Congenital Diseases of the Intestine

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List of Abbreviations

ACTA2	Aorta smooth muscle actin alpha-2
ACTG2	Enteric smooth muscle gamma actin-2
BSA	Bovine serum albumin
bp	Base pair
CADD	Combined annotation dependent depletion
Cald1	Caldesmon-1
CFTR	Cystic fibrosis trans-membrane conductor receptor
c-Kit	Tyrosine protein-kinase kit
CLMP	Coxsackie- and adenovirus receptor-like membrane protein
CNV	Copy number variation
CRISPR-Cas9	Clustered regularly short palindromic repeat/CRISPR-associated protein-9
CSBS	Congenital short bowel syndrome
Csrp1	Cysteine and glycine rich protein-1
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
dpf	Day post fertilisation
EDC	Erasmus Dierexperimenteel Centrum
EDTA	Ethylenediaminetetraacetic acid
F-	Filamentous
FGF9	Fibroblast growth factor-9
FLNA	Filamin A
G-	Globular
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
HE	Hematoxylin & Eosin
hea	Hereditary erythroblastic anemia
HEK	Human embryonic kidney
hISMC	Human intestinal smooth muscle cell
HMIA	Hereditary multiple intestinal atresia
hpf	Hour post fertilisation
IA	Intestinal atresia
Ihh	Indian hedgehog
IUFD	Intrauterine fetal death
KO	Knock-out
LMOD1	Leiomodin-1
MD	Molecular dynamics
METC	Medische Ethische Toetsings Commissie

MIA	Multiple intestinal atresia
MMIHS	Megacystis microcolon intestinal hypoperistalsis syndrome
mRNA	Messenger ribonucleic acid
MYH11	Myosin heavy chain-11
MYLK	Myosin light chain kinase
nAChR	Nicotinic acetylcholine receptor
NGS	Next generation sequencing
NMD	Nonsense-mediated decay
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PUV	Posterior urethral valve
qRT-PCR	Quantitative real time polymerase chain reaction
ROH	Region of homozygosity
RMSD	Root mean square displacement
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
SMC	Smooth muscle cell
SM22α	Smooth muscle protein 22-α
SNP	Single nucleotide polymorphism
siRNA	Small interference ribonucleic acid
SRF	Serum response factor
Tagln	Transgelin
TALEN	Transcription activator-like nuclease
TUNEL	Terminal deoxynucleotide transferase dUTP nick-end labelling
Tpm2	Tropomyosin-2
TPN	Total parenteral nutrition
Ttc7	Tetratricopeptide-7
TTC7A	Tetratricopeptide-7A
USG	Ultrasound sonography
VM	Visceral myopathy
WES	Whole exome sequencing
WT	Wild type
ZO-1	Zonula occludins-1

CHAPTER 1

Introduction

INTRODUCTION

The gastrointestinal (GI) tract is a unique organ which requires coordinated movements of its neuromuscular apparatus, as well as fluid secretion for the mixing and propulsion of food, for breaking down complex food particles during digestion, and for absorption and excretion. It consists of many specialised regions, which all must remain connected and function optimally. Over the last several decades, significant advances have been made in our understanding towards normal development and physiology of the GI tract (Huizinga, Thuneberg et al. 1995, Silberg, Swain et al. 2000, Burns, Pasricha et al. 2004, He, Zhang et al. 2004, Sancho, Batlle et al. 2004).

For example, we currently know that this sophisticated system is made of cells that are derived from all three different embryonic germ layers, the ectoderm, mesoderm and endoderm. The epithelial lining of the GI tract is derived from the endoderm, while the smooth muscle cells are derivatives of the mesoderm. Enteric neurons and glia are formed from neural crest cells, derivatives of the ectoderm (de Santa Barbara, van den Brink et al. 2003, McLin, Henning et al. 2009, Goldstein, Hofstra et al. 2013). The epithelial lining of the intestine mainly functions in nutrient absorption. Meanwhile, coordination between muscular and neuronal structures of the intestine underlies intestinal contractility. Collective and continuous works from all of these cell types are pivotal to maintain normal physiology. Thus, failure in any of the developmental processes necessary for the formation of the GI tract can result in life-threatening congenital diseases.

Although recent advances in medical care and diagnostics have offered significant improvements in the prognosis of patients with GI disorders/diseases, some of these, such as megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS), hereditary multiple intestinal atresia (HMIA) and congenital short bowel syndrome (CSBS), remained lethal for the majority of patients or are even universally fatal. Thus, improvement in diagnostics and therapy of these diseases are urgently needed to provide a better prognosis for the patients and their family. Nonetheless, efforts to advance therapy for patients with these diseases have been hampered primarily by our limited understanding of their pathogenesis and the intrinsic morbidity related to potential treatment options.

Based on the lethality of these congenital diseases of the intestine, and the lack of knowledge towards their pathogenesis, we focused our research on MMIHS, CSBS and HMIA. Since these diseases are inherited a better understanding might start by identifying their genetic cause. Currently, finding disease-causing genes has been greatly facilitated by the rapid progress in technologies such as next generation sequencing (NGS). Moreover, further testing of identified candidate genes by genome editing techniques (in animal models) is currently making forward genetic studies possible. Thus, by utilizing these state-of-art methodologies in genetics, we can unravel the pathogenesis of these diseases. Moreover, since all three are diseases of the newborn, our research may also enlighten our knowledge towards the development and physiology of the intestine.

MEGACYSTIS MICROCOLON INTESTINAL HYPOPERISTALSIS SYNDROME

Clinical Presentation

Megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS) is arguably the most severe cause of intestinal and bladder obstruction in neonates, characterized by severely reduced contractility of these visceral organs (White, Chamberlain et al. 2000, Puri and Shinkai 2005). Berdon et al. first reported the disease in 5 newborns, including 2 sibs (Berdon, Baker et al. 1976), indicating genetic abnormality as a probable cause of this devastating syndrome.

In majority of the cases, the bladder abnormality starts to manifest in the prenatal period, hence a routine 20-week ultrasonography examination often reveals bladder distension. Another MMIHS-related prenatal complication often reported is polyhydramnios (Vintzileos, Eisenfeld et al. 1986, Garber, Shohat et al. 1990, Hsu, Craig et al. 2003, Cuillier, Cartault et al. 2004, Hellmeyer, Herz et al. 2013, Tuzovic, Anyane-Yeboa et al. 2014). At birth, MMIHS patients usually present with abdominal distension due to bladder hypocontractility, inability to urinate, and signs of intestinal obstruction, such as bilious vomiting. The presence of microcolon can be identified through barium enema test and/or surgery (Clark and O'Connor 2007) (Fig. 1).

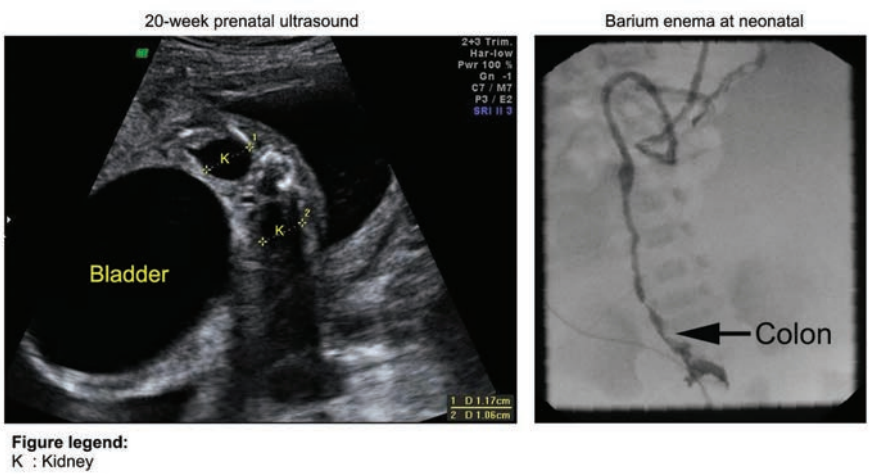

Fig.1: Prenatal USG and neonatal barium enema test in patient with MMIHS. showing a distended bladder and bilateral hydronephrosis. Barium enema test in the same patient identifies microcolon.

In addition to the main characteristics of MMIHS, a list of associated symptoms has been reported, including hydronephrosis, malrotation, Meckel's diverticulum, duodenal web and lax abdominal wall (Patel and Carty 1980, Lorenzo, Twickler et al. 2003, Levin, Soghier et al. 2004, Puri and Shinkai 2005, Akhtar, Alladi et al. 2012). Hydronephrosis might be secondary to the inability of the bladder to excrete urine, but the explanation for most of the other associated symptoms is not yet available. Aganglionic megacolon has also been reported in one case (Chamyan, Debich-Spicer et al. 2001). All these reports show a wide

range of clinical anomalies that might be featured in patients with MMIHS.

Overall, diagnosing MMIHS, in clinical practice, is challenging, especially since several other diseases have similar clinical signs, such as visceral myopathy, intestinal pseudo-obstruction, posterior urethral valve (PUV) obstruction, and prune belly (Schuffler, Pagon et al. 1988, Peters, Bolkier et al. 1990, Ghavamian, Wilcox et al. 1997). Even with the revelation of microcolon, it is still possible that the intestinal and bladder symptoms are separate entities that are caused by different etiologies. Thus, elucidating the genetic etiology of this syndrome is important to provide a reliable parameter that will help clinicians to confirm the diagnosis of MMIHS.

Histopathology

As contraction of the bladder and the intestine requires the coordinated action of smooth muscle cells, neurons and interstitial cells of Cajal (al-Rayess and Ambler 1992, Rolle, O'Briain et al. 2002, Piotrowska, Rolle et al. 2003, Piaseczna Piotrowska, Rolle et al. 2004, Szigeti, Chumpitazi et al. 2010), it is logical to think that failure of contraction is the result of alterations in any (or a combination) of these three cell types. Therefore, it is not surprising that initial histopathology analyses in specimens from MMIHS patients came up with various conclusions that could fit the pathogenesis of MMIHS. In many patients, a neuropathy was excluded (Puri and Shinkai 2005). However, some reports identified decreased to absence of ganglia (Vezina, Morin et al. 1979, Young, Yunis et al. 1981), while others reported increased number of ganglia (Berdon, Baker et al. 1976), and one report suggested axonal dystrophy as the cause of MMIHS in one patient (al-Rayess and Ambler 1992). Abnormal interstitial cells of Cajal has also been reported (Piotrowska, Rolle et al. 2003, Piaseczna Piotrowska, Rolle et al. 2004), while some reported MMIHS as a congenital myopathy of the visceral organs (Rolle, O'Briain et al. 2002). These differences in the interpretation of the histological findings may indicate that different factors can lead to the clinical symptomology of MMIHS.

Treatments and Prognosis

Until now, all treatments for patients with MMIHS can be categorized as symptomatic. Surgery to remove the defective intestine, catheterization and total parenteral nutrition (TPN) to feed the patient are the main potential treatment modalities. Despite numerous efforts and reports of long term survival, most MMIHS patients do not survive their early years of life (Gosemann and Puri 2011). Furthermore, in cases where patients survive, morbidities related to long-term use of parenteral nutrition and catheterization have been reported (Hirato, Nakazato et al. 2003). Multivisceral transplantation is the last line of treatment for MMIHS patients (Masetti, Rodriguez et al. 1999, Loinaz, Mittal et al. 2004), although not every patient will have the opportunity to become the multi organ recipient, and the patients who have the transplantation have a grave risk of immune rejection (Wu, Selvaggi et al. 2011).

Genetics

Since the first publication from Berdon et al., more than 250 MMIHS cases have been reported. Most of these were sporadic, however some were considered familial, as they were born from consanguineous parents. Therefore, MMIHS has been suggested to be an inherited disease with an autosomal recessive pattern of inheritance. In the beginning of our study on MMIHS (2012), only a deletion of chromosome 15 (15q11) (Szigeti, Chumpitazi et al. 2010) and a trisomy of chromosome 18 (Chamyan, Debich-Spicer et al. 2001) had been reported in two separate cases. No other DNA aberrations were described. Besides these chromosomal abnormalities, one candidate gene was reported, α3 nAChR. Knockout of the α3 nicotinic acethylcoline receptor (α3 nAChR) subunit in mice resulted in a MMIHS like phenotype. Richardson et al. performed immunostaining on specimens from 10 patients with MMIHS using an antibody against the α3 nAChR subunit, and observed a significant decrease in protein expression (Xu, Gelber et al. 1999, Richardson, Morgan et al. 2001). Nonetheless, no disease-causing variants in the gene that encodes α3 nAChR have been reported in MMIHS patients to date.

CONGENITAL SHORT BOWEL SYNDROME

Clinical Presentation

Congenital short bowel syndrome (CSBS) is a very rare congenital disease, characterized by an extremely short small intestine (Hamilton, Reilly et al. 1969). Comparing the intestines of normal and CSBS neonates, delivered at term (>35 weeks), the small intestine in the normal neonate is approximately 250 cm in length, while the length of small intestine in CSBS neonates is approximately 50 cm. Despite this obvious anatomical defect, the intestine in most CSBS patients is not obstructed. Thus, the abnormality may remain undetected for several days or weeks.

The length of small intestine correlates with the available area for digestion and absorption (Cheng, O'Grady et al. 2010), hence this severe shortening results in a failure of these functions. Consequently, patients with CSBS usually present with chronic dehydration, chronic diarrhea, malnutrition and failure to thrive. The diagnosis can be confirmed by a barium enema test, or by exploratory laparotomy (Kern, Leece et al. 1990, Schalamon, Schober et al. 1999, Sabharwal, Strouse et al. 2004, Hasosah, Lemberg et al. 2008).

In all CSBS cases, the intestine is malrotated. Volvulus has been identified in four patients (Tanner, Smith et al. 1976, Kern, Leece et al. 1990), while the appendix was absent in three (Iwai, Yanagihara et al. 1985, Sarimurat, Celayir et al. 1998). In most cases, no additional intestinal phenotypes were identified. However, minor dysmorphic features and patent ductus arteriosus coexisted in a few patients (Royer, Ricour et al. 1974, Sansaricq, Chen et al. 1984).

Histopathology

Histology from the intestine of CSBS patients is not always available. However, a few cases were reported and no abnormal histological findings were described. In three CSBS patients, who also suffered from functional intestinal obstruction, histopathology analyses revealed decreased numbers of argyrophilic neurons in the ganglia of the intestine (Tanner, Smith et al. 1976). In line with these early findings, neuronal dysplasia was found in one other CSBS patient.

Treatments and Prognosis

As a consequence of the failure to digest and absorb nutrients, TPN is an imperative treatment for CSBS patients. Ideally, TPN is temporarily prescribed until the intestinal length and function are retained/improved sufficiently. However, 75% of CSBS patients die of starvation or sepsis in their first few days of life. Nonetheless, the survival rates of CSBS patients has increased in the last two decades, arguably due to the recent developments in TPN. The oldest patient we are aware of is currently approximately 30 years of age (patient 4 in van der Werf, Wabbersen et al. 2012)

Genetics

Familial cases make up around 60% of all CSBS cases. Within these familial cases both sexes are equally affected, and consanguinity is described, hence, an autosomal recessive pattern of inheritance has long been suggested. Besides the presumed recessive forms of CSBS, an X-linked form of CSBS was also suggested. Recently, these hypotheses were confirmed by the discovery of disease-causing variants in *CLMP* as the cause of recessive CSBS and variants in *FLNA* as the cause of the X-linked form of CSBS (Van Der Werf, Wabbersen et al. 2012, van der Werf, Sribudiani et al. 2013).

HEREDITARY MULTIPLE INTESTINAL ATRESIA

Clinical Presentation

Intestinal atresia (IA) makes up approximately thirty percent of the causes of intestinal obstruction in neonates (Hajivassiliou 2003). IA is characterized by a discontinued lumen of the intestine at one or multiple sites. As a consequence, patients with IA are not able to digest food, hence lethality is the definitive outcome unless corrections of the atretic sites are performed in the newborn period (Dalla Vecchia, Grosfeld et al. 1998).

Based on the number of the atretic sites and the type of the discontinuity itself, IA can be categorized into 4 different types. In IA type I, although the intestinal tube seems to be connected, the lumen inside of it is disrupted. IA type II refers to a disconnected intestinal tube, without any mesenteric defect. Based on the size of the mesenteric defect and the existence of intestinal malrotation, IA type III can be divided into 2 subtypes. The mesenteric defect is restricted to the atretic site in type IIIa, while most of the mesentery is absent in type IIIb. Since the mesentery also functions as a supporting structure for the

intestine, the intestine in IA type IIIb is often malrotated, causing an appearance often referred to apple-peel-like. Lastly, IA type IV consists of atresia of any type I, II, III or a combination of these, at multiple sites of the intestine, referred to as multiple intestinal atresia (MIA) (Fig. 2).

Compared to the other types of IA, MIA is the rarest, yet the most lethal type of IA. During pregnancy, ultrasonography (USG) on MIA can identify abdominal distension, with multiple dilated intestinal loops. At the postnatal period, a characteristic X-ray, a so-called double-bubble sign, can be indicative, in which the first larger bubble is the stomach, and the second smaller bubble is the dilated proximal duodenum (Ali, Rahman et al. 2011). Nonetheless, it is difficult to distinguish this from classical duodenal atresia cases. Thus, the information cannot differentiate the different types of IA, and the exact diagnosis can only be confirmed during surgery.

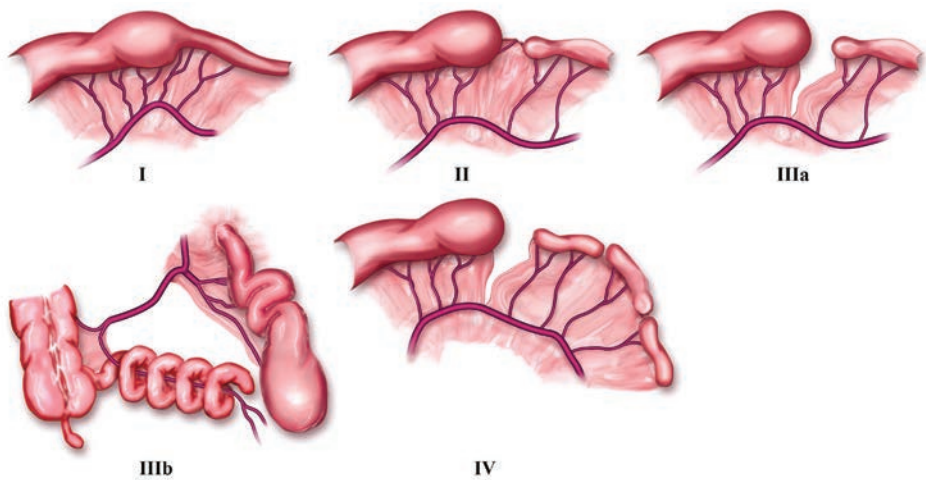

Fig.2: Illustration of different types of intestinal atresia. In type I and II, the lumen of the intestine is disrupted, but there is no defect in the mesentery. Meanwhile, defect in the mesentery is identified in type IIIa, while larger mesentery defect is found in type IIIb, causing volvulus of the intestine. Type IV consists of atretic intestine in more than 1 site.

Many familial cases of MIA have been reported, hence they are often referred to as hereditary multiple intestinal atresia (HMIA). In addition to the intestinal phenotype, many HMIA patients have been described to also have a severe immune deficiency (Gahukamble and Gahukamble 2002, Bilodeau, Prasil et al. 2004, Gilroy, Coccia et al. 2004, Ali, Rahman et al. 2011). The prevalence of this unique combination of comorbidities in hereditary MIA highlights the possibility that the two entities are caused by one common genetic aberration. Hence, unraveling the genetic etiology of this disease might be an entry point in understanding the pathogenesis of HMIA.

Histopathology

In patients diagnosed with HMIA combined with severe immune deficiency, multiple separate lumens, that are lined up with separate mucosa, were described. This is often described as a sieve-like appearance. Many of the lumens from this atretic site are filled with calcified substances and cell debris, while the mucosal villi appear shorter or flattened. Although these individual lumens are lined/connected by separate mucosa and its muscularis mucosa, they are all surrounded by common layers of submucosal and mucularis propria of the intestine (Fig. 3). At the time we initiated this project, there were no descriptions about the histopathology of immune-related organs probably due the low prevalence of this combination of anomalies.

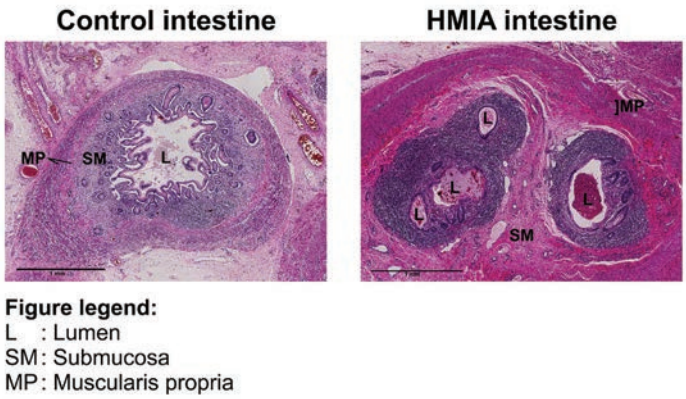

Fig.3: H&E staining in intestinal specimens from control and HMIA patient. Multiple lumen in the atretic site of the intestine is a common feature found in HMIA.

Treatments and Prognosis

Surgical resection of the affected intestinal sites is a mandatory treatment for HMIA. After surgery, patients are usually dependent on long term TPN until intestinal function is restored sufficiently. The general conditions of HMIA patients, who also have severe immune deficiency, usually deteriorate due to the many infections they suffer from. Therefore, intestinal transplantation is considered as an alternative treatment to restore intestinal function. Despite all efforts, mortality rates of HMIA remain close to 100%, with only one reported patient surviving long term (Fischer, Friend et al. 2014).

Genetics

Consanguinity was described in many of the familial cases. This strongly suggests that HMIA is an inherited disease with an autosomal recessive pattern of inheritance. No genes were reported at the start of this project (2012).

Overall aim

All research described in this thesis is focused on understanding the pathophysiology of these three rare congenital diseases of the intestine. For MMIHS and IA, we follow a comparable path, starting a search for the genetic cause of the disease by SNP arrays and or exome sequencing. Subsequently, after identifying the candidate genes, we performed both in vitro and in vivo assays to prove causality and to determine the mechanism underlying disease development. For CSBS we performed mutation analysis of the already known gene and perform functional work to unravel disease mechanism.

THESIS AIM AND OUTLINE

The prevalence of MMIHS, HMIA and CSBS in siblings, and the fact that most of the familial cases are from consanguineous families, strongly suggest that these congenital intestinal diseases have a genetic origin. The aim of the studies described in this thesis is to identify the genetic etiologies of these lethal diseases, and use these findings to unravel the disease pathogenesis.

Outline

Most cases of MMIHS are sporadic. In the study described in **Chapter 2**, we performed a genetic screening in 8 patients with sporadic MMIHS, and identified disease-causing heterozygous missense variants in all patients in the gene for enteric smooth muscle actin γ-2 (*ACTG2*). Using intestinal specimens from controls and three patients, we assessed the gene expression patterns during normal development of the human intestine, and determined the effect of the variants on the pathology of patients' intestines. Molecular modeling and a series of *in vitro* studies, using cell lines, were performed to investigate the mechanisms underlying how the identified variants could lead to sporadic MMIHS.

In **Chapter 3**, we present a genetic study on one MMIHS patient from a consanguineous family. To do so, homozygosity mapping and whole exome sequencing were utilized. A nonsense mutation in Leiomodin-1 (*LMOD1*) was identified in the patient. To confirm the involvement of *LMOD1* in MMIHS our collaborators created a *Lmod1* knockout mouse model. Similar phenotypes in the KO mice, resembling MMIHS in human, were revealed. To investigate the molecular pathogenesis of this disease, primary human intestinal smooth muscle cells were used in a series of *in vitro* assays.

Despite our findings in both sporadic and familial MMIHS cases, the genetic etiology in three MMIHS patients from two separate consanguineous families are yet to be identified. In **Chapter 4**, we describe the identification of two disease-causing-variants in myosin light chain kinase (*MYLK*) as the cause of MMIHS in the two remaining families. In one family, a duplication of 7-bp in exon 22 led to an out-frame-mutation that resulted in loss of MYLK expression in the patient's intestine and bladder specimens. In the other family, a splice-site variant in intron 22 was identified. Mini-gene analysis showed that the variant identified disturbed the splice site in such a way that exon 21 was not recognized

by the splice machinery. Immunohistochemistry was performed to study the effect of the out-of-frame mutation on the pathology of the patient's intestine and bladder specimens.

An overview of the literature on CSBS is given in **Chapter 5**. This overview particularly focuses on the recent genetics findings, in which mutations in Coxsackie-And Adenovirus Receptor-Like Membrane Protein (*CLMP*) and Filamin A (*FLNA*) were identified in CSBS patients. We speculate on how the protein products from these two genes are involved in the tight junction complex. Additionally, a summary of knockout mouse models with a CSBS phenotype is included.

In **Chapter 6**, we describe the identification of a homozygous nonsense variant and compound heterozygous variants in two CSBS families. These findings validate *CLMP* as the disease-causing gene of CSBS, as previously reported.

FLNA was previously reported as the disease-causing gene for the X-linked form of CSBS. Based on this recent finding, we generated a *flna* knockout zebrafish model. Characterization and functional studies on this zebrafish model are presented in **Chapter 7**.

HMIA is the familial form of MIA, and an autosomal recessive pattern of inheritance has been suggested. In **Chapter 8**, a homozygous deletion of the entire exon 2 in tetratricopeptide-7A (*TTC7A*) was identified in two patients from a consanguineous family. Immunohistochemistry was performed to assess the effect of this mutation on the intestinal pathology from both patients. In addition, a *ttc7a* knockout zebrafish model was generated to study the effect of the loss of ttc7a on the structure and function of the zebrafish intestine.

A general discussion, including future perspectives, is presented in **Chapter 9**.

Together, the work in this thesis provides new information that extend our knowledge on the nature and pathogenesis of these congenital diseases of the intestine. Our data can be used in genetic counseling, and also in further research to design therapies for the patients.

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CHAPTER 2

ACTG2 variants impair actin polymerization in sporadic Megacystis Microcolon Intestinal Hypoperistalsis Syndrome

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ABSTRACT

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS) is a rare congenital disorder, in which heterozygous missense variants in the Enteric Smooth Muscle actin γ-2 (*ACTG2*) gene have been recently identified. To investigate the mechanism by which *ACTG2* variants lead to MMIHS, we screened a cohort of eleven MMIHS patients, eight sporadic and three familial cases, and performed immunohistochemistry, molecular modelling and molecular dynamics (MD) simulations, and *in vitro* assays. In all sporadic cases, a heterozygous missense variant in *ACTG2* was identified. ACTG2 expression was detected in all intestinal layers where smooth muscle cells are present in different stages of human development. No histopathological abnormalities were found in the patients. Using molecular modelling and MD simulations, we predicted that *ACTG2* variants lead to significant changes to the protein function. This was confirmed by *in vitro* studies, which showed that the identified variants not only impair ACTG2 polymerization, but also contribute to reduced cell contractility. Taken together, our results confirm the involvement of ACTG2 in sporadic MMIHS, and bring new insights to MMIHS pathogenesis.

INTRODUCTION

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS) (OMIM 249210) is the most severe form of intestinal obstruction in neonates. It was first reported by Berdon et al in 1976, and is characterized by non-obstructive bladder distension, microcolon, and decreased or absent intestinal peristalsis (Berdon, Baker et al. 1976). MMIHS is a rare congenital disorder for which surgical intervention is normally required. However, this frequently results in futile therapy without improvement of the clinical symptoms. Pathological evaluation of intestinal biopsies collected from patients have resulted in different theories regarding the underlying cause of MMIHS. Some reports suggested a muscular problem (Ciftci, Cook et al. 1996, Rolle, O'Briain et al. 2002, Szigeti, Chumpitazi et al. 2010), while others stated that the main defect was neuronal with both increased and decreased number of neurons being detected (Kubota, Ikeda et al. 1989, Richardson, Morgan et al. 2001, Narayanan, Murphy et al. 2007). Abnormalities of the interstitial cells of Cajal have also been associated with MMIHS pathogenesis (Piotrowska, Rolle et al. 2003, Piaseczna Piotrowska, Rolle et al. 2004). However, most of these findings were obtained in one or a few number of patients using different methods and consensus regarding pathogenesis does not exist.

Familial appearance and parental consanguinity have been previously reported for MMIHS suggesting that it is an autosomal recessive disorder (Anneren, Meurling et al. 1991, McLaughlin, Gooch et al. 1993). However, since most MMIHS cases are sporadic, it seems reasonable to hypothesize that locus heterogeneity exists, and that the genetic etiology of sporadic and familial MMIHS cases may differ. Recently, this hypothesis has been confirmed by the identification of pathogenic variants in two genes: the Enteric Smooth Muscle Actin γ-2 (*ACTG2*) gene, and the smooth muscle myosin heavy chain (*MYH11*) gene. Heterozygous missense variants in *ACTG2* were identified as the cause of sporadic MMIHS in three independent studies (Thorson, Diaz-Horta et al. 2014, Tuzovic, Anyane-Yeboa et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014), while a homozygous missense variant in *MYH11* was identified in a newborn patient of consanguineous descent (Gauthier, Ouled Amar Bencheikh et al. 2014). *ACTG2* encodes one of the six actin isoforms present in humans and is specifically expressed in smooth muscle cells of the intestinal and urogenital tracts (Miwa, Manabe et al. 1991, Szucsik and Lessard 1995, Thorson, Diaz-Horta et al. 2014). *MYH11* encodes for the myosin heavy chain protein, one of the components required for smooth muscle contraction. Loss of *Myh11* in mice has been reported to result in a bladder and intestinal phenotype reminiscent to the one seen in MMIHS patients (Morano, Chai et al. 2000), supporting the involvement of this gene in MMIHS pathogenesis.

Interestingly, heterozygous missense variants in *ACTG2* have also been described to cause another intestinal disorder, i.e. Visceral Myopathy (VM; OMIM 155310). Four families were reported with heterozygous *ACTG2* variants affecting three different residues: R40 (missense), R148 (missense), and G269 (tandem base substitution)

(Lehtonen, Sipponen et al. 2012, Wangler, Gonzaga-Jauregui et al. 2014, Klar, Raykova et al. 2015). Since MMIHS and VM are mainly characterized by inadequate contractility of the intestine and variable levels of bladder dysfunction (severe in MMIHS and often mild in VM), it is not surprising that the same gene is involved in the pathogenesis of both disorders. However, it raised the hypothesis that MMIHS and VM are one disease entity with different spectrums of severity.

Despite the identification of *ACTG2* as a disease-causing gene for MMIHS, it is still unclear how variants in *ACTG2* lead to its development. In this study, we confirm the involvement of *ACTG2* in MMIHS, and bring new molecular insights into the mechanism associated with the pathogenesis of this disease.

MATERIALS AND METHODS

Patient information

In this study, a cohort of eleven MMIHS patients was analyzed. Eight of these patients were sporadic cases (S1-S8), two patients were siblings of consanguineous descent (F1 and F2), and one patient was derived from an isolated Dutch community where inbreeding was suspected, and was thus, considered as a familial case (F3). Patient S1 has been previously reported (Talisetti, Longacre et al. 2009). Prenatal ultrasounds showed the presence of a distended bladder in the majority of the patients included in this study. A final MMIHS diagnosis was confirmed in all of them, due to the presence of a microcolon and distended bladder. All patients included in this study were Caucasian, except for patients F1, F2 and F3 of North African ancestry. Written informed consent was given by the families, and ethical approval was obtained from the Erasmus Medical Center ethical committee (Medische Ethische Toetsings commissie - METc 2011/148, ABR form: NL35920.042.11).

Sanger sequencing

Genomic DNA was isolated from peripheral blood lymphocytes using standard methods. DNA obtained from amniotic fluid was extracted using standard DNA isolation protocols for prenatal material (Chemagic DNA Blood Kit Special, Chemagen, Perkin Elmer). Exons 1-10 of *ACTG2* were amplified using 15 ng of genomic DNA and the set of primers described in Supplementary Table 1. PCR products were purified (ExoSap it – GE Healthcare), and Sanger sequencing was performed with dye labelled primers (forward and reverse; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems) on a ABI 3130XL genetic analyzer. Sanger reads were analysed using SeqScape software.

Immunohistochemistry

Fetal human gut tissues were obtained from the MRC-Wellcome Trust Human Developmental Biology Resource (HDBR), in collaboration with UCL Institute of Child Health in London, UK. Paraffin-embedded intestinal tissues from neonatal controls and

patients S3 and S5, were obtained from the Pathology Department repository of the Erasmus University Medical Center. Intestinal paraffin-embedded material from patient S1 was obtained from Stanford Histology/EM Laboratory of the Stanford University Medical Center. All human embryonic and neonatal intestinal tissues used as control were obtained from elective abortions, or from patients in which the cause of death was not intestinal related. Immunostainings were performed using specific antibodies against ACTG2 (1:100; Novus Biologicals), neurofilament (1:600; Monosan), smooth muscle actin α2 (ACTA2; ready to use; Dako), tyrosin protein kinase Kit (c-Kit/CD117) (1:200; Cell Marque), tryptase (1:1600; Dako), and synaptophysin (SP11) (ready to use; Ventana). 3,3'-diaminobenzidine (DAB) chromogen (Ventana) was used for protein visualization, and sections were counterstained with hematoxylin. All images were taken with the Nanozoomer 2.0-HT (Hamamatsu Photonics), and analyzed with the Nanozoomer Digital Pathology viewer software (Hamamatsu Photonics). Negative controls performed with depletion of primary antibody were included for each staining, and showed no signal in any of the material tested (data not shown).

Molecular modelling and molecular dynamics simulations

Molecular dynamics (MD) simulations of ACTG2 were performed using the structure of the chicken actin gamma monomer in complex with Dnase1 (PDB ID: 3W3D) (Sakabe, Sakabe et al. 1983). In order to obtain only the actin subunit for subsequent MD simulations, removal of Dnase1, N-acetyl-D-glucosamine and alpha/beta-D-mannose structures was performed using the VMD software, version 1.9.1 (Humphrey, Dalke et al. 1996). Different ACTG2 mutants were produced using the in-built VMD plugin "mutate residue". Each mutant was then checked for chirality errors and cis peptide bonds to ensure proper geometry via dedicated VMD plugins. An explicit 15 Å -padding TIP3P water box was used to solvate the protein, and the system was neutralized with KCl. All-atom MD simulations were performed with the NAMD software on a 32-core Intel®Xeon(R) CPU E5-2665 (Phillips, Braun et al. 2005). Integration steps of 2 fs were used for minimization, equilibration and productive run in an isothermal-isobaric ensemble with constant number of particles, pressure and temperature (NPT ensemble), in the presence of periodic boundary conditions (PBC) and Particle Mesh Ewalds (PME) for full electrostatics. Temperature of 310 K was ensured by Langevin dynamics with a damping coefficient of 2 ps, and pressure was maintained constant at 1.013 bar by a Langevin Piston. Minimization was performed for 20 ps and productive run for 10 ns. Simulations were performed using the same parameters for mutants and wild-type. Analysis was carried out with standard VMD Analysis tools. Root-mean square deviation (RMSD) was calculated over the alpha-carbon traces every 50 ps.

Expression vectors

pCMV6-Myc-ACTG2 wild-type (WT) was purchased from Origene. All the mutants described were generated by site-directed mutagenesis on pCMV6-Myc-ACTG2 WT

according to the manufacturer's instructions (QuickChange II site-directed mutagenesis kit, Agilent technologies). Primers used are listed in supplementary Table 2. Following mutagenesis, the entire ACTG2 insert was checked by Sanger sequencing.

Cell culture and transfection

The U2OS cell line was cultured in DMEM with high glucose content (Lonza), supplemented with 10% fetal calf serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco). Cells were maintained at 37°C and 5% CO_2. For transient transfection, 300,000 cells were seeded in 6 well plates. Transfection was performed 24 hours after using GeneJuice transfection reagent (Millipore) according to the manufacturer's instructions.

Cell lysates and Western blot analysis

Cells were washed with PBS and incubated with lysis buffer [m-PER (Thermo Scientific) containing protease inhibitors (Roche)], for 30 minutes on ice. Cell lysates were collected by scraping and cleared by centrifugation at 14000 rpm for 10 minutes in a pre-cooled (4°C) centrifuge. Lysates were stored at -80°C before being processed further for SDS-PAGE and immunoblotting. Protein quantification was performed using a BCA kit (Thermo Scientific), and 30 µg of protein was loaded into a criterion TGX precast gel (Bio-Rad). The following antibodies were used: Myc (Cell Signaling) and GAPDH (Millipore). Secondary antibodies used were IRDye 800CW Goat anti-mouse and IRDye 680RD Goat anti-Rabbit (Li-Cor).

Microscopy and image analysis

For immunofluorescence cells were cultured on cover slips. Four percent paraformaldehyde was used as a fixative and cells were permeabilised with 1% BSA and 0.1% Triton X-100 in PBS. Myc antibody (Cell Signaling) was used at a concentration of 1:100, and Phalloidin-rhodamin (Santa Cruz Biotechnology) was used at a concentration of 1:500. Images were taken using a Leica (AOBS) microscope, and analysed with the Leica LAS AF Lite software.

Actin Binding assays

U2OS cells transfected with ACTG2 expressing constructs (WT and mutants) were lysed in an actin buffer (Cytoskeleton) containing 0.1% Triton X-100 (Sigma) and protease inhibitors (Roche). Lysates were centrifuged for 15 min at 14000 rpm in a pre-cooled centrifuge (4°C), and supernatant fractions were incubated with polymerized actin (F-actin), generated according to the manufacturer's instructions (Cytoskeleton™). Polymerized actin was pelleted by centrifugation (100,000 G for 1 h) using the Airfuge, Air-driven Ultracentrifuge (Beckman-Coulter). Supernatant and pellet fractions were further analyzed by Western blotting.

Contractility assays

Twenty-four hours after transfection with ACTG2 expressing constructs (WT and mutants), U2OS cells were trypsinised, and analysed for their ability to contract. A cell contraction kit (Cell Biolabs) was used according to the manufacturer's instructions.

RESULTS

Heterozygous missense variants in *ACTG2* are present in sporadic MMIHS patients

Since previous studies have implicated *ACTG2* in MMIHS development, we screened our cohort of eleven patients for the presence of variants in this gene (Fig.1A). All patients presented with typical MMIHS features, including a microcolon and an enlarged bladder (Table 1). In all sporadic MMIHS cases a heterozygous missense variant in *ACTG2* was identified (Table 2).

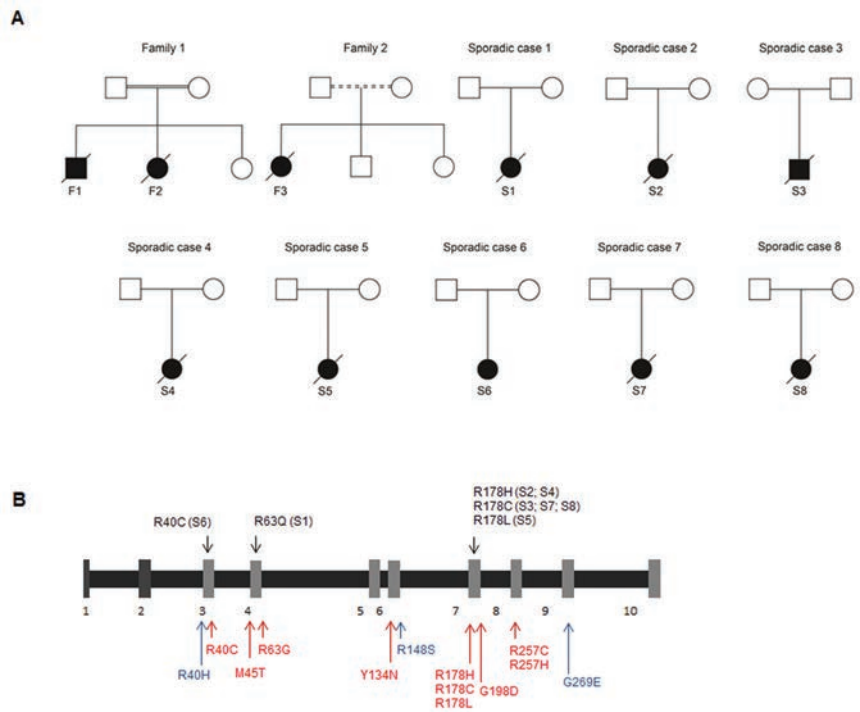

Fig.1: Analysis of *ACTG2* variants in MMIHS patients. A) Pedigrees of the ten families included in this study. **B)** Overview of the ten exons of *ACTG2* with all the variants identified to date. The F110L variant is not represented, as it affects an alternate exon 4 of a predicted *ACTG2* short isoform [14] (Black – in this study; Red – previously reported MMIHS cases; Blue – identified in VM patients).

In three of these cases we were able to confirm the *de novo* status of the variants. However, for the remaining five patients this was not possible due to unavailability of parental DNA. The missense variants identified in our patient cohort are located in three different exons of *ACTG2*, exons 3, 4 and 7 (Fig.1B), and are all predicted to be pathogenic by at

Table 1. Clinical features of the MMIHS patients reported in this study

Patient	Consanguinity	Gender	Prenatal findings	Age of Onset	Mega-cystis	Micro-colon	Hypoperi-stalsis[1]	Malrotation	Performed Surgeries	Additional Symptoms	Outcome	Cause of Death
F1	Yes	Female	Megabladder	Prenatal	+	+	+	+	None	None	Deceased	Termination of pregnancy
F2	Yes	Male	Megabladder	Prenatal	+	NA	NA	NA	None	None	Deceased	Termination of pregnancy
F3	Suspected	Female	Megabladder	Prenatal	+	+	+	+	?	None	Deceased	NA
S1	No	Female	Megabladder, hydronephrosis, hydroureter	Prenatal	+	+	+	+ (volvulus at 5 y.o.)	Gastrostomy, ileostomy, small intestinal resection, entire colon resection, multivisceral organ transplantation	None	Deceased (23 y.o.)	Multiple organ failure
S2	No	Female	?	?	+	+	+	?	?	?	Deceased	?
S3	No	Male	Low urinary tract obstruction with bilateral hydronephrosis, mega-ureter, abdominal wall dehiscence	Prenatal	+	+	+	+	Laparotomy	Lax abdominal wall, undescended testis	Deceased (5 d.o.)	Discontinued treatment
S4	No	Female	Megabladder, bilateral hydronephrosis, polyhydramnios	Prenatal	+	+	+	+	Laparotomy	None	Deceased (5 m.o.)	Discontinued treatment
S5	No	Female	Megabladder, polyhydramnios	Prenatal	+	+	+	+	Laparotomy, suprapubic catheterization	None	Deceased (8 m.o.)	Multiple organ failure
S6	No	Female	Megabladder	Prenatal	+	+	+	+	Gastrostomy, ileostomy, colostomy, cholecystectomy, small intestinal resection	None	Alive (24 y.o.) with parenteral and enteral nutrition	Alive
S7	No	Female	N/A	Prenatal	+	+	?	?	None	?	Deceased	?
S8	No	Female	N/A	Prenatal	+	+	?	?	None	?	Deceased	?

[1)] Detected by manometric study/Barium enema; N/A = Not available; ? = unknown; y.o. = years old; d.o. = days old; m.o.= months old.

Table 2. Overview of all ACTG2 mutations detected in MMIHS and VM patients

Patient	Gene	Position	Exon	AA Position	Effect	Polyphen	MutationTaster	Provean	*De novo*
F1	(-)								
F2	(-)								
F3	(-)								
S1	ACTG2	74129548	4	R63Q	nonsynonymous	Probably damaging	Disease causing	Neutral	?
S2	ACTG2	74140693	7	R178H	nonsynonymous	Benign	Disease causing	Deleterious	Yes
S3	ACTG2	74140692	7	R178C	nonsynonymous	Benign	Disease causing	Deleterious	Yes
S4	ACTG2	74140693	7	R178H	nonsynonymous	Benign	Disease causing	Deleterious	?
S5	ACTG2	74140693	7	R178L	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
S6	ACTG2	74128449	3	R40C	nonsynonymous	Probably damaging	Disease causing	Deleterious	?
S7	ACTG2	74140692	7	R178C	nonsynonymous	Benign	Disease causing	Deleterious	?
S8	ACTG2	74140692	7	R178C	nonsynonymous	Benign	Disease causing	Deleterious	?
MMIHS 1[12]	ACTG2	74140693	7	R178L	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 2[12]	ACTG2	74140692	7	R178C	nonsynonymous	Benign	Disease causing	Deleterious	Yes
MMIHS 3[13]	ACTG2	74141962	8	R257C	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 4[13]	ACTG2	74140693	7	R178H	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
MMIHS 5[13]	ACTG2	74129494	4	M45T	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 6[13]	ACTG2	74136215	6	Y134N	nonsynonymous	Benign	Disease causing	Deleterious	Yes
MMIHS 7[13]	ACTG2	74129547	4	R63G	nonsynonymous	Probably damaging	Disease causing	Deleterious	?
MMIHS 8[13]	ACTG2	74141962	8	R257C	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 9[13]	ACTG2	74128449	3	R40C	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 10[13]	ACTG2	74140753	7	G198D	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 11[13]	ACTG2	74140692	7	R178C	nonsynonymous	Benign	Disease causing	Deleterious	Yes
MMIHS 12[13]	ACTG2	74141962	8	R257C	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS13[13]	ACTG2	74140693	7	R178H	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 14[13*]	ACTG2	74141962	8	R257C	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
MMIHS 15[13**]	ACTG2	74129825	4[#]	F110L	nonsynonymous	?	?	?	?
MMIHS 16[13**]	ACTG2	74129825	4[#]	F110L	nonsynonymous	?	?	?	?
MMIHS 17[13**]	ACTG2	74128450	3	R40H	nonsynonymous	Probably damaging	Disease causing	Neutral	No
MMIHS 18[14]	ACTG2	74141963	8	R257H	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 19[14]	ACTG2	74141963	8	R257H	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 20[14]	ACTG2	74141962	8	R257C	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
MMIHS 21[14]	ACTG2	74140693	7	R178H	nonsynonymous	Probably damaging	Disease causing	Deleterious	Yes
VM 1[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No

VM 2[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 3[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 4[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 5[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 6[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 7[19]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 8[13]	ACTG2	74128450	3	R40H	nonsynonymous	Probably damaging	Disease causing	Neutral	Yes
VM 9[20]	ACTG2	74140602	6	R148S	nonsynonymous	Probably damaging	Disease causing	Deleterious	?
VM 10[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 11[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 12[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 13[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 14[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 15[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No
VM 16[21]	ACTG2	74141999 -74142000	9	G269E	nonsynonymous	Probably damaging	Disease causing	Deleterious	No

? = unknown due to unavailability of parental DNA. F = Familial; S = Sporadic; * = Patient with megacystis and pseudo-obstruction; ** = Patient with pseudo-obstruction.; *** - Familial history of pseudo-obstruction and one MMIHS case. [#] alternative exon of a predicted short *ACTG2* isoform.

least two of three prediction programs (PolyPhen, MutationTaster, and Provean) (Table 2). None of these variants is present in the 1000 Genomes project database or in the Exome Sequencing project, but all of them have been reported before in MMIHS patients (Table 2) [12-14]. No variant in *ACTG2* was identified in the three familial cases (F1, F2 and F3).

