured 12-18h after. Untransfected cells (un) were used as controls.

Immunofluorescence

U2OS cells transfected with pCMV6-FLNA(Myc-DDK)WT, pCMV6-FLNA(Myc-DDK)Mut1 and pCMV6-FLNA(Myc-DDK)Mut2 were cultured on coverslips and treated as described before [24]. Myc-antibody (Cell Signaling Technology, Danvers, Massachusetts, USA) was used at a concentration of 1:100, Phalloidin-rhodamin (Santa Cruz Biotechnology, Dallas, Texas, USA) was used at a concentration of 1:500 and Hoechst (Roche, Basel, Switzerland) was used at a concentration of 1:10,000. Images were taken using a Leica TCS SP5 confocal microscope (Leica Camera, Wetzlar, Germany) and analysed with the Leica LAS AF Lite software.

Animals

The Tupfel long fin (TL) zebrafish strain was used for all *in vivo* experiments described in this manuscript. Adult and larval fish were maintained on a 14h/10h light –dark cycle at 28^0C. Animal experiments were approved by the Animal Experimentation Committee of the Erasmus Medical Center.

Whole mount *in situ* hybridization

RNA isolated from 48 hours post fertilization (hpf) larvae was used as a template for cDNA using a One-Step RT-PCR kit (Qiagen, Hilden, Germany). Primers used for amplification are described in Supplementary table 1. Amplified bands were subcloned into the TOPO TA PCRII cloning vector (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Digoxigenin labelled anti-sense probes (Roche, Basel, Switzerland) were generated by linearizing the plasmids with *Not*I (New England Biolabs, Ipswich, Massachusetts, USA), then transcribed with SP6 polymerase (Roche, Basel, Switzerland). *In situ* hybridization was performed on staged zebrafish larvae as previously described (Walker, 1995).

Generation of the *flna* long isoform knockout zebrafish

TALENs were designed to target exon one of *flna* and recognize the following sequences: 5'-CCCCTATCCAACGCTTC and 3'- GACGCCGACATGCCCGC. To establish the *flna* long knockout mutant fish, 100 pg mRNA of each TALEN was injected into the cell of a one-cell stage embryo collected from wild-type zebrafish crossings. All injected embryos were raised to adulthood and crossed to generate F1. F1 transgenic larvae were raised to adulthood and genotyped. One homozygous fish containing a 6 base-pair deletion followed by a 1 base-pair insertion was crossed with a heterozygous fish containing the same genetic alteration. F2 transgenic fish were generated, raised to adulthood, and genotyped. All *in vivo* experiments described in this manuscript were performed in F3 fish.

Genotyping of transgenic zebrafish

Finclips of transgenic zebrafish were used for DNA isolation using a mixture containing Tris-HCl (pH9.0), KCl, Triton X-100, and protease K (Sigma Aldrich, St. Louis, Missouri, USA). After incubation at 55^0C for one hour, protease K was inactivated at 98^0C for 10 min. Isolated DNA was used as a template for a PCR reaction with a standard program. Primers are described in Supplementary Table 1. The PCR product was subjected to restriction digestion with *Nci*I (New England Biolabs, Ipswich, Massachusetts, USA) for 1h at 37^0C. In parallel, the PCR product was submitted to Sanger sequencing, which was performed with dye labelled primers (Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems, Waltham, Massachusetts, USA) on ABI 3130XL genetic analyzer. Sanger reads were analysed using SeqScape software.

Intestinal length measurements in zebrafish

Zebrafish larvae were collected after fertilization and individually placed in a 24-well plate. Each larva was imaged every twenty-four hours under the microscope (Leica DFC550, Leica Camera, Wetzlar, Germany) for four days. Gut measurements were made using Fiji Image J software and based on the distance from mouth and vent. All larvae were genotyped at the end of the experiment. Statistical significance was calculated using a two-paired t-test.

Intestinal transit assays in zebrafish

Seven-days old zebrafish larvae were fed for two hours with a mixture containing 100 mg of powdered larval feed, 150 µL of fluorescent 2.0 µm polystyrene microspheres (Invitrogen) and 50 µl of water. After two hours, larvae were anesthetized with Tricaine and were checked under the microscope (Leica DFC550, Leica Camera, Wetzlar, Germany) for the presence of fluorescent food in their intestine. Individual larvae were placed in separate wells of a 24-well plate and examined twenty-four hours after feeding under the microscope. All larvae were genotyped at the end of the experiment.

Enteric neuronal density analysis in zebrafish

Zebrafish larvae at five days post fertilization (dpf), previously treated with phenyl thiourea at 1dpf to prevent the pigmentation, were used for enteric neuronal density analysis. Larvae were incubated on ice for 30 minutes before being fixed in 4% Paraformaldehyde (PFA) and washed in 1x phosphate buffer solution/ 0.25% Triton X-100 for 1 hour, at room temperature. Whole mount antibody staining was performed according to previous reports (Uyttebroek et al., 2010). Anti HuC/HuD (1:1000, Invitrogen, Waltham, Massachusetts, USA) was used as primary antibody and the Alexa Fluor 488 Mouse IgG (1:2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA) as secondary antibody. Larvae were observed under the fluorescent microscope (Leica M165FC, Leica Camera, Wetzlar, Germany) using the GFP filter. Images of the intestine were taken, and the number of enteric neurons were counted using Fiji Image J software. All larvae were genotyped at the end of the experiment. Statistical significance was calculated using a one-way ANOVA test.

Immunohistochemistry

Adult zebrafish were sacrificed and fixated in 4% PFA overnight. Fish were fixed in 1.8% low-melting-point agarose and proceeded with paraffin embedding. Ten µm sections were cut with the microtome and immunohistochemical (IHC) staining was performed using the Ventana Benchmark Ultra automated staining system (Ventana Medical System, Tuscon, AZ, USA). Briefly, antigen retrieval was performed on sectioned specimens for 60 min after deparaffinization, using the Cell Conditioning Solution (CC1, Ventana 950-124). After 30 minutes, incubation with the primary antibody was performed at 36˚C (ACTA2 1:2000; GTX124505; Genetex and HuC/D, 1:50; A-21271, Molecular Probes), followed by amplification with the Ultraview amplification kit (Ventana 760-080), and detection with the UltraView Universal DAB detection kit (Ventana 760-500). Sections were counterstained with hematoxylin II (Ventana 790-2208). Images were taken using Olympus DP72 digital camera microscope and analysed with Olympus cellSense software (Olympus, Tokyo, Japan).

RESULTS

FLNA is expressed in the muscle layers of the human small intestine

FLNA is a cytoplasmic protein that is ubiquitously expressed in all tissues of the body. However, not much is known about FLNA expression during human development. Since CIPO is characterized by intestinal dysmotility, we investigated the expression of FLNA in the human intestine (jejunum/ileum) at weeks 17 and 22 of fetal development, at the neonatal stage and postnatally (three-months old). At all developmental stages, FLNA was abundantly expressed in the cytoplasm of smooth muscle cells, including smooth muscle cells of the muscularis mucosa (mm), and the inner circular layer (cm) and outer longitudinal layer (lm) of the muscularis propria (Figure 1).

Figure 1.

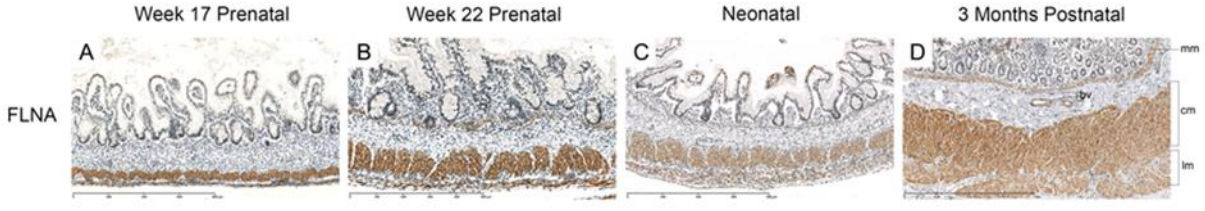

Figure 1: FLNA expression is present in the muscular layer of the human small intestine from early fetal stages. FLNA Immunohistochemistry (brown stain) performed in human intestinal specimens collected at different developmental stages shows that FLNA is expressed in the muscular layer of the small intestine. mm: muscularis mucosa; cm: circular layer of the muscularis propria; lm: longitudinal layer of the muscularis propria.

Expression of the long FLNA isoform is disrupted in CIPO patients

Recently, it has been shown that a deletion of two base pairs in *FLNA* (c.18-19delTC) in a CIPO patient, led to disruption of the long FLNA isoform (Jenkins et al., 2018). Since the two base pair deletions found in other CIPO patients (c.16-17delTC, c.65-66delAC), are also located between the two methionine residues shown to be distinct transcription sites (Figure 2a), we hypothesized a similar outcome for these deletions, i.e. disruption of the long FLNA isoform. To investigate that indeed an alternative initiation codon was used in the presence of the two base pair deletions, we used a mini-gene construct previously described (Gargiulo et al., 2007), where only exon two of *FLNA* (WT) is present, fused to an HA-tag. By expressing constructs containing the WT, c.16-17delTC (Mut1) and c.65-66delAC (Mut2), in HEK293 cells we observed that FLNA WT produced 2 bands, showing the existence of two initiation codons in FLNA (Figure 2b). However, in the presence of any of the deletions, Mut1 or Mut2, only the lower band was detected (Figure 2B), showing that in these cases only the second initiation codon is active.

To further study the impact of the two base pair deletions in the total expression levels of FLNA, we overexpressed a construct containing FLNA WT, Mut1 and Mut2, tagged C-terminally with a Myc-tag, in HEK293 cells. We subsequently determined the levels of *FLNA* messenger RNA (mRNA) and protein, by quantitative real time (qRT)-PCR and Western blot, respectively. Our

results showed that the presence of the two base pair deletions lead to reduced levels of FLNA, both at mRNA and protein levels (Figure 2C and 2D).

Figure 2.

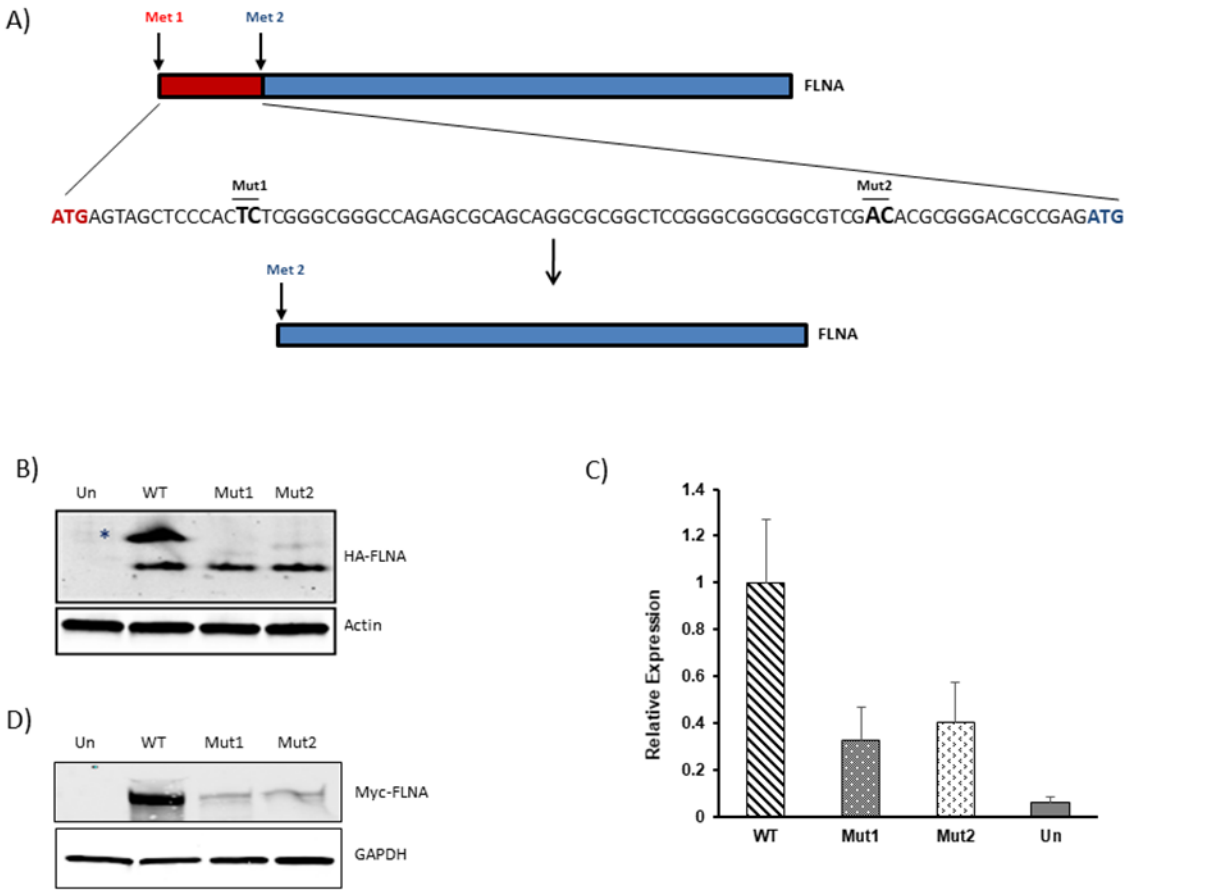

Figure 2: Absence of the long isoform results in decrease of FLNA expression. **A)** Schematic representation of FLNA showing expression of two isoforms from a single transcript. In the presence of the 2-base pair (bp) deletions identified in CIPO patients, only the short isoform is expressed (blue). **B)** Western blot (WB) analysis of a mini gene construct expressing wild-type (WT) and mutant [Mut1(c.65-66delAC); Mut2 (c.16-17delTC)] versions of FLNA, show that two bands are expressed in the WT, while the mutants, express only one band. **C)** qRT-PCR and **D)** WB performed show reduced mRNA and protein levels in the mutants.

The long FLNA isoform is required for contractility of intestinal smooth muscle cells

Our immunohistochemistry studies showed that FLNA is expressed in the smooth muscle of the intestine at different human developmental stages. Since the smooth muscle plays an important role in intestinal peristalsis, we decided to evaluate whether the 2bp deletions in *FLNA* affect contraction of human intestinal smooth muscle cells (hISMCs). For this purpose, we transfected hIMSCs with constructs expressing the WT FLNA protein and the two 2bp deletions associated with CIPO, Mut1 (c.16-17delTC) and Mut2 (c.65-66delAC). Untransfected cells (Un)

were used as control. We then performed *in vitro* contractility assays, based on the contraction of a collagen matrix. Cellular contractility was calculated by measuring the total area occupied by the collagen matrix at time 0 (Initial Area (Ai)) and 24 hours after detachment (Final area (Af)) of the matrix from the wells. Our results showed that overexpression of FLNA leads to an increase in cellular contractility, whereas expression of Mut1 and Mut2 showed no significant effect when compared to the control (Figure 3A and 3B). This result is likely due the lower amounts of FLNA produced when the 2 deletions (Mut1 and Mut2) are present, showing that FLNA levels are instrumental for intestinal smooth muscle contractility.

