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## Genetics dissection and functional studies in Hirschsprung disease

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Publisher's PDF, also known as Version of record

*Publication date:*

2011

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Sribudiani, Y. (2011). *Genetics dissection and functional studies in Hirschsprung disease*. s.n.

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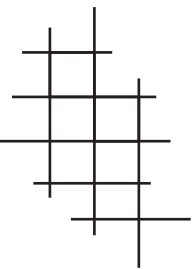
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# **Genetic Dissection and Functional Studies in Hirschsprung Disease**

**Yunia Sribudiani**

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Genetic Dissection and Functional Studies in Hirschsprung Disease  
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This study was financially supported by University of Groningen and  
Research Institute GUIDE.

ISBN/EAN: 978-94-6182-039-6

Cover Illustration: Ella Elviana, [www.ellasworks.blogspot.com](http://www.ellasworks.blogspot.com)  
Cover & book layout: Briyan B Hendro, [www.brianos.daportfolio.com](http://www.brianos.daportfolio.com)  
Printed by: Off Page, Amsterdam, [www.offpage.nl](http://www.offpage.nl)

Printing of this thesis was financially supported by: University of Groningen,  
GUIDE and Department of Genetics - UMCG.

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RIJKSUNIVERSITEIT GRONINGEN

# **Genetic Dissection and Functional Studies in Hirschsprung Disease**

## **Proefschrift**

ter verkrijging van het doctoraat in de  
Medische Wetenschappen  
aan de Rijksuniversiteit Groningen  
op gezag van de  
Rector Magnificus, dr. E. Sterken,  
in het openbaar te verdedigen op  
woensdag 21 december 2011  
om 14.30 uur

door

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geboren op 28 juni 1977  
te Bandung, Indonesië



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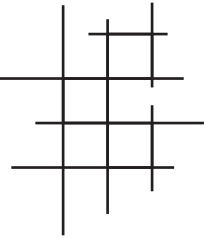
# CONTENTS

Chapter 1	General Introduction	1
Chapter 2	Fine mapping of the 9q31 Hirschsprung disease locus <i>Hum Genet, 2010 Jun; 127(6):675-83</i>	23
Chapter 3	Exome sequencing in a family with Hirschsprung disease <i>Manuscript in preparation</i>	45
Chapter 4	Variant in <i>RET</i> associated with Hirschsprung disease affect binding of transcription factor and gene expression <i>Gastroenterology, 2011 Feb; 140(2):572-582.e2</i>	59
Chapter 5	<i>RET</i> mediated gene expression in ENS precursors shows involvement of <i>RET</i> in innate immune response <i>Submitted</i>	83
Chapter 6	General discussions and future perspective	123
Appendices	Summary	138
	Samenvatting	141
	Ringkasan	145
	List of Abbreviations	149
	Acknowledgments	151



1

**General  
Introduction**



# 1. Hirschsprung Disease: Diagnosis and Treatment

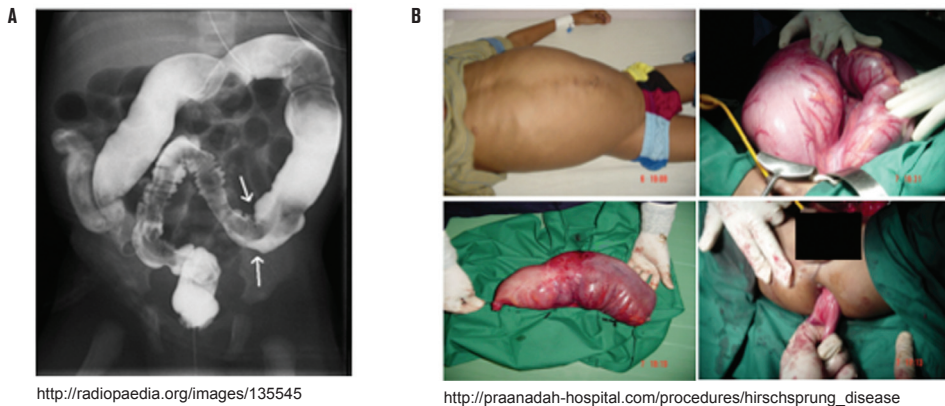
## 1.1 Diagnosis

Hirschsprung (HSCR) disease, also known as congenital megacolon, is characterized by the absence of enteric ganglia (aganglionosis) in the submucosal and myenteric plexuses of the gastrointestinal (GI) tract leading to a life-threatening bowel distention which presents itself usually shortly after birth.<sup>1</sup> HSCR disease was first recognized in 1887 by the Danish pediatrician Harald Hirschsprung. He described two boys who died at age 8 and 11 years with severe constipation. HSCR is clinically characterized by a failure to pass meconium (the first stool) within 48 hours after birth, abdominal distention, vomiting or neonatal enterocolitis.<sup>2</sup> In particular, enterocolitis can prove life-threatening in HSCR. The mortality rate of HSCR disease due to enterocolitis ranges from 0-30%. The variation is most likely due to differences in diagnosing Hirschsprung-associated enterocolitis (HAEC).<sup>3,4</sup> The gold standard for diagnosing HSCR disease is the histopathology of a full-thickness rectal biopsy in which the neurons and glial cells (ganglia) are absent in both the myenteric and submucosal plexus of the GI tract of HSCR patients.<sup>5</sup> Measurement of the pressures in the rectum (rectal manometry) and X-rays made with a barium enema are methods that can provide extra information to the physician for an accurate diagnosis. The dilated (mega)colon is proximal to the aganglionic region, which narrows towards the distal part and an empty rectum is a common finding.<sup>1</sup> In 75% of cases, the diagnosis is made within six weeks of birth and in 90% of cases, the disease is diagnosed before the age of five years.<sup>6</sup>

The length of the aganglionic region varies and based on this HSCR disease is classified into short-segment HSCR (S-HSCR), long-segment HSCR (L-HSCR) and total colonic/intestinal aganglionosis (TCA/TIA). S-HSCR is the most common type (80% of cases) and the length of the aganglionic region does not extend beyond the upper sigmoid. Fifteen percent of the patients have L-HSCR in which the aganglionic region extends beyond the upper sigmoid. In about 5% of cases, the TCA/TIA is diagnosed. In these patients the complete colon and the terminal part of the ileum show an absence of ganglia and in rare cases even the whole bowel can lack ganglia, which is a lethal condition.<sup>1,7-9</sup>

HSCR is the most common developmental disorder of the enteric nervous system (ENS) with a prevalence of 1 in 5000 newborns, although variation of the prevalence is observed between ethnic populations.<sup>10</sup> Interestingly, a sex-dependent penetrance is observed in S-HSCR with a male:female ratio of 4:1. With respect to these findings, it is interesting to report that the transmission of disease-causing alleles in this group is also disturbed, as 78% of the commonly shared *RET* allele, the major risk factor for HSCR development, are maternally de-

rived.<sup>11,12</sup> Whether the male:female ratio is caused by these differences in the transmission of associated alleles is not yet known. HSCR most commonly presents sporadically, although in approximately 20% of cases, several members in a family are affected (familial HSCR). In 70% of cases, HSCR occurs as an isolated trait and in the other 30% HSCR is associated with other congenital malformation or syndromes.



**Figure 1** - A) Barium enema X-ray of HSCR patient's bowel, the arrows shows the beginning of the aganglionic region. B) Treatment of HSCR patient with pull-through surgery to remove the aganglionic region and rejoin the healthy ganglionated colon to the anus.

## 1.2 Treatment

Current treatment of HSCR is surgical resection of the aganglionic part of the gastrointestinal (GI) tract. These operations, so called pull-through operations, are mostly based on the procedures developed by Swenson, Duhamel or Soave. In this surgical procedure the affected region of the colon is removed and the healthy ganglionated region of the colon is rejoined with the anus.<sup>13-15</sup> A one-stage procedure (one operation) is possible when the diagnosis of HSCR is made before the colon dilates. Otherwise a primary colostomy is temporarily required before the patient has the second pull-through surgery to remove the aganglionic region. Although the surgical procedure relieves the patient's constipation and has proven life-saving, the long-term outcome remains rather poor, irrespective of the length of the aganglionic region. A retrospective study performed by Catto-Smith and colleagues showed that fecal incontinence is very common and episodic constipations have also been reported.<sup>16</sup>

These clinical findings have led researchers to think about developing alternative treatments for HSCR disease. One method that seems promising is ENS replacement therapy.

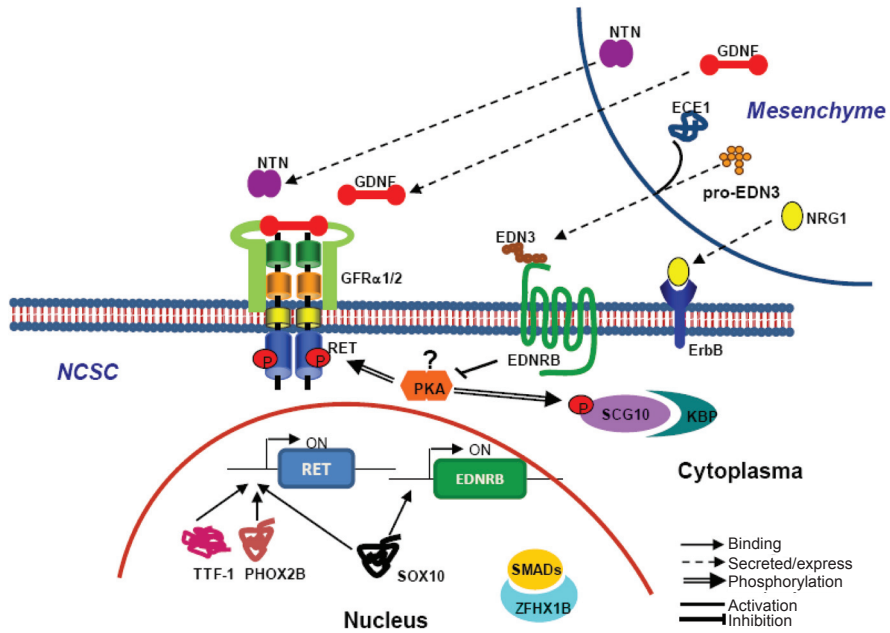
Metzger and colleagues showed that enteric nervous system stem cells (ENSSCs) can be isolated from postnatal human gut mucosal biopsy and used to generate neurosphere-like bodies (NLBs). More importantly, they showed that these NLBs have the same stem cell plasticity as those generated from embryonic human gut tissue and, when grafted onto aganglionic chick hindgut, these NLBs have the ability to migrate and differentiate into glial cells and different types of ENS neurons. Whether these grafted cells function correctly and whether they repair the ENS system needs further investigation. These preliminary results show, however, that ENSSCs transplantation might have therapeutic potential and could, in the future, be considered as an additional therapy for HSCR disease.<sup>17</sup>

## 2. Pathogenesis of Hirschsprung Disease: Genetic Background

### 2.1 Genes involved in the development of HSCR disease

The genetic dissection of HSCR has led to the identification of 12 genes and five susceptibility loci implicated in the development of HSCR disease. These 12 genes are expressed either in the enteric neural crest cells (ENCCs) or in other cells in the gut along the path of ENCCs migration during ENS development. The inheritance of HSCR is considered to be complex. The disease can be transmitted as a dominant trait or as a recessive trait, but in the majority of cases it is probably polygenic with differences in sex ratio, with a male predominance in S-HSCR (4:1), incomplete penetrance and variable expression; associations with a large number of syndromes and congenital malformations have been observed.<sup>1</sup> Linkage analyses of multiplex HSCR families revealed that the *RET* (REarrange during Transfection) gene, located at 10q11.2, is the major risk factor for Hirschsprung disease as almost all families showed linkage with *RET*.<sup>11, 18, 19</sup> Coding sequence mutations in *RET* are responsible for a dominant form of HSCR (with incomplete penetrance) and coding and splice site mutations have been identified in up to 50% of familial cases and 15-35% of sporadic cases.<sup>20</sup> The mutations are scattered throughout the *RET* coding sequence, including large and micro-deletions and a variety of point mutations. *RET* mutations associated with HSCR are believed to cause a loss of function (haploinsufficiency).<sup>21-23</sup> However, *RET* mutations on their own might not result in aganglionosis of part of the GI tract, as the penetrance of the *RET* mutations (in general) is 72% in males and 51% in females. Yet *RET* is believed to be the major gene underlying HSCR, as evidenced primarily in families enriched for L-HSCR.<sup>11</sup> Mutations in the rest of the 11 genes *EDNRB*<sup>24</sup>, *EDN3*<sup>25,26</sup>, *GDNF*<sup>27,28</sup>, *NTN*<sup>29</sup>, *SOX10*<sup>30</sup>, *PHOX2B*<sup>31</sup>, *ECE1*<sup>32</sup>, *KIAA1279/KBP*<sup>33</sup>, *ZFH1B*<sup>34,35</sup>, *TTF-1*<sup>36</sup> and *NRG1*<sup>37</sup> do not exceed 20% of the cases, supporting a genetic

heterogeneity for this disorder. Finally, rare heterozygous germline mutations of *EDNRB* in combination with *RET* germline mutations have also been detected in patients showing the HSCR phenotype.<sup>38</sup> This supports the concept of synergistic heterozygosity for HSCR, i.e. that the disease phenotype could be the result of the cumulative effect of at least two mutations in different genes.



**Figure 2** - Schematic representation of the encoded proteins known to be associated with HSCR development and showing their possible interactions.

Mutations in most of the 11 genes are found mostly in the syndromic cases where HSCR is associated with other congenital malformation (see Table 1). Mutations in *EDNRB*, *EDN3* and *SOX10* were identified in a patient with Shah-Waardenburg syndrome (*WS4*), which is characterized by congenital hearing loss, pigmentary abnormalities of the hair, skin and eyes, and HSCR disease.<sup>30,39</sup> Mutations in *PHOX2B* have been identified in patients with congenital central hypoventilation (*CCHS*) and HSCR disease. This syndrome is also known as Haddad syndrome. *CCHS* is a rare disorder characterized by impairment of autonomic control of spontaneous respiration in the absence of other lung or cardiac disease.<sup>31</sup> Mutations in *ZFH1B* have been identified in patients with Mowat-Wilson syndrome, which is an autosomal dominant disorder characterized by mental retardation, epilepsy, delayed motor development, and HSCR disease.<sup>40</sup> Mutations in *KIAA1279* (now called kinesin-binding protein or



*KBP*) have been identified in patients with Goldberg-Shprintzen (GOSH) syndrome, a rare autosomal recessive disorder characterized by HSCR, microcephaly, mental retardation, and polymicrogia.<sup>33</sup> A mutation in *ECE1* was identified in a single patient with craniofacial and cardiac defects.<sup>32</sup>

Besides mutations in these 11 genes, chromosomal abnormalities are frequently observed and found in 12% of all syndromic HSCR cases. Trisomy chromosome 21 (Down Syndrome (DS)) is the most frequent chromosomal abnormality (>90% cases) associated with HSCR disease. On the other hand, 77% of DS patients have associated gastrointestinal abnormalities, of which 2-15% can be explained by HSCR disease.<sup>41</sup> This might implicate chromosome 21 in the etiology of HSCR, so it is not surprising that DS patients have a 40-fold higher chance of getting HSCR compared to the normal population.<sup>42</sup> The other possible explanation could be that the genetic imbalance disturbs the homeostasis of genes expressed during development that eventually contribute to the disease phenotype.<sup>43</sup> Interestingly, besides these possibilities, a variant in the *RET* gene also seems to be related to the HSCR phenotype in DS patients. It was shown that a common single nucleotide polymorphism (SNP), rs2435357 (C>T), located in the conserved region of intron 1 of the *RET* gene, when homozygous for the T-allele, gives DS patients a 5.3-fold increased risk of developing HSCR compared to DS patients who are homozygous for the wild-type C-allele.<sup>44</sup> Furthermore, the frequencies of the T-allele differ significantly between individuals with DS alone (0.26±0.04), HSCR alone (0.61±0.04) and those with HSCR and DS (0.41±0.04), showing there is association and genetic interaction between *RET* and the chromosome 21 gene dosage.<sup>42</sup>

**Table 1 - HSCR associated genes and their clinical features (Modified from Brooks A et al. 2005).**

Gene Symbol	Position	Inheritance	Phenotype
RET	10q11.2	Dominant, incomplete penetrance	Non-syndromicMEN2A
GDNF	5p13	Non-Mendelian	Non-syndromic
NTN	19p13	Non-Mendelian	Non-syndromic
EDNRB	13q22	Recessive	Shah-Waardenburg
EDN3	20q13	Dominant (de novo in 80%) Recessive	Non-Syndromic Shah-Waardenburg
PHOX2B	4p12	Dominant, incomplete penetrance	Non-syndromic
SOX10	22q13	Dominant (de novo in 90%)	Haddad Syndrome (CCHS)
ECE1	1p36	Dominant (de novo in 75%)	Shah-Waardenburg
ZFHX1B (SIP1)	2q22	Dominant (de novo)	Congenital heart formation
KIAA1279 (KBP)	10q22.1	Dominant (de novo)	Mowat-Wilson
TTF1 (TITF1)	14q13	Recessive	Goldberg-Shprintzen
NRG1	8p21	-	Non-syndromic
		-	Non-syndromic

## 2.2 Non-coding *RET* variants

As mentioned above, *RET* coding mutations have been identified in 50% of the familial cases. However, regardless of the *RET* coding mutation status, almost all familial cases are linked to the *RET* locus.<sup>11, 45</sup> This suggests that non-coding *RET* mutations must play a major role in the remaining familial cases. This idea was corroborated in association studies on sporadic (simplex) cases with and without *RET* coding mutations, performed on several Caucasian populations and an Asian population. These studies revealed a strong association between a certain haplotype (covering 27 kb in total) and the disease. This haplotype starts 4 kb upstream of the *RET* transcription start site, going all the way to the beginning of exon 2. This haplotype is present in 56%-62% of patients, but in only 20% of the controls tested in the Caucasian population. In the Chinese patient population, the frequency of this same haplotype was 85% but 40% in the controls. This last finding might partially explain the higher incidence of HSCR in Asians compared to Caucasians. Several groups have focused their studies on fine-mapping the associated region and tried to verify the location of the causative variant.<sup>12, 36, 46-48</sup>

### SNP -1(G>A) and -5 (A>C)

Two variants located in the *RET* promoter, SNP rs10900296 (G>A) and rs10900297 (A>C) located at -5 and -1 from the *RET* transcription start site respectively, were selected as probably causative. It was shown that the HSCR associated haplotype (AC) decreased the *RET* promoter activity in luciferase assays.<sup>46</sup> Extended functional studies performed by Garcia-Barcelo and colleagues showed that these two variants and their surrounding sequences were the binding site of TTF-1 (Thyroid Transcription Factor 1). The AC-haplotype reduces the binding affinity of TTF-1, hence reducing *RET* promoter activity.<sup>36</sup> However, this effect proved to be cell-line-dependent and it is therefore difficult to conclude whether these two variants really are the causative ones. Another aspect that makes the interpretation difficult is the fact that these two variants are in a region with strong linkage disequilibrium (LD), which might mask the real functional variant(s).<sup>49</sup>

### SNP rs2435357 (C>T)

A similar study was performed by Emison and colleagues. They genotyped 28 SNPs spanning a region of 175 kb in 126 trios. The region covers *RET* and two other downstream genes, *GALNACT-2* and *RASGEF1A*. They identified HSCR-associated SNPs in *RET* (13 out of 17), *GALNACT-2* (3 out of 7) and *RASGEF1A* (2 out of 4). By multi-species alignment, they identified that one of the associated SNPs in *RET*, rs2435357 (C>T), is located in a highly con-

served region called MSC+9.7 (Multi Species Conserved). This variant is not only conserved among nine different mammalian species, but also showed the largest transmission distortion ( $\tau$ : 0.8) in transmission disequilibrium test (TDT) analysis. This data led the authors to postulate that SNP rs2435357 is most likely a disease-causing variant. They supported this idea with reporter (luciferase) assays and showed that, indeed, the MCS+9.7 region, containing the disease-associated variant (T-allele), reduced promoter activity when compared to the wild-type sequence (C-allele).<sup>12</sup> Additional functional studies on SNP rs2435357 by the same group proved that SOX10, a transcription factor, binds to a sequence motif containing this specific variant.<sup>50</sup> In addition *in vivo* functional studies on MCS+9.7 were performed by Grice and colleagues. They generated transgenic mice carrying this conserved region coupled to the *LacZ* reporter gene and studied the spatial temporal *LacZ* expression during embryonic development. They showed that *LacZ* expression, under the regulation of MSC+9.7, has a similar spatial temporal expression as the *RET* gene, indicating this region contributes to *RET* expression regulation.<sup>51</sup>

### **SNP rs2506004 (C>A)**

Burzynski and colleagues sequenced the shared common haplotype region from 10 kb upstream of the *RET* gene through intron 1 (a total of 33 kb) in a patient homozygous for the common risk haplotype and in a control individual homozygous for the most common non-risk haplotype. They identified eight variants, located in highly conserved regions, within putative transcription binding sites. Among these variants, SNP rs2506004 (C>A) proved to be conserved not only in vertebrates but also in an avian species (chicken). They hypothesized that the region containing the SNP might be an enhancer for *RET* expression and that this SNP would result in a reduction of *RET* expression.<sup>48</sup> The variant is also located in the MSC+9.7 region, 217 bp downstream of SNP rs2435357 (C>T). These two SNPs are in strong linkage disequilibrium ( $r^2=0.98$ ). Recently we identified NXF/ARNT2 and SIM2/ARNT2 as the transcription factors and repressors, respectively, for the wild-type C-allele of SNP rs2506004. Moreover, transfection of *Nxf/Arnt2* and *Sim2/Arnt2* constructs into a mouse neuroblastoma cell line (Neuro2A) enhanced and reduced the *RET* endogenous expression, respectively, proving that NXF/ARNT2 and SIM2/ARNT2 can indeed regulate *RET* expression endogenously (see Chapter 4).<sup>52</sup>

Interestingly, the gene encoding one of the above transcription factors, SIM2, is located in the Down syndrome (DS) critical region on chromosome 21. It has been shown that overexpression of *SIM2* reduces the expression of *Drebrin* (*DBN1*), encoding a protein that plays an important role in synaptic plasticity and hence suggested to play a role in the development of DS.<sup>53</sup>

<sup>54</sup> The overrepresentation of *SIM2* in DS patients, due to the trisomy of chromosome 21, might lead to *SIM2* overexpression and, as a consequence, not only down-regulate *DBN1* but also down-regulate *RET* expression. This might then contribute to the development of HSCR and partly explain why DS patients have a 40-fold higher risk of HSCR disease compared to the normal population (Chapter 4).<sup>52</sup> Together these data suggest that these four variants, all identified in the *RET* non-coding sequences, may play important roles in HSCR disease development.

### 2.3 Susceptible HSCR loci

As mentioned above, *RET* coding mutations are the major risk factor for HSCR development. However, a *RET* mutation alone may not be sufficient to cause HSCR as the penetrance of mutant alleles, in general, is incomplete in HSCR patients (72% in males and 51% in females). Non-coding mutations, as described above, also contribute to the phenotype, especially in S-HSCR. Furthermore, mutations in other 11 HSCR genes can only explain a small proportion of the cases. In the majority of sporadic and simplex S-HSCR cases, it is therefore likely that mutations occur in the coding regions of as yet unknown genes.

Several studies have been searching for additional susceptibility loci, with or without them being in conjunction with a *RET* mutation. Bolk and colleagues (2000) performed a linkage analysis in 12 multiplex HSCR families. They split them into two groups: the first consisted of families that were linked to 10q1.2 and all had severe *RET* mutations (six families), while the second group consisted of five families that were also linked to the *RET* region but no clear *RET*-coding sequence mutation had been identified, and one family that was not linked to the *RET* region (and obviously had no *RET* mutation). Bolk and colleagues segregated a new locus at 9q31 in the second group (the six families without *RET* coding mutations). It was hypothesized that 9q31 is probably a modifier locus for development of HSCR disease.<sup>45</sup>

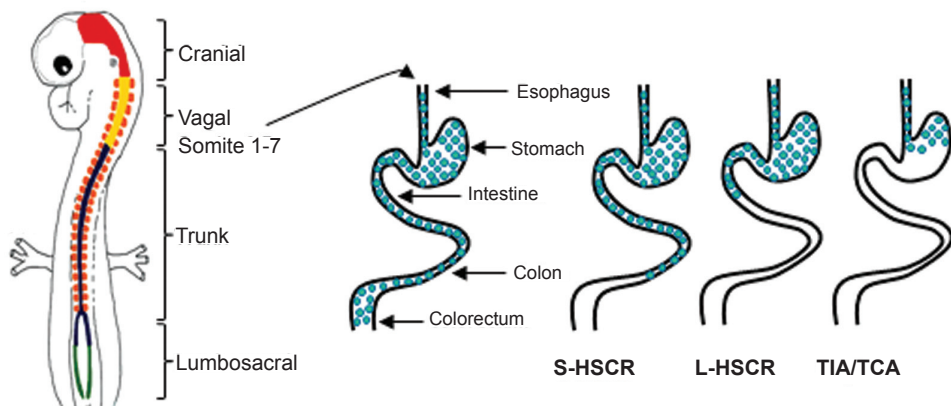
In another study, Gabriel and colleagues (2002) performed a genome-wide scan in 49 nuclear families with S-HSCR. They carried out a sib-pair analysis and found significant allele sharing with markers on 10q11, where *RET* is located ( $p = 2.4 \times 10^{-7}$ ), on 19q12 ( $p = 6.6 \times 10^{-4}$ ) and on 3p21 ( $p = 1.9 \times 10^{-4}$ ), with non-parametric LOD scores of 5.02, 3.46 and 3.23, respectively. To validate these data, they checked the families' mutation status for *RET*. Surprisingly, they only identified *RET* coding sequence mutations in only 40% of *RET*-linked families, suggesting the importance of non-coding variants. The risk ratios, as determined by the associated risk haplotypes at 10q11, 3p21 and 19q21 were 8.3, 4.2 and 5.0-fold, respectively. Using a multiplicative model, the frequency of individuals being heterozygous for these three loci is close to the observed population incidence. Gabriel et al. therefore hypothesized that all three loci were necessary and possibly even sufficient for the observed occurrence of S-HSCR.<sup>11</sup>

A fourth locus was identified by performing a genome-wide scan on 43 Mennonite trios, all belonging to the same large kindred. They identified three loci, two of which were known loci (13q22.3-q31.1 where the *EDNRB* gene is located and 10q11.21 containing the *RET* gene) and one new locus on 16q23.3. The LOD scores of the loci were 55.60, 5.6 and 3.01, respectively, showing that *EDNRB* is the primary susceptibility factor for the disease phenotype in this kindred.<sup>55</sup>

Studying a large multi-generational Dutch family with an isolated HSCR phenotype resulted in the identification of a new susceptibility locus on 4q31-32 (the fifth locus), with a parametric LOD score of 2.7.<sup>56</sup> The low penetrance of the locus in this family suggests that this mutation is necessary but not sufficient for disease development. In this thesis we describe our search for the disease-causing gene on this locus and for modifiers elsewhere in the genome by exome sequencing performed in this family (see Chapter 3).

