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ATP5PO levels regulate enteric nervous system development in zebrafish, linking Hirschsprung disease to Down Syndrome

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ABSTRACT

Hirschsprung disease (HSCR) is a complex genetic disorder characterized by the absence of enteric nervous system (ENS) in the distal region of the intestine. Down Syndrome (DS) patients have a >50-fold higher risk of developing HSCR than the general population, suggesting that overexpression of human chromosome 21 (Hsa21) genes contribute to HSCR etiology. However, identification of responsible genes remains challenging. Here, we describe a genetic screening of potential candidate genes located on Hsa21, using the zebrafish. Candidate genes were located in the DS-HSCR susceptibility region, expressed in the human intestine, were known potential biomarkers for DS prenatal diagnosis, and were present in the zebrafish genome. With this approach, four genes were selected: *RCAN1*, *ITSN1*, *ATP5PO* and *SUMO3*. However, only overexpression of *ATP5PO*, coding for a component of the mitochondrial ATPase, led to significant reduction of ENS cells. Paradoxically, *in vitro* studies showed that overexpression of *ATP5PO* led to a reduction of ATP5PO protein levels. Impaired neuronal differentiation and reduced mitochondrial ATP production, were also detected *in vitro*, after overexpression of *ATP5PO* in a neuroblastoma cell line. Finally, epistasis was observed between *ATP5PO* and *ret*, the most important HSCR gene. Taken together, our results identify *ATP5PO* as the gene responsible for the increased risk of HSCR in DS patients in particular if *RET* variants are also present, and show that a balanced expression of ATP5PO is required for normal ENS development.

1. Introduction

Hirschsprung disease (HSCR, MIM #142623) is a complex congenital disorder characterized by the absence of an enteric nervous system (ENS) in a variable length of the distal gut. This defect is caused by a failure of enteric neural crest cells (ENCCs) to migrate, differentiate,

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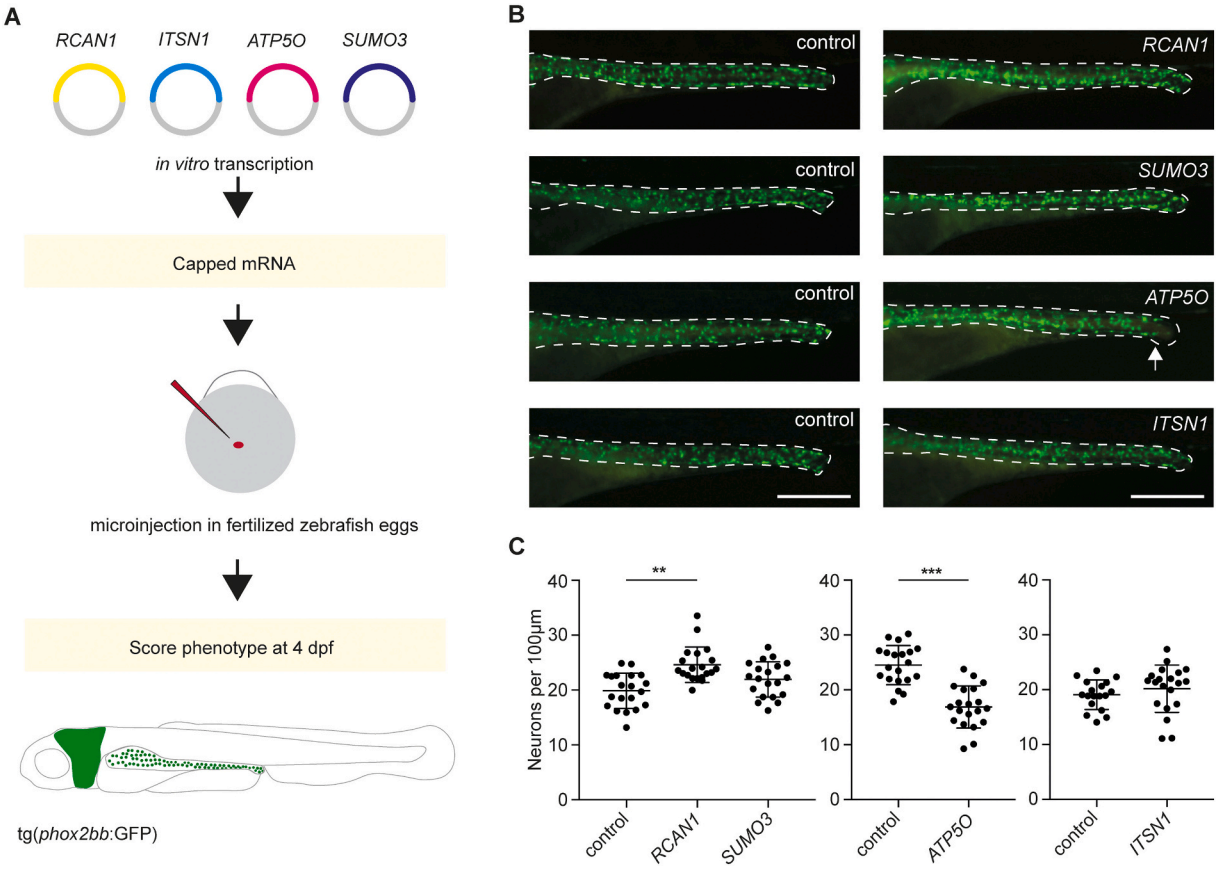

Fig. 1. Systematic approach to assess the effects of overexpression of Hsa21 genes on ENS development in zebrafish.
A. Schematic representation of the experimental setup. B. Representative images of control and larvae injected with human RNA. The arrow depicts the start of the intestine that lacks ENS cells. Scale bar represents 200 µm. C. Quantification of the number of *phox2bb:GFP*+ enteric neurons per 100 µm gut length. **p < 0.01, *** = p < 0.001. Each dot represents one animal; error bars represent standard deviation.

proliferate or survive during colonization of the gastrointestinal (GI) tract. HSCR is considered to be a complex genetic disease and coding variants in >20 genes have been identified to cause or contribute to its development [1]. The major gene involved in HSCR pathogenesis is the rearranged during transfection (*RET*) gene, with a mutation prevalence of 50 % in familial HSCR and 15 % in sporadic cases [2,3]. Common variants in *RET* influence HSCR penetrance and are known to increase disease risk, especially in homozygous carriers [4,5]. Two of these variants are located in intron 1 of *RET* (SNP1 [rs2506004] and SNP2 [rs2435357]) [6,7], and are frequently referred to as a risk haplotype, due to their effect on RET expression [8,9].