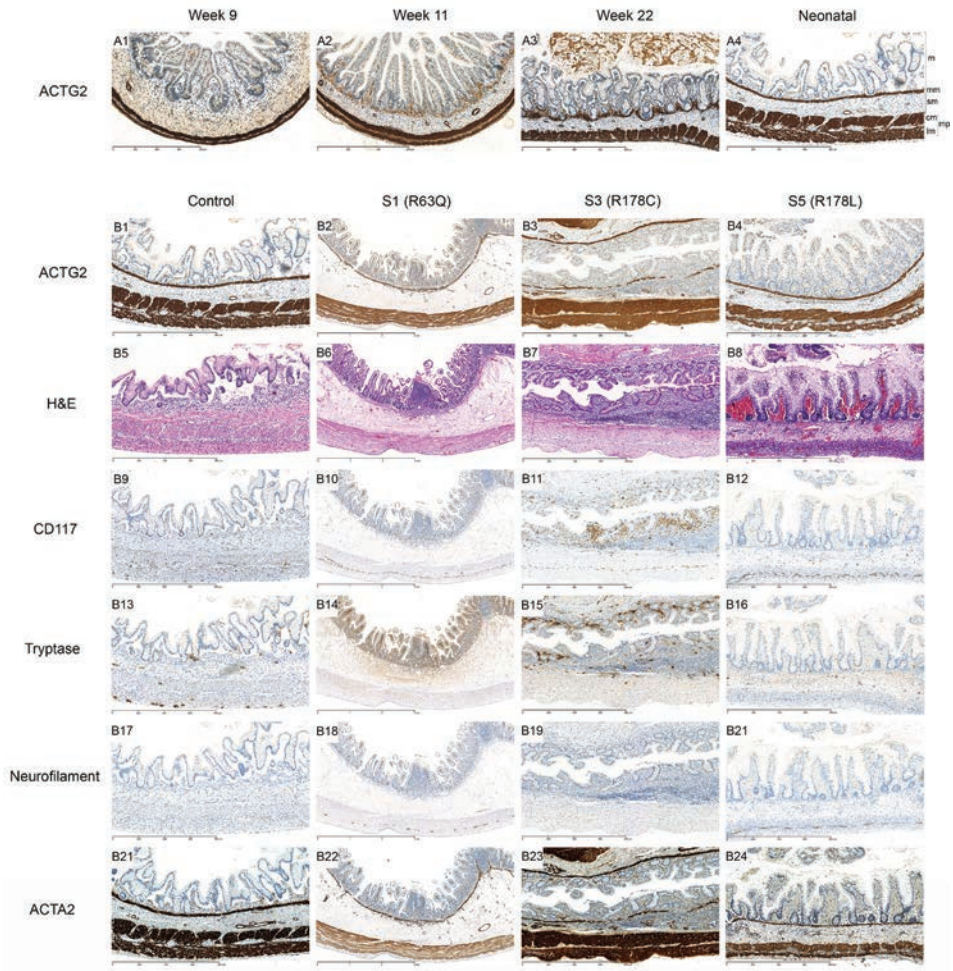

Fig.2: Expression of ACTG2 in human intestinal material. A) Immunohistochemistry analysis shows that ACTG2 is expressed in the human intestine during different stages of development. **B)** Immunohistochemistry performed in intestinal material obtained from three MMIHS patients, S1, S3 and S5, and an age matched control shows a diffuse cellular distribution of ACTG2 in the cytoplasm of smooth muscle cells of the muscularis mucosa, blood vessels and, the circular and longitudinal muscle of muscularis propria in patients and control. No significant changes were detected in any cellular constituents of the intestine of MMIHS patients, shown by hematoxylin and eosin (HE) staining and immunohistochemistry for c-Kit/CD117, Tryptase, Neurofilament and ACTA2. Legends: m: mucosa, mm: muscularis mucosa, sm: submucosa, cm: circular muscle, lm: longitudinal muscle, mp: muscularis propria.

Table 3. RMSD value for ACTG2 WT and mutant proteins

ACTG2 (376 residues)		
ACTG2	**Average (Å)**	**STDEV**
WT	2.856	0.288
R178C	3.476	0.448
R178L	2.779	0.361
R178H	3.168	0.363
R40C	2.536	0.343
R63Q	2.815	0.255
R148S	3.079	0.429

Table 4. RMSD value for the D-loop in ACTG2 WT and mutant proteins

D-loop (residues 41-53)		
ACTG2	**Average (Å)**	**STDEV**
WT	7.061	1.077
R178C	8.405	1.697
R178L	6.376	1.447
R178H	8.012	1.488
R40C	4.955	1.998
R63Q	8.055	1.180
R148S	9.455	2.320

Table 5. RMSD value for the ADP-rybosilation site in the ACTG2 WT and mutant proteins

ADP-ribosylation site (residues 174-182)		
ACTG2	**Average (Å)**	**STDEV**
WT	1.820	0.545
R178C	2.106	0.454
R178L	1.996	0.545
R178H	1.712	0.340
R40C	1.260	0.338
R63Q	1.856	0.381
R148S	1.350	0.240

Table 6. RMSD value for the the myosin binding site in the ACTG2 WT and mutant proteins

Myosin binding site (residues 357-376)		
ACTG2	**Average (Å)**	**STDEV**
WT	2.383	0.636
R178C	2.564	0.361
R178L	2.328	0.407
R178H	2.630	0.517
R40C	3.557	0.506
R63Q	2.560	0.716
R148S	2.827	0.440

ACTG2 is expressed in all smooth muscle cells during human intestinal development

Since MMIHS is an intestinal congenital anomaly, we assessed the expression of ACTG2 in the human intestine (jejunum/ileum) at weeks 9, 11, and 22 of embryonic development, and at neonatal stage. At all developmental stages ACTG2 was abundantly expressed in the cytoplasm of smooth muscle cells, including smooth muscle cells of the muscularis mucosa, inner circular layer and outer longitudinal layer of the muscularis propria (Fig.2A).

ACTG2 expression was also detected in vascular smooth muscle cells of the intestine, and surprisingly, in myofibroblast cells of the mucosal layer. Since myofibroblasts have been described to only express the smooth muscle actin α 2 (ACTA2) (Arnoldi, Hiltbrunner et al. 2013), we cannot exclude the possibility that the antibody used recognizes both the α and γ smooth muscle actin isoforms, and could therefore, be unspecific for ACTG2.

MMIHS patients with *ACTG2* variants do not show abnormal expression of ACTG2, or primary defects in any cellular constituents of the intestine

To define the histopathological findings in MMIHS patients with a *ACTG2* variant, we investigated the expression of ACTG2, neurofilaments, ACTA2, c-Kit/CD117, and tryptase

by immunohistochemistry, in intestinal material obtained from three MMIHS patients (S1, S3 and S5). Intestinal specimens collected during autopsy from an age-matched control were used for comparison. In the three patients analysed we were unable to detect any difference in the expression levels of ACTG2 when compared to the control (Fig.2B). The same result was obtained for neurofilaments and ACTA2 (Fig.2B, Supplementary Fig.1). An increased expression of c-Kit/CD117, a marker for interstitial cells of Cajal, was observed in the intestinal mucosa of patients S1 and S3 (Fig.2B, Supplementary Fig.1), but no change was observed in patient S5. Considering that c-Kit/CD117 is also a marker for mast cells, a tryptase staining was performed, confirming that the increased expression of c-Kit/CD117 in these two patients was due to an increased number of mast cells.

Missense variants in *ACTG2* lead to significant changes to the protein structure

To gain a better understanding of the possible mechanisms underlying the development of MMIHS, we performed molecular modelling and molecular dynamics (MD) simulations for ACTG2. Molecular modelling involves editing protein structures by, for instance, introducing amino acid substitutions into a known crystal structure of a protein. However, since molecular modelling is often insufficient to infer the functional role of such substitutions, MD simulations are often performed. Using this approach, the atoms of the (modeled) crystal structures are allowed to move according to the forces exerted by the surrounding environment (temperature, pressure, water, ions, nearby atoms, electrostatic interactions, hydrophobic interactions, etc). Their motion can be measured at each time-point of the simulation and followed for the entire duration of the movement. In the end, these motions are plotted as the root mean square displacement (RMSD) from the initial crystal structure, over time. Therefore, higher RMSD values at a given time correspond to more structural differences in respect to the starting crystal structure, allowing prediction of the behavior of a (mutated) protein over time.

Since they represent the “skeleton” of the protein, their motion is more restricted than the side chains, which are more likely to suffer irrelevant high deviations associated to their intrinsic flexibility. Up to date, there is no statistical test that can be performed on RMSDs to assess significant differences. However, the persistence and the extent of the differences in RMSD, coupled with visual inspection of the simulated trajectories, are used to determine the relevance of such differences.

Since the human ACTG2 structure was not available, we used in this study the chicken actin gamma monomer, which is 99.2% homologous in primary sequence (they differ only by three residue). We systematically compared the structure of the entire ACTG2 protein or its main relevant sites, for wild type (WT) and mutants during 10 nanoseconds (ns) (Tables 3-6). All mutants displayed structural differences to some extent with respect to WT ACTG2 (Fig.3A, 3B). However, the most striking observations were associated with the R178C mutant, which showed the highest RMSD of the alpha-carbon trace throughout the whole simulation time for the entire protein (Fig.3B, Table 3).

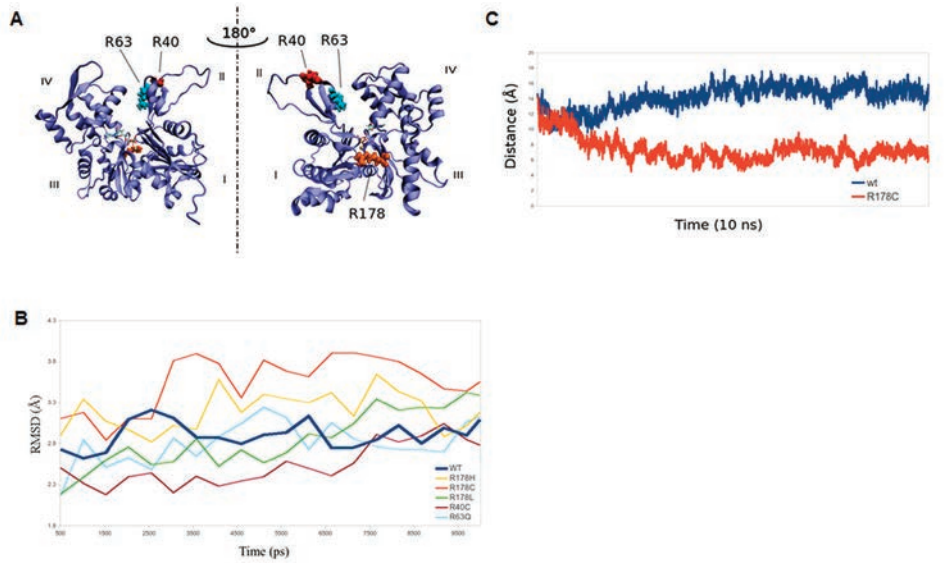

Fig.3: Molecular modelling and MD simulations of ACTG2 WT and mutant proteins. A) Ribbon representation of the secondary structure of ACTG2. Residues of interest have been depicted as Van der Waals spheres. The representation on the bottom has been obtained by 180° rotation around the vertical axis. (B) Root-mean square deviation (RMSD) of ACTG2 alpha-carbon trace (376 residues) for WT and mutant proteins plotted as a function of time every 0.5 ns. The behavior of each protein was analysed every 50 ps. (C) Distance between subdomains II and IV for ACTG2 WT and the R178C mutant shows significant differences over time. Distances were calculated between alpha-carbons of Thr67 (on subdomain II) and Ala205 (on subdomain IV).

We also noticed that subdomains II and IV behaved differently from WT, since they moved closer to each other over time, instead of moving slightly apart (Fig.3A). The average distance during the majority of the simulation time was about half compared to the WT (Fig.3C). Distances were computed between the alpha-carbons of Thr 67 (subdomain II) and of Ala 205 (subdomain IV), with an average of 7.3±1.5 Å for R178C and 14.2±1.5 Å for the WT (Fig.3C). The R40C mutant showed the lowest average RMSD value for the entire protein (2.536 Å compared to 2.856 Å for the WT), reflecting a less dynamic structure with respect to WT ACTG2, as well as for the ADP ribosylation site (residues 174 to 182), with an average RMSD of 1.26 Å (compared to 1.82 Å for the WT) (Fig.3B, Tables 3 and 5). An exception to the generally lower structural deviations of the R40C mutant was observed for the region spanning residues 357-376, which belongs to the myosin-binding site. This region showed a significant displacement, having an average RMSD of 3.557 Å in contrast with 2.383 Å for the WT (Table 6). Moreover, the region spanning residues 41 to 53, belonging to the D-loop, resulted in lower RMSD values for the R40C mutant (4.995±1.998Å) when compared to the WT (7.061±1.077Å, Table 4).

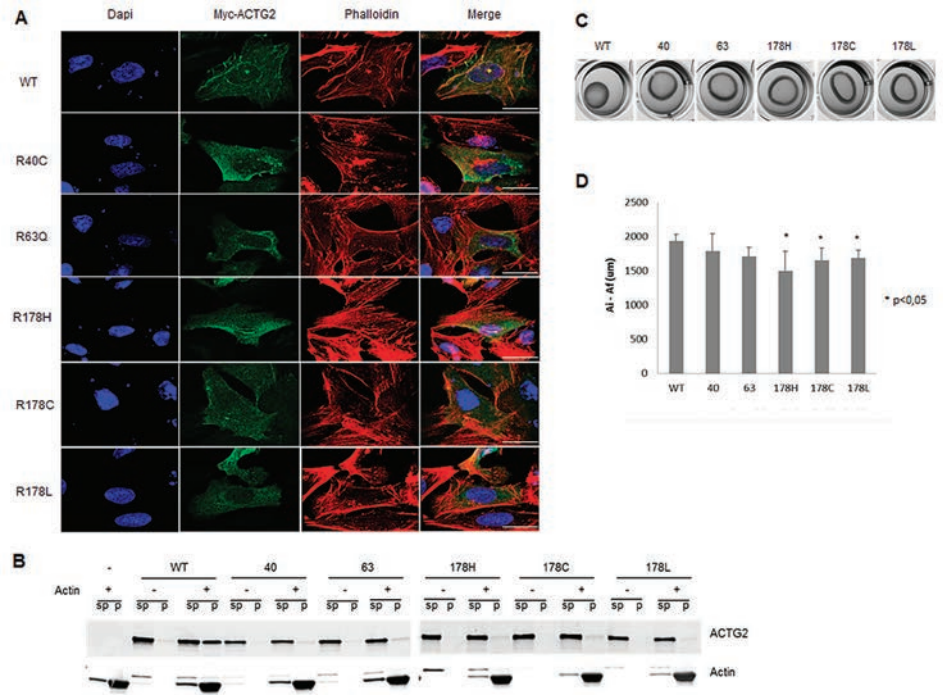

Fig.4: Cellular distribution and function of ACTG2 WT and mutant proteins. A) Confocal images of U2OS cells stained for Phalloidin (a F-actin marker), and expressing a Myc-tagged version of ACTG2 WT and mutant proteins show reduced incorporation of the mutant proteins into the actin filaments. **B)** *In vitro* actin binding assays show that ACTG2 mutant proteins do not precipitate with polymerized actin when subjected to high-speed centrifugation. **C)** Contractility assays performed for ACTG2 WT and mutant proteins show a reduction of cellular contractility for all the mutants studied. **D)** Quantification of the difference between the initial (0 hours) and final (24 hours) area occupied by a collagen matrix confirms reduced cellular contractility for the mutant proteins. *($p<0.05$); Scale bars: 20 µm.

ACTG2 polymerization is impaired by the presence of a missense variant

To further investigate the effect of the missense variants found in MMIHS patients on the overall function of ACTG2, we determined the localization of WT and mutant proteins with phalloidin, a known marker of filamentous actin (F-actin). We over-expressed a tagged version of ACTG2 WT and mutant proteins in a human osteosarcoma cell line (U2OS), and specifically looked for co-localization between ACTG2 and actin fibers. While the WT protein could incorporate into the actin filaments, no co-localization was observed for any of the mutants analyzed (Fig.4A). We further confirmed this finding by performing *in vitro* actin binding assays. In these assays, polymerized actin is pelleted by high-speed centrifugation and the binding of ACTG2 to the actin filaments is determined by co-sedimentation. We found that while ACTG2 WT was able to pellet in the presence of F-actin, all the mutants analyzed remained in the supernatant both in the absence and presence of polymerized actin (Fig.4B).

ACTG2 missense variants reduce cellular contractility

Contraction and relaxation of smooth muscles occur in an involuntary manner and are critical processes for the normal functioning of the vascular, digestive, respiratory and urogenital systems (Lehtonen, Sipponen et al. 2012). ACTG2 is the main actin isoform present in visceral smooth muscle and previous studies showed that its presence is necessary for the controlled dilatation of various muscular structures within the body (Arnoldi, Hiltbrunner et al. 2013). Recent *in vitro* studies have also reported that ACTG2 is involved in cellular contractility, and that variants in *ACTG2* lead to reduced cellular contraction (Lehtonen, Sipponen et al. 2012, Thorson, Diaz-Horta et al. 2014). Therefore, we decided to investigate if the missense variants identified in our cohort of patients also affect cellular contractility. For that, U2OS cells were transfected with ACTG2 WT and mutant vectors, and cell contraction assays were performed *in vitro* based on the contraction of a collagen matrix. Our results showed that all missense variants analyzed affected cellular contractility (Fig.4C), but this effect was only significant ($p<0,05$) for the variants affecting codon 178 (R178H; R178C; R178L; Fig.4D).

The *ACTG2* variant R148S identified in Visceral Myopathy leads to similar *in vitro* changes as the ones described for MMIHS

Since heterozygous *ACTG2* missense variants are also known to cause Visceral Myopathy (VM) [13,19-21], we investigated the effect of one of these variants (R148S) on the structure and function of ACTG2. We performed molecular modelling and MD simulations, and the same set of *in vitro* assays described above for the MMIHS associated variants. Our MD simulations showed that the R148S mutant protein behaved similarly to the WT, and we could only detect an increasing RMSD (for the entire protein trace) towards the end of the simulation (Fig.5A). However, this increasing RMSD is likely due to the high RMSD values for the residues belonging to the D-loop (9.455 Å for the R148S variant in comparison to 7.061 Å for the WT, Table 4), an intrinsically flexible region. Exclusion of the D-loop from the analysis gave RMSD values for the entire R148S mutant protein comparable to the ones obtained for the WT and the R40C mutant (data not shown). The analysis of the 10 ns simulation also showed that the R148S variant led to specific structural modifications of the ACTG2 protein that are comparable to the R40C variant. Both these mutant proteins showed the lowest RMSD average values at the ADP-ribosylation site (1.260 Å and 1.350 Å for the R40C and R148S variants respectively), which reflect a less dynamic structure when compared to the WT (1.820 Å) (Table 5). They also presented the highest alpha-carbon trace displacements at the myosin-binding site (3.557 Å and 2.827 Å for the R40C and R148S respectively) when compared to the WT (2.383 Å, Table 6), suggestive of structural differences at this site that could possible translate into suboptimal interactions with myosin.

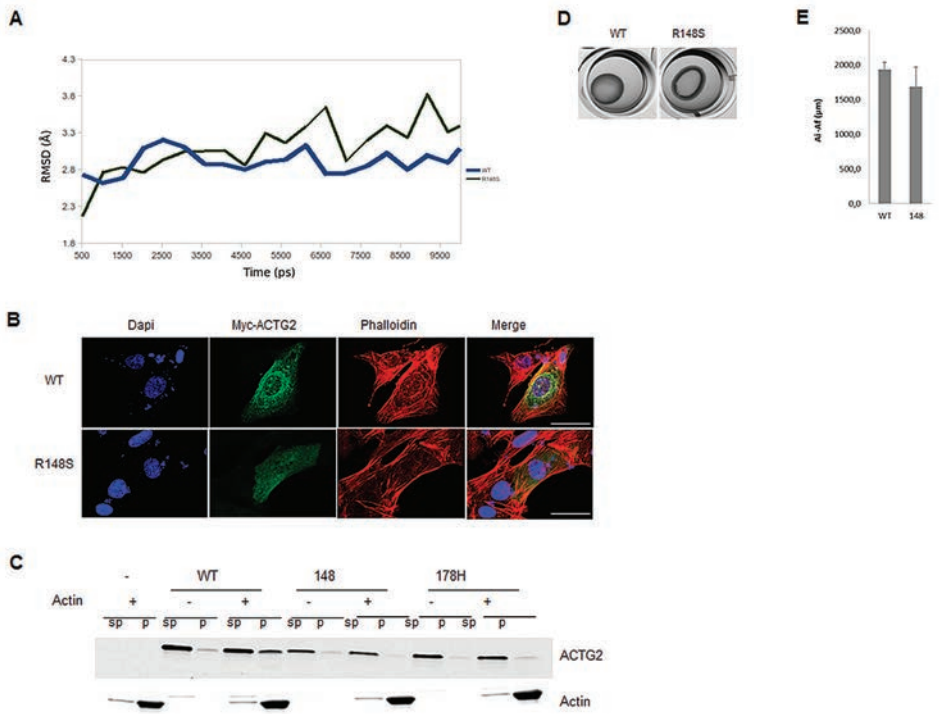

Fig.5: Effect of the R148S to the structure and function of ACTG2. A) Molecular modelling and MD simulations of ACTG2 WT and the R148S mutant show moderate structural differences. **B)** Confocal images of U2OS cells stained for Phalloidin (a F-actin marker), and expressing a Myc-tagged version of ACTG2 WT and of the R148S protein show reduced incorporation of the mutant into the actin filaments. **C)** *In vitro* actin binding assays show that the R148S mutant protein does not precipitate with polymerized actin when subjected to high-speed centrifugation. **D)** Contractility assays performed for ACTG2 WT and the R148S mutant protein show a reduction of cellular contractility for the mutant. **E)** Quantification of the difference between the initial (0 hours) and final (24 hours) area occupied by a collagen matrix confirms a reduced cell contractility for the R148S variant. However, this difference is not significant ($p>0.05$). Scale bars: 20 µm.

Our *in vitro* assays also showed that, similarly to what we observed for the MMIHS variants, the cellular localization of ACTG2 is disrupted by the presence of the R148S variant, as well as its ability to bind to actin filaments (Fig.5B, 5C). Moreover, a reduction in cell contractility was detected in the presence of this variant, as previously described (Fig.5D, 5E) [19].

DISCUSSION

ACTG2 is the main actin isoform expressed in intestinal smooth muscle cells, and it is therefore, not surprising that changes affecting its structure lead to severe disruption of normal intestinal development and function. Recently, heterozygous *ACTG2* variants have been identified as the cause of MMIHS (Thorson, Diaz-Horta et al. 2014, Tuzovic, Anyane-Yeboa et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014). In this manuscript we

confirm the involvement of ACTG2 in this disorder, and show that the heterozygous missense variants associated with MMIHS are pathogenic, disrupting actin polymerization. As a consequence, cellular contractility is affected which could account for the phenotypic abnormalities seen in MMIHS patients. Surprisingly, no differences in the expression levels of ACTG2, or any other major histological abnormalities was detected in the intestinal biopsies of MMIHS patients, despite previous studies have reported pathological abnormalities associated with this disorder (Kubota, Ikeda et al. 1989, Ciftci, Cook et al. 1996, Richardson, Morgan et al. 2001, Rolle, O'Briain et al. 2002, Piotrowska, Rolle et al. 2003, Piaseczna Piotrowska, Rolle et al. 2004, Narayanan, Murphy et al. 2007, Szigeti, Chumpitazi et al. 2010). However, since most of these studies did not provide a genetic etiology for the patients reported, and some abnormal findings, such as increased number of mast cells, can be a secondary effect due to reactive changes normally associated with bowel obstruction, the use of histological analysis for diagnostic purposes still needs to be carefully evaluated. Of particular notice is a recent report describing differences in ACTG2 expression between one MMIHS patient carrying a R178H variant and a control (Tuzovic, Anyane-Yeboa et al. 2014). In our study two patients with a variant on the same codon were also analyzed immunohistochemically (Fig.2B, patients S3 and S5), but we were unable to find any differences when compared with the control. Although we find this discrepancy difficult to explain, we believe that the sample size in both cases is rather small and thus, any inferences must be treated with caution.

To date, all of the *ACTG2* variants associated with MMIHS affect specific residues strongly conserved among different species (Table 2) (Thorson, Diaz-Horta et al. 2014, Tuzovic, Anyane-Yeboa et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014). There also seems to be an over-representation of variants associated with residue 178 in MMIHS (Fig.1B, Table 2), which is suggestive of a possible mutational hot-spot in this region. Since no clear loss of function variants (early stop codons or out of frame insertions/deletions) have been identified in any of the MMIHS patients reported, and no difference in ACTG2 expression levels has been found in patients and controls (Fig.2B), we hypothesize that the variants identified in *ACTG2* are likely to have a dominant negative effect. Based on our functional studies we cannot establish a genotype-phenotype correlation, since all the variants seem to behave in a similar way. However, considering that in our cohort, patients with a variant in residue 178 died a few days after birth, and the only patient alive has a R40 variant (S6), it is tempting to speculate that variants affecting residue 178 have a stronger dominant effect leading to a more severe phenotype. Interestingly, residue 178 of ACTG2 corresponds to residue 179 of ACTA2, which has been reported to be mutated in patients diagnosed with early-onset vascular disease associated with smooth muscle cell dysfunction (Milewicz, Ostergaard et al. 2010), and in a patient with prune belly syndrome (Richer, Milewicz et al. 2012), supporting a stronger effect of this variant on the overall phenotype. Our molecular dynamics (MD) simulations also corroborate this idea, since the structural changes assessed from visual inspection of the MD trajectories, were generally

more predominant for the R178 mutants when compared to the others. However, since our analysis focused only on the whole protein or specific regions (as described in Tables 3-6), it is possible that we have missed subtle changes involving smaller sub-regions that might lead to significant local structural differences in functionally important domains. To further investigate and understand such changes, the crystal structure for each mutant protein should be determined and longer MD simulations (on the μs- to ms-scale) should be performed.

From our cohort of eleven MMIHS patients, three of them did not carry a variant in *ACTG2* (F1, F2 and F3). These patients were considered to be familial cases. Since a homozygous variant in *MYH11* (L1200T) has been recently identified in a patient of consanguineous descent diagnosed with MMIHS and prune belly syndrome (Gauthier, Ouled Amar Bencheikh et al. 2014), we screened this gene in our familial cases. However, no variant was found in any of them (data not shown). The absence of *ACTG2* and *MYH11* variants in these patients strengthens the idea of locus heterogeneity, and suggests the involvement of other genes in MMIHS pathogenesis. These genes would be associated to the familial cases, in which an autosomal recessive pattern of inheritance is expected. Since our immunohistochemistry data shows that ACTG2 localizes to the intestinal smooth muscle, and myosin and actin are two major proteins involved in muscle contractility, MMIHS can be considered to result from a myopathic defect. Therefore, we believe that particular attention should be given to other proteins involved in the contractile apparatus, as impaired function of any of these proteins could also possibly lead to the same syndrome or overlapping clinical entities. Alternately, Filamin A (FLNA), an actin binding protein with a well characterized role in the cytoplasm, could also be considered to be another suitable candidate for MMIHS, especially because of its known involvement in other intestinal motility disorders (Gargiulo, Auricchio et al. 2007, Clayton-Smith, Walters et al. 2009, Kapur, Robertson et al. 2010).

VM and MMIHS are clinically two separate disorders with overlapping features. The main differences rely on the age of onset, MMIHS can be diagnosed prenatally due to a recognizable bladder distension while VM occurs during adolescence, and disease severity with most MMIHS patients surviving only a few days or months after birth. The identification of *ACTG2* missense variants as the cause of both disorders led to their recent classification as ACTG2-related disorders, and suggested that MMIHS and VM could be one entity with a continuum of symptoms that vary in severity (Wangler, Gonzaga-Jauregui et al. 2014). To investigate this possibility, we included one of the VM missense variants previously described (R148S) (Lehtonen, Sipponen et al. 2012), in our study, and analyzed its effect on the structure and function of ACTG2. Our MD simulations and *in vitro* results corroborated this idea, as the R148S variant affected ACTG2 structure and function in a similar way as the MMIHS variants. However, contradictory to what has been previously reported for VM patients carrying a R148S variant (Lehtonen, Sipponen et al. 2012), we were unable to detect the presence of ACTG2 positive inclusions in any of the

MMIHS patients analyzed by immunohistochemistry. We found instead a diffuse cytoplasmic distribution of ACTG2 (Fig.2B). Considering that we were unable to obtain material from a VM patient carrying the R148S variant, we cannot rule out the possibility that the R148S variant leads to specific localization changes of ACTG2 that do not occur in the presence of the MMIHS associated variants. However, based on our results, we have no indication that MMIHS and VM are pathophysiologically different disorders. We can only hypothesize that variants identified in MMIHS cause specific conformational changes that do not occur in VM, but disrupt binding of ACTG2 to key interactors required for intestinal and bladder development, accounting for a more severe phenotype in MMIHS.

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Conflict of Interest: The authors have nothing to disclose.

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Supplementary table 1: ACTG2 sequencing primers.

Primer name	Sequence (5'-3')
ACTG2 1F	CGTCAGCTGGCCTTTTTAGG
ACTG2 1R	CAAAGGACATCAGGGTGTGG
ACTG2 2F	GGTTTCTGGCCACCTTTGG
ACTG2 2R	TAGGGTCCCCTCAGCAACC
ACTG2 3F	TTCACATTTCAGGGCAGAGG
ACTG2 3R	CACAGTGCAGCGCTTACTCC
ACTG2 4F	TGTTTCTGGGGGAAGAAAGG
ACTG2 4R	CAGCCTAGGTTGGAGAAATGG
ACTG2 5F	GAGGAGGGAAGGTCAAATGG
ACTG2 5R	GGCATGGACCACAGACATAGC
ACTG2 6F	TAAATCCCAAGGCCAACAGG
ACTG2 6R	GACCCCAGCTTTTCCCTACC
ACTG2 7F	TCAGAGCTCATTGGTAACAGTCC
ACTG2 7R	AGCTGGTCTCTGGGGAAGG
ACTG2 8F	AGCTACTTGGGAGGCTGAGG
ACTG2 8R	GAGATTGGCAGGGACTGTGG
ACTG2 9F	AACCAGGGATCAGAGGTTGC
ACTG2 9R	GGAGACAACTGAGGCAAGACC
ACTG2 10F	GATTCACCACATTTGTTCTTTGC
ACTG2 10R	TGACACAGTAGAAGGAGCACTAGC

Supplementary table 2: Primers for site-directed mutagenesis of ACTG2 WT. The affected nucleotide is underlined in each sequence.

Primer name	Sequence (5'-3')
ACTG2(R40C)F	GTGGGCCGCCCTTGCCACCAGGGTGTGATGG
ACTG2(R40C)R	CCATCACACCCTGGTGGCAAGGGCGGCCCAC
ACTG2(R63Q)F	TGAGGCTCAGAGCAAGCAAGGGATCCTAACTCTC
ACTG2(R63Q)R	GAGAGTTAGGATCCCTTGCTTGCTCTGAGCCTCA
ACTG2 (R148S)F	CTCTATGCCTCTGGCAGCACGACAGGCATCG
ACTG2(R148S)R	CGATGCCTGTCGTGCTGCCAGAGGCATAGAG
ACTG2(R178H)F	CCATGCCATCATGCACCTGGACTTGGCTGGC
ACTG2(R178H)R	GCCAGCCAAGTCCAGGTGCATGATGGCATGG
ACTG2 (R178C)F	CCATGCCATCATGTGCCTGGACTTGGCTGGC
ACTG2(R178C)R	GCCAGCCAAGTCCAGGCACATGATGGCATGG
ACTG2(R178L)F	CCATGCCATCATGCTCCTGGACTTGGCTGGC
ACTG2(R178L)R	GCCAGCCAAGTCCAGGAGCATGATGGCATGG

Supplementary table 3: RMSD value for ACTG2 WT and mutant proteins.

ACTG2 (376 residues)		
ACTG2	**Average (Å)**	**STDEV**
WT	2.856	0.288
R178C	3.476	0.448
R178L	2.779	0.361
R178H	3.168	0.363

R40C	2.536	0.343
R63Q	2.815	0.255
R148S	3.079	0.429

Supplementary Table 4: RMSD value for the D-loop in ACTG2 WT and mutant proteins.

D-loop (residues 41-53)		
ACTG2	**Average (Å)**	**STDEV**
WT	7.061	1.077
R178C	8.405	1.697
R178L	6.376	1.447
R178H	8.012	1.488
R40C	4.955	1.998
R63Q	8.055	1.180
R148S	9.455	2.320

Supplementary table 5: RMSD value for the ADP- ribosylation site in the ACTG2 WT and mutant proteins.

ADP-ribosylation site (residues 174-182)		
ACTG2	**Average (Å)**	**STDEV**
WT	1.820	0.545
R178C	2.106	0.454
R178L	1.996	0.545
R178H	1.712	0.340
R40C	1.260	0.338
R63Q	1.856	0.381
R148S	1.350	0.240

Supplementary table 6: RMSD value for the myosin binding site in the ACTG2 WT and mutant proteins.

Myosin binding site (residues 357-376)		
ACTG2	**Average (Å)**	**STDEV**
WT	2.383	0.636
R178C	2.564	0.361
R178L	2.328	0.407
R178H	2.630	0.517
R40C	3.557	0.506
R63Q	2.560	0.716
R148S	2.827	0.440

CHAPTER 3

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome in Human and Mice Deficient of Leiomodin-1

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ABSTRACT

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS) is a congenital myopathy characterized by severe obstruction of the bladder and the intestine. Elucidating the etiology of this disease will extend our understanding of the networks crucial for proper functioning of muscle cells in visceral organs. Using a combined strategy of homozygosity mapping and whole exome sequencing, we aimed to identify the deleterious recessive variation responsible for MMIHS in a child from consanguineous parents. We identified a homozygous Leiomodin-1 (*LMOD1*) nonsense mutation in a newborn child. *LMOD1* expression in patient's fibroblasts was reduced. We utilized CRISPR genome editing to engineer a knockout mouse. Western blotting, qRT-PCR, and immunofluorescence microscopy were performed to define expression of the identified gene. Actin filament formation and contractility assays were performed to gain mechanistic insights. Analysis of actin filaments by G-Actin/F-Actin assay indicated that deficiency of LMOD1 caused severely reduced formation of actin filaments and impaired contractility. Characterization of *Lmod1* knockout mice carrying a similar nonsense mutation revealed phenotypes featured in patients with MMIHS. Thus, we conclude that *LMOD1* is a new disease gene for MMIHS. Adding to the previously known MMIHS disease-causing-genes, *ACTG2* and *MYH11*, this study confirms that MMIHS is a heterogeneous disease of the visceral organs, with multiple patterns of inheritance. The striking similarities in pathology in human and mice support the latter as a powerful model to further investigate the underlying mechanistic role of LMOD1 in MMIHS as well as potential therapeutic interventions.

INTRODUCTION

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS) (OMIM 249210) is a rare congenital malformation characterized by a severe obstruction of the intestine and the urinary bladder in neonates. More than 250 cases of MMIHS have been reported since its first description in 1976, (Berdon, Baker et al. 1976, Mc Laughlin and Puri 2013, Wangler and Beaudet 2015). Until now, parenteral nutrition and multivisceral organ transplantation are the only available treatments for this disease. Fatal outcomes were reported in most cases, while morbidities caused by long term administration of parenteral nutrition or catheterization were manifest in surviving patients (Lopez-Munoz, Hernandez-Zarco et al. 2013).

Although most MMIHS cases are considered sporadic, several familial cases with multiple affected sibs from consanguineous families have been reported, suggesting that MMIHS has a genetic cause with two distinct modes of inheritance: *de novo* mutations in sporadic patients and a recessive mode of inheritance in familial patients (Mc Laughlin and Puri 2013). This hypothesis is now partially confirmed as we and other groups identified *de novo* mutations in the enteric smooth muscle gamma actin gene (*ACTG2*) (Thorson, Diaz-Horta et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014, Halim, Hofstra et al. 2016). These mutations were found mainly in sporadic cases of MMIHS, leading to the assumption that other loci are responsible for the presumed recessive form of MMIHS. This notion is strengthened by the recent identification of a homozygous loss-of-function variant in smooth muscle myosin heavy chain-11 (*MYH11*) in an MMIHS patient from a consanguineous family (Gauthier, Ouled Amar Bencheikh et al. 2015). To date, no other reports of MMIHS patients with *MYH11* variants have been published, suggesting more genes may lead to the same phenotypes represented by this syndrome.

In this study, we investigated an MMIHS patient from a consanguineous family in whom no rare variants in *ACTG2* or *MYH11* were found. We describe the identification of a homozygous nonsense mutation in Leiomodin-1 (*LMOD1*), an ill-defined gene that is expressed preferentially in smooth muscle lineages (Nanda and Miano 2012). At the same time, a CRISPR-generated nonsense mutation in exon 1 of murine *Lmod1* yielded an MMIHS-like phenotype. Functional studies support a previously unrecognized role for LMOD1 in visceral smooth muscle function. LMOD1 is a new MMIHS disease gene that expands the genetic heterogeneity of this devastating visceral smooth muscle disease.

MATERIALS AND METHODS

Recruitment of the Study Subject

An MMIHS patient from consanguineous parents was included. Ethical approval for this study was granted by the Erasmus Medical Center ethical committee (Medisch Ethische Toetsings commissie - METc 2011/148, ABR form: NL35920.042.11), and written informed consent was given by the family.

SNP-array analysis

To determine chromosomal abnormalities in the patient and to identify the presence of Runs of Homozygosity (ROH), we processed 250 ng of DNA on the Illumina HumanOmniExpress BeadChip v1 array (Illumina, Inc., San Diego, USA) using standard protocols. Normalized output was generated using Genome Studio version 2011.1 (Illumina, San Diego, CA, USA), ROH and Copy Number variations were analyzed and visualized in Nexus CN8.0 (Biodiscovery Inc, El Segundo, CA, USA). CNV was profiled according to previously described prioritization methods (Brosens, Marsch et al. 2016). As a minimum cut-off for homozygosity mapping, ROH regions had to contain at least 50 probes and be larger than 1 Mb in size in line with criteria described elsewhere (Yu, Zhao et al. 2001, Auton, Bryc et al. 2009). To determine the consanguinity coefficient (f) and the inbreeding coefficient (F) we used the the genomic oligoarray and SNP-array evaluation tool v3.0 (Wierenga, Jiang et al. 2013), and a 50 probe minimum for ROH region.

Whole Exome Sequencing

Genomic DNA was fragmented using acoustic technologies (Covaris, Inc. Woburn, Massachusetts, USA). Exome capture was performed with the SureSelect Human All Exon 50 Mb Targeted exome enrichment kit v4 (Agilent Technologies, Inc., Santa Clara, California) and the library was paired-end sequenced (2x101 bp) using the Illumina TruSeq version 4 procedure. (Illumina, Inc., San Diego, USA). More than 5 Gigabases of raw sequence data was processed using the NARWHAL pipeline and aligned to the hg19 reference genome with Burrows-Wheeler Aligner version 0.6.2. Variants determined using the Bayesian genotyper incorporated in the genome analysis toolkit version 1.2.9 were uploaded to Cartagenia Bench NGS version 4.1.8 (Cartagenia Inc, Boston, MA, USA) for filtering and prioritization. Copy Number Variants (CNV) and ROH regions were exported from Biodiscovery Nexus CN8.0. to Cartagenia Bench CNV and NGS to inspect for overlap between the identified ROH regions with the rare variants.

We excluded variants with a high frequency in our in-house control cohort (n=200, with similar capture, alignment and variant calling procedures), variants with an allele frequency above 0.1% in public databases (ExAC release 0.3, ESP6500SI-V2; 1000 Genomes Phase 3 release v5.20130502 and GoNL SNPs and Indels release 5). Next, we annotated variants using the framework described by Kircher and colleagues (Kircher, Witten et al. 2014) and retained all the variants predicted to affect splicing, nonsense variants, and all coding and non-coding variants, with a CADD score above 20 representing the top 1% ranked variants in the framework. The higher this Phred-base-calling score is, the more likely the variant has a deleterious effect (http://cadd.gs.washington.edu/home, CADD v1.3). Deleterious variant is deposited in the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/) using the submission name “Loss of LMOD1 in MMIHS” and using identifiers as described in this manuscript.

Genes harboring candidate variants are inspected for their mRNA and protein

expression patterns in the Human Integrated Protein Expression Database and the Genotype Tissue Expression project database (Consortium 2013), incorporated in Genecards (Fishilevich, Zimmerman et al. 2016) and overlapped with smooth muscle functioning in the Reactome database (Croft, Mundo et al. 2014).

Sanger sequencing

Genomic DNA was isolated from peripheral blood lymphocytes using standard methods (Chemagic DNA Blood Kit Special, Chemagen, Perkin Elmer, Waltham, Massachusetts, USA). Sanger sequencing across exon 2 of *LMOD1* was performed to confirm the nonsense mutation using sets of primers described in Supplemental table 1. 15 ng of genomic DNA was used to amplify exon 2 of LMOD1 using a standard PCR protocol. PCR products were purified (ExoSap it – GE Healthcare, Little Chalfont, UK) and Sanger sequencing was performed by using dye labelled primers (forward and reverse; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems, Foster City, California, USA) on an ABI 3130XL genetic analyzer. Sequencing reads were analyzed using SeqScape® v2.5 software.

Animal Work

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Rochester in Rochester, NY. All animals were housed in a temperature- and humidity-controlled facility under the supervision of the Department of Laboratory Animal Medicine, University of Rochester Medical Center, Rochester, NY.

CRISPR-Cas9 Editing of *Lmod1*

Three guide-RNAs (gRNAs; Supplementary table 1) targeted to mouse *Lmod1* exon 1 were designed and cloned into the *BbsI* site of pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene #42230). The gRNAs were synthesized by *in vitro* transcription using the HiScribe T7 mRNA synthesis kit (New England Biolabs, Ipswich, Massachusetts, USA) and microinjections were carried out as previously described (Oliver, Yuan et al. 2015). Briefly, fertilized zygotes were microinjected with a mixture of Cas9 mRNA from Trilink Biotechnologies (200 ng/µl) and each individual *in vitro* transcribed gRNA (100ng/µl each; 300 ng/µl in total). Following injection, zygotes were cultured in KSOM+AA medium for 1 hour before transfer into the oviductal ampullae (10-15 zygotes per oviduct) of CD1 mice that had been mated with vasectomized CD1 males the previous night.

Genomic DNA was isolated from mouse tail-snips by overnight proteinase K digestion. PCR was used to detect the deletion on *Lmod1* exon 1 (Supplemental table 1) using AccuStart II Supermix (Quanta, Houston, Texas, USA) at the following cycling conditions: 3 minute incubation at 95˚C, followed by 35 cycles of 30 seconds at 95˚C, 30 seconds at 58 ˚C, and 30 seconds at 72 ˚C, and lastly a 5 minute incubation at 72 ˚C. PCR reactions were analyzed on a 2% agarose gel.

PCR Screening for CRISPR-Cas9 Off-targeting

A gRNA design tool based on an algorithm designed by Feng Zhang's lab (http://crispr.mit.edu/) was used to calculate the ten most highly ranked off-targets for each of the three gRNAs used to develop the *Lmod1*^{KO} allele. PCR primers flanking each off-target sequence were designed and are listed in supplemental table 3. Adapter sequences were added onto the 5' ends of each primer (5'-ACACTGACGACATGGTTCTACA-3' for forward primers, 5'-TACGGTAGCAGAGACTTGGTCT-3' for reverse primers). PCR amplification was carried out for 28 cycles (95˚C for 30 seconds, 53˚C for 30 seconds, 72˚C for 30 seconds) using AccuStart II Supermix (Quanta, Houston, Texas, USA). All 30 PCR reactions were pooled and sent for sequencing to a DNA Services Facility (Research Resources Center at the University of Illinois at Chicago). Sequence analysis was done using CRISPresso (Pinello, Canver et al. 2016).

Quantitative Real Time (qRT)-PCR

RNA extraction from patient's and controls' fibroblasts was performed using a RNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. 1 µg of RNA was converted into cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, California, USA). Mix of cDNA and primers (Supplementary table 1) were used with KAPA SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, Massachusetts, USA), and qRT-PCR was performed using a IQ5 cycler PCR machine (Bio-Rad). Expression levels were corrected with the expression of two housekeeping genes, *COPS5* and *CLK2*, averaged and presented as fold changes. Total RNA was isolated from mouse tissues using miRNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized with an iScript cDNA Synthesis kit (Bio-Rad, Hercules, California, USA). qPCR was performed using PerfeCTa SYBR Green SuperMix (Quanta, Houston, Texas, USA) and PCR primer pairs (Supplemental table 1).

Protein isolation and Western blot analysis

Fibroblasts were washed with PBS and incubated with standard lysis buffer and protease inhibitors (Roche) for 30 minutes on ice. Cell lysates were collected by scraping and cleared by centrifugation at 13.000 rpm for 15 minutes at 4^{o}C.

When using human intestinal SMC, protein isolation was performed based on the manufacturer's instruction. Protein lysates were kept at -80^{o}C before being processed further for SDS-PAGE and immunoblotting. Protein was quantified using a BCA kit (Thermo Scientific), and 30 µg of protein from each sample were loaded into a TGX precast gel (Bio-Rad). The following primary antibodies and their dilutions were used: LMOD1 (Proteintech; 1:200), Actin (Cytoskeleton; 1:250) and GAPDH (Millipore 1:1000). Secondary antibodies used were IRDye 800CW Goat anti-mouse and IRDye 680RD Goat anti-Rabbit (Li-Cor).

When using mouse tissue, whole protein was extracted in Cell Lysis Buffer

(Cell Signaling) supplemented with complete Mini EDTA-free protease inhibitor cocktail tablets (Roche) by mechanical homogenization. Cleared protein lysates were subjected to Western blotting as previously described.[3]we describe a new SRF/MYOCD-dependent, SMC-restricted gene known as Leiomodin 1 (Lmod1 Antibodies used were LMOD1 (Proteintech 15117-1-AP, 1:800), SM22α (Abcam ab14106, 1:5000), and Beta-Tubulin (Cell Signaling 2128, 1:1000).

Immunohistochemistry and Immunofluorescence

Formalin-fixed paraffin-embedded human small intestinal tissues from controls were obtained from the repository of the department of Pathology, Erasmus University Medical Center. Immunostaining was performed using specific antibody directed against LMOD1 (dilution 1:1000; Proteintech). After the addition of the primary antibody, the biotinylated multilink secondary antibody and the avidin-biotin-complex with peroxidase were applied, followed by the addition of 3,3'-diaminobenzidine (DAB) chromogen (Ventana, Tucson, Arizona, USA), and then slides were counterstained with hematoxylin. All images were taken with a Nanozoomer 2.0-HT (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan), and analyzed using the Nanozoomer Digital Pathology (NDP) viewer software (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan).