### 3. Pathogenesis of Hirschsprung Disease: Developmental background

#### 3.1 Enteric nervous system (ENS) development



(Adapted from Dyer, MA, et al., J Clin Pathol 57, 2004)

**Figure 3** - Schematic representation of the neural tube. The enteric neural crest cells (ENCCs) are formed in the vagal region, in somites 1-7. From there they migrate rosto-caudally to colonize the whole gastrointestinal (GI) tract, later forming the enteric nervous system. Defects in ENCCs migration, proliferation or differentiation could lead to HSCR disease. The different lengths of the aganglionic region in the GI tract (S-HSCR, L-HSCR and TCA/TIA) define the severity of the disease.

The enteric nervous system (ENS) is a complex system that contains as many neurons as the spinal cord and it functions independently from the central nervous system (CNS). It coordinates normal bowel motility and changes the blood flow and secretion of water and electrolytes. The intrinsic innervation of the gut wall consists of two interconnected ganglionated nerve plexuses: the myenteric plexus, which is located between the longitudinal and circular muscle layers, and the submucous plexus, which is located between the mucosa and the circular muscle layers.<sup>57</sup> Neurons and glial cells of the ENS all derive from neural crest stem cells (NCSCs), which are pluripotent stem cells. They are formed at the time the neural tube closes, at the border of the neural plate and the non-neural ectoderm. They directly migrate towards different structures in the embryo to form distinct (neural crest-derived) tissues. These tissues include the skin's pigment cells, the craniofacial bones and cartilage, smooth muscle cells, peripheral and enteric neurons, and glial cells of the ENS.<sup>58,59</sup> It is the NCSCs which originate from the vagal region of the hind brain, specifically from somites 1 to 7, that give rise to the vast majority of the parasympathetic neurons and glial cells of the ENS.<sup>60</sup> These precursor cells migrate from the vagal region towards the foregut rostrocaudally in the 4<sup>th</sup> week of gestation in humans, colonize the whole GI tract and reach the end of the hindgut in the 7<sup>th</sup> week.<sup>57,61,62</sup> These precursor cells termed enteric neural crest-derived cells (ENCCs) when they enter the foregut.<sup>57</sup> A small part of the ENS precursor cells derive from the sacral region. These cells migrate in the opposite direction from those from the vagal region and move from the end of the hindgut upwards. The sacral ENS precursor cells contribute to ENS development in the gut by colonizing the distal part of the GI tract, however, the real contribution of the sacral neural crest cells in ENS development is still under debate.<sup>63</sup> For instance, a study in chick embryos suggested that sacral neural crest cells differentiate only into glial cells and not into the enteric neurons.<sup>64</sup>

All in all, the colonization of the whole GI tract by ENCCs and their later differentiation into neurons and glial cells is a very complex process. Timing and harmonization of the necessary transcription factors, growth factors, their receptors and the signals they give is essential for each stage of ENS development.<sup>65,66</sup> Any defect in this process (ENCCs migration, proliferation, differentiation or survival of ENS progenitors) can lead to the development of HSCR.

### **3.2 Signaling pathways and genes involved in ENS development *RET Signaling Pathway***

RET is a transmembrane Tyrosine Kinase Receptor (RTK) that plays an important role in several intracellular signaling cascades that regulate differentiation, proliferation, migration and cell survival. Activation of RET signaling is unique among RTKs as it requires a multi-protein complex instead of the customary direct receptor-ligand interaction. The RET activa-

tion complex comprises a soluble ligand (GDNF, Neurturin, Persephin or Artemin), GFR $\alpha$ 1-4 (glycosyl-phosphatidylinositol membrane-anchored co-receptor of GDNF) and RET itself. A complex containing the ligand and co-receptor heterodimers brings two RET molecules together and triggers auto-phosphorylation of tyrosine residues in the TK-domain of RET (see Figure 2).<sup>67, 68</sup>

RET signaling has been proven critical in ENS development as mice deficient for GDNF, RET and GFR $\alpha$ 1 show severe defects in enteric innervations (i.e. aganglionosis).<sup>21, 22, 27</sup> But how RET signaling is involved in ENS development is only partly known. What *is* known is that GDNF, in particular, plays a crucial role, as it serves as a chemo-attractant for the RET-positive vagal ENCCs. GDNF is highly expressed in the stomach ahead of the migrating ENCCs wave-front and its expression is elevated in the caecum when ENCCs migrate towards the distal part of the gut.<sup>66, 69</sup> Besides GDNF, several other ligands can activate the RET signaling pathway, namely Neurturin (NTN), Artemin (ARTN) and Persephin (PSPN) that bind to GFR $\alpha$ 2, GFR $\alpha$ 3 and GFR $\alpha$ 4, respectively. Mouse *Ntn* and *Gfra2* mutants show ENS defects, revealing their importance in ENS development.<sup>70, 71</sup> Furthermore, HSCR patients carrying *NTN* mutations have also been reported.<sup>29</sup>

Even though the RET signaling pathway is known to play a pivotal role in many processes during ENS development, its downstream effectors, the proteins that transmit signals within the cells, are still largely unknown. In Chapter 5 we describe expression array studies performed on mouse ENCCs to gain more insight into the RET signaling pathways on GDNF stimulation.

### ***EDNRB-EDN3 Signaling Pathway***

A second pathway involved in HSCR is the Endothelin Receptor type B (EDNRB) pathway. This belongs to the guanine nucleotide binding protein (G-protein-coupled) receptor family, which is activated by its ligand Endothelin 3 (EDN3). EDN3-EDNRB signaling is required for the development of melanocytes and enteric neurons.<sup>72</sup> Upstream of EDN3, ECE1 (Endothelin Converting Enzyme), the enzyme that converts the inactive precursor of EDN3 into the active EDN3 peptide, thus activates the EDNRB-EDN3 signaling pathway (see Figure 2).

In mouse embryonic gut, *Ednrb* is expressed in the migrating ENCCs and *Edn3* is mainly expressed in the midgut and the hindgut mesoderm during the early phases of ENCCs migration, and at high levels in the caecum and the proximal colon when the ENCCs colonize the terminal gut region.<sup>73, 74</sup> This pattern of expression suggests that, as for GDNF-RET signaling, EDN3-EDNRB signaling plays an important role in ENCCs migration. Furthermore, it also suggests that EDN3-EDNRB plays a role in cell proliferation as the *Edn3* mutant mouse has



fewer neural crest stem cells compared to the wild-type mouse.<sup>73</sup> Moreover, cell differentiation is inhibited by EDN3-EDNRB signaling.<sup>75</sup> Together these studies showed that EDN3-EDNRB signaling is important for ENCCs migration and proliferation, and for maintaining ENCCs in their progenitor state during ENS development.

By using a system that allows the regulation of expression of *Ednrb* at different stages of development, Shin and colleagues showed that EDNRB is required during a limited period of ENS development in mouse, namely between embryonic days 10 to 12.5.<sup>72</sup> *Edn3*, *Ednrb* and *Ece1* knockout mice show aganglionosis and pigmentary abnormalities, similar to the phenotypic abnormalities seen in human patients carrying mutations in these genes (Shah-Waardenburg syndrome). Mutations on these genes have been identified in approximately 5% of HSCR cases.<sup>1</sup>

### ***RET and EDNRB Interaction***

The RET and the EDNRB signaling pathways were considered independent signaling pathways, however there are several findings that indicate that the two pathways are indirectly connected. A study by Puffenberger and colleagues identified a homozygous *EDNRB* mutation, which was found in association with a specific *RET* haplotype in a large Mennonite kindred with HSCR disease.<sup>76</sup> The data was later corroborated in a genome-wide association study of 43 trios from the same Mennonite population. Joint transmission of a specific *RET* haplotype and the previously identified *EDNRB* mutation with HSCR was found more often than would have been expected by chance.<sup>55</sup> In an Italian patient with HSCR, both a *RET* mutation, inherited from the unaffected mother, and an *EDNRB* mutation, from the unaffected father, were identified.<sup>38</sup> These studies suggest that there might be a genetic interaction between RET and EDNRB.

Studies using animal models support this hypothesis. McCallion and colleagues (2003) intercrossed mice carrying a *Ret* null mutation (*Ret*/*Ret*<sup>-</sup>) and mice carrying an *Ednrb* loss-of-function mutation, *Ednrb*<sup>s</sup>/*Ednrb*<sup>s</sup> or *Ednrb*<sup>s/l</sup>/*Ednrb*<sup>s</sup> (s: piebald, s-l: piebald lethal). Of the offspring, 100% of the males carrying the *Ret*/*Ret*<sup>-</sup> *Ednrb*<sup>s</sup>/*Ednrb*<sup>s</sup> genotype were affected, whereas only 70% of the females were affected with variable lengths of aganglionic colon. In mice offspring carrying the *Ret*/*Ret*<sup>-</sup> *Ednrb*<sup>s/l</sup>/*Ednrb*<sup>s</sup> genotype, a genotype that expresses less EDNRB, both males and females were equally affected, showing that this sex difference is gene-dose dependant. This suggests there is epistasis between *EDNRB* and *RET*.<sup>77</sup> Similar studies showed that, whereas *Ret*<sup>51/51</sup> and *Edn3*<sup>sls</sup> (lethal spotting) mice display colonic aganglionosis, combinations of these mutant alleles lead to almost complete intestinal aganglionosis.<sup>73</sup>

Finally, SOX10, a transcription factor that regulates *RET* expression, also influences *ED-*



*NRB* expression. Mice carrying *Sox10*<sup>-/-</sup> or *Sox10*<sup>+/-</sup> either show reduced or loss of *EDNRB* expression, respectively.<sup>78</sup>

### ***Other Signaling Pathways***

The signaling pathways described above are ones related to known HSCR genes. However, there are many more signaling pathways that play important roles in ENS development, such as the NOTCH, WNT and TGF- $\beta$  /BMPs signaling pathways. NOTCH signaling is involved in various aspects of neurogenesis. It could decrease cell proliferation (although not solely) and it is known to act as a negative regulator for neuron differentiation.<sup>79, 80</sup>

WNT signaling induces cell proliferation and maintains the pluripotency of stem cells. However, a recent study showed that the result of Wnt signaling can differ between different types of stem cells. In ENCCs, for example, WNT signaling is important for cell lineage decision-making rather than for maintaining the pluripotency of the stem cells, which is a function that WNT signaling has in hematopoietic and CNS stem cells. Deletion of *Wnt1* and *Wnt3a* leads to a reduction of neural crest derivatives.<sup>81</sup> The WNT signaling pathway interacts with other signaling pathways, such as the NOTCH and the TGF- $\beta$ /BMP pathways.

BMP are expressed in submucosal and myenteric plexus and BMP4 has been shown to direct or indirectly influence ENCCs migration. Even though BMP4 alone is not sufficient to promote cell migration, in the absence of BMP4 activity, the number of ENCCs that reach the hindgut is reduced. Furthermore, BMP4 and BMP2 are also required *in vitro* for cell aggregation of isolated enteric neurons, which might indicate an important role for BMPs in ganglion formation during ENS development.<sup>82</sup> Chalazonist and colleagues proposed an interaction between GDNF and BMP4 in ENS. This was based on their data that the combination of these two proteins synergistically enhanced enteric neural development.<sup>83</sup> This study indicates there is possible cross-talk between the RET and BMP signaling pathways in ENS development. Another possibility is that GDNF directly influences BMP signaling independent of RET, since it has been suggested that GDNF could also interact with neural cell adhesion molecule (NCAM), which has been known play a role in a number of developmental process including cell migration, neurite outgrowth, and synaptic plasticity.<sup>84</sup>

## **4. Aims and Outline of this Thesis**

The aims of the studies described in this thesis were to: (1) identify new HSCR-causing genes in associated loci reported in previous studies, in particular in 9q31 and 4q31-32.<sup>45,56</sup> (2) per-

form functional assays on a non-coding RET variant (SNP rs2506004) identified by Burzynski et al.<sup>48</sup> to reveal the pathogenic nature of the variant and to determine the molecular mechanism of this SNP with respect to RET expression regulation; and (3) perform gene expression profiling of ENCCs in the presence and absence of GDNF to gain a better insight into the RET signaling pathway in this cell type.

Bolk and colleagues showed that the 9q31 region contains a gene or mutation that acts as a modifier in the development of HSCR disease.<sup>45</sup> This locus was found in families that were linked to the *RET* region but in whom no clear *RET*-coding sequence mutation was identified and in one family that was not linked to the *RET* region. In **Chapter 2**, we focus on fine mapping the 9q31 region by genotyping 301 tagging-SNPs spanning a region of 7 Mb, in 137 Dutch trios (121 patients without a *RET*-coding mutation and 16 patients with *RET*-coding mutations) and in Chinese case-controls (173 patients and 436 controls) study.

In a previous linkage study performed by Brooks and colleagues on a Dutch multi-generational HSCR family, linkage was identified for a region on 4q31-32.<sup>56</sup> In **Chapter 3** we tried to discover the causative gene in this locus using exome sequencing.

Previous studies showed strong association of HSCR with a haplotype containing part of the 5' region of the *RET* gene. This haplotype was transmitted to 60-85% of the HSCR patients, but only to 20-40% of the controls. Some of the variants present on this haplotype have been reported and suggested as HSCR-causative variant(s) by functional studies. For some possible causative variants the pathogenic nature has not yet been examined, in particular, SNP rs2506004 (C>A) that was identified by Burzynski et al. In **Chapter 4**, we describe functional studies of this SNP. By *in silico* analysis we found that the C-allele (wild-type) is located in a Central Midline Element (CME) sequence, which is a binding site for NXF/ARNT2 transcription activators and SIM2/ARNT2 repressors, respectively. Subsequently, we determined whether these transcription factors could indeed regulate *RET* expression and whether the mutant A-allele reduced the enhancer activity of this CME region on *RET* expression.

It has been shown that the GDNF-*RET* signaling pathway plays a central role in ENS development. However, the actual downstream effectors of *RET* signaling, in particular in ENCCs, is still largely unknown. To gain a better insight into the downstream effects of GDNF-*RET* signaling, we performed expression microarray experiments on mouse embryonic gut and also on ENCCs isolated from mouse embryonic gut (Embryonic day 14.5), untreated and treated with GDNF, the ligand of *RET*. We analyzed the microarray data not only with single-gene analysis, but also performed pathway analysis using Gene Set Enrichment Analysis (GSEA) software. The results are presented in **Chapter 5**.

The summary, general discussion and future perspective are presented in **Chapter 6**.

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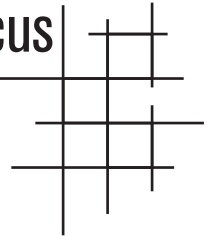






# Fine mapping of the 9q31 Hirschsprung disease locus

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## ABSTRACT

Hirschsprung disease (HSCR) is a congenital disorder characterized by the absence of ganglia along variable lengths of the intestine. The *RET* gene is the major HSCR gene. Reduced penetrance of *RET* mutations and phenotypic variability suggest the involvement of additional modifying genes in the disease. A *RET*-dependent modifier locus was mapped to 9q31 in families bearing no coding sequence (CDS) *RET* mutations. Yet, the 9q31 causative locus is to be identified. To fine-map the 9q31 region, we genotyped 301 tag-SNPs spanning 7 Mb on 137 HSCR Dutch trios. This revealed two HSCR-associated regions that were further investigated in 173 Chinese HSCR patients and 436 controls using the genotype data obtained from a genome-wide association study (GWAS) recently conducted.

Within one of the two identified regions *SVEP1* SNPs were found associated with Dutch HSCR patients in the absence of *RET* mutations. This ratifies the reported linkage to the 9q31 region in HSCR families with no *RET* CDS mutations. However, this finding could not be replicated. In Chinese, HSCR was found associated with *IKBKAP*. In contrast, this association was stronger in patients carrying *RET* CDS mutations with  $p=5.10 \times 10^{-6}$  [OR=3.32 (1.99, 5.59)] after replication. The HSCR-association found for *IKBKAP* in Chinese suggest population specificity and implies that *RET* mutation carriers may have an additional risk. Our finding is supported by the role of *IKBKAP* in the development of the nervous system.

## INTRODUCTION

Hirschsprung disease (HSCR; aganglionic megacolon) is a congenital disorder characterized by the absence of enteric ganglia along a variable length of hindgut. There is significant ethnic variation in the incidence of the disease, and it is most often found among Asians (2.8 per 10,000 live births).<sup>1,2</sup> Non-familial HSCR has a complex pattern of inheritance and manifests with low, sex-dependent penetrance and variability in the length of the aganglionic segment, according to which patients are classified into short segment (S-HSCR; 80%), long segment (L-HSCR; 15%), and total colonic aganglionosis (TCA 5%). The male:female ratio (M:F) is  $\approx$ 4:1 among S-HSCR patients and  $\approx$ 1:1 among L-HSCR patients. HSCR presents mostly sporadically although it can be familial (5-20% of cases) where the recurrence risks to sibs vary from 1.5–33% depending on the gender and the length of the aganglionic segment in the proband, and the gender of the sibling.<sup>3</sup>

The *RET* gene, encoding a tyrosine-kinase receptor, is the major HSCR causing gene and its expression is crucial for the development of the enteric ganglia.<sup>4,5</sup> Mutations in the coding sequence (CDS) of *RET* account for up to 50% of the familial cases and between 15%-20% of the sporadic cases, indicating that additional HSCR-causing mutations exist.<sup>6</sup> Other HSCR genes identified so far mainly code for protein members of interrelated signalling pathways involved in the development of enteric ganglia: *RET*, endothelin receptor B (*EDNRB*), and the transcriptional regulator *SOX10*. Yet, mutations in genes other than *RET* (*GDNF*, *GFRA1*, *NRTN*, *PHOX2B*, *NKX2.1*, *SOX10*, *NRG1*, *EDNRB*, *EDN3*, *ECE-1*, *KIAA1279*, *ZFXH1B*, *NTRK3*, *L1CAM*) account for only 7%.<sup>7-15</sup> Failure to identify *RET* CDS mutations in some of the *RET*-linked families suggests that noncoding *RET* mutations, mutations in regulatory regions of *RET* could also contribute significantly to the disease. Indeed, common *RET* single nucleotide polymorphisms (SNPs) are strongly associated with HSCR, with the largest contribution to risk made by a functional SNP (rs2435357) lying in an enhancer-like sequence in intron 1.<sup>16-23</sup> We have recently reported the genetic interaction of *RET* intron 1 rs2435357 SNP with HSCR-associated *NRG1* SNPs whereby the risk to HSCR is increased by 2.3-fold for the *RET* heterozygous genotype.<sup>24</sup> Reduced penetrance of *RET* and other HSCR genes mutations and variable expression of the HSCR phenotype indicates that the disease may result from the combined effect of several genes whereby the outcome would be altered *RET* expression.<sup>25-28</sup>

*RET* modifiers have been mapped to the following chromosomal regions: (i) 3p21; (ii) 19q12<sup>29</sup>; (iii) 4q31.3-q32.3<sup>30</sup> and (iv) 9q31. Despite these important findings, the genes in these loci are yet to be identified. The 9q31 locus segregated in families of European descent harbouring no or hypomorphic *RET* mutations, yet the families still showed linkage to 10q12 (*RET* locus).<sup>31</sup> To identify the HSCR locus in the 9q31 candidate region, we have conducted dense genotyping of the region a Dutch patient cohort and evaluated the same region in Chinese.

## MATERIALS AND METHODS

The overall study was approved by the institutional review board of The University of Hong Kong together with the Hospital Authority (IRB: UW 07-292).

### Dutch (Groningen) family-based association study

The Dutch patient cohort consisted of HSCR 140 trios that were genotyped using Illumina GoldenGate platform for 370 SNPs spanning the 7 Mb (from 108.5–115.5 Mb) of the 9q31 HSCR-associated region as described in Bolk *et al.* Most of these 370 were tag SNPs. Quality control based on identity-by-descent (IBD) revealed 3 duplicated trios. SNPs with missing genotypes (>5%) and with minor allele frequency (MAF)<5% and/or violating Hardy-Weinberg equilibrium ( $p<0.001$ ) were excluded, leaving a total of 301 SNPs for association study. PLINK<sup>32</sup> was then used to conduct the TDT analysis in the resulting 137 Dutch trios. Sixteen HSCR probands carried *RET* CDS mutations.

### Chinese case-control analysis

#### *Subjects*

For analysis of the 9q31 region in the Chinese sample, we revised the genotype data of the candidate 9q31.3 region obtained from a previously GWAS conducted on a total of 181 HSCR Chinese patients and 386 healthy individuals (discovery group) using *Affymetrix* GeneChip® Human Mapping 500K Array as previously described.<sup>24</sup> To that data, we included 92 additional controls recently genotyped (see below).

For the replication of the association found when patients were stratified according to the *RET* mutation status, we included 21 independent HSCR cases bearing *RET* CDS mutations and 71 control individuals from Mainland China (replication group). Characteristics of the patients are depicted in supplementary Tables 1 and 2.

#### *Revision of the Affymetrix 500K data*

Following the recent recommendations on quality control standards<sup>33</sup> we imposed a more stringent filtering criterion based on heterozygosity to the GWAS described above. Heterozygosity screening aims at the identification of individuals who have more heterozygous genotype calls than the average of the sample set, since the excess of heterozygosity is likely due to cross-contamination of samples. Hence, outliers should be excluded from the analysis. Prior to evaluating the heterozygosity of the samples, we pruned the SNP data set to avoid bias that could have been introduced by large quantities of SNPs in strong LD. In

brief, we pruned the dataset (by removing 1 of a pair of SNPs with  $r^2 > 0.25$  within a window of 200 SNPs and shifts of 50 SNPs forward at each step) using PLINK. In total, 56,000 SNPs in LD with each other remained. The heterozygosity screening excluded outliers with more heterozygous calls than the average (estimated inbreeding coefficient lower than 3 standard deviations from the average).

After this filtering criteria, a total of 173 HSCR cases (31 patients bearing *RET* CDS mutations) and 436 controls were included in the analysis. Restricting by the region analyzed by the Dutch group, 988 SNPs falling within the 9q31 7 Mb candidate region (from 108.5–115.5 Mb) were selected. The mean call rate was 99.09%. After filtering by MAF, genotyping rate and Hardy-Weinberg equilibrium, 981 SNPs were left for analysis.

#### *Correction for population structure and association analysis*

We used the EIGENSOFT package on the whole genome data pruned for LD as described above to detect population substructure. The Tracy-Widom test (implemented in EIGENSOFT) nominated only the first two axes of variation as significant ( $p < 0.05$ ). The first axis of variation corresponded to the ancestral differences between Northern and Southern Chinese as seen in supplementary Figure 1. To correct for these ancestral differences, we applied EIGENSTRAT<sup>34</sup> (also implemented in EIGENSOFT) on the candidate regions based on the two axes of variation nominated by the Tracy-Widom test. The Cochran-Armitage trend test was then used to assess the levels of association of the SNPs with HSCR.

#### *Replication stage*

EIGENSTRAT could not be applied to correct the replication sample for population stratification as no genealogy information can be inferred when only two SNPs are genotyped. Logistic regression was carried out instead to assess the association in the combined sample (discovery + replication samples). We included a covariate for the sample ancestral origin (northern and southern Chinese ancestry) and a covariate to distinguish GWAS and replication samples, thereby controlling for any possible allele frequency differences between known populations and between stages of association analysis. We also tested the interaction terms between each covariate and the genotype, which were found to not be significant ( $p > 0.5$  for both, indicating that the effect is not significantly different between subpopulations or stages). Thus, only the two covariates were included in the final model for combined analysis.

## RESULTS

### Family-based association test on Dutch trios identifies two associated regions on 9q31

Through TDT analysis of 301 SNPs on 137 Dutch trios, we identified 17 SNPs (supplementary Table 3) ( $p < 0.05$ ) showing asymmetrical transmission from parents. Of these, 10 SNPs could be grouped into 2 moderately associated peaks of 5 SNPs each, **9q31A** (110.7–111.1Mb) and **B** (112.1–112.7Mb).

The **9q31A region** encompasses a gene-rich spot which contains 4 genes *IKBKAP*, *C9orf6*, *CTNNAL1* and *C9orf5* within a single LD block that spans about 100kb. Among the 5 associated SNPs, the strongest evidence for association with HSCR was found for **rs12351693** ( $p = 0.023$ ) and **rs10979637** ( $p = 0.023$ ) located within the intronic region of *CTNNAL1* (Table 1 and Figure 1A).

