OVEREXPRESSION OF THE CHROMOSOME 21 GENE ATP5O RESULTS IN FEWER ENTERIC NEURONS; THE MISSING LINK BETWEEN DOWN SYNDROME AND HIRSCHSPRUNG DISEASE?

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Manuscript in preparation
Hirschsprung disease (HSCR) is characterized by the absence of enteric ganglia in the distal region of the gastrointestinal tract, leading to severe intestinal obstruction. Around 12% of patients with HSCR have a chromosomal abnormality, the most of which have Down Syndrome (DS), trisomy 21. Moreover, individuals with DS have a >100-fold higher risk of developing HSCR than the general population. This suggests that overexpression of human chromosome 21 (Hsa21) genes contribute to the etiology of HSCR. To identify the gene(s) contributing to HSCR in DS, we overexpressed candidate genes in a reporter zebrafish, Tg(-8.3bpox2b:Kaede) where neural crest derived cells express the fluorescent kaede protein. We prioritized 21 genes and overexpressed them by microinjecting capped mRNAs in single-cell stage zebrafish embryos and scored them at 5 days post fertilization (dpf). We show that overexpression of ATP5O (ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit) leads to a disturbed enteric nervous system (ENS) with a reduced number of enteric neurons, strongly implicating ATP5O as a contributor to a HSCR phenotype. The ATP5O gene encodes a component of the F-type ATPase found in the mitochondrial matrix and participates in ATP synthesis coupled proton transport. ATP5O does not link to the known HSCR pathways, and although we show expression of the protein in the enteric ganglia, its involvement in disease development and ENS development is yet to be uncovered.
INTRODUCTION

Hirschsprung disease (HSCR, MIM #142623) is a complex congenital gut motility disorder resulting from a failure in the development of the enteric nervous system (ENS) of the gastrointestinal (GI) tract. It is characterized by the absence of enteric ganglia in a variable length of the distal gut. HSCR is recognized by a failure to pass meconium in the first 48 hr after birth, abdominal distention, vomiting, and neonatal enterocolitis. It leads to severe intestinal obstruction and life threatening constipation. The prevalence of HSCR is 1 in 5000 live births and there is an unexplained sex bias of four males to one female. The lack of neurons in the distal part of the GI tract results from a failure of enteric neural crest cells (NCC) to migrate, differentiate, proliferate or survive and thereby colonize the gut and form a functional network of neurons and glia (reviewed by Sasselli et al., 2012).

HSCR is considered as an inherited disease, based on the fact that there are familial cases (~5%), and the 200-fold increased risk of HSCR to siblings of patients. Highly penetrant, coding mutations, in approximately 15 genes, have been identified to cause or contribute to HSCR (for review see). The major gene in HSCR is RET, with a mutation prevalence of 50% in familial HSCR and 15% in sporadic HSCR. However, cumulatively all the mutations in HSCR-associated genes explain only a small fraction of cases. In addition to the high penetrant coding mutations, common low-penetrance polymorphic variants at RET, in the region containing SEMA3C/SEMA3D and in NRG1 are also associated with HSCR.

However, all together the heritability of the vast majority (~80%) of HSCR cases is still to be uncovered. Finding genetic factors that may explain the missing heritability could come from analysis of known HSCR linkage regions, syndromic HSCR cases, or from the chromosomal abnormalities often identified in HSCR patients.

HSCR is associated with chromosomal abnormalities in 12% of all cases. In this study we focused on the most common chromosomal abnormality found in HSCR, Trisomy 21. Trisomy 21, leading to Down Syndrome (DS), is the most frequent cause of learning difficulties with an incidence of 1 in 750 live births. The incidence of DS among HSCR patients ranges from 2% to 10%. Moreover, DS patients have >100 fold higher risk of developing HSCR than the general population. This suggests that overexpression of one or more genes on chromosome 21 may have a substantial contribution to HSCR development in DS-associated HSCR cases. However, none of the established HSCR genes are localized...
on chromosome 21. Existing animal models for DS have not, as yet, been explored in detail for any ENS related defects and despite the vast knowledge available, this association still remains poorly understood.

Here we aimed to identify the gene(s) on chromosome 21 that could contribute to the HSCR phenotype. We injected mRNA of selected Hsa21 genes into a transgenic zebrafish reporter model, and found that elevated levels of one of the chromosome 21 genes, ATP5O, resulted in altered ENS development and a HSCR-like phenotype. Moreover, we show that ATP5O is expressed in the zebrafish gut and in the myenteric and submucosal ganglia of human postnatal colon sections.