HSCR is associated with chromosomal abnormalities in 12 % of the cases, most commonly Trisomy 21, which affects 2 % to 10 % of all HSCR cases [10]. Trisomy 21, leading to Down Syndrome (DS), is the most frequent cause of intellectual disability, with an incidence of 1 in 707 live births in the USA, and 1 in 1.000 live births in the Netherlands [11,12]. Moreover, DS patients have a > 50-fold higher risk of developing HSCR than the general population [10]. This suggests that overexpression of one or more genes located on chromosome 21 (Hsa21) has a substantial contribution to HSCR development. A discrete critical region in Hsa21, <13 Mb, was identified as a susceptibility region for HSCR [13]. Polymorphisms near two genes located in this region, *DSCAM* and *DYRK1A*, have been previously linked to HSCR [13–15]. To evaluate the role of these genes in ENS development, two trisomy 21 mouse models containing many Hsa21-homologous genes, including *Dscam* and *Dyrk1a*, were generated. Although these models showed an abnormal ENS, neither of them presented a typical HSCR phenotype (no aganglionosis in the distal colon was observed) [16]. Moreover, normalizing the copy number of *Dscam* and *Dyrk1a* failed to rescue the ENS defects, challenging the role of these genes in HSCR susceptibility. Another study showed that increased levels of collagen VI alter the cell-autonomous extracellular matrix composition surrounding ENCCs, slowing their migration [17]. Since two genes coding collagen VI components, *COL6A1* and *COL6A2*, are located on Hsa21, it was suggested that this mechanism could explain the ENS phenotype in HSCR-DS [17]. Finally, the BACE1-APP-BACE2 pathway has recently been associated with HSCR, as mutations in *BACE2* were shown to enhance ENCC migration and prevent apoptosis [18,19]. *BACE2* is located in the HSCR-DS susceptibility region of Hsa21, and although it was considered to be a good candidate to explain the ENS defects that occur in DS patients, its role has never been explored *in vivo*.

Here, we report on a targeted screen in zebrafish, to evaluate the effect of a potential set of candidate genes located in Hsa21, in ENS development.

2. Materials and methods

2.1. Zebrafish husbandry and strains

The *Tg(phox2bb:eGFP)* zebrafish line expresses the fluorescent GFP protein in *phox2bb* expressing cells, including those of the ENS [20]. The *ret*$^{sa2684/+}$ zebrafish line was obtained directly from the Zebrafish International Resource Center (ZIRC) [21]. Zebrafish were maintained at 28.5 °C, according to standard zebrafish laboratory protocols [22].

2.2. Whole mount in situ hybridization

atp5po probes were generated and labelled as described before [8].

Table 1
Patient characteristics.

Patients	Surgery	HSCR phenotype	Gender	Age at surgery (months)	WES panel
Controls					
01	Rectosigmoid resection; congenital rectal prolapse	NA	F	5	NA
02	colon ascendens resection; stenosis after NEC	NA	M	3	NA
03	Colon ascendens/ transversum resection; stenosis after NEC	NA	M	3	NA
HSCR+/Trisomy 21+					
04	Pullthrough	short	M	12	Not performed
05	Pullthrough	short	F	4	Not performed
06	Pullthrough	short	M	9	Not performed
HSCR+/Trisomy 21−					
07	Pullthrough	short	M	4	No pathogenic variant
08	Pullthrough	short	F	3	No pathogenic variant
09	Pullthrough	short	F	4	No pathogenic variant

Abbreviations: HSCR = Hirschsprung disease, NEC = Necrotising enterocolitis, NA = not applicable, F = female, M = male, Short = Short-segment HSCR.

2.3. Generation of human capped mRNA

The Hsa21 Gene Expression Set generated by the lab of Prof. Roger Reeves and now available through AddGene (https://www.addgene.org/Roger_Reeves/), was used to generate capped mRNA (Kit #1000000099) [23]. All constructs were verified by Sanger sequencing, digested with *Xho*I and purified using a standard phenol chloroform extraction method, followed by ethanol precipitation. Linearized plasmids were used for *in vitro* synthesis of capped mRNA, using the mMEssage mMachine SP6 kit (Ambion Inc., AM1340). Capped mRNA was purified with the RNeasy mini kit (Qiagen Inc., 74104) and loaded on a 2 % agarose gel to assess RNA quality and integrity.

2.4. CRISPR/Cas9 gene disruption

For disruption of *atp5po* in zebrafish we used the Alt-R™ CRISPR Custom Guide RNAs from integrated DNA technologies (IDT). Alt-R® CRISPR-Cas9 tracrRNA for zebrafish *atp5po* was designed using the tool offered by IDT (Dr.Cas9.ATP5O.1.AD): gRNA sequence: 5′ - CATCAGGGGTCAAGGTCAAG – 3′ PAM: CGG, on-target score: 68, off-target score: 91. Injections were performed as previously described [24,25]. To determine gRNA efficiency, a 497 base pairs (bp) product was amplified using forward 5′ – ACCAAGGCTAAAGTCTCCCC – 3′ and reverse 5′ – TTCAGTGCCACTTTCAGCTC – 3′ primers, located in exon 3 and 5 of *atp5po*, respectively. The PCR product was Sanger sequenced and efficiency was determined using the online tool TIDE (https://tide.nki.nl) [26].

2.5. Imaging and neuronal counting in zebrafish

Fertilized eggs at the 1–2 cell stage (50–100 eggs) were injected with capped mRNA (150 ng and 300 ng), or the CRISPR/Cas9 complex. Non-injected controls were scored using a Leica M165 microscope for any visible phenotype under bright field, and GFP-positive ENCCs were visualized with a GFP filter (Fig. 1). To analyze zebrafish embryos at 4 days post fertilization (dpf), embryos were anesthetized using tricaine in E3 media and mounted on their sides on a 1.8 % agarose gel. Digital images were taken using a Leica M165 microscope. Fluorescent imaging was made under the same settings for each image. Images were processed with the Leica LAS software, and the number of enteric neurons present in the full length of the intestine was counted using ImageJ (FIJI), in a semi-automated way.