*CTNNAL1* encodes alpha-catenin, a protein that modulates the Rho signalling pathway by providing a scaffold for the *ARHGEF1*.<sup>35</sup> The expression of its mouse homologue, *Ctnnal1*, was found to be severely reduced in intestines of *Ret* deficient mice (*Ret<sup>k-/k-</sup>*) when compared to normal mice (*Ret<sup>+/+</sup>*) and consequently, the gene has been considered a putative *RET* modifier.<sup>36</sup>

However, the strongest associations lied in 9q31B and mapped upstream the *TXNDCC8* gene ( $p = 4.8 \times 10^{-3}$  for **rs7038415**), and in the intronic region of *SVEP1* ( $p = 2.9 \times 10^{-3}$  for **rs10816998**) (Table 1). Other associated SNPs within the 9q31B region, mapped to the *MUSK* gene. *MUSK* is required for neuromuscular junction formation in vivo and for the survival and development of discrete neuronal subpopulations.<sup>37</sup> The associations described above were confirmed by performing 1 million **permutations (Table 1 and Figure 1)**.

#### *SVEP1 is associated with HSCR in Dutch patients without RET mutations*

As it was reported that the 9q31 HSCR susceptibility locus segregated exclusively in *RET*-linked families depleted of *RET* CDS mutations<sup>31</sup>, we stratified the cases according to their *RET* CDS mutation status. We defined *RET* CDS mutations as DNA changes in the coding region or exon/intron boundaries of *RET* that lead to frame-shift, non-sense or missense mutations in the *RET* protein and that have not been found in at least 400 control chromosomes. PolyPhen<sup>38</sup> was used to predict the effect of the missense mutations on the *RET* protein. Truncating mutations were considered highly deleterious.

The 301 SNPs in the 9q31 region were checked again for association. The rs10816998 and rs7038415 SNPs within *SVEP1* were strongly associated with HSCR in families without *RET* mutations. After stratification of the patients, the OR attained a significance level of

$p=5.33 \times 10^{-5}$  with OR=2.379 (95% CI:1.542, 3.671) for rs10816998 and  $p=7.50 \times 10^{-5}$  with OR=2.345 (95% CI:1.518, 3.622) for rs7038415. These association values survived the conservative Bonferroni correction for multiple testing ( $\approx 300$  SNPs). This finding in the Dutch population is in line with the reported linkage of 9q31 with HSCR in patients of European descent bearing no or hypomorphic *RET* mutations.<sup>31</sup>

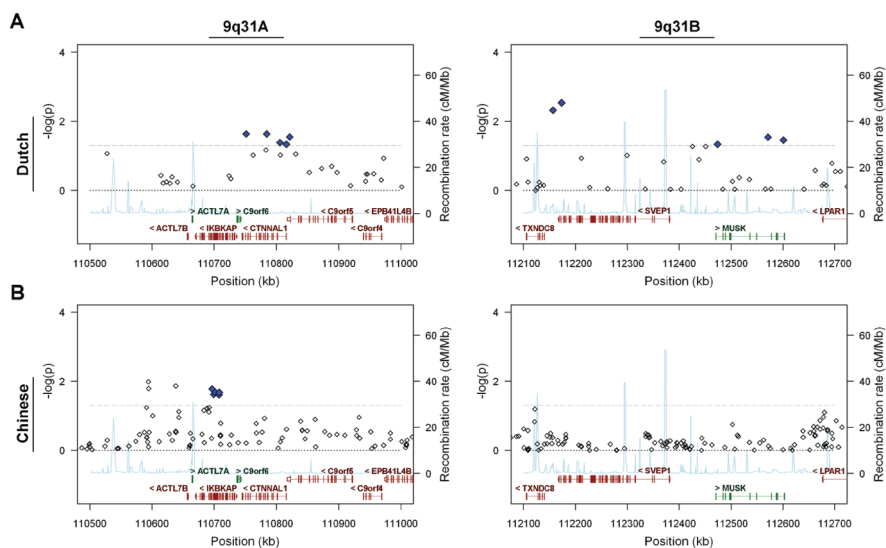
The *SVEP1* SNPs rs10816998 and rs7038415, were then genotyped in 107 independent Dutch patients without *RET* CDS mutations and 183 controls. Unfortunately, the p value of the replication did not reach statistical significance.

**Table 1 - 9q31A and B SNPs associated with HSCR in Dutch trios samples**

9q31A	Position (bp)	Allele		Overlapping gene	Location	p-value <sup>a</sup>
		A	B			
rs12351693	110751336	G	A	CTNNAL1	intron 13	0.023
rs10979637	110784448	C	A	CTNNAL1	intron 6	0.023
rs12555920	110805558	T	C	CTNNAL1	intron 1	0.041
rs1333344	110816045	A	G	CTNNAL1	downstream	0.046
				C9orf5	upstream	
rs1044905	110821316	T	C	C9orf5	3' UTR	0.028
<b>9q31B</b>						
rs7038415	112157004	A	C	TXNDC8	upstream	0.0048
				SVEP1	downstream	
rs10816998	112173562	G	A	SVEP1	intron 46	0.0029
rs3010815	112473999	T	C	MUSK	intron 1	0.046
rs2766998	112571406	T	C	MUSK	intron 9	0.029
rs3780529	112601072	C	A	MUSK	intron14	0.035

A: minor allele; B: major allele; a: p-value for the transmission disequilibrium test (TDT).





**Figure 1** - Association analysis on 9q31A (110500-111000kb) (*left panel*) and 9q31B (112000-112500kb) (*right panel*). (A) TDT analysis on 137 Dutch HSCR trios and (B) Case-control study on Chinese using 500k. Associated SNPs listed in Table 1 and 2 are represented as blue diamonds. Recombination rate and genes in the region are also shown (green, “+” strand; red, “-” strand).

## Revision of the population-based GWAS on Chinese identifies *IKBKAP* as a HSCR candidate gene

We used the GWAS data (above described) to investigate the association observed in the Dutch. After EIGENSTRAT correction for stratification (see material and methods and supplementary material), genotypes of 981 SNPs spanning the entire 9q31 region were tested for association on 173 HSCR patients (31 with *RET* CDS mutations) and 436 controls.

In the Chinese dataset, 6 SNPs within the **9q31A** region showed association with HSCR. No association was found in **9q31B**. The loci displaying the strongest statistical evidence for association were **rs10979596** and **rs10979597** (in intron 25 of the *IKBKAP* gene) which are in complete LD with each other ( $r^2=1$ ) (Figure 1B and Table 2). Four additional SNPs within *IKBKAP* also showed marginally significant association ( $p<0.03$ ) with HSCR. These include the *IKBKAP* I816L missense polymorphism (rs2230793,  $p=0.024$ ) and 3 intronic SNPs (Table 2). Mutations in *IKBKAP* (inhibitor of kappa light polypeptide gene enhancer in B-cells kinase complex-associated protein, involved in transcription elongation) are associated with a neurodevelopmental disease, the Riley-Day syndrome, also known as familial dysautonomia (FD).<sup>39,40</sup> FD is an autosomal recessive disorder that occurs almost exclusively in persons of Ashkenazi Jewish descent. This neuropathy results from depletion of subsets of autonomic

and sensory neurons.<sup>41</sup> FD patients are born with fewer neurons in their dorsal root and sympathetic ganglia than their peers, and over time, neurons become more scarce.<sup>42</sup> Most importantly, some patients with FD also suffer from gastrointestinal dysfunction shortly after birth and interestingly, the co-occurrence of both FD and HSCR has been reported.<sup>43</sup> Interestingly, the region encompassing our 6 *IKBKAP* associated SNPs includes the region where FD-causing mutations lie. *Ikbkap* is expressed in the rat developing gut and *IKBKAP* in the human colon, supporting a biological role for this gene in the development of enteric nervous system.<sup>42, 44</sup>

**Table 2 - 9q31A SNPs associated with HSCR in Chinese**

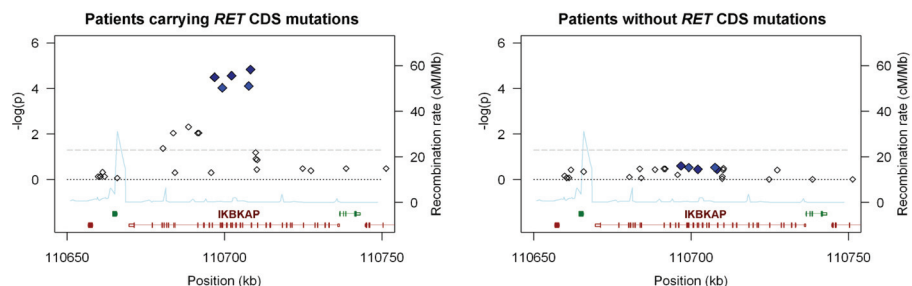
9q31A	Position (bp)	Allele		IKBKAP	OR (95% CI)	Frequencies		
		A	B			Cases	Controls	<i>p</i> -value <sup>a</sup>
rs10979596	110696824	G	T	intron 25	1.53 (1.10, 2.13)	0.20	0.14	0.017
rs10979597	110696853	A	T	intron 25	1.53 (1.10, 2.13)	0.20	0.14	0.017
rs2230793	110699304	G	T	1816L exon 23	1.58 (1.20, 2.09)	0.32	0.23	0.024
rs2275630	110702228	G	A	intron 19	1.68 (1.12, 2.51)	0.13	0.08	0.021
rs10979607	110707646	G	A	intron 14	1.49 (1.08, 2.06)	0.20	0.14	0.025
rs4369056	110708259	T	C	intron 14	1.58 (1.21, 2.08)	0.33	0.24	0.021

A: minor allele; B: major allele; <sup>a</sup>: corrected trend test computed using EIGENSTRAT.

#### *The association of IKBKAP is stronger in Chinese HSCR patients with RET mutations*

The Chinese HSCR patients were also stratified according to the *RET* CDS mutation status. We then tested again the 981 SNPs in the 9q31 region for association by independently comparing *RET* CDS mutation carriers (31 Chinese HSCR patients; discovery group) against 436 controls as well as non-carriers (142 HSCR patients) versus the same control set.

Twelve SNPs were found associated with HSCR in *RET* mutations carriers ( $p < 0.01$ ). Importantly, 6 of these SNPs mapped to the *IKBKAP* loci in the 9q31A and had been found associated with HSCR before the stratification of the patients according to the mutation status (Figure 2). This data suggested that the initial association detected was mainly driven by this subset of *RET* mutation carriers and that the original marginal association had been masked prior stratification because of the small number of carriers. Among the 12 SNPs segregating with *RET* mutations, four *IKBKAP* SNPs survived the conservative Bonferroni correction for multiple testing ( $\approx 1000$  SNPs) (Table 3). The frequency of the risk SNPs alleles tended to increase with the severity of the *RET* mutations, yet the small sample does not allow to draw any conclusion (Table 3).



**Figure 2** - Association analysis on *RET* Chinese CDS mutation carriers (left) and Chinese non-carriers (right). Blue diamonds represent associated SNPs before stratification by mutation status. Dark blue diamonds indicate the 4 SNPs that survived the Bonferroni correction multiple for testing (only 3 dark blue diamonds can be observed as there is overlapping between two SNPs).

**Table 3** - IKBKAP SNPs associated<sup>a</sup> with HSCR patients with *RET* CDS mutations

	Risk allele	MAF of risk allele					OR (95% CI)	Trend test p-value <sup>d</sup>
		RET <sup>b</sup>	Highly deleterious	Damaging	Tolerating	Controls		
rs4369056	T	0.52	0.75	0.50	0.47	0.24	3.44 (2.04, 5.80)	$1.48 \times 10^{-5}$
rs2275630	G	0.25	0.38	0.30	0.19	0.08	3.83 (2.03, 7.23)	$2.79 \times 10^{-5}$
rs10979596 <sup>c</sup>	G	0.34	0.63	0.35	0.32	0.14	3.21 (1.83, 5.62)	$3.25 \times 10^{-5}$
rs10979597 <sup>c</sup>	A							

<sup>a</sup>: Survived Bonferroni correction for multiple testing; <sup>b</sup>: Frequency in HSCR patients carrying *RET* CDS mutations; <sup>c</sup>: same frequencies for these two SNPs as  $r^2=1$ ; <sup>d</sup>: corrected trend test computed using EIGENSTRAT; MAF: minor allele frequency; Note that the MAFs increase with the severity of the *RET* mutation.

To validate the association of the *IKBKAP* SNPs with HSCR patients carrying *RET* CDS mutations, we genotyped (by PCR followed by direct sequencing) the 2 perfectly linked SNPs (rs10979596 and rs10979597) in an independent set of 21 HSCR patients (replication group) bearing *RET* mutations and 71 controls collected from mainland China. These SNPs were chosen for replication because they fall in the same amplicon and on the basis of genetic homogeneity between Northern and Southern Chinese. We successfully detected the association of rs10979596 and rs10979597 in the replication sample, with logistic regression association value of  $p=9.45 \times 10^{-3}$  and OR=3.38 (95% CI: 1.35, 8.49) (Table 4). Logistic regression was also used for the combined analysis of HSCR samples bearing *RET* mutations (discovery, -31 patients- and replication -21 patients). This yielded a highly significant association of

$p=2.71 \times 10^{-6}$  and  $OR=3.15$  (95% CI:1.95, 5.09) for the heterozygous genotype under an additive model. Considering the possible population stratification, we employed the same logistic regression approach conditioning on the strata defined by sample origins (Northern and Southern Chinese), obtaining a significant level of association of  $p=5.75 \times 10^{-6}$  with  $OR=3.29$  (95% CI:1.97, 5.51) (Table 4). Results of the latter approach were very similar, indicating that samples collected inside and outside Hong Kong (discovery and replication phases) were homogeneous, with little stratification.

**Table 4** - Summary of the statistics for the *IKBKAP* rs10979596 and rs10979597 markers in *RET* mutation carriers

Phase	N		MAF		Logistic <sup>b</sup>			
	<i>RET</i> <sup>a</sup>	Controls	<i>RET</i> <sup>a</sup>	Controls	OR (95% CI)	<i>p</i> (adjusted)	OR (95% CI)	<i>p</i> (non-adjusted)
500K	31	436	0.34	0.14	3.31 (1.77, 6.18)	$1.78 \times 10^{-4}$	3.38 (1.86, 6.12)	$6.13 \times 10^{-5}$
Replication	21	71	0.29	0.11	NA	NA	3.38 (1.35, 8.49)	$9.45 \times 10^{-3}$
Combined	52	507	0.319	0.13	3.32 (1.99, 5.59)	$5.10 \times 10^{-6}$	3.15 (1.95, 5.09)	$2.71 \times 10^{-6}$

NA: all cases and control used in the replication stage had the same ancestral origin of northern Chinese;

<sup>a</sup>: HSCR patients carrying *RET* CDS mutations

<sup>b</sup>: P-value adjusted for population substructure in GWAS (500K) analysis and for both the substructure and strata defining analysis stages (500K and replication) in combined analysis

## DISCUSSION

This study aims at the identification of a HSCR susceptibility locus in the 9q31 region. As the association of this region was initially reported in families of European descent, we first explored the region in a Dutch cohort. As the evaluation of an association in two populations of different ethnic origins would definitively increase confidence in the finding, we also explored the 9q31 region in the Chinese. We reasoned that this strategy would allow us to determine i) whether the 9q31 locus exerted an effect in patients of European descent other than those in whom the locus was initially identified, ii) assess whether the association of the 9q31 locus with HSCR is population specific, and if not; iii) use population differences in LD to fine-map the gene of interest.

Our data show an initial strong association of *SVEP1* SNPs with Dutch HSCR patients

bearing no *RET* CDS mutations. This finding ratifies the reported linkage to the 9q31 region in HSCR families with no *RET* CDS mutations. The case-control replication of the *SVEP1* association in the Dutch did not reach statistical significance indicating that the initial association was spurious or that there is a population stratification issue in the samples used for replication. This point would need further exploration in a much larger sample. The role of *SVEP1* is not well characterized. Study of its possible implications in the development of the nervous system should follow.

Intriguingly, the analysis of the region in the Chinese population demonstrated the existence of a HSCR-associated locus (*IKBKAP*) that, although within 9q31, is not correlated at all with the *SVEP1* locus identified in the Dutch. The association of *IKBKAP* SNPs with HSCR in Chinese became much more prominent in those patients with *RET* CDS mutations which is at odds with the initial findings described by Bolk and our own findings in the Dutch. Importantly, the *IKBKAP* association with HSCR bearing *RET* CDS mutations was replicated in the same Chinese population. No *IKBKAP* association was detected in the Dutch (supplementary Table 4).

We investigated whether the lack of cross-population replication of the associations of *SVEP1* and *IKBKAP* SNPs with HSCR could be due to population LD and allele frequencies differences, in which case, some true associations may not be replicated, regardless of the sample size of the study. Close examination of the *SVEP1* and *IKBKAP* regions showed no major LD differences between CEU and CHB HapMap populations indicating that lack of replication across populations was not due to differences in LD structure (Supplementary Figures 2 and 3). Lack of replication could also be attributed to a false positive result on the Chinese sample although we think this is unlikely since the initial association found in Chinese was replicated in an independent sample.

In view of these results, we would conclude that the 9q31 locus that appears associated with HSCR in families bearing no *RET* CDS mutations is indeed population specific, as the findings in Chinese point to a different locus with a completely different genetic behavior than that identified in the Dutch. As 9q31 locus was reported to segregate exclusively with *RET*-linked families of Caucasian origin whose members had weak or hypomorphic *RET* CDS mutations, the investigation of *SVEP1* rare variants in these type of patients is warranted.

The association of *IKBKAP* in Chinese implies that *RET* mutation carriers may have an additional risk. As yet, no genotype-phenotype correlation could be found which, after all, is in line with the genetic complexity inherent to HSCR. Had parental DNA been available, we might have been able to provide an argument for the role of *IKBKAP* SNPs on the penetrance of inherited *RET* CDS mutations. SNPs may modulate the penetrance or phenotype of a par-

ticular mutation through epistasis as exemplified by the exclusive action of the *FGFR2* and *MAP3K1* SNPs in *BRCA2* mutation carriers.<sup>45</sup>

## FUNDING

This work was supported by grants from the Hong Kong Research Grants Council HKU 765008M and HKU 775907M and The University of Hong Kong Seed Funding 200709159003 and 200611159152 to MGB and PT respectively. Support was also obtained from the University Grants Committee of Hong Kong (AoE/M-04/04) and from The University of Hong Kong Genomics Strategic Research Theme. SSC and PCS are supported by NIH Grant EY-12562. YS is supported by a grant from the Bernouille Foundation and GB was supported by a NWO grant (no. 901-04-225).

## ACKNOWLEDGEMENTS

We would like to express our gratitude to all the subjects who participated in the study. This work supported by grants from Hong Kong Research Grants Council HKU 765008M and HKU 775907M and the University of Hong Kong Seed Funding 200709159003 and 200611159152 to MGB and PT, respectively. Support was also obtained from the University Grants Committee of Hong Kong (AoE/M-04/04) and from the University of Hong Kong Genomics Strategic Research Theme. SSC and PCS are supported by NIH Grant EY-12562. YS is supported by grant from the Bernouille Foundation and GB was supported by NOW grant (no.901-04-225).

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**Supplementary Table 1 - Characteristics of the Chinese HSCR patients included in the study**

500K n=173 <sup>a</sup>	S-HSCR n=154		L-HSCR n=8		TCA n=10		Undetermined n=1	
	Males	Females	Males	Females	Males	Females	Males	Females
126 {27} (9)	28 {3} (3)	2	6 {1}	7 {2}	3	0	1 {1}	
With RET CDS mutation n=31	S-HSCR n=28		L-HSCR n=1		TCA n=2			
Males	Females	Males	Females	Males	Females	Males	Females	
21 (2)	7 {2} (1)	0	1	0	2			
Replication n=21	S-HSCR n=14		L-HSCR n=4		TCA n=1		Undetermined n=2	
Males	Females	Males	Females	Males	Females	Males	Females	
13	1	4{1}	0	1	0	2	0	

<sup>a</sup>number of patients after quality control filtering. { } additional anomalies; ( ) Down syndrome; U: undetermined

**Supplementary Table 2 - Characteristics of the Dutch HSCR patients included in the study**

n=132	S-HSCR n=84		L-HSCR n=24		TCA n=5		Undetermined n=19	
	Males	Females	Males	Females	Males	Females	Males	Females
65	19 {3}	5	4	1	18	1		

{ } additional anomalies; U: undetermined

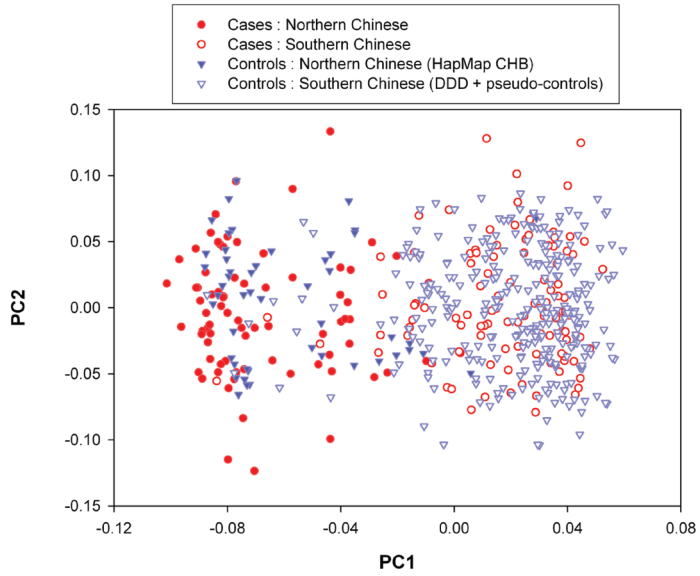
**Supplementary Table 3**

SNP	Position (bp)	Allele		T:U <sup>a</sup>	OR (95% CI)	p-value
		A	B			
rs10978770	109050289	G	A	83:54	1.54 (1.09, 2.17)	0.013
rs7038659	109095510	A	G	15:33	0.45 (0.25, 0.84)	0.0094
rs180533	109124082	T	C	16:34	0.47 (0.26, 0.85)	0.011
rs12351693	110751336	G	A	20:8	2.50 (1.10, 5.68)	0.023
rs10979637	110784448	C	A	20:8	2.50 (1.10, 5.68)	0.023
rs12555920	110805558	T	C	20:9	2.22 (1.01, 4.88)	0.041
rs1333344	110816045	A	G	50:72	0.69 (0.48, 1.00)	0.046
rs1044905	110821316	T	C	48:72	0.67 (0.46, 0.96)	0.028
rs10119758	111324700	A	G	52:30	1.73 (1.11, 2.72)	0.015
rs9987721	111331292	T	C	52:30	1.73 (1.11, 2.72)	0.015
rs7038415	112157004	A	C	76:45	1.69 (1.17, 2.44)	0.0048
rs10816998	112173562	G	A	78:45	1.73 (1.20, 2.50)	0.0029
rs3010815	112473999	T	C	55:78	0.71 (0.50, 1.00)	0.046
rs2766998	112571406	T	C	32:52	0.62 (0.40, 0.96)	0.029
rs3780529	112601072	C	A	41:24	1.71 (1.03, 2.83)	0.035
rs384408	113584922	A	G	18:32	0.56 (0.32, 1.00)	0.048
rs1411681	114223965	T	G	10:21	0.48 (0.22, 1.01)	0.048

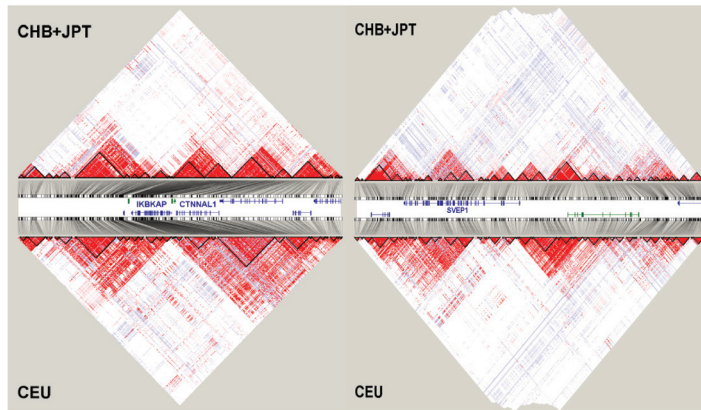
A: Minor allele; B: Major allele; <sup>a</sup>: 'T' and 'U' indicate transmitted and untransmitted minor allele of each SNP

**Supplementary Table 4 - Replication of the 9 associated SNPs on Dutch cases and controls**

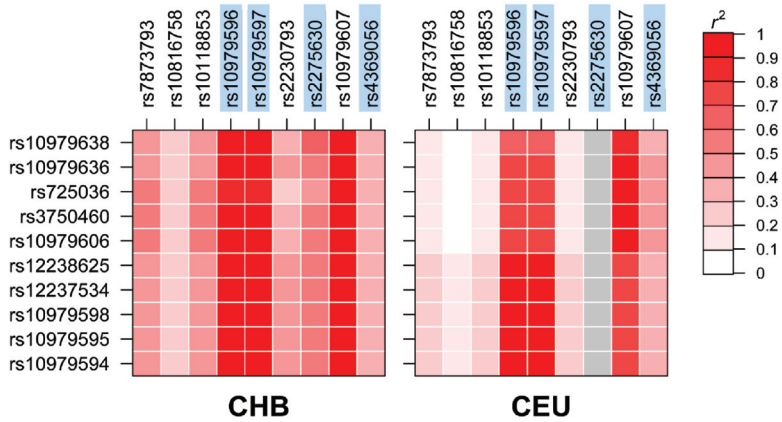
SNP ID	Position		Minor allele	Major allele	MAF		p-value
					Case	Control (HapMap CEU)	
rs7873793	110683696	intron 31	A	G	0.20	0.19	0.84
rs10816758	110688553	intron 29	G	A	0.41	0.32	0.18
rs10118853	110691843	intron 28	A	C	0.20	0.19	0.81
rs10979596	110696824	intron 25	G	T	0.048	0.075	0.39
rs10979597	110696853	intron 25	A	T	0.048	0.075	0.39
rs2230793	110699304	exon 23	G	T	0.23	0.21	0.65
rs2275630	110702228	intron 19	G	A	0.00	0	NA
rs10979607	110707646	intron 14	G	A	0.048	0.08	0.33
rs4369056	110708259	intron 14	T	C	0.095	0.15	0.24



**Figure 1** - Plot of principal components identified by EIGENSOFT.



**Figure 2** - LD structure across the 9q31A (right) and 9q31B (left) regions in CEU and CHB+JPT



**Figure 3** - LD structure of *IKBKAP* SNPs in CHB and CEU. The associated SNPs in Chinese which fail to replicate in Dutch are shown along the x axis (highlighted in blue:  $p$ -value passing multiple testing). SNPs in moderate to high LD in CHB (y-axis) can also be tagged effectively ( $r^2 > 0.8$ ) by at least one of these nine associated SNPs (x-axis) in CEU, demonstrating the population-specific association. LD information for monomorphic SNP (rs2275630) in CEU is not shown.