METHODS

Prioritizing Hsa21 candidate HSCR Genes
In this study we first prioritized candidate genes based on genetic data and literature. The genetic data we used was: conservation of the genes between human and mouse; expression of the genes in mouse enteric NCC (in-house RNA sequencing data); whether genes encode transcription factors and; presence of the genes in segmental duplicated regions of chromosome 21 in DS/HSCR patients. In our literature search we took in consideration: previous studies on associations between DS and HSCR; Hsa21 genes that are involved in ENS and gut development; genes related to neuronal development; genes involved in neuronal signaling; known animal models of DS.

Hsa21 clone sets
To be able to microinject capped human mRNAs into 1-cell stage Tg(-8.3bphox2b:Kaede) zebrafish, the set of prioritized Hsa21 genes were sub-cloned in pCS2+ and were grown overnight followed by plasmid isolation and purification using the NucleoBond® Xtra plasmid purification system (Marchery-Nagel, Nagel, 2012). All the constructs were verified by DNA sequencing. A pSG5-huAPP-695 construct was used for the APP clone.

Zebrafish husbandry and strains
The Tg(-8.3bphox2b:Kaede) zebrafish line expresses the fluorescent Kaede protein in phox2b expressing cells, including those of the ENS. ret^sa2684/+ zebrafish line was obtained directly from Zebrafish International Resource Center (ZIRC). Both
the Tg(-8.3bphox2b:Kaede) and retsa2684/+ zebrafish lines were maintained by pairwise mating. A cross between retsa2684/+ and Tg(-8.3bphox2b:Kaede) was performed to generate Tg(-8.3bphox2b:Kaede); retsa2684/+ fish. Zebrafish were maintained at 28°C according to the standard zebrafish laboratory protocols. Embryos were scored for ENS defects and abnormal phenotypes at 5 dpf as described below. The institutional review board for experimental animals of Erasmus MC, Rotterdam approved the use of zebrafish embryos for this study. All procedures and fish experiments were performed in accordance with Dutch animal welfare legislations and those of the Erasmus Dierexperimenteel Centrum (EDC).

In vitro transcription of mRNA and microinjections into zebrafish embryos
In order to generate capped mRNA for microinjections, the plasmids were linearized with an appropriate restriction enzyme. After digestion, the plasmid DNA was cleaned using a phenol chloroform extraction method followed by ethanol precipitation. The linearized plasmids were used for in vitro synthesis of capped mRNA using the mMEssage mMMachine SP6 kit (Ambion Inc., AM1340). Total RNA was purified using the RNeasy mini kit (Qiagen, Inc., 74104) and loaded on a 2% agarose gel to assess RNA quality and integrity. Capped mRNA quantification was done using the Nanodrop8000 (Thermo). RNA samples were stored at -80°C. Capped mRNA was diluted in nuclease free water and microinjections were done in 1-cell stage zebrafish embryo to overexpress the genes, as described previously. Different dosages of each mRNA (5pg, 10pg, 50pg, 100pg, 150pg, 200pg and 250pg) were injected to determine their effect on ENS development. The mRNA-injected animals were raised in E3 media until 5dpf at 28°C. Non-injected control (NIC) embryos served as positive controls for survival.

Imaging and neuronal counting in zebrafish
Zebrafish embryos injected with capped mRNA and NIC were scored using a Leica MZ16FA microscope for any visible phenotype under bright field and by using a GFP filter to image phox2b-positive enteric NCC. To analyze the zebrafish embryos, they were anesthetized using tricaine in E3 media and then mounted on 0.3% agarose gel for capturing the images. The digital images were made using a Leica MZ16FA microscope. Fluorescent imaging was made under the same settings for each image. Images were processed with Leica LAS and Adobe Photoshop CS software. To count the number of enteric neurons, we used an in-house made
algorithm with image analysis software from FIJI in a semi-automated way (Figure 1C,D).