2.6. Zebrafish genotyping

Tg(phox2bb:GFP); *ret*$^{sa2684/+}$ embryos grew until 4 dpf for phenotyping (as described in the imaging and neuronal counting section). Larvae were lysed individually for post-hoc genotyping. A genotyping PCR was performed to distinguish heterozygous mutants from wildtype (wt) fish, using the allele-specific forward primers, *ret*-wt-F1 (5′-GATCTCGTTCGCCTGGC-3′) and *ret*-mut-F1 (5-′GATCTCGTTCGCCTGGT-3′), with the reverse primer *ret*-wt- R1 (5′-GGGGGCGTGTGACTAATTT-3′). Products were analyzed on a 2 % agarose gel.

2.7. Immunohistochemistry on human colon material

Human colon sections from controls, HSCR patients and DS patients with HSCR, were obtained from the repository of the Pathology Department of the Erasmus University Medical Center (for patient characteristics see Table 1). Immunohistochemical (IHC) staining was performed using the Ventana Benchmark Ultra automated staining system (Ventana Medical System, Tuscon, AZ, USA). Briefly, sectioned specimens were processed for 60 min after deparaffinization for antigen retrieval, using the Cell Conditioning Solution (CC1, Ventana 950-124). After 30 min, incubation with the primary antibody was performed at 36 °C (ATP5PO 1:200; HPA041394; Atlas antibodies), followed by amplification with the Ultraview amplification kit (Ventana 760-080) and detection with the UltraView Universal DAB detection kit (Ventana 760-500). Sections were counterstained with hematoxylin II (Ventana 790-2208).

2.8. Cell culture and transfections

The SH-SY5Y neuroblastoma cell line (ATCC # HTB-11) was cultured according to the ATCC's protocol (LGC Standards, Middlesex, UK). A stable ATP5PO transfected SH-SY5Y cell-line was established, using G418 selection. After optimising the amount of G418 to be used, 2×10^6 SH-SY5Y cells were plated in a 10 cm dish. Twenty-four hours after, cells were transfected with a ATP5PO construct using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions. A dish with untransfected cells was used as a negative control. Culture medium containing G418 was replenished every 3 days until all the untransfected cells died. Colonies formed from the surviving cells were isolated and continued to grow separately. They were tested for ATP5PO RNA and protein expression levels.

2.9. Neuronal differentiation

Neuronal differentiation was induced according to a previously described protocol [27]. Briefly, 3×10^5 cells were seeded on laminin (Sigma) coated wells or coverslips, of/in a six wells plate (10 μg/ml in PBS). Twenty-four hours after seeding, control cells were harvested (0 h) and 50 nM of insulin growth factor (IGF-1; Peprotech) was added to the remaining wells to start differentiation (72 h). Medium was replenished

after 48 h. Seventy-two hours after treatment, cells were trypsinized, resuspended in 1 ml complete medium, and centrifuged at 1.000 rpm for 5 min. Cell pellets were washed with 1 ml PBS and resuspended in either RNA lysis buffer, protein lysis buffer or ATP assay buffer.

2.10. Reverse transcriptase quantitative (RT-q) PCR

RNA was isolated using the Qiagen mini-RNA isolation kit according to the manufacturer's instructions. Thereafter, cDNA was generated using the iScript™ cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. For RT-qPCR, iTAQ™ Universal SYBR® Green Supermix (Biorad) was used on a CFX Opus 96 Real-Time PCR system (Biorad). Primers targeting *ATP5PO*, neuron-specific class III beta-tubulin (*TUJ1*) and growth associated protein 43 (*GAP43*) were used (Table S1). Expression of target genes was normalized by the expression of the housekeeping gene Heterogeneous nuclear ribonucleoprotein L (*HNRNPL*). For the RT-qPCR of endogenous zebrafish *atp5po*, *eef1a1* and *actb1* were used as housekeeping genes (Table S1).

2.11. Western blot

After lysis, cells were centrifuged at 14.000 rpm for 10 min at 4 °C to remove nuclei and cellular debris. Supernatants were collected and prepared for Western blotting as previously described [28]. Primary antibodies specific for ATP5PO (HPA041394 Atlas antibodies, 1:100) and B-actin (sc-69879 Santa Cruz, 1:1000) were used, followed by incubation with the secondary antibodies, IRDye 800CW Goat anti-mouse and IRDye 680RD Goat anti-Rabbit (Li-Cor).

2.12. Immunofluorescence

Cells on coverslips were fixed with 4 % paraformaldehyde (PFA) and permeabilized with 1 % BSA and 0.1 % Triton X-100 in PBS. Cells were incubated with phalloidin-rhodamine (Santa Cruz, 1:500), TUJ1 (1:1000) and Hoechst 33342 (Invitrogen). Images were taken using a Leica SP5 Intravital microscope and analyzed with the Leica LAS AF Lite software.

2.13. ATP production/activity

ATP levels were determined using the ATP Colorimetric assay kit (Sigma-Aldrich, MAK190) according to the manufacturer's instructions. Protein concentrations were measured to correct for the input amount.

2.14. Statistical analysis

Results are presented as means ± standard deviation (SD). Data were analyzed by unpaired *t*-test (comparisons of two groups) or two-way ANOVA with Tukey multiple comparisons test (comparison of >2 groups with two independent variables) for statistical significance. For the epistasis experiment, the online tool MedCalc (MedCalc software ltd., Ostend, Belgium), was used together with the "N-1" Chi-squared test for categorization. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Prioritizing Hsa21 candidate HSCR genes

To identify novel HSCR genes in Hsa21, we chose a targeted approach. Hsa21 contains 218 genes (NCBI) of which 117 are located in the previously identified DS-HSCR critical region [13]. From these genes, we selected the ones expressed at >20 counts per million in the human intestine at gestational week 12 [29]. Forty-seven genes remained. Interestingly, a previous study aimed at identifying potential biomarkers for prenatal diagnosis of DS, showed that five of these 47 genes are overexpressed in DS amniocytes [16,17]. These genes were

Table 2
Gene selection of Hsa21 genes for analysis in zebrafish.