Mouse tissues were fixed in methanol/H_2O/acetic acid (60:30:10, v/v) solution overnight, embedded in paraffin, and sectioned at 5-micron thickness. Following deparaffinization, antigen retrieval was performed using a pressure cooker set at high pressure for 5 minutes in a pH 9 solution (Dako S2367) for LMOD1 primary antibody. For TUNEL and Ki-67 staining, slides were similarly subjected to high pressure steam in a pH 6 solution (Dako S1699) for 5 and 10 minutes, respectively. For LMOD1 staining set (LMOD1 Proteintech 15117-1-AP, 1:100; SM actin-Cy3, Sigma #C6198) and Ki-67 staining set (Ki-67 antibody: BioLegend #652402, 1:100; smooth muscle myosin, Biomedical Tech BT-562, 1:100), cells were washed in 1X PBS prior to overnight incubation with primary antibody (antibody diluent: Dako S0809) at 4˚C. The next day, slides were washed in 1X PBS prior to incubation with secondary antibody (AF-488 anti-rabbit (Thermo Scientific #A11008), AF-488 anti-mouse (Thermo Scientific #A11001), or AF-594 anti-rabbit (Thermo Scientific #A11012)) for 1 hour at room temperature. Cells were washed again before being mounted using ProLong Gold Antifade (Molecular Probes P36935). TUNEL staining was carried out using the *In Situ* Cell Death Detection Kit, Fluorescein (Sigma #11684795910) according to manufacturer's instructions, followed by staining with smooth muscle alpha actin-Cy3 conjugated antibody. All images were taken with a confocal microscope (Olympus FV 1000, Olympus, Tokyo, Japan) with voltages set using wild type controls for each tissue, such that wild type and experimental samples were imaged with the same parameters within each tissue group.

For immunofluorescence of hISMC, hISMC were cultured on cover slips. Seventy-two hours after being transfected with either scrambled siRNA or siLMOD1, cells

were fixed with four percent paraformaldehyde (PFA) and permeabilized with 1% BSA and 0.1% Triton X-100 in PBS. LMOD1 antibody (Proteintech, 1:25) and Phalloidin-rhodamine (Santa Cruz Biotechnology, 1:500) Images were taken using a Leica (AOBS) microscope, and analyzed with the Leica LAS AF Lite software.

Cell culture and transfections

Primary human intestinal smooth muscle cells (hISMC) were obtained from the Sciencell research laboratories, and cultured in smooth muscle cell (SMC) medium mixed with 1% smooth muscle growth supplement and 1% penicillin/streptomycin (Sciencell, Carlsbad, California, USA). Cells were maintained at 37°C and 5% CO_2. 24 hours before the transfection, 300,000 cells were seeded in 6 well plates. Cells were transfected with either Scrambled siRNA or siLMOD1 (Ambion, 100 nM per well) using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After 6 hours, the medium was refreshed with a normal SMC medium, and cells were allowed to grow for 72 hours before analysis.

G-actin/F-actin analysis

Seventy-two hours after hISMC were transfected with scrambled siRNA or siLMOD1, the cells were harvested and lysed in a detergent-bases lysis buffer that preserves the monomeric (G-) and filamentous (F-) forms of actin. F-actin was pelleted by ultracentrifugation, while G-actin remained in supernatant. Samples of supernatant and pellet were loaded into an SDS-PAGE system and underwent Western blot analysis (Cytoskeleton, Denver, Connecticut, USA).

Contractility assays

Twenty-four hours after transfection with scrambled siRNA or siLMOD1, hISMC were trypsinized, and used for collagen contractility assay. A cell contraction kit (Cell Biolabs, San Diego, California, USA) was used according to the manufacturer's instruction.

Myography Assay

Primary jejunum was isolated from mice and placed in ice-cold PSS buffer (130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, 0.026 mM EDTA, 2.5 mM $CaCl_2$). Jejunum ring segments were mounted on a 610m myograph (DMT) in PSS buffer with oxygenation, and the temperature was brought up to 37°C for approximately 30 minutes. Passive tension was applied and buffer was exchanged before tissues were allowed to equilibrate for an additional 20 minutes. Tissues were treated with K-PSS buffer (PSS with 60 mM KCl, 74.7 mM NaCl) or 100 μM carbachol in order to induce contraction. For KCl treatment, the mean peak contractile force was taken for several independent rounds of contraction. N = 6 animals for KCl treatment and n = 4 for carbachol treatment.

RESULTS

Clinical information of the patient

The proband was a female child from a consanguineous couple of Dutch origin. During a routine ultrasound sonography (USG) examination at 20 weeks of pregnancy, bilateral hydronephrosis and a distended bladder were detected (Fig. 1A). Polyhydramnios was later acknowledged, and delivery was medically induced at 34-weeks due to mechanical problems of the mother.

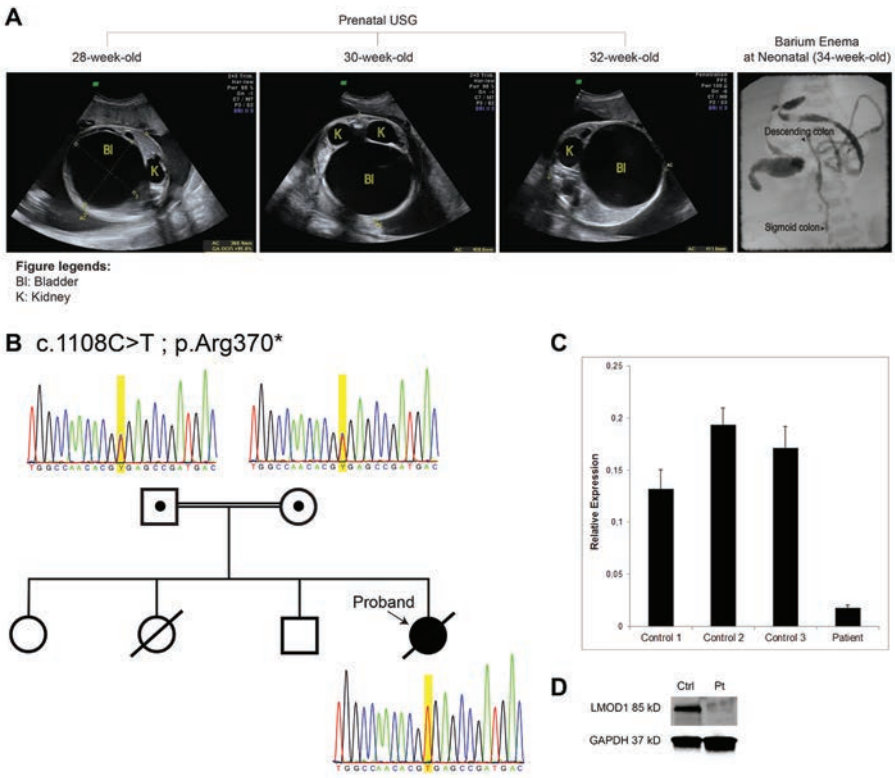

Fig.1: Identification of loss-of-function mutation in *LMOD1* gene in MMIHS patient. (A) Barium enema test confirmed the clinical diagnosis of MMIHS in patient. **(B)** Pedigree of the studied family, with their Sanger Sequencing results showing heterozygous variant in parents and homozygous variant in proband. **(C)** Quantitative real time (qRT) PCR analysis using RNA from controls and patients' fibroblasts showed severely reduced expression of *LMOD1* at RNA level. **(D)** Protein expression study by Western blot using control's and patient's fibroblasts confirmed the reduced expression of *LMOD1* in patient at protein level.

Physical examination at the neonatal period noted distended and lax abdominal wall, causing the intestine to be externally palpable. Further USG investigation did not reveal abnormalities of the brain and the heart. Bilateral hydronephrosis was noted,

along with bilateral distension of proximal ureter and distended bladder. No intestinal peristaltic movement was acknowledged. Barium enema testing indicated the presence of a microcolon (Fig. 1A). Conventional treatments given included parenteral nutrition, gut feeding, stomach suction and catheterization for the atonic bladder. As the patient succumbed at 5-days-old due to sepsis, there were no further clinical measures or interventions.

Although the patient was the first child in the family diagnosed with MMIHS, the parents acknowledged a previous intrauterine fetal death (IUFD) in which routine 20-week USG detected abdominal distension of the fetus. Meanwhile, two other siblings in this family are healthy and do not show any signs of bladder and intestinal abnormalities.

Copy Number Variation (CNV) profiling and homozygosity mapping

In the parental samples, 24 regions that are larger than 1 Mb, including ROH regions and heterozygous regions, were detected. Seven CNVs were present, all common polymorphisms inherited from unaffected parents (see supplementary table 4) Two homozygous regions were larger than 10 Mb: (hg19 chr2:95,395,757-115,363,347 and hg19 chr1:193,573,186-210,528,162). Genotype information derived from the SNP-array was indicative of consanguinity with an f of 0.375 and F of 0.1875 with 528 Mb of ROH.

A homozygous nonsense mutation in *LMOD1* was identified in the MMIHS patient from a consanguineous family

Whole exome sequencing analysis identified no deleterious variants in the known disease genes, *ACTG2* and *MYH11*. In total, 526 variants had a minor allele frequency below 0.1%. Prioritization to yield the most likely deleterious variants resulted in 7 nonsense variants, 10 that were predicted to affect splicing and 53 other variants had a CADD score of twenty or higher. (n= 70 variants in total). Only two out of 70 variants were homozygous, one nonsense (premature stop mutation) in *LMOD1* (NM_012134.2:c.1108C>T; p.Arg370*) and an intronic variant predicted to affect splicing in *CREG2* (NM_153836.3: c.442-5G>A). Of these two genes, the *LMOD1* gene has mRNA and protein expression in human bladder and intestine (Croft, Mundo et al. 2014, Fishilevich, Zimmerman et al. 2016).

We identified a homozygous mutation in exon 2 of *LMOD1*, that converted a cytosine at the position g.201.897.806 into a thymine, resulting in a premature termination codon. Confirmation with Sanger sequencing was obtained, and sequence analyses of both parents revealed the heterozygous variant, thus confirming the recessive mode of inheritance (Fig. 1B). This genetic finding was registered in ClinVAr (http://www.ncbi.nlm.nih.gov/clinvar/) under the following accesion number: SCV000299346.

To assess the effect of this variant, we examined the expression of *LMOD1* mRNA and protein in patient versus control fibroblasts. qRT-PCR and Western blot analyses revealed severe reduction/loss of LMOD1 expression in patient fibroblasts compared with that in the control (Fig. 1C-D).

***LMOD1* is expressed in all SMC throughout the developmental stages of human intestine**

SMC are the building blocks of muscle structures in human intestine, including muscularis mucosa, the circular and longitudinal muscles that form the muscularis propria, and the blood vessels primarily found in submucosa. Immunostaining showed strong LMOD1 expression within the SMC that constitute these structures. To test the continuity of LMOD1 expression, we examined human intestine from prenatal to neonatal stages of development. In all stages, LMOD1 is expressed by all SMC of the intestine (Fig. 2A-F).

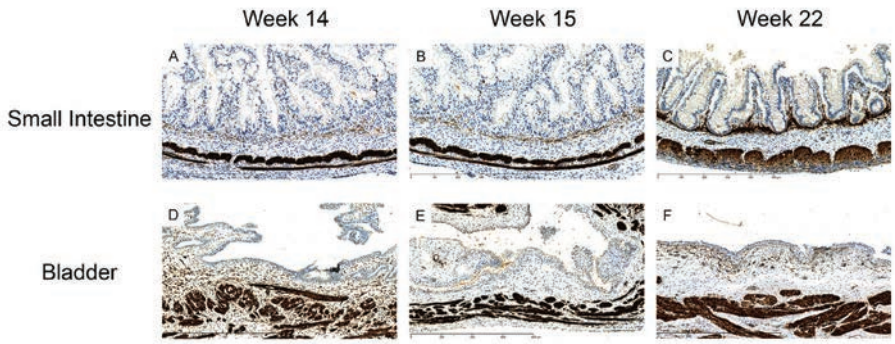

Fig.2: Expression of LMOD1 in embryonic human intestine. (A-E) LMOD1 is expressed in all SMC that constitute structures of the intestine, including muscularis mucosa, blood vessels, and both muscle layers of the mucularis propria. This expression is constant throughout various stages of development that are included in this study.

CRISPR-Cas9 Mediated generation of a nonsense codon in *Lmod1* is neonatal lethal in mice.

In order to determine the importance of LMOD1 in SMC function in an experimental animal model, we used two component CRISPR genome editing (Miano, Zhu et al. 2016) to target exon 1 of the *Lmod1* gene in mice. PCR genotyping around the region of exon 1 targeted by all three sgRNAs and Sanger sequencing of neonates revealed a 151 base pair deletion that created a frameshift and the introduction of a premature termination codon (PTC) within exon 1 of *Lmod1* (Fig. 3A). This PTC results in a truncated 19-amino acid peptide, the first 8 of which are native to the wild type LMOD1 protein (Fig. 3B). We used PCR to detect different *Lmod1* alleles and found the expected Mendelian frequency (Fig. 3C-D) indicating all death due to the nonsense codon was postnatal.

Western blotting of *Lmod1*$^{+/-}$ mice showed a 2-fold decrease in expression in stomach tissue (Fig. 3E), as well as aorta and bladder (data not shown). Heterozygous mice and humans As expected, no detectable LMOD1 protein was observed in any SMC-rich tissue of *Lmod1*$^{-/-}$ mice (Fig. 3 and data not shown). To test if the PTC results in degradation of the *Lmod1* transcript via nonsense mediated decay (Maquat 2004), we

analyzed steady-state mRNA using qRT-PCR. Surprisingly, results revealed no significant difference between Lmod1$^{+/+}$*Lmod1*$^{+/-}$, *Lmod1*$^{-/-}$ mice (Fig. 3F). Similar results were obtained in qRT-PCR experiments using primers targeted to the 5' and 3' regions of the *Lmod1* transcript (data not shown). These data suggest that the *Lmod1* transcript is not targeted for nonsense-mediated decay (NMD) in knockout mice.

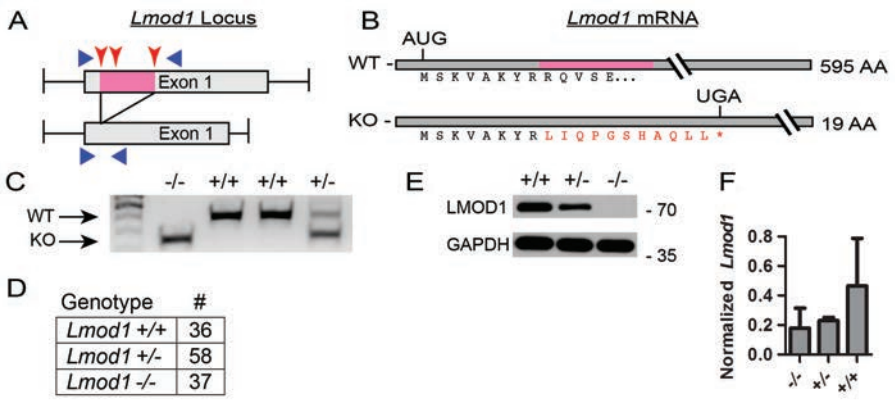

Fig.3: Cas9-mediated Deletion of *Lmod1* exon 1. (A) Schematic of *Lmod1* exon 1 deletion strategy. Injection of capped Cas9 mRNA and three gRNAs (red arrowheads) resulted in deletion of 151 basepairs (pink) within *Lmod1* exon 1. PCR primers (blue arrowheads) were used to confirm deletion. **(B).** Deletion of 151 basepair region (pink) results in frameshift mutation and the introduction of premature termination codon within *Lmod1* exon 1, generating a truncated 19 amino acid peptide. **(C)** Agarose gel analysis of *Lmod1 KO* mice. PCR products indicating wildtype, heterozygous or homozygous deletion of *Lmod1* were generated using the primers in Fig1A. **(D)** Genotyping data from a heterozygous *Lmod1+/-* x *Lmod1 +/-* mating. **(E)** Western blot showing complete absence of LMOD1 protein in the stomach of *Lmod1*$^{-/-}$ mice. **(F)** RT-qPCR analysis reveals no significant difference in *Lmod1* mRNA expression in knockout mice, indicating that the transcript most likely is not targeted for nonsense-medicated decay.

While the *Lmod1* mutation is transmitted at the expected Mendelian frequency, *Lmod1*$^{-/-}$ neonatal mice display gross distension of the abdominal cavity shortly following birth (Fig. 4A). Closer observation and dissection revealed dramatic distension of the stomach and urinary bladder (Fig. 4A-B), the latter of which is consistent with clinical observations in human MMIHS patients.

Hydronephrosis was also noted in some animals, likely due to failed micturition. Histopathological analysis revealed thinning and compaction of the visceral smooth muscle in both stomach and bladder, along with varied, often severe atrophy of the epithelium (Fig. 4C). Flattened mucosa and diminished gastric pits were also acknowledged in *Lmod1*$^{-/-}$ stomach.Immunofluorescence microscopy revealed undetectable LMOD1 protein within the SMC tissue of knockout stomach and bladder, consistent with earlier Western blotting data (Fig. 4C).

To further assess the *Lmod1*$^{-/-}$ phenotype, we conducted histopathological analysis on each of the three primary segments of intestine (Fig. 5A). Atrophy of the

epithelial layer, while not as dramatic as that observed in stomach and bladder, was still noticeable, particularly in the duodenum. However, unlike the bladder and stomach, no dramatic architectural changes were detected in the intestinal segments, although distension of the duodenum

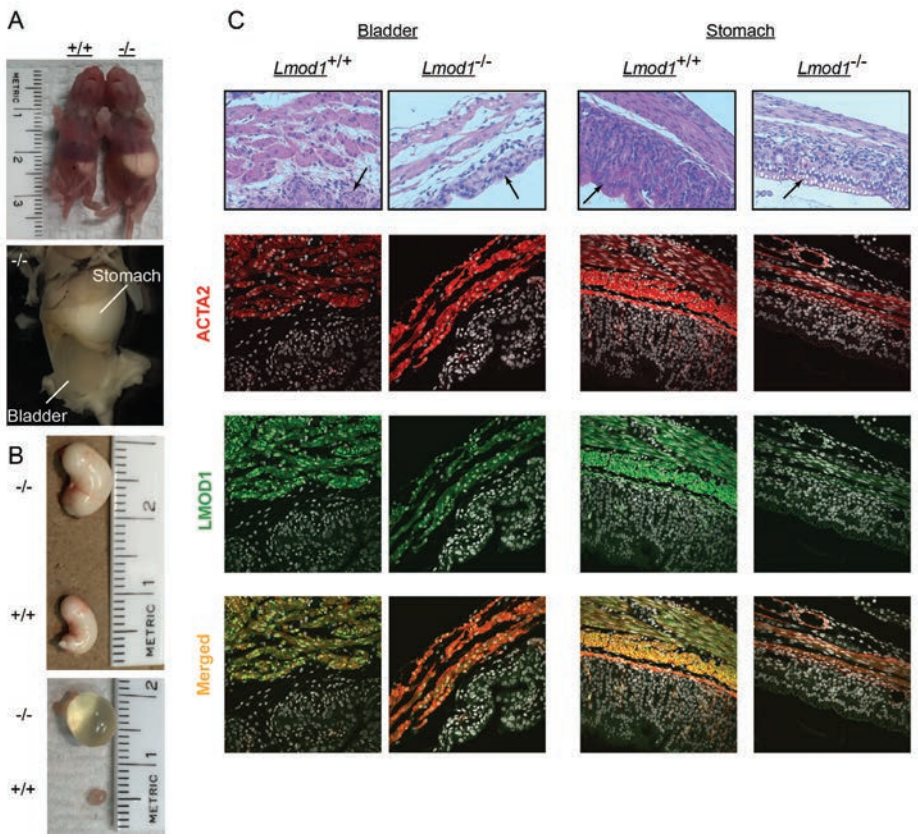

Fig.4: LMOD1 deletion results in gross distension of stomach and bladder. (A) Photograph of gross *Lmod1* knockout pups showing abdominal distension (top), notably the severely distended stomach and bladder (bottom panel) present in all *Lmod1*$^{-/-}$ animals. **(B)** Gross pathology of distended stomach and bladder of *Lmod1*$^{-/-}$ mice. **(C)** *Histology of Lmod1*$^{-/-}$ stomach and bladder compared with *wt*. H&E-stained sections reveal severe atrophy of epithelial cell in stomach and bladder of *Lmod1*$^{-/-}$ mice (top row) and immunofluorescence microscopy reveals absence of LMOD1 from visceral smooth muscle (bottom three rows) of the same tissues.

was evident in some animals upon dissection (Fig. 5A). Similar to both the stomach and bladder, no LMOD1 protein was visible using immunofluorescence microscopy.

To assess changes in SMC-specific gene expression following *Lmod1* deletion, we analyzed protein and mRNA from *Lmod1*$^{-/-}$ mice; no significant changes in MYH11, CNN1, ACTA2, or TAGLN were observed.

In order to assess any increase in proliferation following *Lmod1* deletion, we conducted Ki-67 staining on intestinal segments from *Lmod1*$^{-/-}$ mice. We found a higher

percentage of Ki-67^{+} cells in the outer SMC layer of the duodenum and jejunum (Fig. 5B-C). Collectively, these data indicate that *Lmod1* deletion bestows an MMIHS-like phenotype in mice, characterized by gastroparesis, megacystis, and hydronephrosis. Interestingly, vascular and respiratory function appeared normal. (data not shown).

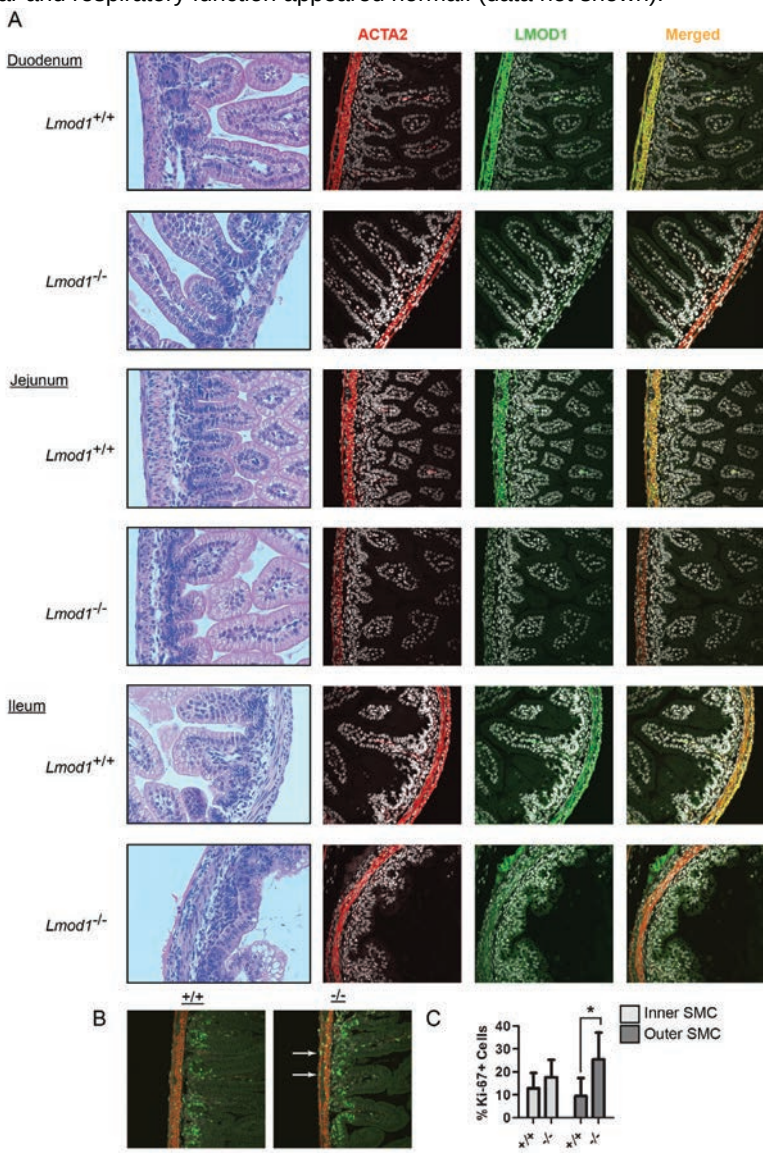

Fig.5: Loss of LMOD does not cause structural abnormalities of the duodenum, jejunum and ileum. (A) Histology of *Lmod1*$^{-}$ intestinal segments. H&E-stained sections of duodenum, jejunum and ileum (left column) and immunofluorescence microscopy reveals absence of LMOD1 from visceral smooth muscle of intestinal segments from *Lmod1*$^{-/-}$ mice. **(B)** Representative photographs of Ki-67 staining (green) of inner and outer SMC layers (red; stained with anti-SM actin) of the duodenum and ileum of *Lmod1* knockout mice. White arrowheads indicate an increase in Ki-67 positive cells in the outer SMC layer of *Lmod1*$^{-/-}$ duodenum and jejunum. **C.** Quantitation of Ki-67 staining shown in part B.

Deficiency of LMOD1 impairs the formation of actin filaments in hISMC and mouse intestine, and reduced cellular contractility

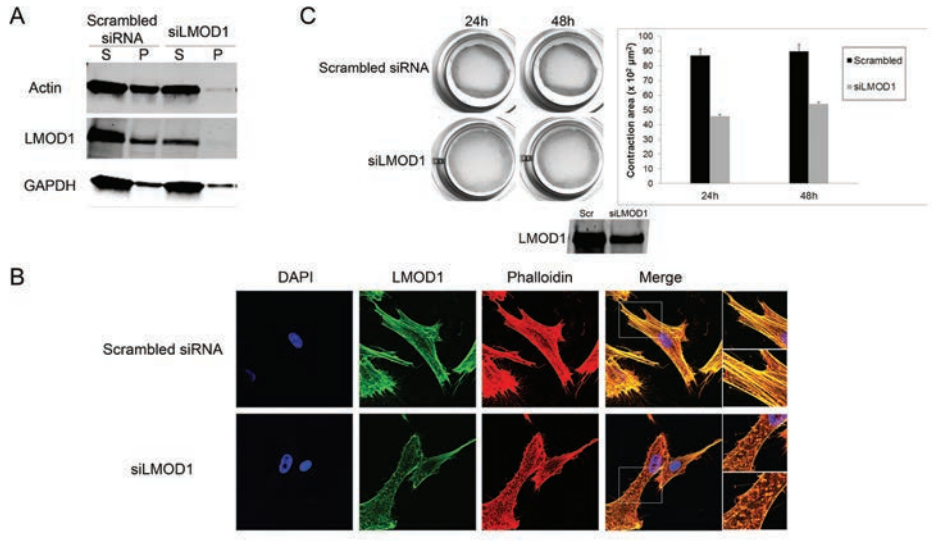

Fig.6: Knockdown of *LMOD1* on hISMC disturb the formation of actin filaments and contractility. **(A)** *In vitro* G-actin : F-actin assays show that when *LMOD1* is knocked down, the amount of actin filaments that pelleted by ultracentrifugation are severely reduced. **(B)** Confocal images of hISMC stained for LMOD1 and Phalloidin on cells transfected with Scrambled siRNA or siLMOD1, show that reduced expression of LMOD1 cause reduced formation of actin filaments. **(C)** Collagen contractility assays on hISMC transfected with scrambled siRNA or siLMOD1 show impaired contractility when *LMOD1* is knocked down.

Since LMOD1 has been described as an actin filament nucleator in SMC (Boczkowska, Rebowski et al. 2015), we aimed to test the effect of LMOD1 deficiency in hiSMC. siRNA against human *LMOD1* was used on cultured hiSMC and Western blot analysis confirmed knockdown of LMOD1 protein. Immunoblotting with smooth muscle alpha actin (ACTA2) showed that the formation of actin filaments in LMOD1-deficient cells was severely reduced compared to controls (Fig. 6A). LMOD1 and Phalloidin staining showed severe reduction in the number and size of actin filaments in LMOD1-deficient hiSMC (Fig. 6B). Results from G-actin/F-actin and immunofluorescence analyses demonstrated that knockdown of *LMOD1* in hiSMC impairs actin filament formation.

To further investigate the effect of the reduction of actin filaments on the function of hISMC, we performed a contractility assay. We revealed reduction of cellular contractility by more than 40% in LMOD-1 deficient hiSMC compared to controls (Fig. 6C)

Lmod1-KO mice exhibit defects in filamentous actin formation and SMC contraction

Our data in hiSMC revealed decreased formation of filamentous actin (F-actin) following siRNA knockdown of *LMOD1*. Therefore, we wished to examine F actin in primary tissues from *Lmod1*-KO mice. Ultracentrifugation of bladder tissue lysates and subsequent G/F actin analysis revealed an approximate 2-fold decrease in F actin following LMOD1 deletion (Fig. 7A-B). These data support the hypothesis that the functional and structural defects in *LMOD1*$^{-/-}$ MMIHS results from a decrease in F actin formation.

Since MMIHS is characterized by functional obstruction of the urinary bladder and hypoperistalsis in the intestine, we wished to test the hypothesis that *Lmod1* deletion results in decreased SMC contractility. We conducted myography experiments on primary jejunum ring segments; the results revealed significantly reduced contractile force in *Lmod1*$^{-/-}$ mice in response to both KCl and carbachol treatment. Importantly, the *Lmod1*$^{-/-}$ tissues did not exhibit a complete absence of contraction (Fig. 7C-D).

Cumulatively, these data suggest that LMOD1 deletion results in decreased formation of F actin and impaired contraction in visceral smooth muscle. Together, these phenotypes most likely lead to the functional obstruction of the urinary bladder and intestine observed in *LMOD1*$^{-/-}$ MMIHS.

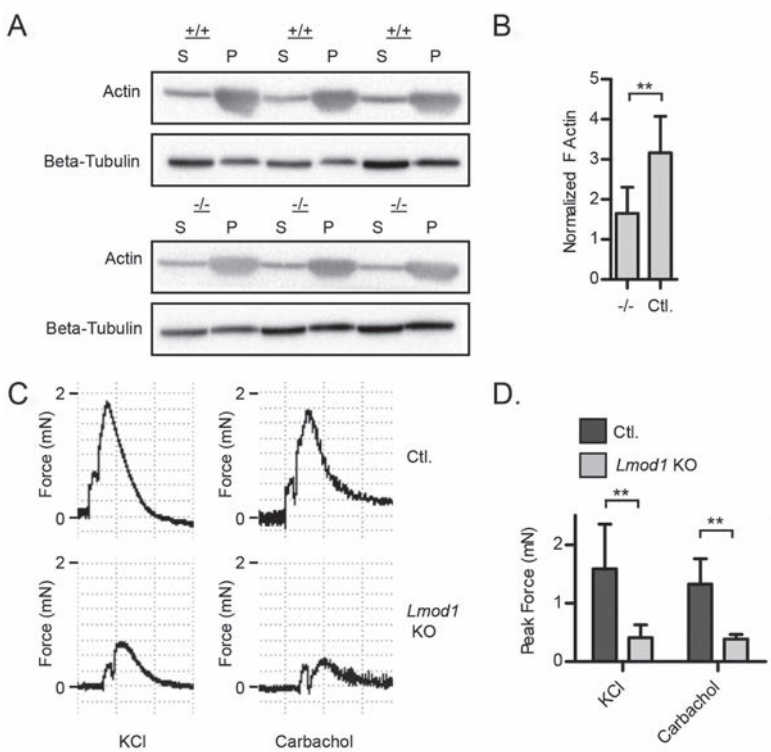

Fig.7: Deficit of Lmod1 in mice impairs filamentous actin formation and SMC contraction. (A-B) G-actin : F-actin assays show significant reduction of actin filaments in *Lmod1*-KO mice. (C-D) Myography assays show reduction of SMC contractility in *Lmod1*-KO mice.

DISCUSSION

We identified a single-nucleotide nonsense mutation within exon 2 of *LMOD1* in a patient with MMIHS, a rare congenital disease of the intestine and urinary bladder. Our study revealed that this mutation severely decreased the expression of *LMOD1* at both the mRNA and the protein level in patient fibroblasts. Moreover, the gross morphology of *Lmod1* KO mice is strikingly similar with the MMIHS phenotype in human, indicating LMOD1 is a new disease gene for MMIHS. Our studies indicate that loss of LMOD1 decreases filamentous actin formation and impairs SMC contraction *in vivo.*

LMOD1 is one of three Leiomodins, a family of tandem monomer-actin-binding nucleators (Chereau, Boczkowska et al. 2008, Chen, Ni et al. 2015). *LMOD1* is preferentially expressed in SMC lineages and is regulated by the serum response factor-myocardin transcriptional switch (Nanda and Miano 2012). *LMOD2* and *LMOD3* are preferentially expressed by cardiac and skeletal muscle, respectively (Tsukada, Pappas et al. 2010, Yuen, Sandaradura et al. 2014). Knockout of *Lmod2* in mice resulted in cardiomyopathy while disruption of *Lmod3* expression mimics nemaline myopathy in human (Yuen, Sandaradura et al. 2014, Pappas, Mayfield et al. 2015, Tian, Ding et al. 2015).

Despite preferential expression of LMOD1 in SMC has long been known, the importance of LMOD1 in SMC of visceral organs remained unclear. Our immunohistochemistry results using human intestine from different developmental stages showed continuous expression of LMOD1 in all SMC that inhabit the intestine (Fig. 2). Since MMIHS is a congenital disease, early expression of LMOD1 may serve an important role during the development of human intestine and urinary bladder, therefore any disturbances may affect these organs and possibly contribute to MMIHS. This expression pattern is similar to previous findings in the intestine of mouse embryo (Nanda and Miano 2012), hence the necessity to create an *Lmod1* knockout mouse in order to investigate further.

As expected, gross morphology of *Lmod1* KO mice is strikingly similar to MMIHS in humans. However, significant architectural changes were only observed in the stomach, not in the continuing three proximal segments of the intestine, in *Lmod1*$^{-/-}$ mice. Data from *Lmod1*$^{-/-}$ E18.5 mouse embryo showed none of the structural changes in the stomach, confirming that this abnormality is only acquired after birth, and likely due to overdilation of the stomach by its contents that could not be efficiently passed through to the continuing organs. Interestingly, and entirely consistent with clinical observations of MMIHS, we did observe distension of the urinary bladder in e18.5 embryos. These data suggest that in principal, loss of LMOD1 is insufficient to alter embryogenesis of the bladder and intestine, but may be detrimental to the functional contractility of these organs. Of note, there does not appear to be severe pathology in other SMC containing tissues such as blood vessels and airways of the lung though further investigation is needed using more restrictive means of deleting Lmod1 to vascular or respiratory SMC.

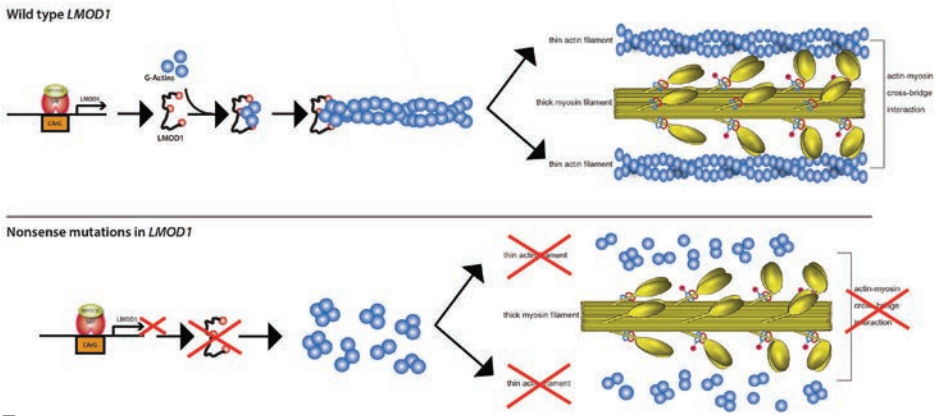

Fig. 8: Schematic representation of LMOD1's function in the formation of actin filaments, and the predicted effect of LMOD1 mutations on this mechanism. In wild-type, SRF/MYOCD complex interacts with CArG regions in LMOD1 gene, leads to *LMOD1* expression. Then, as an actin filament nucleator, LMOD1 protein initiates the formation of actin filaments. These actin filaments will form a cross-bridge interaction with thick myosin filaments, and cause contractions. In mutant LMOD1, although SRF/MYOCD complex can bind with CArG regions in its gene, expression of LMOD1 is disrupted. Thus, formation of actin filaments is severely impaired. The absent/decreased number of actin filaments impair actin-myosin interaction, leading to the failure of contraction in cells.

Muscle contraction in visceral organs is the product of coordinated molecular processes inside SMC. The center of this elaborate mechanism is the cross-bridge interaction between actin thin filaments and myosin thick filaments. Monomer (G-) actin molecules polymerize to form an actin filament; to start this process, actin monomers must first form actin nuclei, a process referred to as actin nucleation. Although this process can occur spontaneously, it is energetically inefficient (Sept and McCammon 2001), and cells employ actin nucleator proteins to enhance this reaction.

As tandem-monomer-binding nucleator, Leiomodins have actin binding motifs that bring G-actins together and create a molecular foundation for actin polymerization to form actin thin filaments (Fig. 8) (Qualmann and Kessels 2009). Reports have suggested that the process of actin polymerization and depolymerization relates with the continuous cycle of contraction and relaxation in SMC. By using actin fractionation assays and phalloidin staining, earlier publications have suggested that contraction stimulation on SMC induces actin polymerization, hence increases the amount of F-actin. Conversely, actin filaments are reduced when relaxation occurs (Hirshman, Zhu et al. 2005, Chen, Pavlish et al. 2006).

Our functional data suggest that loss of LMOD1 decreases of the number of actin filaments in hISMC (Fig. 6A-B) and in primary mouse jejunum (Fig. 7A-B). These indicate that LMOD1 is indeed an important factor that regulate the actin polymerization rates in hISMC. Based on this findings and our current knowledge of the importance of actin filament formation in contractility, the decreased actin filaments in LMOD1-deficient cells may well be the cause of the impaired cellular contractility in hISMC (Fig. 6C) and in

primary mouse jejunum (Fig. 7C-D).

Our myography data shows a significant decrease in contractile force in mouse intestine lacking LMOD1. Interestingly, we noted a distinct lack of structural integrity in mouse tissues during these studies; the intestinal segments were more delicate to the touch in KO tissues during dissection, requiring extreme care to handle. Perhaps most striking was the inability of the KO jejunum segments to maintain passive tension during the initial stages of myography. Whereas control tissues exhibited tensile strength and maintained passive tension in response to dilation, KO tissues were unable to do so. Despite this, KO tissues were able to undergo repeated cycles of contraction and relaxation, suggesting that the distension/dilation observed in stomach, duodenum, and urinary bladder results from some combination of defective contraction and decreased structural integrity.

In addition to the data we collected on the involvement of LMOD1 in MMIHS, indirect evidence on its involvement comes from another mouse model lacking serum response factor (SRF), a regulator of LMOD1 transcription. In both cell and mouse model, knockdown of SRF result in reduction of LMOD1 expression (Nanda and Miano 2012). As expected, smooth muscle-specific inactivation of SRF in mouse models lead to severe dilation of the gastrointestinal tract and urinary bladder (Angstenberger, Wegener et al. 2007, Mericskay, Blanc et al. 2007). Reduction of SRF also showed a decrease in the number of actin filaments formed in colon SMC from mutant mouse (Angstenberger, Wegener et al. 2007). Altogether, these findings support our hypothesis about LMOD1 as the disease-causing-gene of MMIHS.

LMOD1 appears to interact with tropomyosins (Kostyukova 2007), however, mice null for the major SMC tropomyosin isoform (*Tpm2*) are embryonic lethal thus precluding analyses in postnatal visceral SMC functions (EN: 6550). It will be interesting to assess whether SMC-specific KO of *Tpm2* has an MMIHS-like phenotype. Additionally, several other null phenotypes, among proteins that presumably are all tied to contractile activity, have been described. Chronic intestinal pseudo-obstruction with defective intestinal SMC contraction is seen in mice lacking Smoothelin A (EN: 6546) (Niessen, Rensen et al. 2005). These results should be contrasted with the relatively normal GI/bladder phenotypes observed in mice lacking other SMC cyto-contractile genes such as *Cnn1* (EN: 2683), *Telokin* (EN: 4318), *Acta2* (EN: 3018), *Cald1* (EN: 6548), *Csrp1* (EN: 6549), and *Tagln* (EN: 3131). The reason for such different null phenotypes among proteins that presumably are all tied to contractile activity is unclear.

Mutations in two other genes, *ACTG2* and *MYH11*, have been identified previously (Thorson, Diaz-Horta et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014, Gauthier, Ouled Amar Bencheikh et al. 2015, Halim, Hofstra et al. 2016). *In vitro* assays showed that disease-causing-variants in *ACTG2* impair actin polymerization (Halim, Hofstra et al. 2016). The *Actg2* null mouse, which based on human data would have a MMIHS-like phenotype, has yet to be generated. Meanwhile, knockout of *Myh11* in mouse causes the inability of SMC to maintain the initial high-force phase of muscle contraction which

precipitates hypocontractility of the bladder and the intestine (EN:2957) (Morano, Chai et al. 2000).

We conclude that this discovery adds *LMOD1* to the list consisting *ACTG2* and *MYH11*, as MMIHS disease-causing-genes. This shows that MMIHS is a heterogeneous disease of the smooth muscle of visceral organs, with multiple patterns of inheritance.