**Whole mount *in situ* hybridization in zebrafish**

A fragment of 686bp of zebrafish *atp5o* cDNA was amplified using RT-PCR using primer pair 5’-TTTCATCCAGACCAGTACG-3’ (forward) and 5’-GGTATCCCTGATCAGCTTGG-3’ (reverse). The amplified PCR product was ligated directly into the pCR®II-TOPO vector using the Dual Promoter TA cloning kit (Invitrogen). Positive clones were confirmed by DNA sequencing for the orientation and the correct sequence and used to generate antisense and sense

Figure 1. Schematic overview of experimental procedure. A) Schema for overexpression of prioritized candidate genes from Hsa21. B) Tg(-8.3bpox2b:Kaede) zebrafish line in bright field and under GFP filter, *phox2b* expressing neural crest cells are marked with fluorescent kaede protein. C) The enteric neurons of the intestinal region corresponding to 8 myotomes from the urogenital opening were selected as shown. D) Using FIJI software, the enteric neurons were counted as represented in the picture.
probes to detect atp5o mRNA expression. Whole-mount in situ hybridization was carried out as previously described\textsuperscript{19}. We used the DIG RNA labelling kit (Roche) to generate digoxygenin-labeled riboprobes against atp5o. Stained embryos were mounted in 70% glycerol. The images were acquired using a Leica MZ16FA microscope.

**Zebrafish genotyping**

\textit{Tg(-8.3bphox2b:Kaede); ret\textsuperscript{in2684/+} embryos were grown until 5dpf for phenotyping. DNA was extracted from individual embryos and genotyping PCR was performed to distinguish mutants from wildtype using the gene-specific primers ret-wt-F1 (5’GATCTCGTTCGCCTGGC3’), ret-mut-F1 (5’GATCTCGTTCGCCTGGT3’) and ret-wt-R1 (5’GGGCGGCGTGACTAATT3’).}

**Immunohistochemistry on human colon material**

Control postnatal human colon tissues were obtained from the Pathology Department repository of the Erasmus University Medical Center. Immunohistochemical (IHC) staining was performed using the Ventana Benchmark Ultra automated staining system (Ventana Medical System, Tuscon, AZ, USA). Briefly, after deparaffination the sectioned specimens for IHC detection of \textit{ATP5O} were processed for 60 min antigen retrieval using Cell Conditioning Solution (CC1, Ventana 950-124). After 30 minutes incubation with the primary antibody at 36°C (ATP5O 1:200), detection with UltraView Universal DAB detection kit (Ventana 760-500) was performed after amplification with Ultrasview amplification kit (Ventana 760-080). The sections were counterstained with hematoxylin II (Ventana 790-2208).

**Epistasis between ATP5O and ret in zebrafish**

1 ng of translation-blocking antisense morpholino against ret\textsuperscript{20} and 50 pg of \textit{ATP5O} capped mRNA were co-injected in 1 cell-stage \textit{Tg(-8.3bphox2b:Kaede)} embryos. Embryos injected with either ret morpholino or \textit{ATP5O} capped mRNA served as controls. At 5 dpf, embryos were imaged and enteric neurons present in the three myotome-length long, distal-most intestine were counted and compared.

**Cell culture and transfections**

The SK-N-SH Neuroblastoma cell line (ATCC # HTB-11) was cultured according to the ATCC’s protocol (LGC Standards, Middlesex, UK) and incubated at 37°C,
supplied with 5% of CO\textsubscript{2}. Approximately 10\textsuperscript{6} cells were cultured in 1 well of a 6-wells plate for 24 hr prior to transient transfections. Cells were transfected with 1\mu g of DNA construct containing \textit{ATP5O} (pCS2+/\textit{ATP5O}) or empty vector (pCS2+) and we used untransfected (UT) cells as a negative control. Transfections were done using 4\mu l Geneljice transfection reagent (Novagen, 70967, Millipore) according to the manufacturer’s instructions. Cells were starved in serum free media for 48 hr prior to harvesting and analysis.

\textbf{Cell apoptosis and cell proliferation assay}

Cell apoptosis was assessed by FACS analysis using PE Annexin V Apoptosis Detection Kit I (BD Pharmingen\textsuperscript{TM}) as per manufacturer’s instructions. Cells were washed with PBS. Early apoptotic cells were identified as PE Annexin V-positive and 7AAD-negative, while cells positive for both, PE Annexin V and 7AAD were marked as apoptotic cells. For cell cycle staining assays, ethanol fixed cells were stained with propidium iodide (PI) for 30 min at room temperature. Stained cells were analyzed on a FACS flow cytometer (BD Biosciences, San Jose, CA) and for both assays data analysis was performed using FlowJo.

\textbf{Statistical analysis}

Results are presented as means ± standard deviation (SD). Data were analyzed by unpaired two-tailed t-test (comparisons of two groups) for the statistical significance.