Gene name	Expressed human intestine [29]	Biomarkers for DS [13]	Zebrafish orthologue [54]
ATP5PO	Yes	Yes	Yes
ITSN1	Yes	Yes	Yes
RCAN1	Yes	Yes	Yes
SUMO3	Yes	Yes	Yes
SON	Yes	Yes	No
ADARB1	Yes	No	
AGPAT3	Yes	No	
BRWD1	Yes	No	
C2CD2	Yes	No	
CBR1	Yes	No	
CLIC6	Yes	No	
COL18A1	Yes	No	
COL6A1	Yes	No	
CRYZL1	Yes	No	
CSTB	Yes	No	
DYRK1A	Yes	No	
ETS2	Yes	No	
GART	Yes	No	
HLCS	Yes	No	
HMGN1	Yes	No	
IFNAR1	Yes	No	
IFNGR2	Yes	No	
MORC3	Yes	No	
MRPS6	Yes	No	
NDUFV3	Yes	No	
PDE9A	Yes	No	
PDXK	Yes	No	
PFKL	Yes	No	
PKNOX1	Yes	No	
POFUT2	Yes	No	
PSMG1	Yes	No	
PTTG1IP	Yes	No	
PWP2	Yes	No	
RIPK4	Yes	No	
RRP1	Yes	No	
RRP1B	Yes	No	
SETD4	Yes	No	
SLC19A1	Yes	No	
SLC37A1	Yes	No	
SLC5A3	Yes	No	
TFF3	Yes	No	
TMEM50B	Yes	No	
TMPRSS2	Yes	No	
TRAPPC10	Yes	No	
TTC3	Yes	No	
U2AF1	Yes	No	
UBE2G2	Yes	No	
ABCG1	No		
AIRE	No		
B3GALT5	No		
BACE2	No		
CBR3	No		
CBS	No		
CHAF1B	No		
DNAJC28	No		
DONSON	No		
DSCAM	No		
ERG	No		
FAM207A	No		
FAM3B	No		
ICOSLG	No		
ITGB2	No		
LRRC3	No		
MX1	No		
MX2	No		
PCBP3	No		
PCP4	No		
PIGP	No		
PRDM15	No		
RSPH1	No		
RUNX1	No		
SH3BGR	No		
SIK1	No		

(*continued on next page*)

Table 2 (*continued*)

Gene name	Expressed human intestine [29]	Biomarkers for DS [13]	Zebrafish orthologue [54]
TFF1	No		
TMPRSS3	No		
TRPM2	No		
TSPEAR	No		
WDR4	No		

considered to be excellent candidates to start the screening. Finally, genes that lacked a zebrafish orthologue were excluded (Table 2), resulting in a final selection of four genes: *RCAN1*, *ITSN1*, *ATP5PO* and *SUMO3*.

3.2. Overexpression of ATP5PO leads to a reduction in the number or enteric neurons in zebrafish

To evaluate if any of the 4 genes selected could be a HSCR gene, capped mRNAs encoding *RCAN1*, *ITSN1*, *ATP5PO* or *SUMO3* were injected into fertilized 1-cell-stage eggs from *Tg(−phox2bb:GFP)* zebrafish. *RCAN1* and *SUMO3* were overexpressed in the same clutch of eggs, while *ATP5P0* and *ITSN1* were overexpressed in different days. For each overexpression, uninjected controls collected from the same clutch of eggs were used for comparison. At 4 dpf, injected fish were examined for the presence of enteric neurons in the gut (Fig. 1A; Table 1). We chose 4 dpf since at this time point, the ENS is formed, covering the full intestine. For *ITSN1* and *SUMO3* mRNA overexpression, no ENS phenotype was observed (Fig. 1B). Although we cannot exclude that the lack of