Figure 3.

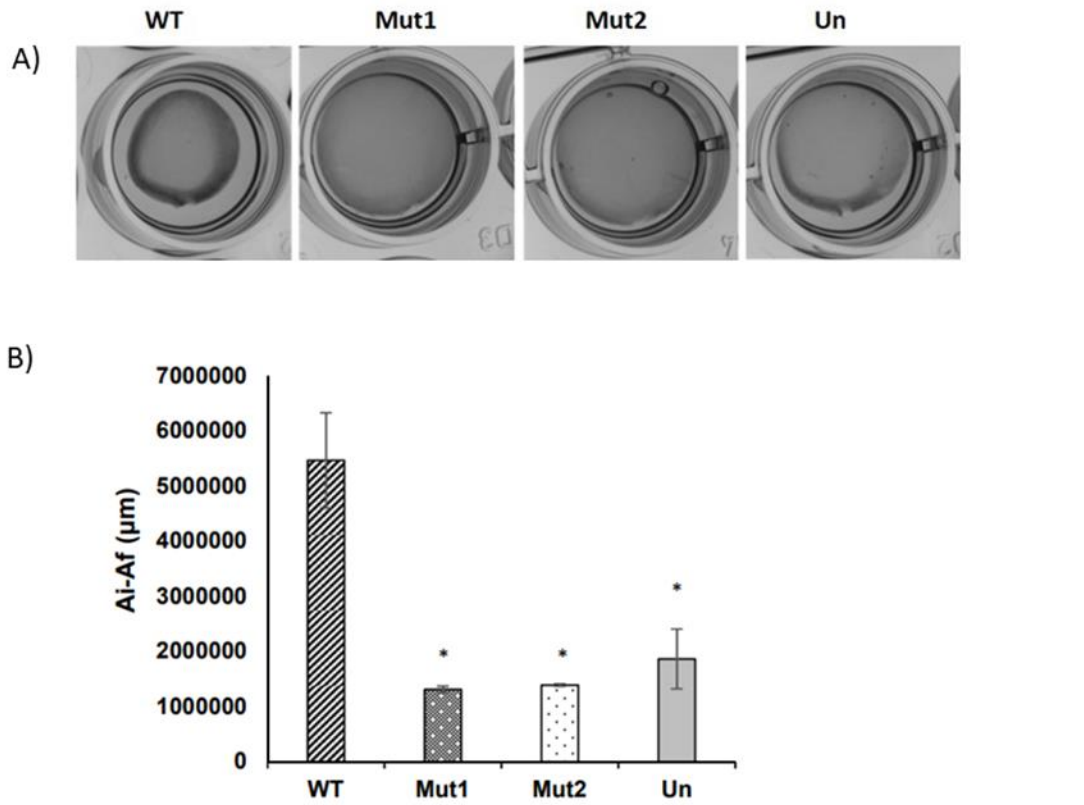

Figure 3: The long FLNA isoform is required for intestinal smooth muscle contractility, in vitro. **A)** Contractility assays performed in human intestinal smooth muscle cells (hISMC) transfected with FLNA WT, Mut1 (c.16-17delTC) and Mut2 (c.65-66delAC), show an increase in contractility upon expression of FLNA WT, when compared to untrasnfected cells (Un). However this effect was lost in the presence of both mutants, Mut1 and Mut2. **B**) Quantification of the difference between the initial (0h) and final (24h) area occupied by the collagen matrix, confirms altered contractility of hISMCs overexpressing Mut1 and Mut2. *p<0,05. Ai: initial area; Af: final area.

Generation of the *flna* long isoform knockout zebrafish line

To evaluate the effect of the long FLNA isoform on the development of the intestine, we used the zebrafish as an *in vivo* model. RT-PCR results showed that *flna* is expressed in early

embryonic stages starting at the four-cell stage, with increased expression throughout development (Figure 4A). *In situ* hybridization also showed that *flna* is expressed in the brain and intestine of the zebrafish at all stages analysed (Figure 4B).

To generate the transgenic zebrafish line expressing only the short Flna isoform, we used two TALENs designed to target the region between the two methionine residues located in the first exon of *flna*. Considering that disruption of *Nci*I restriction site would occur if the TALENs had successfully annealed, we used this enzyme to distinguish WT from transgenic fish (Supplementary Figure 1A). A deletion of six base pairs followed by an insertion of one base pair in a homozygous (*flna long isoform*$^{-/-}$) and in a heterozygous (*flna long isoform*$^{+/-}$) state, was identified in one male and one female fish, respectively (Supplementary Figure 1B). This in-frame deletion followed by an insertion, resulted in the appearance of an early stop codon at the end of exon 1, replicating the effect of the 2bp deletions found in CIPO patients. Mutant fish were subsequently crossed to generate homozygous offspring lacking the long Flna isoform (*flna* long isoform$^{-/-}$). Western blot analysis showed that Flna protein was present in heterozygous and homozygous larvae (Supplementary Figure 1C), showing that the short Flna isoform was still expressed.

Figure 4.

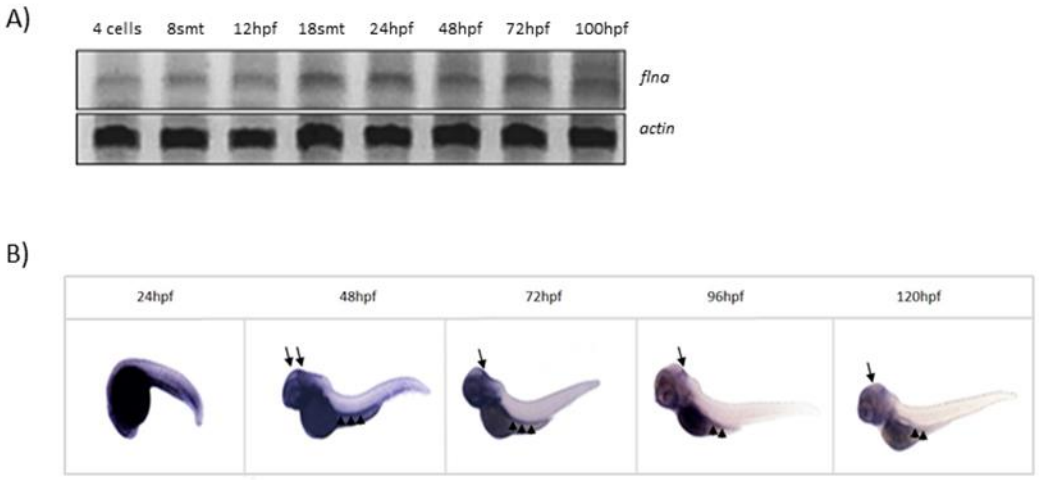

Figure 4: *flna* expression is detected in the zebrafish from early embryonic stages. **A)** RT-qPCR performed in RNA isolated from zebrafish embryos at different embryonic stages shows that flna transcripts are present from as early as the 4-cell stage, and its expression increases during development. **B)** *In situ* hybridization shows that flna is highly expressed in the brain (arrows) and gut (arrowheads) of the zebrafish at different stages of development. smt - somites; hpf - hours post fertilization.

The long FLNA isoform is required for intestinal elongation.

Since one of the features commonly seen in CIPO patients with *FLNA* mutations is the presence of CSBS, a dramatically shortened small intestine, we investigated the effect of disruption of the long FLNA isoform on the total length of the intestine of zebrafish larvae. Unlike humans, zebrafish do not have X and Y chromosomes, and carry two copies of the *flna* gene. Therefore, *flna long*$^{+/-}$ fish were analysed together with homozygous mutant fish. The intestinal length of approximately 150 offspring (F3) was measured every day, for four days and revealed that the *flna long isoform*$^{-/-}$ zebrafish had a significantly shorter intestine compared to WT (0.318 mm ± 0.014 vs. 0.334 mm ± 0.015, respectively) (Figure 5A). Disruption of only one of the *flna* alleles had no effect on the total length of the gut (0.331 mm ± 0.016 for *flna long isoform*$^{+/-}$ vs. 0.334 mm ± 0.015 for WT) (Figure 5A). To exclude the possibility that the shortened intestine found in *flna long isoform*$^{-/-}$ fish was the result of an overall reduction in body length, the total length of each larva was also measured. However, the relative gut length-body length ratio remained significantly shorter in the *flna long isoform*$^{-/-}$ mutant fish (Figure 5B).

Figure 5.

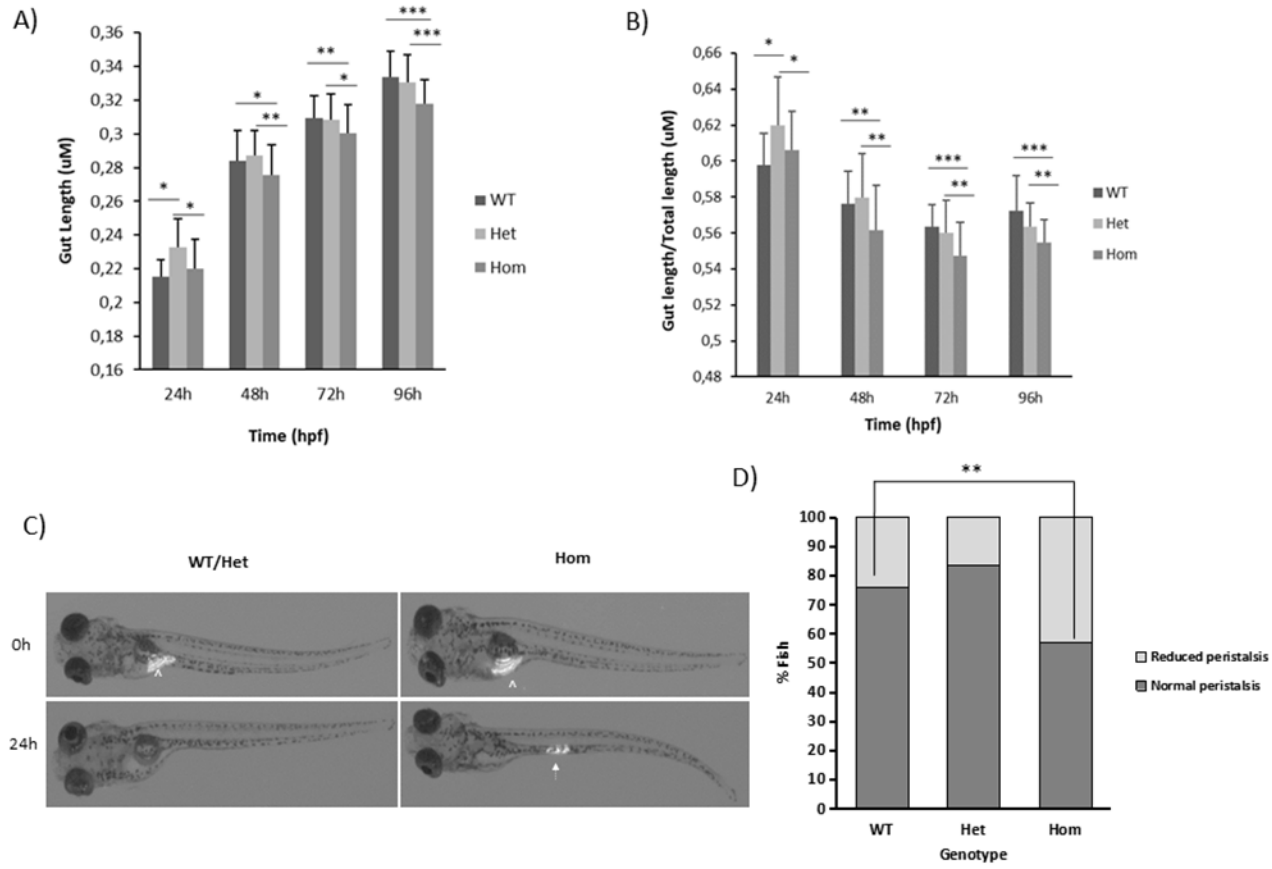

Figure 5: *flna long*$^{-/-}$ zebrafish have shorter small intestines and increased transit time. **A)** Measurement of the intestinal length of WT and transgenic fish shows that *flna long isoform*$^{-/-}$ (hom) embryos have significantly shorter intestines, when compared to WT and *flna long isoform*$^{+/-}$ (het). **B)** No effect of the body length was detected. **C)** WT, *flna long isoform*$^{+/-}$ and *flna long isoform*$^{-/-}$ larvae were screened for the

presence of fluorescence microspheres in the intestinal bulb (arrowheads). **D)** *flna long isoform* $^{-/-}$ (hom) larvae have longer transit time (43%) compared to WT (24%) and *flna long isoform*$^{+/-}$ (17%; het) fish. Hpf - hours post-fertilization; *p<0,05; ** p<0,005; ***p<0,00005.

Intestinal transit is reduced in the *flna long isoform*$^{-/-}$ transgenic zebrafish

Smooth muscle contractility is required for normal intestinal function. As our *in vitro* results showed that contractility of hiSMCs in the absence of the long FLNA isoform is affected, we decided to perform intestinal transit assays using our *flna* transgenic line. For these studies, 7-day old larvae (25 WT, 24 *flna* long isoform$^{+/-}$ and 35 *flna* long isoform$^{-/-}$) were fed for two hours with fluorescently labelled larval feed and imaged to select the ones that showed fluorescence in their intestinal bulb (Figure 5C). Twenty-four hours later, these larvae were imaged again, and we observed that while the majority of WT and heterozygous fish no longer showed fluorescence within the GI tract (76% and 83% respectively), only 57% of homozygous fish showed the same (Figure 5C and 5D).

To exclude the possibility that the motility defect observed in the *flna* long isoform$^{-/-}$ was a result of reduced numbers of enteric neurons present in the Enteric Nervous System (ENS), we decided to quantify these cells in the gut of WT, heterozygous and homozygous 5 dpf larvae (n=16, 46 and 11 respectively). We found no significant differences (31.043 neurons ± 3.226 vs. 30.504 neurons ± 3.757 vs. 31.552 neurons ± 2.384, respectively) between the groups (Figure 6A and 6B). Additional immunohistochemistry studies using the pan-neuronal marker HuC/D, revealed normal distribution of enteric neurons in between the inner and outer smooth muscle layers (sml) for the *flna* long isoform$^{-/-}$ fish (Figure 6C, green arrow).

Figure 6.