\textbf{RESULTS}

\textbf{Prioritization of candidate genes and generation of cDNA clones}

To test which gene(s) on chromosome 21 contribute(s) to HSCR in DS patients, a selection of the most promising candidate genes was made. A total of 169 genes were initially assembled for screening. They consisted of 149 Hsa21 genes that are conserved between human and mouse and another 20 genes that are non-conserved, but are potentially interesting, and human specific\textsuperscript{12,13}. From these 169 candidates we selected genes encoding transcription factors, genes involved in neuronal development and genes reported as involved in DS with or without gut abnormalities (such as HSCR). Following these criteria, we generated a subset of 65 candidate genes and among them we further prioritized the genes based on
their expression in E14.5 mouse enteric NCC (in-house RNA sequencing data), and based on functional evidence from studies in other model organisms. This pipeline resulted in a shortlist of 28 genes (Table 1) and we were able to synthesize 21 capped mRNAs (technical difficulties made us exclude 7 genes). A list of prioritized genes is presented in Table 1. A schematic of the experimental design is shown in Figure 1A.

Table 1. List of prioritized 28 genes in Hsa21 for overexpression.

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Overview of the 28 prioritized genes for overexpression. mRNA was injected into the Tg(-8.3bphox2b:Kaede) zebrafish for the first 21 genes. The last 7 genes were omitted due to failed mRNA generation.
**Tg(-8.3bphox2b:Kaede); ret^sa2684/+ mutants display ENS defect**

In humans, loss of function mutations in the RET gene result in HSCR. In this study we used the ret^sa2684/+ zebrafish that was identified in a ENU mutagenesis project as a positive control for a HSCR-like phenotype. The ret^sa2684/+ line was crossed with the Tg(-8.3bphox2b:Kaede) reporter zebrafish line and the number of enteric neurons was scored at 5dpf followed by genotyping. The ret^sa2684/+ mutant embryos contained significantly less enteric neurons in the gut, indicating an HSCR-like phenotype, when compared to control animals (Figure 2A-D). The quantification of enteric neurons, corresponding to 8 myotomes from the urogenital opening, demonstrated a significant reduction in number of enteric neurons in ret^sa2684/+ fish (88 ± 41) compared to WT fish (158 ± 23) (p<0.0001, Figure 2G). These data show that the Tg(-8.3bphox2b:Kaede) is a suitable animal model for HSCR-like aganglionosis.

**Overexpression of selected candidate gene mRNA in a zebrafish model**

Capped mRNAs of the 21 selected Hsa21 genes were injected into Tg(-8.3bphox2b:Kaede) zebrafish (Figure 1A,B), which were subsequently examined at 5 dpf (as described in the Methods section). The mRNA dosage was titrated in a range of 5pg to 250pg, to find the optimal dosage for each mRNA based on the lethality and phenotype observed. Injections of mRNAs resulted in normal ENS phenotypes for all mRNAs, except one. Only when overexpressing ATP5O (100pg), a reduction in the number of enteric neurons was observed along the entire intestine with normal gross morphology when compared to the non-injected controls (Figure 2E,F). The percentage of zebrafish displaying reduction in enteric neurons remained similar at higher dosage (150pg). Counting the enteric neurons within the gut, corresponding to 8 myotomes from the urogenital opening,

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**Figure 2. Reduced numbers of enteric neurons in the ret^sa2684/+ mutant fish and ATP5O mRNA injected fish.**
A,B) The control Tg(-8.3bphox2b:kaede) fish at 5 dpf in the bright field and under GFP filter showing fluorescently tagged phox2b expressing cells and the gut is completely colonized with enteric neurons until the urogenital opening. The asterisk indicates the urogenital opening. C,D) Enteric neurons along the gut of the Tg(-8.3bphox2b:Kaede); ret^sa2684/+. Heterozygous ret mutant displayed less neurons in the gut at 5dpf and discontinuity of colonization of the gut is indicated by arrowhead. E,F) The zebrafish injected with ATP5O mRNA at 100pg dosage show less enteric neurons in the gut. G) Quantification of enteric neurons in the intestine corresponding to 8 myotomes of 5dpf ret mutant compared to the control fish, marked significant reduction. H) Quantification of enteric neuronal count of non-injected controls compared to the ATP5O overexpressed embryos. A total of 37.5% of embryos injected with 100pg of ATP5O displayed reduction in enteric neurons.
OVEREXPRESSION OF ATP5O AS THE LINK BETWEEN DS AND HSCR?
the average count for the controls was \(178 \pm 23\) neurons (Figure 2H). We classified a gut as hypo-neuronal when the fish contained 2 SD less enteric neurons compared to the average control zebrafish. For the fish injected with \(ATP50\) mRNA we found that \(37.5\% \ (15/40)\) of the embryos displayed such a reduction in the number of enteric neurons in the gut. The enteric neuron count in the affected embryos displaying reduced enteric neurons was \(113 \pm 18\) (Figure 2H), showing that elevated levels of \(ATP50\) interfere with normal development of the ENS.