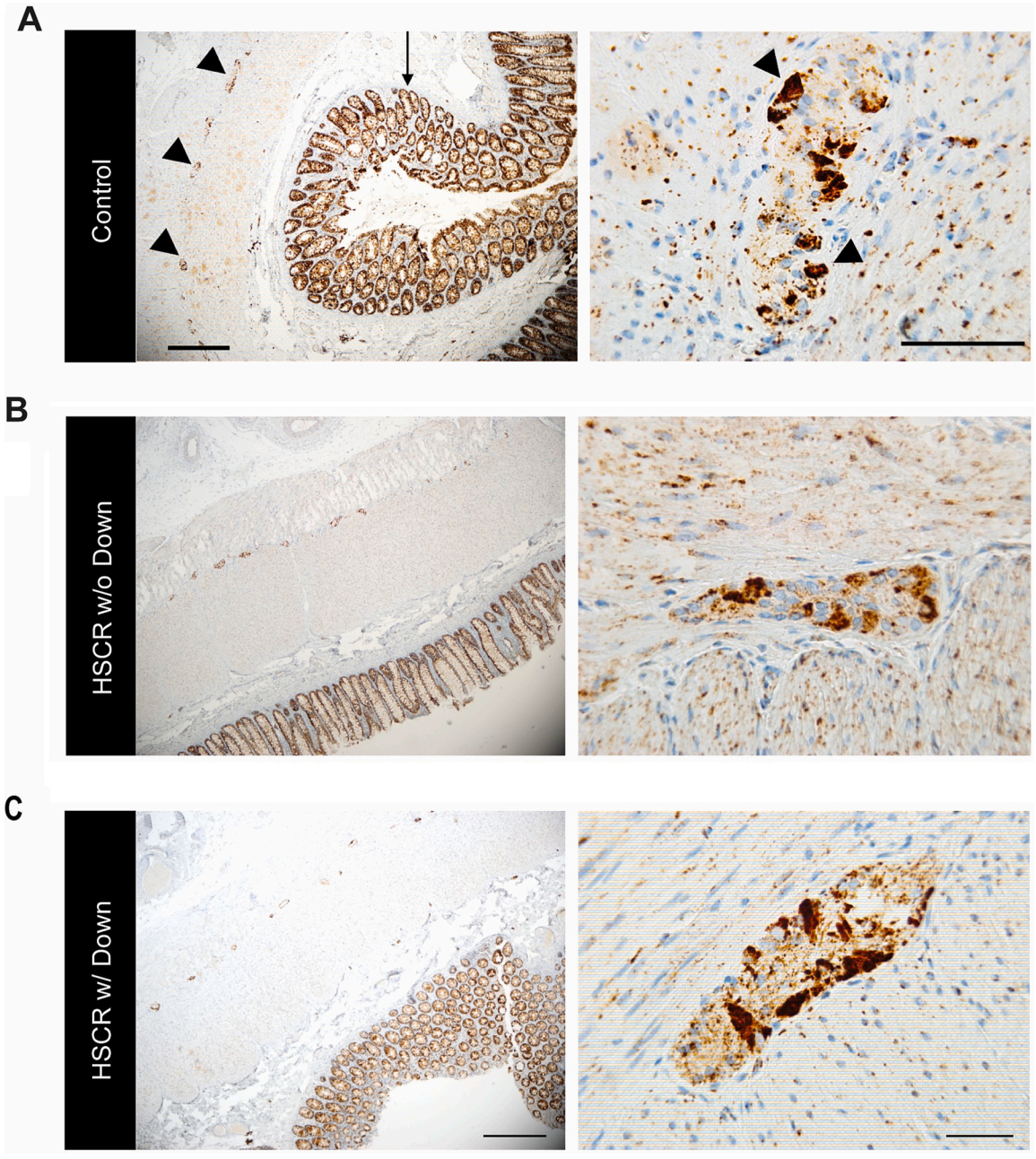

Fig. 2. ATP5PO is expressed in the myenteric plexus of the human colon.
A. Intestine of a healthy individual showing ATP5PO staining in the myenteric plexus (arrowheads) and the epithelium (arrow). Scale bars represent 100 μm. B. Representative images of the (ganglionic) intestines of a HSCR patient without DS, and C. a HSCR patient with DS. Similar expression patterns of ATP5PO were detected. Scale bars represent 100 μm (left panels) and 50 μm (right panels); n = 3 per group.

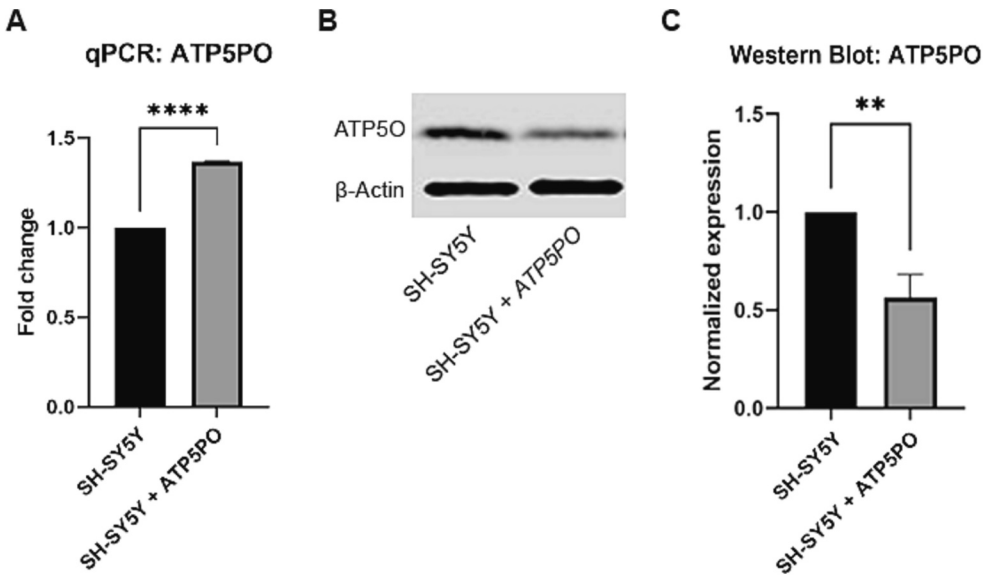

Fig. 3. *ATP5PO* RNA overexpression leads to reduced ATP5PO protein.
A. RT-qPCR data showing *ATP5PO* RNA expression levels in SH-SY5Y and SH-SY5Y-*ATP5PO* stable cells. Mean of two technical replicates is visualized. Error bar represents standard deviation; *p < 0.05. Experiment was performed in triplicate. B. Representative image of western blot. C. Western blot quantification showing reduced ATP5PO protein expression in SH-SY5Y-*ATP5PO* cells, normalized to b-actin. Mean of three independent experiments is plotted. Error bar represents standard deviation; **p < 0.01.

phenotype is due to use of human mRNA and not zebrafish mRNA, the high conservation rate of these genes, 70 and 80 % homology respectively, suggests that this is unlikely. Overexpression of *RCAN1* and *ATP5PO* mRNA on the other hand, showed a significant effect. While *RCAN1* unexpectedly increased the number of neurons present in the GI tract by 24 % ($p = 0.001$; Fig. 1C), overexpression of *ATP5PO* had the opposite effect, resulting in a significant reduction (31 %) in the number of enteric neurons present in the distal gut, when compared to non-injected controls ($p < 0.001$, Fig. 1C). For all groups, the gross morphology of injected larvae appeared to be normal (Fig. S1A). The presence of microinjected human *ATP5PO* mRNA in zebrafish was verified by RT-qPCR at 2, 3 and 4 dpf (Fig. S2A). However, the injected human ATP5PO protein was not detected (Fig. S2B). Based on the results obtained, ATP5PO was selected for further analysis.

3.3. *ATP5PO is expressed in the postnatal human colon of controls, HSCR patients and DS-HSCR patients, as well as in the zebrafish intestine*

To assess whether *ATP5PO* is expressed in the human ENS, we performed immunohistochemistry on postnatal colon of healthy individuals. Our results showed that *ATP5PO* is specifically detected in the ganglia of the submucosal (Fig. 2A) and myenteric plexuses (Fig. 2B), as well as in the colonic epithelium. Moreover, expression patterns of ATP5PO were analyzed in the ganglionic segment of the intestine of HSCR and DS-HSCR patients. However, no differences in protein distribution or density were observed (Fig. 2B).

atp5po expression was also evaluated in zebrafish by *in situ* hybridization, performed at different developmental stages (1-4dpf). We observed that *atp5po* expression was present early in development, since 1dpf, in the brain and the intestine. However, increased expression of a*tp5po* was detected at day 2 and 3 (Fig. S3A). To further confirm the presence of *atp5po* in the ENS, we used single cell RNA sequencing data recently publish by us, for the intestine of 5 dpf zebrafish [30]. Using this data, we were able to see that a*tp5po* is indeed expressed in the gut, but was also present in almost all the ENS clusters identified (Fig. S3B).