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Supplemental table 1

Primer	Sequence
Sequencing forward mutation in exon 2 Human *LMOD1*	GGAGGTGGCCAAGAAAGAGG
Sequencing reverse mutation in exon 2 Human *LMOD1*	ATCATGAAGCCAGGGTCTCC
qRT-PCR forward human *LMOD1*	GAGGCCATGCTCAACTTCTG
qRT-PCR reverse human *LMOD1*	CTCTCCATTCTTGGCATCTG
qRT-PCR forward human *COPS5*	CCAGGAACCATTTGTAGCAG
qRT-PCR reverse human *COPS5*	GTAGCCCTTTGGGTATGTCC
qRT-PCR forward human *CLK2*	TCGTTAGCACCTTAGGAGAGG
qRT-PCR reverse human *CLK2*	TGATCTTCAGGGCAACTCG
PCR forward deletion in exon 1 mouse *Lmod1*	GCCCAAAGAGCTGCAGTGC
PCR reverse deletion in exon 1 mouse *Lmod1*	CTCACATCCACAGACATCTCTCTC
qRT-PCR forward Exon 1 mouse *Lmod1*	TGTGGATGAAAGCAAGCAAGTG
qRT-PCR reverse Exon 2 mouse *Lmod1*	AATACCTCTGATGACCTTCTCCTC
qRT-PCR forward Exon 2 mouse *Lmod1*	CTGCCATCCGTTCTAGCAAC
qRT-PCR reverse Exon 3 mouse *Lmod1*	CAAGAGTCTGGGCAGTCATG
qRT-PCR forward mouse *Gusb*	CATCAGAAGCCGATTATCCAGAG
qRT-PCR reverse mouse *Gusb*	TGTTTCCGATTACTCTCAGCG

Supplemental table 2

Guide RNA	Sequence
gRNA1	CCAAAGTAGCTAAGTACCGG
gRNA2	GGTCTTCACTCACCTGCCGC
gRNA3	TCCCGGTTGAATGAGCCCGA

Supplemental table 3

gRNA	Chromosome Coordinates	Strand	Primer Orientation	Primer Sequence	Possible Mismatch	Mismatch Profile	Nearest Gene
1	chr2:-168304560	-	Forward	TTTCTTCTTCCAACAGCGTTTC	CTACAGTGGCTAAGTACCCGTGG	2:4:8:19	*Nfatc2*
			Reverse	GGTCCTGACCACAAAGTCATA			
1	chr2:+119860458	+	Forward	CAAACCTGCCCAGTCTACAT	TCAGAGTAGTTAAGTACCAGTGG	1:4:10:19	*Pla2g4b, Jmjd7*
			Reverse	CTTCCTTCTCCCAGACACTTC			
1	chr2:+155459613	+	Forward	TGGTGACAGGAAGAGTGGA	CCAACCTGGCTAAGTACCGCAAG	5:6:8:20	*Myh7b*
			Reverse	CACTCCTTGTGCTAAGAGATGAA			
1	chr3:+81759039	+	Forward	CTTCTTGCCTTCTTCCCTCTG	CCAAAGTAACTCAGTACCGTTGG	9:12:20	*Ctso*
			Reverse	CCGTTTCTTTACCCTCCATGAT			
1	chr11:-69611753	-	Forward	GGTTCTCTAAGGGTGAGTGTAAG	CCACAGTGGCCATGTACCGGGAG	4:8:11:13	*Fgf11*
			Reverse	GCTCAGGGCATGTGTGT			
1	chr7:+36220567	+	Forward	TGGTCCTTCTGTCTCCTTAGAT	GCAAAGCAGCTCAGTACCAGAAG	1:7:12:19	
			Reverse	GCTCCCGCACTTACCTTT			
1	chr5:+44105813	+	Forward	TACTGGCTATGCACCAAAGG	CCACAGTAGCCAAGTACCTTCAG	4:11:19:20	*Cc2d2a*
			Reverse	CCATCTTACAATCGTGGCTCT			
1	chr10:-94052876	-	Forward	CCAGGCAATTAGGTCTGGATT	CCAAAGTAACTAATTATTGGGAG	9:14:17:18	*Tmc33*
			Reverse	CTTGTGTTGTCTGTTGGTGTTC			
1	chr6:+32136129	+	Forward	GGGAAGCAAGCCAGTAAAGA	GCAAAGTAGTTAAGTACCTGCAG	1:10:19	
			Reverse	CTGCATCACCATCAGGAAAGA			
1	chr9:+40970879	+	Forward	ATAGAACGTGGTGTGGAAGTG	TCAAAATAGCTAGGTACCGGAGG	1:06:13	
			Reverse	ACTTCCACACTAAACTGGGTGT			
2	chr15:+60655990	+	Forward	GGGAGGGAAGGAAATAGAAACT	GGACTAGACTCACCTGCCGCAAG	3:06:07	*Fam84b*
			Reverse	GCGAACTCGCCCTAGAAG			
2	chr12:+85262753	+	Forward	TACCTTCCCAGCTCCAACT	CGCCCGCACTCACCTGCCGCTGG	1:3:5:6	*Numb*
			Reverse	GGACAGGAAGAGTCAACCAATC			
2	chr6:+85290322	+	Forward	CCAAATAGAGGAGACCCTTACC	GGTGGACGCTCACCTGCCGCTGG	4:5:6:8	*Rab11fip5*
			Reverse	GGCCCTTGACCTCTTCTTC			
2	chr8:-123093143	-	Forward	AGGAGGACAGATAGACAGACTT	GGTCTTCCTTCTCCTGCCGCAGG	8:09:12	
			Reverse	GTGCCCAGCCTCATCTC			
2	chr1:-174475299	-	Forward	GCTCTTCCAGCTTCTGTTTCT	GGTTCTCACGCACCTGCCGTCGG	4:5:10:20	*Ccdc19*
			Reverse	TGTGGACCTTGAAGCATGAC			
2	chr11:+54864689	+	Forward	CTTTCTGACTCCCAAGGAAAGA	GGTCCTCAACCTCCTGCCGCAGG	5:9:10:12	*Ccdc69*
			Reverse	ACTGTGCCAAGAGTGAGTG			
2	chr5:-121812464	-	Forward	CCTACCTCACCTTGTTCTTGTT	GCTCTTTACACACCTGCCACAGG	2:7:10:19	*Gm15800, mKIAA0614*

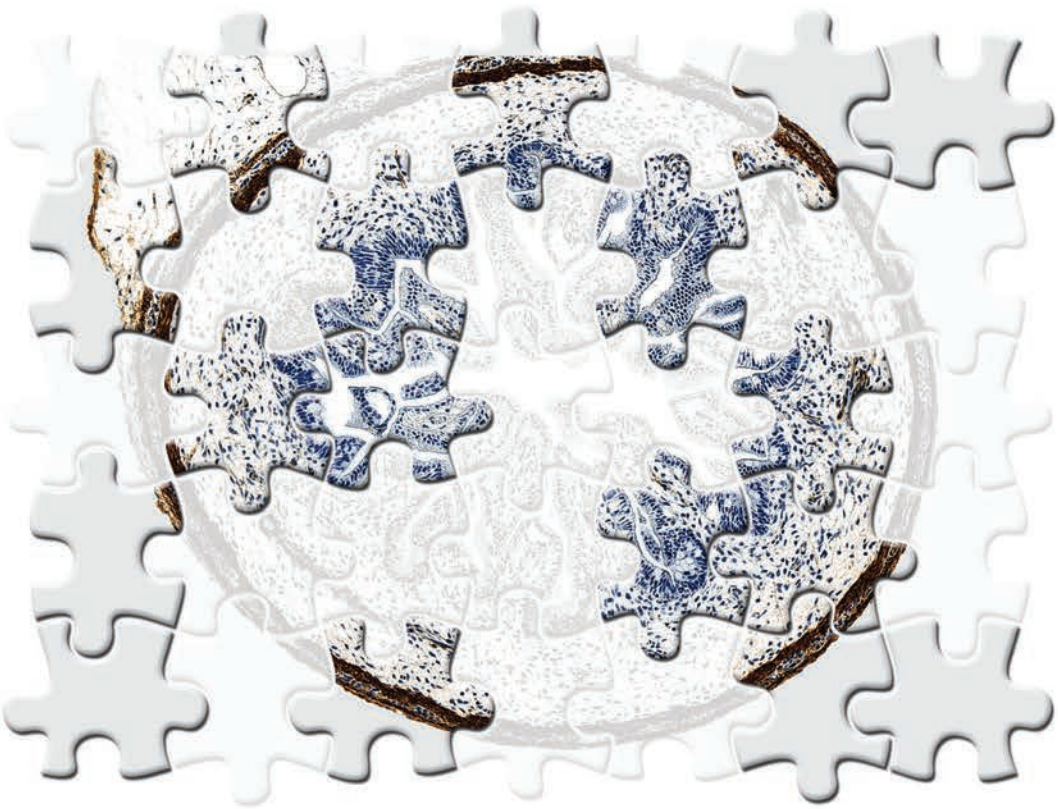
			Reverse	AGCTAGGGTCAGCATCTCA			
2	chr19:-46384378	-	Forward	CAGGCATGTGCAATATTTGGG	GGTCTGCACCCACCTGCAGCAGG	6:10:18	*Nfkb2*
			Reverse	GCAGATAGCCCACGTCATTTA			
2	chr15:-83161031	-	Forward	AGAGAGGGAGAGAGAGAGAGA	TGGCTTCTCTCACCTGCTGCAGG	1:3:8:18	*Arfgap3*
			Reverse	CAGGAGACCACAGGACATTG			
2	chr2:-167433844	-	Forward	GGTGAGGAATTCAGGGCTTC	GGACCACACTCACCTGCCACCAG	3:5:6:19	*Ube2v1, Ube2v2*
			Reverse	GGCCTTTCTCTAGGCATCTTT			
3	chr6:-50334908	-	Forward	GCTTCACCTAAGGGACTTGTAG	TCCTGGTAGAATGAGCCTGATGG	4:08:18	*Osbp13*
			Reverse	AGGTCTTACTGACTCCCACATA			
3	chr2:+120960142	+	Forward	GGCTGTCCTAGAACTCACTTTG	TCCTTGTGGAATGAGCTCGAGAG	4:5:8:17	*Lcmt2*
			Reverse	GAGCGTCCTTAGGGAGATAGAT			
3	chr18:+16761798	+	Forward	AGAGATGGTCTGAGCAAAGATAAG	TCACAGTTGACAGAGCCCGAGGG	3:5:11:12	*Cdh2*
			Reverse	CAGAGGGAGTAATGTTGGGATAAG			
3	chr19:+15937358	+	Forward	GAGGTCTGTGTGCAAAGGT	TCTCTGATGAATGAGCCCCACAG	3:5:7:19	
			Reverse	GCTTCGGTTACTGCTGAGTT			
3	chr3:-60477413	-	Forward	CACACTGATGCCCAGGATAAA	TCCAAGTGGAATGAGCCAGAAAG	4:5:8:18	
			Reverse	ACAGAGAAGTTACATGCAGAGAAG			
3	chr9:-57442270	-	Forward	TTGATCTGCTCCTTCAGGTATTC	TCCCTGTTGTAGGAGCCCGGCAG	5:10:12:20	*Ulk3*
			Reverse	CTCAGGTCTGTGGGACATAAATAG			
3	chr2:-148578529	-	Forward	CATATAGTGGAGAGAAGCTCTTGG	TCCTGGTTGAATGATCCCCATAG	4:15:19	*Cst11*
			Reverse	AGTGGTTGGCACAGTTCTAC			
3	chr2:-147438139	-	Forward	TCAACAGAGCACAGCAGAC	TCCTGGAAGAATGAGCCAGACAG	4:7:8:18	
			Reverse	TTATGACAGCTTGAACCCTACC			
3	chr13:-52994868	-	Forward	CCGGTTTCTCAGTGTCCTTT	TCTCGGTGGCATGTGCCCGAGAG	3:8:10:14	*Auh*
			Reverse	GGGTCATTTCGCTCTGAGTT			
3	chr16:-10681231	-	Forward	GGTGTGAAGACAGCGACTAT	TCCTTGTTGAATTAGCCCCAAGG	4:5:13:19	*Clec16a, mKIAA0350*
			Reverse	GAGAGCAGTGGGTTGAGAAT			

Supplemental table 4

Gene	Coding Effect	Zygosity	Read depth	Type	Location	Exon	HGVS cDNA-level
FNDC1	missense	heterozygous	24	snp	exonic	14	NM_032532.2: c.4436C>G
ITGA8	missense	heterozygous	34	snp	exonic	2	NM_003638.1: c.245C>T
LMOD1	stopgain	homozygous	65	snp	exonic	2	NM_012134.2: c.1108C>T
NEB	missense	heterozygous	20	snp	exonic	125	NM_001271208.1: c.19396C>T
PPP1R13L	missense	heterozygous	22	snp	exonic	6	NM_001142502.1: c.823T>G
RYR1	missense	heterozygous	26	snp	exonic	21	NM_000540.2: c.2654G>A
SMTN	missense	heterozygous	65	snp	exonic	9	NM_001207018.1: c.1504G>A
SMTN	missense	heterozygous	54	snp	exonic	9	NM_001207018.1: c.1558G>A
SMTN	missense	heterozygous	76	snp	exonic	10	NM_001207018.1: c.1762C>G
TCF3	missense	heterozygous	60	snp	exonic	18	NM_003200.3: c.1600G>A

CHAPTER 4

Loss-of-function Mutations in Myosin Light Chain Kinase Cause Recessive Megacystis Microcolon Intestinal Hypoperistalsis Syndrome



Danny Halim, Erwin Brosens, Françoise Muller, Michael F. Wangler, Arthur Beaudet, Michael Doukas, Hans J. Stoop, Bianca de Graaf, Rutger W.W. Brouwer, Wilfred F.J. van IJcken, Raj Kapur, Jean-François Oury, Jonathan Rosenblatt, Alice S. Brooks, Alan J. Burns, Dick Tibboel, Robert M.W. Hofstra, Maria M. Alves[#]

Manuscript in preparation

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ABSTRACT

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS) is a congenital disease characterized by the loss of smooth muscle contraction in the bladder and the intestine. Two genes: *ACTG2* and *MYH11*, have been identified as the disease-causing genes of MMIHS. Nonetheless, the genetic cause of this disease in some patients remains unknown. By using homozygosity mapping and whole exome sequencing, we determined the deleterious variation shared between three MMIHS patients from two different consanguineous families. Splicing assay and immunohistochemistry were used to confirm the pathogenicity of the identified variants. We identified homozygous variants in the Myosin light chain kinase (*MYLK*) gene in both families. A duplication of seven base pairs was found in one family, while s splice-site variant was identified in the other family. Expression studies indicated that these variants severely reduced *MYLK* expression. Histopathology analyses in one patient excluded other significant changes in the structure and cellular contents of the intestine and bladder. Our results show that *MYLK* is a new disease-causing gene of recessive MMIHS, confirming MMIHS is a heterogeneous disease of the visceral organs, with multiple patterns of inheritance.

INTRODUCTION

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS [OMIM 249210]) is a congenital disease of the visceral organs, characterized by severe hypocontractility of the urinary bladder and the intestine (Berdon, Baker et al. 1976, Wymer, Anderson et al. 2016). Total parenteral nutrition and multivisceral transplantation are the only currently available treatments for MMIHS patients (Yokoyama, Fujimoto et al. 1989, Masetti, Rodriguez et al. 1999, Raofi, Beatty et al. 2008, Lopez-Munoz, Hernandez-Zarco et al. 2013). Although long term survival has been documented, most patients with MMIHS do not survive through the early years of their life (Hirakawa, Ueno et al. 2009, Lopez-Munoz, Hernandez-Zarco et al. 2013, Halim, Hofstra et al. 2016).

Initial studies suspected that defects in either muscles, neurons or interstitial cells of Cajal are the underlying cause of MMIHS (al-Rayess and Ambler 1992, Rolle, O'Briain et al. 2002, Piotrowska, Rolle et al. 2003, Piaseczna Piotrowska, Rolle et al. 2004, Szigeti, Chumpitazi et al. 2010). However, recent findings have reported mutations in three muscle-related, including enteric smooth muscle actin γ2 (*ACTG2*), myosin heavy chain-11 (*MYH11*) and Leiomodin-1 (*LMOD1*), as the genetic etiologies of this lethal syndrome (Wangler and Beaudet 1993, Thorson, Diaz-Horta et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014, Gauthier, Ouled Amar Bencheikh et al. 2015, Tuzovic, Tang et al. 2015, Halim, Hofstra et al. 2016). De novo mutations in *ACTG2* lead to the autosomal dominant form of MMIHS, while mutations in *LMOD1* and *MYH11* cause recessive forms of MMIHS. Altogether, these discoveries have led to the hypothesis that MMIHS is caused by disruption of actin-myosin interactions in smooth muscle cells (SMC) of the visceral organs.

Despite these findings, the genetic etiology in some patients with MMIHS remained unknown, leading us to hypothesize that another locus might be responsible. Here, we describe genetic studies in three MMIHS patients from two consanguineous families, for which no variants in two known MMIHS genes was identified.

Considering that consanguinity is known to increase regions of shared haplotypes, identical by descent (IBD) regions, we used a combined strategy of homozygosity mapping and WES to identify deleterious variations in these consanguineous patients. Two different loss of function variants in the gene encoding myosin light chain kinase (MYLK) (OMIM 600922) were found in these families (Bittles 2001); (Lander and Botstein 1987).

MATERIALS AND METHODS

Patient inclusion

We included three patients suspected of having a recessive form of MMIHS. From family I, two patients were analyzed, whereas from family II, one patient was examined. Family anamnesis suggested that the parents were consanguineous. DNA of all patients was isolated from peripheral blood lymphocytes. Paraffin-embedded intestinal and bladder specimens were obtained from patient 1. This study was approved by the Erasmus Medical

Center ethical committee (Medisch Ethische Toetsings commissie - METc 2011/148, ABR form: NL35920.042.11). Written informed consents were granted by the families.

SNP-array analysis

250 ng of DNA from patient 1 was processed according to standard protocols and hybridized to the CytoSNP-850K v0 array (Illumina, Inc., San Diego, USA). The normalized output generated with Illumina's Genome Studio program version 2011.1 (Illumina, San Diego, CA, USA) was visualised with Nexus CN8.0 (Biodiscovery Inc, El Segundo, CA, USA) using analysis settings and Copy Number Variation (CNV) prioritization methods as previously described (Brosens, Marsch et al. 2016). The genotype information of the SNP-array was used to confirm family relations (patient 1 and her parents) and determine Runs of Homozygosity, the consanguinity coefficient (f), and the inbreeding coefficient (F).

Homozygosity mapping

The genotype information collected from the SNP-arrays was used to identify ROH possibly harbouring homozygous recessive variants (Kearney, Kearney et al. 2011). As ROH can vary in length between populations and in different individuals of the same population, strict criteria have to be used to increase the likelihood of detecting a recessive variant in these regions. A suggested criterium is at least 50 SNPs (minor allele frequency of at least 5%) in a 1 cM region (Auton, Bryc et al. 2009), roughly 500kb-2000kb (Yu, Zhao et al. 2001). Here we use a 50 SNP minimum and at least a 1Mb ROH length.

Whole Exome Sequencing

Fragmentation of DNA (Covaris, Inc. Woburn, Massachusetts, USA), Target capture (SureSelect Human All Exon 50 Mb Targeted exome enrichment kit v4, Agilent Technologies, Inc., Santa Clara, California), library preparation (paired-end, 101 bp, TruSeq version 4 protocol, Illumina, Inc., San Diego, USA) and processing of raw sequence data using the NARWHAL pipeline was done as described before (Brouwer, van den Hout et al. 2012). Reads were aligned to the hg19 reference genome using Burrows-Wheeler Aligner version 0.6.2. Variants were determined using the Bayesian genotyper that is incorporated in the genome analysis toolkit version 1.2.9. The results were then uploaded to the Cartagenia Bench NGS version 4.1.7 (Cartagenia Inc, Boston, MA, USA) for filtering. Copy Number Variants (CNV) and ROH regions were transferred from Biodiscovery Nexus CN7.5. into Cartagenia Bench CNV and NGS in order to determine the overlap between the identified ROH regions and rare variants.

High frequency in our in-house control cohort (n=280, with similar capture, alignment and variant calling procedures and n=500 additional in-house controls) were ruled out. Furthermore, variants with an allele frequency above 0.1% in public databases (ExAC release 0.3, ESP6500SI-V2; 1000 Genomes Phase 3 release v5.20130502 and GoNL SNPs and Indels release 5) were excluded. Only variants predicted to affect splicing,

nonsense variants, and coding and non-coding variants with a CADD score above 20 (CADD v1.3) were retained.

Sanger sequencing

Sanger sequencing was used to validate the presence of the *MYLK* variants identified. For that, 15 ng of genomic DNA was used to replicate each amplicon using standard PCR methods, and the primers set listed in Supplemental table 4. PCR products were purified using ExoSap it (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and Sanger sequencing was conducted using dye labelled primers (forward and reverse; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems, Waltham, MA, USA) on the ABI 3130XL genetic analyser. Sequencing reads were performed by using SeqScape® v2.5 software (Thermo Fisher Scientific, Waltham, MA, USA).

Cell culture and transfection

Human embryonic kidney cells (HEK293) cells were cultured in DMEM (Lonza, Basel, Switzerland), complemented with 10% FCS (Sigma-Aldrich, St. Louis, Missouri, USA) and 1% Penicillin-Streptomycin (Gibco, Waltham, MA, USA). Cells were incubated at 37˚C and 5% CO_2. For transient transfection, 300,000 cells were plated in a 6-well-plate (Nunc, Rochester, NY, USA). Twenty-four hours after, cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) as a transfection reagent according to the manufacturer's instructions.

Generation of splicing constructs

Wild-type (WT) and mutant constructs were generated using 15 ng of genomic DNA from control and patient, as template, respectively. Primer sets were designed to include the genomic sequence that stretch from the last part of intron 21 of *MYLK* to the beginning of intron 22 (Supplemental table 4). After confirmation of successful amplification by gel electrophoresis, PCR products were subjected to enzyme restriction with NotI and PstI (New England Biolabs, MA, USA), ligated into pSPL3 using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA), and transformed into *E.coli* One Shot TOP10 competent cells (Thermo Fisher Scientific, Waltham, MA, USA). Constructs were isolated with a MIDI prep kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. All constructs were Sanger sequenced to confirm successful ligation and transformation.

Splicing assays

48 hours after transfection of HEK293 cells with splicing constructs, cells were collected and used to isolate mRNA using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Reverse transcriptase (RT)-PCR was performed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). To evaluate the produced transcripts from WT, mutant, empty vector and nontransfected cells, forward

SD6 and reverse SA2 primers (Supplemental table 4) were used in a standard PCR protocol. PCR products were analysed by electrophoresis on a 3% agarose gel, and visualised using ChemiDoc XR+ System (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Formalin-fixed paraffin-embedded human small intestinal and bladder tissues obtained from controls were retrieved from the Biobank of the Pathology Department, Erasmus University Medical Center. Intestinal and bladder specimens from patient 1 were obtained from the Department of Pathology of the Hôpital Universitaire Robert Debré. Specific antibodies directed against MYLK (1:100; Thermo Fisher Scientific, Waltham, MA, USA), Neurofilament (1:600; Monosan, Uden, The Netherlands), Smooth muscle actin α2 (ACTA2) (Ready to use; Dako, Glostrup, Denmark), Tyrosine protein kinase kit (c-Kit/CD117) (1:200; Cell Marque, Rocklin, CA, USA), and Tryptase (1:1600; Dako, Glostrup, Denmark) were applied. The biotinylated multilink secondary antibody and the avidin-biotin-complex with peroxidase were then used, followed by application of 3,3'-diaminobenzidine (DAB) chromogen (Ventana, Tucson, AZ, USA). All slides were counterstained with hematoxylin. Images were generated with a Nanozoomer 2.0-HT, and analyzed with the Nanozoomer Digital Pathology (NDP) viewer software (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan).

RESULTS

Clinical features of the patients

Three patients derived from two different consanguineous families. Patient 1 was the second child to consanguineous parents of North African origin. Prenatal 13-week ultrasound acknowledged the presence of distended bladder, and a generalized subcutaneous edema (Fig. 1A). Hence, parents opted for termination of pregnancy at 15-weeks of gestation. Autopsy of the fetus confirmed the diagnosis of MMIHS. Patient 2 was the younger brother of patient 1.

Prenatal 24-week ultrasound detected the distended bladder, with hydronephrosis and severe oligohydramnios (Fig. 1B). The parents opted not to terminate the pregnancy, and labor was prematurely occured at 31-weeks of gestation. The neonate experienced respiratory distress, and succumbed. Further anamnesis on the family history revealed that the older sister of patient 1 was also acknowledged of having distended bladder prior to her intra uterine death at 30-weeks of gestation.

Patient 3 was the first child of a couple of Indian origin. Antenatal period was complicated with polyhydramnios. Nevertheless, the baby was born at term by normal delivery, without any neonatal complications. At 2-days-old, she was admitted back to the hospital due to bilious vomiting. Barium enema testing confirmed intestinal obstruction, and suspected intestinal malrotation as the cause (Fig. 1C-E). Surgery at 3-day-old released the small intestinal congenital bands, and corrected the intestinal malrotation

with ileostomy. The surgery also revealed the unused distal microcolon. Additionally, the bladder was catheterized. Histopathology analysis of the mid-ileum identified the presence of ganglia. Since the ileostomy remained non-functional until 13 days after the initial surgery, second surgery was performed. Adhesions between the intestinal loops were revealed and released. The first ileostomy was removed, and a new double barrel ileostomy was made.

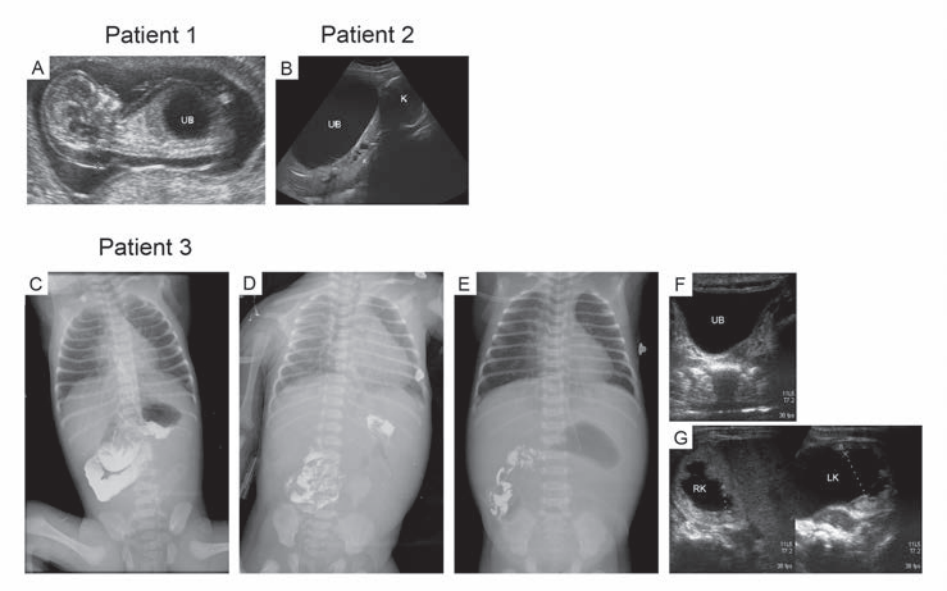

Fig.1: Barium enema and ultrasonography examinations in the three MMIHS patients. (A) Prenatal ultrasonography on patient 2 at 13 weeks revealed distended bladder and generalized subcutaneous edema. **(B)** Prenatal ultrasonography on patient 3 at 24 weeks revealed distended bladder, hydronephrosis and severe oligohydramnios.**(C-E)** Barium enema testing suggested intestinal obstruction and malrotation. **(F and G)** Ultrasonography on patient 1 at neonatal revealed dilatation of the bladder and bilateral hydronephrosis. Legends: K: kidney, UB: urinary bladder.

Despite all efforts, the new ileostomy was never functional, and the patient experienced sepsis. Abdominal ultrasound revealed distension of the bladder and bilateral hydroureteronephrosis (Fig. 1F-G), and the diagnosis of MMIHS was made. The parents were made aware of the poor prognosis, and they opted to have the patient discharged from the hospital. Further follow-up on the family acknowledged that the younger sister of patient 1 was also diagnosed with MMIHS.

Homozygosity mapping confirms consanguinity in family I

Homozygosity mapping has been used to successfully determine causal genes in suspected autosomal recessive disorders both in inbred and outbred populations. Considering this, we performed homozygosity mapping in Family I as a starting point of our genetic analysis. Familial relations were confirmed and, as suggested by the anamnesis, the genotype

information indicated consanguinity (f of 0.25 and F of 0.125 with 372 Mb of ROH). Fifty-five homozygous regions were identified in patient 1 of at least one Mb in length and harboring at least 50 probes. Of these 55 regions, 38 were present in the parents in a heterozygous state (Supplemental table 1). No deleterious CNVs were identified in patient 2 (Supplemental table 2).

Whole Exome Sequencing identified deleterious variants in *MYLK* in patient 1 and 3

As consanguinity increases the risk for autosomal recessive disorders (Hamamy, Antonarakis et al. 2011, Hamamy 2012), we screened patient 1 for deleterious recessive mutations using WES. Segregation of identified variants was investigated in her affected brother, patient 2, and unaffected sister. Out of 1075 rare variants found (MAF below 0.1%), 98 were located in one of the identified 38 ROH regions.

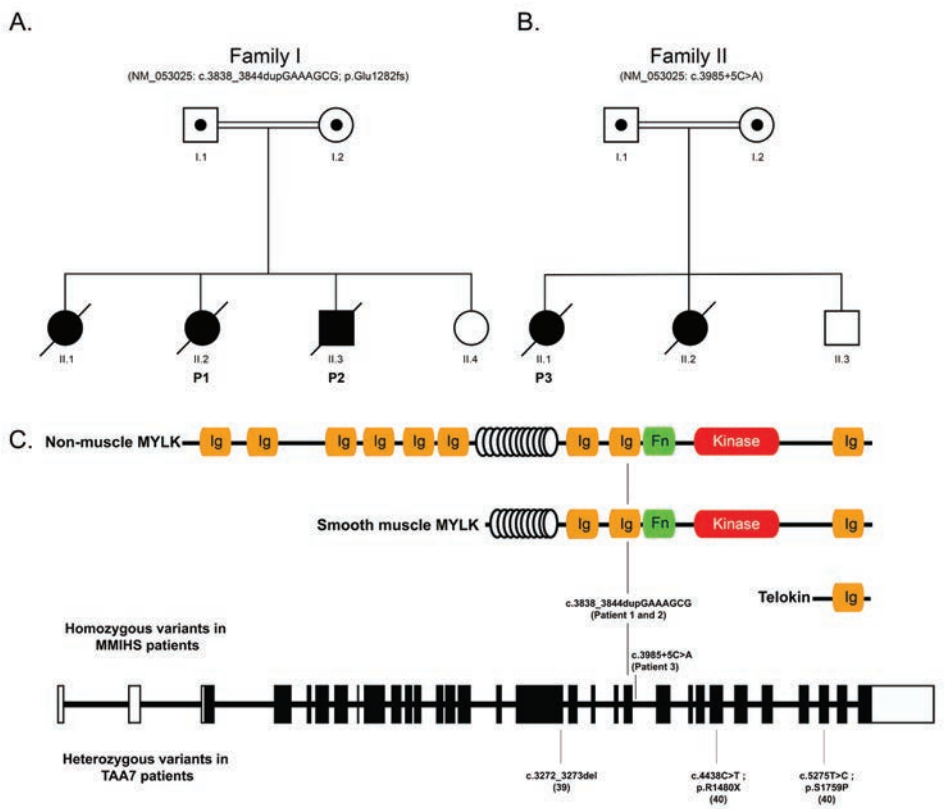

Fig.2: Identification of the homozygous mutations in *MYLK* in MMIHS patients. (A) Out-of-frame mutation is identified in patient 2 and patient 3, while the presence of this variant in a heterozygous state in their parents confirmed the recessive pattern of inheritance. **(B)** Splice-site mutation is identified in patient 3. (C) An overview of MYLK with its 33 exons and its three major isoforms, with all the identified variants in MMIHS and TAA7 patients.

51 of these variants were inherited recessively. To screen for known recessive disease gene involvement, we uploaded the 38 ROH regions, present only in the patient and not in the parents, in the genomic oligoarray and SNP-array evaluation tool v3.0 (Wierenga, Jiang et al. 2013) and screened for recessive candidate syndrome genes using Human Phenotype Ontology terms “abnormality of the gastrointestinal tract” and “abnormality of the genitourinary system”. One-hundred genes were prioritized, none of which had rare recessive deleterious variants in patient 1. Eleven out of 51 recessive variants were predicted to be deleterious: two variants were predicted to affect splicing, two frameshift mutations and 7 missense variants (see supplemental table 3). Of these, one affected gene was involved in the biological process known to be affected in MMIHS: SMC contraction. This variant, a frameshift insertion in exon 23 of the *MYLK* gene (NM_053025.3: c.3838_3844dupGAAAGCG) was present in a 2,898,353 bp ROH region (hg19, chr3:120,385,537-124,830,052). Sanger sequencing confirmed this homozygous variation in the affected patients 1 and 2 and absence in their unaffected sister, while heterozygous in their parents (Fig. 2A). Sanger sequencing of the healthy sibling showed no mutant allele was inherited. This genetic finding was submitted to ClinVAr (http://www.ncbi.nlm.nih.gov/clinvar/) and was given the following accesion number: SCV000299345.

Patient 3 was also submitted to WES and data analysis was performed as described before (Wangler, Gonzaga-Jauregui et al. 2014). We identified splice variant in intron 22-23 of *MYLK* as the only homozygous rare variant in this patient (NM_053025: c.3985+5C>A).

MYLK is expressed in all SMC throughout the developmental stages of the human intestine and bladder

As we hypothesized that rare variants in *MYLK* cause MMIHS we assume that *MYLK* is physiologically expressed during the development of the intestine. Hence, loss of *MYLK* expression during embryogenesis might hamper normal development of these organs. By using a specific antibody against MYLK, we performed immunostainings on human intestinal and bladder specimens collected at different developmental stages. We show that MYLK is strongly expressed in all SMC that form all muscular structures of the intestine and bladder, across all the developmental stages included in this study (Fig. 3A-F).

Homozygous out-of-frame variant in patient 1 severely reduces _MYLK_ expression in the intestine and bladder

To assess the effect of the out-of-frame variant in the expression of *MYLK*, we immunostained the intestinal and bladder specimens collected from patient 1. Intestinal tissue collected from age-matched controls were used for comparisons.

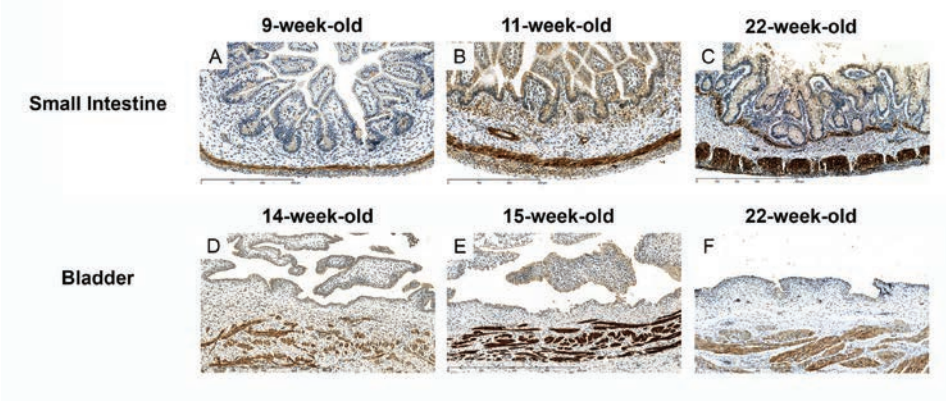

Fig.3: Expression of *MYLK* in human intestine and bladder. (A-F) *MYLK* is expressed in SMC that constitute muscular structures of the intestine and the bladder, including muscularis mucosa, blood vessels, circular and longitudinal muscles of the muscularis propria. Similar patterns and levels of expression were present throughout all developmental stages included in this study.

As expected, we observed that *MYLK* is strongly expressed in SMC of the intestine of controls. However, no expression was detected in the small intestine, rectum and bladder of the patient (Fig. 4A-E), confirming pathogenicity of the duplication found.

Homozygous splice variant in patient 3 severely impairs splicing of exon 22 of *MYLK*

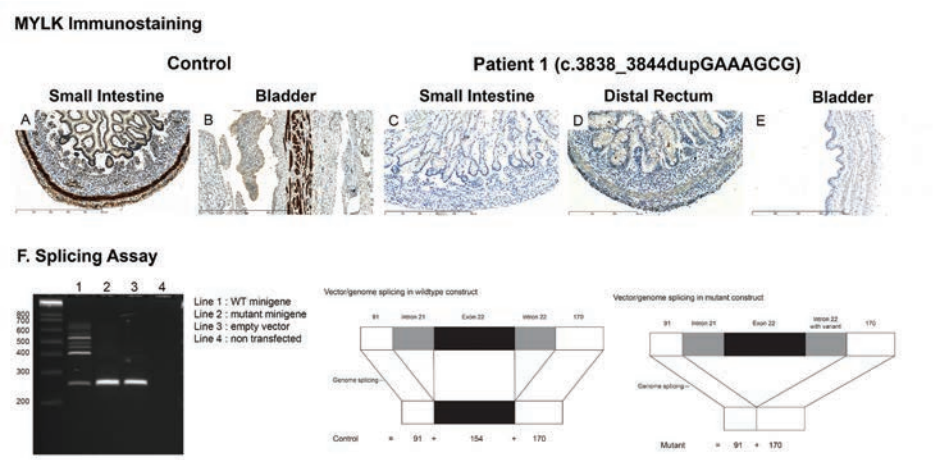

Fig.4: The identified mutations caused the loss of *MYLK* expression in patients with MMIHS (A-E) Immunohistochemistry results showed that the out-of-frame mutation in patient 2 eliminated *MYLK* expression in the intestine and the bladder. **(F)** Splicing assay revealed the diminished transcripts in mutant mini-gene consisting of the splice variant found in patient 1.

To test the effect of the presumed splice site variant found in patient 3 (NM_053025: c.3985+5C>A), we performed splicing assays. For that, HEK293 cells were transfected

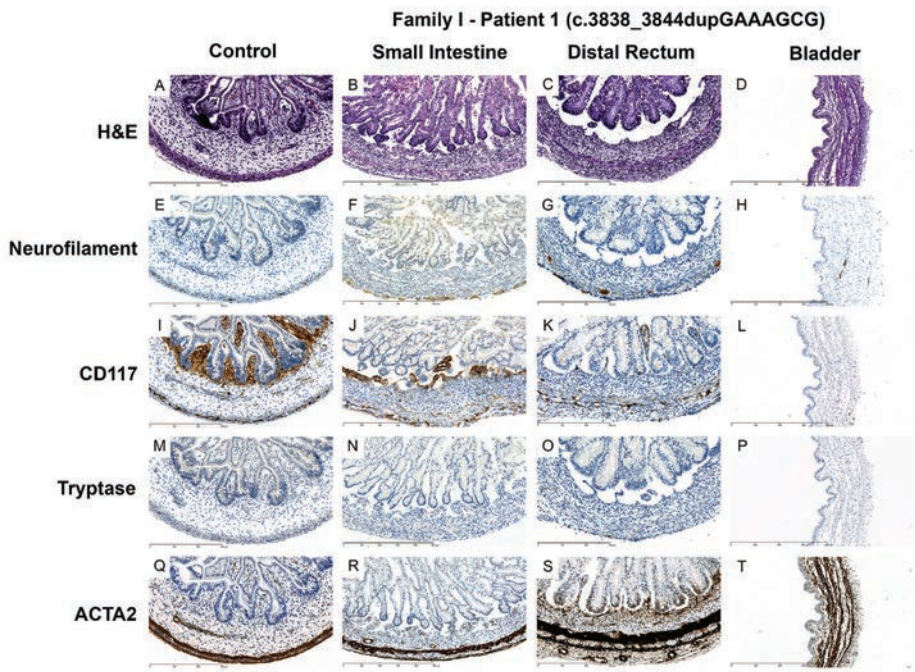

Fig.5: Immunohistochemistry of the intestine and bladder specimens from patient 1 showed no structural and cellular defects due to the out-of-frame mutation. (A-T) H&E staining and immunostainings using specific antibodies against ACTA2, Neurofilament, c-Kit/CD117, Tryptase and ACTA2 on control and patient specimens showed were not significantly different.

with one of the following splicing constructs generated: wild type, mutant, empty vector, and non-transfected. Non-transfected cells were also included as a control. The expected transcript of the wild type construct is 414 bp, while the empty vector should produce a transcript of 261 bp. Our results showed that the wild type construct produced the transcript with the expected size (414 bp). However, several other transcripts were also generated, suggesting that exon 22 is normally target of alternative splicing. Conversely, the mutant construct eliminated all the transcription products seen in the wild type situation, and produced only a band identical to the one showed by the empty vector. This result suggests that the identified variant leads to loss of a splice site in intron 22 of *MYLK*, and is likely to lead to the absence of exon 22 in the final expression product (Fig. 4F).

No structural abnormalities were found in the intestine and bladder of patient 1

To investigate the impact of the loss of *MYLK* expression on the histopathology of the intestine and bladder, we immunostained specimens collected from the small intestine, rectum and bladder of patient 1 using antibodies against Neurofilament, c-Kit/CD117,

Tryptase and ACTA2. Intestinal specimens obtained from the autopsy of age-matched controls was used for comparison. Our results did not detect any abnormal expressions of the investigated proteins when compared with controls (Fig. 5), suggesting that absence of MYLK does not significantly alter the structure of the intestine and bladder.

DISCUSSION

Along with neurons and interstitial cells of Cajal, SMC are crucial for the contractility of the intestine and the bladder. SMC contraction results from an elaborate molecular process, involving the cross-bridge interaction between thin actin filaments and thick myosin filaments. Therefore, loss of any of the proteins involved in this process is likely to affect cell physiology, resulting in loss of contractility. In this manuscript we report the identification of pathogenic variants in one of the genes involved in this process, *MYLK,* in three patients diagnosed with MMIHS, a congenital disease that is characterized by inability of the bladder and intestine to contract. MYLK is an important protein kinase that phosphorylates the regulatory light chain (RLC) of myosin II in SMC (Kamm and Stull 2001, Hong, Haldeman et al. 2011), leading to its activation. Therefore, it is not surprising that altered expression of this protein may cause loss of contractility in the bladder and intestine, as experienced by MMIHS patients.

In humans, three major MYLK isoforms exist, including the long MYLK isoform (~210 kD) referred to as non-muscle MYLK (nmMYLK), the short MYLK isoform (~130 kD) referred to as smooth muscle MYLK (smMYLK), and a very small isoform called telokin (~17 kD) (Hong, Haldeman et al. 2011).The nmMYLK and the smMYLK isoforms are composed by several important functional domains, including actin binding domains, a kinase and a regulatory domain. However, the nmMYLK has 922 additional amino acid residues at the N-terminal region that results in the presence of six additional immunoglobulin (Ig) modules and two actin binding motifs (Garcia, Lazar et al. 1997, Kamm and Stull 2001).

Until now, the functional role of the additional domains present in nmMYLK remains unclear. However, as implied by their names, smMYLK predominantly functions to phosphorylate RLC of smooth muscle myosin (SMM) in SMC, while nmMYLK is involved in both non-muscle and SMC contraction (Hong, Brizendine et al. 2015).Two different deleterious variants were identified in the two families included in this study, that based on their genomic location may affect both nmMYLK and smMYLK. In patient I.1 from family I, we identified a splice site variant that disrupts splicing of exon 22. In patient II.1 and II.2 from family II, we found an out-of-frame duplication in exon 22 that results in the absence of *MYLK* expression in the intestine and the bladder. Exon 22 encodes an immunoglobulin (Ig)-like domain that has been suggested to have an actin binding function. However, MYLK immunostainings on intestinal and bladder specimens from patient II.1 showed that the out-of-frame duplication in exon 22 abolishes expression of *MYLK* in SMC of the bladder and the intestine (Fig. 4A-E), indicating loss of the whole protein. Hence, we remain open to the possibility that the region encoded by exon 22 might have an important

role in the function of MYLK in visceral organs.

As showed by our results, MYLK is continuously expressed during the development of the intestine (Fig. 3A-F), but to our surprise, no abnormalities in the structure and cellular constituents of the intestine and bladder were evident with the loss of *MYLK* (Fig. 5A-T). This finding suggests that although MYLK plays an important role in the regulation of SMC contractility in visceral organs, it is not involved in the embryogenesis of these organs. These results also suggest that the acquired phenotypes found in MMIHS patients with *MYLK* variants, are solely due to the loss of SMC contractile ability, and not because of abnormalities in the structure of the visceral organs. Based on our current understanding, we suspect that loss of *MYLK* causes failure of phosphorylation of the RLC, leading to a reduced interaction between actin and myosin, and consequently to reduced contractility of SMC (Fig. 6).

This hypothesis is supported by the presence of severe gut dysmotility, accompanied with abnormal urinary bladder function in a conditional knockout mouse for the smMYLK isoform (He, Peng et al. 2008). The similarities between the phenotype presented by the mouse with the one showed by MMIHS patients, strongly support our conclusion that *MYLK* is a new disease-causing-gene for MMIHS.

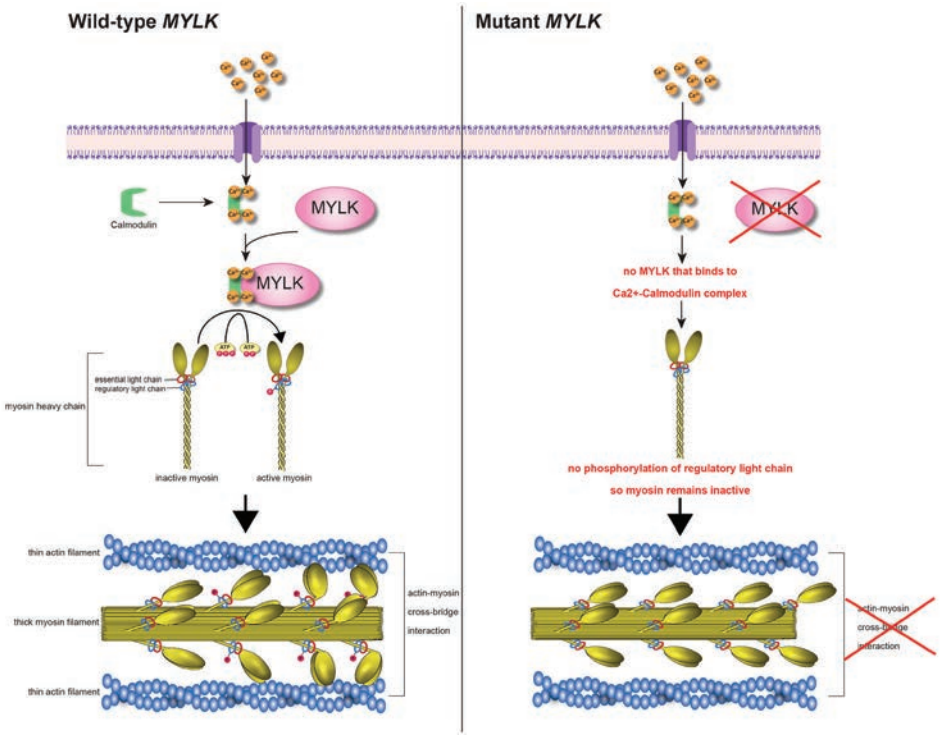

Fig.6: Schematic illustration of the role of MYLK in the molecular mechanism of SMC contraction, and the effect of *MYLK* variant on this mechanism. In wild-type, MYLK phosphorylates regulatory MLC, causing the interaction between actin and myosin filaments that leads to the SMC contraction. In mutants, no phosphorylation of regulatory MLC occurs, thus myosin cannot interact with actin filaments, and SMC contraction fails.

The answer to the question on how microcolon could develop in MMIHS patients remains unclear. Nonetheless, research on human fetal lung muscle contraction indicates that fetal lung smooth muscle contraction may provide an important physical force that plays a role in lung development (McCray 1993). Therefore, we speculate that gastrointestinal SMC contraction may provide an important physical stimulation for the development of colon, thus when its absent, microcolon develops.

Since SMC do not only constitute the muscular structures in the intestine and the bladder, but also blood vessel walls, we expected that the identified homozygous variants would cause extra-visceral phenotypes in patients. Interestingly, the phenotypes that were acquired in our patients are restricted to the intestine and the bladder. To our knowledge, two possibilities may cause such specific defects. The first possible explanation is the fact that since none of our patients survived, we were unable to assess extra-visceral phenotypes that may be visible at later stages in life. However, it could also be that other kinases salvage the contraction in non-muscle and SMC inhabiting extra-visceral organs (Goeckeler, Masaracchia et al. 2000, Totsukawa, Yamakita et al. 2000, Niiro and Ikebe 2001). Both hypothesis are supported by previous evidence. Heterozygous *MYLK* variants found in patients with familial thoracic aortic aneurysm 7 (TAA7 [OMIM 613780]) are in support of the first, since in these patients aneurysms often occur without being preceded by gradual dilatation of the aorta (Wang, Guo et al. 2010, Hannuksela, Stattin et al. 2016).This suggests that it takes time for the reduction of MYLK to cause its toll on major blood vessels. On the other hand, data collected from mouse models are in support of the latter possibility. Isolation of aortic cells from mice where the expression of all three MYLK isoforms was abolished resulted in induced RLC phosphorylation (Somlyo, Wang et al. 2004). In conclusion, our findings add *MYLK* to the list of existing MMIHS-causing genes, which already includes *ACTG2*, *MYH11* and *LMOD1*. Interestingly, all these four genes encode for products involved in the cross-bridge interactions between actin and myosin filaments necessary for SMC contraction. Alterations in the expression of *ACTG2* or *LMOD1* impair the formation of actin filaments, while loss of *MYH11* and *MYLK* are likely to impair formation of thick myosin filaments. Hence, it is tempting to state that MMIHS is caused by a defect in the actin-myosin interaction which eventually leads to failure of visceral smooth muscle contraction.

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Conflict of Interest: The authors have nothing to disclose.

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Supplemental table 1: “new” ROH regions in patient 2

Chromosome	ROH region	Size (kb)
1	chr1:242944438-244084838	1140400
1	chr1:145416055-147826789	2410734
1	chr1:150856155-165465569	14609414
1	chr1:70267535-120553638	50286103
2	chr2:193957725-194971610	1013885
2	chr2:228026937-231049859	3022922
2	chr2:132604119-139882966	7278847
2	chr2:231059532-240330999	9271467
3	chr3:120385537-121923035	1537498
3	chr3:121931699-124830052	2898353
4	chr4:87160439-91509561	4349122
4	chr4:77709496-86985942	9276446
5	chr5:92602370-93717798	1115428
5	chr5:41416674-42624527	1207853
5	chr5:87090359-88357858	1267499
5	chr5:179620442-180915260	1294818
5	chr5:1483897-8953381	7469484
6	chr6:15067080-19041846	3974766
6	chr6:19050234-25173355	6123121
7	chr7:122810117-123980215	1170098
7	chr7:101954468-103191686	1237218
7	chr7:40483744-43163338	2679594
7	chr7:29178667-40443341	11264674
8	chr8:0-2683108	2683108
8	chr8:46886735-119798778	72912043
9	chr9:70984372-84389822	13405450
11	chr11:54794237-56482051	1687814
11	chr11:79726393-91682741	11956348
12	chr12:42347636-71689131	29341495
13	chr13:23646794-24668110	1021316
15	chr15:36662007-54162140	17500133
16	chr16:68580385-73593216	5012831
17	chr17:44287381-45879796	1592415
17	chr17:41687610-44278785	2591175
18	chr18:334419-3513265	3178846
19	chr19:55569398-59128983	3559585
20	chr20:0-1613328	1613328
22	chr22:18922645-21800198	2877553

Supplemental table 2: Identified rare CNVs in patient 2

CNV	Zygosity	Segregation	overlap in DDD controls[a]	Genes[b]	Authors classification
hg19 chr2:g. (152,849,322_152,849,625)_ (152,880,509_152,881,908)del	Heterozygous	Paternal	No[c]	CACNB4	Uncertain-likely benign
hg19 chr7:g. (40,442,062_40,444,621)_ (40,481,539_40,485,949)del	Homozygous	Parental	Yes	C7orf10	Benign
hg19 chr1:g. (46,216,575_46,221,440)_ (46,465,662_46,476,587)dup	Heterozygous	Maternal	Yes	MAST2	Benign
hg19 chr16:g. (80,441,420_80,442,198)_ (80,551,598_80,554,948)del	Heterozygous	Maternal	Yes	-	Benign
hg19 chr17:g. (77,364,581_77,364,843)_ (77,384,557_77,387,550)dup	Heterozygous	De novo	Yes	RBFOX3	Uncertain-likely benign

[a] CNVs present in the control groups of the developmental delay studies (Cooper, Coe et al. 2011, Kaminsky, Kaul et al. 2011, Coe, Witherspoon et al. 2014) and hundred percent CNV overlap, [b] Genes located in the maximum CNV interval, [c] No CNVs are described in the Developmental delay control group, there are losses present. However, these are much larger ranging from several hundred Kb to several Mb in length.