**Expression of \(atp5o\) in zebrafish**

Whole mount \textit{in situ} hybridization (ISH) was used to determine the spatio-temporal expression pattern of \(atp5o\) between 1dpf and 5dpf of zebrafish development. RNA \textit{in situ} hybridization revealed expression of \(atp5o\) in different organs at different developmental stages. At 1dpf, \(atp5o\) expression was seen ubiquitously (Figure 3B,C). At 2dpf the expression was restricted to the cerebellum, the otolith and the whole gut (Figure 3E,F). Between 3dpf to 5dpf \(atp5o\) was predominantly expressed in the intestine and cerebellum (Figure 3E,F,H,I,K,L,N,O). At 5dpf, high \(atp5o\) expression was observed in the proximal and mid intestine along with the caudal vein (Figure 3N). The sense probe did not show any staining at 1dpf – 5dpf stages (Figure 3A,D,G,J,M), confirming the specificity of the probe.

**Expression of \(ATP50\) in postnatal human colon**

To assess whether \(ATP50\) is also expressed in the human colon, immunohistochemistry was performed on postnatal colon from healthy individuals. \(ATP50\) was specifically detected in the ganglia present in the submucosal (Figure 4A,B) and myenteric plexuses (Figure 4C,D). In addition, \(ATP50\) was also detected in the colon epithelium. These results suggest that \(ATP50\) may be important for ENS development in humans as well.

**In vitro assays for cell apoptosis and cell cycle analysis**

To examine whether \(ATP50\) overexpression affects early apoptosis or the cell cycle and thereby leads to less neurons in zebrafish gut, we used a human neuroblastoma cell line (SK-N-SH) and assayed cell apoptosis and cell cycle. SK-N-SH cells expressing \(ATP50\) were cultured in the absence of serum for 48 hr and tested for both alterations in apoptosis and cell cycle. Flow cytometry analysis
didn’t indicate any significant changes in the early and late apoptosis in cells expressing ATP5O, when compared to other conditions (Supplementary Figure 1A). In order to identify the impact of ATP5O overexpression on cell cycle using

**Figure 3. Spatio-temporal expression of atp5o in zebrafish.** atp5o expression at indicated developmental stages ranging from 1–5dpf in zebrafish embryos (lateral view) detected using ISH. atp5o is expressed along the GI tract in all the stages. It is expressed ubiquitously at 1dpf (B,C) and the expression becomes restricted to cerebellum, otolith and whole gut by 2dpf (E,F). Arrowheads indicate expression in the brain and arrow marks indicate expression in the gastrointestinal tract (E,F,H,I,K,L,N,O). The sense probe shows no staining at 1–5dpf developmental stages as shown (A,D,G,J,M).
flow cytometry, we observed a slight increase in the fraction of cells in the G1 phase as a result of ATP5O overexpression (Supplementary Figure 1B). Although the observed difference is not statistically significant, it could indicate that there can be some effect on the cell cycle arrest in ATP5O-overexpressing cells.

**Epistasis between ATP5O and ret in zebrafish**
To investigate whether ATP5O interacts with RET during the development of the ENS and in the pathogenesis of HSCR in DS, we knocked down ret and overexpressed ATP5O simultaneously by co-injecting ret translation-blocking morpholino (1 ng) and ATP5O capped mRNA (50 pg) and compared enteric neurons in distal intestine at 5 dpf to controls injected with either ret morpholino or ATP5O mRNA alone (Figure 5). The doses were chosen so that neither was sufficient to induce severe ENS defect by itself, and any synergistic effect between ret knockdown and ATP5O overexpression would readily be observed. The ret morpholino caused a mild decrease in enteric neuron number in the distal intestine compared to ATP5O mRNA control. However, co-injection of ret morpholino and ATP5O mRNA did not result in further significant reduction, suggesting limited or no synergistic effect between ret knockdown and ATP5O overexpression.