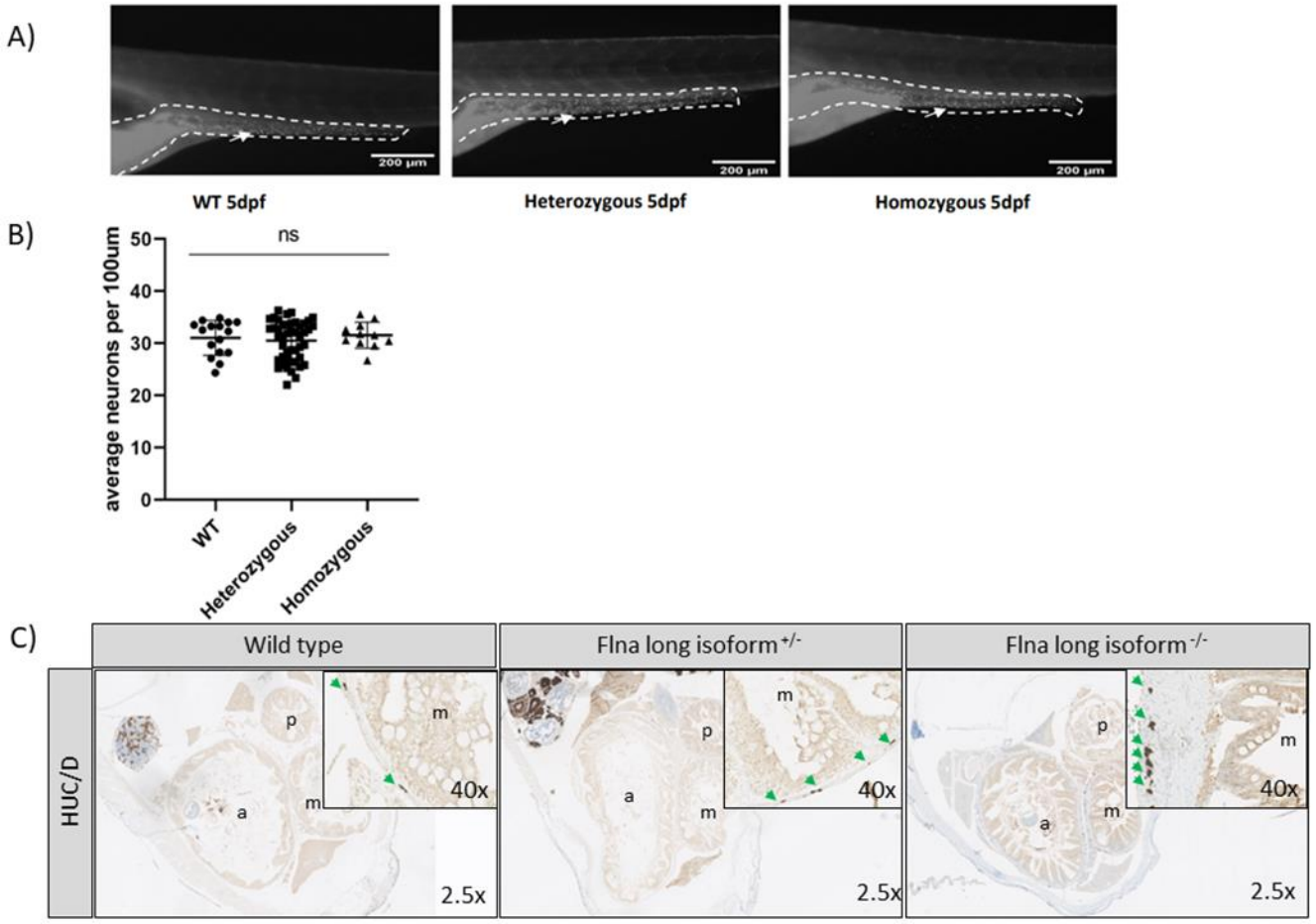

Figure 6. **Enteric neuronal numbers and localization are not affected in *flna long isoform*$^{-/-}$ larvae. A)** Whole mount staining of WT, *flna long isoform*$^{+/-}$ (heterozygous) and *flna long isoform*$^{-/-}$ (homozygous) 5dpf fish, was performed with an HuC/D antibody, to label enteric neurons (pointed by white arrow). **B)** Quantification of the number of enteric neurons present in WT, heterozygous and homozygous larvae showed no difference on the neuronal density of the gut between genotypes. Ns= not significant. **C)** HuC/D stainings performed in adult fish showed a similar distribution and localization of enteric neurons for WT, heterozygous and homozygous fish (green arrow).

Diffuse abnormal layering of the intestinal smooth muscle is observed in *flna* long isoform$^{-/-}$ adult fish.

Previous studies have shown that CIPO patients in which the long FLNA isoform is missing, presented a diffused abnormal layering of small intestinal smooth muscle (Kapur et al., 2010). To investigate if similar changes were present in our *flna* long isoform$^{-/-}$ adult fish, haematoxylin staining was performed in full-thickness sections. Our results showed that the muscularis propria of WT and mutant zebrafish was composed by two distinct layers: outer layer or longitudinal smooth muscle (lsm), and inner layer or circular smooth muscle (csm). This is in agreement with

previous reports (Georgijevic et al., 2007). However, in the *flna* long isoform$^{-/-}$ fish, we interestingly found hypertrophy of the sml (Figure 7A). Additional staining for α-smooth muscle actin (ACTA2), confirmed a thicker and diffuse abnormal layering of the muscularis propia in the posterior intestine of homozygous fish and showed the presence of a prominent muscularis mucosae layer that is not visible in the WT or heterozygous fish (Figure 7B, red arrow). These results show that structural and architectural changes are present in the intestinal smooth muscle of the *flna* long isoform$^{-/-}$ adult fish.

Expression of smooth muscle markers are affected in *flna* long isoform$^{-/-}$ fish.

To further investigate the reasoning behind the histological changes detected in the homozygous fish, we determined the expression levels of intestinal smooth muscle markers, *acta2*, *sm22α-b* and *calponin1*, by qRT-PCR. The first two, are early developmental markers, whereas calponin 1 is specific for differentiated smooth muscle. We decided to look at the expression of these markers at two time points, at 7dp when the larvae start eating and the intestinal is functional, and in the adult stage. The levels of *flna* present in larvae and adult fish, were also determined for the three genotypes. Our results showed that changes in the expression of smooth muscle markers are indeed present in the *flna long isoform*$^{-/-}$ fish. *acta2* was significantly downregulated in homozygous larvae, when compared to heterozygous and control fish. However, in adult stages, an increase in the expression level of acta2 was significantly detected. For *sm22α-b*, a decrease expression was observed in homozygous larvae and adult fish when compared to WT fish, while *calponin1*, was increased (Figure 7C). Interestingly, the levels of *flna* were significantly upregulated in both heterozygous and homozygous larvae, and this pattern was maintained in adulthood but only in the *flna* long isoform$^{-/-}$ fish (Figure 7C).

Figure 7.

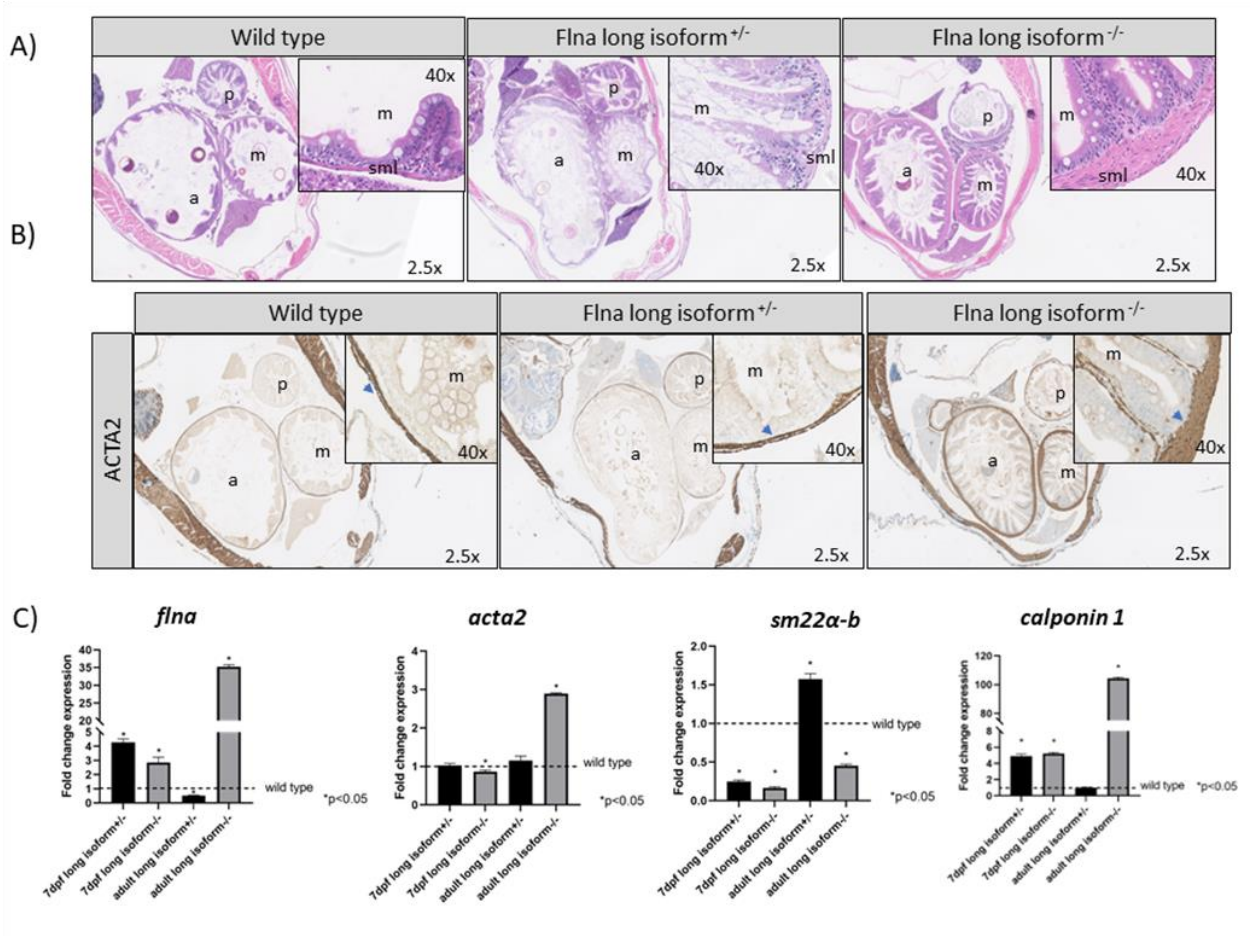

Figure 7: *flna long isoform*$^{-/-}$ zebrafish show hypertrophy and abnormal layering of the intestinal smooth muscle, as well as altered expression of smooth muscle markers. **A)** Hematoxylin staining showed the presence of a thick and diffuse abnormal layering in muscularis propria of homozygous adult fish. Low to high magnification are presented. **B)** Acta2 staining confirmed hypertrophy of the intestinal smooth muscle in *flna long isoform*$^{-/-}$ adult fish, and revealed an extra muscularis mucosae layer (blue arrow; 1, 2, 3) that is not present in the WT or heterozygous fish. Low to high magnification are presented. **C)** qRT-PCR revealed altered expression (*p<0.05) of intestinal smooth muscle markers in 7dpf homozygous larvae and adult zebrafish. a: anterior gut lumen; b: middle gut lumen; p: posterior gut lumen; sml: smooth muscle layer.

DISCUSSION

FLNA has been extensively studied as pathogenic variants in its coding region have been associated with a broad spectrum of disorders collectively referred to as X-linked filaminopathies (4). To date, loss and gain of function variants in *FLNA* are associated with eight syndromes, including CIPO, a heterogeneous gastrointestinal disorder characterized by compromised intestinal motility (Gargiulo et al., 2007; Jenkins et al., 2018; Van Der Werf et al., 2013).

Interestingly, these variants are two base pair deletions located in between two different transcription starting sites, and they have recently been shown to block expression of the long FLNA isoform (Jenkins et al., 2018). In addition, the long FLNA isoform was observed to be the predominant one in ileum and colon, as well as in colonic smooth muscle (Jenkins et al., 2018). Based on our results, abundant expression of FLNA was indeed detected in the small intestine during human embryonic development, specifically in the smooth muscle cells of the muscularis mucosa and muscularis propria (Figure 1). However, we were unable to distinguish expression of the different FLNA isoforms, due to the absence of specific commercially available antibodies. Our mini-gene assays confirmed the presence of two initiation codons in FLNA and showed that CIPO causing *FLNA* variants, lead to the adoption of the second initiation codon, resulting in the expression of only the short isoform (Figure 2B). . Considering that the long isoform has been shown to be the major FLNA isoform expressed in intestinal smooth muscle (12), it is not surprising that these patients only have intestinal complaints, with the majority of them being diagnosed with CIPO. Taken together, it is tempting to say that loss of the long FLNA isoform in CIPO patients affects intestinal SMCs. Such concept has already been suggested before based on the analysis of the neuromuscular histopathology of CIPO patients with *FLNA* variants (13). Therefore, our results are in line with previous findings, confirming a myopathic nature for CIPO in these cases.

Previous studies have shown that disruption of the first 32 amino acids of FLNA, does not impact FLNA co-localization with actin, neither its actin binding ability (21). However, selective disruption of *Flna* in the smooth muscle of adult mice, showed that Flna is involved in signalling cascades for smooth muscle contractility (Retailleau et al., 2016). In line with this study, we observed that expression of only the short isoform, leads to expression changes that impact cellular contractility (Figure 3A and 3B), suggesting that FLNA levels are instrumental for intestinal smooth muscle contraction To further investigate these findings, we generated a transgenic zebrafish line in which expression of the long *flna* isoform was abolished. We observed that *flna long isoform*$^{-/-}$ larvae had significantly shortened intestines (Figure 5A), suggesting that the long FLNA isoform is important for intestinal elongation. This data also explains why the majority of CIPO patients carrying *FLNA* mutations are born with CSBS. To our surprise, the reduction observed in intestinal length was limited to 5%, which is far below the reduction observed in CSBS patients (>75%). This discrepancy can be attributed to anatomical differences, as the fish gut is just a simple linear tube with no defined regions, while in humans it is very elongated and requires complex patterns of rotation and looping to reach its final configuration. We also cannot exclude the involvement of other proteins with a redundant function in the zebrafish. Previous studies have shown that three other genes are known to encode for *flna* homologs in the fish, which include *flnb*, *flnca*, and *flncb* (Bandaru et al., 2014; Ruparelia et al., 2012). Although the function of all flna proteins is not yet clear, shared characteristics between all homologs suggest possible functional redundancy. Our *flna long isoform*$^{-/-}$ larvae also showed increased intestinal transit time, when compared to WT and *flna long isoform*$^{+/-}$ fish (Figure 5C and 5D), suggesting that intestinal motility is affected by the loss of the long FLNA isoform. Histological examination

of the intestine of adult homozygous fish revealed hypertrophy of the smooth layer and showed the presence of what seems to be an extra muscularis mucosae layer (Figure 7A and B). Similar findings have been described for CIPO patients carrying *FLNA* variants (13). Moreover, we observed altered expression of intestinal smooth muscle markers in *flna long isoform*$^{-/-}$ larvae and adult fish, that can potential underlie the structural changes identified (Figure 7C). Since we found no differences in the distribution and number of enteric neurons present in WT, *flna long isoform*$^{+/-}$ and *flna long isoform*$^{-/-}$ (Figure 6), it seems that the GI defects observed in CIPO patients with *FLNA* deletions, are likely due to a defect in smooth muscle contractility caused by the absence of the long FLNA isoform. However, we cannot exclude that possible defects in the anatomy or physiology of the ENS might still exist..