Supplemental table 3: Rare recessive mutations predicted deleterious in ROH regions

Gene	Type	Exon	Effect	HGVS genomic-level	cDNA	Protein	CADD PHRED
DENND4B	Ins	18	F	NC_000001.10:g.153907306_153907307insGCTGCTGC	c.2702_2703 insGCAGCAGC	p.Gln904Hisfs*48	32
LENEP	SNV	1	MS	NC_000001.10:g.154966258C>G	c.175C>G	p.Leu59Val	23.6
MYLK	Ins	23	F	NC_000003.11:g.123383093_123383099dup	c.3838_3844 dupGAAAGCG	p.Glu1282Glyfs*51	36
AD-AMTS16	SNV	16	MS	NC_000005.9:g.5239937C>T	c.2422C>T	p.Arg808Trp	25.7
ID4	SNV	1	MS	NC_000006.11:g.19838067C>G	c.82C>G	p.Leu28Val	25.7
ZNF596	Del	4	CSP	NC_000008.10:g.193799_193812delCTGCAAGGTGAGCT	c.217_223+7 delCTGCAAGGT-GAGCT	.	23.2
C8orf34	SNV	13	S, SP	NC_000008.10:g.69728122T>C	c.1551T>C	p.=	6.825
PKHD1L1	SNV	71	MS	NC_000008.10:g.110523018A>G	c.11408A>G	p.His3803Arg	24.2
CCDC81	SNV	4	MS	NC_000011.9:g.86103688C>T	c.404C>T	p.Ser135Leu	28.3
NSF	SNV	20	MS	NC_000017.10:g.44832731G>A	c.2209G>A	p.Gly737Arg	33
USP29	SNV	4	MS	NC_000019.9:g.57642673G>A	c.2630G>A	p.Gly877Glu	24.4

Depicted are the rare recessive mutations predicted deleterious in the new ROH regions of patient P1. All minor allele frequencies of variants are below 0.1% in the public databases and in-house cohorts described in the method section. Ins; insertion, Del; deletion, SNV, Single nucleotide variant, MS; missense, F; frameshift , S; synonymous variant, SP predicted to affect splicing, CSP; Canonical splice site

Supplemental table 4 : List of primers used in this research

Primer	Sequence
Sequencing forward mutation in exon 22 Human *MYLK*	TCAGGGAAGCTGGACTCTGG
Sequencing reverse mutation in exon 22 Human *MYLK*	CAGGGAGTCTGTGGGTTGC
PCR forward minigene construct	GCCCCCTTCCTTTCCTAGCC
PCR reverse minigene construct	GCAGGGAGTCTGTGGGTTGC
Forward SD6	TCTGAGTCACCTGGACAACC
Reverse SA2	GCTCACAAATACCACTGAGAT

CHAPTER 5

Congenital Short Bowel Syndrome: From Clinical and Genetic Diagnosis to the Molecular Mechanisms Involved in Intestinal Elongation

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ABSTRACT

Congenital short bowel syndrome (CSBS) is a rare gastrointestinal disorder in which the mean length of the small intestine is substantially reduced when compared to its normal counterpart. Families with several affected members have been described and CSBS has been suggested to have a genetic basis. Recently, our group found mutations in *CLMP* as the cause of the recessive form of CSBS, and mutations in *FLNA* as the cause of the X-linked form of the disease. These findings have improved the quality of genetic counseling for CSBS patients and made prenatal diagnostics possible. Moreover, they provided a reliable starting point to further investigate the pathogenesis of CSBS, and to better understand the development of the small intestine. In this review, we present our current knowledge on CSBS and discuss hypotheses on how the recent genetic findings can help understand the cause of CSBS.

INTRODUCTION

Short bowel syndrome (SBS) refers to the sum of functional alterations that are the result of a critical reduction in the length of the intestine. In the absence of adequate treatment, SBS presents as chronic diarrhea, chronic dehydration, malnutrition, weight loss, and nutriment and electrolyte deficiency. In most cases, SBS occurs as a result of surgical intervention for other diseases, such as necrotizing enterocolitis and intestinal atresia. However, in a small number of cases the small intestine is already shortened at birth, leading to a diagnosis of Congenital Short Bowel Syndrome (CSBS). CSBS is a heritable gastrointestinal disorder, first described by Hamilton *et al* in 1969 (Hamilton, Reilly et al. 1969). For many years the underlying genetic cause of the disease was unknown. Recently, mutations in *CLMP* were identified to cause the autosomal recessive form of CSBS (Van Der Werf, Wabbersen et al. 2012), and mutations in *FLNA* as the cause of the X-linked form of the disease (van der Werf, Sribudiani et al. 2013). These findings brought new insights into disease pathogenesis, but the mechanisms in which CLMP and FLNA contribute to intestinal elongation are still unknown.

In this review we describe the clinical aspects of CSBS, the recent genetic findings, and the aetiological aspects of this gastrointestinal disorder. Moreover, we hypothesize about the mechanisms underlying the development of CSBS and the signaling pathways that might be essential for development and elongation of the small intestine, based in previously described mouse models.

Clinical presentation

CSBS patients are characterized by the presence of a substantially shortened small intestine at birth, approximately 50 cm, when compared to 250 cm in neonates delivered at term (> 35 weeks of gestation). As a consequence, they have a reduced absorptive surface of the small intestine and suffer from malabsorption (Sabharwal, Strouse et al. 2004). CSBS can be detected by radiography, but the diagnosis is usually done by laparotomy. Patients with CSBS often present within a few days after birth with bile-stained vomiting and diarrhoea or failure to thrive, but in some cases the diagnosis has been made later in life when an exploratory laparotomy was performed for significant gastrointestinal complaint (Siva, Brasington et al. 2002). Malrotation of the bowel is always present, and although this can point to an independent developmental defect, it can also be just a consequence of the shortened small intestine. The cecum is often positioned in the left upper quadrant of the abdomen close to the splenic flexure (Hamilton, Reilly et al. 1969, Konvolinka 1970, Schnoy, Bein et al. 1978, Iwai, Yanagihara et al. 1985, Dorney, Byrne et al. 1986, Ordonez, Sondheimer et al. 2006), but it can also be located in the lower left quadrant of the abdomen when nonrotation of the bowel is observed (Tiu, Chou et al. 1984). In three reported patients the appendix was absent (Iwai, Yanagihara et al. 1985, Sarimurat, Celayir et al. 1998, Sabharwal, Strouse et al. 2004), and volvulus was found in four patients (Yutani, Sakurai et al. 1973, Tanner, Smith et al. 1976). In a few cases, not

only the small intestine was shortened, but also the colon was affected (Yutani, Sakurai et al. 1973, Iwai, Yanagihara et al. 1985, Sarimurat, Celayir et al. 1998, Sabharwal, Strouse et al. 2004). Another gastrointestinal anomaly that has been described in ten CSBS patients is hypertrophic pyloric stenosis (Kern and Harris 1973, Royer, Ricour et al. 1974, Nezelof, Jaubert et al. 1976, Tanner, Smith et al. 1976, Schnoy, Bein et al. 1978, Sansaricq, Chen et al. 1984). However, it has been suggested that hypertrophic pyloric stenosis is not part of the general developmental defect of the gastrointestinal tract, but a physiological consequence from the attempts of the remnant small intestine to slow down the gastric emptying and improve absorptive capacity. CSBS patients usually have normal intellectual ability (Huysman, Tibboel et al. 1991, Schalamon, Schober et al. 1999) and do not present any extra-intestinal symptoms. However, in three patients a patent ductus arteriosus was found (Royer, Ricour et al. 1974, Sansaricq, Chen et al. 1984), and in two patients minor dysmorphic features were reported (De Backer, Parizel et al. 1997, Ordonez, Sondheimer et al. 2006).

Histological findings in CSBS patients

Based on the literature available, it is difficult to assess if abnormal peristalsis is associated with CSBS pathogenesis, or if it is an independent event to the presence of a short bowel (Matter and Balda 2003, Shehata, Chang et al. 2011, van der Werf, Sribudiani et al. 2013). In most CSBS patients the bowel wall seems macroscopically normal, but an abnormal histology has been described in some patients. Tanner et al performed silver staining in patients material and found an abnormally high number of neurons in the ganglia (Tanner, Smith et al. 1976). This result led the authors to suggest that the normal fall-out of ganglion cells does not occur in CSBS patients. They also found that the neuronal nuclei showed clumped chromatin, which is characteristic for neuroblasts, and the intrinsic argyrophilic ganglion cells were absent or reduced in number. However, these results lacked a quantitative analysis of the data and comparison to suitable controls in order to support hyperganglionosis as the cause of reduced intestinal motility. In an independent study, Schalamon *et al* also observed an abnormal bowel wall with signs of neuronal intestinal dysplasia in two siblings with CSBS (Van Der Werf, Wabbersen et al. 2012). In another CSBS patient heterotopic gastric mucosa was found (Shehata, Chang et al. 2011). Conversely, in other cases, no abnormalities of the nerves plexus were seen on routine acetylcholinesterase staining (Hamilton, Reilly et al. 1969, Dumke and Schnoy 1974, Sansaricq, Chen et al. 1984, Dorney, Byrne et al. 1986, Peng, Chen et al. 1993, Sarimurat, Celayir et al. 1998, Erez, Reish et al. 2001). Nezelof et al described three cases with several congenital malformations, which included a shortened small intestine and heterotopia. In these patients a normal myenteric plexus was observed by cytoenzymatic and silver stainings (Nezelof, Jaubert et al. 1976). In another study, Kapur et al reported an extensive pathologic analysis performed on intestinal tissue collected from five male patients diagnosed with CSBS and X-linked intestinal pseudo-obstruction. They observed

that these patients had diffused abnormal layering of the small intestinal smooth muscle, in which the muscularis propria layer was formed by three perpendicular muscle laminae, instead of two. Such abnormal structure was restricted to the small intestine without any extension to the colon (Kapur, Robertson et al. 2010). Based on this report, a myopathic cause for the abnormal intestinal peristalsis found in CSBS patients was suggested. Despite the contradictory results, these histological findings can account for the motility abnormalities described in CSBS patients, but to date it still remains unclear whether the reduced peristalsis observed in these patients results from a neuronal or a myopathic defect.

To our knowledge, there has never been a precise histological confirmation to define which part of the small intestine is affected in CSBS patients. It is possible that every part of the small intestine is shortened in general, but one cannot rule out the possibility that only one specific part of the small intestine is affected. If this is the case, a correlation between the type of the remaining small intestine and prognosis of CSBS patients can be established. Since different parts of the small intestine have different histology and function (Figure 1), it would not be surprising that depending on the region affected, different degrees of severity for CSBS could exist. Findings in acquired SBS cases support this idea, as it has been shown that the residual length of the jejunum and ileum with the presence of ileocecal valve (ICV), are important factors to determine the outcome of the disease (Koffeman, van Gemert et al. 2003). Therefore, we believe that identifying the part of the small intestine that is affected in CSBS patients should be a priority.

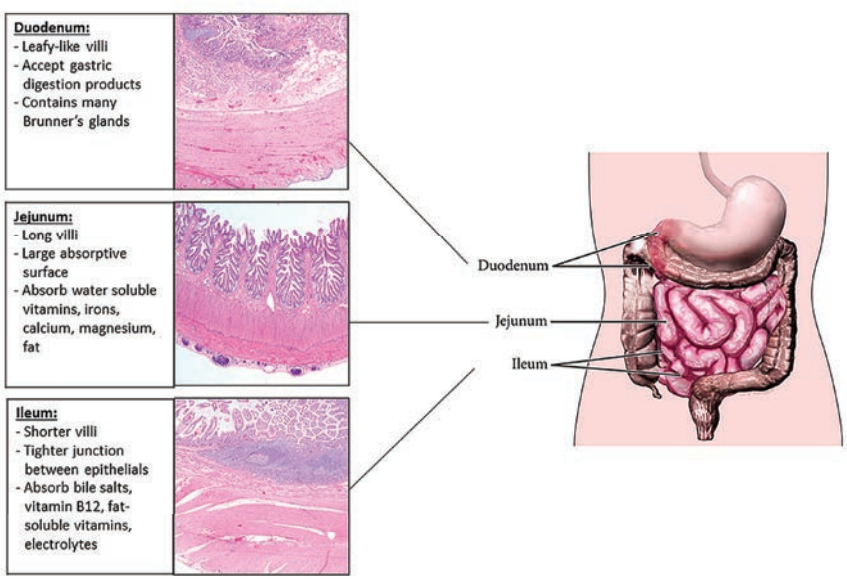

Fig.1: Histological characteristics and function of each part of the small intestine.

Treatment management and outcome

To date, there is no cure for CSBS and patients need total parenteral nutrition for long-term survival until sufficient bowel length and functions are gained. In some cases, total parenteral nutrition has to be continued for the first two years of life (Dorney, Byrne et al. 1986, Ordonez, Sondheimer et al. 2006), and oral feeding is introduced gradually. With time, the function of the remnant small intestine in CSBS patients improves, both in length and absorption capacity, leading to better absorption of fat and vitamin B12 (Hamilton, Reilly et al. 1969, Yutani, Sakurai et al. 1973, Dorney, Byrne et al. 1986). The weight and height of CSBS patients are frequently below the 50th percentile (Hamilton, Reilly et al. 1969, Sansaricq, Chen et al. 1984, Dorney, Byrne et al. 1986, Schalamon, Schober et al. 1999, Chu, Luo et al. 2004), but no nutritional deficiencies are observed (Dorney, Byrne et al. 1986, Ordonez, Sondheimer et al. 2006, Hasosah, Lemberg et al. 2008).

Parenteral nutrition has brought a new lease of life to an otherwise fatal condition. However, its use is often associated with very high rates of complications, such as sepsis and liver failure (Sansaricq, Chen et al. 1984). Small bowel transplantation can also be considered, but due to its relatively poor overall survival it is viewed only as a last resort treatment option. It has been recommended that CSBS patients should be managed in a multidisciplinary manner in a centre specialized in the care of children with intestinal failure (Ordonez, Sondheimer et al. 2006), but despite considerable efforts to improve treatment, most patients die of starvation or sepsis within the first few days of life, and only a quarter of the reported patients survived for more than one year (Table 1). Thus, an improved understanding of the mechanisms underlying the development of CSBS is necessary to develop better methods to treat this condition.

Disease aetiology

To understand the causes underlying the pathogenesis of CSBS, a better understanding of the mechanisms involved in the development of the small intestine is required. In this section, we describe the embryonic events necessary for intestinal growth and elongation. During embryogenesis, the primitive gut tube is divided in three regions: the foregut, the midgut and the hindgut, each of them with their own arterial supply (the celiac artery, the superior mesenteric artery and the inferior mesenteric artery, respectively). The small intestine (jejunum and ileum) originates from the midgut, as well as the distal duodenum, cecum, ascending colon, and the proximal two-thirds of the transverse colon. In the fifth week of embryonic development the future ileum is elongating rapidly (Figure 2), but as the abdominal cavity grows slower, the midgut forms an anteroposterior loop called the primary intestinal loop. The cranial limb of this loop includes the ileum and the caudal limb includes the ascending and transverse colons. In the sixth week of development, the primary intestinal loop herniate into the umbilicus forced by elongation of the loop and growth of other abdominal organs. At this time the loop rotates 90 degrees counter clockwise around the axis of the superior mesenteric artery. The future ileum is now

lying on the right, and the cecum on the left. The cecum and the appendix continue to differentiate and the small intestine elongates further forming the jejunal-ileal loops. The attached mesentery accompanies this intestinal growth but it does so at a lower rate. Recent studies have shown that it is the differential growth rate between the intestine and the mesentery that creates specific patterns of looping and rotation of the gut in different species (Savin, Kurpios et al. 2011). Therefore, any disturbance of this differential growth rate may lead to abnormal growth patterns and result in intestinal malrotation. During the tenth week of gestation, the intestinal loop returns rapidly to the abdominal cavity. The small intestine returns first and the ascending and transverse colons follow later. It is not known what causes this retraction, but an increase in the size of the abdominal cavity and a relatively decrease in the size of the liver and kidneys seem to play an important role. To reach the definitive configuration of the small and large intestines, the intestinal loop rotates another 180 degrees counter-clockwise (Moore and Persaud 2003, Schoenwolf and Larsen 2009, Carlson 2014).

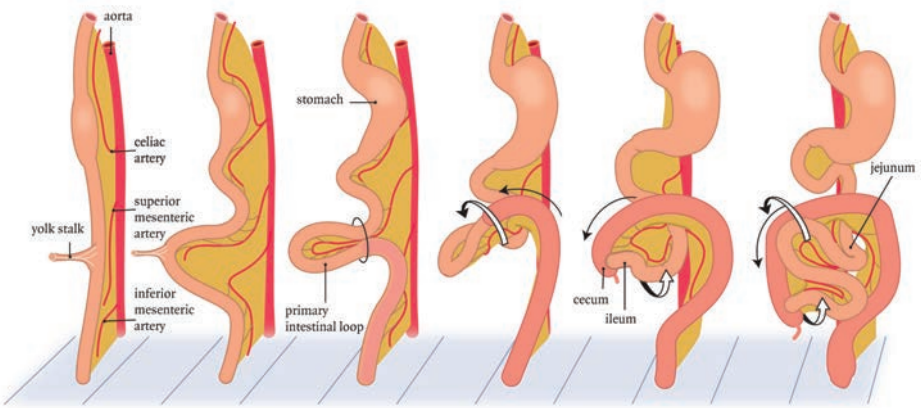

Fig.2: Embryogenesis of the human small intestine.

Following this intricate growth, looping and rotation of the gut, intestinal patterning and regionalization takes place. All intestinal organs derived from the foregut, midgut and hindgut consist of similar layers, which include mucosa, muscularis mucosa, submucosa, submucosal plexus, muscularis propria, myenteric plexus and serosal layer. Formation of these layers requires extensive proliferation and differentiation of cells that compose the epithelial and muscle of the intestine. The neuronal network responsible for intestinal innervation is mainly formed by enteric neural crest cells (ENCCs) that migrate from the neural tube in a rostral to caudal direction to promote neuronal colonization of the intestinal tract (Goldstein, Hofstra et al. 2013). Interestingly, cell differentiation and radial patterning of the gut is largely dictated by signals exchanged between the different cell types that constitute the various layers. For example, the Hedgehog signaling initiated in

the intestinal epithelium affects cell proliferation and differentiation of cells derived from all embryonic layers (endoderm, mesoderm and ectoderm), playing an important role in the establishment of the villus-crypt patterning, smooth muscle cells proliferation, and ENCCs proliferation, differentiation and migration (Ramalho-Santos, Melton et al. 2000, De Santa Barbara, Williams et al. 2005). Several other morphogenetic pathways that regulate cellular interactions and that are necessary for intestinal elongation are discussed further in section 8 of this review. Nonetheless, it is important to acknowledge here the existence of such interactions in the context of CSBS, since the gut abnormalities found in these patients may well arise due to dysregulation of these cellular interactions.

Genetics

Familial occurrence of CSBS was described in the very first case report in 1969. Hamilton et al reported a French-Canadian couple who were not related and who had five daughters, two of them diagnosed with CSBS. One of the affected girls died at the age of 1 month and 1 week, and prior to death a laparotomy showed a small intestine of 30 cm in length (Hamilton, Reilly et al. 1969). More case reports followed and a familial occurrence was described in approximately 60% of the cases published in the literature. In most of these cases siblings were affected, and in approximately 25% of the cases the parents were consanguineous (Table 1). It has therefore been suggested by several authors that genetic factors were involved in CSBS and an autosomal recessive pattern of inheritance was proposed by many of them (Royer, Ricour et al. 1974, Schnoy, Bein et al. 1978, Shawis, Rangecroft et al. 1984, Wei and Lai 1985, Dorney, Byrne et al. 1986, Huysman, Tibboel et al. 1991, Aviram, Erez et al. 1998, Erez, Reish et al. 2001). However, since only boys were found affected in some families, an X-linked pattern of inheritance has also been suggested (Nezelof, Jaubert et al. 1976, Sansaricq, Chen et al. 1984, Kern, Leece et al. 1990, Duveau, Bardot-Labbe et al. 2000). In this section, we focus on the genetic findings described in CSBS patients.

Table 1. Overview of the reported cases with CSBS as the main symptom.

Sex	Small bowel length (cm)	Reference	Year of publication	Age at time of presentation	Age at death	Familial	Consan-guinity
F	40	[1]	1969	4 months	Alive at time of publication	Yes	No
F	30	[1]	1969	Unknown	1 month	Yes	No
M	30	[8]	1970	3 months	5 months	Unknown	Unknown
F	42	[14]	1973	3 days	35 days	No	No
M	70	[15]	1973	7 weeks	5 months	Unknown	Unknown
F	25	[17]	1974	1 month	21 days	Yes	Unknown
M	70	[17]	1974	Unknown	4 days	Yes	Unknown
M	45	[17]	1974	15 days	7 months	Yes	Yes
F	40	[17]	1974	5 days	2 months	No	No
M	106	[24]	1974	22 days	25 days	Yes	Yes
M	75	[7]	1976	6 days	6 months	No	No

M	70	[16]	1976	18 days	22 days	Yes	No
M	Unknown	[16]	1976	7 days	16 days	Yes	No
M	50	[16]	1976	15 days	7 months	Yes	No
M	24	[11]	1984	32 days	55 days	Yes	No
M	27	[11]	1984	2 days	5 months	Yes	No
F	45	[39]	1984	Unknown	3 months	Yes	Yes
F	45	[39]	1984	1 day	6 weeks	Yes	Yes
M	72	[18]	1984	6 weeks	Alive at time of publication	Yes	No
M	65	[18]	1984	18 days	2 months	Yes	No
M	24	[40]	1985	3 days	55 days	Yes	No
M	27	[40]	1985	2 days	5 months	Yes	No
M	45	[7]	1985	5 weeks	2 months	No	No
M	69	[6]	1986	5 weeks	Alive at time of publication	No	Yes
M	112	[42]	1990	1 month	6 weeks	Yes	No
M	70	[42]	1990	6 hours	6 months	Yes	No
M	237*	[42]	1990	3 months	Alive at time of publication	Yes	No
F	54	[19]	1991	2 months	Alive at time of publication	No	No
F	30	[26]	1993	2 days	4 months	Yes	No
M	39	[26]	1993	Unknown	6 months	Yes	No
F	30	[26]	1993	Unknown	2 months	No	No
F	30	[43]	1996	1 day	Alive at time of publication	No	No
F	50	[21]	1997	1 day	Alive at time of publication	No	No
F	25	[12]	1998	4 days	6 months	No	No
M	47	[20]	1999	9 days	Alive at time of publication	Yes	Yes
M	42	[25]	2001	2 days	5 months	Yes	No
F	51	[25]	2001	3 days	2 weeks	Yes	No
M	95	[25]	2001	2 months	Alive at time of publication	No	Yes
M	35	[25]	2001	2 days	2 months	Yes	No
M	228,6**	[5]	2002	15 years	Alive at time of publication	No	No
M	56	[4]	2004	4 months	Alive at time of publication	No	No
F	20	[29]	2004	4 days	Alive at time of publication	No	No
F	30	[9]	2006	days	Alive at time of publication	Yes	No
M	50	[30]	2008	6 weeks	Alive at time of publication	Yes	Unknown
M	20-25	[44]	2010	26 days	1 month	No	No
M	unknown	[3]	2013	Unknown	Alive at time of publication	Yes	No

*At 14 years of age; **At 15 years of age.

Chromosomal abnormalities

Two patients have been described with chromosomal abnormalities. Hou et al reported a female patient with multiple congenital anomalies, such as congenital short bowel, malrotation, and patent ductus arteriosus, in addition to major malformations, such as left upper amelia, dextrocardia and asplenia. Chromosomal investigation of this patient showed a mosaic pattern with complex rearrangements of chromosome 4: 85% of the peripheral lymphocytes showed a normal female cell line (46,XX), while 12% of the cells

showed a pattern with one normal chromosome 4 and a ring chromosome 4 (46,XX-4,+r(4)(p16→q22.3). The ring caused a deletion of the long arm of chromosome 4. Approximately 4% of the cells from this patient had a pattern with a partial trisomy of chromosome 4: one normal chromosome 4, one ring chromosome 4 and one chromosome 4 with the same deletion of the long arm of chromosome 4 (47,XX,4,+r(4)(p16→q22.3),+del(4)(pter→q22.3:)) (Hou and Wang 1996). De Backer et al also described a female CSBS patient with a *de novo* balanced translocation between chromosome 2 and 11 (46,XX,t(2,11)(q32.2,p12)) (De Backer, Parizel et al. 1997). However, no functional implications of these chromosomal abnormalities have been reported in these cases that could explain the development of CSBS.

Loss-of-function mutations in *CLMP* cause recessive CSBS

In seven CSBS patients from five unrelated families, different homozygous and compound heterozygous loss-of-function mutations have been identified in the gene encoding for the Coxsackie- and adenovirus receptor-like membrane protein (CLMP) (Van Der Werf, Wabbersen et al. 2012). The reported length of the small intestine of these patients was 30 to 54 cm and they all presented malrotation of the bowel. Neuronal intestinal dysplasia was reported in two patients (from one family) with a more complex mutation, presumably an inversion (Schalamon, Schober et al. 1999, Van Der Werf, Wabbersen et al. 2012).

CLMP, located on chromosome 11 (11q24.1), encodes a transmembrane protein that co-localizes with tight junction proteins and acts as an adhesion molecule (Raschperger, Engstrom et al. 2004, Van Der Werf, Wabbersen et al. 2012). It is expressed in the intestine during different stages of human development, and knockout of its orthologue in zebrafish resulted in developmental defects of several organs, including the intestine. As tight junction proteins play an important role in proliferation (Balda and Matter 2000, Matter and Balda 2003), we hypothesized that loss-of-function of CLMP results in less proliferation of the small intestinal cells during human development, leading to a shortened small intestine at birth (Van Der Werf, Wabbersen et al. 2012). Recently, this hypothesis was tested using an *in vitro* approach where a mutant CLMP (V124D), was over-expressed in a human intestinal epithelial cell line (T84). This mutant has been previously reported to mislocalized to the cytoplasm (Van Der Werf, Wabbersen et al. 2012), but *in vitro* assays failed to confirm the role of CLMP in cell viability, proliferation and migration in this cell line (van der Werf, Hsiao et al. 2013). Thus, the role of CLMP in small intestinal development and its function still remain unclear.

Mutations in *FLNA* cause X-linked CSBS

Mutations in Filamin A (*FLNA*) have been associated with a wide spectrum of disorders characterized by a variable phenotype. Loss-of-function mutations are found in patients with bilateral periventricular nodular heterotopia, a neuronal migration disorder characterized by seizures affecting mainly females, as it is often lethal in males (Fox,

Lamperti et al. 1998). Mutations that alter the function of *FLNA* are associated with three different disorders: otopalatodigital syndromes type 1 and 2, frontometaphyseal dysplasia, and Melnick Needles syndrome. These syndromes constitute a phenotypic spectrum that includes skeletal dysplasia, craniofacial-, cardiac-, genito-urinary and intestinal anomalies, and central nervous system defects (Robertson, Twigg et al. 2003). In addition, missense mutations in *FLNA* are associated with X-linked cardiac valvular dystrophy (Kyndt, Gueffet et al. 2007, Bernstein, Bernstein et al. 2011). CSBS has also been described in some of the patients reported with loss-of-function mutations in *FLNA*. However, these patients presented multiple congenital anomalies and the short bowel was described as part of the disease phenotype. A male patient, stillborn at 33 weeks of gestation, was reported with a duplication of the first 28 exons of *FLNA*. Prenatal ultrasounds showed normal growth with a single umbilical artery, umbilical vein varix, and persistent dilatation of the bowel first seen at 20 weeks gestation. These findings were confirmed by an autopsy of the foetus, which also detected a bifid uvula, an atrial septal defect, and a malrotated short small intestine of 45 cm (164 cm would be the expected length at this gestational stage). This duplication was also detected in his mother, who was diagnosed with a bifid uvula and patent ductus arteriosus, and in his maternal uncle, who had multiple congenital anomalies including a bifid uvula, intestinal malrotation, undescended testes, partial agenesis of the corpus callosum, patent ductus arteriosus, patent foramen ovale, ventricular septal defect and periventricular heterotopia, and a small intestine measuring only 115 cm at the age of 10 years (Kapur, Robertson et al. 2010). Another patient with a hemizygous nonsense mutation in *FLNA* (c.7021C>T, Q2341X) was diagnosed prenatally with a left diaphragmatic defect, which caused a displacement of the spleen, left hepatic lobe, and portions of the stomach and small intestine into the left hemithorax. He also had dysmorphic facial features, spina bifida occulta, natal tooth, periventricular heterotopia, a posterior fossa arachnoid cyst, and proximally placed thumbs. He died at 6 weeks of age and his small intestine measured only 68 cm (Kapur, Robertson et al. 2010). Recently, a family has been described with two affected male siblings where a novel no-stop mutation in *FLNA* (c.7941_7942delCT, p.(*2648Serext*100)) was identified upon genetic screening. The same mutation was detected in their male cousin. These patients were diagnosed with congenital short bowel syndrome, “wandering spleen”, periventricular nodular heterotopia, persistent ductus arteriosus, and urinary tract abnormalities (Oegema, Hulst et al. 2013). All these reports confirm that CSBS patients with mutations in *FLNA* have, in general, multiple congenital anomalies and not only a congenital shortened small intestine. Recently, however, a mutation in the second exon of *FLNA* has been identified in two unrelated male patients where CSBS appeared as an isolated symptom without other major congenital anomalies (van der Werf, Sribudiani et al. 2013). In these patients, a two-base-pair (bp) deletion in *FLNA* (c.16-17delCT) was identified. In another male patient previously described with Chronic Idiopathic Intestinal Pseudo-obstruction, a 2-bp deletion was also found in the second exon of *FLNA* (c.65-66delAC). This patient was diagnosed

with malrotation, pyloric hypertrophy, intestinal pseudo-obstruction, and CSBS (Auricchio, Brancolini et al. 1996, Gargiulo, Auricchio et al. 2007). In these three patients the length of the small intestine ranged from 55 to 235 cm, and the age of diagnosis varied from 1 day to 15 years (Kern and Harris 1973, Kern, Leece et al. 1990, Siva, Brasington et al. 2002), suggesting that in some cases, the small intestine is less reduced in length and the diagnosis is made later in life when compared to CSBS patients with *CLMP* mutations. These 2-bp deletions are located between two nearby methionines at the N-terminus of *FLNA*. Previous studies showed that translation of FLNA occurs from both methionines, resulting in two protein isoforms (Gargiulo, Auricchio et al. 2007). In the presence of this 2-bp deletion the longer isoform is not translated anymore, but there is still expression of the shorter FLNA isoform. We hypothesized that this is the reason why these deletions are not lethal for males *in utero*, and they only develop CSBS. Screening of exon 2 of *FLNA* is therefore recommended in such cases. In X-linked families where CSBS is associated with multiple congenital anomalies it is advisable to screen the entire *FLNA* for mutations.

FLNA encodes a cytoskeletal protein that binds to actin and has a well-characterized role in the cytoplasm. It regulates cell shape by cross-linking actin filaments, and plays an important role in cell signaling and migration in response to environmental changes (Robertson 2005). A role for FLNA has also been recently discovered in the nucleoli, where it inhibits ribosomal RNA transcription (Deng, Lopez-Camacho et al. 2012). FLNA has been reported to play an important role in vascular development and cardiac morphogenesis (Feng, Chen et al. 2006), but its role is still unclear in intestinal development. Nishita et al reported that FLNA is able to interact with the tyrosine kinase-like orphan receptor 2 (Ror2), and showed that this interaction is required for filopodia formation and migration (Nishita, Yoo et al. 2006). Since disruption of Ror2 expression has been shown to lead to a shortened small intestine in mice (Yamada, Udagawa et al. 2010), it is tempting to hypothesize that *FLNA* mutations leading to CSBS disrupt the FLNA-Ror2 interaction and impair cell migration. However, further studies are required to clarify the role of FLNA in intestinal elongation.

Link between CLMP, FLNA and CSBS

We now know that mutations in *CLMP* and *FLNA* underlie CSBS pathology (Van Der Werf, Wabbersen et al. 2012, van der Werf, Sribudiani et al. 2013). However, it is still unclear whether CLMP and FLNA interact with each other (directly or indirectly) and, therefore, whether mutations in one of these two genes result in a similar course of events during development of the small intestine. In this section, we discuss different hypotheses to explain CSBS pathogenesis, and speculate about a possible link between CLMP, FLNA and each of these hypotheses.

Embryologic and intrauterine events

As we mentioned in section 4, during the 7th and 10th week of embryonic development,

the primitive digestive tube needs to return to the intraumbilical coelom. Hamilton et al suggested that in CSBS this process is prevented. As a consequence, the primitive bowel is forced to stay in the abdominal cavity and the cranial portion of the bowel is not able to elongate, leading to a shortened small intestine (Hamilton, Reilly et al. 1969). In another report, Aviram et al observed the presence of bowel loops inside the umbilical cord on a prenatal sonography of a patient with CSBS, showing that the gut was able to elongate, but the return of the intestine to the abdominal cavity was in fact delayed. They speculated that the incomplete dextral rotation and elongation of the bowel caused this delay (Aviram, Erez et al. 1998). Delayed return of the intestine into the abdominal cavity is also associated with volvulus and intestinal obstruction (Finley, Burlbaw et al. 1992). However, volvulus has only been described in four CSBS cases, and adhesions, atresia, stenosis and scars are rarely found in CSBS patients. In a case report of a premature neonate in whom the small bowel was absent, there was also no evidence of an abdominal wall defect or other intra-abdominal anomalies on prenatal sonography (Besner, Bates et al. 2005). Antenatal intussusception followed by auto-anastomosis and auto-amputation is also suggested as a cause of CSBS (Sarimurat, Celayir et al. 1998). However, failure of intestinal elongation can also be the cause, rather than the outcome of the observed malrotation in CSBS patients (Kern and Harris 1973). In cases were auto-anastomoses were found, intrauterine events like volvulus and infarction can be a reasonable explanation for the shortened small intestine (Sabharwal, Strouse et al. 2004). Another hypothesis is that vascular events underlie CSBS (Kern and Harris 1973). Intrauterine infarction of the bowel may lead to reabsorption of the ischemic bowel and result in a decreased length of the remaining gut. Considering the proposed embryologic and intrauterine events one could hypothesize that CLMP might play a role in these events. As mentioned earlier, tight junction proteins play an important role in proliferation (Balda and Matter 2000, Matter and Balda 2003). Hence, we hypothesized that loss-of-function of CLMP results in less proliferation of the small intestinal cells during human development, leading to a shortened small intestine at birth. The processes and pathways that may be disturbed by the loss of CLMP still remain unknown.

FLNA can also be linked to embryologic and intrauterine events, since it is known to play an important role in vascular development. Patients with *FLNA* mutations have been reported with developmental anomalies of the blood vessels (Kakita, Hayashi et al. 2002, Guerrini, Mei et al. 2004), and omphalocoele (Robertson 2005). An intrauterine vascular event causing small intestinal infarction (Kern and Harris 1973) might, therefore, be a reasonable explanation for the development of CSBS, supporting the hypothesis that the developmental defect seen in CSBS patients originates in the embryonic stage at which the bowel is accommodated in the intraumbilical coelom (Hamilton, Reilly et al. 1969).

Lack of neurotransmitters and hormones

As abnormal peristalsis has been observed in some CSBS patients, Sansaricq *et al* hypothesised that CSBS patients may lack synthesis of neurotransmitters (Sansaricq, Chen et al. 1984). However, abnormal peristalsis is not reported in all patients. Schalamon *et al* also suggested that CSBS patients lack growth-stimulating hormones like epidermal growth factor, insulin-like growth factor, and human growth hormone, but they were unable to detect abnormal hormone levels in their patients (Schalamon, Schober et al. 1999).

How mutations in *CLMP* and *FLNA* can lead to disturbed or lack of neurotransmitters is unclear. However, as these genes do not encode or even have an (direct) effect on the production of neurotransmitters, it is unlikely that a lack of neurotransmitters is the cause for the shortened small intestine observed in CSBS patients.

CLMP* and *FLNA

CSBS patients with mutations in *CLMP* seem to have a phenotype more restricted to the intestine, whereas patients with mutations in *FLNA* are more likely to have multiple congenital anomalies. However, CSBS patients with a deletion in the second exon of *FLNA* are very similar to patients with mutations in *CLMP*. This observation together with the fact that both gene products are involved in similar cellular processes, such as cell-cell contact and actin organization, suggest that FLNA and CLMP might be involved in the same protein network that is essential for intestinal development.

FLNA is an actin-binding protein and its actin-binding domain is located in its N-terminal region (Robertson 2005, Nakamura, Osborn et al. 2007, Qiu, Nomiyama et al. 2011). Gargiulo et al showed abnormal actin organisation in a lymphoblastoid cell line of a patient with a c.65-66delAC deletion in FLNA (Gargiulo, Auricchio et al. 2007). There is therefore evidence that the cytoskeletal actin organisation is disturbed in CSBS patients with mutations in FLNA. However, it is still not known whether patients with mutations in CLMP have a similar problem. Raschperger et al showed that CLMP co-localizes with actin filaments (Raschperger, Engstrom et al. 2004). They speculated that CLMP interacts with a protein that directly binds to actin filaments, which would bring CLMP to the tight junctions by anchoring CLMP to the actin cytoskeleton. They suggested that ZO-1 could be such an interacting protein. As FLNA also binds to actin filaments and is known to play a role in anchoring transmembrane proteins to the cytoskeleton for correct targeting to the cell membrane, such as integrin beta and the cystic fibrosis trans-membrane conductance regulator (CFTR) (Playford, Nurminen et al. 2010, Qiu, Nomiyama et al. 2011), it is tempting to suggest that FLNA is the link between CLMP and the actin cytoskeleton and is responsible for the proper localization of CLMP to the tight junctions (Figure 3). Another possibility is that FLNA plays a role in the internalization of CLMP into the plasma membrane. As FLNA is known to control the internalization of the chemokine receptor 2B in different dynamic membrane structures (Minsaas, Planaguma et al. 2010), one can speculate that mutations in *FLNA* influence the expression levels of

CLMP on the plasma membrane. Further research is needed to determine whether CLMP and FLNA interact with each other as part of the same protein network and, if so, which other proteins are involved in this network. However, we cannot exclude the possibility that different pathways underlie X-linked CSBS and autosomal recessive CSBS, with different disease mechanisms leading to a similar disease phenotype.

It is also not known whether more genes are involved in the pathogenesis of CSBS. We did not identify a mutation in all patients screened for *CLMP* and the second exon of *FLNA*. However, we did not screen all exons of *FLNA* in all patients analysed, which means that FLNA might still play a role in disease development in some of these patients. Based on the finding of an abnormal karyotype with a ring chromosome 4 in one CSBS patient with multiple congenital anomalies (Hou and Wang 1996), a gene on the long arm of chromosome 4 might also be involved. Further research on the protein networks of CLMP and FLNA might help find more candidate genes for CSBS.

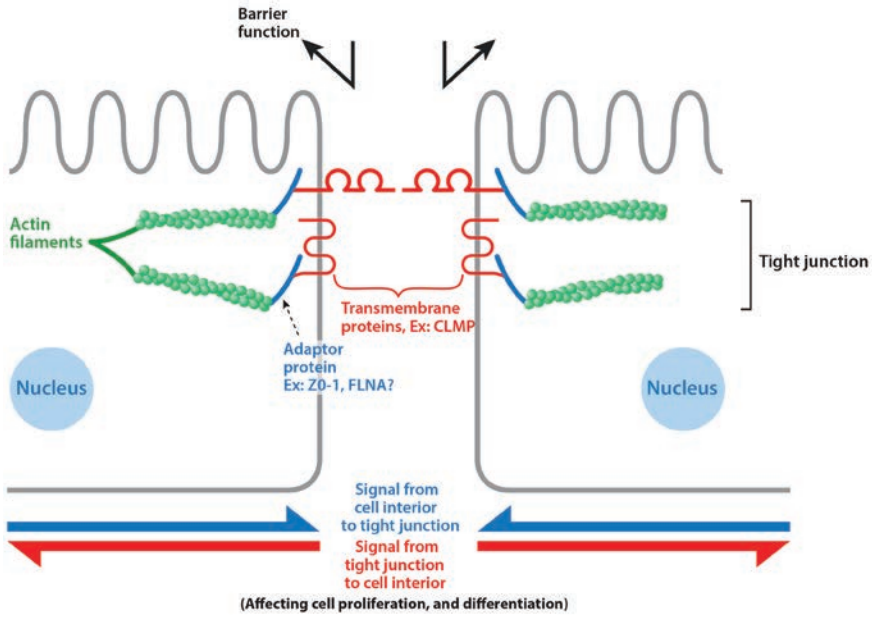

Fig.3: Tight junction complexes and the possible link between CLMP and FLNA

Mouse models for CSBS and intestinal elongations

In order to get new insights about the pathogenesis of CSBS, observations on *CLMP* and *FLNA* knockout animal models might be important. They can help us identify which cell types are affected, and also determine if there is a general shortening or if specific parts of the intestine are affected. To date, there is no mouse model available for *CLMP*, only a zebrafish model that was recently reported by our group (Van Der Werf, Wabbersen et al. 2012). In this model, there was an overall reduction of the body length accompanied by a drastic reduction in the size of the small intestine. Histological findings showed that

there was a significant difference in gut morphology in knockout zebrafish, marked by the absence of goblet cells in the mid intestine. This result confirmed the importance of CLMP for small intestine development and elongation, but further investigation is required to determine the role of CLMP in intestinal embryogenesis.

For *FLNA*, two mouse models have been reported: a conditional knockout model, and a N-ethyl-N-nitrosourea induced model called *Dilp2* (Feng, Chen et al. 2006, Hart, Morgan et al. 2006). Both of them showed complete loss of *Flna* and resulted in embryonic lethality. Vascular defects were also reported in both of these models, and in one of them there was a delayed resorption of the umbilical hernia (Hart, Morgan et al. 2006). Feng et al further investigated the reasons associated to the vascular phenotype, and showed that migration and motility of different cell types were not affected in *Flna*-null embryos (Feng, Chen et al. 2006). However, abnormal epithelial and endothelial organization, and aberrant adherent junctions were observed in several tissues, including developing blood vessels, heart, and brain. A defect in intestinal elongation was not reported in any of these models, but it is possible that a more detailed examination of these embryos could shed some light into the role of FLNA in normal intestinal development.

With respect to intestinal elongation, several mouse models have been reported with a shortened small intestine: *Fgf9*$^{-/-}$; *Shh*$^{-/-}$ *Ihh*$^{-/-}$; *Notch*$^{-/-}$; *Wnt5a*$^{-/-}$; *Ror2*$^{-/-}$; *Sfrp1*$^{-/-}$*Sfrp2*$^{-/-}$ *Sfrp5*$^{-/-}$ and *Hlx*$^{-/-}$ (Hentsch, Lyons et al. 1996, Geske, Zhang et al. 2008, Yamada, Udagawa et al. 2010, Bakker, Raghoebir et al. 2012). All these genes encode for proteins involved in highly conserved signaling pathways known to play a crucial role in normal embryonic development (Geske, Zhang et al. 2008). In this section, we describe these proteins and associated pathways, and discuss their contribution towards the elongation of the small intestine.

Fibroblast Growth Factor 9 (*FGF9*)

FGF9, also known as glia activating factor, is part of a large family of polypeptide growth factors that are involved in a variety of biological processes, such as embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion (Itoh and Ornitz 2008). A knockout mouse model for *Fgf9* showed a disproportional small intestine, suggesting that Fgf9 is particularly important for small intestinal morphogenesis. However, this effect was only seen after embryonic day 14.5 (E14.5), suggesting that Fgf9 regulates small intestinal elongation during late stage embryogenesis (Geske, Zhang et al. 2008). The mechanism by which FGF9 regulates this process is not totally known, but Geske et al showed that this effect might be due to a significant increase of the transforming growth factor beta (TGFβ) signaling pathway in the absence of FGF9 (Geske, Zhang et al. 2008). TGFβ signals are well known to drive the transition of mesenchymal fibroblasts to myoblasts by activation of intermediate molecules, such as Smad2 and Smad3 (Waite and Eng 2003). However, this process is mostly seen as the small intestine transitions to the postnatal period, when the proliferative properties of the mesenchymal fibroblasts are less

required. In the *Fgf9*$^{-/-}$ mouse model, an increase in TGFβ signaling was detected at earlier stages of embryogenesis, leading to a premature differentiation of the mesenchymal fibroblasts into myofibroblasts and hence, to decreased proliferation (Geske, Zhang et al. 2008). As a consequence, the normal elongation of the small intestine was impaired.

Sonic (*Shh*) and Indian Hedgehog (*Ihh*)

The mammalian family of lipid-modified hedgehog (Hh) signals are composed of three members: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh). Each of them is thought to signal through a common mechanism that involves binding and inactivation of Ptch1, a multi-pass transmembrane receptor. As a consequence, the seven-pass membrane protein smoothened (Smo) is activated, leading to a transcriptional response controlled by the Gli family of transcription factors (Zhang, Ramalho-Santos et al. 2001). Hh signaling is involved in proliferation, patterning and differentiation of many tissues (McMahon, Ingham et al. 2003). In the mammalian gut, Shh and Ihh are known to be co-expressed in the endodermal epithelium from early developmental stages (E8.5) (Bitgood and McMahon 1995), and dysregulation of this pathway has already been implicated in both congenital defects and cancers arising from the gastrointestinal tract (Bitgood and McMahon 1995, de Santa Barbara, van den Brink et al. 2002). However, mouse models where the expression of *Shh* or *Ihh* was abolished only showed limited anomalies, likely due to functional redundancy between these two proteins (Ramalho-Santos, Melton et al. 2000). Mao et al. generated a double mutant mouse model where expression of both *Shh* and *Ihh* was abolished (Mao, Kim et al. 2010). They showed that at E11.5 the digestive tract of double mutant embryos was normal in shape, orientation and location in the embryo, but was dramatically reduced in size relative to wild-type controls. At the end of gestation this difference was even more striking as the intestine of *Shh* and *Ihh* null embryos completely failed to expand (Mao, Kim et al. 2010). However, the primary patterning of the gut into distinct organ segments was not affected. They further investigated the reasons behind the phenotype and showed that double null embryos present a dramatically reduced number of mesenchymal progenitors necessary for normal endodermal-mesenchymal interplay in the mammalian gut. Vasculature integrity was intact and no significant change in the levels of necrotic and apoptotic markers were detected in these embryos, suggesting that the decreased number of mesenchymal progenitors was a primary effect of absent Hh signaling. Considering that intestinal smooth muscle cells are derived from local mesenchymal progenitors, the authors also investigated the effect of absent expression of Shh and Ihh for smooth muscle development (Mao, Kim et al. 2010). They showed that double mutant embryos do not express smooth muscle α actin (SMA), and have impaired smooth muscle differentiation due to reduced expansion of the early mesenchymal progenitor pool. Taken together, these results showed that Hh signaling is a mitogenic factor necessary for expansion of gut mesenchymal progenitors including those of the smooth muscle compartment, and is thus, essential for embryonic gut development.

Notch

The *Notch* gene encodes for a transmembrane receptor protein known to activate a signaling cascade critical for normal embryonic development and tissue homeostasis (D'Souza, Meloty-Kapella et al. 2010). In mammals there are four Notch receptors (Notch 1-4), which upon ligand binding, trigger a series of proteolytic cleavages to release the intracellular domain of Notch (NICD), a biologically active signal transducer (Kopan and Ilagan 2009). NICD translocates to the nucleus and binds to a transcription factor, recombining binding protein suppressor of hairless (RBP-J), and to a co-activator, Mastermind, activating transcription of several target genes involved in cell proliferation or apoptosis (Collu, Hidalgo-Sastre et al. 2014). The Notch signaling pathway is widely used in different cell types and cellular processes. Therefore, it is not surprising that defects in this pathway are associated with developmental disorders and cancers (Koch and Radtke 2007, Turnpenny, Alman et al. 2007).