**Other phenotypic effects of injection of DSCAM and SIM2 mRNA**
The use of this zebrafish model and its optical transparency allowed us to detect other gross developmental abnormalities upon overexpression of the prioritized genes. Injection of two candidate genes (SIM2 and DSCAM) resulted in an abnormal phenotype. Overexpression of SIM2 (100 pg) resulted in notochord defects in 66% of the injected embryos at 5dpf (Figure 6A,C) and 33% among them also displayed craniofacial abnormalities (Supplementary figure 2A,B). Overexpression of DSCAM (200 pg) resulted in deformed notochord and myotomes in 68% of the embryos at 5dpf (Figure 6B,D). The majority of these embryos also lacked the swim bladder. Microinjection of higher dosages (>200 pg for DSCAM and >100 pg for SIM2) of these mRNAs induced lethality.
DISCUSSION

This study reports a role for a chromosome 21 gene, \textit{ATP5O}, in the development of the ENS in zebrafish using an mRNA overexpression screen, as overexpression of \textit{ATP5O} results in reduced numbers of enteric neurons in the zebrafish gut. This phenotype is comparable to that of the \textit{ret}^{ iso2684/+} zebrafish line that carries a mutation in \textit{ret}, a known HSCR gene in humans. This makes us hypothesize that elevated levels of \textit{ATP5O}, as likely in the case of DS, could contribute to the high prevalence of HSCR among DS patients.

The zebrafish as a model organism for human enteric neuropathies

The intestinal architecture and anatomy of zebrafish closely resembles that of mammals\textsuperscript{21}. The zebrafish gut undergoes rapid development and by 5dpf the whole GI tract is functional\textsuperscript{22}. In contrast to amniotes, the zebrafish gut is simpler,

![Figure 4. Expression of \textit{ATP5O} in postnatal human colon. Expression of \textit{ATP5O} detected by immunohistochemistry on paraffin embedded post-natal colon sections. Arrowheads indicate expression of \textit{ATP5O} in submucosal plexus (A,B) and myenteric plexus (C,D). \textit{ATP5O} is also expressed in the gut epithelia as shown by arrows (A, B).]
it lacks submucosal layer and myenteric neurons are arranged as neuronal pairs or single neurons\textsuperscript{21}. The zebrafish ENS is also derived from NCC, as in other vertebrates\textsuperscript{23}. In zebrafish, NCC migrate as two parallel chains of cells to colonize the whole gut and differentiate into enteric neurons and glia\textsuperscript{24}. Despite these differences, the organization of the ENS, which modulates functions such as motility, homeostasis and secretion, is comparable but less complex compared to mammals making it a good model for human GI diseases\textsuperscript{25}. Previous studies have shown that perturbation of zebrafish orthologues of known human HSCR genes using morpholino mediated knockdown, but also some mutant zebrafish for genes not connected to HSCR, leads to loss of enteric neurons in zebrafish gut and recapitulates the human HSCR phenotype\textsuperscript{10,20,24,26-29}. In particular \textit{RET} is known to be the major player in HSCR and in ENS development\textsuperscript{4,30}. For these reasons we included the \textit{ret\textsuperscript{sa2684/+}} mutant zebrafish line as positive control. Indeed when quantifying the number of neurons in the hindgut, the most distal part of zebrafish intestine, the region in which mostly the aganglionosis in HSCR patients is observed, the number of neurons in this mutant fish was reduced.\textit{ATP5O} overexpression results in reduced enteric neurons\textit{ATP5O} overexpression results in reduced enteric neurons

Microinjection of \textit{ATP5O} mRNA resulted in reduced numbers of enteric neurons in the zebrafish gut comparable to what was found in case of the \textit{Tg(-8.3bphox2b:Kaede); ret\textsuperscript{sa2684/+}} zebrafish (Figure 2A-H). \textit{ATP5O} was the only gene for which overexpression resulted in ENS defects. The fact that overexpression results in fewer enteric neurons might not be a real surprise as mouse \textit{Atp5o} is highly expressed in mouse enteric NCC (in-house RNA sequencing data). \textit{ATP5O} is also expressed in the ganglia of submucosal and myenteric plexuses as shown in our studies using control postnatal colon sections. Similarly, \textit{atp5o} is also expressed in the zebrafish gut during early embryonic development and the ENS also forms during this period. Furthermore, meta-analysis of DS phenotypes in segmental trisomy’s and its association with congenital gut abnormalities such as HSCR, duodenal stenosis and intestinal atresia suggested a critical GI region of <13 MB. This region also includes \textit{ATP5O}\textsuperscript{14}. Previous identity-by-descent (IBD) and association mapping in a large (inbred) Mennonite population also showed that \textit{ATP5O} is within the IBD region associated with HSCR\textsuperscript{31}. All these data suggest that \textit{ATP5O} might well be responsible, or at least contribute to, the HSCR phenotype often seen in DS patients.
The role of ATP5O in HSCR