3.4. *Overexpression of ATP5PO RNA reduces ATP5PO protein levels in vitro*

To study the effect of ATP5PO overexpression *in vitro*, a stable mutant neuroblastoma cell line was generated (herein referred to as SH-SY5Y-*ATP5PO*). We observed that clones overexpressing *ATP5PO* at mRNA levels, showed reduced expression of ATP5PO protein on western blot (Fig. 3A–C). This result is in line with a previous study showing that post-transcriptional effects, such as significant protein downregulation of the mitochondrial proteome, including ATP5PO, occur in Trisomy 21 [31].

To evaluate if disruption of *ATP5PO* also resulted in an ENS phenotype, we decided to knockout the expression of the endogenous gene in zebrafish, using CRISPR/Cas9. This approach induced mosaic insertions and deletions confirmed by Sanger sequencing, that would result in a frameshift and disrupt expression of *atp5po* (Fig. S4). As expected, reduced numbers of enteric neurons at 4 dpf were observed in the distal intestine of crispant fish (Fig. S5), a phenotype comparable to the one observed upon overexpression of human *ATP5PO* RNA (Fig. 1B and C). However, the phenotype was often more severe, with severe loss of enteric neurons in the distal intestine and overall morphological alterations observed (Fig. S1B). These observations are consistent with the idea that loss of Atp5po protein affects ENS development.

3.5. *ATP5PO overexpression leads to reduced neuronal differentiation and impaired ATP production*

To determine the functional effect of *ATP5PO* RNA overexpression/ATP5PO protein downregulation, we induced neuronal differentiation in SH-SY5Y and SH-SY5Y-*ATP5PO* cells, using insulin growth factor 1 (IGF-1), as previously described [27]. Seventy-two hours after treatment, morphological changes were observed in nearly all control cells, which presented with extended neurites and an elongated cell body (Fig. 4A). Overexpressing cells on the other hand, showed enlarged cell bodies resembling the morphology of undifferentiated cells, with only a minority of cells presenting with neurites and elongated cell bodies (Fig. 4A). Interestingly, expression levels of TUJ1, a known neuronal marker, were substantially reduced in SH-SY5Y-*ATP5PO* cells when