In summary, our data confirm the presence of two FLNA isoforms and show that the long isoform plays an instrumental role in intestinal elongation and smooth muscle contractility, bringing new insights to CIPO pathogenesis. We also confirm that the zebrafish is an excellent animal model to study intestinal development, specifically congenital intestinal myopathies, as the phenotype of our homozygous fish showed great similarities to the one previously described for CIPO patients (13).

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Conflict of Interest: No conflicts of interest exist.

Abbreviations:

CIPO	Chronic intestinal pseudo-obstruction
CSBS	Congenital Short Bowel Syndrome
Dpf	days post fertilization
FLNA	Filamin A
HEK	Human Embryonic Kidney
HIMSCs	Human Intestinal Smooth Muscle Cells
Hpf	hours post fertilization
HSCR	Hirschsprung disease
MMIHS	Megacystis Microcolon Intestinal Hypoperistaltic Syndrome

References

Bandaru, S., Zhou, A. X., Rouhi, P., Zhang, Y., Bergo, M. O., Cao, Y., & Akyürek, L. M. (2014). Targeting filamin B induces tumor growth and metastasis via enhanced activity of matrix metalloproteinase-9 and secretion of VEGF-A. *Oncogenesis*, *3*(9). https://doi.org/10.1038/oncsis.2014.33

Bernstein, J. A., Bernstein, D., Hehr, U., & Hudgins, L. (2011). Familial cardiac valvulopathy due to filamin A mutation. *American Journal of Medical Genetics, Part A*, *155*(9), 2236–2241. https://doi.org/10.1002/ajmg.a.34132

Gamboa, H. E., & Sood, M. (2019). Pediatric Intestinal Pseudo-obstruction in the Era of Genetic Sequencing. *Current Gastroenterology Reports*, *21*(12). https://doi.org/10.1007/s11894-019-0737-y

Gargiulo, A., Auricchio, R., Barone, M. V., Cotugno, G., Reardon, W., Milla, P. J., Ballabio, A., Ciccodicola, A., & Auricchio, A. (2007). Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. *American Journal of Human Genetics*, *80*(4), 751–758. https://doi.org/10.1086/513321

Georgijevic, S., Subramanian, Y., Rollins, E. L., Starovic-Subota, O., Tang, A. C. Y., & Childs, S. J. (2007). Spatiotemporal expression of smooth muscle markers in developing zebrafish gut. *Developmental Dynamics*, *236*(6), 1623–1632. https://doi.org/10.1002/dvdy.21165

Halim, D., Hofstra, R. M. W., Signorile, L., Verdijk, R. M., Van Der Werf, C. S., Sribudiani, Y., Brouwer, R. W. W., van IJcken, W. F. J., Dahl, N., Verheij, J. B. G. M., Baumann, C., Kerner, J., Van Bever, Y., Galjart, N., Wijnen, R. M. H., Tibboel, D., Burns, A. J., Muller, F., Brooks, A. S., & Alves, M. M. (2016). ACTG2 variants impair actin polymerization in sporadic Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *Human Molecular Genetics*, *25*(3), 571–583. https://doi.org/10.1093/hmg/ddv497

Jenkins, Z. A., Macharg, A., Chang, C. Y., van Kogelenberg, M., Morgan, T., Frentz, S., Wei, W., Pilch, J., Hannibal, M., Foulds, N., McGillivray, G., Leventer, R. J., García-Miñaúr, S., Sugito, S., Nightingale, S., Markie, D. M., Dudding, T., Kapur, R. P., & Robertson, S. P. (2018). Differential regulation of two FLNA transcripts explains some of the phenotypic heterogeneity in the loss-of-function filaminopathies. *Human Mutation*, *39*(1), 103–113. https://doi.org/10.1002/humu.23355

Kapur, R. P., Robertson, S. P., Hannibal, M. C., Finn, L. S., Morgan, T., Van Kogelenberg, M., & Loren, D. J. (2010). Diffuse abnormal layering of small intestinal smooth muscle is present in patients with FLNA mutations and X-linked intestinal pseudo-obstruction. *American Journal of Surgical Pathology*, *34*(10), 1528–1543. https://doi.org/10.1097/PAS.0b013e3181f0ae47

Kyndt, F., Gueffet, J. P., Probst, V., Jaafar, P., Legendre, A., Le Bouffant, F., Toquet, C., Roy, E., McGregor, L., Lynch, S. A., Newbury-Ecob, R., Tran, V., Young, I., Trochu, J. N., Le Marec, H., & Schott, J. J. (2007). Mutations in the gene encoding filamin A as a cause for familial cardiac valvular dystrophy. *Circulation*, *115*(1), 40–49. https://doi.org/10.1161/CIRCULATIONAHA.106.622621

Nakamura, F., Stossel, T. P., & Hartwig, J. H. (2011). The filamins: Organizers of cell structure and function. *Cell Adhesion and Migration*, *5*(2), 160–169. https://doi.org/10.4161/cam.5.2.14401

Oegema, R., Hulst, J. M., Theuns-Valks, S. D. M., van Unen, L. M. A., Schot, R., Mancini, G. M. S., Schipper, M. E. I., de Wit, M. C. Y., Sibbles, B. J., de Coo, I. F. M., Nanninga, V., Hofstra, R. M. W., Halley, D. J. J., &

Brooks, A. S. (2013). Novel no-stop FLNA mutation causes multi-organ involvement in males. *American Journal of Medical Genetics, Part A*, *161*(9), 2376–2384. https://doi.org/10.1002/ajmg.a.36109

Onoprishvili, I., Ali, S., Andria, M. L., Shpigel, A., & Simon, E. J. (2008). Filamin A mutant lacking actin-binding domain restores mu opioid receptor regulation in melanoma cells. *Neurochemical Research*, *33*(10), 2054–2061. https://doi.org/10.1007/s11064-008-9684-y

Retailleau, K., Arhatte, M., Demolombe, S., Peyronnet, R., Baudrie, V., Jodar, M., Bourreau, J., Henrion, D., Offermanns, S., Nakamura, F., Feng, Y., Patel, A., Duprat, F., & Honoré, E. (2016). Arterial Myogenic Activation through Smooth Muscle Filamin A. *Cell Reports*, *14*(9), 2050–2058. https://doi.org/10.1016/j.celrep.2016.02.019

Robertson, S. P. (2005). Filamin A: Phenotypic diversity. *Current Opinion in Genetics and Development*, *15*(3 SPEC. ISS.), 301–307. https://doi.org/10.1016/j.gde.2005.04.001

Ruparelia, A. A., Zhao, M., Currie, P. D., & Bryson-Richardson, R. J. (2012). Characterization and investigation of zebrafish models of filamin-related myofibrillar myopathy. *Human Molecular Genetics*, *21*(18), 4073–4083. https://doi.org/10.1093/hmg/dds231

Thapar, N., Saliakellis, E., Benninga, M. A., Borrelli, O., Curry, J., Faure, C., De Giorgio, R., Gupte, G., Knowles, C. H., Staiano, A., Vandenplas, Y., & Di Lorenzo, C. (2018). Paediatric Intestinal Pseudo-obstruction: Evidence and Consensus-based Recommendations from an ESPGHAN-Led Expert Group. *Journal of Pediatric Gastroenterology and Nutrition*, *66*(6), 991–1019. https://doi.org/10.1097/MPG.0000000000001982

Uyttebroek, L., Shepherd, I. T., Harrisson, F., Hubens, G., Blust, R., Timmermans, J. P., & van Nassauw, L. (2010). Neurochemical coding of enteric neurons in adult and embryonic zebrafish (Danio rerio). *Journal of Comparative Neurology*, *518*(21), 4419–4438. https://doi.org/10.1002/cne.22464

Van Der Werf, C. S., Sribudiani, Y., Verheij, J. B. G. M., Carroll, M., O'Loughlin, E., Chen, C. H., Brooks, A. S., Liszewski, M. K., Atkinson, J. P., & Hofstra, R. M. W. (2013). Congenital short bowel syndrome as the presenting symptom in male patients with FLNA mutations. *Genetics in Medicine*, *15*(4), 310–313. https://doi.org/10.1038/gim.2012.123

Wade, E. M., Halliday, B. J., Jenkins, Z. A., O'Neill, A. C., & Robertson, S. P. (2020). The X-linked filaminopathies: Synergistic insights from clinical and molecular analysis. *Human Mutation*, *41*(5), 865–883. https://doi.org/10.1002/humu.24002

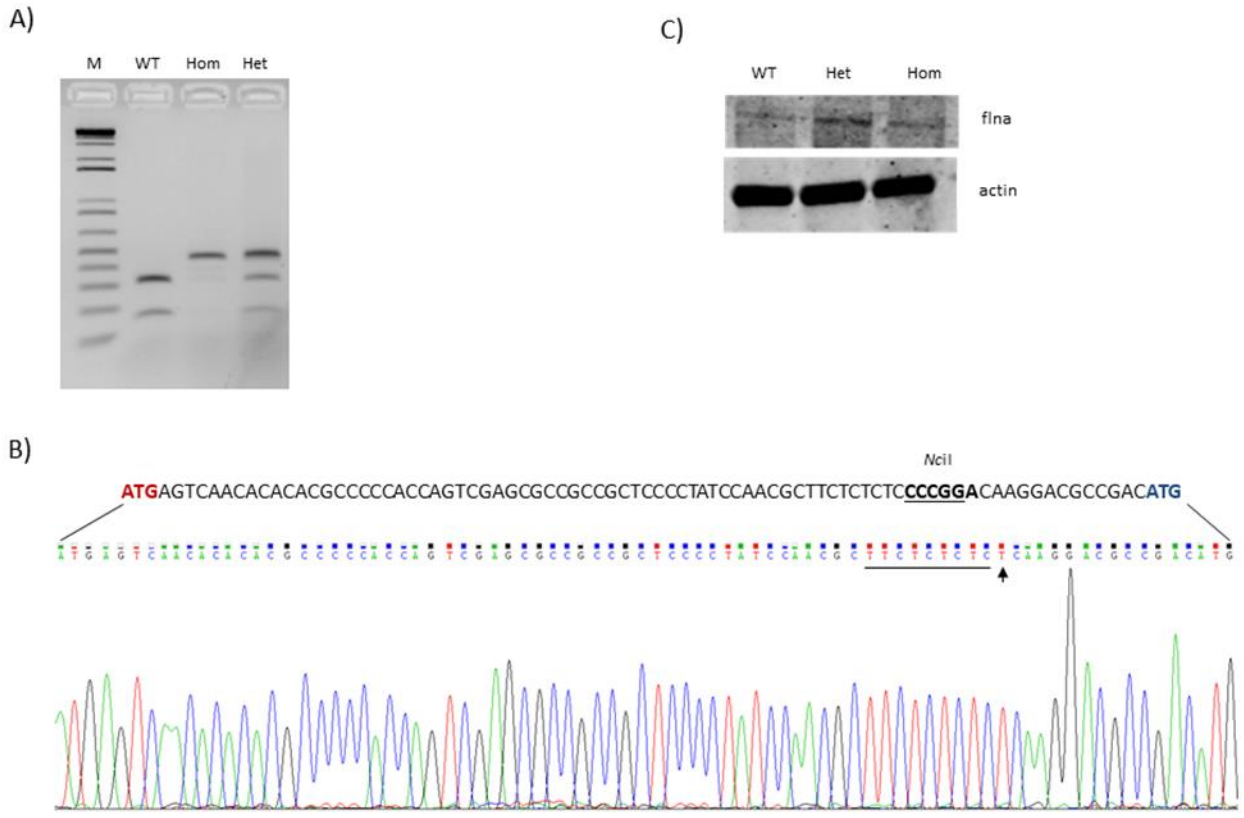
Walker, J. M. (1995). In situ hybridization protocols (methods in molecular biology, Vol. 33). In *Trends in Genetics* (Vol. 11, Issue 8). https://doi.org/10.1016/0168-9525(95)90187-6

Supplementary Materials

Supplementary Table 1. Primers used in this study.

Primer	Sequence
FLNAmutF(c.16-17)_SDM	AGTAGCTCCCACTCGGGCGGGCCA
FLNAmutR(c.16-17)_SDM	TGGCCCGCCCGAGTGGGAGCTACT
FLNAmutF(c.65-66)_SDM	CGGCGGCGTCGACGCGGGACGC
FLNAmutR(c.65-66)_SDM	GCGTCCCGCGTCGACGCCGCCG
FLNAqPCR3F	CTGCCCATCACCAACTTCAG
FLNAqPCR4R	GTCCCAAGAGTCCCAGTCAG
flna_F_fish-genotyping	CACCCGAATTGCAGTTTCTC
flna_R_fish-genotyping	CAATGGAGACCAGCTTGATG
flna-qPCR-F	GTTCCGCAAGTACAACCAGC
flna-qPCR-R	TCTTCTGCTTGCTTTCCTCG
acta2-qPCR-F	TCAAGATAATCGCTCCACCTG
acta2-qPCR-R	TTGCTGATCCACATCTGCTG
sm22α-b-qPCR-F	AGACGGATGCGTGTTGTG
sm22α-b-qPCR-R	TCTGCTTAAAGGCCATGCTG
calponin1-qPCR-F	CAGAAAACTTCATGGAGGGGGC
calponin1-qPCR-R	GAGTGTGGTTCATGTCCTCG

Supplementary Figure 1.