ATP5O is a mitochondrial gene, which encodes the ATP synthase H+ transporting, mitochondrial F1 complex, O subunit protein and is also known as Oligomycin Sensitivity Conferral Protein (OSCP). It is a component of ATP synthase (F(1)F(0) ATP synthase or Complex V) found in the mitochondrial matrix. ATP synthase is composed of an extramembranous catalytic core (F1) and a peripheral membrane proton channel (F0). The encoded protein appears to be part of the connector linking these two subunits and may be involved in transmission of conformational changes or proton conductance. It produces ATP from ADP via oxidative phosphorylation in the presence of a proton gradient across the mitochondrial membrane. Electron transport complexes of the respiratory chain generate this gradient\textsuperscript{32,33}. The gene ontology (GO) annotation of ATP5O associates it with drug binding and transporter activity. It was hypothesized that overexpression of ATP5O could interfere with the normal subunit composition of ATP synthase, resulting in an impairment of oxidative phosphorylation\textsuperscript{34}. An imbalance of expression, as generated in our zebrafish, could potentially impair the subunit composition of ATP synthase, leading to oxidative phosphorylation disruption and

Figure 5. Epistasis between ATP5O and ret. Quantification of enteric neurons at 5dpf in the distal most intestine corresponding to 3 myotomes of zebrafish embryos for epistatic interaction between ATP5O and ret. Embryos were injected with ATP5O (50ng), ret MO (1ng) and a combination of both and the enteric neuronal count is plotted in the graph. There are no significant differences between ATP5O (50pg) vs 1 ng ret MO (p=0.6587), 1 ng ret MO vs ATP5O (50pg) + 1ng ret MO (p=0.5437) and ATP5O (50pg) vs ATP5O (50pg) + 1 ng ret MO (p=0.2146).
eventual perturbed proliferation of these cells. We found a slight but not significant effect on cell cycle arrest (G1 phase) upon overexpression of ATP5O in SK-N-SH cells. It has also been shown that disruption of oxidative phosphorylation can have a neurotoxic effect on neuronal progenitor cells\textsuperscript{35}, and overproduction of ATP synthase in Escherichia coli has already been implicated in cell division and growth\textsuperscript{36}. During early ENS development, the enteric NCCs migrate, proliferate extensively and differentiate into neurons and glia. ATP5O overexpression could potentially affect enteric NCC proliferation and lead to fewer neurons in zebrafish gut, as observed in our experiments.

Figure 6. Notochord defects in SIM2 and DSCAM mRNA injected zebrafish. Overexpression of SIM2 and DSCAM lead to defects in the notochord, as represented by arrows in the bright field images of 5dpf embryos as compared to respective controls (A, B). The notochord is discontinuous and deformed in embryos in which SIM2 and DSCAM are overexpressed (C, D).
**ATP5O does not interact with ret**

A previous study showed over-representation of the enhancer polymorphism RET+9.7 (rs2435357:C>T) in DS-HSCR\(^3^7\). The disease-associated allele was significantly different between individuals with DS alone, HSCR alone, and those with HSCR and DS, demonstrating an association and interaction between RET and chromosome 21 gene dosage. However, our zebrafish data did not demonstrate any interaction between ret and ATP5O in ENS development, suggesting that they acted independently in separate pathways.