# 3

## Exome Sequencing in a Family with Hirschsprung Disease

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## ABSTRACT

Hirschsprung disease is a congenital disorder characterized by the absence of enteric ganglia in a variable length of the intestinal tract. A linkage study performed on a large, multi-generational, Dutch family with five family members affected with Hirschsprung disease revealed significant linkage to 4q31.3-q32.3. We have sequenced the exomes of two patients from this family, focusing our search for mutations on this linkage region. We assumed an autosomal dominant model of inheritance with low penetrance, suggesting that the mutation in the linkage region is necessary but not sufficient to cause disease development. Exome sequencing revealed only one possible mutation in the linkage region, a single nucleotide change in exon 20 of the *LRB* gene. This variant was validated in all five affected family members. As this variant is located downstream of *MAB21L2*, a gene which plays a role in enteric neural crest cells (ENCCs) migration during ENS development, we hypothesized that this mutation might regulate *MAB21L2* expression in ENS, thereby disturbing ENS development. However, we identified this variant in four individuals out of 220 screened controls and once in 87 Hirschsprung patients. It is therefore unlikely that it is disease-associated. As this was the only variant we identified in the linkage region, we must have missed the causative mutation, which is quite possible since the coverage of the coding sequences, in general, was only approximately 65%.

## INTRODUCTION

Hirschsprung (HSCR) disease is a congenital disorder characterized by the absence of enteric ganglia in a variable length of the intestinal tract. Based on the length of the aganglionic region, HSCR is classified into three types: short-segment (S-HSCR), long-segment (L-HSCR) and total colonic aganglionosis (TCA). The estimated incidence of HSCR varies in different populations; it is lowest in Caucasians (1:5000) and highest in Asians (1.4:5000). Mostly, HSCR occurs sporadically and most cases are diagnosed with S-HSCR and only in a minority of cases (5-15%) is more than one patient seen in a family (familial HSCR).<sup>1</sup>

The pattern of inheritance of sporadic cases can be multifactorial, recessive or dominant with low penetrance, while familial cases can be either recessive or autosomal dominant, in both cases with incomplete penetrance<sup>2</sup>. So far, 12 genes and five loci have been implicated in HSCR and *RET* is considered to be the major gene in HSCR etiology. The 12 genes are: *RET*<sup>3</sup>, *EDNRB*<sup>4</sup>, *EDN3*<sup>5,6</sup>, *GDNF*<sup>7,8</sup>, *NTN*<sup>9</sup>, *SOX10*<sup>10,11</sup>, *PHOX2B*<sup>12</sup>, *ECE1*<sup>13</sup>, *KIAA1279/KBP*<sup>14</sup>, *ZFHX1B*<sup>15,16</sup>, *TTF-1/TITF1*<sup>17,18</sup> and *NRG1*<sup>19</sup>. Besides *RET*, most other genes are involved in what is called syndromic-HSCR, HSCR patients who also suffer from other congenital malformations besides HSCR. Altogether, mutations in these 12 genes explain no more than ~20% of the total heritability, suggesting that other disease associated genes must exist.

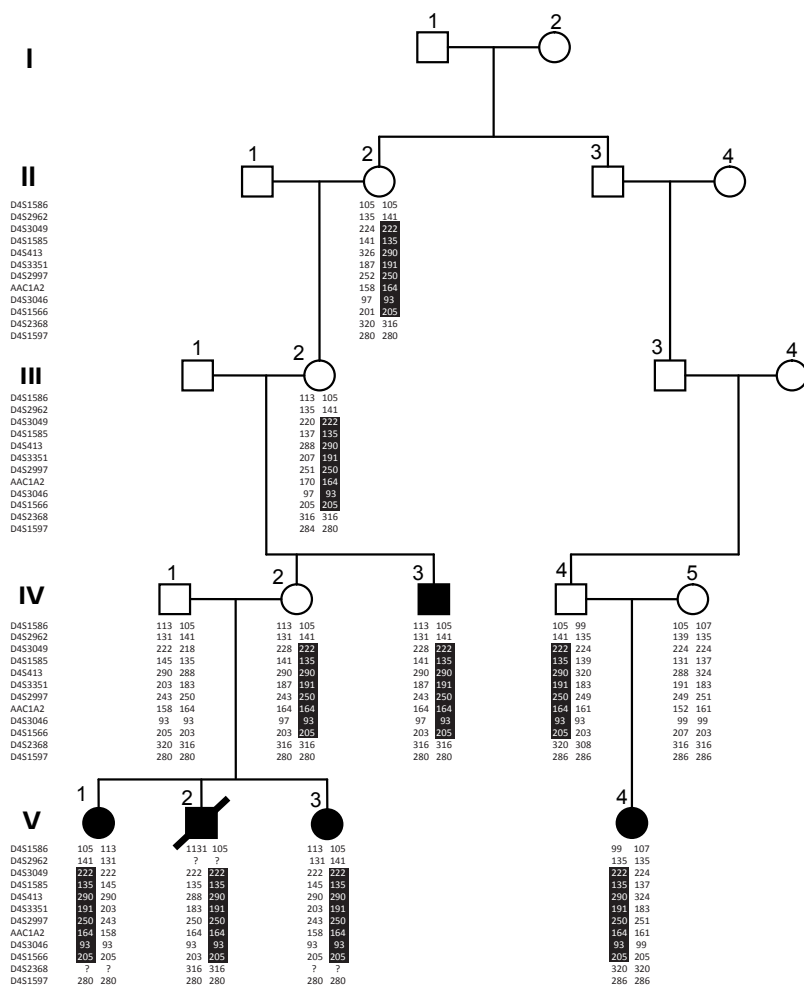
In a previous linkage study, Brooks and colleagues identified a linkage region on 4q31.3-q32.3 in a multi-generational, Dutch HSCR family with five affected members.<sup>20</sup> The pattern of inheritance in this family seems to be autosomal dominant with low penetrance, indicating that the mutation in this region is necessary but not sufficient to cause the disease. It therefore seemed likely that in addition to the chromosome 4 mutation, other modifier genes or variants contribute to the development of the disease. This 4q linkage region contains at least 57 genes. From a functional point of view, Brooks *et al.* picked the *MAB21L2* gene as the most promising candidate, however no coding mutation was found in this gene.

We have now sequenced all exons (the exome) of two affected family members from different branches of the family in an attempt to identify the disease-associated mutation in the linkage region. Furthermore, we looked for possibly other modifier mutations elsewhere in the coding regions in the genome.

# MATERIALS AND METHODS

## Patients and DNA Samples

DNA from the five affected individuals, from 220 normal controls and from 87 HSCR patients was isolated from peripheral blood leucocytes using a standard protocol. For exome sequencing, we selected two affected members from different branches of the family (V-1 and V-4, see Figure-1). Validation of some identified variants by Sanger sequencing was performed in these two individuals and on three more affected family members (IV-3, V-2 and V-3).



**Figure 1** - Pedigree structure and haplotypes. Patients are represented as black symbols. The segregating haplotype, 4q31-q32, is depicted. The minimum critical region at chromosome 4 spans 11.7 cM between D4S3049 and D4S1566 (adapted from Brooks et al. 2006).

## Exome Sequencing and Pipeline Data Analysis

Five microgram of DNA from each individual was used for exome sequencing. For exome capturing and enrichment, we used the hybrid capture SureSelect Human All Exon kit (Agilent Technologies, Amsterdam, the Netherlands) which targets most human exons (approximately 38 Mb). The captured fragments were sequenced on a Genome Analyzer II (Illumina, San Diego, USA). The alignment of the sequencing results, the SNP calling and data filtering was performed using a pipeline set up by the Genomics Coordination Centre (GCC), hosted by the Department of Genetics, UMCG, Groningen. The pipeline is based on the genome analysis toolkit (GATK) version 1.0.4418 developed at the Broad institute (Boston, USA). dbSNP v1.29 is incorporated into this pipeline for filtering of known SNPs. The SNPs that passed the threshold of all the criteria of the pipeline and that were unknown in dbSNP v1.29 were further filtered by checking whether they were known in a later version of dbSNP (dbSNP v1.31) or mentioned in the 1000 Genomes database. SNPs were further checked for their presence in both the sequenced patients and in an unrelated sample that was run at the same time on the Genome Analyzer II (Illumina). The list of variants of this unrelated sample we call "in-house controls". This last step was incorporated into the whole filtering process to reduce the number of false-positives introduced during the enrichment and sequencing processes. As the pattern of inheritance is autosomal dominant, we focused on searching for heterozygous mutations.

## Deletion-Insertion Polymorphism (DIP) Analysis

Deletion-insertion polymorphism (DIP) analysis was performed using a program called CLC bio (CLCbio, Muehlital, Germany). It was performed only for chromosome 4. Again, as the pattern of inheritance seems to be autosomal dominant, we focused on searching for heterozygous DIPs and we set the minimum variant frequency at 30%, with a window length of 11 bp, a maximum number of gaps and mismatches of 2, and a coverage of at least 10.

## Validation of Candidate Variants (PCR and Sequencing)

PCR reactions were performed in a final volume of 15  $\mu$ l, containing 100 ng of DNA, 1.5  $\mu$ l of 10X PCR buffer (Invitrogen, Leek, the Netherlands), 15mM  $MgCl_2$ , 0.15  $\mu$ l of 25mM dNTPs, 1 unit of Taq Polymerase (Invitrogen) and 1  $\mu$ l of 20 pmol/ $\mu$ l of each primer. The following primers were used for *LRBA* 5'CCACATAACTTAAGGTTGATTC3' and 5'GATATAAGGAGATGTGGCTG3'; for *ARMC9* 5' TGGCCAGGTTTGTCTTGC3' and 5' GGCTTCCTAAAGGCCTCTTG3'; for *CD27* 5' CTGGGATTACAGGCATGAG3' and 5' GC-CTGAGACAGTTCTTACG3'. The PCR conditions were as follows: pre-heating 1 min at 95°C, followed by 35 cycles of 45 seconds at 95°C, 45 seconds at 60°C and 45 seconds at 72°C. The

final elongation step was 10 min at 72°C. PCR purification was performed by using EXOSAP IT according to the manufacturer’s manual (GE Healthcare, Eindhoven, the Netherlands). For Sanger sequencing we used 5 µl of purified PCR product.

## RESULTS

### Exome Sequencing

A total of 2.9-3.0 GB of paired-end sequence data was generated per individual, with a mean read length of 101 bps. About 84.4% of bases were mapped to the target exons and, on average, 64.8% of the exome was covered at least 10 times. A summary of the exome sequencing performance is shown in Table 1.

13,403 and 14,637 variants per individual passed the pipeline’s thresholds for SNP calling. After filtering with dbSNP build 129, version 131 and the 1000 Genome database, using the SeattleSeq annotation, 501 and 544 novel variants were identified per individual. To identify potentially pathogenic variants, we focused only on missense, splice-site, and nonsense mutations also insertions and deletions (indels). An overview of our SNP calling results is shown in Table 2. DIP analysis on chromosome 4 did not identify any heterozygous deletion or insertion.

**Table 1** - Summary of the statistics of exome sequencing analysis of two HSCR patients.

	Patient V-1	Patient V-4
Raw data yield (bp)	2.974.801.379	3.023.364.300
Target region (bp)	38.925.539	38.925.539
Base-pairs mapped to genome	2.325.231.999	2.336.642.676
Base-pairs mapped to target region	1.303.155.126	1.382.373.365
Base-pairs mapped to target and flanking regions	2.036.529.862	2.065.636.143
Mean read length (bp)	101	101
Mean depth of target region	37.34	38.71
Coverage of target region	33.47	35.51
Fraction of targets covered $\geq$ 2X (%)	80.14	83.29
Fraction of targets covered $\geq$ 10X (%)	62.85	66.70
Fraction of targets covered $\geq$ 20X (%)	50.39	53.70
Fraction of targets covered $\geq$ 30X (%)	40.29	42.82
Capture specificity (%)	43.80	45.72

**Table 2** - Overview of the SNP calling and filtering steps in two HSCR patients.

	Patient V-1	Patient V-4
<b>Variants identified (pipeline)</b>	<b>13,403</b>	<b>14,637</b>
<b>Unknown SNPs (in dbSNP129)</b>	<b>743</b>	<b>859</b>
<b>Unknown SNP in dbSNP131 and the 1000 genome database</b>	<b>501</b>	<b>544</b>
*coding-synonymous	115	102
*coding-not Mod 3	1	1
*intergenic	24	20
*intron	149	172
*missense	196	210
*nonsense	6	11
*near-gene	1	None
*splice-sites (SS)	1	3
*utr-3	4	19
*utr-5	4	6
<b>Missense+SS+nonsense variants</b>	<b>203</b>	<b>224</b>
Missense+SS+nonsense variants present in both patients	10	10
Variants - probably damaging (Polyphen)	3	3
Variants - benign (Polyphen)	6	6
Variants - unknown (Polyphen)	2	2
No. of genes in overlapped variants	9	9
Private missense + SS + nonsense variants	193	214

### Selection of Possible Disease-Causing SNPs

As the two individuals we sequenced are related they should have mutations in common. Ten new variants proved to be present in both individuals (genome-wide) (Table 3). Three of them, however, were also identified in the “in-house controls” and were therefore not considered as possible disease-associated candidate variants. Three of the seven remaining SNPs that were still possibly disease-causing were predicted as probably damaging in Polyphen-2 (Polymorphism Phenotyping v2). One of the three variants was in the linkage region, in exon 20 of *LRBA* (LPS Responsive beige-like Protein Kinase A anchor). A second possibly damaging variant was located in exon 19 of *ARMC9* (chromosome 2) and the third was located in exon 1 of the *OR1F1* gene (chromosome 16).

## Validate of Candidate Variants

To confirm a role for these missense mutations in the disease process, we screened all five affected individuals for the presence of these variants. The variant in exon 20 of the *LRBA* gene was confirmed in all five affected family members, while the variant in *ARMC9* was confirmed in three of the five (V-1, V-3 and V-4). The variant on *CDC27* was not confirmed in any of the five individuals, showing that an allele-balance (AB) threshold less than 0.6 is crucial to defining the real variant and notifying a false-positive one. For this reason we did not validate the remaining four candidate variants as all of them had an AB value of more than 0.6. The variant in exon 20 of *LRBA* is a missense mutation that changes an Asparagine to a Serine (N > S). This amino acid (N) is conserved in humans, mouse and zebrafish (Figure 2A).

		Exon 20			
A	784	YNVLF	FEILIEQIGTQVIHKQHPDPDSSVKIQ	N	PQILKV 821 Homo sapiens (LRBA)
	784	YNVLF	FEILIEQICTQVIHKQHPDPDSTVKIQ	N	PQILKV 821 Mus musculus (LRBA)
	142	YNVLF	FEILTEQICTQVIHKQHPDPDSTVKIQ	N	PQILKV 162 Danio rerio (LRBA-like)
	142	YNVLF	FEILTEQICTQVIHKQHPDPDSTVKIQ	N	PQILKV 162 Danio rerio (LRBA-like)
		Exon 19			
B	536	REEARAMGMEDIL	R	CFIKEGNAEMIRQIEFIIKQLNSE	563 Homo sapiens (ARMC9)
	536	REEARAMGMEDIL	R	CFIKEGNAEMIRQIEFIIKQLNSE	564 Mus musculus (ARMC9)

**Figure 2 - A)** Comparative alignment of part of the protein sequence of LRBA (using human, mouse and zebrafish data). The amino acid encoded by the *LRBA* variant (N > S) is colored in grey. **B)** Comparative alignment of part of the *ARMC9* amino acid sequence of human and mouse. The amino acid encoded by the *ARMC9* variant (R > C) is colored in red. Zebrafish has a predicted ortholog of *ARMC9*, however the precise protein sequence is not yet available.

**Table 3** - Summary of candidate variants.

Chr	Position	Ref	Variant	Mutation	Amino Acid Changes	Polyphen-2	Gene Symbol	Case V-1		Case V-4	
								C	AB	C	AB
2	232156084	C	T	missense	ARG > CYS	PD	ARMC9	20	0.4	21	0.48
4	151791682	T	C	missense	ASN > SER	PD	LRBA	38	0.42	36	0.45
7	142460335	A	G	missense	LYS > GLU	Benign	PRSS1	30	0.7	45	0.64
7	142460369	G	A	missense	SER > ASN	Benign	PRSS1	27	0.77	50	0.72
10	135438967	C	T	missense	ARG > GLN	PD	FRG2B *	15	0.6	37	0.54
11	123814439	A	G	missense	ILE > THR	Benign	OR6T1	31	0.7	32	0.34
16	3254293	G	A	missense	GLY > GLU	PD	OR1F1	11	0.64	13	0.54
17	16068396	G	A	missense	SER > LEU	Benign	NCOR1*	42	0.67	30	0.67
17	44144993	C	G	missense	ARG > PRO	Benign	KIAA1267 *	40	0	38	0
17	45234303	G	C	missense	ALA > GLY	Benign	CDC27	55	0.6	51	0.69

\* Present in "in-house controls"

Chr: Chromosome, Ref: Reference, PD: Probably damaging, C: Coverage, AB: Allele Balance = Ref.seq/(Ref.seq+Variant.Seq)



## ***LRBA* Variant in Controls and HSCR Patients**

We checked for the prevalence of the *LRBA* variant in a control population and in a HSCR population. The HSCR patients that were screened do not carry a *RET* coding sequence mutation. The *LRBA* variant was identified heterozygously in four of the 220 healthy individual (4/440 chromosomes: 0.9%) and heterozygously in one of the 87 HSCR patient (1/174 chromosomes: 0.57%).

## ***ARMC9* Variant in Controls**

We checked for the prevalence of *ARMC9* variant in a control population and we identified the variant heterozygously in six healthy individual (6/296 chromosomes: 2%).

## **DISCUSSION**

We identified a possible disease-associated variant in exon 20 of the *LRBA* gene in two cases from a large, multi-generational HSCR family on which we performed exome sequencing. We confirmed the presence of this variant in all five affected family members by Sanger sequencing. However, this variant proved to be present in the control population (0.9%, 4/440 chromosomes), and as the incidence rate of this variant is lower in our HSCR population (0.5%, 1/174 chromosomes) compared to the control population, it is unlikely that the variant is a strong HSCR-predisposing mutation or even a modifier gene for HSCR. A second variant was identified in *ARMC9*. The variant was identified in three of the five affected members (V-1, V-3 and V-4). However, the variant was also present in the control population (0.57%, 6/298 chromosomes) which again makes it unlikely that this variant is a disease-associated.

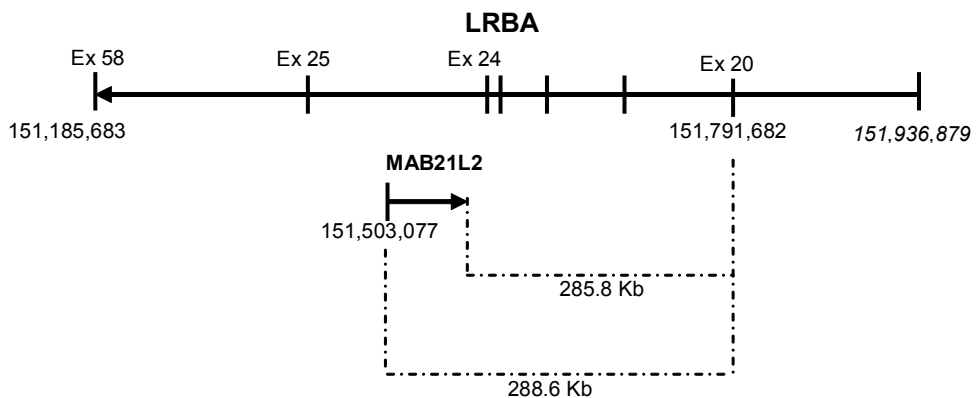
As the *LRBA* variant was the only variant we found on chromosome 4 that was present in both the exome-sequenced cases, we assume that we have probably missed the causative variant that all the cases in this family should have in common on chromosome 4. This was likely due to technical issues. One of the criteria we used in SNP calling was a minimum coverage of 10. The statistical data presented in Table 1 show that only 62.8% and 66.70% of the target sequences were covered with a minimum number of 10 reads. In fact, we found that when we lowered the coverage to 2, we still only had a coverage of 80.14% and 83.29%, respectively. In short, this means that by taking a coverage of 10, we do miss mutations in almost 40% of the target sequences (Table 1). Furthermore, the enrichment kit used in this experiment covers 81.33% of all human exons. So, in addition to the missed 40% of target sequences, we also missed about 19% of all exomic data. These figures led us to conclude that we might well have

missed the real disease-causing mutation.

Although we did not find any common mutation on chromosome 4, we wondered whether we could still select the best possible candidate gene associated with HSCR based on function in order to perform additional analysis. This led us to *MAB21L2*, which was mentioned earlier. *MAB21L2* is expressed in the neural crest derivatives, including the progenitor cells of the neurons and the glial of the enteric nervous system.<sup>21</sup> *MAB21L2* is a downstream target of the TGF- $\beta$ /SMADs signaling pathway, which is important for ENS development. Knock-down of the *MAB21L2* ortholog in zebrafish showed that subsequent ENCCs migration along the intestine is perturbed and a reduction of cell differentiation was seen, all indicating that normal *MAB21L2* function is required for normal ENCCs migration during ENS development (I. Sheperd, personal communication).

As mentioned earlier, *MAB21L2* has already been sequenced and no coding mutation was found.<sup>20</sup> We therefore decided to extend our sequencing efforts to the promoter region, and in fact we sequenced 16 Kb upstream of *MAB21L2* in all five affected family members. However, again, no mutation was identified (data not shown). One could argue that our analysis more or less excluded *MAB21L2* as the best candidate HSCR gene. However, this might not be completely true as will become clear from the following reasoning.

*MAB21L2* is located within the *LRBA* gene, shown in Figure 3. The *MAB21L2* is transcribed from the anti-sense strand of *LRBA* gene. This unique nested gene pair (*LRBA* and *MAB21L2*) is conserved in mouse, chick, frog and zebrafish.



**Figure 3** - Schematic overview of the *LRBA* and the *MAB21L2* genes and their position in the genome. The variation in exon 20 of *LRBA* is located 288.6 Kb downstream of the start site of *MAB21L2* gene.

Moreover Tsang and colleagues identified five paralogous, non-coding elements (pNEs) that are scattered throughout *LRBA*. They are located both upstream and downstream of *MAB21L2* and could regulate the expression of a reporter gene in a tissue-specific manner during mouse embryonic development, comparable to the expression patterns of *MAB21L2*.<sup>22</sup> Mutations in the *LRBA* locus might also have a regulatory effect on the gene that is transcribed from the anti-sense strand, namely *MAB21L2*. As we screened only part of the *LRBA/MAB21L2* locus, we cannot exclude that there are mutations in the locus that have an influence on *MAB21L2* expression.

We therefore propose to re-sequence the affected family members, but with a higher coverage and for a larger targeted sequence, including the entire *LRBA* genomic region (thereby including the multiple species conserved region that are involved in expression regulation of *MAB21L2*)

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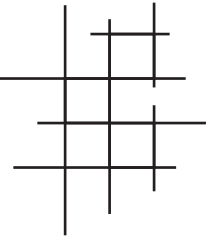
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# 4

## Variants in *RET* Associated With Hirschsprung Disease Affect Binding of Transcription Factors and Gene Expression

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## ABSTRACT

**Background & Aims:** Two non-coding *RET* variations, the T allele of SNP rs2435357 (Enh1:C>T) and the A allele of SNP rs2506004 (Enh2:C>A), are strongly associated with Hirschsprung disease (HSCR) susceptibility. These SNPs are in strong linkage disequilibrium and located in an enhancer element in intron 1 of the *RET* gene. For Enh1 it has been shown that the disease-associated T allele results in reduced expression in Luciferase experiments, via disturbed SOX10 binding, compared to the non-disease associated C allele. We aimed to determine whether Enh2-A is also a functional variant.

**Methods:** We evaluated the function of Enh2-A by Luciferase assays of constructs containing both alleles, separately or in combinations. We performed *in silico* analysis to identify a possible transcription activator or repressor that binds to Enh2-C (non-disease associated variant).