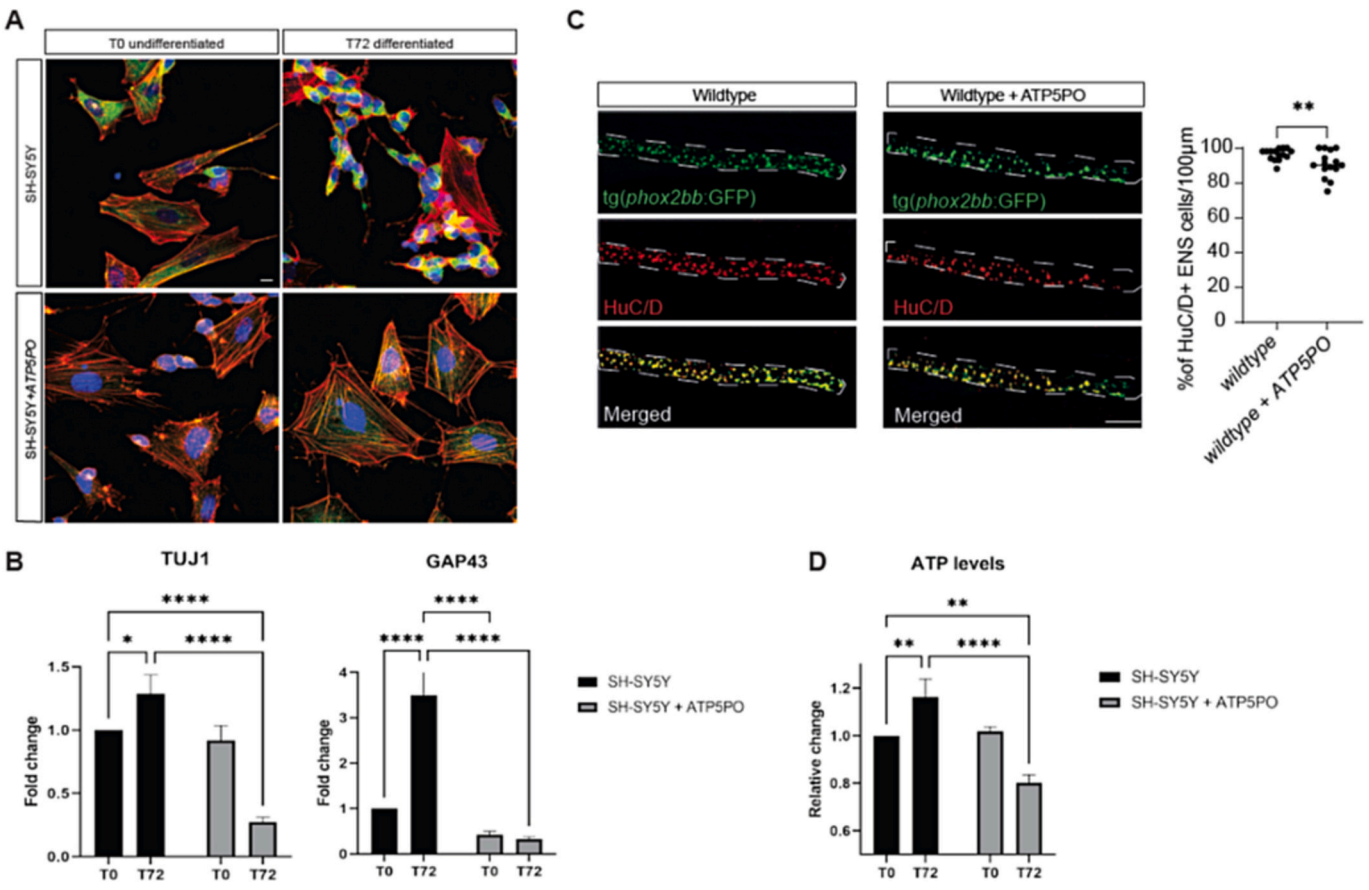

Fig. 4. Overexpression of *ATP5PO* impairs neuronal differentiation and production of ATP levels.
A. Representative images of undifferentiated (T0) and differentiated (T72) control SH-SY5Y and SH-SY5Y-*ATP5PO* cells. Phalloidin in red, TUJ1 in green and DAPI in blue. A differentiated neuronal morphology was only observed in control cells. Moreover, TUJ1 expression was reduced in SH-SY5Y-*ATP5PO* cells when compared to SH-SY5Y. Scale bar represents 50 µm. B. RT-qPCR data showing increased expression of neuronal markers (*TUJ1* and *GAP43*) in control SH-SY5Y cells 72 h after differentiation, but not in SH-SY5Y-*ATP5PO* cells. Mean of two technical replicates is visualized. (two-way ANOVA *TUJ1*: effect of *ATP5PO* overexpression $p < 0.01$, effect of differentiation $p < 0.05$, and interaction effect between genotype and differentiation $p < 0.01$; *GAP43*: effect of *ATP5PO* overexpression $p < 0.0001$, effect of differentiation $p < 0.0001$, and interaction effect between genotype and differentiation $p < 0.0001$) C. HuC/D stainings performed at 4 dpf showed that overexpression of *ATP5PO* in zebrafish, leads to reduced neuronal differentiation. D. ATP levels in control SH-SY5Y cells increase, as expected, upon induction of differentiation, whereas in SH-SY5Y-*ATP5PO* cells there is a decrease in ATP levels; $^{**}p < 0.01$, $^{****}p < 0.0001$. A–B and D Average of three independent experiments is visualized. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to controls, pointing to reduced differentiation of these cells when ATP5PO is overexpressed (Fig. 4A). RT-qPCR analysis of *GAP43*, a known neuronal differentiation gene [27], and *TUJ1*, confirmed our results, showing reduced expression of neuronal differentiation markers in the SH-SY5Y-*ATP5PO* cells (Fig. 4B). Overexpression of *ATP5PO in vivo* also showed that impaired neuronal differentiation was the underlying cause of the ENS defect observed (Fig. 4C).

Considering that neuronal differentiation is a cellular process demanding high energy levels, and *ATP5PO* is a component of the ATP synthase located in the mitochondrial matrix, we decided to evaluate ATP levels in SH-SY5Y and SH-SY5Y-*ATP5PO* overexpressing cells. Our results showed that similar levels of ATP are produced in both cells, in a normal undifferentiated state (Fig. 4D). However, upon treatment with IGF-1, an increase in ATP levels was observed only in control cells, whereas the SH-SY5Y-*ATP5PO* cells showed reduced ATP levels (two-way ANOVA: effect of *ATP5PO* overexpression $p < 0.05$ and interaction effect between genotype and differentiation $p < 0.01$) (Fig. 4D).

3.6. Epistasis between ATP5PO and ret in zebrafish

Most DS-HSCR patients contain single nucleotide polymorphisms (SNPs) in intron 1 of *RET* (SNP1 [rs2506004] or SNP2 [rs2435357]), which have been shown to reduce RET expression and increase the risk of ENS defects [32]. Combination of these SNPs with overexpression of (a) Hsa21 gene(s) might tip the balance to HSCR development [33]. To investigate whether *ATP5PO* shows epistasis with *RET* during ENS development and thereby contributes to HSCR pathogenesis in DS patients, we overexpressed *ATP5PO* mRNA (300 ng) in an outcross of Tg (*phox2bb:GFP*); $ret^{sa2684/+}$ zebrafish. We then compared the presence of enteric neurons in the distal intestine of $ret^{sa2684/+}$, *wildtype*, $ret^{sa2684/+}$ + *ATP5PO* and *wildtype* + *ATP5PO* larvae at 4 dpf, according to our previously established characterization (Fig. 5) [24,34]. Interestingly, synergistic effects between *ret* and *ATP5PO* could be observed, as seen by the significant increase of larvae presenting a HSCR phenotype reminiscent of human total colonic aganglionosis, after overexpression of *ATP5PO* (13,15 % of uninjected $ret^{sa2684/+}$ *vs.* 50 % of *ATP5PO* RNA injected $ret^{sa2684/+}$; $p = 0.0014$; Fig. 5).

4. Discussion

The majority of individuals with DS have GI complaints [35], some of which might be related to the presence of an abnormal ENS. Here, we found that *ATP5PO,* a gene located in the DS-HSCR critical region of chromosome 21, is involved in ENS development, as overexpression or disruption of this gene in zebrafish, leads to a HSCR phenotype. Although this result seemed surprising, the fact that both overexpression