**Additional phenotypes due to overexpression of Hsa21 genes**

In this overexpression screen of 21 candidate genes from Hsa21, we also identified phenotypic defects other than that of the ENS for DSCAM and SIM2. Zebrafish dscam is highly expressed in the developing brain. It is thought to be involved in shaping the nervous system and early morphogenesis of the zebrafish embryo\(^3^8\). The expression pattern of sim2 in zebrafish has been reported using whole mount ISH; it is expressed mainly in the diencephalon, the midbrain and the pharyngeal arches\(^3^9\). Overexpression of DSCAM and SIM2 in zebrafish displayed defects mainly in notochord development and in the floor plate, exhibiting discontinuity with some twists and folds upon overexpression of these genes. The notochord is essential for proper vertebrate development by producing secreted factors that signal to the surrounding tissues. It is also important for specification of the ventral fates in the CNS and it plays an important role in patterning and in a proper structural integrity. The defects in notochord development are possibly due to uneven cell patterning or selective cell death or defects in signaling pathways required for normal notochord development (reviewed by Stemple, 2005\(^4^0\)). Moreover, besides notochord defects we also observed craniofacial abnormalities on overexpression of SIM2 in a subset of embryos at 5dpf along with notochord defects (Supplementary Figure 2A,B), indicating its prospective contribution to the phenotype observed in DS affected individuals. To our surprise, some candidates (such as DSCAM, SIM2, and APP) already associated with ENS phenotypes, based on previous genetic studies and murine models did not display any visible ENS phenotype following their overexpression in the zebrafish model. DSCAM has been highlighted as a predisposing locus to HSCR in patients with DS\(^1^4,3^1,4^1\). Our previous studies, using in vitro methods, have shown that overexpression of SIM2 leads to a down regulation of the RET gene\(^8\). Similarly, a transgenic APP mouse model displayed reduction in myenteric neuronal density and delay in gut transit\(^4^2\). These
are characteristic features of HSCR in humans, but we were not able to recapitulate similar phenotypes in the zebrafish model upon their overexpression. This could be due to the fact that the regulatory mechanism required for efficient translation of certain human RNAs was not equally efficient in zebrafish, or that the human protein does not have the same effect as the zebrafish protein or alternatively the threshold dosage of RNA required resulting in a phenotype may not have been achieved in our study. Furthermore, we cannot rule out any unknown feedback mechanisms resulting in the net neutrality of overexpression. On the other hand, a phenotypic effect may simply require a combinatorial overexpression of more than one gene.

Within the list of 21 genes we did not include COL6A4 although it was recently shown that overexpression of Col6a4 in transgenic mice could lead to a HSCR-like phenotype\textsuperscript{43}. The reason for not including it was the fact that we had not found any direct or indirect evidence for the involvement of Col6a4 with ENS development nor did we see the gene being expressed in the mouse enteric NCC at E14.5 (in-house RNA sequencing data).

Conclusions
Although the association of DS with HSCR is well recognized, the causative link between them is not well understood. The majority of DS affected individuals exhibit GI abnormalities\textsuperscript{44}, which might be related to abnormal ENS development. Here, we used a transgenic zebrafish line, whose ENS is marked with the fluorescent Kaede protein, to assay the functional effects of overexpression of Hsa21 candidate genes. We found that ATP5O affects ENS development in zebrafish. The use of a vertebrate model to find the missing link between DS and HSCR opens the door for larger screens and better understanding of this complex association.

ACKNOWLEDGEMENTS

This study was supported by research grant from ZonMW (TOP-subsidie 40-00812-98-10042) and the Maag Lever Darm stichting (WO09-62). We thank Prof. Bart de Strooper (Leuven, Belgium) for providing us with the APP plasmid; Herma van der Linde and Maria Alves for technical advice and helpful discussions.
REFERENCES

**SUPPLEMENTARY INFORMATION**

Supplementary Figure 1. *In vitro* apoptosis and cell cycle assays. SK-N-SH neuroblastoma cells were transected with construct containing *ATP5O* and the cells were starved for 48 hours and assayed for apoptosis and cell cycle assays by FACS analysis. Data are represented as mean ± SD for two independent experiments. A) Cells that were PE Annexin V-positive and 7AAD-negative were classified as early apoptotic, while cells positive for both PE Annexin V and 7AAD were marked as apoptotic. There were no differences between *ATP5O*-transfected cells and controls. B) Cell cycle analysis using propidium iodide (PI) DNA staining indicated no major differences between the cell phases, but there was a slight increase in G1 phase for *ATP5O*-transfected cells as compared to the controls.
Supplementary Figure 2. Craniofacial abnormalities by SIM2 overexpression. A) Control embryo at 5dpf. (B) SIM2 (100pg) overexpressing embryo at 5dpf displaying craniofacial abnormality as shown by arrows in 33% of them along with the notochord phenotype. The lower jaw appears dislodged compared to the control.