In the intestine, genetic analyses of zebrafish and mouse mutants have revealed a requirement of the Notch signaling pathway for cell expansion and proper lineage allocation of epithelial progenitors (Crosnier, Vargesson et al. 2005, Fre, Huyghe et al. 2005). In addition, this pathway is particularly active in the developing intestinal mesenchyme, specifically in sub-epithelial fibroblasts, and its dysregulation was shown to play a role in intestinal elongation. Selective disruption of the Notch pathway in the mesenchyme by the use of a conditional knockout mouse for the effector gene *RBP-J*, or constitutive activity of Notch by forced expression of NICD, led to a reduction of intestinal length (Kim, Kim et al. 2011). In both cases, a progressive loss of sub-epithelial fibroblasts was detected, but the phenotype observed was more severe in the presence of constitutive Notch activation. A close inspection of the mutant embryos showed that despite similarities in the phenotype, the mechanisms leading to a reduction of sub-epithelial fibroblasts were different in each case. While the absence of Notch signaling led to reduced proliferation of sub-epithelial fibroblasts but no significant increase in apoptosis, over-activation of this pathway resulted in apoptosis, and consequently cell death (Kim, Kim et al. 2011). These observations showed a critical role of Notch signaling for the development of the intestinal mesenchyme, and revealed that tight regulation of this pathway is needed to fine-tune its effects during intestinal elongation.

Wnt5a

The Wnt proteins are secreted glycoproteins known to activate various intracellular signaling cascades upon binding to their receptors, Frizzled (Fzd) and/or transmembrane co-receptors, such as the lipoprotein receptor-related protein 5/6 (Lrp5/6), Ror2, and the related to receptor tyrosine kinase (Ryk) [89]. Wnt signaling pathway is divided in two general categories - canonical and non-canonical - based on transcriptional involvement of β-catenin. Independent of the pathway activated, Wnt signaling is essential for diverse processes, including cell fate, proliferation, differentiation, migration, polarity and asym-

metric cell division (Clevers and Nusse 2012).

Wnt5a is one of the ligands involved in the activation of the non-canonical Wnt signaling pathway. It is known to bind Ror2 and is required for normal embryogenesis, playing a pivotal role in the elongation process of several organs, including the small intestine (Reya and Clevers 2005, Green, Kuntz et al. 2008, Cervantes, Yamaguchi et al. 2009). Loss of either Wnt5a or Ror2 expression in mice was reported to lead to a dramatic shortening of the small intestine (Cervantes, Yamaguchi et al. 2009, Yamada, Udagawa et al. 2010, Bakker, Raghoebir et al. 2012). Accordingly, expression levels of Wnt5a and Ror2 have been shown to peak during the critical period of midgut elongation in mice, which is between E10.5 and E13.5 [93]. Moreover, Bakker et al showed that Wnt5a expression during mouse intestinal embryogenesis is tightly orchestrated in certain time frames, and overexpression or loss of expression during the critical period of midgut elongation (before E13.5) leads to intestinal elongation defects (Bakker, Raghoebir et al. 2012). It is still not clear how Wnt5a and Ror2 regulate intestinal elongation, but at the cellular level, they are known to be involved in cell migration and proliferation (He, Xiong et al. 2008). Therefore, a decrease in cell proliferation and migration induced by the absence of Wnt5a or Ror2, might lead to a shortened small intestine.

Wnt5a is also known to activate the Wnt/Jun N Kinase (JNK) signaling pathway, defined as the planar cell polarity pathway, as it mediates orientation of the cell movements during development (Qian, Jones et al. 2007). Previous studies showed that JNK plays an irreplaceable role in preserving endoderm cell-cell adhesion and maintaining the stability of microtubules, which are required for normal intestinal elongation. However, activation of this pathway has to be tightly regulated for proper GI tract development. The secreted Frizzled-related protein 1 (Sfrp1) has been shown to directly interact with Wnt5a, playing a key role regulating its activity (Matsuyama, Aizawa et al. 2009). Sfrp1 together with Sfrp2 and Sfrp5, belong to the type 1 subfamily of Sfrp antagonists of the Wnt signaling [96]. Since there is functional redundancy between the members of each subfamily (Satoh, Matsuyama et al. 2008), loss of Sfrp1, 2 and 5 in a compound mutant mouse model (*Sfrp1*$^{-/-}$ *Sfrp2*$^{-/-}$ *Sfrp5*$^{+/-}$) led to dysregulation of the Wnt5a signaling pathway detected by elevated levels of phosphorylated c-Jun in the epithelium of the small intestine. As a consequence, mutant embryos exhibited a shorten body axis and unsurprisingly, a dramatic reduction in gut length (Matsuyama, Aizawa et al. 2009). On the other hand, absence of active JNK signaling led to dissociation of endoderm cells and perturbation of the cytoskeleton due to microtubule destabilisation, resulting as well, in an impairment of gut elongation (Dush and Nascone-Yoder 2013).

Hlx

The divergent murine homeo box gene *Hlx* encodes for a transcription factor that during embryogenesis is mainly expressed in tissues of mesodermal origin, such as visceral mesenchyme, skeletal myoblasts, sclerotome and limb mesenchyme (Allen, Lints et

al. 1991, Lints, Hartley et al. 1996). *Hlx* expression is detected around E9.5 of mouse development in the midgut and hindgut, and from E10.5 to E12.5 in the liver, gall bladder, and gut. Homozygous disruption of *Hlx* led to a dramatic impair of visceral organogenesis specifically of the liver (only reached 3% of its normal size) and the gut (only reached a quarter of its normal length), suggesting that neither of these organs were able to go through the dramatic expansion characteristic of normal organogenesis in the absence of Hlx [74]. A detailed examination of the *Hlx*$^{-/-}$ embryos showed a normal appearance with characteristic midgut and hindgut structures at E10.5. However, from E11.5 to E14.5 the extensive looping and midgut umbilical hernia observed in wild-type embryos was absent in the *Hlx*$^{-/-}$ mice. These mutants exhibited only a single intestinal loop at E13.5 and E14.5, but the mesenchyme became normally stratified into histologically distinct layers [74]. The mechanism by which Hlx controls visceral organogenesis is still not well understood. Henscht et al suggested that Hlx controls a mesenchymal-epithelial interaction critical for liver and gut extension. This interaction is likely mediated by mitogenic factors or matrix components secreted by the mesenchyme, and is required for proliferation of both liver and gut epithelia (Hentsch, Lyons et al. 1996).

Concluding remarks

Genetic studies have identified two genes, *CLMP* and *FLNA*, that when mutated lead to the development of CSBS. These findings have improved the quality of genetic counseling for CSBS patients and made prenatal diagnostics possible. However, the mechanisms by which CLMP and FLNA lead to CSBS are still unknown. Mouse models have also identified several other genes that are instrumental for intestinal elongation. All these genes encode for proteins that play an instrumental role in major signaling pathways required for embryonic development. However, the involvement of these genes in the development of CSBS has not yet been reported. Based on our current knowledge, it is difficult to place CLMP and FLNA in one of the complex networks involved in intestinal elongation, but it is tempting to speculate that CLMP and FLNA can either control or be controlled by one (or more) of these signal transduction pathways. All these findings provide new insights into CSBS pathogenesis, and represent a first step to identify major processes required for intestinal development and elongation.

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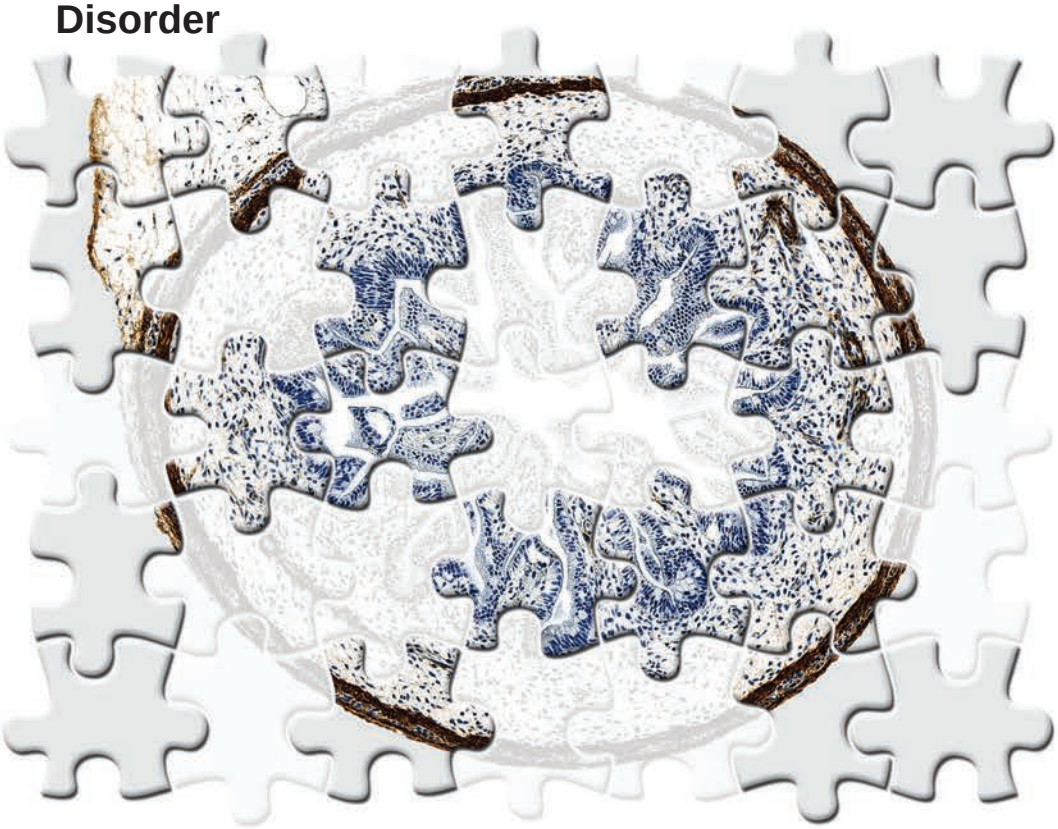
CHAPTER 6

Genetic Screening of Congenital Short Bowel Syndrome Patients Confirms *CLMP* as the Major Gene Involved in the Recessive Form of This Disorder



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ABSTRACT

Congenital Short Bowel Syndrome (CSBS) is an intestinal pediatric disorder, where patients are born with a dramatic shortened small intestine. Pathogenic variants in *CLMP* were recently identified to cause an autosomal recessive form of the disease. However, due to the rare nature of CSBS, only a small number of patients have been reported to date with variants in this gene. In this report, we describe novel inherited variants in *CLMP* in three CSBS patients derived from two unrelated families, confirming *CLMP* as the major gene involved in the development of the recessive form of CSBS.

INTRODUCTION

During embryogenesis, the small intestine experiences major growth expanding several times the total length of the whole body (Moore and Persaud 2003). This is a complex process that starts around the fifth week of human development, when the midgut forms a simple linear tube that runs down the midline of the embryo, and is completed by the twentieth week of development. Several pathways have been reported to play a role in intestinal elongation (van der Werf, Halim et al. 2015), but to date the molecular mechanism(s) responsible for this process remains unclear.

In Congenital Short Bowel Syndrome (CSBS) intestinal elongation is impaired, and patients are born with a substantially shortened small intestine (approximately 50 cm in length instead of 250 cm in a normal counterpart) (Hamilton, Reilly et al. 1969). It is a rare disorder for which no cure is available. Total parenteral nutrition is required for long-term survival, but despite considerable efforts to improve treatment, most patients die of starvation or sepsis within the first few days of life (Howard, Ament et al. 1995). CSBS was first described by Hamilton *et al.* in 1969, and it has always been considered to have a genetic cause (Hamilton, Reilly et al. 1969). However, it was only recently that two genes were identified as the cause of CSBS: *CLMP* and *FLNA* (Van Der Werf, Wabbersen et al. 2012, van der Werf, Sribudiani et al. 2013). *CLMP* encodes for the Coxsackie-and adenovirus receptor-like membrane protein, an adhesion molecule that co-localizes with tight junction proteins, but whose function is still unknown (Raschperger, Engstrom et al. 2004). *FLNA* encodes for Filamin-A, an actin binding protein known to regulate cell shape and to control cell signalling and migration (Robertson 2005). Pathogenic variants present in *CLMP* and *FLNA* have been linked to the recessive and X-linked forms of CSBS, respectively.

Here, we report the genetic screening of three CSBS patients derived from two unrelated families, and describe the identification of three novel variants in *CLMP*.

MATERIALS AND METHODS

Patient information

In this study, three female patients diagnosed with CSBS were investigated (Table 1). Two of these patients were siblings of consanguineous descent (P1 and P2). The third patient (P3) was previously described, when a *de novo* translocation was found between chromosome 2 and 11 (46,XX,t(2,11)(q32.2,p12)) (De Backer, Parizel et al. 1997). However, no gene was identified as the causative factor at that time. A complete description of the patients can be found in supplementary data. Written informed consent was given by the two families reported, and ethical approval was obtained from the Erasmus Medical Center ethical committee (Medisch Ethische Toetsings commissie - METc 2009/364, ABR nr: NL31708.042.10).

Table 1: Clinical and molecular features of the CSBS patients included in this study.

	P1	P2	P3
Gender	female	female	female
Age of diagnostic	3 months	38 days	1 week
Ethnicity	Iranian	Iranian	Caucasian
Consanguinity	+	+	No
Malrotation	+	+	+
Length of the small intestine (cm)	76	26	50
Additional features	Intestinal dysmotility UPJO*	Intestinal dysmotility UPJO*	Very mild mental retardation
***CLMP* variants**	c.508C>T; p.(R170*)	c.508C>T; (p.R170*)	c.410G>A; p.(C137Y) c.29-2A>G
Zygosity	Homozygous	Homozygous	Compound heterozygous

*UPJO: Ureteropelvic junction obstruction

Genetic analysis

Genomic DNA was isolated from peripheral blood lymphocytes using standard methods. Exons 1-7 of *CLMP* (ENST00000448775) were amplified using 30 ng of genomic DNA, as described before[5]. PCR products were purified (ExoSap it – GE Healthcare, Eindhoven, The Netherlands), and Sanger sequencing was performed with dye labelled primers (forward and reverse; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems, Bleiswijk, The Netherlands) on an ABI 3130XL genetic analyzer. Sanger reads were analysed using SeqScape software and compared to the reference *CLMP* genomic sequence, ENSG00000166250. Genetic data was submitted to ClinVAr (http://www.ncbi.nlm.nih.gov/clinvar/) and the following accession numbers were obtained: SCV000264793 [p.(R170*)] and SCV000264792 [p.(C137Y) and (c.29-2A>G)].

Exon trapping assay

The exon trap assay was performed as previously described[5]. Primers used to amplify exon 2 of CLMP and its flanking regions (±150 bp) containing the wild type (WT) and the mutant allele, are described in supplementary table 1 (CLMPtrapF and CLMPtrapR).

Expression vectors

Generation of the expression vectors used is described in supplementary data.

Immunofluorescence

Chinese Hamster cells (CHO-K1) were cultured as described before[5]. Five hundred thousand cells were seeded in a 6-well plate, and transiently transfected with pcDNA-HA-CLMP WT or pcDNA-HA-CLMP C137Y expressing vectors, using GeneJuice (Millipore, Amsterdam, The Netherlands) as transfection reagent. Twenty four hours after transfection, immunofluorescence was performed as previously described[5]. Images were taken in a Leica (AOBS) microscope, and analysed with the Leica LAS AF Lite software.

RESULTS AND DISCUSSION

As the three patients included in this study are females, an X-linked pattern of inheritance involving *FLNA* was considered unlikely, and only *CLMP* was screened. In all patients we identified previously unreported variants in *CLMP*. None of these variants is listed in any of the available human genome variant databases.

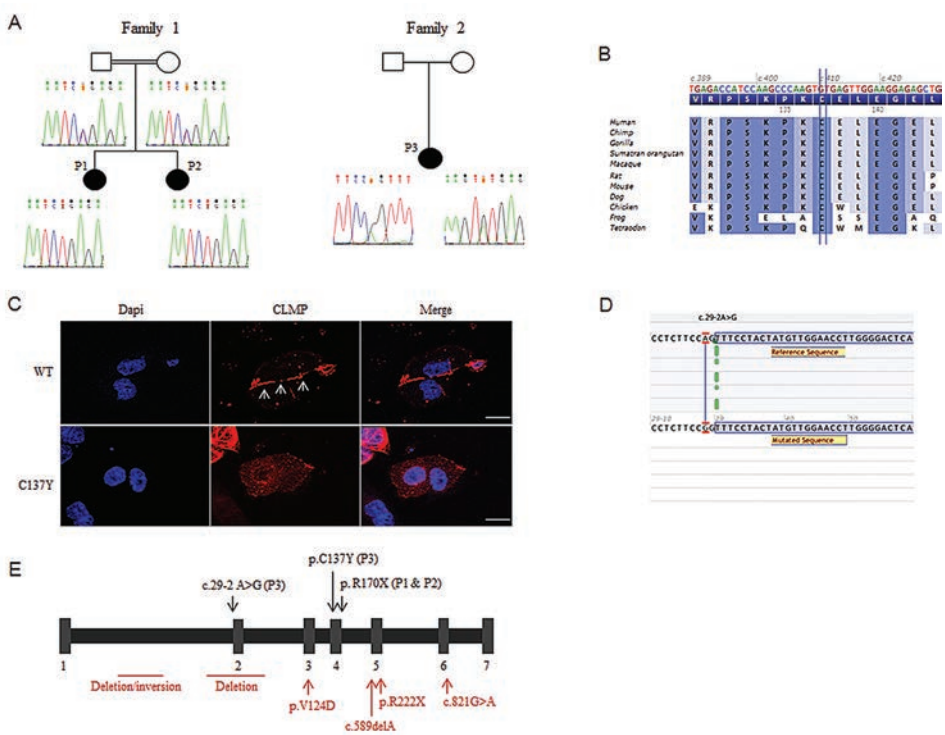

Fig.1: Genetic analysis of three CSBS patients. **A)** Pedigree of the two families included in this study with Sanger sequencing results showing the presence of homozygous or compound heterozygous variants in *CLMP*. **B)** Conservation alignments showed that the missense variant p.(C137Y) leads to a change of a cysteine residue highly conserved among vertebrates. **C)** CHO-K1 cells transfected with constructs expressing wild-type (WT) CLMP or the p.(C137Y) variant, show different cellular distribution of CLMP. While the WT protein normally localizes to the tight junctions (see arrows), the mutated protein has a punctuated distribution through the cytoplasm. Scale bars: 30µm. **D)** Splicing predictions suggested that the intronic variant identified in patient 3 likely affects normal splicing, as it is predicted to disrupt an acceptor-splicing site (c.29-2A>G). **E)** Exon trapping assay confirmed disruption of exon 2 splicing by the c.29-2A>G variant. **F)** Schematic overview of all the variants identified to date in *CLMP*. In black are the variants reported in this report, while in red are the variants previously described. Figures 1B and 1D were generated using Alamut prediction software.

Patients P1 and P2 possess a homozygous nonsense variant in exon 4 [c.508C>T; p.(R170*)], leading to the appearance of an early stop codon (Fig.1A). This nonsense variant was inherited from the parents, who were found to be heterozygous. Patient P3 is compound heterozygote, and has two heterozygous variants in *CLMP*. Although we suspect that both variants were inherited from the parents, we were unable to

investigate segregation of the variants in the family due to unavailability of parental DNA. In this patient a missense variant located in exon 4 was identified leading to an amino acid substitution [c.410G>A; p.(C137Y)], together with a possible splice site variant located two base pairs upstream of exon two (c.29-2A>G) (Fig.1A).

Prediction programs used to infer pathogenicity (PolyPhen-2, MutationTaster, SIFT, Human Splicing Finder and Alamut Visual) showed that both variants are likely to disturb protein function. The C137Y variant is located in a highly conserved region present in all mammals (Fig.1B), and affects the cellular localization of CLMP to the tight junctions (Fig.1C). The c.29-2A>G variant is predicted to disrupt normal splicing of exon 2 of *CLMP* (Fig.1D), and we were able to confirm this effect using an exon trapping *in vitro* assay (Fig. 1E). Considering that both variants functionally affect CLMP, we believe that the *de novo* translocation previously identified in this patient[9] is an independent event unrelated to CSBS.

Currently, only seven patients have been described carrying pathogenic variants in CLMP[5]. All the variants reported were loss of function, leading to the absence of CLMP, or to the expression of a mutant protein with a disrupted function (Fig.1F). In this report, we expand the mutational spectrum of *CLMP* variants involved in CSBS, consolidating the involvement of this gene in the development of the recessive form of this disease. We also show that it might be worthwhile to screen previously described patients for whom an X-linked pattern of inheritance has been disregarded, for the presence of variants in *CLMP*, as this might shed some light on the genetic basis of the disease in these cases. To date, the role of *CLMP* in intestinal elongation is still largely unknown, making it difficult to determine the molecular mechanisms underlying the development of CSBS. We believe that the results here described reinforce the need to further study the role of *CLMP* in intestinal elongation, as this might contribute for the development of new therapeutic strategies for CSBS patients.

Acknowledgments: The authors would like to thank all families involved in this study. The authors would also like to thank Katherine MacKenzie for technical assistance with the confocal microscope. In addition, we would like to dedicate this short report in memory of the paediatric gastroenterologist involved in the diagnosis and care of one of the patients described, Dr. André Deprettere. This study was supported by a grant from the Stichting Sophia Kinderziekenhuis Fonds (MMA and RMWH), a Mrace grant from the Erasmus University Medical Center (RMWH), and by the Newlife Foundation as well as the MRC (UK, AHC).

Conflict of Interest: The authors have nothing to disclose.

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Supplementary Material and Methods

Patient information

Patients P1 and P2 were both admitted to the hospital shortly after birth due to bilious vomiting and diarrhea. Patient P2 had a distended abdomen, but an otherwise normal physical exam. Abdominal x-ray showed air fluid level, and transit of small bowel showed a distended loop. A final diagnosis of malrotation and the presence of a congenital short bowel measuring 76 and 26 cm in P1 and P2 respectively, was made by laparotomy.

Patient P3 was admitted to the hospital in the first days after birth due to diarrhea, vomiting and poor weight increase, as previously described[9]. Radiological examination showed malrotation and the presence of a congenital short bowel. The final diagnosis was made by exploratory laparotomy that confirmed the presence of a small intestine measuring only 50 cm in length.

All patients included in this study are still alive at the time of publication, and for all of them additional symptoms have been reported. Patients P2 and P3 presented with intestinal dysmotility and ureteropelvic junction obstruction, while patient P3 was diagnosed with mild mental retardation. Mild dysmorphic features were also initially reported for patient P3[9]. However, they were later disregarded, as they resulted from malnutrition during the neonatal period.

Expression vectors

pcDNA-HA-CLMP WT was generated by PCR amplification of CLMP using the primers CLMP-HAF and CLMP-HAR (supplementary table 1). BamHI-NotI restriction sites included respectively in the forward and reverse primers (see underlined nucleotides in the primer sequence), were used to clone CLMP into pcDNA-HA vector. pcDNA-HA-CLMP (C137Y) was originated by site directed mutagenesis according to the manufacturer's instructions (QuickChange II site-directed mutagenesis kit, Agilent technologies). Primers used to insert the C137Y variant were the CLMPmutF and CLMPmutR (Supplementary table1; the mutated nucleotide is underlined in the primer sequence). The following mutagenesis, the entire CLMP insert was checked by Sanger sequencing.

Supplementary Table 1: Primers used in this study.

Primer name	Sequence (5'-3')
CLMPHAF	CATATAGGATCCATGTCCCTCCTCCTTCTCCTC
CLMPHAR	CATATAGCGGCCGCTCAGACCGTTTGGAAGGCTCTG
CLMPmutF	CCATCCAAGCCCAAGTATGAGTTGGAAGGAGAGC
CLMPmutR	GCTCTCCTTCCAACTCATACTTGGGCTTGGATGG

CHAPTER 7

The Long Filamin-A Isoform is Essential for Intestinal Elongation and Function

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Work in progress

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ABSTRACT

Pathogenic variants in Filamin A (*FLNA*) have been associated with a wide spectrum of disorders, and more recently with an X-linked form of Congenital Short Bowel Syndrome (CSBS). Since the role of FLNA in intestinal development is largely unknown, we performed expression studies at different human fetal stages, and a combination of *in vitro* and *in vivo* assays, to understand the molecular mechanisms associated with *FLNA* variants underlying CSBS. Our results show that *FLNA* is expressed in the muscular layer of the small intestine from early human fetal stages. Moreover, *FLNA* variants associated with CSBS block expression of one of its two isoforms, possibly explaining why these patients only have intestinal complaints. Finally, by generating a transgenic zebrafish line using TALEN, we show that the long flna isoform is required for intestinal elongation and function. *flna* mutant fish have stastistically significant reduction of gut length, but are otherwise phenotypically indistinguishable from wild-types. Moreover, intestinal transit assays showed an increased transit time for the transgenic zebrafish. Taken together our results bring new insights to the role of FLNA during intestinal development and elongation, and contribute to a better understanding of CSBS pathogenesis.

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 (Onoprishvili, Ali et al. 2008, Nakamura, Stossel et al. 2011). FLNA has several known binding partners, including ion channels, receptors, intracellular signaling molecules and transcription factors (Nakamura, Stossel et al. 2011). Therefore, it is not surprising that loss of function variants affecting the normal expression levels of FLNA result in a wide range of abnormalities associated with different human disorders (Robertson 2005, Kyndt, Gueffet et al. 2007, Bernstein, Bernstein et al. 2011, Oegema, Hulst et al. 2013, van der Werf, Sribudiani et al. 2013).

Recently, *FLNA* was associated with the development of an X-linked form of Congenital Short Bowel Syndrome (CSBS), when a two base pair deletion (c.16-17delTC) was identified in three male patients born with a dramatically shortened small intestine (van der Werf, Sribudiani et al. 2013). This deletion is located in exon two of *FLNA* and results in a frameshift with the subsequent appearance of an early stop codon a few base pairs later. In a previous study, a similar deletion (c.65-66delAC) was identified in two related male patients diagnosed with Chronic idiopathic intestinal pseudo-obstruction (CIIP) associated with CSBS (Gargiulo, Auricchio et al. 2007). Considering that in both these studies intestinal abnormalities were reported without other major congenital abnormalities, it was suggested that two FLNA isoforms exist, and in the presence of the two base pair deletion, only the expression of the long isoform was affected (Gargiulo, Auricchio et al. 2007). This would explain why these deletions are not lethal for males *in utero* and that they only have intestinal complaints. However, the role of FLNA in intestinal development is largely unknown, making it difficult to pinpoint the pathogenic mechanism involved in the development of CSBS.

Here we bring new insights into the role of FLNA, as we further investigate the existence of two FLNA isoforms and provide evidence that the long FLNA isoform is required for intestinal elongation.

MATERIALS AND METHODS

Immunohistochemistry

Paraffin-embedded human small intestinal specimens from controls were collected from the department of Pathology, Erasmus University Medical Center. Slides were immunostained using a specific antibody against human FLNA dilution 1:1000; Eurogentec). After incubation with the primary antibody, samples were treated with anti-rabbit secondary antibody and the avidin-biotin-complex with peroxidase. 3,3'-diaminobenzidine (DAB) chromogen (Ventana) was applied, and hematoxylin was used as counterstain. Images were acquired using the Nanozoomer 2.0-HT (Hamamatsu Photonics), and analyzed with the Nanozoomer Digital Pathology (viewer software (Hamamatsu Photonics).

Expression vectors and site-directed mutagenesis

pAAV2.1-CMV-EGFP-FLNA wild-type (WT) and pAAV2.1-CMV-EGFP-FLNA mutant 1 (c.65-66delAC) constructs were kindly provided by Dr. Alberto Auricchio from the University of Napoli. pAAV2.1-CMV-EGFP-FLNA mutant 2 construct containing the other 2 base pair deletion (c.16-17delTC), was made by site–directed mutagenesis according to the instructions provided by the manufacturers (Stratagene), using the WT construct. The following primers were used: FLNAmutF: GAGTAGCTCCCACTCGGGCGGGCCAGAG and FLNAmutR: CTCTGGCCCGCCCGAGTGGGAGCTACTC.

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. Cells were incubated at 37^0C in the presence of 5% CO2. For transient transfection, 300,000 cells were cultured in a 6 well plate. Twenty-four hours after, cells were transfected with 1μg of plasmid DNA using GeneJuice (Millipore) 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) and 1X protease inhibitors (Roche). Cell lysate preparation, protein quantification and Western blot analysis were performed as described before (Halim, Hofstra et al. 2016). The following antibodies were used: HA (Cell Signaling) and actin (Santa Cruz Biotechnologies). Secondary antibodies used were IRDye 800CW Goat anti-mouse and IRDye 680RD Goat anti-Rabbit (Li-Cor).

RNA isolation, cDNA preparation and RT-PCR

Zebrafish embryos collected at different embryonic stages were lysed in Trizol, and RNA isolation was performed with the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. 1 μg of RNA was used for cDNA preparation with the iScript cDNA Synthesis Kit (Bio-Rad). A Reverse transcriptase PCR was performed using 100 ng/μl of cDNA as template and a standard protocol. The primers used for amplification were: flnazebF: ATGCCCGCTACAGAGAAAGA; flnazebR: ATGGGCATGGAGATGGAATA; actinzebF: TGAAGTACCCCATCGAGCAGG; actinzeb R: GCAAGATTCCATACCCAAGAAGG.

Whole mount *in situ* hybridization

RNA isolated from 48 hours post fertilization (hpf) embryos was used as a template for cDNA using a One-Step RT-PCR kit (Qiagen). Primers used for amplification were: flnazebprobeF: TCCAAAATCACAGGTGACGA; flnazebprobeR: TACACCAGCTTCTGCCCTCT. Amplified bands were subcloned into the TOPO TA PCRII cloning vector (ThermoFisher). Digoxigenin labelled anti-sense probes (Roche) were generated by linearizing the plasmids with *Not*I (NEB), then transcribed with SP6 polymerase (Roche). *In situ* hybridization was performed

on staged zebrafish embryos as previously described (Thisse, Thisse et al. 1993).

Generation of the *flna* long isoform knockout zebrafish

TALENs were designed in order 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 embryos collected from wild-type zebrafish crossings. The Tupfel long fin (TL) zebrafish line was used for all *in vivo* experiments described in this manuscript. All injected embryos were raised to adulthood and crossed to generate F1. F1 transgenic embryos 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

Tail clip of transgenic zebrafish was performed followed by DNA isolation using a mixture containing Tris-HCl (pH9.0), KCl, Triton X-100, and protease K (Sigma Aldrich). 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 and the following primers: flnazebF: CACCCGAATTGCAGTTTCTC and flnazebR: CATCAAGCTGGTCTCCATTG. The PCR product was subjected to restriction digestion with *Nci*I (New England Biolabs) for 1h at 37^0C, and submitted to Sanger sequencing, which was performed with dye labelled primers (flnazebF and flnazebR; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems) on a ABI 3130XL genetic analyzer. Sanger reads were analysed using SeqScape software.

Intestinal length measurements in zebrafish

Zebrafish embryos were collected after fertilization and individually placed in a 24-well plate. Each larvae was imaged every twenty-four hours under the microscope (Leica DFC550) for four days. Gut measurements were made using Fiji software, and were based on the distance between mouth and anus. All embryos were genotyped at the end of the experiment. Statistical significance was calculated using a two-paired ttest.

Intestinal transit assays in zebrafish

Seven day 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 ul of water. After two hours, the larvae were anesthetized with Tricaine and were individually checked under the microscope (Leica DFC550) for the presence of fluorescence. Individual larvae were placed in separate wells of a 24-well plate, and

examined once again under the microscope twenty-four hours after feeding. All larvae were genotyped at the end of the experiment.

RESULTS

FLNA is expressed in the muscle layer of the 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 CSBS affects elongation of the small intestine, 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, and the inner circular layer and outer longitudinal layer of the muscularis propria (Fig. 1).

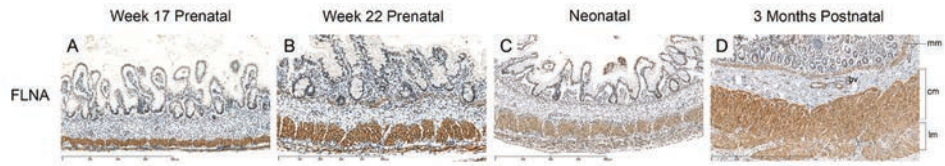

Fig.1: FLNA expression is localized in the muscular layer of the human small intestine since early fetal stages. Immunohistochemistry performed in human intestinal specimens collected at different developmental stages shows that FLNA is expressed in the muscular layer of the small intestine. mm: muscularis mucosa; cm: circular layer of the muscularis propria; lm: longitudinal layer of the muscularis propria.

The two base pair deletions found in patients with CSBS disrupt expression of the long FLNA isoform

Considering that the two base pair deletions identified in CSBS are located between two methionine residues (Fig. 2A), it was hypothesized that two FLNA isoforms exist, and that in the presence of these deletions only the expression of the long isoform was affected. A previous study already confirmed that the c.65-66delAC deletion identified in two related male patients diagnosed with CIIP associated with CSBS, leads to expression of only one FLNA isoform (Gargiulo, Auricchio et al. 2007). The authors observed this effect by introducing the 2bp deletion in a mini-gene construct where only exon 2 of *FLNA* (WT) was present fused to a HA-tag. Here, we made use of the same WT construct to evaluate the effect of the c.16-17delTC deletion. We used the c.65-66delAC construct as control. By expressing the three constructs (WT, c.65-66delAC, and c.16-17delTC) in HEK293 cells we observed that the WT construct produced 2 bands, suggesting that two different FLNA isoforms are normally expressed (Fig. 2B). In the presence of both deletions, c.65-66delAC, and c.16-17delTC, expression of only the lower band was detected (Fig. 2B).

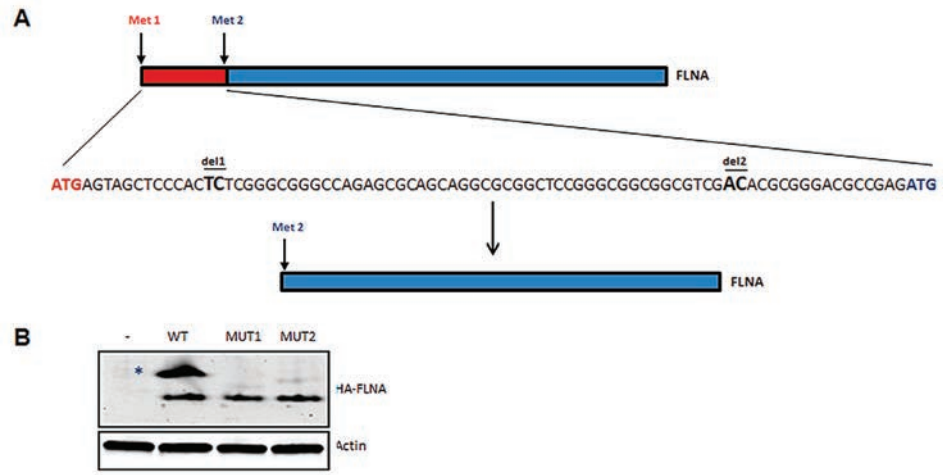

Fig.2: Two FLNA isoforms are normally expressed from a single transcript. A) Schematic representation of FLNA where two isoforms are depicted: long FLNA isoform (red + blue), and short FLNA isoform (blue). Amplification of the region between the two methionine residues show DNA sequence of FLNA. In the presence of the 2-base pair (bp) deletions identified in CSBS patients (del1: c.16-17delTC; del2: c.65.66delAC), an early stop codon appears leading to the expression of only the short isoform (blue). B) Western blot analysis using a mini gene construct expressing wild-type (WT) and mutant [Mut1(c.65-66delAC); Mut2 (c.16-17delTC)] versions of FLNA fused to an HA-tag, show that while in the WT situation two bands are normally expressed, in the presence of either one of the deletions identified in CSBS patients, only one band is present.

In light of these results, we concluded that in the presence of the c.16-17delTC deletion, as well as the c.65-66delAC, only the short FLNA isoform is expressed.

Generation of the *flna* long isoform knockout zebrafish

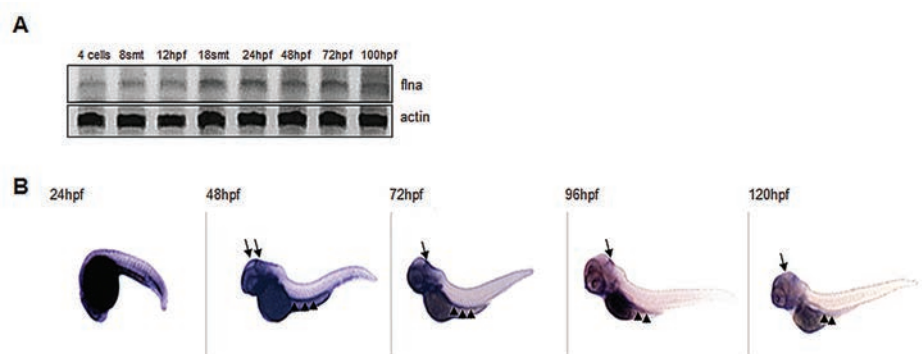

Fig.3: *flna* expression is detected in the zebrafish from early embryonic stages. A) RT-PCR performed in RNA isolated from zebrafish embryos at different embryonic stages shows that *flna* transcripts are present from as early as the 4 cell stage, and its expression seems to increase during development. B) *In situ* hybridization shows that *flna* is highly expressed in the brain and the gut of the zebrafish at different stages of development. smt - somites; hpf - hours post fertilization.

To evaluate the effect of the long FLNA isoform on the development and elongation of the intestine, we used the zebrafish as an *in vivo* model. We started by determining where and when flna was expressed, and for that the expression levels of flna were analysed by RT-PCR at different embryonic stages. Our results showed that flna is expressed in early embryonic stages starting at the four cell stage, and that its expression seems to increase during development (Fig. 3A). *In situ* hybridization also showed that flna is abundantly expressed in the brain and intestine of the zebrafish at all stages analyzed (Fig. 3B).

To generate the transgenic zebrafish line where only the short isoform is expressed, 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 have successfully annealed, we used this enzyme to differentiate between wild-type (WT) and transgenic fish (Fig.4A). A deletion of six base pairs followed by 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 (Fig. 4B). This out-of-frame deletion followed by an insertion resulted in the appearance of an early stop codon in the end of exon 1, leading to a similar effect to the deletions described for CSBS patients.

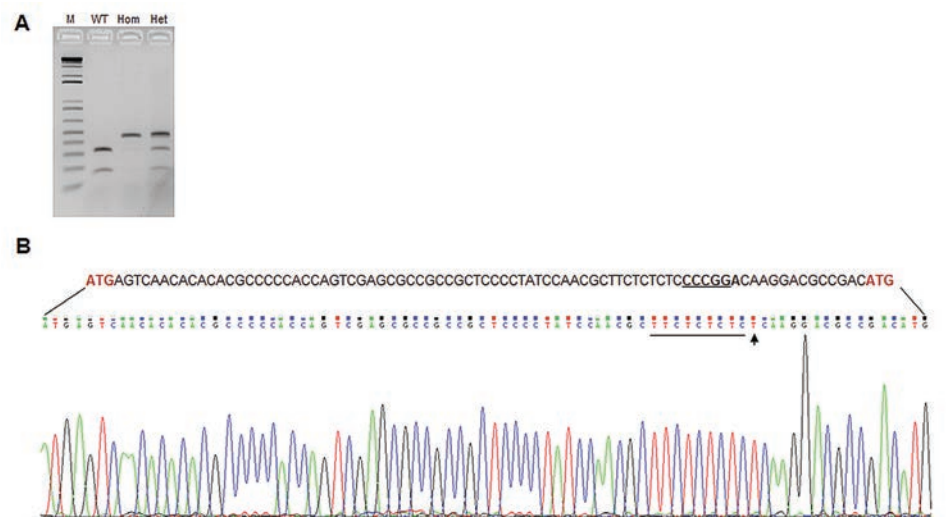

Fig.4: Generation of the long *flna* knockout zebrafish line. A) Genotype of the transgenic *flna* zebrafish generated with TALENs. Wild-type (WT) and transgenic fish can be distinguished after treatment with *Nci*I restriction enzyme. In the WT situation a restriction site for *Nci*I is present between 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).

The long FLNA isoform is required for intestinal elongation

Since the main feature of CSBS patients is a dramatically shortened small intestine, we

investigated the effect of the disruption of the long FLNA isoform on the total length of the intestine in zebrafish. We crossed the *flna long isoform*$^{-/-}$ fish to generate offspring where the out-frame deletion plus insertion was present in a homozygous state. Unlike humans, the zebrafish does not have X and Y chromosomes, and two copies of *flna* are normally present. Therefore, *flna long*$^{+/-}$ fish were also crossed with WT TL in order to evaluate the effect of the out-of frame variant in a heterozygous state. 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 statistically significant shorter intestine than the WT (0.318 mm ± 0.014 vs. 0.334 mm ± 0.015, respectively) (Fig. 5A).

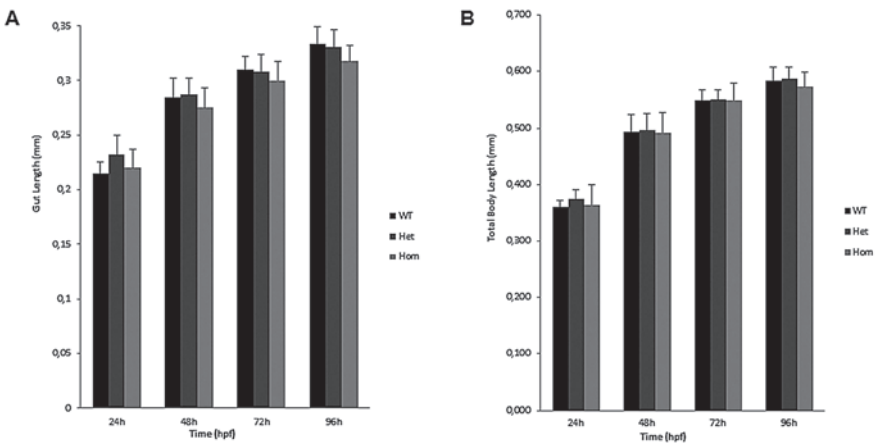

Fig.5: *flna long*$^{-/-}$ zebrafish embryos have a shorter small intestine. A) Measurement of the intestinal length of a total of 150 WT and transgenic fish show that *flna long*$^{-/-}$ (hom) embryos have significantly shorter intestine when compared to WT and *flna long*$^{+/-}$ (het). No difference was observed between WT and *flna long*$^{+/-}$ (het) embryos. B) Measurement of the total body length of WT and transgenic zebrafish reveals no difference between WT, *flna long*$^{-/-}$ (hom) and *flna long*$^{+/-}$ (het) embryos. hpf - hours post fertilization. *p<0,05; ** p<0,006; ***p<0,000003.

However, 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) (Fig. 5A). In order to exclude 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 zebrafish was also measured every day for four days. Our results showed that homozygous, heterozygous and WT zebrafish were undistinguishable in total body length (0.573 mm ± 0.026; 0.587 mm ± 0.020 and 0.583 mm ± 0.025, respectively) (Fig. 5B).

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

CSBS patients carrying the c.65-66delAC deletion were also diagnosed with CIIP (Gargiulo *et al.*, 2007). To investigate whether the intestinal obstruction experienced by these patients might be related to the expression of the long FLNA isoform, we performed intestinal transit assays using the *flna long isoform*$^{-/-}$, *flna long isoform*$^{+/-}$, and WT zebrafish

larvae. For these studies, 7 day old larvae were fed for two hours with fluorescently labelled larval feed, and imaged to select the ones that showed fluorescence in their intestinal bulb (Fig. 6A). Twenty-four hours after, these larvae were imaged again and we observed that, while almost all WT and heterozygous fish no longer had fluorescence within the GI tract (6 out of 25 ; 24% for WT and 4 out of 24 ; 16,6% for *flna long isoform*$^{+/-}$), for a substantial amount of homozygous fish (15 out of 35 ; 42,8%) fluorescence was still present in the gut (Fig.6A, 6B).

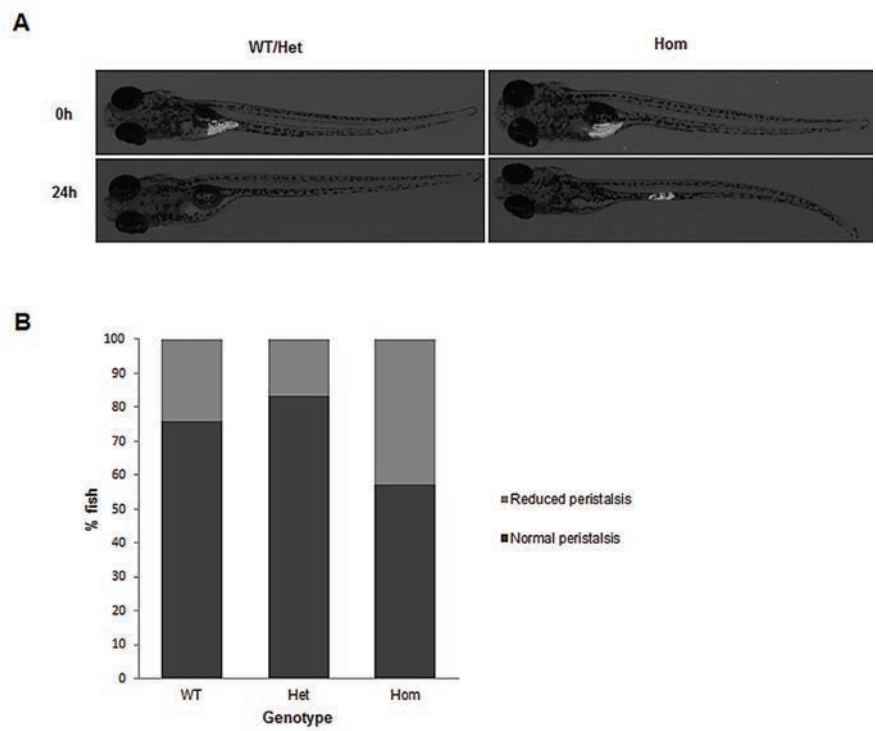

Fig.6: Intestinal transit assays show that *flna long*$^{-/-}$ larvae have reduced intestinal motility. A) WT, *flna long*$^{+/-}$ and *flna long*$^{-/-}$ larvae fed for two hours with a mixture of powdered larval feed and fluorescence microspheres, were screened for the presence of fluorescence in the intestinal bulb (see arrows). Positive larvae were separated and imaged again 24 hours after feeding. While most of the WT and heterozygous larvae no longer had fluorescence signal in the gut, a substantial number of *flna long*$^{-/-}$ larvae still had fluorescence (see arrow). B) Quantification of the number of zebrafish larvae for which fluorescence was detected in the intestinal tube 24 hours after feeding. 43% of the *flna long*$^{-/-}$ (hom) larvae have delayed intestinal motility when compared to WT (24%) and *flna long*$^{+/-}$ (17%; het) fish. * intestinal bulb.

This result shows that intestinal transit is delayed in the homozygous mutant fish (it is only delayed in some of the homozytoes – you need to say something about that), and suggests? that the long isoform of flna is required for normal intestinal peristalsis.