**Results:** Luciferase assays showed that not only the Enh1-T allele but also the Enh2-A allele decreased Luciferase expression. *In silico* analysis identified the sequences of Enh2-C and its surroundings sequences (-ACGTG-) as a potential binding site for the NXF/ARNT2 and SIM2/ARNT2 heterodimers. The binding affinity of NXF/ARNT2 to Enh2-C was confirmed by Electrophoresis Mobility Shift Assays and Supershift. Transfections of NXF/ARNT2 or SIM2/ARNT2 into neuroblastoma cell lines increased and decreased *RET* expression, respectively.

**Conclusions:** Our results show that more than one SNP on an associated haplotype can influence disease development. Our data strongly implicate the involvement of NXF, ARNT2 and SIM2 in *RET* expression regulation, thereby contributing to HSCR development.

**Abbreviation:** CME, central nervous system midline enhancer; ENS, enteric nervous system; HSCR, Hirschsprung disease; MCS, multi-species conserved sequences; NLBs, neurosphere-like bodies; NPE, nuclear protein extract; NCSCs, neural crest stem cells; SNP, single nucleotide polymorphism

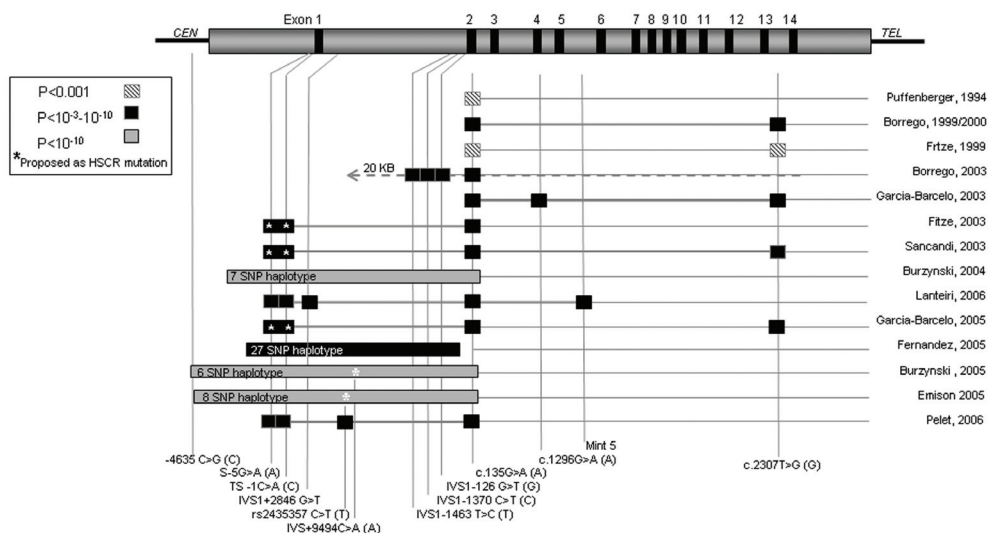
**Keywords:** Hirschsprung disease, *RET* expression, Down Syndrome

## INTRODUCTION

Loss-of-function mutations in the *RET* gene are associated with Hirschsprung's disease (HSCR), a congenital disorder characterized by the absence of ganglia in the distal part of the gut. Genetic dissection of HSCR has led not only to the identification of mutations in *RET*, but also to coding mutations in 10 other genes. Of these eleven genes, *RET* is believed to be the major factor in the etiology of HSCR. Coding sequence mutations in *RET* have been found in up to 50% of familial and 15 to 35% of sporadic cases of HSCR.<sup>1</sup> However, genome-wide linkage analysis on multigenerational HSCR families and sibpairs studies on smaller HSCR families showed that in almost all of the families, *RET* seems to play a role<sup>2,3</sup>. This led to the hypothesis that non-coding *RET* mutations likely play a role in these familial cases. It further led us to hypothesize that non-coding variants may also play a role in the sporadic cases.

Association studies carried out on several European and Asian patient populations, mostly without *RET* mutations, indeed showed a very strong association with a specific *RET* haplotype (Figure 1)<sup>1,4-15</sup>. In fact, regardless of the population, the same haplotype, spanning ~27 Kb (4 Kb of the 5' UTR and exon 1; 23 Kb of intron 1 and exon 2) is present in most HSCR patients. It is now believed that the mutation(s) on this haplotype are probably present in intron 1, within an enhancer region<sup>13,14</sup>. In both zebra fish and mice it was shown that the reporter gene expression patterns driven by this enhancer element (called MCS+9.7) are similar to *RET*, both in a tissue and in a time-wise manner<sup>16,17</sup>. There are two SNPs in this enhancer sequence: rs2435357 (Enh1:C>T) (also called RET+3)<sup>13</sup> and rs2506004 (Enh2:C>A) (also called IVS+9494)<sup>14</sup>, of which the Enh1-T and the Enh2-A alleles both are strongly associated with HSCR<sup>13,14</sup>. Emison proposed that Enh1-T was most likely the disease-associated allele based on conservation and functional arguments<sup>14,18</sup>. However, the Enh2-A allele, an allele in strong linkage disequilibrium with Enh1-T ( $r = 0.969$ ), was also present in the constructs used for the functional interpretation of Enh1-T<sup>14</sup>. Thus we cannot totally exclude that this Enh2-A allele also contributes to disease development. We therefore investigated the role of this SNP in *RET* expression regulation.



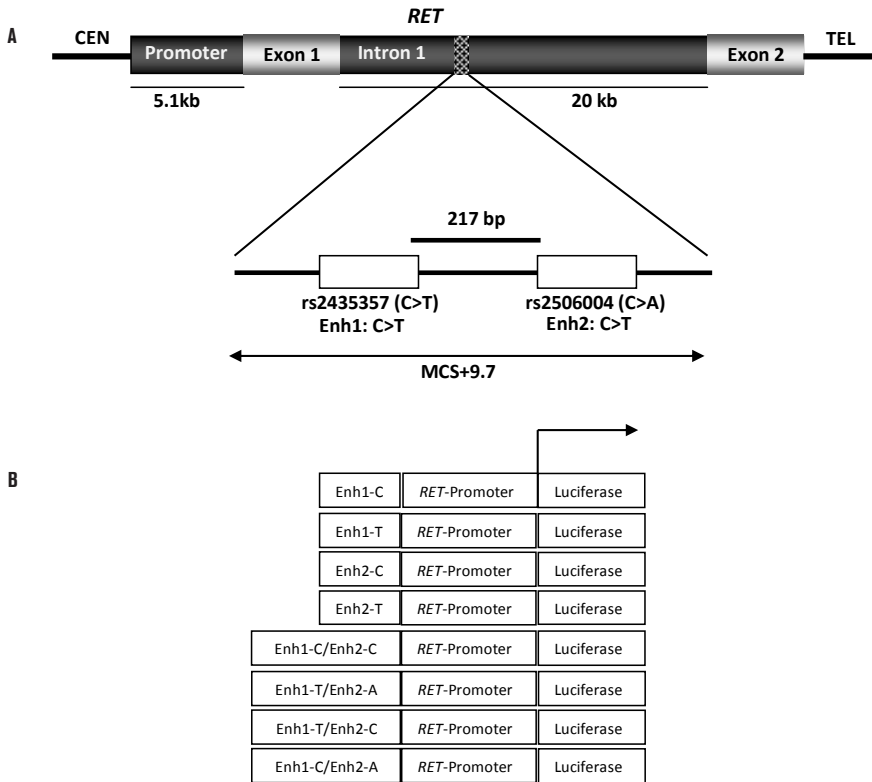


**Figure 1** - An overview of the reported Hirschsprung (HSCR) disease-associated SNPs

## MATERIALS AND METHODS

### Construction of Vectors

The enhancer elements, containing one or both HSCR-associated SNPs, were PCR amplified using patients DNA as template, and cloned into KpnI/XhoI sites of a pGL3-*Luciferase* construct already containing the RET promoter or the SV40 promoter (Promega, Madison, WI, USA). Constructs containing a combination of non-disease- and disease-associated variants for both SNPs were generated in RETprom+Enh1-C/Enh2-C by site-directed mutagenesis (QuickChange XL Site-Directed Mutagenesis kit, Stratagene, Texas, USA). For primers see Supplementary Table 2. The sequences of the inserts were checked by direct sequencing.



**Figure 2** - Schematic overview of the *RET* gene and the constructs used in this study. (A) SNP rs2435357 (Enh1:C>T) and SNP rs2506004 (Enh2:C>A) are both located in a conserved region of intron 1, called MCS+9.7, separated by 217 bp. (B) *RET* and SV40 promoter constructs coupled to *Luciferase* and enhancer sequences used in this study.

## Luciferase Assays

Transient transfections were performed using a mouse neuroblastoma cell line (Neuro-2A) which was cultured according to the ATCC protocol.  $0.5 \times 10^6$  Neuro2A cells were plated in 35 mm dishes for 48 hours prior to transfection. Cells were co-transfected with 100 ng of pRL-SV40-*Renilla Luciferase* (Promega, Madison, USA) as an internal control and 1 mg of the reporter constructs containing different enhancer elements (Figure 2B) using 4 ml of Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol. Luciferase activity was analyzed with a Dual-Luciferase-Reporter Assay (Promega) 24 hours after transfection. The ratios of Firefly Luciferase compared to Renilla Luciferase (normalized-Luciferase unit) were used to determine the activity of each promoter and enhancer element.

The results presented in the graphs are fold changes of normalized-Luciferase expression of each construct to the control (pGL3-RET or pGL3-SV40 promoter). Activation or repression of Luciferase expression by *NXF/ARNT2* and *SIM2/ARNT2*, respectively were performed by co-transfection the *NXF/ARNT2* (1mg/500ng) or *SIM2/ARNT2* (1mg/500ng) with 1 mg of reporter construct containing different enhancer elements. In all cases the amount of the transfected DNA was the same, if necessary this was achieved by adding pCDNA3. The assays were performed in three, independent, triplicate experiments. For the dosages effect experiment, three different dosages of *NXF/ARNT2* or *SIM2/ARNT2* (100/50 ng, 500/250ng and 1 mg/ 500 ng) were used.

### Statistical Analysis

Statistical analysis of the Luciferase assay results was performed using the one-way ANOVA test. The results represent the mean  $\pm$  S.E.M. Statistical analysis was performed by GraphPad Prism 4. P-values of  $< 0.05$  were considered significant.

### Protein Analysis

Western blotting was performed to check the level of RET expression in the Neuro2A cell line, after *NXF/ARNT2* or *SIM2/ARNT2* transfections. Transient transfection was performed as described above. For the mock control, cells were only transfected with 1.5 mg pcDNA3. After 24 hours cells were harvested and total cell lysates were isolated using CellLytic™ (Sigma Aldrich, Saint Louis, USA) according to the manufacture's protocol. Protein concentrations were determined using the BCA protein assay (Pierce Biotechnologies, Rockford, IL, USA) and measured on a NanoDrop® ND-1000 (Thermo Scientific, Waltham, MA, USA). 30 mg of cell lysate was used for Western blotting. Primary antibodies anti-RET, and anti-b-Actin were applied and visualized with the respective IgG-HRP secondary antibodies (see supplementary Table 3).

### Neural Crest Stem Cells (NCSCs) Isolation and Culture

C57BL/6 mouse embryos E11.5, E12.5, E13.5, E14.5 and E15.5, were sacrificed and the gut (jejunum to rectum) was used for the isolation of NCSCs. The gut was mechanically dissected and tissues were incubated in collagenaseXI /dispaseII enzyme solution (collagenase XI [750U/ml], Sigma Aldrich, Saint Louis, USA; dispase II [250 mg/ml], Roche, IN, USA) in PBS, incubated at 37°C for up to 5 minutes. Digested tissue was triturated until homogenous cell suspensions were created. Cell suspensions were washed and seeded onto 35 mm Petri dishes coated with

fibronectin 2 mg/cm<sup>2</sup> (Sigma Aldrich) in 2 ml of DMEM-F12 medium (PAA) supplemented by N2 supplement 1:100, B27 supplement 1:50 (Invitrogen, Carlsbad, CA, USA), Penicillin (100 IU/ml)/ streptomycin (100 µg/ml) (PAA), 20 ng/ml FGF, 20 ng/ml EGF (Peprotech EC, London, UK) . Half of the medium was refreshed every two days and fresh EGF and FGF were added to the medium. The neural crest-derived cells were kept in culture as neurosphere-like bodies (NLBs) for 14 days.

## RT-PCR

Total RNA was isolated from NLBs cultures using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Two micrograms of RNA were transcribed into cDNA with the Ready-To-Go First Strand Synthesis kit (GE-Health care) according to the manufacturer's instruction. PCRs were performed by using 1.25 ml of cDNA for 35 cycles using the primers given in supplementary Table 4.

## Immunohistochemistry

Immunostaining was performed on cryosections (12 µm) of frozen NLBs. NLBs were centrifuged at 180g in a microtube and then frozen in OCT embedding compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan). Cryosections of NLBs were fixed in 4% PFA for 10 minutes at room temperature and rinsed three times in PBS before application of blocking solution (PBS containing 10% donkey serum, 0.1% Triton X-100) for 30 minutes at room temperature. Primary antibodies anti-RET, NXF, SIM2 and ARNT2 were applied and visualized with the respective fluorochrome-conjugated secondary antibodies (see supplementary Table 5). Cell nuclei were stained with 4'-6-diamidino-2-phenylindole [DAPI] solution (Invitrogen; 100 ng/ml) and cover-slipped with Kaiser's gelatine (Merck, Darmstadt, Germany). The images were captured on a Nikon inverted fluorescence microscope (Nikon, Dusseldorf, Germany).

## EMSA and Supershift Assays

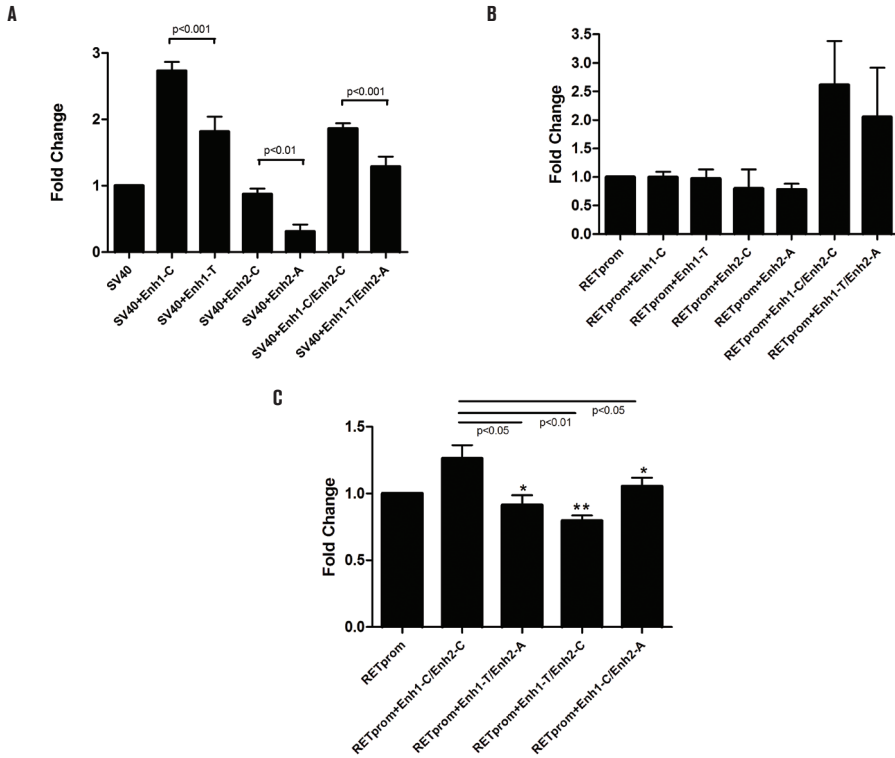
Nuclear protein extracted from Neuro2A cells transfected with *NXF/ARNT2*, *SIM2/ARNT2*, *SIM2*, *NXF* and *ARNT2* constructs were isolated using a Nuclear Protein extraction kit (Sigma Aldrich, Saint Louis, USA). Complementary oligonucleotides (30 bp) containing either Enh2-C or Enh2-A were designed (see Supplementary Table 6). Sense and antisense oligonucleotide were 3'end labeled (Biotin3'end-DNA labeling kit, Pierce Biotechnologies, Rockford, IL, USA) according to the manufacturer's protocol. Binding experiments were performed using an EMSA kit (Pierce) according to the manufacturer's protocol using 20 µg of nuclear protein

and 1 ml of 25 fmol of biotin labeled oligonucleotides. In the competitive reactions a 1000 times excess of unlabeled oligonucleotides was added. In the Supershift experiments, 1 ml rabbit polyclonal NXF antibody (Abcam, Cambridge, UK) was mixed with 10 mg of total lysate of a NXF and ARNT2 positive cell line (SK-N-SH). Gelshifts were visualized using the LightShift® Chemiluminescent EMSA kit (Pierce).

## RESULTS

### Mutations in either Ehn1 or Ehn2 reduce reporter gene expression

Constructs expressing Luciferase, driven by the *RET* or *SV40* promoter coupled to the *RET* enhancer sequence containing SNP rs245357 (Enh1:C>T) or SNP rs2506004 (Enh2:C>A), were transfected into Neuro2A cells. The inserts containing Enh1, Enh2 and Enh1/Enh2 differ in length; 300bp, 400bp and 500 bp respectively. These differences might have an effect on binding affinity. Therefore, we compared the expression of the mutant constructs to their WT counterparts. Constructs with the *SV40* promoter showed that both the disease-associated alleles (Enh1-T/Enh2-A) separately or in combination, decreased the Luciferase expression approximately two-fold (Figure 3A). Constructs in which the *RET* enhancer sequence was coupled to the *RET* promoter, however, showed that only when the two disease variants were both present, were able to decrease Luciferase expression (Figure 3B). In these *RET* promoter experiments, we did not test whether one or both mutant alleles were necessary for the reduction of Luciferase expression. Therefore, by using site-directed mutagenesis, we made constructs that contain a combination of one non-disease-associated variant and its counterpart disease-associated variant (Enh1-C/Enh2-A or Enh1-T/Enh2-C). We could show that a mutation in either variant decreased the Luciferase expression (Figure 3C).



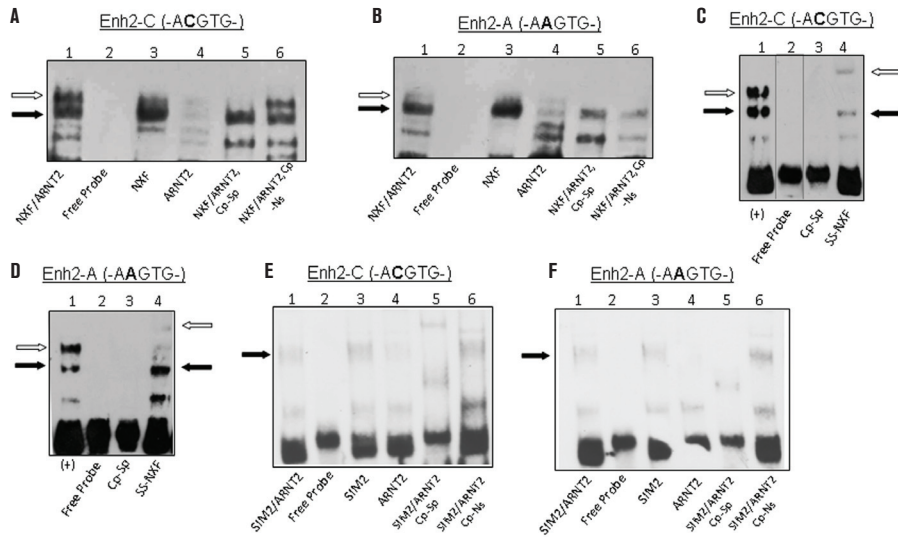
**Figure 3** - Panel (A) shows under SV40 promoter regulation, both the Enh1-T and the Enh2-A separately or in combination decreased the Luciferase expression approximately 2-fold. (B) Under *RET* promoter regulation, only when both diseases associated SNPs are present they can decrease Luciferase expression. (C) Constructs with the *RET* promoter coupled to the enhancer sequence containing combination of a non-disease associated variant and its disease-associated counterpart (Enh1-T/Enh2-C and Enh1-C/Enh2-A), showed that both SNPs could significantly decrease Luciferase expression. The bars indicate the mean  $\pm$  S.E.M of the fold changes of Luciferase expression for each construct.  $p < 0.05$  was considered significant.

### In silico analysis of the MCS+9.7 sequence

*MatInspector* ([www.genomatix.de](http://www.genomatix.de)) analysis of the MCS+9.7 sequences showed that the Enh2-C allele with its surrounding sequences -ACGTG- (also known as the Central Nervous System Midline Element (CME)) is a core binding site of the heterodimers NXF/ARNT2 and SIM2/ARNT2. These heterodimers have the same core binding sequence, but have opposite effects. NXF/ARNT2 is an expression activator, whereas SIM2/ARNT2 represses gene transcription<sup>19</sup>.

## Reduced NXF/ARNT2 binding to Enh2-A compared to Enh2-C

To check whether the NXF/ARNT2 heterodimer could indeed bind to the Enh2-C sequence, we performed Electro Mobility Shift Assay (EMSA). Oligonucleotides containing the Enh2-C allele or the Enh2-A allele were mixed with the nuclear protein extract (NPE) of Neuro2A cells that were transiently transfected with either the *NXF* or *ARNT2* constructs or with both constructs together. Protein binding was determined to be sequence specific if the excess of unlabeled oligonucleotides could replace the labeled oligonucleotides (Cp-Sp) while the excess of unrelated-unlabeled oligonucleotides could not replace it (Ns-SP). NPE of Neuro2A transfected with all these constructs could bind to both oligonucleotides containing Enh2-C or Enh2-A (Figure 4A and B: lane 1, 3 and 4). Only one band of the NPE of Neuro2A transfected with NXF/ARNT2 (Figure 4A lane 1, white arrow) could be completely deleted by an excess of unlabeled oligonucleotides and stay the same when an excess unrelated-unlabeled was used in the competition assay (Figure 4A lane 5 and 6). Furthermore, this specific band has less or no binding to the oligonucleotides containing Enh2-A (Figure 4B lane 1, white arrow). To confirm whether this protein complex bound to Enh2-C is NXF/ARNT2, Supershift assays were performed using an anti-NXF antibody and a total lysate of a SK-N-SH, a human neuroblastoma cell line positive for NXF/ARNT2. Total lysates of SK-N-SH had the same binding pattern as the NPE of Neuro2A transfected by NXF/ARNT2 (Figure 4C and D, lane 1 arrow white and black). When the antibody anti-NXF was applied, the upper band was shifted and the lower band was blocked (Figure 4C and D, lane 4) showing that these two bands contain the NXF protein. Notably the polyclonal ARNT2 antibody was not able to either block or supershift the upper band (data was not shown). This could be because the epitope of ARNT2 is hidden and is not recognized by the antibody. Based on these results, we conclude that the upper band (white arrow) is the NXF/ARNT2 heterodimer. Based on EMSA results (Figure 4A lane 3) and Supershift results (Figure 4C lane 4, black arrow) we assume that the lower band most likely is NXF alone. Similar experiment was performed using NPE of Neuro2A cells transiently transfected with SIM2/ARNT2, SIM2 and ARNT2 (Figure 4E and F). It was shown that SIM2/ARNT2 and SIM2-alone gave a specific band for both Enh2-C and Enh2-A suggesting that only SIM2 binds to the enhancer sequence, independent of the mutation status (Figure 4E and F, lane 1 and 3).



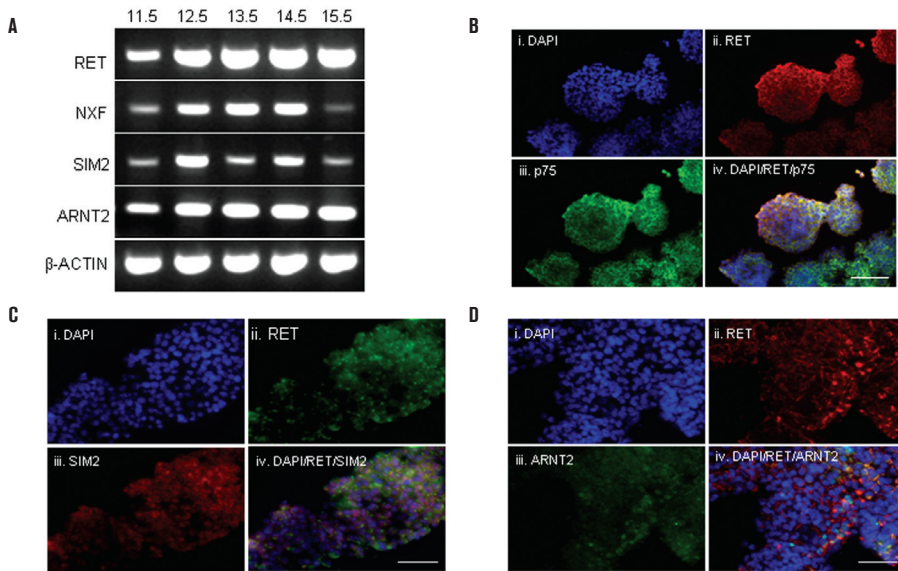
**Figure 4** - Panel (A) and (B) show EMSA using labeled oligonucleotides containing Enh2-C and Enh2-A with nuclear protein extract (NPE) of Neuro2A cells transfected with *NXF/ARNT2*, *NXF* or *ARNT2* constructs: an extra band (white arrow) appeared when oligonucleotides containing Enh2-C allele were used in combination with NPE containing both *NXF* and *ARNT2*. In a competition assay (Cp-Sp) in lane 5, the extra band as seen in lane 1 disappeared, while excess of unrelated, unlabeled oligonucleotide (Cp-NS), could not compete the specific labeled oligonucleotide (lane 6). (C) and (D) EMSA-Supershift (SS) assays using Enh2-C and Enh2-A labeled oligonucleotide with total cell lysates of the SK-N-SH cell line and anti-NXF antibody showed that the highest band was shifted upwards and the second lower band became weaker (was blocked) (lane 4). Based on Fig. 4C, the highest band (white arrow), which is shifted by the *NXF* antibody, is the *NXF/ARNT2* complex, and the second band (black arrow), for which the binding is blocked in lane 4, most likely is *NXF*. (E) and (F) EMSA using labeled oligonucleotides containing Enh2-C and Enh2-A with NPE of Neuro2A transfected with *SIM2/ARNT2*, *SIM2* or *ARNT2* construct. *SIM2* has the same binding affinity to the enhancer sequences containing Enh2-C or Enh2-A (lane 1 and 3), the specificity of this binding is confirmed in competition specific and non-specific assays (lane 5 and 6).