Supplementary Figure 1: Generation of the long *flna* knockout zebrafish line. A) Genotype of the transgenic *flna* zebrafish generated with TALENs. Wild-type (WT) and transgenic fish can be distinguished after treatment with *Nci*I restriction enzyme. In the WT situation a restriction site for *Nci*I is present between the two methionine residues. Therefore, treatment with this enzyme generates two bands. If the TALENs had successfully annealed, the restriction site for *Nci*I is disrupted, leading to a single uncut band [homozygous (hom)], or to a combination of both cut and uncut bands [heterozygous (het)]. **B)** Sanger sequencing of the transgenic *flna long*$^{-/-}$ zebrafish detected a homozygous deletion of 6-base pairs (bp) (underlined), disrupting the *Nci*I restriction site (GGGCC), followed by an insertion of one bp, thymine (arrow). **C)** Western blot showing Flna expression in WT, *flna long*$^{+/-}$ (het), and *flna long*$^{-/-}$ (hom) zebrafish.

Chapter 6.

General Discussion and Future Perspective

Pediatric intestinal pseudo-obstruction (PIPO) is a form of congenital intestinal motility disorders without the presence of any lesion restricting or occluding the intestinal lumen (Di Nardo et al., 2017; Zenzeri et al., 2020). The cause of PIPO can be divided into primary, secondary and idiopathic. Primary PIPO has a genetic nature and many causative genes have been identified due to recent advances in genetic testing (Gamboa & Sood, 2019). However, there is still a strong missing heritability, as for the majority of patients, the genetic cause leading to disease is unknown. The studies described in this thesis aimed to identify new causative genes involved in the pathogenesis of primary neuropathic and myopathic PIPO using genetic (Chapter 2) and epigenetic (Chapter 4) approaches. They also highlight the use of various disease models to delineate cellular and molecular changes caused by variants identified in PIPO related genes (Chapter 2, 3, 4, 5).

Identification of novel PIPO genes

Whole exome sequencing (WES) has contributed to the identification of more than 1000 Mendelian genes and made an accelerated pace into clinical diagnostics in recent years. Diagnostic yield of WES in perinatal and postnatal cohorts has ranged from 8.5 to 80% and 25 to 50%, respectively. Therefore, WES is now a first-tier investigation for various clinical presentations (Aggarwal, 2021; Bertier et al., 2017; Corominas et al., 2022). Particularly in PIPO where the majority of cases is sporadic with likely a *de novo* pattern of inheritance, WES analysis has identified several new causative genes, for example *LMOD1* (Halim, Wilson, et al., 2017), *MYLK* (Halim, Brosens, et al., 2017), *MYH11* (Dong et al., 2019), *MYL9* (Moreno et al., 2018), *RAD21* (Bonora et al., 2015), and *SGOL1* (Chetaille et al., 2014). However, there are still many cases left without genetic diagnosis and unexplained disease mechanisms. In **Chapter 2**, we identified a *de novo* heterozygous deletion of 10 base pairs, in the Transcription Factor Activating Protein-2 Beta gene (*TFAP2B*) (NM_003221.4: c.602-5_606delTCTAGTTCCA) in a PIPO patient, using WES. This variant was classified as a variant of unknown significance (VUS) according to the American College of Medical Genetics and Genomics (ACMG) guidelines for variant interpretation. Intriguingly, three loss of function variants in *TFAP2B* have been reported in control cohorts in GnomAD. However, considering that mild GI complaints are normally missed, we cannot exclude that these cases could be mistakenly identified as controls. A similar effect is frequently seen in family members who carry the same pathogenic variant as the affected proband, but only present with mild GI symptoms (Sribudiani et al., 2018). This phenomenon called 'penetrance', is commonly present in intestinal motility disorders. For example, reduced penetrance has been documented in non-syndromic familial HSCR cases with autosomal

dominant or recessive inheritance (Alves et al., 2013), as despite the presence of the variant, only part of the population develops the disease.

Epigenetic modifications have been shown to contribute to PIPO pathogenesis, especially in the context of neuropathic PIPO. In Hirschsprung disease (HSCR), DNA methylation of genes involved in the development of the ENS has already been described. A CG-rich region within the *RET* promoter has been shown to regulate its expression levels in peripheral white blood cells (Munnes et al. 1998). On a higher level, it has also been shown that *de novo* methyltransferases such as DNMT3B, play a role in neural crest development and thus, if aberrant, can contribute to a more severe disease phenotype, especially in combination with rare variants in known HSCR-related genes (Torroglosa et al. 2014). These DNMTs, as well as other epigenetic regulators such as chromatin modifiers, are known to be highly tissue and cell-type specific (Bonn et al. 2012, Lokk et al. 2014, Torroglosa et al. 2016). Therefore, it is tempting to say that epigenetic modifications occurring in ENCCs might underlie the unexplained genetic diagnosis of HSCR. In **Chapter 4**, DNA from enteric neuronal cells was isolated from HSCR patients and controls. Genome wide methylation analysis was then performed to investigate the presence of differentially methylated regions (DMRs) in transcription starting sites (TSS), gene bodies (intragenic) and CPG islands. A global hypermethylation in TSS and gene bodies was observed in all patients analyzed, which can potentially impact expression levels of specific genes. Gene ontology analysis revealed that differential methylated genes were involved in pathways related to structural and cellular development of the ENS, such as axonogenesis and synapse organization. However, since the function of intragenic methylation have yet to be further elucidated, we decided to focus specifically on TSS, as promoter methylation has been widely accepted to cause gene inactivation via transcription repression (Li & Zhang, 2014; Medvedeva et al., 2013). Using this approach, we identified 5 hypermethylated genes, namely *CSFR1, ZNF124*, *SLC6A16, MAB21L2,* and *ENTPD7*. The first three genes have been linked to central nervous system disorders (Ho et al., 2019; Luo et al., 2021; Park et al., 2019; Siamoglou et al., 2023), while *ENTPD7* is known to affect cell senescence (Tordella et al., 2016). *MAB21L2* on the other hand, was previously shown by our lab to be required for ENS development in zebrafish (Sribudiani et al., 2018). Studies in mice showed that *Mab21l2* is required for eye, ventral body wall (Yamada et al., 2004), heart, and liver development (Saito et al., 2012). In humans, mutations in *MAB21L2* have been linked to eye disorders (Deml et al., 2015). However, this gene has not been linked to intestinal disorders. Therefore, we decided to focus on the DMR identified in the TSS of *MAB21L2* for further studies.

Cellular and molecular changes caused by identified variants in PIPO related genes.

To assess the involvement of newly identified variants and genes to a specific disease phenotype, functional genetic studies should be performed (Lappalainen & MacArthur, 2021). Such studies are important tools to understand disease pathogenesis, and to investigate underlying molecular mechanisms associated with a specific variant found in genetic diagnosis (Richards et al., 2015).

In this thesis, each chapter highlighted the use of various *in vitro* and *in vivo* disease models to delineate phenotypes, as well as determine the cellular and molecular changes caused by the variants identified.

1. *The use of in vivo models to study neuropathic and myopathic PIPO*

To recapitulate the complexity of the cellular environment and biological responses, *in vivo* models are required. Animal models are frequently used to study human genetic diseases, as access to patients and patient derived material is sometimes difficult to obtain, especially in the case of rare genetic diseases. In addition, observations and studies in animal models can be made at different stages of disease but also, during embryonic development (Guénet, 2011). Due to the ability to obtain biopsies and fresh material from *in vivo* models, they provide a greater depth of mechanistic understanding through histology and molecular biology studies (Taormina et al., 2019). *In vivo* studies using rodents have contributed to the understanding of the disease mechanisms involved in primary PIPO (Bondurand & Southard-Smith, 2016; Halim, Brosens, et al., 2017; He et al., 2008). Especially for HSCR, mouse models have provided insights into genes and developmental processes that are crucial for ENS formation. Moreover, studies involving a single gene mutation, double mutants and modifiers are also possible, providing means to investigate the genetic basis of oligogenic inheritance in HSCR (Bondurand & Southard-Smith, 2016). For myopathic PIPO, a conditional *Mylk*$^{-/-}$ and *Lmod1*$^{-/-}$ mouse models, were used to support the involvement of these gene in MMIHS pathogenesis, as they were characterized by severe gut dysmotility and abnormal bladder function, (Halim, Brosens, et al., 2017; He et al., 2008). However, working with mouse models is expensive and requires ethical approval licenses, which is a long trajectory and can be troublesome. In addition, the generation of a specific mouse model can take more than one year, as each breeding round takes minimally 11 weeks, and they produce, on average, only six to eight pups per breed (Díaz et al., 2020; Guénet, 2011). The zebrafish (*Danio rerio*) on the other hand, have a high fecundity (average of 200 eggs in one breeding pair) with fast developing embryos, where all major organ systems are formed in 4 days (Kimmel et al., 1995). The zebrafish genome has also been sequenced and around 71.4% of human proteins have at least one zebrafish ortholog (K. Howe et al., 2013). The embryonic and larval zebrafish are virtually transparent and their development occurs *ex-utero*. These characteristics are especially relevant for the study of developmental processes, as *in vivo* visualization of different organs is possible in a non-invasive way (Kuil, Chauhan, et al., 2021). One of these organs is the intestine. This fact, together with rapid advancements in gene editing techniques make the zebrafish a highly suitable model to study intestinal development, and thus bring new insights into the molecular mechanisms underlying primary PIPO pathogenesis. In this thesis, we used the zebrafish to study the effect of new genes identified in primary PIPO patients.

In **Chapter 2**, we showed that target knockout of *Tfap2b* with CRISPR/Cas9 or morpholinos led to an overall reduction in the number of enteric neurons, as well as delayed intestinal transit time. We also found that the long FLNA isoform is required for intestinal elongation and motility in zebrafish, as it is likely required for smooth muscle contraction (**Chapter 3**). We also investigated the methylation pattern of *MAB21L2* during ENS development in the zebrafish, and observed that while in wildtype *this* gene is methylated after intestinal colonization by ENCCs, 72 hours post fertilization (hpf), in *ret* mutant zebrafish *mab21l2* methylation occurs at 24 hpf (**Chapter 4**). In addition, we demonstrated that *ret* x *mab21l2* heterozygote zebrafish exhibit an aggravated HSCR phenotype, with an increased rate of total aganglionic fish, when compared to *ret* heterozygotes alone. This observation is indicative of epistasis between *RET* and *MAB21L2*, suggesting that these two genes are involved in the same signaling pathway. Taken together, these studies showed that *TFAP2B*, *FLNA* and *MAB21L2* are new candidate genes for primary PIPO, as they are required for intestinal development and function. They also confirmed that the zebrafish is an excellent model for congenital intestinal motility disorders, leading to a better understanding of disease pathogenesis caused by genetic and epigenetic defects.

2. The use of hiPSC to model HSCR

Human diseases are complex, and different responses can be observed in the same organ depending on disease location, influences of physical and chemical microenvironment, immune and inflammatory responses, and whether the condition is acute or chronic. Particularly from a genetic point of view, disease manifestations can also differ depending on the genetic variation present in different patients. When developing an *in vitro* disease model, some of the basic elements to consider are the source and type of cells to be used, as well as the chemical and physical stimuli necessary to promote a specific phenotype (Benam et al., 2015). Several well-known immortalized mammalian cell lines such as HEK293, Neuro-2a, and SH-SY5Y, are commonly used *in vitro* to investigate the cellular and molecular effects of a genetic variant identified in neuropathic PIPO (Bonora et al., 2015; Sribudiani et al., 2018). However, as they have an embryonic or oncogenic origin, they tend to have high proliferation rates, which clearly contrast with neuronal characteristics. To study an enteric neuropathy, access to primary cells such as enteric neuronal progenitors directly isolated from the mammalian intestine (Cheng et al., 2015; Hotta et al., 2016) would therefore, be preferred. However, technical difficulties associated with the isolation of this specific cell population, *in vitro* culture and expansion, and even the ability to perform genetic modifications which mimic the effect of variant identified in the patient, represent a challenge. Particularly when modeling a complex genetic condition such as HSCR, in which rare coding variants, predisposing haplotypes, and Copy Number Variations (CNV), are involved (Alves et al., 2013; Kuil, MacKenzie, et al., 2021), creating *in vitro* and also *in vivo*

models to study disease pathogenesis, is even more difficult. The use of inducible pluripotent stem cells (iPSCs) has addressed this problem, as they are derived from patients and thus, recapitulate phenotypes, as well as cellular and molecular changes present in an organism, representing their genetic complexity. They also have the potential to be differentiated into almost every cell of the human body, including ENCCs (Barber et al., 2019; Gogolou et al., 2021; Lee et al., 2010; Zeltner et al., 2014). Based on this fact, we generated iPSCs derived from HSCR patients and investigated the effect of variants present in *RET, GFRA1, ZEB2* and *EDNRB/EDN3* in differentiation, migration, and proliferation of enteric neuronal progenitors (**Chapter 5)**. We also evaluated the potential of these cells to form an ENS composed by enteric neurons and glia. Based on our results, we showed that variants in *RET*, *GFRA1*, *ZEB2* and *EDNRB/EDN3* affect ENCC specification by intervening in the developmental timing of neuronal and glial commitment. In addition, we showed that all ENCCs derived from HSCR patients' iPSCs presented defective migration, and the majority of them also had proliferation defects. These results showed that patient derived hiPSCs could precisely delineate cellular and molecular changes occurring during HSCR development, and thus serve as an excellent model to study this disease.

The successful generation of enteric neuronal progenitors from patient derived hiPSC brings a future perspective into strategies aiming for cell therapy, particularly for HSCR. Since surgery to remove the aganglionic gut is still insufficient to treat intestinal dysmotility, replacing the missing ENS becomes the ultimate treatment for patients affected by this disease. Recent advances in generating enteric neuronal progenitors have shown that different types of stem cells could potentially serve as a source for enteric neural transplantation. Initial studies started off by isolating enteric neural stem cells (ENSCs) from ganglionic and aganglionic human colon of HSCR patients and expanding them in culture. Surprisingly, these cells were able to migrate and differentiate into neurons and glia when transplanted into embryonic hindgut. However, while endogenous ENSCs represent an autologous source of cells which will not impact the host immune system, and in theory could provide all neuronal subtypes present in the human gut, they are notoriously difficult to isolate (Almond et al., 2007; Hotta et al., 2016; Rollo et al., 2016; Wilkinson et al., 2015). Human embryonic stem cells (hESCs) have the capability to differentiate into multiple subtypes and can be unlimitedly expanded. However, their non-autologous origin could trigger undesirable immune responses in the recipient. The use of hESCs has also raised ethical problems (Ilic & Ogilvie, 2016). Here, hiPSCs could overcome these obstacles. However, the idea to transplant enteric neuronal progenitors derived from hiPSCs should likely be preceded by genetic correction. This conclusion is based on our results, that assorting for a pure population of ENCCS was not sufficient to change the cellular and molecular defects associated to a specific genetic variant (Chapter 5). The need for genetic editing brings potential additional risks (Davies, 2019; Shinwari et al., 2018), including malignant formation (Hacein-Bey-Abina et al., 2008; S. J. Howe et al., 2008). The search for

other regenerative methods is currently a hot topic in the field, especially after identification of Schwann cell precursors in the aganglionic bowel of HSCR patients (Windster et al., in print). Considering that these cells are able to differentiate into enteric neurons (El-Nachef & Bronner, 2020; Kamenev et al., 2021; Uesaka et al., 2015, 2021), identification of compounds that could stimulate neurogenesis is currently on-going. Moreover, as the intestinal environment can further influence differentiation of these cells, characterization of supporting non-neuronal cells present in the human gut, might also be beneficial in the future.