DISCUSSION

FLNA has been extensively studied since loss-of-function variants in its coding gene are generally lethal in males, and have been associated with several disorders, such as periventricular nodular heterotopia (OMIM 300049) (Poussaint, Fox et al. 2000, Sheen, Dixon et al. 2001), otopalatodigital syndrome type I (OMIM 311300) (Robertson, Twigg et al. 2003, Hidalgo-Bravo, Pompa-Mera et al. 2005) and type II (OMIM 304120) (Marino-Enriquez, Lapunzina et al. 2007), frontometaphyseal dysplasia (OMIM 305620) (Robertson, Twigg et al. 2003, Zenker, Rauch et al. 2004), and Melnick-Needles syndrome (OMIM 309350) (Parrini, Mei et al. 2015). Recently two out-of-frame deletions located between two methionines of *FLNA* were identified in five patients diagnosed with CSBS and CIIP (Gargiulo, Auricchio et al. 2007, van der Werf, Sribudiani et al. 2013). Considering that these patients mainly had gastrointestinal complaints, it was hypothesized that the deletions identified change the transcription starting site of *FLNA* to the second methionine, and result in the expression of only the short FLNA isoform (Gargiulo, Auricchio et al. 2007, van der Werf, Sribudiani et al. 2013). This hypothesis is corroborated by the results of our mini-gene assays, which showed that similar to the findings presented for the c.65-66delAC variant (Gargiulo et al. 2007), the c.16-17delTC deletion leads to loss of expression of the FLNA long isoform (Fig. 2). Based on this result it is tempting to speculate that the FLNA long isoform is required for intestinal elongation. To test this hypothesis, we generated a transgenic zebrafish line whereby the presence of an out-of-frame deletion plus insertion had a similar effect to the deletions described in CSBS patients, and led to the expression of the short flna isoform only. The length of the intestine, as well as total body length, was measured for homozygous, heterozygous, and WT zebrafish from 1-4 dpf, and we observed that while the majority of *flna long isoform*$^{-/-}$ embryos had significantly shortened intestines, there was no difference in the total body length of wt, heterozygous and homozygous fish (Fig. 5). These findings confirm the importance of the long FLNA isoform for intestinal elongation, and indicate that its function is conserved between species. To our surprise, the reduction observed in intestinal length for the *flna long isoform*$^{-/-}$ embryos is limited to 5%, which is far below the reduction observed in CSBS patients (>75%). This discrepancy can be attributed to differences between species, as the fish gut is just a straight simple 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. However, we also cannot exclude the involvement of another protein in the zebrafish with a redundant function. Previous studies have shown that three other genes are known to encode for *flna* homologs in the zebrafish, which include *flnb*, *flnca*, and *flncb*. Although the function of all flna proteins in the zebrafish is not yet clear, shared characteristics between all of the homologs suggest possible functional redundancy. Such redundancy has also been suggested to exist between human FLNA and FLNB during neuronal migration (Sheen, Feng et al. 2002). However, the reasons why such redundancy is not able to compensate for the loss of the long FLNA isoform, still needs to be elucidated. Speculation over species

specific issues may provide a reasonable explanation, but this is yet to be confirmed by further investigation on the function of FLNA and its interacting proteins in these species.
CIIP has been reported as a co-morbidity in CSBS patients with *FLNA* variants (Gargiulo, Auricchio et al. 2007, Kapur, Robertson et al. 2010). Thus, we made use of the *flna long isoform*$^{-/-}$ zebrafish to assess the effect of the deletion on intestinal motility. Data from gastrointestinal transit assays performed in WT, *flna long isoform*$^{+/-}$ and *flna long isoform*$^{-/-}$ zebrafish showed that intestinal transit time is significantly longer in the *flna long isoform*$^{-/-}$ fish (Fig. 6). This suggests that the CIIP phenotype observed in CSBS patients might well be directly related to the FLNA deletions. An involvement of the Enteric Nervous System (ENS) has been suggested to contribute to the reduced intestinal motility observed (Camilleri, Carbone et al. 1991, Pingault, Guiochon-Mantel et al. 2000). However, histological analysis performed on bowel tissues of these patients show contradictory results. While some CSBS patients have signs of ENS defects, others show abnormal gut smooth muscle. Based on our immunohistochemistry data, we clearly show that *FLNA* is expressed only in the smooth muscle cells of the human intestine during various developmental stages (Fig. 1), suggesting that expression of this protein is essential for the function of human intestinal smooth muscle cells. Since the muscular structures make up a major part of the intestine, and play an indispensable role in intestinal contractility, it is logical to hypothesise that the loss of FLNA long isoform may also cause failures in intestinal contractility. A recent study where Flna expression was specifically abolished in smooth muscle cells of an adult mouse showed that Flna is involved in signalling cascades that underlie smooth muscle contractility (Retailleau, Arhatte et al. 2016). Thus, it is very likely that a muscular defect is the cause of the intestinal obstruction diagnosed in CSBS patients with *FLNA* variants, but further investigations in the *flna long isoform*$^{-/-}$ zebrafish are currently on-going to test this hypothesis.

Besides *FLNA*, one other gene has been identified as the cause of the recessive form of CSBS, CXADR like membrane protein (*CLMP*) (Van Der Werf, Wabbersen et al. 2012). To date, no relationship between FLNA and CLMP has been found. However, FLNA is known to directly interact with actin filaments and is involved in the transport of various proteins, including receptors and tight junctions, to the cell membrane (Feng and Walsh 2004). Considering that *CLMP* encodes for a tight junction protein (Raschperger, Thyberg et al. 2006, Sze, Lee et al. 2008), it is tempting to speculate that FLNA interacts with CLMP in smooth muscle cells of the gut, and is required for the proper cellular localization of CLMP. Further studies are currently on-going to investigate a possible relationship between these two proteins in order to bring new insights into the molecular mechanisms required for intestinal elongation and functioning.

Acknowledgements
The authors thank Dr. Alberto Auricchio for kindly providing the pAAV2.1-CMV-EGFP-FLNA wild-type (WT) and pAAV2.1-CMV-EGFP-FLNA mutant 1 (c.65-66delAC) constructs.

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CHAPTER 8

Loss of function mutations in *TTC7A* cause hereditary multiple intestinal atresia in patients and disrupt intestinal lumen in zebrafish

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Work in progress

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Abstract

Hereditary Multiple Intestinal Atresia (HMIA) is a familial form of multiple intestinal atresia that often comorbid with severe immunodeficiency. Recently, mutations in *TTC7A* have been identified in patients with this disease. Further investigation has been hampered by the unavailability of animal models that recapitulate the human intestinal phenotype. We studied two sibs who were diagnosed with HMIA. Intestinal specimens from both patients were immunostained to analyze the histopathology of the intestine. Moreover, by utilizing Transcription Activator Like Effector Nucleases (TALEN) genome editing, a transgenic zebrafish model was generated. Histological and functional characterization of the transgenic zebrafish larvae was performed to further evaluate the effect of the mutations to the intestine. We identified a homozygous deletion of 13,123 bp that includes exon 2 of *TTC7A* in 2 sibs from a consanguineous family. Histopathology of patients' intestinal specimens showed a distinctive phenotype of multiple lumens that configure a sieve-like appearance, without any clear abnormalities in the muscular and neuronal structures of the intestine. Histology of zebrafish larvae, carrying an out-of-frame mutations in *ttc7a,* showed dramatic segmental narrowing of the intestinal lumen. Motility assays showed decreased gastrointestinal transit time (GIT) in the group of *ttc7a* mutant fish. We corroborate the role of TTC7A as the gene for HMIA and we show that the *ttc7a* mutant zebrafish is a suitable animal model to study the pathogenesis of intestinal atresia in human, and to elucidate normal embryogenesis of intestinal lumen formation.

Introduction

Multiple intestinal atresia (MIA) (OMIM 243150) is a rare cause of neonatal intestinal obstruction that is characterized by discontinuity of the intestinal lumen at multiple sites of the small and large intestine (Guttman, Braun et al. 1973). Some cases of MIA are familial, and often referred as hereditary MIA (HMIA). Interestingly, in most HMIA cases, immunodeficiency has been reported as a comorbidity.

Like the treatment for any other type of intestinal atresia, surgical removal of the atretic intestine, followed by anastomosis of the residual intestine is the standard treatment (Dalla Vecchia, Grosfeld et al. 1998). Despite successful surgery, post-surgical recurrence of intestinal obstruction and prolonged dysmotility are often observed in patients (Avitzur, Guo et al. 2014). This condition is worsened by the inability of patients' immune system to resist infections (Ali, Rahman et al. 2011).

Although HMIA has long been suggested as a genetic disease with an autosomal recessive pattern of inheritance (Bilodeau, Prasil et al. 2004), recently, variants in Tetratricopeptide-7A (*TTC7A*) were identified in patients with HMIA (Samuels, Majewski et al. 2013). *TTC7A* is a gene that encodes a protein known to hold an important role in normal iron haemostasis (White, McNulty et al. 2005). This important finding is then followed by in vitro studies that suggest the importance of TTC7A protein in the regulation of intestinal polarity and cell adhesion (Avitzur, Guo et al. 2014, Bigorgne, Farin et al. 2014). Nevertheless, the mechanism on how the change in intestinal polarity and cell adhesion could lead to the narrowing of intestinal lumen and immunodeficiency remains unclear.

Unfortunately, the effort to study the disease mechanism further has been hampered mainly by the unavailability of animal models, since *Ttc7* knockout mouse models do not recapitulate the phenotype in HMIA patients (White, McNulty et al. 2005, Kasahara, Shimizu et al. 2008). For example, in one of the *Ttc7* mutant mice known as the flaky skin (fsn) mice, the deficit of TTC7 protein leads to hypochromic anemia, and skin abnormalities that resemble human psoriasis (Sundberg, Dunstan et al. 1994, Helms, Pelsue et al. 2005). Beamer et al. suggested that a general impairment of iron uptake by the intestinal epithelial cells leads to the anemia (Beamer, Pelsue et al. 1995). However, none of these mouse models acquire any intestinal phenotype that resemble intestinal atresia in human, suggesting that a species-specific difference likely exists. As an alternative, zebrafish has long been used in intestinal research due to similarities in its gastrointestinal (GI) system to the GI system in mammals.

In this study we corroborate the role of TTC7A as the gene for HMIA and based on our finding in patients with HMIA, we generate and characterize a *ttc7a* knockout zebrafish model.

Materials and Methods

Human sample collection and DNA isolation

We studied two sibs from a consanguineous family who were diagnosed with HMIA and immunodeficiency at the Erasmus University Medical Center in Rotterdam, The Netherlands. Genomic DNA was isolated from peripheral blood lymphocytes using standard protocol (Chemagic DNA Blood Kit Special, Chemagen) (Perkin Elmer, Waltham, Massachusetts, USA). Based on earlier reports about the mutations in *TTC7A* in patients with HMIA and immunodeficiency, genetic screening in a diagnostic setting was performed on the two siblings.

Sanger sequencing

Sanger sequence of exons 1-20 in *TTC7A* was performed using the set of primers described in Supplementary table 1. 15ng of genomic DNA was used to amplify each exon, using a standard PCR protocol. PCR products were purified (ExoSap it – GE Healthcare, Fairfield, Connecticut, USA), and Sanger sequencing was performed with dye labelled primers (forward and reverse; Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems) on an ABI 3130XL genetic analyzer. Sequencing results were analysed using SeqScape software version 2.6 (Applied Biosystems, Foster City, California, USA).

Immunohistochemistry

Human intestinal tissues from controls and patients II.1 and II.2 were embedded in paraffin, and obtained from the Department of Pathology at the Erasmus University Medical Center. Hematoxylin and eosin staining was performed using a standard protocol. Immunostainings were performed using specific antibodies against Neurofilament (dilution 1:600; Monosan), Smooth muscle actin-α2 (ACTA2; ready to use; Dako), Tyrosin protein kinase Kit (c-Kit/CD117) (dilution 1:200; Cell Marque). All images were taken with a Nanozoomer 2.0-HT (Hamamatsu Photonics, Shizuoka, Japan), and analyzed using the Nanozoomer Digital Pathology (NDP) viewer software (Hamamatsu Photonics, Shizuoka, Japan).

Generation of *ttc7a* mutant zebrafish line

The Transcription Activator Like Effector Nuclease (TALEN) genome editing technique was used to create a double strand break in exon 3 of *ttc7a* in zebrafish. Once injected zebrafish embryos reached adulthood, genomic DNAs was isolated from the tails using standard method, and used directly to amplify exon 3 of *ttc7a*. The PCR products underwent purification (Exosapt it – GE Healthcare, Little Chalfont, UK) and Sanger sequencing with dye labelled primers using ABI 3130 XL Genetic analyzer. Sequencing results were analyzed with SeqScape software version 2.6 (Applied Biosystems, Foster City, California, USA). Heterozygous fishes with an out-of-frame deletion or insertion were then bred and crossed to create homozygous *ttc7a* out-of-frame mutants' larvae that were used in this research.

Histology of zebrafish larvae and image analysis

Adult heterozygous out-of-frame ttc7a mutants were crossed, and larvae were kept until 5 dpf. The 5 dpf larvae were then euthanized using Tricaine methanesulfonate (Sigma Aldrich, St. Louis, Missouri, USA), and tail clipped for genotyping. The larvae were fixed in 4% paraformaldehyde. After fixation, zebrafish larvae underwent serial dehydration, and embedded in paraffin. 5 µm serial transverse sections were stained with hematoxylin and eosin using standard protocol. All histological images were taken using a Nanozoomer 2.0-HT, and analyzed with Nanozoomer Digital Pathology (NDP) viewer software (Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan).

In vivo analysis of gut function

Study protocols involving the use of zebrafish larvae for *in vivo* analysis of gut function using a fluorescent pellet were approved by the Erasmus Animal Experiments Committee (Erasmus Dierexperimenteel Centrum – EDC). To make the fluorescence pellet, we use FluoSpheres® Carboxylate-Modified Microspheres, 2.0 µm, Nile Red fluorescent (535/575), 2% solids according to the manufacturer's instruction (Thermo Fisher Scientific, Waltham, Massachusetts, USA). In brief, 200 µl of FluoSpheres is mixed with 100 µg of fish pellet and 100 µl of water. Then the stirred mixtures formed a paste, were air dried, and scrapped into a fine powder. As previously suggested (Field, Kelley et al. 2009), we fed this fluorescence pellet into a mixed population of zebrafish larvae, results from crossings of heterozygous out-of-frame *ttc7a* mutants. The larvae were kept in the incubator at 28˚C. After 2-3 hours, we assessed the larvae using fluorescence stereo microscope Leica M165FC (Leica, Wetzlar, Germany) to select larvae with their intestinal bulb filled completely with fluorescent pellet. Thereafter, larvae with a full intestinal bulb were individually placed in a well of a 24-well-plate, and kept in the incubator at 28˚C for 24 hours. Final assessment were taken using the fluorescence microscope. Then, the zebrafish larvae were euthanized and genotyped.

Furthermore, to record the phenotype of the *ttc7a* mutant zebrafish, we also performed live imaging using intravital microscopy on 7 dpf zebrafish larvae that underwent the *in vivo* assay of gut function using fluorescent pellets. 5-6 hours after feeding, larvae were assessed under the fluorescent stereo microscope, and larvae that were filled with fluorescent pellet were selected to be used. To embed these larvae, low melting agarose was (Bio-Rad) diluted in embryo E3 buffer to make 1% low melting agarose embedding solution. The larvae were embedded, and viewed using the Leica SP5 upright confocal microscope with 20x water lens (Leica, Wetzlar, Germany). Standard parameters were then set to perform a time lapse recording at 15-20 minutes interval, for the total duration of 9 hours. Results were analysed with Fiji software (Schindelin, Arganda-Carreras et al. 2012).

We applied these assays in three groups, including wildtype, heterozygous mutant and homozygous mutant *ttc7a* zebrafish larvae.

Results

Patients Information

Patient 1 was a male infant who, born at an estimate 35-weeks of gestation. Both parents are Dutch, and believed to be non-consanguineous. During pregnancy, polyhydramnios was identified, and ultrasonography suggested multiple dilated bowel loops. Thus, the child was immediately admitted to the paediatric surgery intensive care unit after birth. Physical examination acknowledged no signs of major abnormalities. However, evaluation by plain abdominal X-ray showed multiple fluid levels. Consequently, either jejunal or ileal atresia was suspected.

Explorative laparotomy was performed. The entire small intestine looked macroscopically normal, without any evidence of a disconnected intestinal tube or a mesenteric defect. However, Further exploration revealed a duodenal web. This was resected and the duodenum was subsequently closed. Intestinal lumen was atretic from the duodenal region that was attached to the Treitz ligament, until 5 cm prior the end of distal ileum, where a considerably normal lumen was observed. Other atretic sites at the caecum and the flexura lienalis were revealed. Microcolon appearance was identitied at the descending colon. After surgery, he received total parenteral nutrition (TPN) and ventilation. He was considered for bone marrow and intestinal transplantation, but the disease progressed thus his condition quickly deteriorated. The patient died at an age of 40 days.

The second patient was the younger female sibling of the first patient. She was born at an estimate 35 weeks and 5 days of gestation. Clinical signs and operative findings were identical to her late brother. After being informed of the operative findings, the parents opted not to continue her treatment.

Table 1: Hematologic evaluation of lymphocyte counts

Cell type	Value ($x10^9$/l)		
	Patient 1	Patient 2	Normal range
Lymphocyte	1.09	4.45	0.7-7.3
B-lymphocyte	0.16	0.98	0.4-1.1
T-lymphocyte	0.50	2.54	0.6-5.0
CD4+ T-lymphocyte	0.35	2.23	0.4-3.5
CD8+ T-lymphocyte	0.03	0.18	0.2-1.9
HLA-DR+ T-lymphocyte	0..02	<0.01	0.03-0.4
Natural killer (NK) cell	0.17	0.71	0.1-1.9

In both patients, measurement of lymphocyte subpopulations revealed that $CD8^+$ T-lymphocytes (cytotoxic T-cells) and HLA-DR^+ T-lymphocytes were lower than normal (Table 1).

Homozygous deletion of exon 2 of *TTC7A* in 2 sibs with HMIA

Sanger sequencing identified a homozygous deletion of 13.123 bp from intron 1 until intron 2 (g.47.169.356_47.182.479del), including the entire length of exon 2 (Fig. 1).

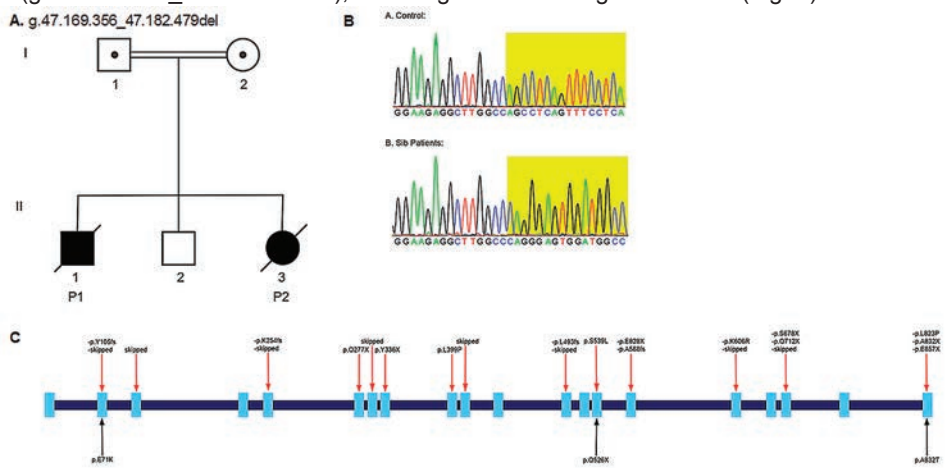


Fig. 1: (A-B) Pedigree of the studied family, with their Sanger Sequencing results showing homozygous deletion in the siblings with HMIA. **(C)** Schematic illustration of all the reported variants in TTC7A, with red arrows indicate variants found in HMIA, while black arrows indicate variants identified in inflammatory bowel disease.

Patients' atretic intestine featured multiple lumen without abnormalities in other major cellular constituents of the gut

H&E stainings of the atretic intestine revealed multiple lumen with focal villous atrophy, crypts loss and increased number of apoptotic cells. By ACTA2 stainings, we acknowledged that half of the lumens have their own individual muscularis mucosa, while the other half does not. Surrounding them all, there was a common muscularis propria, including circular and longitudinal mucosa. Similarly, CD117 staining also showed the presences of interstitial cells of Cajal between circular and longitudinal muscles. The myenteric plexus in the patients' intestine looked normal. Hence, other than abnormal intestinal lumens and mucosa, we could not detect any other cellular abnormalities in the patients' intestine (Fig. 2A-L).

The intestinal lumen in homozygous *ttc7a* mutant zebrafish is significantly narrowed

Macroscopically, homozygous *ttc7a* mutant zebrafish looked identical to wildtype zebrafish. H&E staining in wt *ttc7a* larvae (n=5) and mutant *ttc7a* larvae (n=5) at 5 dpf identified a narrowing of the intestinal lumen in homozygous *ttc7a* mutant zebrafish throughout the intestine (Fig. 3A-M). Apart from the mucosa in the intestinal bulb, the intestinal mucosa at the other parts of the intestine are flattened. In 4 out of 5 mutant larvae, intestinal stenosis was found at 1 segment of the intestine (Fig. 3K). None of the observed *wt* larvae acquired this phenotype.

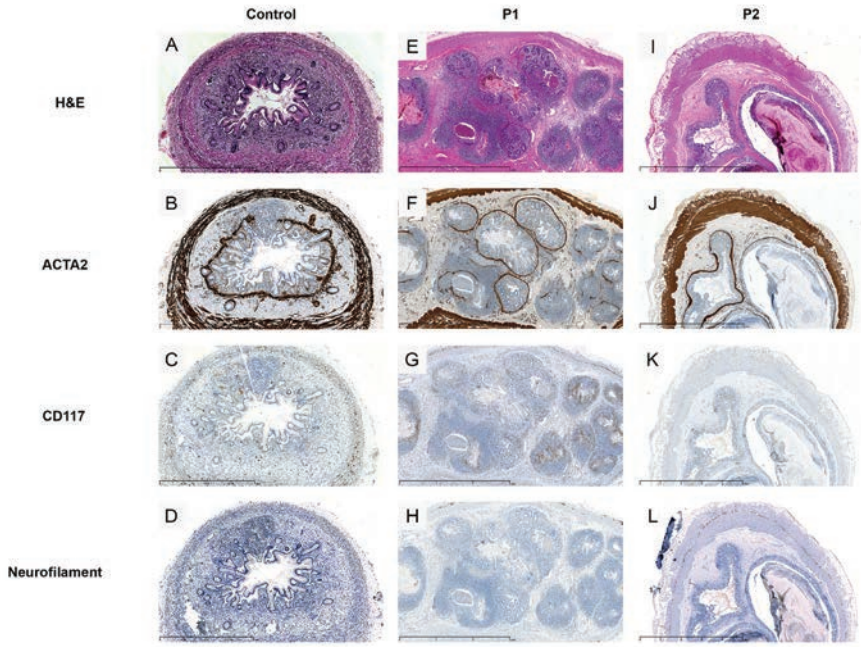


Fig. 2: Immunohistochemistry in age-matched control and patients' intestine. (A-L) Immunohistochemistry performed in intestinal material obtained from two HMIA patients, P1 and P2, and an age matched control. Apart from the phenotype of multiple lumen in patients' atretic intestine No significant changes were detected in any cellular constituents of the intestine of MMIHS patients, shown by immunohistochemistry for ACTA2, c-Kit/CD117 and Neurofilament.

Gastrointestinal transit time in *ttc7a* mutant zebrafish was significantly longer compared towildtype and heterozygous fish

Results from the *motility* assays assays (Fig. 4A). showed that as many as 45,6% (n=57) of the homozygous mutant *ttc7a* larvae did not completely passed the fluorescence

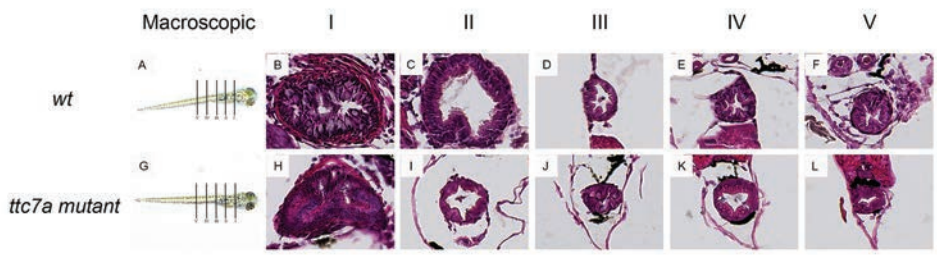


Fig. 3: Macroscopy and histology of wildtype and *ttc7a* homozygous mutant. (A and G) Macroscopically, there is no noticeable different between the wt and ttc7a mutant fishes at 5 dpf. Histologically, the intestinal lumen in ttc7a mutant fishes is narrowed, and the mucosa is flattened when compared with the intestine in *wt* fishes.

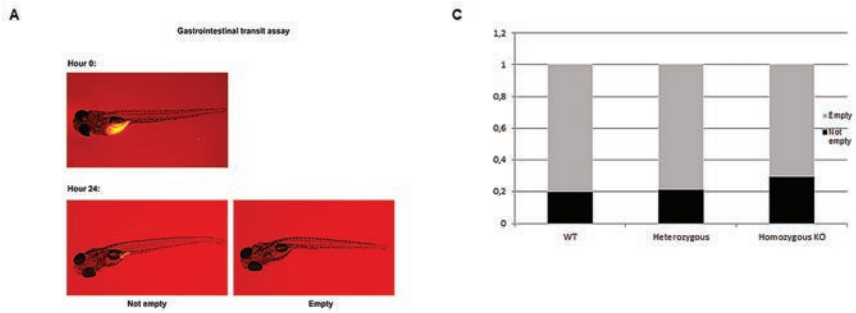
pellets within 24 hours, compared to 37,5% (n=27) in wt *ttc7a* larvae and 37,8% (n=140) in heterozygous mutant *ttc7a* larvae. These differences are statistically significant (P<0,00006; Chi^2 test), showing a slower gastrointestinal transit time in homozygous mutant *ttc7a* zebrafish (Fig. 4B-C).

Intravital fluorescence microscopy time lapse imaging revealed segmental defects in the intestine that delays food passage in homozygous mutant *ttc7a* zebrafish

Time-lapse movies show that the affected intestinal segment prevents the food to pass.

Discussion

In this study, we identified a homozygous deletion of the entire exon 2 in *TTC7A* in 2 siblings that were diagnosed with HMIA and immunodeficiency. This result is in line with the findings from other groups where different variants in the same gene were found in patients with identical conditions(Chen, Giliani et al. 2013, Samuels, Majewski et al. 2013). Thus, this confirms *TTC7A* as the disease-causing gene in patients with HMIA and immunodeficiency.



B

	WT	Heterozygous	Homozygous KO
Not empty	27	53	26
Empty	45	87	31
Total	72	140	57

Fig. 4: Gastrointestinal transit assays in wildtype, heterozygous and homozygous *ttc7a* knockout fishes at 7 dpf. (A) Fishes that have its intestinal bulb filled with fluorescence pellet were included in the assays. (B-C) Compared with wildtype and heterozygous fishes, the *tt7a* homozygous mutant fishes show slower gastrointestinal transit time.

Subsequently, we analysed intestinal specimens from both siblings with HMIA. H&E and antibodies stainings were performed against neurofilaments, interstitial cells of Cajal and smooth muscle cells. Apart from the multiple lumen phenotype, no other cellular abnormalities are seen, suggesting that the intestinal phenotype in HMIA is solely because of the failure of intestinal lumen formation, and not causing any abnormalities in other major cellular constituents of the gut. The mechanism on how the variants in TTC7A lead to this phenotype is only partly understood.

Bigorgne et al. and Yavitzur et al. respectively showed that loss of *TTC7A* leads to abnormal polarity and loss of cellular adhesion (Avitzur, Guo et al. 2014, Bigorgne,

Farin et al. 2014). Furthermore, by using Rho kinase (ROCK) inhibitor, the abnormal polarity in patient-derived intestinal organoids was reversed to normal, suggesting that the function of TTC7A in humans serves as an endogenous ROCK inhibitor (Bigorgne, Farin et al. 2014). Interestingly, the experiment that was used to show the abnormal polarity in HMIA patient-derived intestinal organoids was also used to test the polarity in IBD patient-derived intestinal organoids (Louw and Barnard 1955). The authors show that the abnormal polarity in IBD patient-derived intestinal organoids are also reversible by the addition of ROCK inhibitor. The question of how the same molecular mechanism may lead to these two different diseases is yet unanswered.

Meanwhile, Yavitzur et al. showed that human TTC7A also plays an important role in the translocation of Phosphatidylinositol-4 Kinase (Pi4K) to the plasma membrane, in order to phosphorylate Phosphatidylinositol (Pi) (Avitzur, Guo et al. 2014). The level of phophorylated Pi has been suggested as an important molecule in the maintenance of cellular polarity and survival. How to link both these processes to the function of the gene remains largely unknown. As the name implied, TTC7A is a protein consisting of 9 tetratricopeptide repeat regions (TPR), each is formed by a sequence of 33-37 amino acids. In mouse, an ortholog of human *TTC7A*, referred to as *Ttc7*, is known to play an important role in normal iron homeostasis (White, McNulty et al. 2005). Whether iron homeostatis plays a role in gthese processes is yet unknown.

Animals models may help further in our understanding on how this protein is involved in the disease and in these cellular processes. Two mouse models already exist for *Ttc7a.* The first model is called *hea,* it has a deletion of the exons 1-14. It is called *hea* as it is a model for hereditary erythroblastic anemia (White, McNulty et al. 2005, Kasahara, Shimizu et al. 2008). The second mouse model is called *fsn, it has* a insertion of 183 bp between exon 14 and exon 15. It is called *fsn* as it is a model for flaky skin (Sundberg, Dunstan et al. 1994, Beamer, Pelsue et al. 1995, Helms, Pelsue et al. 2005, White, McNulty et al. 2005, Takabayashi, Iwashita et al. 2007). Both models suffer from anemia. Additonally, the *fsn* mice model, also acquire psoriasiform dermatitis, hence it has been known as the mouse model for human psoriasis. The mechanism that leads to the differences between each *Ttc7* mutant mouse model is yet unknown. Nevertheless, except a decrease in the absorption of iron from intestinal lumen, none of the *Ttc7* mutant mice show any signs of abnormal intestine.

Unfortunately, the use of *Ttc7* knockout mice to study the disease in more detail proved difficult as a species-specific function of TTC7A in human and mice seems to exist. Hence, another animal model that might have a more similarities with human is needed to further investigate the disease mechanism of HMIA.

Zebrafish (*Danio rerio*) has long been used in intestinal-related research due to the similarities in its gastrointestinal system with mammals, including humans. Similar with human intestine, zebrafish intestine is also formed by intestinal epithelium, smooth muscle cells and neurons (Wallace and Pack 2003, Wallace, Akhter et al. 2005). Moreover, the

failure of a single intestinal lumen formation in zebrafish that resulted in multiple lumen has also been reported (Bagnat, Cheung et al. 2007). For this reason, we opted to generate a *ttc7a* mutant zebrafish model. We could not identify multiple lumens in the intestine of *ttc7a* homozygous mutant zebrafish, however, the diameter of the intestinal lumen in the mutant fish is significantly narrowed. This indicates that ttc7a plays an important role in the intestinal lumen formation of zebrafish. When these mutant fishes underwent transit assays, live imaging shows that the narrowed intestinal lumen causes a slower intestinal transit time. Altogether, we conclude that zebrafish is an interesting animal model to study the intestinal lumen formation, and might shed light on the disease mechanism of HMIA.

Our zebrafish model might give answers the question whether there are more intestinal abnormalities than the narrowing of the lumen, such as abnormal digestion and/or absorption of the intestine. Moreover, the question on how the TTC7A variants could lead to immunodeficiency, and whether the abnormal immune system is somehow related to the intestinal phenotype might be answered.

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Conflict of Interest: The authors have nothing to disclose.

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Supplementary table 1: List of primers used in this study

Primer	Sequence
Sequencing forward in exon 1 Human *TTC7A*	CCGGGTCTCCACTTCTTGG
Sequencing reverse in exon 1 Human *TTC7A*	GCAAAAGCCGTTTCTCTTCC
Sequencing forward in exon 2 Human *TTC7A*	GGGAACTCTGTCTTCCCAACC
Sequencing reverse in exon 2 Human *TTC7A*	AGGGAGGACTGGACAGATGC
Sequencing forward in exon 3 Human *TTC7A*	GGAGGAGGGGAGTTCTGAGC
Sequencing reverse in exon 3 Human *TTC7A*	CGGCATCCACCTTAGACACC
Sequencing forward in exon 4 Human *TTC7A*	GGGTGATGCACTGTGATTCC
Sequencing reverse in exon 4 Human *TTC7A*	TCTTTGGGGGACAGAGAAGG
Sequencing forward in exon 5 Human *TTC7A*	GTTGGATGGGGAGAGAATGC
Sequencing reverse in exon 5 Human *TTC7A*	GTTCCCACCAATCCTCTTGG
Sequencing forward in exon 6 Human *TTC7A*	AGTCTGACACCAGCCCAAGG
Sequencing reverse in exon 6 Human *TTC7A*	CTCCCACCAAAGCTGACTCC
Sequencing forward in exon 7 Human *TTC7A*	GGCAGATTTAGCTGGGATGG
Sequencing reverse in exon 7 Human *TTC7A*	GGCTCCTGCTGAGACTGAGG
Sequencing forward in exon 8 Human *TTC7A*	CTGTAGCCAACCAGCAGAGC
Sequencing reverse in exon 8 Human *TTC7A*	CCCTGGGAGACAGAGTTTGG
Sequencing forward in exon 9 Human *TTC7A*	GCTTGACCCCTCAGAACTGC
Sequencing reverse in exon 9 Human *TTC7A*	GGTGGAGAAGGGACAAATGG
Sequencing forward in exon 10 Human *TTC7A*	TGAACTGAGGGCCTGAGAGG
Sequencing reverse in exon 10 Human *TTC7A*	ACCAGCGGCAGGATAATACC
Sequencing forward in exon 11 Human *TTC7A*	TTGAATTCCTTGGGGAGACC
Sequencing reverse in exon 11 Human *TTC7A*	CATGCACCCACACATACACG
Sequencing forward in exon 12 Human *TTC7A*	CTTGGGTTCATGCTCTCAGC
Sequencing reverse in exon 12 Human *TTC7A*	TGGTGTCTATGGAGGCATGG
Sequencing forward in exon 13 Human *TTC7A*	GTATGTGGCCAGGCAGAGG
Sequencing reverse in exon 13 Human *TTC7A*	TGTGAATCTGCCACCAAAGG
Sequencing forward in exon 14 Human *TTC7A*	GCCCCTACCTCAGTGAGTGC
Sequencing reverse in exon 14 Human *TTC7A*	CTTGCAGAGGGTCACACAGC
Sequencing forward in exon 15 Human *TTC7A*	TGAGTGCCCTTGTTGACTGG
Sequencing reverse in exon 15 Human *TTC7A*	GTCCTCACCCCTGTCTCTCC
Sequencing forward in exon 16 Human *TTC7A*	TGGAGTGCTTCATGCTTTGG
Sequencing reverse in exon 16 Human *TTC7A*	ACCCAGCTGTGGATTGTTGC
Sequencing forward in exon 17 Human *TTC7A*	GTGAGGCTGTCCCATTCTCC
Sequencing reverse in exon 17 Human *TTC7A*	CTCTTTCTTCACCCCCATGC
Sequencing forward in exon 18 Human *TTC7A*	TGTGGACCTCTGACCACTGC
Sequencing reverse in exon 18 Human *TTC7A*	GGACCTCCAATCCTCTCACG
Sequencing forward in exon 19 Human *TTC7A*	GACTCCCCACCACTCACACC
Sequencing reverse in exon 19 Human *TTC7A*	CAACAGAGCAGGAGGGAAGC
Sequencing forward in exon 20 Human *TTC7A*	CTACTCACCCTGCCCTGTGC
Sequencing reverse in exon 20 Human *TTC7A*	TCACCACAAAACTCTCCAAAGG
Long range PCR sequencing forward in exon 1 Human *TTC7A*	CAGAGGCGGAGGTTGCTCCTGTACG
Long range PCR sequencing reverse in exon 3 Human *TTC7A*	TCACCACAAAACTCTCCAAAGG
Sequencing reverse in intron 1 Human *TTC7A*	CCGCGAGAGAAAAATCACAT
Sequencing reverse in intron 2 Human *TTC7A*	AGAGGCATTGGAGGAGTTCA
Sequencing forward in intron 2 Zebrafish *ttc7a*	GTTCCAGTCCTCCTTCTTCAG
Sequencing reverse in intron 3 Zebrafish *ttc7a*	CTTTGCTCCCCAAACGAGCTTCTC

CHAPTER 9

Discussion

DISCUSSION

Intestinal obstruction is one of the problems in neonates that requires surgery and intensive treatment (Hajivassiliou 2003). In many cases, surgical and post-operative care is sufficient. However, in some cases, current treatments are unable to correct the anatomical and functional defects of the intestine (Georgeson and Breaux 1992, Sondheimer, Cadnapaphornchai et al. 1998, Spencer, Neaga et al. 2005, Wales, de Silva et al. 2005).

For example, in patients who are diagnosed with megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS), surgical resection of the defected intestine and bladder catheterization is not sufficient to repair the functional contractility of the visceral organs (Soh, Fukuzawa et al. 2015). Similarly, patients with hereditary multiple IA (HMIA) have a very poor prognosis, despite having the atretic intestine resected (Boyd, Chamberlain et al. 1994, Bilodeau, Prasil et al. 2004). Meanwhile, although the intestinal length of many elderly CSBS patients is more than 50 cm, most of these patients never regain normal intestinal length and function.

The mechanisms of how these diseases can be lethal were far from clear. Consequently, the efforts to improve the treatment and prognosis of the patients have also been hampered by our lack of knowledge towards the disease pathogenesis. Driven by the facts of our limited understanding on the molecular pathogenesis of MMIHS, CSBS and HMIA, and the lethality of these diseases, we focused our study on these three diseases. In our studies, we used genetics as a way to understand disease pathogenesis. Equipped with state-of-art genetic methodologies and the use of genome editing tools to create animal models, we extended our knowledge of the molecular mechanisms behind the disease phenotypes.

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS)

ACTG2 variants cause a dominant form of MMIHS

Twelve patients who were diagnosed with MMIHS, including eight sporadic cases and four familial cases, were genetically screened. In all sporadic cases, we identified heterozygous missense variants in enteric smooth muscle γ-2 (*ACTG2*), a gene that encodes one of the six actin isoforms that is preferentially expressed in enteric smooth muscle cells (SMC). In three patients, we confirmed that the mutations were de novo. Our findings are in line with reports from five other groups that described the heterozygous *ACTG2* variants in MMIHS patients (Thorson, Diaz-Horta et al. 2014, Wangler, Gonzaga-Jauregui et al. 2014, Tuzovic, Tang et al. 2015, Lu, Xiao et al. 2016, Matera, Rusmini et al. 2016). These discoveries confirmed the abnormal muscle contractile apparatus as the cause in most sporadic MMIHS cases. Interestingly, two heterozygous variants in ACTG2, including R148S and G269E, have also been identified in patients diagnosed with visceral myopathy (VM), a disease that resembles MMIHS, but is less severe (Lehtonen, Sipponen et al. 2012, Klar, Raykova et al. 2015). To date, none of the variants found in MMIHS

patients were ever found in VM patients, and vice versa. Hence, the correlation between the variants and the severity is very likely.

We addressed this possible genotype-phenotype correlation partly in our functional studies, in which we included the R148S variant as a positive control. To our surprise, the results from molecular dynamics simulation, collagen contractility and actin polymerization assays did not show any significant differences between the variants in MMIHS patients with R148S variant. In brief, all variants seemed to equally impair the function of the mutant ACTG2 protein in binding to actin filaments, thus reducing cellular contractility (Halim, Hofstra et al. 2016). On the other hand, patients with the R148S variant survive longer than MMIHS patients with other *ACTG2* variants. Based on the survival differences, we still believe that the correlation between variants with disease severity does exist. We hypothesize that the similarity between the the effects of one *ACTG2* variant with the others are due to the insensitivity of the artificial systems used in functional studies. In this case, an *in vivo* model would certainly offer a more sensitive approach to any differences in the variants. Moreover, generation of an animal model with variants in ACTG2 would also give more clues on whether the MMIHS phenotypes are due to the decreased of normal ACTG2 protein, or because of the dominant negative effect of the mutant ACTG2 protein. For example, if we can show that an animal model carrying a heterozygous stop variant in *ACTG2* could survive, while another animal model carrying a heterozygous MMIHS-causing missense variants could not survive, then we can suggest that the MMIHS phenotype is likely because of the dominant negative effect of the mutant ACTG2 protein. This information is highly important as each condition requires different therapeutic approach that can be applicable for future therapy in patients.

MYH11, MYLK and LMOD1 variants as the cause of the recessive form of MMIHS

At the beginning of our study in 2012, any expectations to find more than one gene in a rare disease such as MMIHS would have been purely speculative. Nonetheless, this hypothesis became highly plausible when we did not identify any rare variants in both *ACTG2* and myosin heavy chain-11 (*MYH11*) in our familial cases. In one familial patient, we identified a homozygous nonsense variant in Leiomodin-1 (*LMOD1*), a gene that encodes an actin filament nucleator that preferentially is expressed in SMC (Nanda and Miano 2012, Boczkowska, Rebowski et al. 2015). Knockout of *LMOD1* in mice results in a phenotype similar to MMIHS in human, including distension of the bladder and the stomach. The molecular mechanism that led to this phenotype was provided by functional studies in human intestinal smooth muscle cells that shows a defect in actin filament formation due to the knockdown of *LMOD1*. As *LMOD1* is not only expressed by the SMC of the visceral organs, but is also expressed by SMC of the blood vessels, we also expected to see abnormalities in the blood vessels of the *Lmod1* knockout mice. To our surprise, we could not identify any abnormalities of the blood vessels in mice lacking Lmod1. One possibility that may explain this phenomenon is the existence of another actin filament nucleator that

might have a redundant function with Lmod1 in murine vascular SMC. Previous studies on the mechanisms of vascular SMC migration has reported a complex comprising an actin filament nucleator and actin-related protein 2/3 (ARP2/3) as an important complex in vascular SMC migration (Kaverina, Stradal et al. 2003, Williams, San Martin et al. 2012). Moreover, experiments in tracheal smooth muscle also showed that ARP2/3 complex formation is necessary for the development of tension in tracheal smooth muscle (Zhang, Wu et al. 2005). It could be that this or another protein takes over the function of LMOD1. Nevertheless, we could not identify any abnormal bladder nor intestinal functions in the parents of the MMIHS patient (both carriers) nor in the heterozygous mice, suggesting that reduced LMOD1 expression is not sufficient to cause any abnormal phenotypes in both human and mice.

In two other families, a homozygous splice site and a homozygous out-of-frame variant in myosin light chain kinase (*MYLK*) were identified, respectively. Expression studies showed that these variants eliminate the normal expression of *MYLK* in these patients. Together with the phenotypes in the already published smooth-muscle specific *Mylk* knockout mouse model, *MYLK* is convincingly proven to be a gene responsible for recessive MMIHS. The parents who all are carriers of the variant identified show no signs of bladder nor gastrointestinal problems. Interestingly, heterozygous variants in *MYLK* have been reported as the cause of familial thoracic aortic aneurysm and dissection (FTAAD) (Wang, Guo et al. 2010, Hannuksela, Stattin et al. 2016). Different from FTAAD patients without *MYLK* variants, aortic aneurysm and dissection of the aorta in patients with *MYLK* variants occur rapidly, without a preceding chronic dilatation of the aorta (Wang, Guo et al. 2010). The mechanism underlying how this disease accelerates rapidly is still unclear. However, this characteristic acceleration of aorta dilatation in patients with a heterozygous *MYLK* variant has given an evidence-based reason for clinicians to consider prophylactic surgical repair of the aorta for these patients (Milewicz and Regalado 1993).

Whether the *MYLK* carriers in the two families are at risk for FTAAD is still unclear. Within the families, no reports were made of aneurysms or any vessel problems. Nonetheless, based on the identification of *MYLK* variants in MMIHS patients, and the earlier reports about the heterozygous *MYLK* variants as the cause of an unforeseen thoracic aortic aneurysm, we advised the carriers to undergo a routine medical examination.

In accordance with our findings, no gastrointestinal dysmotility was ever reported in FTAAD patients with heterozygous *MYLK* variants. We hypothesize that a certain threshold must be passed to acquire any gastrointestinal disturbances.

Actin-myosin cross-bridge interaction is fundamental in the functional contractility of the bladder and the intestine

It has been suggested that MMIHS might well be a neuropathy. However, all four MMIHS disease-causing genes (*ACTG2*, *MYH11*, *LMOD1*, *MYLK*) identified so far, encode proteins that are involved in smooth muscle contractility. More specifically, these proteins

are all involved in the actomyosin cross-bridge interaction that underlies smooth muscle contraction. Along with other published studies on the identified MMIHS genes, our studies suggest that ACTG2, LMOD1, MYLK and MYH11 form key molecules in actin-myosin interaction in visceral SMC specifically (Figure 1).

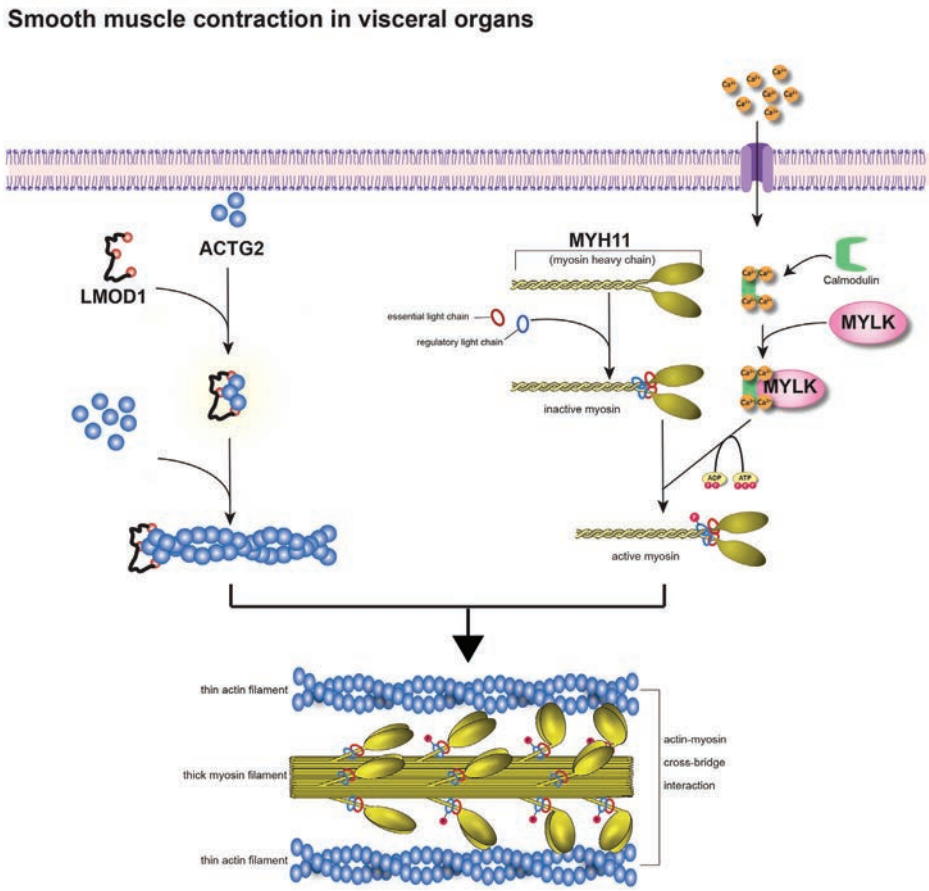

Fig.1: Hypothesis on the molecular mechanism of smooth muscle contraction in visceral organs

This makes a case for clinicians and scientists to prioritize the screening of acto-myosin related genes whenever studying patients with MMIHS. Hence, in cases where genetic screening fails to identify variants in known MMIHS genes, we recommend searching for variants in other genes that are related to the proteins that are closely related to the acto-myosin interaction pathway, such as calcium channels and Calmodulin. On the other hand, findings of any candidate variants in unknown genes should be followed by functional studies linking the encoded protein with acto-myosin interaction pathway.