### ***RET*, *NXF*, *SIM2* and *ARNT2* are expressed in NCSCs isolate from mouse embryonic gut**

Neurosphere-like bodies (NLBs), propagated in vitro from the gut of mouse embryos E11.5-E15.5 (Figure 5A) were checked for expression of *RET*, *NXF*, *ARNT2* and *SIM2* by RT-PCR. The result showed that *RET*, *SIM2*, *NXF* and *ARNT2* are expressed in all stages analyzed. The *RET* expression increase in time, while *NXF* and *SIM2* expression reach the highest level on E12.5 to 14.5 and decrease on E15.5. The neural progenitor status of *RET* positive cells within NLBs cultures was demonstrated by co-immunostaining with the neural pro-



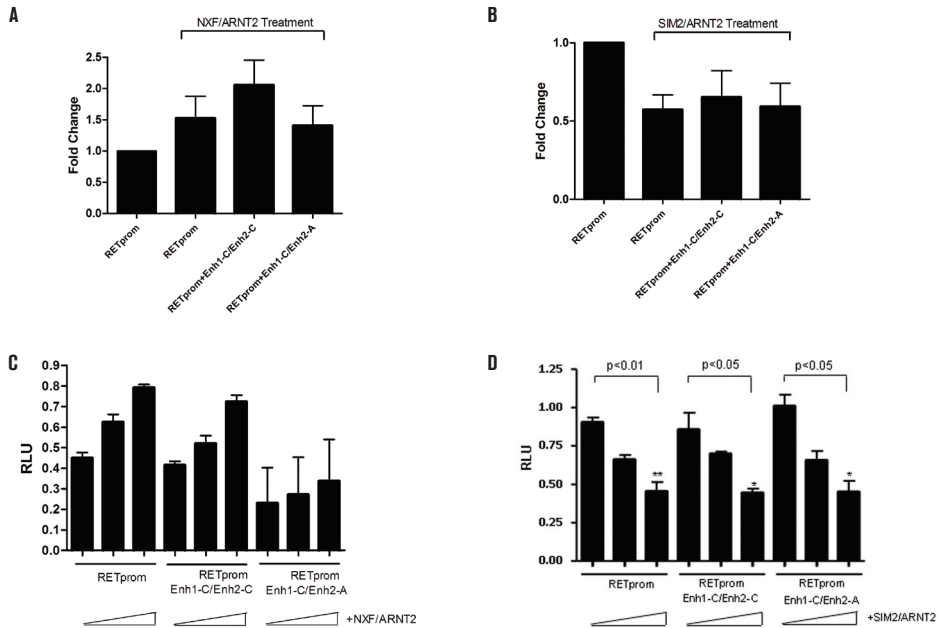
genitor marker p75 (Figure 5B). In addition, the majority of SIM2 and ARNT2-positive cells co-expressed RET (Figure 5C and 5D). The only available commercial NXF antibody revealed no satisfying results on mouse tissue and it therefore could not be used for immunostaining (data not shown).



**Figure 5** - Panel (A): By using RT-PCR method it is showed that *RET*, *NXF*, *SIM2* and *ARNT2* are expressed in NCSCs isolated from mouse embryonic gut: E11.5, 12.5, 13.5, 14.5 and 15.5. (B) Progenitor status of RET positive cells in neurosphere-like body (NLBs) cultures by co-immunostaining with the neural crest marker p75: i) DAPI staining, ii) RET expression, iii) p75 expression and iv) co-expression of RET and p75. C) SIM2 co-expression with RET in NLBs cultures: i) DAPI staining, ii) RET expression, iii) SIM2 expression and iv) co-expression of RET and SIM2. D) ARNT2 co-expression with RET in NLBs cultures: i) DAPI staining, ii) RET expression, iii) ARNT2 expression and iv) co-expression of RET and ARNT2. Scale bars: 100  $\mu$ m in B (iv), 50  $\mu$ m in C (iv) and D (iv).

### ***NXF/ARNT2* and *SIM2/ARNT2* increases and decrease *RET* expression respectively via the *RET* promoter and the enh2 sequences.**

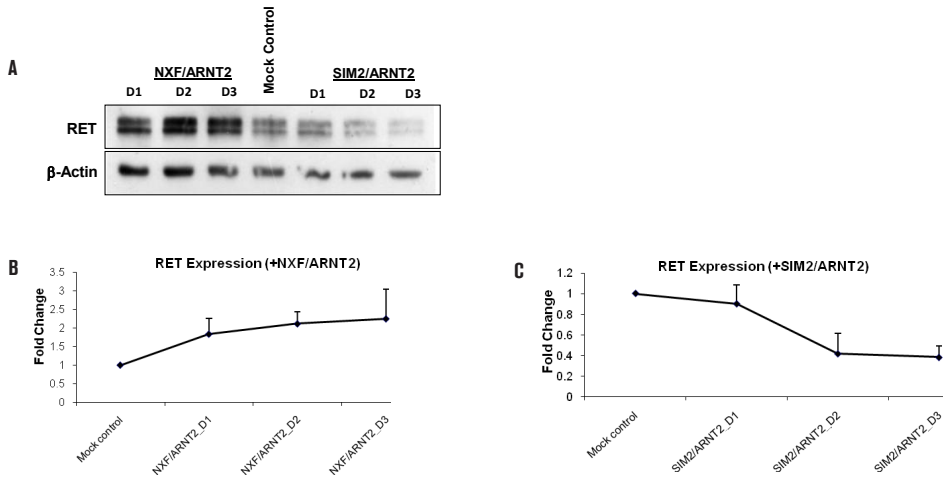
Not only the *RET* enhancer sequence (MCS +9.7) but also the *RET* promoter (starting 5.1 kb upstream of the *RET* start site) contains six CME (Central Nervous System Midline Element) variants. *NXF/ARNT2* and *SIM2/ARNT2* might regulate *RET* expression via binding to the enhancer region containing the Enh2-C allele and the *RET* promoter. To prove this, we performed co-transfections of *NXF/ARNT2* or *SIM2/ARNT2* with the *RET* promoter (*RET*-prom) constructs with or without the enhancer sequences containing Enh2-C or Enh2-A, into Neuro2A, a cell line not endogenously expressing *NXF*, *SIM2* and *ARNT2*. Figure 6A shows that co-transfection of *NXF/ARNT2* with the *RET* promoter construct increases the Luciferase expression 1.25-fold, while co-transfection with *RET*prom+Enh2-C allele, the Luciferase levels go up 1.75-fold. The *RET*prom+Enh2-A allele results in a Luciferase expression level comparable to that of the *RET*prom construct without the enhancer sequence. This shows that *NXF/ARNT2* can regulate *RET* expression via both the promoter and the enhancer sequences. On the contrary, the co-transfection with *SIM2/ARNT2* decreased the Luciferase expression level two-fold for the *RET*prom construct (without enhancer). Similar levels of Luciferase expression were obtained for the constructs containing the *RET* promoter coupled to the enhancer (for both the Enh2-C and the Ehn2-A allele). These data suggest that the binding of *SIM2/ARNT2* to either of Enh2-C or Enh2-A is ablating the enhancer effect. Therefore the expression levels of the *RET*prom with Enh2-C or Ehn2-A are equal to those of the *RET*prom alone, in the present of *SIM2/ARNT2* expression. Furthermore, by increasing the amount of constructs transfected, we could induce an increase of up- or down-regulation of gene expression (Figure 6C and D). This shows that expression regulated by *NXF/ARNT2* and *SIM2/ARNT2*, via the *RET* promoter and the enhancer sequence, depends on dosage (Figure 5C).



**Figure 6** - Panel (A) shows the effect of *NXF/ARNT2* to Luciferase expression of constructs containing the *RET* promoter or the *RET* promoter and enhancer containing Enh2-C or Enh2-A. (B) Effect of *SIM2/ARNT2* to Luciferase expression of constructs containing the *RET* promoter or the *RET* promoter and enhancer containing Enh2-C and Enh2-A variants. (C) and (D) a possible (not significant) dosage effect of *NXF/ARNT2* and *SIM2/ARNT2* on Luciferase expression driven by the *RET* promoter and enhancer regulation. Three dosages of *NXF/ARNT2* and *SIM2/ARNT2* constructs were used. The bars indicate the mean  $\pm$  S.E.M of fold changes of Luciferase expression for each construct after co-transfection of *NXF/ARNT2* or *SIM2/ARNT2*. In figures C and D the bars indicate the mean  $\pm$  S.E.M of normalized-Luciferase expression for each construct after co-transfection with three different dosages of *NXF/ARNT2* or *SIM2/ARNT2*.  $p < 0.05$  was considered significant.

### Level of endogenous RET protein expression is up- and down-regulated after *NXF/ARNT2* & *SIM2/ARNT2* transfection, respectively

To check the effect of *NXF/ARNT2* and *SIM2/ARNT2* on endogenous RET expression, we transfected three different dosages of the *NXF/ARNT2* or *SIM2/ARNT2* constructs into Neuro2A cells. The level of RET endogenous expression was checked by Westernblot. The transfection of the highest dosage of *NXF/ARNT2* in the cell line enhanced the RET expression level 2.25 times, whereas the transfection of the highest dosage of *SIM2/ARNT2* constructs reduced the RET expression level 2.6 times (Figure 7).



**Figure 7** - Panel A shows Western blotting of total cell lysate of Neuro2A cells transfected with three different dosages of *NXF/ARNT2* or *SIM2/ARNT2*: D1 (100/50 ng), D2 (500/250 ng) and D3 (1000/500 ng) using anti-RET and anti- $\beta$ -Actin primary antibodies. (B) Densitometric analysis of the Western blots. The highest dosage of *NXF/ARNT2* enhanced RET expression 2.25-fold compared to the RET expression in the mock controls. (C) Densitometric analysis of the Western blots. The highest dosage of *SIM2/ARNT2* decreases RET expression 2.6-fold compared to the RET expression in the mock controls. The bars indicate the mean  $\pm$  S.E.M of fold changes for RET endogenous expression after transfection of *NXF/ARNT2* or *SIM2/ARNT2*.  $p < 0.05$  was considered significant.

## DISCUSSION

Recent studies showed that the T allele of SNP rs2435357 (Enh1-T), a SNP present in an enhancer element (MCS+9.7) in intron 1 of the *RET* gene, contributes to HSCR development. It was shown in reporter assays that the construct containing the non-disease associated allele, Enh1-C, contrasts to that with the disease-associated allele, Enh1-T, by enhancing luciferase expression 6-fold<sup>14</sup>. Furthermore, *in vitro* assays showed that the effect Enh1-T has on *RET* expression is caused by a disruption of a SOX10 binding site within the enhancer element<sup>18</sup>. However, in the sequence used in all the experiments, a second SNP is also present, namely rs2506004<sup>14</sup>. The A allele of this SNP is also strongly associated with HSCR,<sup>13</sup> what is not surprising as they are only 217 base pairs apart. We investigated whether the disease-associated allele, Enh2-A, also played a role in regulating *RET* expression.

In order to prove whether Enh1-T and Enh2-A, separately or in combination, function in

gene expression regulation, we generated Luciferase-reporter constructs containing the two alleles of only Enh1 or Enh2 and constructs containing alleles of the two SNPs, under the regulation of the *SV40* or the *RET* promoter (RETprom). Luciferase assays showed that the disease-associated allele of each SNP, separately or in combination, under *SV40* regulation, could reduce Luciferase expression (Figure 3A). However, under *RET* promoter regulation, only the construct containing both non-disease associated SNPs together could yield the enhancer effect, while the disease-associated SNPs, together, decreased Luciferase expression (Figure 3B). It is likely that the constructs used to show the effect for the SNPs independently were lacking sequences necessary for the binding of *RET*-specific transcription factors. This experiment still left the possibility that only one of the SNPs is the functional variant. To define the function of each SNP, we generated constructs containing the non-disease associated allele of one SNP with the disease-associated allele of the other SNP, under *RET* promoter regulation. Luciferase assays of these constructs showed that Enh1-T or Enh2-A in the existence of its non-disease associated counterpart could significantly reduce Luciferase expression. The effect of the Enh1-T allele is bigger than for the Enh2-A allele, but the combination of both SNPs did not double the effect (Figure 3C). This experiment showed that disease-associated alleles of both of these SNPs can contribute to the development of HSCR.

To elucidate the effect of Enh2-A on the functioning of the enhancer, and to identify a protein candidate that could bind to this SNP, we analyzed the enhancer sequences containing the non-disease and disease-associated variants by *MatInspector* software ([www.genom-atix.de](http://www.genom-atix.de)). The Enh2-C (non-disease) allele and its surrounding sequence proved to be present in the -ACGTG- sequence, also known as the Central Nervous System Midline Element (CME).<sup>20</sup> This CME sequence element and three other CME variants (-GCGTG-, -TCGTG- and -CCGTG-) are binding site of NXF/ARNT2 and the SIM2/ARNT2 heterodimers.<sup>19</sup>

NXF is a basic-Helix-Loop-helix/Per-Arnt-Sim (bHLH/PAS) protein, which is mainly expressed in neuronal tissues. It was shown in a previous study that NXF can form a heterodimer with ARNT2. Moreover, it was proven that NXF/ARNT2 competes with the SIM2/ARNT2 heterodimer in binding to the CME sequences.<sup>19</sup> These two heterodimers regulate gene expression for the *Drebrin* gene as transcription activator and repressor, respectively, via binding to the CME sequences in the *Drebrin*'s promoter. We therefore hypothesized that both NXF/ARNT2 and SIM2/ARNT2 are also involved in regulating *RET* expression via the binding of these two heterodimers to the CME in the Enh2 sequence. Moreover, as we detected six CME variants in the promoter of *RET*, we cannot exclude that these sites also play a role in the expression regulation. By EMSAs and a Supershift assay, using a NXF antibody, we showed

that the NXF/ARNT2 heterodimer could indeed bind to the Enh2-C (non-disease associated) allele and that the heterodimer has less binding affinity to Enh2-A, the disease-associated allele (Figure 4A, 4B, 4C and 4D, lane 1). EMSAs of SIM2/ARNT2 showed that also SIM2 could bind to Enh2 but that it had the same binding affinity to the wild type and the mutant Enh2 sequences.

A first step to prove our hypothesis was to show that these proteins are expressed in the (migrating) neural crest stem cells (NCSC), the progenitors of the cells that are lacking in the hindgut of HSCR patients. Therefore, we checked by RT-PCR and immunohistochemical staining the presence of NXF, SIM2 and ARNT2 in NCSC isolated from embryonic guts (E11.5 until E15.5). By day 15.5 the migration of the NCSCs and the formation of the enteric nervous system is complete in the mouse. We show that RET expression is up-regulated during this period, while SIM2 and NXF were expressed in all stages analyzed with the highest expression at E12.5 to E14.5. Furthermore, immunostaining of neural progenitor cells isolated from mouse embryonic gut day 13.5 showed that both SIM2 and ARTN2 are expressed (Figure 5C and D).

To check whether NXF/ARNT2 and SIM2/ARNT2 can indeed activate and inhibit the expression of RET, via the *RET* promoter and/or via the enhancer region containing the Enh2-C allele, we co-transfected *NXF/ARNT2* or *SIM2/ARNT2* constructs with the *RET* promoter-*Luciferase* constructs, with or without the enhancer sequences containing the Enh2-C or Enh2-A alleles, into Neuro2A cell lines. The results show that NXF/ARNT2 could enhance the Luciferase expression via the *RET* promoter and via the enhancer sequence containing Enh2-C. The disease-associated allele, Enh2-A, reduces the Luciferase expression to the level of the *RET* promoter only (Figure 6A). In contrast, co-transfection of *SIM2/ARNT2* resulted in a decrease of Luciferase expression.

Furthermore, the level of Luciferase expression proved to depend on the dosage of NXF/ARNT2 and SIM2/ARNT2, as by increasing concentrations of NXF/ARNT2 or SIM2/ARNT2 a further up- or down-regulation of Luciferase expression was measured, respectively (Figure 6C and D). To further strengthen these data, we also performed a comparable dosage-effect experiment in mouse Neuro2A cells, checking endogenous RET expression. The results confirmed the patterns of gene expression found in our initial Luciferase assays. The highest dosage of *NXF/ARNT2* increased RET expression levels by 2.25-fold, while the highest dosage of *SIM2/ARNT2* reduced RET expression levels by 2.6-fold (Figure 7).

SIM2 is located in the critical region for Down Syndrome on chromosome 21 and it is expressed in the fetal brain, particularly in regions crucial for cognitive processes. Moreover,

it was shown that overexpression of SIM2 in rat and mouse hippocampus impairs the ability of learning and memory by inhibiting synaptic plasticity.<sup>21,22</sup> Interestingly, in Down syndrome patients who have three copies of the SIM2 gene, the structure of the brain regions expressing SIM2 are altered.<sup>23</sup> What this overexpression of SIM2 leads to is only partly known, although it was shown that overexpression of this gene leads to reduced expression of Drebrin, a protein playing an important role in synaptic plasticity.<sup>18</sup> Its expression is known to be decreased in the brains of Down syndrome patients.<sup>24</sup> We have now shown that SIM2 overexpression results in a down-regulation of RET. This finding might partly explain why we see a 40-fold higher risk for HSCR disease in Down syndrome patients than in the general population of newborns.

In this study we have proved that not one but at least two SNPs on the HSCR-associated haplotype can contribute to the disease phenotype. However it is still unclear when exactly these SNPs act in the disease development. They might deregulate proliferation during the first stages of embryogenesis, or impair migration of the NCSCs, or play a role during proliferation when the cells reach their final destination in the gut. We cannot exclude the possibility that the different disease-associated SNPs play a role at different stages of development, or are involved in different processes. Previous studies showed evidence for the involvement of at least two additional SNPs on the same haplotype. These SNPs are located at -5 and -1 from the start site of the RET gene (Figure 1). It was also shown that the TTF-1 protein binds to this region and that TTF-1 can activate the RET promoter<sup>10,12</sup>. Our new data and previously published data hold an important message for the study of complex diseases, namely that more than one SNP on an associated haplotype might influence disease development. So far, almost all studies have stopped when a SNP was found with a proven functional effect. We have shown that even when such a SNP has been found, there may be more SNPs involved in the disease development. This means that polygenic diseases are even more complex than originally thought.

## Acknowledgements

We thank Jackie Senior for editing the manuscript. We thank Prof.Dr. Isabella Ceccherini (Laboratorio di Genetica Molecolare, Istituto G Gaslini, Genoa, Italy) for providing us with the pGL3-RETpromoter plasmid, Dr. Sabourin (Department of Cellular and Molecular Medicine, University of Ottawa, Canada) for providing the pCDNA3-NXF (rat) and pCDNA1-ARNT2 (rat) expression constructs, and Dr. Cheng Xu (Department of Embryology, Carnegie Institution of Washington, Baltimore, USA) for providing the pCDNA3-SIM2 (mouse) expressing construct.

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## SUPPLEMENTARY DATA

**Supplementary Table 1 - Primers used for PCR, length of product and construct name**

Enhancer containing HSCR-associated variant	Primers	Product length (bp)	Constructs
Enh1-C and Enh1-T	F : 5'-TAGTGGTACCTTGACATGGACATGATCTG -3' R: 5'-TAGTCTCGAGTGCTTCCGGCTTCTATGCAA -3'	300	RET <sub>prom</sub> +Enh1-C And RET <sub>prom</sub> +Enh1-T
Enh2-C and Enh2-A	F : 5'-TAGTGGTACCGCCAGTGAGGCTGGTGATTA-3' R: 5'-TAGTCTCGAGGATCCCTGCACGCACGAACA-3'	490	RET <sub>prom</sub> +Enh2-C And RET <sub>prom</sub> +Enh2-A
Enh1-C/Enh2-C and Enh1-T/Enh2-A	F: 5'-GTTGGGTACCGCCAGGGCCAGTGAAC-3' R: 5'-TGGGCTCGAGGGCAGCCCAAGGCTAC-3'	400	RET <sub>prom</sub> +Enh1-C/ Enh2-C And RET <sub>prom</sub> +Enh1-T/ Enh2-A

**Supplementary Table 2 - Primers used for site-directed-mutagenesis**

Construct used for template	Primers	Constructs
Enh1-C/Enh2-C	F: 5'-CAGTGACCCTTACATGGTCATCCACAGGCCACTTG -3' R: 5'-CAAGTGGCCTGTGGATGACCATGTAAGGGTCACTG -3'	RET <sub>prom</sub> +Enh1-T/Enh2-C
Enh1-C/Enh2-C	F: 5'-CTGGTCTGGGTATGGAAGTGTGGGTGGCCATCG-3' R: 5'-CGATGGCCACCCACACTTCCATACCCAGACCGAG -3'	RET <sub>prom</sub> +Enh1-C/Enh2-A

**Supplementary Table 3 - Antibodies used in Westernblot**

	Antibodies	Host	Dilution	Supplier
<b>Primary</b>	RET	Goat	1 : 500	R&D system, MN, USA
	β-Actin	mouse	1 : 10.000	MP Biomedical, Irvine, CA, USA
<b>Secondary</b>	Anti-goat IgG-HRP	Rat	1 : 3000	MP Biomedical, Irvine, CA, USA
	Anti-mouse IgG-HRP	Goat	1 : 5000	Bio-Rad

**Supplementary Table 4 - Primers used for RT-PCR amplification and product length**

Gene	Primers	Product length	Tm= °C
RET	F : 5'- CCACTGGGCCTCTATTTCTC -3' R: 5'- TGCCTCCTTCCGCTTAAAC -3'	799	50
NXF	F : 5'- TTGACCCTGCTGACCATCTC -3' R: 5'- GTCTGGGAAGGTAGCACTGCTG -3'	505	59.3
SIM2	F: 5'- TCACCTGCTCCAAGAGTACGA -3' R: 5'- TGGCTGACATCCAGTTTGTC -3'	765	50
ARNT2	F: 5'-ACCAGCAGGAATGACCATAC -3' R : 5'-CTTGCCCTTTCAGCTTAACC-3'	317	50
β-ACTIN	F : 5'-ATATCGCTGCGCTGGTCGTC -3' R : 5'-AGGATGGCGTGAGGGAGAGC -3'	517	50

**Supplementary Table 5 - Antibodies used in Immunohistochemistry**

	Antibodies	Host	Dilution	Supplier
<b>Primary</b>	RET	Goat	1 : 50	R&D system, MN, USA
	SIM2a	Goat	1 : 200	MP Biomedical, Irvine, CA, USA
	ARNT2	Rabbit	1 : 50	Santa Cruz
	p75 <sup>NTR</sup>	Rabbit	1 : 250	Promega, Mannheim, Germany
<b>Secondary</b>	Anti-Goat IgG Alexa 488	Donkey	1 : 500	Invitrogen, Carlsbad, CA, USA
	Anti-Rabbit IgG Alexa 488	Donkey	1 : 500	Invitrogen, Carlsbad, CA, USA
	Anti-Goat-IgG CY3	Donkey	1 : 500	Invitrogen, Carlsbad, CA, USA
	Anti-Rabbit IgG CY3	Donkey	1 : 500	Invitrogen, Carlsbad, CA, USA

**Supplementary Table 6 - Oligonucleotides containing Enh-2C and Enh-2A used for EMSA**

Oligonucleotides containing	Sequences
<b>Enh2-C</b>	Sense : 5'- GGTCTGGGTATGGACGTGTGGGTGGTGGCC -3' Anti-sense : 5'-GGCCACCACCCACACGTCCATACCCAGACC-3'
<b>Enh2-A</b>	Sense : 5'- GGTCTGGGTATGGAAGTGTGGGTGGTGGCC -3' Anti-sense : 5'- GGCCACCACCCACACTTCCATACCCAGACC -3'

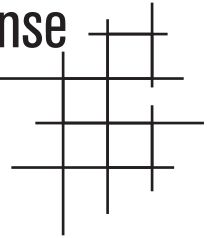




# 5

## RET Mediated Gene Expression in ENS Precursors shows Involvement of RET in Innate Immune Response

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**Submitted**

## ABSTRACT

Signaling of the RET receptor is crucial for the migration, proliferation, differentiation and survival of neural crest cells (ENCCs) that form the enteric nervous system (ENS). Disturbances in RET signaling are associated with ENS defects, as is seen in patients with Hirschsprung disease.

To gain new insight into the pathways involved in or triggered by RET in ENCCs, we performed gene expression profiling with RNA isolated from GDNF- (the RET ligand) stimulated and non-stimulated mouse ENCCs. Microarray data on ENCCs was compared with microarray data generated from mouse embryonic gut E14.5 and analyzed by gene set enrichment analysis (GSEA) and single-gene analysis methods. Pathways that were up-regulated upon GDNF stimulation included those for ATP- and biosynthesis while those significantly down-regulated upon GDNF stimulation were several known development-dependent pathways, such as the Notch, Tgf- $\beta$ /Bmp and Wnt signaling pathways. Moreover, we saw that the Toll-like receptor (TLRs) signaling pathway, important for apoptosis and the innate immune response, was down-regulated upon GDNF stimulation. This is interesting as Hirschsprung patients often suffer from inflammation of the colon and small intestine (enterocolitis). By qPCR we were able to confirm down-regulation of *Tlr2*, *Tlr4* and *Tlr5* and downstream effectors of the TLRs, such as *Tnf $\alpha$*  and *Il-6*, pointing toward the involvement of Ret signaling in the immuno-modulatory capacity of ENCCs.