Overall conclusions

Despite the discovery of novel candidate genes involved in intestinal dysmotility, the cause of PIPO in the majority of patients is still unknown. Searching for new genes involved in disease pathogenesis through WES and by the evaluation of epigenetic modifications, is thus still required to bring new insights into PIPO pathogenesis. In this thesis, by using a combination of techniques, we were able to identify two new genes for neuropathic PIPO, *TFAP2B* and *MAB21L2*. We have also further characterized the role of *FLNA* in intestinal development and function, adding new insights to myopathic PIPO. In addition, the use of hiPSCs derived from HSCR patients was discussed, specifically to study the molecular mechanisms affected by variants in four known HSCR genes: *RET*, *ZEB2*, *GFRA1* and *EDNRB*/*EDN3*. As these cells contain the same genetic information present in the patients, the effects of each mutated gene are likely to represent the actual disease course and are thus, more informative.

Taken together, this thesis brings new insights into genetic/epigenetics approaches to identify new PIPO genes, and into functional studies using animal models and iPSCs, which can have transformative consequences for disease diagnosis, risk prediction, and development of new therapies.

References

Aggarwal, S. (2021). Role of whole exome sequencing for unidentified genetic syndromes. *Current Opinion in Obstetrics and Gynecology*, *33*(2), 112–122. https://doi.org/10.1097/GCO.0000000000000688

Almond, S., Lindley, R. M., Kenny, S. E., Connell, M. G., & Edgar, D. H. (2007). Characterisation and transplantation of enteric nervous system progenitor cells. *Gut*, *56*(4), 489–496. https://doi.org/10.1136/gut.2006.094565

Alves, M. M., Sribudiani, Y., Brouwer, R. W. W., Amiel, J., Antiñolo, G., Borrego, S., Ceccherini, I., Chakravarti, A., Fernández, R. M., Garcia-Barcelo, M. M., Griseri, P., Lyonnet, S., Tam, P. K., Van IJcken, W. F. J., Eggen, B. J. L., Te Meerman, G. J., & Hofstra, R. M. W. (2013). Contribution of rare and common variants

determine complex diseases-Hirschsprung disease as a model. *Developmental Biology*, *382*(1), 320–329. https://doi.org/10.1016/j.ydbio.2013.05.019

Barber, K., Studer, L., & Fattahi, F. (2019). Derivation of enteric neuron lineages from human pluripotent stem cells. *Nature Protocols*, *14*(4), 1261–1279. https://doi.org/10.1038/s41596-019-0141-y

Benam, K. H., Dauth, S., Hassell, B., Herland, A., Jain, A., Jang, K. J., Karalis, K., Kim, H. J., MacQueen, L., Mahmoodian, R., Musah, S., Torisawa, Y. S., Van Der Meer, A. D., Villenave, R., Yadid, M., Parker, K. K., & Ingber, D. E. (2015). Engineered in vitro disease models. *Annual Review of Pathology: Mechanisms of Disease*, *10*, 195–262. https://doi.org/10.1146/annurev-pathol-012414-040418

Bertier, G., Sénécal, K., Borry, P., & Vears, D. F. (2017). Unsolved challenges in pediatric whole-exome sequencing: A literature analysis. *Critical Reviews in Clinical Laboratory Sciences*, *54*(2), 134–142. https://doi.org/10.1080/10408363.2016.1275516

Bondurand, N., & Southard-Smith, E. M. (2016). Mouse models of Hirschsprung disease and other developmental disorders of the enteric nervous system: Old and new players. *Developmental Biology*, *417*(2), 139–157. https://doi.org/10.1016/j.ydbio.2016.06.042

Bonora, E., Bianco, F., Cordeddu, L., Bamshad, M., Francescatto, L., Dowless, D., Stanghellini, V., Cogliandro, R. F., Lindberg, G., Mungan, Z., Cefle, K., Ozcelik, T., Palanduz, S., Ozturk, S., Gedikbasi, A., Gori, A., Pippucci, T., Graziano, C., Volta, U., … De Giorgio, R. (2015). Mutations in RAD21 disrupt regulation of apob in patients with chronic intestinal pseudo-obstruction. *Gastroenterology*, *148*(4), 771-782.e11. https://doi.org/10.1053/j.gastro.2014.12.034

Cheng, L. S., Hotta, R., Graham, H. K., Nagy, N., Goldstein, A. M., & Belkind-Gerson, J. (2015). Endoscopic delivery of enteric neural stem cells to treat Hirschsprung disease. *Neurogastroenterology and Motility*, *27*(10), 1509–1514. https://doi.org/10.1111/nmo.12635

Chetaille, P., Preuss, C., Burkhard, S., Côté, J. M., Houde, C., Castilloux, J., Piché, J., Gosset, N., Leclerc, S., Wünnemann, F., Thibeault, M., Gagnon, C., Galli, A., Tuck, E., Hickson, G. R., Amine, N. El, Boufaied, I., Lemyre, E., De Santa Barbara, P., … Andelfinger, G. (2014). Mutations in SGOL1 cause a novel cohesinopathy affecting heart and gut rhythm. *Nature Genetics*, *46*(11), 1245–1248. https://doi.org/10.1038/ng.3113

Corominas, J., Smeekens, S. P., Nelen, M. R., Yntema, H. G., Kamsteeg, E. J., Pfundt, R., & Gilissen, C. (2022). Clinical exome sequencing—Mistakes and caveats. *Human Mutation*, *43*(8), 1041–1055. https://doi.org/10.1002/humu.24360

Davies, B. (2019). The technical risks of human gene editing. *Human Reproduction*, *34*(11), 2104–2111. https://doi.org/10.1093/humrep/dez162

Deml, B., Kariminejad, A., Borujerdi, R. H. R., Muheisen, S., Reis, L. M., & Semina, E. V. (2015). Mutations in MAB21L2 Result in Ocular Coloboma, Microcornea and Cataracts. *PLoS Genetics*, *11*(2), 1–26. https://doi.org/10.1371/journal.pgen.1005002

Di Nardo, G., Di Lorenzo, C., Lauro, A., Stanghellini, V., Thapar, N., Karunaratne, T. B., Volta, U., & De Giorgio, R. (2017). Chronic intestinal pseudo-obstruction in children and adults: diagnosis and therapeutic options. *Neurogastroenterology and Motility*, *29*(1). https://doi.org/10.1111/nmo.12945

Díaz, L., Zambrano, E., Flores, M. E., Contreras, M., Crispín, J. C., Alemán, G., Bravo, C., Armenta, A., Valdés, V. J., Tovar, A., Gamba, G., Barrios-Payán, J., & Bobadilla, N. A. (2020). Ethical Considerations in Animal Research: The Principle of 3R's. *Revista de Investigacion Clinica; Organo Del Hospital de Enfermedades de La Nutricion*, *73*(4), 199–209. https://doi.org/10.24875/RIC.20000380

Dong, W., Baldwin, C., Choi, J., Milunsky, J. M., Zhang, J., Bilguvar, K., Lifton, R. P., & Milunsky, A. (2019). Identification of a dominant MYH11 causal variant in chronic intestinal pseudo-obstruction: Results of whole-exome sequencing. *Clinical Genetics*, *96*(5), 473–477. https://doi.org/10.1111/cge.13617

El-Nachef, W. N., & Bronner, M. E. (2020). De novo enteric neurogenesis in post-embryonic zebrafish from Schwann cell precursors rather than resident cell types. *Development (Cambridge)*, *147*(13). https://doi.org/10.1242/dev.186619

Gamboa, H. E., & Sood, M. (2019). Pediatric Intestinal Pseudo-obstruction in the Era of Genetic Sequencing. *Current Gastroenterology Reports*, *21*(12). https://doi.org/10.1007/s11894-019-0737-y

Gogolou, A., Frith, T. J. R., & Tsakiridis, A. (2021). Generating Enteric Nervous System Progenitors from Human Pluripotent Stem Cells. *Current Protocols*, *1*(6), 1–20. https://doi.org/10.1002/cpz1.137

Guénet, J. L. (2011). Animal models of human genetic diseases: Do they need to be faithful to be useful? *Molecular Genetics and Genomics*, *286*(1), 1–20. https://doi.org/10.1007/s00438-011-0627-y

Hacein-Bey-Abina, S., Garrigue, A., Wang, G. P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., MacIntyre, E., Dal Cortivo, L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., … Cavazzana-Calvo, M. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *Journal of Clinical Investigation*, *118*(9), 3132–3142. https://doi.org/10.1172/JCI35700

Halim, D., Brosens, E., Muller, F., Wangler, M. F., Beaudet, A. L., Lupski, J. R., Akdemir, Z. H. C., Doukas, M., Stoop, H. J., de Graaf, B. M., Brouwer, R. W. W., van Ijcken, W. F. J., Oury, J. F., Rosenblatt, J., Burns, A. J., Tibboel, D., Hofstra, R. M. W., & Alves, M. M. (2017). Loss-of-Function Variants in MYLK Cause Recessive Megacystis Microcolon Intestinal Hypoperistalsis Syndrome. *American Journal of Human Genetics*, *101*(1), 123–129. https://doi.org/10.1016/j.ajhg.2017.05.011

Halim, D., Wilson, M. P., Oliver, D., Brosens, E., Verheij, J. B. G. M., Han, Y., Nanda, V., Lyu, Q., Doukas, M., Stoop, H., Brouwer, R. W. W., Van Ijcken, W. F. J., Slivano, O. J., Burns, A. J., Christie, C. K., De Mesy Bentley, K. L., Brooks, A. S., Tibboel, D., Xu, S., … Miano, J. M. (2017). Loss of LMOD1 impairs smooth muscle cytocontractility and causes megacystis microcolon intestinal hypoperistalsis syndrome in humans and mice. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(13), E2739–E2747. https://doi.org/10.1073/pnas.1620507114

He, W. Q., Peng, Y. J., Zhang, W. C., Lv, N., Tang, J., Chen, C., Zhang, C. H., Gao, S., Chen, H. Q., Zhi, G., Feil, R., Kamm, K. E., Stull, J. T., Gao, X., & Zhu, M. S. (2008). Myosin Light Chain Kinase Is Central to Smooth

Muscle Contraction and Required for Gastrointestinal Motility in Mice. *Gastroenterology*, *135*(2), 610–620. https://doi.org/10.1053/j.gastro.2008.05.032

Ho, V. M., Hovsepian, D. A., & Shieh, P. B. (2019). Myelopathy in a patient with leukodystrophy due to CSF1R mutation . *Neurology Genetics*, *5*(6), e376. https://doi.org/10.1212/nxg.0000000000000376

Hotta, R., Cheng, L. S., Graham, H. K., Pan, W., Nagy, N., Belkind-Gerson, J., & Goldstein, A. M. (2016). Isogenic enteric neural progenitor cells can replace missing neurons and glia in mice with Hirschsprung disease. *Neurogastroenterology and Motility*, *28*(4), 498–512. https://doi.org/10.1111/nmo.12744

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., Koch, R., Rauch, G. J., White, S., … Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503. https://doi.org/10.1038/nature12111

Howe, S. J., Mansour, M. R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M. H., Pike-Overzet, K., Chatters, S. J., De Ridder, D., Gilmour, K. C., Adams, S., Thornhill, S. I., Parsley, K. L., Staal, F. J. T., Gale, R. E., Linch, D. C., Bayford, J., Brown, L., … Thrasher, A. J. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *Journal of Clinical Investigation*, *118*(9), 3143–3150. https://doi.org/10.1172/JCI35798

Ilic, D., & Ogilvie, C. (2016). P LURIPOTENT S TEM C ELLS Concise Review : Human Embryonic Stem Cells — What Have We Done ? What Are We Doing ? Where Are We Going ? *Stem Cells*, *35*(1), 17–25.

Kamenev, D., Sunadome, K., Shirokov, M., Chagin, A. S., Singh, A., Irion, U., Adameyko, I., Fried, K., & Dyachuk, V. (2021). Schwann cell precursors generate sympathoadrenal system during zebrafish development. *Journal of Neuroscience Research*, *99*(10), 2540–2557. https://doi.org/10.1002/jnr.24909

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics*, *203*(3), 253–310. https://doi.org/10.1002/aja.1002030302

Kuil, L. E., Chauhan, R. K., Cheng, W. W., Hofstra, R. M. W., & Alves, M. M. (2021). Zebrafish: A Model Organism for Studying Enteric Nervous System Development and Disease. *Frontiers in Cell and Developmental Biology*, *8*(January), 1–15. https://doi.org/10.3389/fcell.2020.629073

Kuil, L. E., MacKenzie, K. C., Tang, C. S., Windster, J. D., Le, T. L., Karim, A., de Graaf, B. M., van der Helm, R., van Bever, Y., Sloots, C. E. J., Meeussen, C., Tibboel, D., de Klein, A., Wijnen, R. M. H., Amiel, J., Lyonnet, S., Garcia-Barcelo, M. M., Tam, P. K. H., Alves, M. M., … Brosens, E. (2021). Size matters: Large copy number losses in Hirschsprung disease patients reveal genes involved in enteric nervous system development. *PLoS Genetics*, *17*(8). https://doi.org/10.1371/journal.pgen.1009698

Lappalainen, T., & MacArthur, D. G. (2021). From variant to function in human disease genetics. *Science*, *373*(6562), 1464–1468. https://doi.org/10.1126/science.abi8207

Lee, G., Chambers, S. M., Tomishima, M. J., & Studer, L. (2010). Derivation of neural crest cells from human pluripotent stem cells. *Nature Protocols*, *5*(4), 688–701. https://doi.org/10.1038/nprot.2010.35

Li, E., & Zhang, Y. (2014). DNA methylation in mammals. *Cold Spring Harbor Perspectives in Biology*, *6*(5). https://doi.org/10.1101/cshperspect.a019133

Luo, Z., Dong, X., Yu, J., Xia, Y., Berry, K. P., Rao, R., Xu, L., Xue, P., Chen, T., Lin, Y., Yu, J., Huang, G., Li, H., Zhou, W., & Lu, Q. R. (2021). Genomic and Transcriptomic Analyses Reveals ZNF124 as a Critical Regulator in Highly Aggressive Medulloblastomas. *Frontiers in Cell and Developmental Biology*, *9*(February), 1–12. https://doi.org/10.3389/fcell.2021.634056