All this, of course, does not exclude the possibility that some MMIHS cases present with a neuropathy. However, one should exclude variants in smooth muscle-related

genes first before looking at any possible variants in neuronal-related genes in MMIHS patients. Thus, in clinical setting, we suggest a genetic screening on known MMIHS genes and muscle-related genes, before applying a more universal genetic screening, such as exome sequencing, to find new candidate variants.

Information from MMIHS research may be used for genetic counseling and the development of future therapy for MMIHS patients

From all the histopathological analyses that we performed in this research, we concluded that the identified disease-causing variants did not cause any significant changes in the pathology of the intestine and the bladder (Halim, Hofstra et al. 2016). Thus, it seemed that the phenotypes found in these patients are solely due to the impaired functional contractility of these visceral organs. Clinically, this implies that surgery to resect the affected intestine and the hopes that the residual intestine would regain normal function may not considered to be realistic after all. Unfortunately, conventional therapy that includes long term total parenteral nutrition (TPN) and catheterization also come with formidable risks, such as recurrent sepsis and multiorgan failure(Bishay, Pichler et al. 2012, Naini and Lassman 2012, Niel-Weise, van den Broek et al. 2012). The only available treatment to date that seemed to offer any possibility of survival is multivisceral transplantation (Abu-Elmagd, Kosmach-Park et al. 2012). Reports from Loinaz et al. and Masetti et al. described an acceptable rate of success in MMIHS patients that received such transplantations (Masetti, Rodriguez et al. 1999, Loinaz, Rodriguez et al. 2005). However, like in any other types of organ transplantation, the two major problems with multivisceral organ transplantation are the availability of donor organs and the recipient's immune rejection (Kubal, Mangus et al. 2015, Mathew, Tryphonopoulos et al. 2015, Smith, Skeans et al. 2015). Thus, we can conclude that current therapeutic options for MMIHS patients are far from ideal.

Our data does, however, provide important information for genetic counseling. For example, so far all tested *ACTG2* variants in MMIHS proved to be *de novo*. This lowers the recurrence risk on MMIHS in the next pregnancy to almost zero (~1%). For the variants in the three other genes, which have a recessive pattern of inheritance the recurrence risk on an affected child in the next pregnancy is 25%.

As most MMIHS cases have a lethal outcome, we propose to include these three genes in a targeted sequencing panel that might be used for genetic screening in consanguineous couples (Rehm 2013). When mutations are identified in both parents they can opt for pre-implantation genetic diagnosis, genetic profiling of embryos prior to implantation or they might opt for prenatal screening during early pregnancy (Mastenbroek, Twisk et al. 2007).

Another option for genetic testing might be a search for a *de novo* mutation in *ACTG2* in the prenatal setting, upon an MMIHS-suggestive ultrasonography. Identification of a *de novo ACTG2* variant would provide a reliable foundation to distinguish MMIHS from other diseases that have similar prenatal findings, but are less severe than MMIHS, such

as prune belly syndrome and visceral myopathy (Fisk, Dhillon et al. 1990, Ghavamian, Wilcox et al. 1997, Cromie, Lee et al. 2001). This will enable the parents to consider pregnancy termination more carefully.

Significant development in genome targeting techniques have been made, making gene therapy the technology to eventually correct any genetic diseases (Naldini 2015), including MMIHS. Even though this approach might seem plausible, several limitations must first be solved, such as the efficiency of genetic corrections in living tissues and the length of time needed to obtain any beneficial effects (Ginn, Alexander et al. 2013).

As mentioned earlier, it is also possible that the *ACTG2* variants lead to MMIHS in a dominant negative manner. Even though this hypothesis is yet to be proven through the creation of an animal model, if this can be confirmed, then another possible method that may have a therapeutic potentials is antisense oligonucleotides (Bennett and Swayze 2010, Kole, Krainer et al. 2012). By designing a specific oligonucleotide against the mutant mRNA, we may prevent the translation of mutant protein and rescue the phenotypes. Nevertheless, any treatments for these patients must have an immediate effect before disease progression becomes irreversible.

Congenital Short Bowel Syndrome (CSBS)

CLMP and FLNA are the disease-causing genes of CSBS, and losing any of the two might cause more than just a shortened intestine

In agreement with the earlier report from our group (Van Der Werf, Wabbersen et al. 2012), we identified *CLMP* variants in three additional CSBS patients from two independent families. In two of the three patients, intestinal dysmotility was reported (Alves, Halim et al. 2016). Unfortunately, intestinal specimens were not available, hence no pathological analysis was performed. Extensive analyses of the pathology of the intestine of CSBS patients with *CLMP* variants may give an answer to the question whether the intestinal dysmotility is related to the loss of CLMP itself. Thus, a (conditional) knock-out animal model of *CLMP* might be informative.

Van der Werf et al. described *FLNA* mutation as the cause of X-linked CSBS (van der Werf, Sribudiani et al. 2013). To investigate this further, we generated a *flna* knockout zebrafish model and characterized it. Even though we could not see a reduction in intestinal length as dramatic as that described in CSBS patients (≥75%), we did see that the intestinal length in the homozygous knockout fish had shortened by approximately 5% ($P<0,05$). Moreover, functional studies using a fluorescent pellet in a gastrointestinal transit assay, showed significant reduction of the motility rate in homozygous knockout fish compared to *wt*. This indicates that in addition to the shortening of the intestine, bowel motility may also be disturbed by the loss of flna. This finding seemed to agree with the report on *FLNA* mutations in five male patients with intestinal pseudo-obstruction, in which short bowel was also reported as a comorbidity (Kapur, Robertson et al. 2010).

As *FLNA* is expressed in SMC of the intestine, one could hypothesize that

muscle-related proteins play an important role in intestinal elongation. Based on mouse studies, Kaufman and Bard suggested that muscle contraction in the duodenum and the proximal jejunum plays an important role in the process of intestinal withdrawal into the peritoneal cavity from physiological umbilical hernia (Kaufman and Bard 1999). Interestingly, malrotation is always reported as a comorbidity in CSBS. Based on the finding of *FLNA* variants in CSBS patients, it is intriguing to speculate that the shortened bowel and malrotation in CSBS patients may be due to a common genetic entity. In addition to *FLNA* and *CLMP*, mutations in *FOXF1* have also been reported to cause malrotation and congenital short bowel phenotypes in patients, supporting the hypothesis that the two phenotypes are linked (Stankiewicz, Sen et al. 2009).

So far, it is difficult to prove whether malrotation is primary or secondary to short bowel phenotype. It seems reasonable to hypothesize that malrotation is secondary to short bowel, since the reduced contraction in CSBS patients with *FLNA* mutations may disturb the movement that positions the intestine in its normal configuration at the end of embryogenesis. However, it can also be that malrotation causes a physical force that inhibits the elongation of the intestine during embryogenesis. Thus, the question of whether malrotation is primary or secondary to CSBS remains unanswered.

Since the cellular mechanisms in intestinal rotation during normal embryogenesis in mammals are not the same with in zebrafish (Horne-Badovinac, Lin et al. 2001, Horne-Badovinac, Rebagliati et al. 2003, Davis, Kurpios et al. 2008), as expected, we did not identify the occurrence of malrotation in the *flna* knockout zebrafish model. Therefore, studies in larger animal models are needed to investigate this further.

Implications of our studies on future research to improve CSBS therapy

The fact that (acquired) short bowel syndrome patients survive much better than congenital short bowel syndrome patients with comparable intestinal length suggests that the length of the intestine may not be the only problem in CSBS patients. An intestinal transplantation to replace or elongate the short intestine by specific surgical procedures as bowel lengthening by plication of the bowel may be a therapeutic approach that might offer a more permanent solution. Current development in the creation of patients' own induced pluripotent stem cells (iPS) gives hope on organ engineering in the near future. Results from research on the use of intestinal submucosa and chitosan as scaffolds are encouraging (Chen and Badylak 2001, Zakhem, Raghavan et al. 2012, Zakhem, Elbahrawy et al. 2015). Hence, the next challenge that scientists need to overcome is to combine the scaffold with all the cellular constituents of the intestine and the bladder, to engineer a full thickness organ that can be transplanted back into patients to achieve normal function. Clearly, this is yet far from reality.

Hereditary Multiple Intestinal Atresia (HMIA)

In two sibs from a consanguineous family with HMIA and severe immunodeficiency, we identified a homozygous deletion of 13.123 bp from intron 1 to intron 2, including the entire exon 2 of the tetratricopeptide repeat-7a (*TTC7A*) gene. It encodes a family member of the proteins that are characterized by the presence of tetratricopeptide repeat (TPR) domains. Immunohistochemistry was performed and results showed a typical sieve-like appearance of multiple lumens in the atretic intestine. This finding is in line with previous reports that described variants in *TTC7A* in patients with MIA and immunodeficiency (Chen, Giliani et al. 2013, Samuels, Majewski et al. 2013, Agarwal, Northrop et al. 2014, Bigorgne, Farin et al. 2014). To understand the molecular pathogenesis underlying how *TTC7A* mutations result in a MIA phenotype, Lemoine et al. and Bigorgne et al. utilized intestinal organoids from patients' intestinal stem cells (Bigorgne, Farin et al. 2014, Lemoine, Pachlopnik-Schmid et al. 2014). They observed that patients' organoids have abnormal apicobasal polarity, that can be reversed by the addition of RhoA kinase (ROCK) inhibitor. Although the reversal of apicobasal polarity by the addition of a ROCK inhibitor seemed encouraging, an animal model is required to study the pathogenesis of this disease further and to test potential therapies. This might prove difficult as three different mouse models, all deficient for *Ttc7,* do not recapitulate the human MIA and immunodeficiency phenotypes (Helms, Pelsue et al. 2005, White, McNulty et al. 2005, Kasahara, Shimizu et al. 2008). Species-specific issue may be the reason for these differences, hence an alternative animal model is required to study this disease further.

An alternative animal model might be the zebrafish as it has long been used in intestinal research. The architecture of zebrafish intestine is similar to the intestinal architecture in larger mammals (Wallace, Akhter et al. 2005). Moreover, MIA-like phenotypes, including multiple lumens, have been observed in studies of other genes in zebrafish (Bagnat, Cheung et al. 2007). Hence, we opted to create a *ttc7a* mutant zebrafish model to study the pathogenesis of MIA and the physiology of intestinal recanalization.

Zebrafish is a reliable model to study the process of intestinal recanalization

Although the homozygous *ttc7a* mutant zebrafish does not have the MIA phenotype with multiple intestinal lumens, histology shows that the intestinal lumen in these fish, at dpf 5, is dramatically narrowed when compared to *wt* fish. Similar to the phenotype in human, the intestinal villi in the *ttc7a* mutant fish, appeared flattened. We observed that certain parts of the intestine showed a severe decrease in luminal size, leaving almost no patent lumen. When the homozygous *ttc7a* mutant fish underwent a gastrointestinal transit assay using a fluorescent pellet, all these fish could transport and excrete the digested pellet, although longer time was required to complete this process. This indicates that although the size of the lumen is significantly decreased, the intestinal lumen is not completely blocked as one would expect to see in intestinal atresia. It is unclear if the size of the lumen, or whether abnormal contractility, cause the longer transit time in these fish. Ongoing research is

currently being performed to investigate the cause of the slower transit time.

Whether HMIA is directly related to the observed immunodeficiency, or if these two entities have a different molecular pathogenesis, even though they are caused by the same genetic defect, remain unanswered. Findings of *TTC7A* mutations in patients with immunodeficiency, without MIA may indicate that these two conditions have independent disease mechanisms. To study the effect of ttc7a mutations on the immune system, ongoing studies using crossings of ttc7a mutant fish with a T-cell specific tyrosine kinase (*lck*) reporter line that expresses GFP in their lymphocytes, are currently ongoing.

Overall conclusion

In this thesis, we describe genetics and functional studies that were performed to identify mutations that cause three different congenital intestinal diseases: MMIHS, CSBS and HMIA. Our studies clearly show strategies and challenges in elucidating the pathogenesis of these diseases.

Although rare diseases are often monogenic, our work on MMIHS and CSBS strongly suggest that we have to keep an open mind for the possibility of finding more than one disease-causing genes and that multiple patterns of inheritance in rare diseases are possible. We successfully identified mutations in three different causal genes of MMIHS (**Chapter 2, 3 and 4**). In our studies on *MYLK* and *LMOD1*, we used the genetics data to unravel the molecular pathogenesis of this disease. Importantly, we showed that by studying MMIHS pathogenesis, we obtained clues about the important molecular mechanism that regulates normal visceral SMC contraction.

We managed to validate the findings of CLMP variants as the cause of recessive CSBS (**Chapter 6**). Correspondingly, we gained insights into the effect of flna depletion on the intestinal digestive function through a *flna* knockout zebrafish model (**Chapter 7**). Finally, we gained insights into the process of intestinal recanalization by creating a *ttc7a* mutant zebrafish, based on findings in HMIA patients (**Chapter 8**). Altogether, we showed that genetic research is an efficient way to advance our understanding of disease mechanisms and normal physiology of the gastrointestinal tract.

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APPENDIX

Summary
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SUMMARY

Intestinal obstruction is the most common surgical emergency in neonates. Despite a steady increase in the prognosis of these patients, in cases where the underlying diseases are megacystis microcolon intestinal hypoperistalsis syndrome (MMIHS), congenital short bowel syndrome (CSBS), or hereditary multiple intestinal atresia (HMIA), death remains as the most frequent outcome. Based on the prevalence of familial cases, these three congenital diseases have long been suggested to have a genetic origin. The work presented in this thesis consists of genetic studies, followed by functional studies to elucidate the aetiology of these three diseases (MMIHS, CSBS, and HMIA).

In **chapter one**, we summarize what was already known of the three diseases when we started our studies in 2012. This includes the clinical presentation, histopathology, treatment, prognosis and genetics.

Megacystis Microcolon Intestinal Hypoperistalsis Syndrome (MMIHS),

Chapter two describes the functional study of missense variants identified in the Enteric Smooth Muscle Actin γ-2 (*ACTG2*) gene that cause sporadic MMIHS. As implied within the name of the disease, patients with MMIHS have a distension of the bladder in combination with a decreased or absence of intestinal peristalsis in combination with microcolon, all of which can be identified during the neonatal period. In line with the findings of several other groups, we identified heterozygous variants in ACTG2 in eight sporadic MMIHS patients. Immunohistochemistry shows that ACTG2 is expressed in all developmental stages of the human intestine we examined. Interestingly, no visible abnormalities are seen in the intestinal histopathology of the patients we analysed. Results from molecular dynamics and *in vitro* assays indicate that mutations in *ACTG2* impair its protein binding potentials to actin filaments, decreasing contractility. This make us speculate that these changes underlie the MMIHS phenotype.

Since we could not find *ACTG2* variants in the familial MMIHS patients, we hypothesised that another locus or loci are responsible for the incidence of familial MMIHS. Therefore, we performed exome sequencing and homozygosity mapping in several MMIHS patients from consanguineous families.

In **chapter 3**, we describe the identification of a homozygous nonsense mutation in the Leiomodin-1 (*LMOD1*) gene. *LMOD1* encodes a protein that has been suggested to function as an actin filament nucleator in smooth muscle cells. To prove that *LMOD1* as the disease-causing gene in familial MMIHS, we generated a *Lmod1*-KO mouse model and show that the phenotype in these mice is strikingly similar to MMIHS as seen in the patients. Mechanistic investigation using human intestinal smooth muscle cells and the jejunum from the *Lmod1*-KO mice show that the loss of LMOD1 reduces the formation of actin filaments. This leads to a decreased contractility, and might cause the inability of the bladder and the intestine to excrete metabolic products.

Despite this finding, the genetic aetiology in three patients from two separate consanguineous

families remained unidentified. Thus we analysed exome and homozygosity mapping data from these three patients, and we identified homozygous deleterious variants in the myosin light chain kinase (*MYLK*) gene in both family, including a duplication of seven base pairs in exon 22 in one family, and a splice variant in intron 22 in the other family; these are described in **chapter 4**. *MYLK* encodes a protein that phosphorylates the regulatory myosin light chain. This phosphorylation reaction causes thick myosin filaments to interact with thin actin filaments, a process that underlies contraction in muscle cells. Therefore, loss of MYLK likely disrupts this fundamental mechanism, and cause MMIHS in these patients. Data from a published smooth muscle-specific *Mylk*-KO mouse model show a similar phenotype shared between mouse and human, supporting our hypothesis that *MYLK* is indeed another gene that can cause a recessive form of MMIHS. These findings prove that MMIHS is a heterogeneous disease with multiple patterns of inheritance.

Congenital Short Bowel Syndrome (CSBS)

Patients who are diagnosed with CSBS are born with a short small intestine, which is approximately ≥75% shorter than the small intestinal length in normal neonates. Recently, our group identified two genes related to CSBS: coxsackie- and adenovirus receptor-like membrane protein (*CLMP*) and filamin A (*FLNA*). CLMP is a membrane protein that has been suggested to play a role in the tight junction complex, while FLNA is known to regulate the cross link of actin filaments in the cytoplasm. In **chapter 5**, we provide an overview of the literature on CSBS, including the latest clinical and genetic findings. We speculate on how the recent genetic data could influence our understanding towards the CSBS pathogenesis.

Since the original publication of *CLMP* as the CSBS gene, no publications have reported other *CLMP* variants in CSBS patients. We describe the identification of three novel variants in three CSBS patients in **chapter 6**. In two siblings, a homozygous nonsense variant in exon 4 was identified [c.508C>T; p.(R170*)]. Meanwhile, compound heterozygous variants consisting of a missense variant in exon 4 [c.410G>A; p.(C137Y)] and a splice variant in intron 2 (c.29-2A>G) were identified in another patient. Functional studies using Chinese hamster ovary (CHO-K1) cells shows that the missense variant impairs the expression of CLMP in the tight junction complex.

To further understand how the identified *FLNA* variant in CSBS patients lead to the shortened intestine, we generated and characterised a transgenic zebrafish with an out of frame mutation, a mutation comparable to the mutations found in CSBC, in *flna*. The findings are described in **chapter 7**. Even though the shortening of the *flna* mutant zebrafish intestine is not as dramatic as found in CSBS patients, the shortening remains significant, suggesting a conserved function of *FLNA* in the elongation of the intestine. Furthermore, results from gastrointestinal transit (GIT) assay shows that mutant *flna* fish require a longer GIT time, indicating that the intestine is not only shortened, but also less motile in these mutant fishes.

Hereditary multiple intestinal atresia (HMIA)

HMIA is a familial form of multiple intestinal atresia that often comorbids with immunodeficiency. To date, death is an almost certain outcome in patients who are diagnosed with this disease. Similar to findings of other groups, we identified a variant in Tetratricopeptide-7A (*TTC7A*) in 2 siblings who were diagnosed with HMIA and immunodeficiency. To further investigate the effect of TTC7A loss in the intestinal phenotype, we generated and characterized a transgenic *ttc7a* mutant zebrafish and described our findings in **chapter 8**. Despite macroscopically similar with wildtype zebrafish, the histology of the *tt7a* mutants shows dramatic narrowing of the intestinal lumen, suggesting the loss of *ttc7a* in zebrafish disrupts intestinal lumen formation. Furthermore, GIT time in mutant fishes is longer than in WT fish indicating that the narrowing of the lumen affects motility. Altogether, our data suggest that the ttc7a mutant zebrafish is a good model to further study the pathogenesis of HMIA and of intestinal lumen formation.

Lastly, in **chapter 8**, we discuss how all of the above-mentioned findings affect our understanding towards the disease pathogenesis and physiology of the intestine. Furthermore, we speculate on how further research might be directed to improve our understanding of these diseases.

SAMENVATTING

Voor een kinderchirurg is intestinale obstructie de meest geziene aandoening bij pasgeborenen. Ondanks het feit dat de prognose voor deze patiëntjes steeds beter wordt is de kans op overleven vrijwel nihil voor baby's met het 'Megacystis Microcolon Intestinale Hypoperistalsis Syndroom' (MMIHS), met aangeboren korte darmsyndroom (gemeenschappelijke basisbeginselen) of met erfelijke meerdere intestinale atresieën (HMIA). Omdat bij deze drie ziektebeelden familiale gevallen voorkomen wordt al langere tijd gesuggereerd dat de ziekte aangeboren moet zijn, dus erfelijk. Het werk zoals beschreven in dit proefschrift bestaat uit genetische studies, gevolgd door functionele studies, die allemaal tot doel hebben de etiologie van deze drie ziekten (MMIHS, gemeenschappelijke basisbeginselen en HMIA) beter te leren begrijpen.

In hoofdstuk één vatten we samen wat al bekend was over deze drie ziekten toen we in 2012 aan onze studies begonnen. We bespreken de klinische symptomen, de histopathologie, de behandeling, de prognose en de genetica.

Megacystis Microcolon Intestinale Hypoperistalse Syndroom (MMIHS)

Hoofdstuk twee beschrijft een aantal (functionele) onderzoeken die we hebben gedaan naar de gevonden aminozuurveranderingen (missense varianten) in het *ACTG2* gen. Deze mutaties werden gevonden in sporadische MMIHS patiënten. Zoals de naam al impliceert hebben patiënten met MMIHS een uitzetting van de blaas, in combinatie met een verminderde of afwezige darmperistaltiek, en een microcolon. Al deze symptomen kunnen worden geïdentificeerd in de neonatale periode. In lijn met de bevindingen van een aantal andere groepen, identificeerden we heterozygote varianten in het *ACTG2* gen in acht sporadische MMIHS patiënten. Immunohistochemie toont aan dat het *ACTG2* gen tot expressie komt in alle ontwikkelingsstadia van de menselijke darm die we hebben onderzocht. Het gen lijkt dus cruciaal voor de ontwikkelende darm. Interessant is dat er geen zichtbare afwijkingen konden worden waargenomen in de darmbiopsies van de onderzochte MMIHS patiënten. De resultaten van moleculaire dynamica en de uitgevoerde *in vitro* testen tonen aan dat de mutaties in het *ACTG2* gen resulteren in het minder goed binden van het afwijkende *ACTG2* eiwit aan actine filamenten. Ook konden we laten zien dat de mutaties zorgen voor minder contractiliteit. Dit bevestigt ons idee dat deze veranderingen ten grondslag liggen aan het MMIHS fenotype.

Aangezien we geen *ACTG2* varianten konden vinden in de familiale MMIHS patiënten, veronderstelden wij dat er naast het *ACTG2* gen nog een ander gen of andere genen verantwoordelijk moeten zijn voor het optreden van familiale MMIHS. Daarom voerden we 'exoom sequencing' en 'homozygosity mapping' uit in familiale MMIHS patiënten, meer specifiek in families waarin bloedverwantschap voorkomt. In **hoofdstuk drie** beschrijven we de identificatie van een homozygote stop mutatie in het Leiomodin-1 (*LMOD1*) gen. Het *LMOD1* gen codeert voor een eiwit dat functioneert als een 'actine filament nucleator' in gladde spiercellen. Om te bewijzen dat *LMOD1* het ziekteverwekkende gen voor familiale

MMIHS is, genereerden wij een *LMOD1*-KO muis en konden we laten zien dat deze muizen een aandoening hebben die opvallend veel lijkt op MMIHS. Mechanistisch onderzoek met menselijke intestinale gladde spiercellen en het jejunum van de *LMOD1* KO muizen toont aan dat het verlies van *LMOD1* de vorming van actine filamenten vermindert wat leidt tot een verminderde contractiliteit wat weer een verklaring kan zijn voor het onvermogen van de blaas en de darm om goed te functioneren.
Naast het *LMOD1* gen identificeerden we ook homozygote varianten in het *MYLK* gen. We vonden een verdubbeling van zeven basenparen in exon 22 binnen één gezin, en een splice variant in intron 22 in een andere familie. Deze bevindingen worden beschreven in **hoofdstuk 4**. Het *MYLK* gen codeert voor een eiwit dat de 'myosine lichte keten' fosforyleert. Deze fosforyleringsreactie veroorzaakt een interactie tussen de 'dikke myosine filamenten' met de 'dunne actine filamenten', het proces dat ten grondslag ligt aan spiercontractiliteit. Daarom is het verlies van *MYLK* waarschijnlijk de oorzaak van MMIHS bij deze patiënten. Gegevens van een reeds beschreven 'gladde spier-specifieke *MYLK*-KO' muismodel met een vergelijkbaar fenotype ondersteunt onze hypothese dat verlies van functiemutaties in *MYLK* inderdaad een recessieve vorm van MMIHS kan veroorzaken. Deze bevindingen bewijzen verder dat MMIHS een heterogene ziekte is met meerdere patronen van overerving.

Aangeboren korte dunne darm syndroom (CSBS)
Patiënten die gediagnosticeerd zijn met CSBS worden geboren met een veel te korte dunne darm, en wel een dunne darm die ongeveer ≥75% korter is dan de dunne darm van gezonde pasgeborenen. Onlangs heeft onze groep mutaties in twee genen geïdentificeerd die CSBS verklaren: coxsackie- en adenovirus receptor-achtig membraaneiwit (*CLMP*) en filamin A (*FLNA*). *CLMP* is een membraaneiwit dat een rol speelt in het 'tight junction complex'. *FLNA* is betrokken bij het verbinden van actine filamenten in het cytoplasma. In **hoofdstuk vijf** geven we een overzicht van de literatuur over CSBS, we bespreken onder andere de nieuwste klinische en genetische bevindingen.
Sinds de eerste publicatie over *CLMP* als het gen voor CSBS, zijn er geen andere publicaties meer verschenen over *CLMP* varianten in CSBS. In **hoofdstuk zes** beschrijven we de identificatie van drie nieuwe varianten in drie CSBS patiënten. We identificeerden een homozygote stop variant in exon 4 in één familie en we hebben twee heterozygote varianten gevonden in een andere patiënt (familie), te weten een aminozuur verandering en een splice site variant. Dat de aminozuur variant echt de ziekte zou kunnen veroorzaken werd bevestigd door functioneel werk in Chinese hamster ovarium (CHO-K1) cellen. Hieruit blijkt dat de aminozuur verandering de expressie van *CLMP* in het tight junction complex vermindert.Behalve *CLMP* hebben we ook mutaties gevonden in *FLNA*. Om te begrijpen hoe de geïdentificeerde *FLNA* variant CSBS veroorzaakt, hebben we een transgene zebravis gemaakt en onderzocht. Deze vis heeft een uit-frame mutatie, een mutatie die vergelijkbaar is met de mutaties die gevonden

zijn in CSBS patiënten. De resultaten van ons onderzoek zijn beschreven in **hoofdstuk zeven**. De mutante zebravis heeft net als de patiënten een verkorte darm, maar de verkorting is niet zo dramatisch als in CSBS. Wel laat het zien dat de functie van *FLNA* geconserveerd is tot in de zebravis. Behalve dat de darm iets korter is konden we ook aantonen dat de tijd dat voedsel nodig heeft om de gehele darm te passeren langer is.

Erfelijke meerdere intestinale atresieën (HMIA)

HMIA is een erfelijke vorm van multiple intestinale atresie, een ziekte die vaak samen voorkomt met een immunodeficiëntie. Tot op heden overleden alle patiënten met een dergelijk ziektebeeld. Vergelijkbaar met de bevindingen van andere groepen, identificeerden we een variant in tetratricopeptide-7A (*TTC7A*) in twee kinderen uit één gezin die beiden met HMIA en immunodeficiëntie werden gediagnosticeerd. Om het effect van *TTC7A* verlies te onderzoeken, genereerden we een transgene *TTC7A* zebravis. We beschrijven onze bevindingen over deze zebravis in **hoofdstuk acht**. Ondanks dat de vis er macroscopisch vergelijkbaar uitziet als de wildtype zebravis, laat de histologie van de *TTC7A* mutanten een dramatische vernauwing van het darmlumen zien. Dit suggereert dat het verlies van *TTC7A* in de zebravis de intestinale lumen vorming sterk beïnvloed. Bovendien is de tijd die voedsel nodig heeft de darm te passeren veel langer dan die van de wild type vis. Dit geeft aan dat de vernauwing van het lumen de motiliteit beïnvloedt. Onze data suggereren dat de *TTC7A* mutante zebravis een goed model is voor de pathogenese van HMIA en voor de intestinale lumen vorming.

Tenslotte, in **hoofdstuk negen** bespreken we hoe alle bovengenoemde bevindingen ons begrip met betrekking tot de pathogenese van de drie genoemde ziektebeelden en van de darm heeft veranderd. Verder speculeren we over de richting van vervolgonderzoek om onze kennis met betrekking tot deze ziekten nog verder te verbeteren.

RINGKASAN

Obstruksi usus adalah masalah kesehatan yang paling sering dihadapi dalam kasus kegawatdaruratan pada bayi baru lahir. Meskipun prognosis pasien penderita obstruksi usus terus meningkat, pada kasus-kasus yang disebabkan oleh sindrom megasistis mikrokolon intestinal hipoperistalsis (MMIHS), sindrom kelainan kongenital usus pendek (CSBS), ataupun kelainan bawaan atresia usus multipel (HMIA), kematian seringkali menjadi hasil akhir yang sulit dihindari pada kebanyakan kasus. Berdasarkan laporan yang ada tentang prevalensi kasus familial dari penyakit-penyakit diatas, kelainan genetik telah lama diduga sebagai penyebab utama dari kondisi ini. Thesis ini memaparkan hasil penelitian genetik pada pasien yang didiagnosis dengan penyakit-penyakit diatas, yang dilanjutkan dengan investigasi mengenai fungsi dari gen-gen yang terlibat, demi mengungkap mekanisme terjadinya penyakit-penyakit tersebut pada pasien.

Bab 1 memaparkan tentang hal-hal yang telah diketahui tentang ketiga penyakit bawaan ini sebelum dimulainya penelitian kami pada tahun 2012. Penjelasan ini mencakup tentang gejala klinis, hasil pemeriksaaan histopatologi, metode pengobatan yang telah ada, serta prognosis dan pengetahuan tentang latar belakang genetik dari ketiga penyakit ini.

Sindrom Megasistis Mikrokolon Intestinal Hipoperistalsis (MMIHS)

Bab 2 memaparkan tentang hasil uji fungsi mutasi missense pada gen actin otot polos usus γ-2 (*ACTG2*) yang menyebabkan kasus MMIHS sporadik. Seperti yang telah tersirat dalam nama sindrom ini, pasien yang didiagnosis dengan MMIHS menunjukkan gejala klinis yang khas, seperti distensi kandung kemih, yang disertai dengan berkurangnya atau hilangnya gerakan peristalsis usus, serta mikrokolon, yang kesemuanya dapat diidentifikasi pada masa bayi baru lahir. Sejalan dengan hasil penemuan beberapa grup peneliti lainnya, kami menemukan varian heterozigot pada gen ACTG2 di delapan kasus sporadik MMIHS. Hasil pemeriksaan immunohistochemistry menunjukkan bahwa *ACTG2* diekspresikan di setiap tahap perkembangan usus manusia yang diikutsertakan dalam penelitian ini. Menariknya, kami tidak dapat mendeteksi adanya kelainan dari hasil pemeriksaan histopatologi usus pasien MMIHS dengan mutasi di gen *ACTG2*. Hasil pemeriksaan molekuler dinamik dan eksperimen *in vitro* mengindikasikan bahwa mutasi di gen *ACTG2* yang ditemukan pada pasien MMIHS mengakibatkan penurunan kemampuan protein mutan ACTG2 untuk berikatan dengan filamen aktin, sehingga menurunkan kontraktilitas. Oleh karena itu, kami berspekulasi bahwa rentetan mekanisme inilah yang mengakibatkan terjadinya MMIHS pada pasien.

Mengingat kami tidak menemukan varian *ACTG2* pada kasus familial MMIHS, kami membuat hipotesis tentang kemungkinan adanya lokus lain yang bertanggungjawab atas terjadinya kasus familial MMIHS. Untuk itu, kami menerapkan metode pemeriksaan genetika terbaru, yaitu exome sequencing dan pemetaan homozigot, pada beberapa pasien MMIHS dari orangtua yang berkerabat.

Dalam **bab 3**, kami mempresentasikan penemuan mutasi *nonsense* homozigot di gen

Leiomodin-1 (*LMOD1*). Penelitian pada protein LMOD1 sebelumnya menganjurkan bahwa protein ini berfungsi sebagai nukleator filamen aktin di sel otot polos. Untuk membuktikan bahwa *LMOD1* adalah gen yang menjadi penyebab kasus familial MMIHS, kami membuat model tikus transgenik yang memiliki mutasi pada gen *LMOD1*, sehingga tidak dapat mengekspresikan protein LMOD1. Kami menemukan bahwa fenotipe yang dimiliki tikus transgenik ini sangat mirip dengan fenotipe yang diidentifikasi pada pasien MMIHS. Investigasi lebih lanjut tentang bagaimana mutasi di gen *LMOD1* dapat menyebabkan MMIHS dilakukan dengan menggunakan sel otot polos usus manusia dan jejunum tikus transgenik tanpa ekspresi gen *Lmod1*. Hasil dari berbagai eksperimen menunjukkan bahwa berkurangnya atau hilangnya protein LMOD1 mengakibatkan turunnya pembentukan filamen aktin. Selanjutnya, hal ini berakibat pada menurunnya kontraktilitas, dan berujung pada ketidakmampuan kandung kemih dan usus untuk mengekskresikan sisa produk metabolisme tubuh.

Meski kami telah menemukan mutasi di gen *LMOD1* sebagai penyebab kasus familial MMIHS, kelainan genetika pada tiga pasien MMIHS dari dua pasang orangtua yang berkerabat masih belum ditemukan. Oleh karena itu, kami menganalisis hasil *exome sequencing* dan pemetaan homozigot dari ketiga pasien ini. Di ketiga pasien ini, kami mengidentifikasi mutasi homozigot di gen *myosin light chain kinase* (*MYLK*) yang mengganggu ekspresi dari gen tersebut. Pada satu keluarga, mutasi yang kami temukan adalah duplikasi tujuh pasang basa nukleotida di ekson 22. Di satu keluarga lainnya, kami menemukan varian pada *splice site* yang terletak di intron 22. Kesemuanya ini dijabarkan dalam **bab 4**. Protein MYLK diketahui berfungsi untuk memfosforilasi *regulatory myosin light chain*. Proses fosforilasi ini mengakibatkan rantai tebal miosin berinteraksi dengan rantai tipis aktin, dan menyebabkan sel otot berkontraksi. Oleh karena itu, hilangnya ekspresi gen *MYLK* dapat mengakibatkan terganggunya proses fundamental ini, dan berujung pada didapatkannya MMIHS di ketiga pasien ini. Dari informasi yang telah ada tentang tikus transgenik yang kehilangan ekspresi gen *Mylk*, kami mengetahui bahwa tikus yang kehilangan ekspresi gen ini memiliki fenotipe yang menyerupai MMIHS pada pasien. Berdasarkan hal ini, maka kami menyimpulkan bahwa selain *LMOD1*, *MYLK* adalah gen yang juga berkaitan dengan terjadinya MMIHS pada pasien, dan diturunkan secara resesif. Penemuan ini juga membuktikan bahwa MMIHS adalah penyakit heterogen yang diturunkan dengan pola genetika yang beragam.

Sindrom Kelainan Kongenital Usus Pendek (CSBS)

Pasien yang didiagnosis dengan CSBS dilahirkan dengan panjang usus halus yang jauh lebih pendek dibandingkan panjangnya usus halus pada bayi normal, yaitu sekitar ≥75% lebih pendek. Baru-baru ini, grup kami mempublikasikan penemuan dua gen yang terkait dengan CSBS, yaitu *coxsackie- and adenovirus receptor-like membrane protein* (*CLMP*) dan filamin A (*FLNA*). CLMP adalah protein yang berperan dalam pembentukan struktur sambungan erat (*tight junction*) antar sel, sedangkan FLNA telah diketahui memiliki

fungsi dalam mengatur pola filamen aktin yang saling bersilangan di sitoplasma sel. Dalam bab 5, kami menyajikan studi pustaka yang telah ada mengenai CSBS, termasuk perkembangan terkini dalam bidang klinis dan penemuan genetika. Kami berspekulasi tentang bagaimana penemuan genetika terbaru ini akan mempengaruhi pemahaman kita mengenai mekanisme terjadinya CSBS pada pasien.

Di tahun 2012, sejak dipublikasikannya *CLMP* sebagai gen yang bertanggungjawab atas terjadinya CSBS pada pasien, belum ada publikasi yang melaporkan varian lainnya di gen *CLMP* dari pasien CSBS. Dalam bab 6, kami memaparkan penemuan tiga varian baru di gen *CLMP* pada tiga pasien yang didiagnosis dengan CSBS. Pada dua pasien yang merupakan saudara kandung, kami mengidentifikasi satu varian homozigot *nonsense* di exon 4 [c.508C>T; p.(R170*)]. Selain itu, kami juga menemukan 2 varian *CLMP* lainnya pada seorang pasien CSBS, termasuk varian *missense* di exon 4 [c.410G>A; p.(C137Y)], dan varian pada *splice site* di intron 2 (c.29-2A>G). Uji fungsi dari varian missense dengan menggunakan sel ovarium hamster China (CHO-K1) menunjukkan bahwa varian ini merusak ekspresi protein CLMP pada struktur sambungan erat.

Untuk memahami lebih jauh tentang mekanisme yang menjelaskan bagaimana varian *FLNA* pada pasien CSBS menyebabkan pemendekan usus, kami membuat dan mengkarakterisasi model ikan zebra transgenik dengan mutasi *out-of-frame* di gen *flna*. Hasil dari proyek penelitian ini kami presentasikan dalam **bab 7**. Meskipun hasil yang ada menunjukkan bahwa pemendekan usus pada ikan zebra transgenik tidak sebesar pemendekan usus pada pasien CSBS, secara statistik, pemendekan usus pada ikan zebra transgenik ini masih signifikan. Hal ini menandakan adanya kemiripan fungsi protein FLNA pada proses pemanjangan usus di manusia dan ikan zebra. Lebih lanjut, hasil dari uji transit usus menunjukkan bahwa ikan yang memiliki mutasi di gen *flna* memiliki waktu transit usus yang lebih lama dibandingkan dengan waktu transit usus pada ikan normal, mengindikasikan bahwa hilangnya protein flna pada ikan zebra bukan hanya mengakibatkan pemendekan usus, namun juga mengurangi motilitas usus.

Kelainan Bawaan Atresia Usus Multipel (HMIA)

HMIA adalah tipe familial dari kasus atresia usus multipel yang seringkali disertai dengan defisiensi fungsi imunitas. Hingga saat ini, kematian adalah hasil akhir yang hampir pasti didapat pada pasien HMIA. Sejalan dengan hasil penemuan yang telah dipublikasikan oleh grup penelitian lainnya, kami menemukan sebuah varian di gen *Tetratricopeptide-7A* (*TTC7A*) pada dua orang saudara kandung yang didiagnosis dengan HMIA dan defisiensi fungsi imunitas. Demi melakukan penyelidikan yang lebih mendalam tentang mekanisme terjadinya kelainan usus yang ditemukan pada pasien HMIA, kami membuat dan mengkarakterisasi ikan zebra transgenik yang memiliki mutasi pada gen *ttc7a*. Hasil yang kami dapatkan dalam proyek penelitian ini dipaparkan di **bab 8**. Meskipun secara kasat mata ikan mutan *ttc7a* tidak memiliki perbedaan signifikan dibandingkan dengan ikan normal, namun pemeriksaan histologi menunjukkan adanya penyempitan lumen

usus yang signifikan pada ikan mutan *ttc7a*. Penyelidikan lebih jauh terhadap fungsi usus dari ikan mutan *ttc7a* menunjukkan bahwa waktu transit usus pada ikan mutan *ttc7a* lebih lama dibandingkan dengan waktu transit usus pada ikan normal. Hal ini mengindikasikan bahwa hilangnya ekspresi gen *ttc7a* pada ikan zebra mengganggu proses pembentukan lumen dan motilitas usus. Berdasarkan seluruh hasil penelitian ini, kami menyimpulkan bahwa ikan mutan *ttc7a* yang kami buat adalah modalitas yang baik untuk digunakan dalam studi lebih lanjut tentang patogenesis HMIA dan proses pembentukan lumen usus.

Sebagai penutup, kami mendiskusikan bagaimana seluruh penemuan kami mempengaruhi pemahaman ilmiah yang ada tentang patogenesis and fisiologi usus. Lebih lanjut, kami berspekulasi tentang bagaimana penelitian ketiga penyakit ini di masa depan harus diarahkan.

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List of Publications

Alves M.M., **Halim D.***, Maroofian R.*, de Graaf B.M., Rooman R., van der Werf C.S., Van de Vijver E., Mehrjardi M.Y., Aflatoonian M., Chioza B.A., Baple E.L., Dehghani M., Crosby A.H., Hofstra R.M. 'Genetic screening of Congenital Short Bowel Syndrome patients confirms CLMP as the major gene involved in the recessive form of this disorder', Eur J Hum Genet. 2016 Jun. doi: 10.1038/ejhg.2016.58.

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Curriculum Vitae

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Educations

2012 - Present: Erasmus Medical Center, Rotterdam, the Netherlands

- PhD student at the Department of Clinical Genetics.

2006 – 2008: Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia

- Clinical Clerkship

2001 – 2006: Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia

- Bachelor in Medical Sciences.
- Thesis-research in Department of Pharmacy: "*The Effect of Ethanol Extract of Phaleria macrocarpa on Viability of Leukemia Cell Line L1210*", under the supervision of Dr. Ajeng Diantini.

Professional experiences

May 2012-December 2016: Erasmus Medical Center, Rotterdam, The Netherlands

- PhD Study in the Department of Clinical Genetics, research project: Genetics and Pathogenesis of Congenital Diseases of The Intestine, under the supervision of Prof. dr. Robert MW Hofstra ; Prof. dr. Dick Tibboel ; Dr. Alan J. Burns.

2011-2012: Roswell Park Cancer Institute, Buffalo, New York, United States

- Postgraduate Training in the Department of Cancer Prevention and Control, research project: *Translational Research of Finasteride and Dutasteride on the Prevention of Prostate Cancer*, under the supervision of Prof. dr. Clement Ip and Dr. Yue Wu.

2009-2010: Stem Cell Research Working Group, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia

- As a research staff in collaboration with Stem Cell and Cancer Institute, Jakarta, Indonesia, working *on "A Comparison of Cryopreservation Methods: Slow-Cooling vs Rapid-Cooling Based on Cell Viability, Oxidative Stress, Apoptosis and $CD34^+$ Enumeration of Human Umbilical Cord Blood*

Mononucleated Cells", (8 months) under the supervision of Dr. Ferry Sandra and Dr. Tono Djuwantono.

2007-2008: Promedika (Healthcare Management Consulting and Training Services, Bandung, Indonesia

- As a junior consultant in the project to formulate a strategic plan on how to transform RSPI Sulianti Saroso, Jakarta to become The Indonesian Institute of Infectious Diseases

2004-2006: Health Research Unit,Universitas Padjadjaran, Bandung, Indonesia

- As an assistant in Tissue Culture laboratory in research on: 1)*"The Protective Effect of Sargassum sp. on Ultraviolet A & B Irradiation-Mediated Oxidative Damage in Fibroblast Cells"*, 2005-2006, under the supervision of Dr. Savitri Restu Wardhani and Prof. dr. Tri Hanggono Achmad; 2) *"Optimation on Primary Culture of Fibroblast Cells Isolated from Chick Embryo and Human Preputium",* 2004-2005, under the supervision of Prof. dr. Tri Hanggono Achmad.

2004: Sanbe Farma Ltd, Bandung, Indonesia

- As a research apprentice in molecular biology laboratory of Biotech and Research Division, research on *"Mutation of P53 Gene in Patients with History of Familial Breast Cancer",* (3 months) under supervision of Dr. Joseph H.M. Hilgers, Dr. Kadarsyah and Dr. Eng. Sukma Nuswantara.

Additional Medical Training

- Department of Digestive Surgery, Lund Medical School, Lund University, Lund, Sweden, July - August 2006.
- Department of Transplantation Surgery, Graz Medical University, Graz, Austria, October 2005.
- Pius Branzeu Center for Laparoscopic and Microsurgery, Timisoara, Romania, September 2005.

PhD Portfolio

Summary of PhD training and teaching activities

Name PhD student: Danny Halim Erasmus MC Department: Clinical Genetics Research School: MGC	PhD period: 2012 - 2016 Promotor(s): Prof.dr. R.M.W Hofstra; Prof.dr. D. Tibboel; Dr. A.J. Burns Supervisor: Prof.dr. R.M.W Hofstra

1. PhD training

	Year	Workload (ECTS)
General courses		
- Safely Working in the Laboratory	2012	0,5
- Genetics	2012	3
- Laboratory animal science	2012	3
- Biochemistry and Biophysics	2013	3
- Literature Course	2014	2
- Cell and Developmental Biology	2014	3
- Biomedical English Writing and Communication	2015	2
- Research Integrity	2015	0,5
- Statistics	2015	2
Specific courses		
- Next Generation Sequence Data Analysis	2013	2
Seminars and workshops		
- 20th MGC PhD Workshop, Luxembourg	2013	1
- 21st MGC PhD Workshop, Münster	2014	1
Presentations		
- Oral presentation 26th International Symposium on Paediatric Surgical Research, Cape Town	2013	0,5
- Oral presentation MGC PhD Workshop, Münster	2014	0,5
- Oral presentation Bandung International Biomedical Conference, Bandung	2014	0,5
- Poster presentation International Enteric Nervous System Meeting, Rotterdam	2015	0,25
- Poster presentation European Society of Human Genetics Conference, Glasgow	2015	0,25
- Oral presentation MGC Annual Symposium, Leiden	2016	0,5

(Inter)national conferences		
- 26th International Symposium on Paediatric Surgical Research, Cape Town	2013	1
- Bandung International Biomedical Conference, Bandung	2014 2015	1 1
- International Enteric Nervous System Meeting, Rotterdam	2015	1
- European Society of Human Genetics Conference, Glasgow		
Other		
- Organizing committee 20th MGC PhD Workshop, Luxembourg	2013	2
2. Teaching	**Year**	**Workload (ECTS)**
- Supervising international medical student (Mariagiulia Dal Cero from Bologna, Italy)	2014	2
- Supervising BSc student (Noor Vermeij from Hogeschool Rotterdam)	2014 - 2015	2

TOTAL ECTS: 35,5

Dankwoord

First of all, thank God I will finally get this! This PhD study has been nothing less than a journey to me. Through countless ups and downs, lucks and challenges, happiness and disappointments, and hardships, this moment is finally here. And through this journey, I have been blessed with the presences and kindness of special people whom I would like to express my sincere gratitude to.

Dear **Prof.dr. Hofstra**, dear **Robert**, you have no idea how much your constant support and guidance meant to me. Through hours of discussions, you taught me so many things: not only science-related matters, but also politics and personal life. More than a good scientist, you are also a wonderful human being. So many times, I and my family talked about how thankful we were for your help and kindness. Robert, truly, I am forever in your debt. I hope one day I can make you proud, Sir! Thank you!

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