In conclusion, GDNF stimulation promotes proliferation of ENCCs rather than differentiation. RET seems to be involved in regulating the innate immune response of ENCCs via the TLRs pathway which is possibly linking RET to enterocolitis.

## INTRODUCTION

The *RET* proto-oncogene encodes a transmembrane glycoprotein belonging to the receptor tyrosine kinase (RTKs) family. During vertebrate embryogenesis, *RET* is expressed in motor and catecholaminergic neurons of the central nervous system (CNS), in the developing excretory system and in all lineages of the peripheral nervous system (PNS), including neural crest cells (ENCCs). ENCCs originating from the vagal region of the hindbrain contribute to the vast majority of parasympathetic neurons and glial cells of the enteric nervous system (ENS).<sup>1</sup> In mouse, vagal neural crest cells migrate in a rostrocaudal direction to the foregut at E9.5 and reach the hindgut at E15.5, while sacral crest cells migrate in the opposite direction at E13.5.<sup>1-3</sup> In humans, ENCCs start to invade the foregut at week 4 and reach the terminal hindgut at week 7 of gestation.<sup>4</sup> This process of proliferation and migration of ENCCs into the whole gastrointestinal (GI) tract, followed by differentiation into ENS neurons and glial cells, is very complex. The timing and harmonious action of transcription factors, growth factor, their receptors and the signaling effectors is essential for each stage of development of the ENS.<sup>5,6</sup> The RET, Retinoic Acid (RA), WNT, NOTCH, BMP and FGF signaling pathways are some of the pathways that are known to play important roles in ENCCs development in the gut.

Defects in proper migration, proliferation or differentiation of ENS progenitors can lead to the development of Hirschsprung disease (HSCR), the most common ENS-related disorder. It is characterized by the absence of neurons and glial cells in the myenteric and submucosal plexus of the enteric nervous system and is mostly limited to the most distal part of the colon. The inheritance of HSCR is complex: the disease can be transmitted as a dominant trait, or as a recessive trait, but in the majority of cases it is believed to be polygenic. There are clear differences in the sex ratio of patients, with a male predominance in short-Hirschsprung disease (S-HSCR) (4:1). The disease has an incomplete penetrance, variable expression and is associated with a large number of syndromes and congenital malformations.<sup>7</sup> Over the last 15 years, at least 12 genes that play an important role in the development of HSCR disease have been identified. The *RET* gene is considered to be the major disease risk factor as coding sequence mutations are identified in up to 50% of familial cases and in 15-35% of sporadic HSCR cases.<sup>8</sup> In fact, the RET signaling pathway is believed to be one of the most important signaling cascades in regulating proper migration, proliferation, differentiation and survival of ENCCs during ENS development.<sup>1,9</sup> This is corroborated by several animal studies as mice deficient for the Ret receptor but also for the Ret co-receptor *Gfra1*, or the Ret ligand *Gdnf*, all show severe defects in enteric innervation.<sup>10-13</sup>

Little is known about the signaling cascades triggered by RET in ENCCs or the actual



downstream effectors of RET signaling in these cells. We therefore analyzed the RET network and its downstream effectors in ENCCs in detail. ENCCs were isolated from E14.5 mouse embryonic gut and cultured in the presence or absence of GDNF followed by expression profiling. We also performed gene expression profiling of mouse gut (E14.5). This study has identified genes and signaling pathways that are triggered by the RET signaling pathway in ENCCs and that can further contribute to our understanding of RET signaling pathway and its role in HSCR development.

## **MATERIALS AND METHODS**

### ***Animals***

Transgenic mice carrying Wnt1-Cre-recombinase and a LoxP/R26-YFP site were obtained from the Jackson Laboratory, California, USA, and maintained on a C57BL/6 background. The age of the embryos was set at E0.5 on the day when the vaginal plug was seen. Mouse embryos carrying the transgenes produce green fluorescent ENCCs, and are easily identified by the green cranial face and green gut under a fluorescence microscope (Figure 1B).

### ***ENCCs isolation, in vitro culturing and GDNF stimulation***

Pregnant female transgenic mice were sacrificed at stage E14.5. The embryos were removed and transferred into cold PBS solution. Under the microscope, only the fluorescent embryos indicating transgene expression were selected. We then carefully dissected the gut (foregut to hindgut) and transferred these into cold PBS. The embryonic gut from a single embryo was dissected and treated with 1 ml of collagenase/dispase enzyme solution (Sigma Aldrich, Saint Louis, USA) as described before.<sup>14</sup> The resulting cell suspension was diluted in 1 ml of DMEM/F-12 medium supplemented by 1% N2 (Invitrogen, Carlsbad, CA, USA), 2% B27 (Invitrogen), 20ng/ml FGF (Peprotech EC, London, UK), 20 ng/ml EGF (Peprotech EC) and 1% Penicillin-Streptomycin (Invitrogen). The cell suspension was filtered using sterile cell strainers with 40  $\mu$ m nylon mesh (Becton Dickinson, Oxford, UK). Single YFP-ENCCs were sorted via a MoFlow cell sorter (Beckman Coulter, London, UK) and directly seeded into a 24-wells plate coated with fibronectin (2  $\mu$ g/cm<sup>2</sup>, Sigma Aldrich) filled with 0.5 ml of culture medium. YFP-ENCCs cells from one gut were divided into two wells of 24-wells plate. One well was filled with normal medium and one with medium containing GDNF (50ng/ml). The plates were incubated at 37oC for 14 days in a humidified atmosphere with 5% CO<sub>2</sub>. Half of the medium was changed every two days and fresh GDNF (only for GDNF treated cells),

FGF and EGF were added. Small neurosphere-like bodies (NBLs) appeared after 3-5 days and were kept in culture for 14 days without splitting.

### ***RNA isolation and gene expression profiling***

Cells were washed twice with 1 ml of RNase free 1X PBS. RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacture's instruction. The RNA yield, purity and integrity were determined using the Agilent 2100 BioAnalyzer with 6000 Nano Chips (Agilent Technologies, Amsterdam, the Netherlands). Custom DNA microarray experiments and RNA quality controls were performed by ServiceXS (Leiden, the Netherlands) using the GENEChip Mouse Genome 430 2.0 arrays of Affymetrix. These array experiments were performed on RNA isolated from nine independent ENCCs cultures, untreated and treated with GDNF (in total 18 samples) and eight arrays were hybridized with RNA isolated from the whole gut of embryonic mice, C57BL/6 wild-type E14.5.

### ***Data analysis***

Student's *t*-tests, with Bonferroni multiple testing corrections ( $p < 0.05$ ) were used for the single gene analysis. Gene Set Enrichment Analysis (GSEA) was performed using GSEA 2.0 (Broad Institute, Cambridge, MA, USA).<sup>15, 16</sup> In our analysis, we assembled genes sets from well-accepted and widely used curated pathway databases, such as the KEGG and TRANSFAC.<sup>17, 18</sup> We used a nominal *p*-value  $< 0.05$  and a false discovery rate (FDR)  $< 0.10$  to define sets of genes that were significantly differentially expressed between two conditions (with or without GDNF).

### ***ENCCs GDNF activation and immunomodulatory capacity***

ENCCs ( $3-4 \times 10^4$  cells/well) were seeded into 24-well plates. After one night of culturing, GDNF 50 ng/ml of medium (Peprotech EC) was added and incubated for 48 hours, untreated cells served as control. Supernatants were collected and secreted protein levels of Tnfa and Il-6 were measured by mouse Tnfa and Il-6 ELISAs LEGEND MAX™ (Biolegend, San Diego, USA). ELISAs were performed on three independent experiments in triplicate ( $n = 9$ ). The cells from which we used the supernatant for the ELISAs were also used to isolate RNA for a qRT-PCR experiment (see below). For this, cells were washed with 1X PBS and RNA was isolated using the GeneJet™ RNA purification kit (Fermentas, St. Leon-Rot, Germany) and cDNA was synthesized by RevertAid™ H Minus First Strand cDNA Synthesis (Fermentas).

## **qRT-PCR**

RNA levels of *Tlr2*, *Tlr4*, *Tlr5*, *Tnf $\alpha$* , *Il-6*, *Il1- $\beta$* , *p50* (*Nf- $\kappa$ b component*) and *Ifn $\gamma$*  were quantified by real-time PCR using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Primers were designed to cross exon-exon boundaries for either the forward or the reverse primer. The length of the amplicons designed were around 51-209 bp and the annealing temperature was always 60°C. The primers we used are listed in Supplementary Table 1. qRT-PCRs were performed on three independent experiments in triplicate (n=9) with iTaq™SYBR®Green Supermix ROX (Bio-Rad, Veenendaal, the Netherlands). For data normalization we used the expression data of the house-keeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Data were analyzed using the method described by Livak.<sup>19</sup>

## **Immunohistochemistry**

Immunostaining was performed on cryosections (12  $\mu$ m) of mouse embryonic gut E14.5. Cryosections of mouse embryonic gut were either fixed in 4% PFA (Sox10), ice-cold acetone (Ret) or 70% ethanol/glycine (Gfap) for 10 minutes at respective temperatures. When acetone was used for fixation, sections were dried for a further 4 hours. All slides were rinsed three times in PBS before applying a blocking solution (PBS containing 10% donkey serum, 0.1% Triton X-100) for 30 minutes at room temperature. For Sox10 staining, sections were additionally pretreated by boiling in citrate buffer (10mM, pH 6.0; Sigma) for 10 minutes. Primary antibodies, anti-mouse Ret, Sox10, Gfap, Tlr2, Tlr4 and Tlr5 were applied and visualized with the respective fluorochrome-conjugated secondary antibodies (see Supplementary Table 2). Cell nuclei were stained with 4'-6-diamidino-2-phenylindole [DAPI] solution (Invitrogen; 100 ng/ml) and cover-slipped with Kaiser's gelatine (Merck, Darmstadt, Germany). The images were captured on a Nikon inverted fluorescence microscope (Nikon, Dusseldorf, Germany).

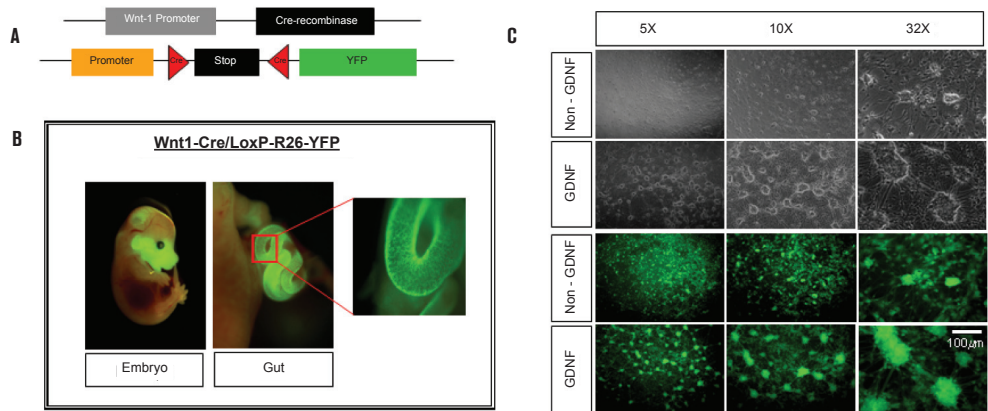
## **Statistical analysis of qRT-PCR**

Statistical analysis of the qRT-PCR results was performed using the Student's *t*-test. The results represent the means  $\pm$  standard deviation (SD). Statistical analysis was performed by GraphPad Prism 4. P values <0.05 and <0.005 (one-tailed distribution) were considered significant.

# RESULTS

## ENCCs isolation

The applied cell culture protocol resulted in almost pure ENCCs cultures as confirmed by fluorescence microscopy (see Figure 1C). After treatment with GDNF, we noticed that neurosphere-like bodies (NLBs) were larger in size (diameter: 100  $\mu\text{m}$ ) than the untreated control culture (diameter: 30-50  $\mu\text{m}$ ) (Figure 1C).

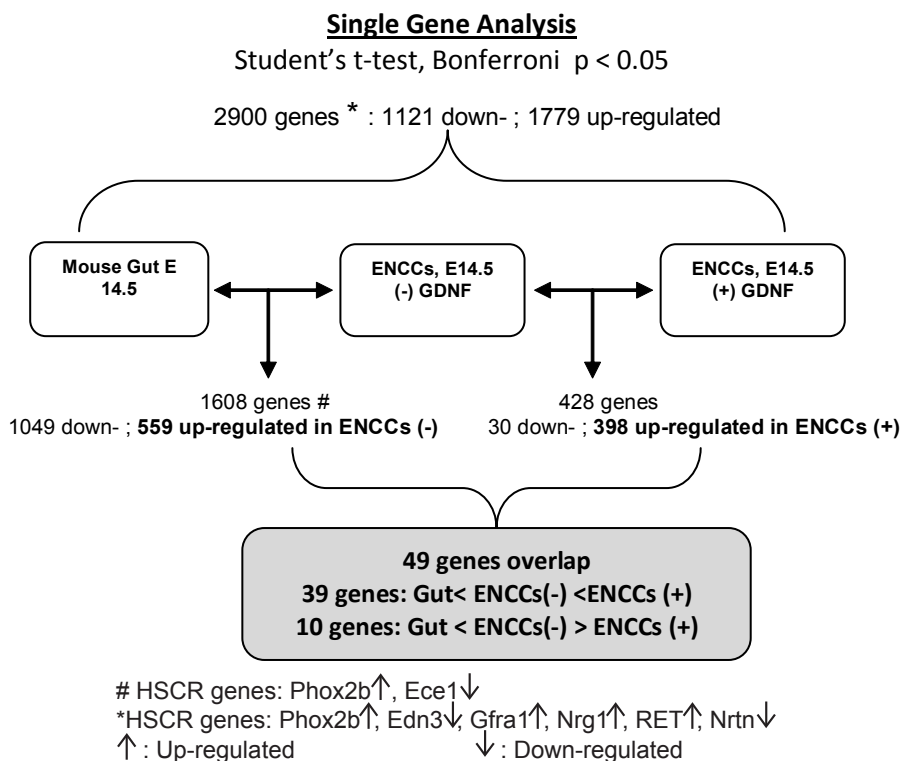


**Figure 1** – A) Schematic overview of the *Wnt1-Cre-recombinase* and *Loxp-YFP* cassettes. B) *Wnt1-Cre/Loxp-YFP* embryonic mouse E14.5 showing YFP-positive cells in the neural crest-derived craniofacial structures, and in the gut. C) ENCCs after 14 days of culture, with and without GDNF treatment (5X, 10X and 32X magnification).

## Expression array analysis

### *Single Gene Analysis (Student's t-test, Bonferroni $p < 0.05$ )*

Microarray data of gene expression profiling obtained from mouse embryonic gut, ENCCs untreated and treated with GDNF were used for single gene analysis using the Student's *t*-test with Bonferroni multiple correction testing  $p < 0.05$ . A schematic overview of the experiments and of the single gene analysis is given in Figure 2. The data collected from single gene analysis can be divided into five groups. The first group consists of genes that are significantly differentially expressed in ENCCs treated with GDNF versus those untreated. From the 428 genes in this list (see Supplementary Table 3), the top 25 are presented in Table 1. Genes that up-regulated (398 genes) were those important for the GTP metabolic process, for GTPase activity, cell-cell signaling, synaptic vesicle transport, neuron projection and genes important for neurogenesis, such as *Spock2*, *Vgf*, *Cend1* and *Dner* (Delta/Notch-like EGF-related Receptor).



**Figure 2** – Schematic overview of the experiments performed and the outcome of the single gene analysis

The second group consists of genes that are expressed significantly different in ENCCs compared to the gut. We identified 1608 genes belong to this group, among these were two known HSCR genes *Phox2b* and *Ece1* that were up-regulated and down-regulated in ENCCs compared to gut, respectively (see Table 2). There were 559 genes that up-regulated in ENCCs compared to gut, the top 25 genes are presented in Table 3. Among these genes are *Sox2* and *Gfra2*.

The third group consists of genes that are up-regulated in ENCCs compared to the gut and further up-regulated in ENCCs upon GDNF treatment. There are 39 genes belonging to this group (see Supplementary Table 4). Among these genes are *Scg10* (*Stmn2*) that has recently been proposed as a candidate gene for HSCR<sup>20,21</sup> and *Snca* ( $\alpha$ -synuclein) a gene known to be involved in Parkinson's disease.<sup>22</sup>

The fourth group consist of genes that are up-regulated in ENCCs compared to the gut, but down-regulated in ENCCs upon GDNF treatment. Only 10 such genes belong to this group (see Supplementary Table 5), including *Rora* (Retinoic acid receptor-related orphan receptor alpha), a transcription factor important for cell survival and differentiation of Purkinje cells.<sup>23</sup>

The fifth group consists of the genes that were expressed significantly different in ENCCs treated with GDNF compared to the gut. There were 2900 genes belong to this group. Among these were the known HSCR genes: *Ret*, *Phox2b*, *Gfra1*, *Nrtn* and *Nrg1* were all up-regulated in ENCCs whereas *Edn3* was down-regulated in ENCCs (see Table 2).

**Table 1** - Single Gene Analysis: Top 25 genes that were expressed significantly higher in ENCCs treated with GDNF compare to untreated ENCCs (Bonferroni :  $p < 0.05$ )

No.	Gene Symbol	Gene Name	Human Chromosome
1	<i>Trnp1</i>	TMF1-regulated nuclear protein 1	1p36.11
2	<i>Spock2</i>	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	10q25.3
3	<i>Tmem59l</i>	transmembrane protein 59-like	19p12
4	<i>Aplp1</i>	amyloid beta (A4) precursor-like protein 1	19q13.12
5	<i>Mblac2</i>	metallo-beta-lactamase domain containing 2	5q14.3
6	<i>Vgf</i>	VGF nerve growth factor inducible	7q22
7	<i>Dner</i>	delta/notch-like EGF repeat containing	2q36.3
8	<i>Rlbp1l2 ( CLVS2)</i>	clavesin 2	6q22.31
9	<i>2900062L11Rik</i>	-	-
10	<i>Rnase1</i>	ribonuclease L (2',5'-oligoadenylate synthetase-dependent)	1q25
11	<i>Slc25a22</i>	solute carrier family 25 (mitochondrial carrier: glutamate), member 22	11p15.5
12	<i>A730017C20Rik</i> (no human ortholog)	-	-
13	<i>Rnf157</i>	ring finger protein 157	17q25.3
14	<i>Resp18</i>	regulated endocrine-specific protein 18	2q35
15	<i>B3galnt1</i>	beta-1,3-N-acetylgalactosaminyltransferase 1 (globoside blood group)	3q25
16	<i>Zdhhc22</i>	zinc finger, DHHC-type containing 22	14q24.3
17	<i>Cyp4x1</i>	cytochrome P450, family 4, subfamily X, polypeptide 1	1p33
18	<i>Stard7</i>	StAR-related lipid transfer (START) domain containing 7	2p11.1
19	<i>Wnk3</i>	WNK lysine deficient protein kinase 3	xp11.22
20	<i>6530401D17Rik</i> (human orth: <i>SPCS3</i> )	signal peptidase complex subunit 3 homolog ( <i>S. cerevisiae</i> )	4q34.2
21	<i>A730017C20Rik</i>	-	-
22	<i>Pmvk</i>	phosphomevalonate kinase	1q21.3
23	<i>Samd10</i>	sterile alpha motif domain containing 10	20q13.33
24	<i>Cend1</i>	cell cycle exit and neuronal differentiation 1	11p15.5
25	<i>Cmas</i>	cytidine monophosphate N-acetylneuraminic acid synthetase	12p12.1

**Table 2** - Observed expression differences of HSCR-associated genes

HSCR Gene	ENCCs/Gut	p-Value	ENCCs(+GDNF)/Gut	p-Value
<i>Ret</i>	-	-	Up-regulated	0.00025
<i>Phox2b</i>	Up-regulated	0.03796	Up-regulated	0.00375
<i>Edn3</i>	-	-	Down-regulated	0.00106
<i>Gfra1</i>	-	-	Up-regulated	0.00125
<i>Ece1</i>	Down-regulated	3.14E-08	-	-
<i>Nrg1</i>	-	-	Up-regulated	2.02E-12
<i>Nrtn</i>	-	-	Down-	0.00789

**Table 3** - Single Gene Analysis: Top 25 genes that were expressed significantly higher in ENCCs compared to the gut (Bonferroni :  $p < 0.05$ )

No.	Gene Symbol	Gene Name	Human Chromosome
1	<i>Ptpn21</i>	protein tyrosine phosphatase, non-receptor type 21	14q31
2	<i>Mdm2</i>	transformed mouse 3T3 cell double minute 2	12q13-q14
3	<i>Cdkn1a</i>	cyclin-dependent kinase inhibitor 1A	6p21.1
4	<i>Tcfap2a</i>	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	6p24.3
5	<i>(C4orf34)</i>	-	-
6	<i>Atf6</i>	activating transcription factor 6	1q22-q23
7	<i>Afap1</i>	actin filament associated protein 1	4p16
8	<i>Trim11</i>	tripartite motif-containing 11	1q42.13
9	<i>BC031353</i>	KIAA1370	15q21.2-q21.3
10	<i>Rora</i>	Retinoic acid receptor-related orphan receptor alpha	15q22.2
11	<i>Pcdh7</i>	protocadherin 7	4p15
12	<i>Tm9sf3</i>	transmembrane 9 superfamily member 3	10q24.2
13	<i>1110059G02Rik</i>	-	-
14	<i>Cpne2</i>	copine II	16q13
15	<i>Cep170</i>	centrosomal protein 170kDa	1q44
16	<i>Tmem111</i>	transmembrane protein 111	3p25.3
17	<i>Nek7</i>	NIMA (never in mitosis gene a)-related kinase 7	1q31.3
18	<i>Sox2</i>	SRY (sex determining region Y)-box 2	3q26.33
19	<i>Bnip2</i>	BCL2/adenovirus E1B 19kDa interacting protein 2	15q21.3
20	<i>Casp12</i>	caspase 12 (gene/pseudogene)	11q22.3
21	<i>Ndst1</i>	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1	5q33.1
22	<i>App</i>	Amyloid beta (A4) precursor protein	21q21.2
23	<i>Gfra2</i>	GDNF family receptor alpha 2	8p21.3
24	<i>Lphn3</i>	latrophilin 3	4q13.1
25	<i>1110032E23Rik</i>	-	-

We identified differential expression of a number of transcription factors that are known to play an important role in embryonic and ENS development, such as transcription factors belonging to the Sox (SRY-related HMG-box), Fox (Forkhead) and Hox (homeobox) transcription factors family. We also identified differential expression of *Dlx1* and *Dlx2* which are required for coordinating and programming neurite maturation and migration<sup>24</sup> and *Etv1* that is known to play an important role in differentiation of dopaminergic neurons in *C.elegans* and mouse.<sup>25</sup> The summary of transcription factor expression differences is presented in Table 4.

**Table 4** - Transcription factors that up- and down-regulated in ENCCs (+GDNF)/ENCCs, ENCCs/Gut and ENCCs(+GDNF)/Gut

Gene	ENCCs(+GDNF) /ENCCs	p-Value	ENCCs/ Gut	p-Value	ENCCs(+GDNF) /Gut	p-Value
<i>Sox2</i>	-	-	Up-	3.30E-07	Up-	0.00280
<i>Sox4</i>	-	-	Up-	0.0134	-	-
<i>Sox6</i>	-	-	Up-	0.00247	-	-
<i>Sox17</i>	-	-	Down-	0.02739	-	-
<i>Sox18</i>	-	-	Down-	0.02430	Down-	0.00163
<i>Sox21</i>	-	-	-	-	Down-	0.00085
<i>Phox2b</i>	-	-	Up-	0.03796	Up-	0.00375
<i>Foxk2</i>	Up-	0.00514	-	-	-	-
<i>Foxf1a</i>	-	-	-	-	Down-	0.04777
<i>Foxp2</i>	-	-	-	-	Down-	0.04632
<i>Foxd3</i>	-	-	Up-	0.00092	-	-
<i>Dlx1</i>	-	-	-	-	Up-	5.87E-05
<i>Dlx2</i>	-	-	Up-	0.01264	-	-
<i>Hoxb4</i>	-	-	-	-	Up-	0.00205
<i>Hoxb5</i>	-	-	-	-	Up-	6.21E-05
<i>Hoxa9</i>	-	-	-	-	Down-	0.013288
<i>Hoxc8</i>	-	-	-	-	Down-	0.007206
<i>Etv1</i>	-	-	-	-	Up-	4.13E-07
<i>Etv5</i>	-	-	-	-	Up-	3.35E-05
<i>Ets2</i>	-	-	-	-	Down-	0.041972
<i>Cdx1</i>	-	-	Down-	0.009328	Down-	0.003229
<i>Cdx2</i>	-	-	Down-	0.003811	Down-	0.003811



## Pathways Analysis using the Gene Set Enrichment Analysis (GSEA) method

The pathway analysis we performed showed that some pathways previously implicated in ENS as well as in embryonic development, such as Tgf $\beta$ /Bmp, Notch and Wnt were down-regulated in ENCCs on GDNF stimulation (Table 5). The Notch signaling pathway for instance is known to be involved in various aspects of neurogenesis, although it is also involved in cell proliferation.<sup>26, 27</sup> Within the Notch pathway we saw down-regulation of *Notch1*, *Notch2*, *Jag1*, and the Notch co-activators *Maml1* and *Maml2*. With respect to the Wnt signaling pathway, it is known that Wnt signaling in ENCCs is important for cell lineage decision-making rather than maintaining cells in their progenitor state.<sup>28</sup> *Wnt* (2, 5B, 9A and 10B), and Wnt receptor *Fzd* (2 and 6) are some of the genes in the Wnt signaling pathway that were down-regulated by GDNF. The Tgf- $\beta$ /Bmp signaling pathway plays a role in many cellular processes including cell proliferation, apoptosis, differentiation and migration. *Tgf- $\beta$* , *Tgf $\beta$ 1*, *Smads*, *Bmps*, *Bmpr1*, *Rock1* are some of the Tgf- $\beta$ /Bmp signaling pathway genes that are down-regulated by GDNF. Rock signaling is involved in regulating neural precursor cell (NPC) migration. Knock down of *Rock1* and *Rock2* in mouse promotes NPC migration to the olfactory bulb.<sup>29</sup> With respect to *Bmps*, *Bmp2* and *Bmp44*, these proteins are known to be involved in neuronal differentiation of post-migratory enteric neural crest.<sup>30, 31</sup> *Bmp2* is also known as a stimulator of *Mash1* expression, which is an important protein for neurogenesis.<sup>32</sup> *Rock1*, together with the Semaphorins and the Plexin protein family (a Semaphorin co-receptor) are all part of the axon guidance signaling pathway. We found that this axon signaling pathway is down-regulated during GDNF stimulation, suggesting that neuronal differentiation of ENCCs is suppressed upon RET activation.