Medvedeva, Y. A., Khamis, A. M., Kulakovskiy, I. V., Ba-Alawi, W., Bhuyan, Md. S. I., Hideya, K., Lassman, T., Harbers, M., Forrest, A. R. R., Bajic, V. A., & Consortium, T. F. (2013). Effects of cytosine methylation on transcription factor binding sites. *BMC Genomics*, *15*(119), 1–12. https://doi.org/10.1134/S0006350909060013

Moreno, C. A., Sobreira, N., Pugh, E., Zhang, P., Steel, G., Torres, F. R., & Cavalcanti, D. P. (2018). Homozygous deletion in MYL9 expands the molecular basis of megacystis-microcolon-intestinal hypoperistalsis syndrome. *European Journal of Human Genetics*, *26*(5), 669–675. https://doi.org/10.1038/s41431-017-0055-5

Park, S. J., Kim, H., Kim, S. H., Joe, E. H., & Jou, I. (2019). Epigenetic downregulation of STAT6 increases HIF-1α expression via mTOR/S6K/S6, leading to enhanced hypoxic viability of glioma cells. *Acta Neuropathologica Communications*, *7*(1), 149. https://doi.org/10.1186/s40478-019-0798-z

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., & Rehm, H. L. (2015). Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, *17*(5), 405–424. https://doi.org/10.1038/gim.2015.30

Rollo, B. N., Zhang, D., Stamp, L. A., Menheniott, T. R., Stathopoulos, L., Denham, M., Dottori, M., King, S. K., Hutson, J. M., & Newgreen, D. F. (2016). Enteric Neural Cells From Hirschsprung Disease Patients Form Ganglia in Autologous Aneuronal Colon. *Cmgh*, *2*(1), 92–109. https://doi.org/10.1016/j.jcmgh.2015.09.007

Saito, Y., Kojima, T., & Takahashi, N. (2012). Mab21l2 is essential for embryonic heart and liver development. *PLoS ONE*, *7*(3). https://doi.org/10.1371/journal.pone.0032991

Shinwari, Z. K., Tanveer, F., & Khalil, A. T. (2018). Ethical issues regarding crispr-mediated genome editing. *Current Issues in Molecular Biology*, *26*, 103–110. https://doi.org/10.21775/CIMB.026.103

Siamoglou, S., Boers, R., Koromina, M., Boers, J., Tsironi, A., Chatzilygeroudi, T., Lazaris, V., Verigou, E., Kourakli, A., van IJcken, W. F. J., Gribnau, J., Symeonidis, A., & Patrinos, G. P. (2023). Genome-wide analysis toward the epigenetic aetiology of myelodysplastic syndrome disease progression and pharmacoepigenomic basis of hypomethylating agents drug treatment response. *Human Genomics*, *17*(1), 1–12. https://doi.org/10.1186/s40246-023-00483-7

Sribudiani, Y., Chauhan, R. K., Alves, M. M., Petrova, L., Brosens, E., Harrison, C., Wabbersen, T., de Graaf, B. M., Rügenbrink, T., Burzynski, G., Brouwer, R. W. W., van IJcken, W. F. J., Maas, S. M., de Klein, A., Osinga, J., Eggen, B. J. L., Burns, A. J., Brooks, A. S., Shepherd, I. T., & Hofstra, R. M. W. (2018).

Identification of Variants in RET and IHH Pathway Members in a Large Family With History of Hirschsprung Disease. *Gastroenterology*, *155*(1), 118-129.e6. https://doi.org/10.1053/j.gastro.2018.03.034

Taormina, G., Ferrante, F., Vieni, S., Grassi, N., Russo, A., & Mirisola, M. G. (2019). Longevity: Lesson from model organisms. *Genes*, *10*(7). https://doi.org/10.3390/genes10070518

Tordella, L., Khan, S., Hohmeyer, A., Banito, A., Klotz, S., Raguz, S., Martin, N., Dhamarlingam, G., Carroll, T., González Meljem, J. M., Deswal, S., Martínez-Barbera, J. P., García-Escudero, R., Zuber, J., Zender, L., & Gil, J. (2016). SWI/SNF regulates a transcriptional program that induces senescence to prevent liver cancer. *Genes and Development*, *30*(19), 2187–2198. https://doi.org/10.1101/gad.286112.116

Uesaka, T., Nagashimada, M., & Enomoto, H. (2015). Neuronal differentiation in schwann cell lineage underlies postnatal neurogenesis in the enteric nervous system. *Journal of Neuroscience*, *35*(27), 9879–9888. https://doi.org/10.1523/JNEUROSCI.1239-15.2015

Uesaka, T., Okamoto, M., Nagashimada, M., Tsuda, Y., Kihara, M., Kiyonari, H., & Enomoto, H. (2021). Enhanced enteric neurogenesis by Schwann cell precursors in mouse models of Hirschsprung disease. *Glia*, *69*(11), 2575–2590. https://doi.org/10.1002/glia.24059

Wilkinson, D. J., Bethell, G. S., Shukla, R., Kenny, S. E., & Edgar, D. H. (2015). Isolation of enteric nervous system progenitor cells from the aganglionic gut of patients with Hirschsprung's disease. *PLoS ONE*, *10*(5), 1–15. https://doi.org/10.1371/journal.pone.0125724

Yamada, R., Mizutani-Koseki, Y., Koseki, H., & Takahashi, N. (2004). Requirement for Mab21l2 during development of murine retina and ventral body wall. *Developmental Biology*, *274*(2), 295–307. https://doi.org/10.1016/j.ydbio.2004.07.016

Zeltner, N., Lafaille, F. G., Fattahi, F., & Studer, L. (2014). Feeder-free derivation of neural crest progenitor cells from human pluripotent stem cells. *Journal of Visualized Experiments*, *87*, 1–9. https://doi.org/10.3791/51609

Zenzeri, L., Tambucci, R., Quitadamo, P., Giorgio, V., De Giorgio, R., & Di Nardo, G. (2020). Update on chronic intestinal pseudo-obstruction. *Current Opinion in Gastroenterology*, *36*(3), 230–237. https://doi.org/10.1097/MOG.0000000000000630

Summary

This thesis focuses on basic and translational research of primary pediatric intestinal pseudo-obstruction (PIPO) caused by defects in the enteric nervous system (ENS) development (neuropathic PIPO) and smooth muscle function (myopathic PIPO). **In Chapter 2**, we described the genetic identification of *TFAP2B*, as a new candidate gene for neuropathic PIPO. Whole exome sequencing (WES) analysis performed in one PIPO patient revealed a heterozygous 10 base pairs deletion in *TFAP2B* (NM_003221.4: c.602-5_606delTCTAGTTCCA). Although this variant was initially classified as a variant of unknown significance, its location near a splicing site led us to perform further functional *in vitro* analysis. This analysis showed that the deletion led to exon 4 skipping and to the appearance of a premature stop codon. Based on these functional assays, we classified the *de novo* deletion as pathogenic. Furthermore, *in vivo* studies using zebrafish showed that *tfap2b* crispants and *tfap2b*$^{+/-}$ F2 fish had a reduction of enteric neurons and delayed intestinal transit time, confirming the involvement of *TFAP2B* in gastrointestinal (GI) development and function.

In Chapter 3, we focused on the study of Filamin A (*FLNA*). Pathogenic variants in this gene have been associated with a broad spectrum of disorders, including X-linked myopathic PIPO. However, the pathomechanism by which variants in *FLNA* lead to PIPO is not clear. In this chapter, we addressed this issue and performed *in vitro* and *in vivo* studies to bring new insights into the role of FLNA in GI development and function. As FLNA variants associated with PIPO are two bp deletions located in between two different transcription starting sites, we first showed that these variants block expression of one of the two FLNA isoforms, the long one. We also showed that expression of FLNA was detected in the small intestine during human embryonic development, specifically in smooth muscle cells of the muscularis mucosa and muscularis propria. Considering that the long isoform has been shown to be the major FLNA isoform expressed in intestinal smooth muscle, it is not surprising that these patients only have intestinal complaints, with the majority of them being diagnosed with PIPO. Furthermore, we observed that in the presence of only the short isoform, expression changes occur that impact cellular contractility, suggesting

that FLNA levels are instrumental for intestinal smooth muscle contraction. To further investigate these findings, we generated a transgenic zebrafish line in which expression of the long flna isoform was abolished. We observed that *flna* long isoform$^{-/-}$ larvae had significantly shortened intestines, suggesting that the long FLNA isoform is important for intestinal elongation. This data explains why the majority of PIPO patients carrying *FLNA* variants are born with congenital short bowel syndrome. In addition, increased intestinal transit time was observed in the *flna* long isoform$^{-/-}$ larvae, when compared to WT and *flna* long isoform$^{+/-}$ fish, suggesting that intestinal motility is affected by loss of the long FLNA isoform. Histological examination of the intestine of adult homozygous fish revealed hypertrophy of the smooth muscle layer and showed the presence of what seems to be an extra muscularis mucosa layer. Moreover, altered expression of intestinal smooth muscle markers was observed in these larvae, as well as adult fish, which can potentially underlie the structural changes identified. Since we found no differences in the distribution and number of enteric neurons present in the *flna* long isoform$^{-/-}$ zebrafish, we concluded that the GI defects observed, are likely due to impaired smooth muscle contractility and not to an ENS defect, suggesting that variants in FLNA are involved in myopathic CIPO.

Whole exome sequencing (WES) has proven to be instrumental in the identification of several sthus, are waiting to be solved. Epigenetic modifications, such as DNA methylation, have emerged as possible contributors to PIPO pathogenesis, and thus could possibly explain the missing heritability present in this disease. In the context of neuropathic PIPO, Hirschsprung disease (HSCR) is the most common (1:3500-5000 live births) and best studied disorder. In **Chapter 4**, we focused on HSCR and on the contribution of DNA methylation for its development. We isolated DNA from enteric neural cells collected from HSCR patients and controls, and performed genome wide methylation analysis. Our results showed that global hypermethylation in transcription starting sites (TSS) and gene bodies (intragenic), was present. Gene ontology enrichment analysis revealed that differential methylated genes were involved in pathways related to structural and cellular development of the ENS, such as axonogenesis and synapse organization. However, since the function of intragenic methylation has yet to be further elucidated, we decided to focus specifically on TSS, as promoter methylation has been widely accepted to cause gene inactivation via transcription repression. From all the differentially methylated regions (DMRs), the one present in *MAB21L2* caught our attention, as we previously showed that this gene is required for ENS development in zebrafish. However, *MAB21L2* has never been linked to HSCR, as no variants in this gene were found in patients. To further investigate the role of *MAB21L2* in HSCR pathogenesis, we evaluated the methylation pattern of this gene during ENS development, by isolating DNA from the gut of zebrafish larvae at different time points (1-120 hours post-fertilization (hpf)). We observed that *mab21l2* is methylated after 72 hpf in wildtype (WT) fish, a time point where migrating ENCCs just have completed gut

colonization. In a *ret* zebrafish model of HSCR presenting with total colonic aganglionosis, we observed that mab21l2 methylation occurred at 24 hpf. Interestingly, our study also showed that *ret* X *mab21l2* heterozygote zebrafish exhibited an aggravated HSCR phenotype, with an increased rate of total intestinal aganglionosis when compared to *ret* heterozygotes alone. This observation is indicative of epistasis between *RET* and *MAB21L2*, and thus suggests that both genes are involved in the same pathway. However, more insights are needed to understand this interaction.

Creating a disease model for a complex genetic condition such as HSCR, in which rare coding variants, predisposing haplotypes, Copy Number Variants (CNV) and penetrance, contribute to the disease phenotype, is challenging. Inducible pluripotent stem cells (iPSCs) derived from patients bring new opportunities to study complex disorders, as they can recapitulate phenotypes as well as cellular and molecular changes present in an organism, due to their genetic complexity. Previous studies showed that human iPSCs can be differentiated into enteric neural crest cells (ENCCs) and further establish an ENS. Therefore, iPSC derived from HSCR patients which can differentiate into neuronal progenitors, offer a unique opportunity to capture the cellular and molecular changes occurring during development. **In Chapter 5**, we generated patient derived iPSCs to investigate the effect of novel variants identified in four HSCR genes, *RET, GFRA1, ZEB2* and *EDNRB/EDN3*. We assessed the effect of these variants in the differentiation, migration, and proliferation of enteric neuronal progenitors, as well as in further specification of these cells into enteric neurons and glia, as these processes are the hallmarks of HSCR pathogenesis. Using a previously reported differentiation protocol based on the use of unsorted populations of ENCCs, we observed that while GFRA1 and EDNRB/EDN3 clones were able to successfully differentiate into ENCCs, RET and ZEB2 clones failed to achieve this state. However, sorting for double positive p75/HNK1 cells proved to induce ENCC differentiation of all clones. Interestingly, neurospheres formed by sorted ENCCs from controls, showed lower expression levels of vagal markers, when compared to unsorted populations. This result was unexpected, as several previously published protocols relied on a sorting procedure to obtain a 'pure' ENCC population. However, little is known about the impact of the sorting process on the quality and characteristic of ENCCs. Based on our study, it is tempting to hypothesize that supportive cells are present in unsorted populations. These cells are likely to provide guidance factors and morphogens to maintain optimal ENCC differentiation and growth, but further characterization of these "supporting" cells is needed.

Based on our results, all ENCCs derived from patient iPSCs presented defective migration and the majority showed increased proliferation rate. Further specification of ENCCs into enteric neurons and glia was also affected, as the variants identified seemed to impair neuronal and glial

commitment. Taken together, these results showed that hiPSC derived ENCCs can be used as an *in vitro* disease model, as they clearly possess specific cellular/molecular changes induced by the presence of the variant. In addition, the knowledge obtained on disease mechanisms of HSCR could potentially be translated into strategies aiming for cell therapy. Since surgery to remove the aganglionic segment in HSCR patients is still insufficient to treat dysmotility, replacing the missing ENS becomes an ultimate treatment for these patients. However, the idea to transplant enteric neuronal progenitors derived from hiPSCs as an autologous cell source, should likely be preceded by genetic correction. This conclusion is based on our results in which sorting for a "pure" ENCC population is not enough to change cellular and molecular defects associated with a specific variant.

Taken together, this thesis brings new insights into the etiology of PIPO, as well as to the understanding of its pathogenesis, by the identification of new causing genes and underlying cellular and molecular mechanisms affected in the neuropathic and myopathic forms of this disorder.

Samenvatting

Deze thesis richt zich op fundamenteel en translationeel onderzoek naar primaire pediatrische intestinale pseudo-obstructie (PIPO), veroorzaakt door defecten in de ontwikkeling van het enterisch zenuwstelsel (ENS) (neuropathische PIPO) en de functie van gladde spieren (myopathische PIPO).