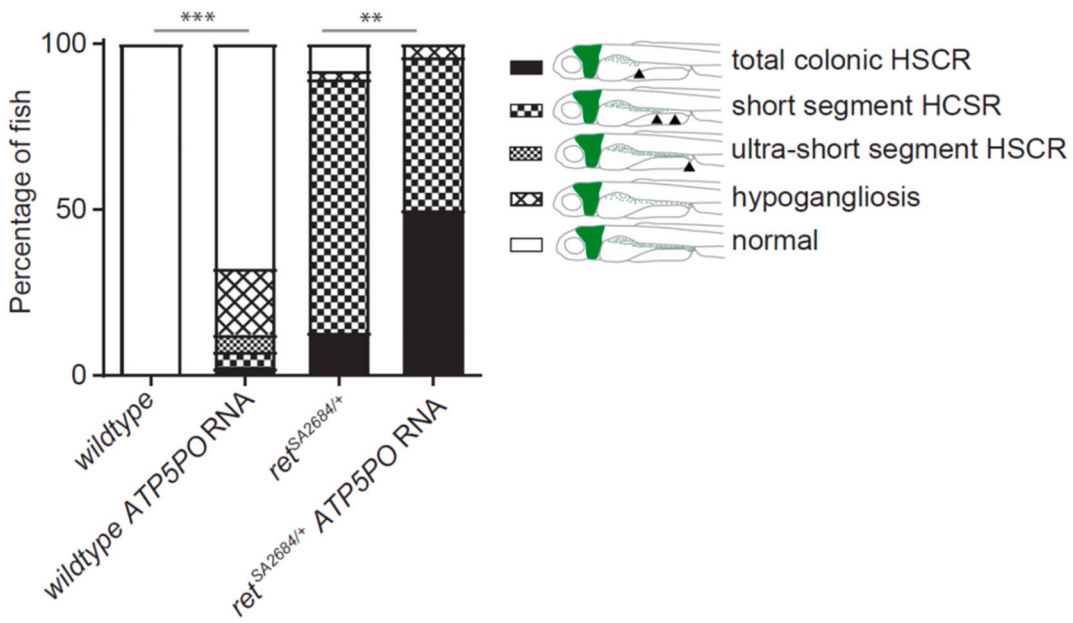

Fig. 5. *ATP5PO* overexpression shows epistasis with RET.
Quantification of the percentage of larvae presenting with ENS phenotypes reminiscent of HSCR in the different experimental groups. Due to *ATP5PO* overexpression in the $ret^{SA2684/+}$ background, more larvae significantly present with a phenotype reminiscent of total colonic HSCR (black bars), compared to $ret^{SA2684/+}$ alone. ***p < 0.001, **p < 0.01.

or knockout of *ATP5PO* led to a similar effect, *i.e.*, reduction of its protein levels, explains why the functional consequences of both are the same. Based on our results, we cannot distinguish if the ENS defect is a direct or indirect consequence of *ATP5PO* overexpression. However, we can say that changes in ATP5PO expression seem to be detrimental to maintain enteric neuronal numbers during development. *ATP5PO* encodes for the ATP synthase H+ transporting, mitochondrial F1 complex, O subunit protein, also known as Oligomycin Sensitivity Conferral Protein (OSCP), and plays an important role in the energy production machinery of the cells. Our results showed that *ATP5PO* mRNA overexpression leads to reduced ATP5PO protein, and results in impaired neuronal differentiation *in vitro* and *in vivo*, as well as reduced ATP production. As the metabolic requirements of cells change during cellular differentiation, resulting in a greater need for mitochondrial ATP production [36], it is not surprising that any change affecting the function of the ATP synthase has a significant impact on the cells, resulting in reduced differentiation.

To our knowledge, this is the first time that a gene coding for an ATPase component is described to be involved in HSCR. However, in neurodegenerative diseases such as Alzheimer disease (AD), ATP5PO expression is known to be reduced in both, humans and in a mouse model of the disease [37] (reviewed in [38]). Furthermore, in Parkinson's disease, autism and other neurodevelopmental disorders, there is evidence of the involvement of the ATP synthase in disease pathogenesis [39–42]. In our bodies less ATP leads to more oxidative stress causing increased cell death. Neurons and their mitochondria, are more prone to this process, since they are metabolically very active. Considering that neuronal differentiation is one of the possible mechanisms underlying HSCR, it is not surprising that individuals with DS, who have an extra copy of Hsa21 and overexpress *ATP5PO*, are more susceptible to an abnormal ENS development. It is tempting to consider that altered ATP5PO levels also affect the brain, contributing to the intellectual disability observed in people with DS.

Interestingly, we observed an increase in the number of enteric neurons upon overexpression of *RCAN1*, a gene expressed in neurons that has been implicated in DS and AD pathology [43,44]. It has been suggested that the short-term overexpression of this gene has a protective effect on neurons, including regulation of the growth cones. Such an effect is in line with the increased neuronal density observed in our injected larvae [43,45]. However, long-term overexpression of *RCAN1* has been described to have detrimental effects on neurons, namely increased apoptosis, mitochondrial dysfunction and oxidative stress, which occur in DS and AD pathologies [46–48]. Such effects were not observed in our fish, due to the relatively short term (four days) in which our experiments were performed but, should definitely not be ignored. Therefore, although we considered that the study of this gene is outside of the scope of this manuscript, further studies should be pursued to determine if *RCAN1* plays a role in ENS development.

HSCR is known to be a complex genetic disorder for which several contributing genes have been described. In some cases, variants in different genes have been reported in the same patient, and it is this combination that underlies disease pathogenesis [8,49–52]. Here, we found that *ATP5PO* and *ret* show an epistatic interaction, at least in the zebrafish. Considering that the risk haplotype located in intron 1 of *RET* [6,7] is commonly found in DS patients with HSCR [32], the interaction between these two genes might explain why only a subgroup of DS patients develops intestinal aganglionosis. Such finding is of particular interest, as it could help diagnose HSCR in a vulnerable patient population known to have delayed diagnosis and consequently poorer outcomes, than those with HSCR alone [53]. Therefore, we believe that it is worthwhile to include the screening of the *RET* risk alleles in prenatal genetic analysis of DS patients, especially if the parents are carriers for these alleles.

CRediT authorship contribution statement

L.E. Kuil: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **R.K. Chauhan:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **B.M. de Graaf:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **W.W. Cheng:** Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **N.J.M. Kakiailatu:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **R. Lasabuda:** Investigation, Methodology, Writing – review & editing. **C. Verhaeghe:**

Investigation, Methodology, Writing – review & editing. **J.D. Windster:** Investigation, Methodology, Visualization, Writing – review & editing. **D. Schriemer:** Investigation, Methodology, Writing – review & editing. **Z. Azmani:** Investigation, Methodology, Writing – review & editing. **A. S. Brooks:** Data curation, Investigation, Writing – review & editing. **S. Edie:** Methodology, Writing – review & editing. **R.H. Reeves:** Methodology, Writing – review & editing. **B.J.L. Eggen:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **I.T. Shepherd:** Data curation, Methodology, Supervision, Writing – review & editing. **A.J. Burns:** Data curation, Supervision, Writing – review & editing. **R.M.W. Hofstra:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision. **V. Melotte:** Conceptualization, Data curation, Supervision, Writing – review & editing. **E. Brosens:** Conceptualization, Data curation, Supervision, Writing – review & editing. **M.M. Alves:** Conceptualization, Data curation, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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