In **hoofdstuk 2** beschreven we de genetische identificatie van TFAP2B als een nieuw kandidaatgen voor neuropathische PIPO. Whole exome sequencing (WES) analyse uitgevoerd bij één PIPO-patiënt onthulde een heterozygote deletie van 10 baseparen in TFAP2B (NM_003221.4: c.602-5_606delTCTAGTTCCA). Hoewel deze variant aanvankelijk werd geclassificeerd als een variant van onbekende betekenis, toonde opvolgende functionele *in vitro* analyses aan dat de deletie leidde tot exon 4 'skipping' en het verschijnen van een vroegtijdig stopcodon. Op basis van deze functionele assays, hebben we de *de novo* deletie als pathogeen geclassificeerd. Bovendien toonden *in vivo* studies in zebravissen aan dat tfap2b-knockout en tfap2b$^{+/-}$ F2 vissen een vermindering van enterische neuronen hadden en een vertraagde intestinale transittijd, wat de betrokkenheid van TFAP2B in de ontwikkeling en functie van het maag-darmkanaal (GI) bevestigt.

In **hoofdstuk 3** hebben we ons gericht op het onderzoek naar Filamin A (FLNA). Pathogene varianten in dit gen zijn geassocieerd met een breed scala van aandoeningen, waaronder X-gebonden myopathische PIPO. Het mechanisme waardoor varianten in FLNA leiden tot PIPO is echter niet duidelijk. In dit hoofdstuk hebben we *in vitro* en *in vivo* studies uitgevoerd om nieuwe inzichten te verkrijgen in de rol van FLNA in GI-ontwikkeling en functie. Aangezien FLNA-varianten geassocieerd met PIPO twee baseparen deleties zijn, die zich bevinden tussen twee verschillende transcriptiestartplaatsen, hebben we eerst aangetoond dat deze de expressie van de lange FLNA variant blokkeren. We toonden ook aan dat FLNA tot expressie komt in de dunne darm tijdens de human embryonale ontwikkeling, specifiek in de gladde spiercellen van de muscularis mucosa en muscularis propria,. Aangezien de lange FLNA-variant is aangetoond als de belangrijkste tot expressie komende variant in gladde darmspieren, is het niet verwonderlijk dat deze patiënten alleen darmklachten hebben, waarbij de meerderheid van hen wordt gediagnosticeerd met PIPO.

Bovendien hebben we waargenomen dat in de aanwezigheid van alleen de korte variant, veranderingen in de expressie optreden die de contractiliteit van de cellen beïnvloeden, wat suggereert dat FLNA-niveaus essentieel zijn voor de contractie van gladde darmspieren. Om deze bevindingen verder te onderzoeken, hebben we een transgene zebravislijn gegenereerd waarin de expressie van de lange flna variant werd geblokkeerd. We observeerden dat flna-lange variant$^{-/-}$ larven aanzienlijk verkorte darmen hadden, wat suggereert dat de lange FLNA-variant belangrijk is voor intestinale groei. Dit verklaart waarom de meeste PIPO-patiënten met FLNA-varianten, worden geboren met het congenitaal kortedarmsyndroom. Daarnaast werd bij de flna lange variant$^{-/-}$ larven een toegenomen intestinale transittijd waargenomen t.o.v. de WT- en flna-lange variant$^{+/-}$ vissen, wat suggereert dat de intestinale motiliteit wordt beïnvloed door het verlies van de lange FLNA-variant. Histologisch onderzoek van de darm van volwassen homozygote vissen onthulde hypertrofie van de gladde spierlaag en toonde de aanwezigheid van wat lijkt op een extra muscularis mucosa-laag. Bovendien werd een veranderde expressie van markers van gladde darmspieren waargenomen in de larven en volwassen vissen, wat mogelijk ten grondslag ligt aan de geïdentificeerde structurele veranderingen. Aangezien we geen verschillen vonden in de distributie en het aantal enterische neuronen aanwezig in de flna lange variant$^{-/-}$ zebravissen, concludeerden we dat de waargenomen GI-defecten waarschijnlijk te wijten zijn aan verminderde samentrekbaarheid van de gladde spieren en niet aan een ENS-defect, wat suggereert dat varianten in FLNA betrokken zijn bij myopathische CIPO.

Whole exome sequencing (WES) is van cruciaal belang gebleken bij de identificatie van PIPO-gerelateerde genen. Echter bij de meerderheid van de PIPO patiënten is de (genetische) oorzaak niet bekend. Epigenetische modificaties, zoals DNA-methylering, zijn naar voren gekomen als mogelijke bijdragende factoren aan de pathogenese van PIPO en zouden de ontbrekende erfelijkheid bij deze ziekte mogelijk kunnen verklaren. In de context van neuropathische PIPO is de ziekte van Hirschsprung (HSCR) de meest voorkomende (1:3500-5000 levendgeborenen) en best bestudeerde aandoening.

In **Hoofdstuk 4** hebben we ons gericht op HSCR en de bijdrage van DNA-methylering aan de ontwikkeling ervan. We isoleerden DNA uit enterische neurale cellen verzameld van HSCR-patiënten en controles, en voerden genoomwijde methylatieanalyse uit. Onze resultaten toonden aan dat er sprake was van globale hypermethylering in transcription start sites (TSS) en genlichamen (intragenisch). Gen-ontologieverrijkingsanalyse toonde aan dat differentieel gemethyleerde genen betrokken waren bij pathways gerelateerd aan ENS ontwikkeling, zoals axonogenese en synapsorganisatie. Omdat de functie van intragenische methylering echter nog verder moet worden onderzocht, besloten we ons specifiek te richten op TSS, aangezien promotor-methylering algemeen wordt geaccepteerd als een oorzaak van geninactivatie. Van alle differentieel gemethyleerde gebieden (DMR's) trok de DMR aanwezig in MAB21L2 onze aandacht, omdat we eerder hadden aangetoond dat dit gen vereist is voor ENS-ontwikkeling bij zebravissen. MAB21L2 is echter nooit in verband gebracht met HSCR, aangezien er bij patiënten geen varianten in dit gen zijn gevonden. Om de rol van MAB21L2 in HSCR-pathogenese verder te

onderzoeken, hebben we het methylatiepatroon van dit gen tijdens ENS-ontwikkeling bepaald. Dit toonde aan dat mab21l2 na 72 hpf in wildtype (WT) vissen werd gemethyleerd, een tijdstip waarop migrerende ENCC's net de colonisatie van de darm hadden voltooid. In een ret zebravismodel van HSCR met totale colone aganglionose, observeerden we dat mab21l2-methylering plaatsvond bij 24 hpf. Interessant genoeg toonde onze studie ook aan dat ret X mab21l2 heterozygote zebravissen een sterker HSCR-fenotype vertoonden, met een verhoogde frequentie van totale intestinale aganglionose in vergelijking met ret heterozygoten alleen. Deze observatie suggereert dat beide genen elkaar versterken in de ontwikkeling van HSCR. Er is echter meer onderzoek nodig om deze interactie volledig te begrijpen.

Het creëren van een ziektemodel voor een complexe genetische aandoening zoals HSCR, waarbij zeldzame coderende varianten, predisponerende haplotypes, Copy Number Variants (CNV) en penetrantie, bijdragen aan het ziektefenotypeis uitdagend. Geïnduceerde pluripotente stamcellen (iPSCs) afkomstig van patiënten bieden nieuwe mogelijkheden om complexe aandoeningen te bestuderen, omdat ze cellulaire en moleculaire veranderingen aanwezig in een organisme kunnen nabootsen vanwege hun genetische complexiteit. Eerdere studies toonden aan dat humane iPSCs kunnen differentiëren in enterische neurale voorlopercellen (ENCC's) en verder een ENS kunnen vormen, waardoor ze gebruikt kunnen worden om cellulaire en moleculaire veranderingen tijdens ENS ontwikkeling te bestuderen.

In **Hoofdstuk 5** genereerden we patiënt-afgeleide iPSCs om het effect van nieuwe varianten geïdentificeerd in vier HSCR-genen, RET, GFRA1, ZEB2 en EDNRB/EDN3, te onderzoeken. We beoordeelden het effect van deze varianten op de differentiatie, migratie en proliferatie van enterische neuronale voorlopercellen, evenals op de verdere specificatie van deze cellen in enterische neuronen en gliacellen.

Met behulp van een eerder gerapporteerd differentiatie protocol, gebaseerd op het gebruik van ongesorteerde populaties van ENCC's, vonden we dat GFRA1 en EDNRB/EDN3 clones succesvol konden differentiëren in ENCCs, terwijl dat niet het geval was voor RET en ZEB2 clones. Maar na het sorten van de cellen die dubbel positief waren voor p75/HNK1, lukte het wel. Interessant genoeg toonden de neurosferen, gevormd door gesorteerde ENCC's van controles, lagere expressieniveaus van vagale markers in vergelijking met de ongesorteerde populaties. Dit resultaat was onverwacht, aangezien verschillende eerder gepubliceerde protocollen gebruik maakte van een sorteermethode om een 'pure' ENCC-populatie te verkrijgen. Er is echter weinig bekend over de impact van het sorteerproces op de kwaliteit en kenmerken van ENCC's. Op basis van onze studie is het verleidelijk om te veronderstellen dat ondersteunende cellen aanwezig zijn in ongesorteerde populaties die waarschijnlijk begeleidende factoren secreteren om optimale ENCC-differentiatie en groei te behouden. Toch is meer onderzoek naar deze ondersteunende cellen nog nodig alvorens hier echt een uitspraak over te kunnen doen.

Alle ENCC's die waren afgeleid van patiënt-iPSCs vertoonde een afwijkende migratie capaciteit, en bij de meeste was er een verhoogde proliferatiesnelheid te zien. Verder werd de verdere

ontwikkeling van ENCC's tot enterische neuronen en glia cellen ook beïnvloed, aangezien de geïdentificeerde varianten de neuronale en gliale differentiatie leken te belemmeren. Samengevat toonden deze resultaten aan dat hiPSC-afgeleide ENCC's gebruikt kunnen worden als een *in vitro* ziektemodel, omdat ze duidelijk specifieke cellulaire en moleculaire veranderingen vertonen die worden veroorzaakt door de aanwezigheid van de variant. Bovendien kan de verkregen kennis over de ziekteprocessen van HSCR mogelijk worden vertaald naar strategieën die gericht zijn op celtherapie. Aangezien chirurgie om het aganglionische segment bij HSCR-patiënten te verwijderen nog steeds onvoldoende is om de motiliteitsstoornis te behandelen, zou het vervangen van het ontbrekende ENS de ultieme behandeling voor deze patiënten kunnen zijn. Het idee om enterische neuronale voorlopercellen, afgeleid van hiPSCs, als een autologe bron voor celtransplantatie te gebruiken, zou echter waarschijnlijk moeten worden voorafgegaan door genetische correctie. Deze conclusie is gebaseerd op onze resultaten, waarin het sorteren voor een "pure" ENCC-populatie niet voldoende bleek om de cellulaire en moleculaire defecten, geassocieerd met een specifieke variant, te corrigeren.

Samenvattend biedt deze thesis nieuwe inzichten in de pathologie van PIPO. Door nieuwe ziekte-veroorzakende genen te identificeren en de onderliggende cellulaire en moleculaire mechanismen te ontrafelen zijn we in staat geweest om nieuwe ziektemodellen en toekomstige potentiële therapeutische toepassingen te identificeren.

Appendix

Curriculum Vitae

Almira Zada

Work Experience

September 2018 – February 2023	PhD Student Erasmus MC, Department of Clinical Genetics Gastro Intestinal Genetics Group
April 2014 - recent	Assistant Professor Faculty of Medicine Universitas Padjadjaran, Bandung, Indonesia

Education

September 2005- September 2009	Bachelor of Medicine Faculty of Medicine Universitas Diponegoro, Semarang Indonesia
October 2009- September 2011	Medical Doctor Faculty of Medicine Universitas Diponegoro, Semarang, Indonesia
October 2011- December 2013	Master of Science (Majoring in Genetic Counseling) Faculty of Medicine Universitas Diponegoro, Semarang, Indonesia

Internship

November 2012- November 2013	Master internship Radboud UMC, Department of Clinical Genetics Supervision: Nicole de Leeuw and Helger Yntema Thesis: *Whole Genome SNP Array Analysis in Intellectually Disabled Patients in Indonesia*

List of Publication

Zada A, Kuil LE, de Graaf BM, Kakiailatu N, Windster JD, Brooks AS, van Slegtenhorst M, de Koning B, Wijnen RMH, Melotte V, Hofstra RMW, Brosens E, Alves MM. *TFAP2B* Haploinsufficiency Impacts Gastrointestinal Function and Leads to Pediatric Intestinal Pseudo-obstruction. Front Cell Dev Biol. 2022 Jul 8;10:901824. doi: 10.3389/fcell.2022.901824. PMID: 35874825; PMCID: PMC9304996.

Zada A, Zhao Y, Halim D, Windster J, van der Linde HC, Glodener J, Overkleeft S, de Graaf BM, Verdijk RM, Brooks AS, Shepherd I, Gao Y, Burns AJ, Hofstra RMW, Alves MM. The long Filamin-A isoform is required for intestinal development and motility: implications for chronic intestinal pseudo-obstruction. Hum Mol Genet. 2023 Jan 1;32(1):151-160. doi: 10.1093/hmg/ddac199. PMID: 35981053; PMCID: PMC9838097.

PhD Portfolio

Courses

Erasmus MC - CC02 Biostatistical Methods I: Basic Principles (2019)

Erasmus MC - Scientific Integrity (2019)

Optical Imaging Center -Course: Functional Imaging and Super Resolution (2019)

The SCORE PhD course: Stem Cells, Organoids and Regenerative Medicine (2019)

Biomedical Writing Course for PhD Candidates (2021)

Technology Facility course day: Genomics and Transcriptomics (2022)

Technology Facility course day: Imaging (2022)

Safe Laboratory Technique (2022)

Basic Human Genetics Course (2022)

Conferences and Workshops

28th MGC Symposium-Leiden (2018)

26th MGC Workshop 2019-Maastricht (2019)

European Society of Human Genetics Conference 2019 (2019)

29th MGC Symposium (2019)

Sophia Research Day 2021 (2021)

27th MGC Workshop 2021 (2021)

European Human Genetics Virtual Conference 2021 (2021)

30th MGC Symposium (2021)

Technology Facility course day: Genomics and Transcriptomics (2022)

Technology Facility course day: Imaging (2022)

Sophia Research Day 2022 (2022)

The Enteric Nervous System Symposium 2022 (2022)

1st Biomedical Science PhD Day 2022 (2022)

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