Adherens junctions, tight junctions (cell-cell adhesion) and focal adhesion (cell-matrix adhesion) play an important role in cell migration. In this study, genes that belong to these signaling pathways were down-regulated on GDNF treatment. Down-regulation could lead to loss of, or less tight, cell-cell and cell-matrix contact, a crucial process for cell migration and proliferation. *Tjp1* (tight junction 1) and *Snai2* (Slug) are genes that were down-regulated and these are members of the tight and adherens junction signaling pathways, respectively. Slug is known to play a role in ENCCs migration and homozygous deletion leads to the development of Waardenburg syndrome type 2D, an auditory-pigmentary syndrome.<sup>33</sup>

We also observed that cell cycle signaling pathways are down-regulated on GDNF stimulation. Histone deacetylases (*Hdac1* and *Hdac2*), *Mdm2*, *Cdkn1a* (*p21*), *Cdkn2b* (*p15*), *Rb1* are all on the list of cell cycle signaling genes that are down-regulated on GDNF treatment. HDACs have a role in cell growth arrest, differentiation and cell death, while *Mdm2* inhibits the G0/G1-S phase transition of human diploid cells, and over-expression of *MDM2* would induce

growth arrest in normal cells. Mdm2, p21, p15 and Rb1 are negative regulators of the cell cycle. Down-regulation of the expression of these genes will most likely reduce their growth-inhibitory function and induce cell proliferation as a consequence. Although GDNF treatment results in a down-regulation of many genes involved in the cell cycle and cell growth, there are also some down-regulated genes that play a role in cell division, such as *Ccnd* (*cyclin D*) and *Cdks* (*cyclin-dependent kinase*). The harmonization of negative and positive regulators of the cell cycle process eventually results in cell division or cell cycle arrest. In this context it is interesting to note that the ENCCs cultures with and without GDNF (Figure 1C) show a clear difference, namely that GDNF-treated ENCCs have bigger Neurosphere-Like Bodies (NLBs) than those not treated with GDNF, pointing towards the higher proliferative state of these treated cells

On the other hand, signaling pathways that play pivotal roles in apoptosis, such as the p53 and Toll-like receptors (TLrs) pathways are down-regulated on GDNF treatment. The down-regulation of genes involved in cell cycle arrest and apoptosis upon Ret activation could point toward a model in which ENCCs proliferate and survive better during ENS development.

Finally the JAK-STAT signaling pathway is known to be activated by RET.<sup>34</sup> Our microarray results, to our surprise, showed that this pathway is down-regulated on GDNF treatment. However, most of the genes that are down-regulated in this signaling pathway are ones important for cytokines and their receptors' expression. Among these are *Il-15*, *Il-12b*, *Il-6r*, *Il-28R*, *Ifn $\alpha$ 1*, and *Ifn $\gamma$ 1*. Together with the TLrs signaling pathways, it seems that GDNF activation reduces the immunomodulatory capacity of ENCCs.

**Table 5 - Pathways analysis (ENCCs\_GDNFneg Vs ENCCs\_GDNFpos)**

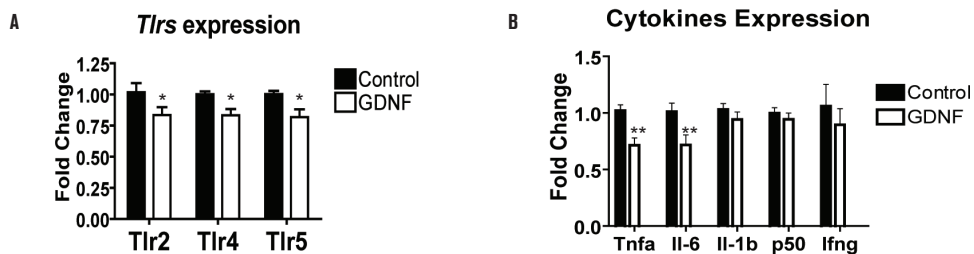
Database	Pathway	P-Value	FDR	#Genes	
<b>KEGG</b>	<u>Up-regulated in GDNF (+)</u>				
	Citrate Cycle	0.01	0.06	12	
	Oxidative phosphorylation	< 0.001	0.025	39	
	Peroxisome	0.001	0.040	19	
	Steroid Biosynthesis	0.004	0.039	6	
	Valin-Leucin-Isoleucin	0.006	0.070	11	
	PPAR	0.002	0.049	15	
	Butanoate metabolism	0.009	0.029	16	
	Propanoate metabolism	0.005	0.079	12	
	Parkinson's disease	<0.001	0.043	35	
	<u>Down-regulated in GDNF (+)</u>				
	ECM Receptor	0.005	0.075	21	
	Adherens Junction	< 0.001	< 0.001	28	
	Axon Guidance	< 0.001	0.052	44	
	Cell cycle	< 0.001	0.056	53	
	Chronic Myeloid	< 0.001	0.054	38	
	Focal Adhesion	< 0.001	0.026	68	
	JAK-STAT	0.001	0.084	56	
	Lysosome	0.003	0.089	24	
	N-Glycan Biosynthesis	0.01	0.076	17	
	Notch Signaling	< 0.001	0.001	19	
	P53 Signaling	< 0.001	< 0.001	32	
	Ribosome	< 0.001	< 0.001	58	
	Spliceosome	< 0.001	0.007	45	
	TGF- $\beta$ Signaling	< 0.001	< 0.001	30	
	Tight Junction	0.005	0.08	41	
	TOLL-Like Receptor	0.009	0.08	40	
	Wnt Signaling	0.003	0.07	42	
	Pathways in Cancer	< 0.001	0.057	95	
	Spliceosome	< 0.001	0.076	45	
	<b>TRANSFAC</b>	<u>Up-regulated in GDNF (+)</u>			
		SF1	< 0.001	0.005	41
ATF3		0.002	0.017	34	
TCF11 (MAFG)		0.006	0.082	41	
AHR-ARNT		0.007	0.016	5	
EGR3		0.02	0.04	11	
LXR		0.015	0.033	10	
NFE2		< 0.001	0.015	50	
NRSF (a.k.a REST)		< 0.001	< 0.001	23	
PAX4		0.01	0.10	30	
RFX1		< 0.001	0.017	35	
<u>Down-regulated in GDNF (+)</u>					
CDX2		< 0.001	0.008	63	
CEBP		< 0.001	0.002	69	
PPAR $\gamma$		< 0.001	0.004	20	
SOX5		< 0.001	0.008	70	
TEF1		< 0.001	0.008	66	
CI21		< 0.001	< 0.001	60	
ETS		< 0.001	0.007	70	
FOXO1		< 0.001	0.006	77	
FREAC2		< 0.001	0.007	70	
HP1		< 0.001	0.007	61	
IPF1		< 0.001	0.008	83	
NFAT	< 0.001	0.008	88		
NFYC	< 0.001	0.005	55		
PAX5	< 0.001	0.004	41		

FDR : False Discovery Rate ; # Number of genes in leading edge subset of GSEA results

## GDNF stimulation significantly reduces Tlr2, Tlr4, Tlr5, Tnf and Il-6 mRNA levels in ENCCs

Pathway analysis showed that the Tlr signaling pathway is down-regulated in ENCCs on GDNF stimulation (see Table 5). In particular, among the genes listed in the Tlr pathway, Tlr2 and Tlr5 were notably down-regulated after GDNF treatment. To confirm these data we performed qRT-PCR on *Tlr2* and *Tlr5* in ENCCs treated and untreated with GDNF. As it is known from previous studies that Tlr4 is also expressed in the myenteric plexus<sup>35,36</sup>, we included *Tlr4* in this experiment as a positive control. As activation of Tlr signaling is known to activate Nf- $\kappa$ b (p65-p50 complex) and initiate the innate immune response by inducing expression of pro-inflammatory cytokines, we also measured the mRNA level of several pro-inflammatory cytokines such as *Tnf $\alpha$* , *Ifn $\gamma$* , *Il-6* and *Il-1 $\beta$* .

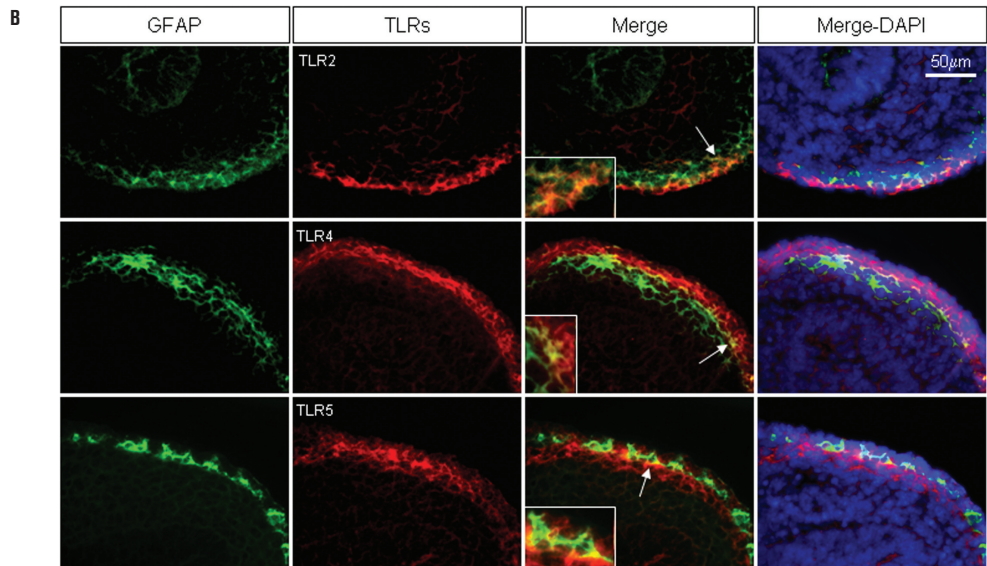
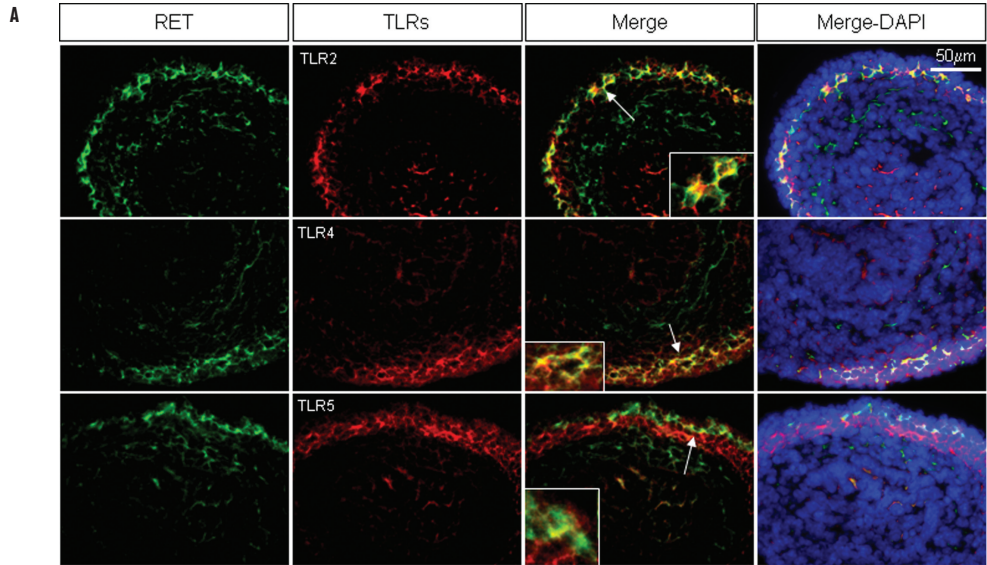
d significantly reduced in ENCCs on GDNF stimulation compared to the untreated cells. The results are presented in Figure 3A-B. *Tnf $\alpha$*  expression levels in the ENCCs were not detectable by ELISA. *Il-6*, which was detectable by ELISA, showed no significant difference in the level of expression between the control and the GDNF-treated cells.

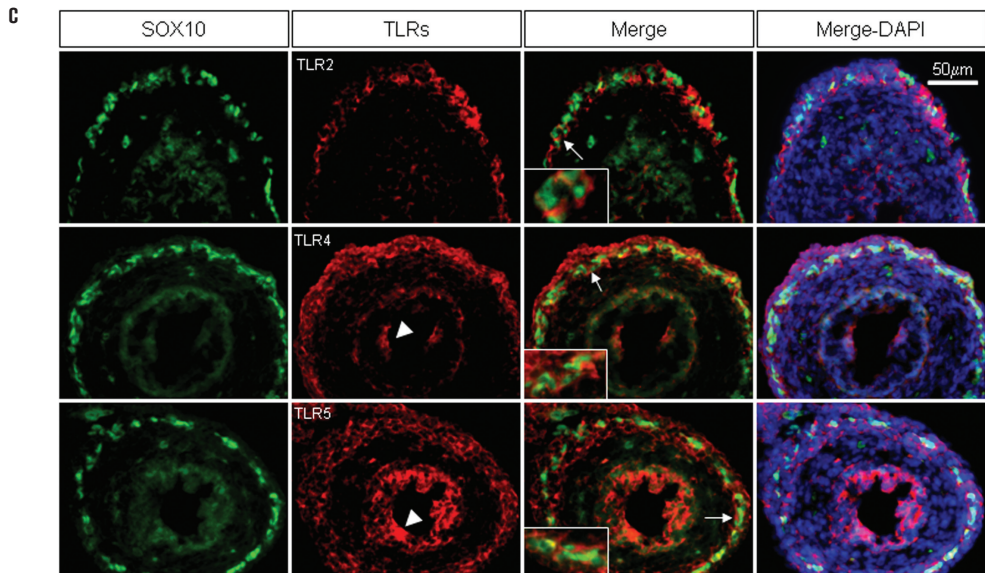


**Figure 3** - GDNF significantly reduces the mRNA level of: A) *TLR2*, *TLR4* and *TLR5* in ENCCs, B) *TNF $\alpha$*  and *Il-6* in ENCCs. The bars indicate the means  $\pm$  SD of the fold changes of the mRNA level for each gene between control and GDNF treated cells. \* $p < 0.05$  and \*\* $p < 0.005$  were considered significant (one-tailed distribution).

## Tlr2, 4 and 5 are expressed in enteric glial, neurons and ENS progenitor cells of the embryonic myenteric plexus

Tlr2, Tlr4 and Tlr5 are expressed in the myenteric plexus of mouse embryonic colon E14.5. In particular, the Tlrs could be demonstrated in the glial cells, as shown by co-staining with *Gfap*, and in neurons as shown by co-staining with *Ret*. Moreover they were already detected in the ENS progenitors as shown by co-staining with *Sox10* (Figure 4A-C). In all the stainings, Tlrs did not overlap completely with *Ret*, *Gfap* or *Sox10*. It seems that parts of the smooth muscle layer were also positively-stained for Tlrs, especially for Tlr5. This is in line with previous reports describing similar tissue expression of Tlr4.<sup>36</sup>





**Figure 4** - Tlr2, Tlr4 and Tlr5 are expressed in the myenteric plexus of embryonic mouse gut E14.5. A) Co-staining of Tlrs with Ret, B) Co-staining of Tlrs with the glial marker (Gfap), and C) Co-staining of Tlrs with ENS progenitor marker Sox10. The merged images of each Tlr with Ret, Gfap and Sox10 staining are shown in the third column of each panel. The magnifications of co-stained Tlrs are shown in the respective insets. Tlr4 and Tlr5 are also expressed on colon epithelial cells as shown by arrowheads in Figure C.

## DISCUSSION

Several genes have been implicated in HSCR development, including *RET*, *GDNF*, *EDNRB*, *EDN3*, *SOX10*, *NTN*, *ECE1*, *ZFXH1B*, *KBP*, *TTF1* and *NRG1*. The majority of these genes can be divided into three groups: the ones involved in the RET pathways (*RET*, *GDNF*, *NTN*), the ones involved in EDNRB pathways (*EDNRB*, *EDN3*, *ECE-1*), and transcription factors that can affect both RET and/or EDNRB pathways (*SOX10*, *ZFXH1B*, *PHOX2B*). Increasing evidence has shown that the proteins encoded by these genes are interconnected. A genome-wide association study, using 43 trios from a genetically isolated Mennonite population, reported statistically significant joint transmission of RET and EDNRB alleles.<sup>37</sup> Furthermore, mice homozygous for the recessive hypomorphic allele of *Ednrb* (*Ednrb<sup>S</sup>* or *Piebald*) and heterozygous for a *Ret* null mutation (*Ret<sup>+/-</sup>*) showed high frequencies of aganglionosis. These findings are suggestive of epistasis between EDNRB and RET and this idea was further supported by *Ret/Ednrb* mouse crosses.<sup>38</sup> Similar studies showed that, whereas *Ret<sup>51/51</sup>* and *Edn3<sup>ls/ls</sup>* (lethal



spotting) mice display colonic aganglionosis, combinations of these mutant alleles lead to almost complete intestinal aganglionosis.<sup>39</sup>

Furthermore, the transcription factor SOX10 is involved in the regulation of both *RET* and *EDNRB* expression.<sup>40-42</sup> PHOX2B is also a transcription factor that is expressed in several classes of differentiating neurons of both the peripheral and central nervous systems. Mice with a homozygous disruption of *Phox2b* show lack of enteric ganglia and no Ret expression which suggests that Phox2b might play a regulatory role in *Ret* expression.<sup>43</sup> All these data indicate a central role for RET in the development of both HSCR and ENS. It was these findings that encouraged us to undertake this study. By determining the *RET*-associated gene networks in ENS progenitors we hoped to gain insight not only into ENS development but also a better understanding of how HSCR might develop, eventually leading to HSCR candidate genes.

To gain such insight we performed gene expression profiling studies on RNA isolated from mouse ENCCs treated and untreated with GDNF, the ligand for the RET receptor, and compared these data to each other and to the expression profile from the whole mouse embryonic gut (all E14.5). The data were used to perform pathway analysis using GSEA and for a single gene analysis by a Student's *t*-test.

## Single Gene Analysis

With our single gene analysis we identified a large number of genes that were differentially expressed in ENCCs upon GDNF treatment. Moreover, the gene expression profiles from the mouse gut helped us to identify ENCC-specific genes. The number of genes we identified, as differentially expressed in these experiments was very high, even after multiple testing correction. The reason we did identify so many significantly differentially expressed genes is mainly due to the large number of replicates we analysed.

The data generated gives new insights in which genes are important for ENS, development, hence important for HSCR development. By comparing genes profile of ENCCs to that of mouse embryonic gut, we identified two genes that directly can be linked to RET namely, Phox2b which acts as a transcription factor for Ret,<sup>43, 44</sup> and Gfra2 that is a known co-receptor of Ret.<sup>45</sup> Moreover, we also identified Sox2 which in a recent study proved to be an excellent ENS progenitor cell marker.<sup>46</sup> Genes belonging to this group, i.e genes expressed in ENCCs but not in the gut, might therefore be considered candidate ENS progenitor markers. Among the down-regulated genes, the known HSCR gene, *Ece1*, was identified. Furthermore, by comparing genes profile of ENCCs treated with GDNF to that of the mouse embryonic gut, we identified six of 12 known-HSCR genes (see Table 2).

We, also identified genes that were up-regulated in ENCCs compared to those in the gut and further up-regulated or down-regulated upon GDNF treatment (see Supplementary Table 4 and 5). It suggests that these genes are ENCCs specific and are downstream effectors of Ret. *Snca* ( $\alpha$ -synuclein) was among the genes that were up-regulated (higher in NCC when compared to the gut and further up-regulated upon GDNF treatment). This suggests that defects in RET signaling will most likely reduce the expression of  $\alpha$ -synuclein. Interestingly, in Parkinson's patients, the presence of Lewy bodies (aggregation of  $\alpha$ -synuclein) in their gut is associated with chronic constipation. These findings suggest that  $\alpha$ -synuclein might play an important role in ENS functioning.<sup>47, 48</sup>

Several comparable expression profiling studies, all aiming at a better understanding of ENS and HSCR development and to find new candidate HSCR genes, have been performed. Different study designs have been used: Heanue and colleagues compared gene expression of mouse embryonic gut from wild type (WT) mice with those of *Ret* knock-out mice (*Ret*<sup>-/-</sup>)<sup>49</sup>; Vohra and colleagues performed a study with a design comparable to Heanue et al. However, they analysed mouse intestines of two different developmental stages (E14 and P0)<sup>50</sup>; Iwashita and colleagues compared gene expression profiles of neural crest stem cells (NCSCs) with the profiles made of total mouse embryos while Ngan and colleagues compared gene expression profiles of ENCCs isolated from mice WT with those from ENCCs isolated from *Ret* mutant mice (E11.5). These ENCCs were treated and untreated with GDNF at two different time-points (8 and 16 hours) before RNA isolation.<sup>51, 52</sup> Each study has its own advantages and disadvantages. The use of knock-out mice (Heanue) clearly has an advantage as also the influence of other endogenous RET ligands can be measured. However using gut instead of ENCCs might be considered a drawback as the ENS progenitor cells are less than 5% of the total cell population in the mouse embryonic gut. The use of NCSCs without culturing as described by Iwashita has an advantage over cultured ENCCs (Ngan et al. and our study) as it omits the potential of *in vitro* influences on gene expression. However to study the RET-GDNF genes network in ENCCs (or NCSCs) without GDNF treatment might not cover completely the dynamic changes of RET-dependent genes expression. Furthermore, the use of expression profiles from gut (our study) compared to those from total embryos (Iwashita) for instance might have some advantages as differences in expression of genes in non-ENCCs of the gut might be identified as well. The study by Ngan et al. was very similar to our study, although they did not compare the ENCCs profiles with the expression profile of the gut or total embryo. Because of this it is difficult to identify ENCCs specific genes. In our study we could detect six of the 12 known-HSCR genes by comparing the gene expression profiles of ENCCs (+GDNF) to the expression profiles of the gut, whereas ENCCs treated versus un-



treated did not detect any of these 12 known-HSCR genes. This shows that this comparison is very informative. The strength of our study is the use almost pure ENCCs isolated from mice carrying Wnt1-Cre-recombinase and a LoXP/R26-YFP, and the fact that we used large numbers of replicates. The last resulted in, even after Bonferroni multiple testing corrections ( $p < 0.05$ ), large numbers of significantly down or up regulated genes.

When comparing our results with those generated by the other studies we saw that 70% of genes (33/47) that were thoroughly examined by Heannue et al. (expression in the ENS by *in situ* hybridization) were present in our data set. Among these are *Tgfb2*, *Mab21l1*, *Sncg*, *Etv1*, *L1cam* and *Fgf13*. From the 38 genes thoroughly examined by Vohra et al (expression in the ENS by *in situ* hybridization) 52% of genes (20/38) proved to be present in our gene set. Among these genes are *Elavl4*, *Ncam2*, *Hoxb5*, *Ndr4*, *Stmn3* and *Dlx2*. The comparison with the study of Iwashita et al. we saw 27% of genes (29/107 annotated genes) were present in our data. Among these are *Ret*, *Gfra1*, *Gfra2*, *Emb* and *Syng1*. The comparison with the study of Ngan et al., we saw 58.6% of genes (17/29) were present in our data. Among these are *Mapk8ip2*, *Tesc*, *Kcnd2* and *Vip*.

These comparisons show considerable overlap between the studies. We did not find major differences between these 4 studies and our study. The differences found, different gene sets, most likely are the result of the different study designs used.

## Pathway analysis

GSE analysis based on the data of the KEGG database showed that upon GDNF stimulation, several sets of genes were significantly up- or down-regulated in the ENCCs (results shown in Table 5, see also supplementary Figure 1A). The Pathways that were up-regulated were those which are important for energy supply (citrate cycle and oxidative phosphorylation), for steroid and valin-leucin-isoleucin biosynthesis and lipid metabolism (peroxisome and peroxisome proliferators-activated receptors/ PPARs). Signaling pathways that are considered important for ENS development such as the Notch, Tgf $\beta$ /Bmp and Wnt signaling were all significantly down-regulated upon GDNF stimulation. Sets of genes important for cell-cell and cell-matrix contacts (e.g. adherens junctions, tight junction and focal adhesion formation), cell cycle arrest, apoptosis (p53, Toll-like receptor signaling) and innate immune system signaling (Toll-like receptor signaling) were also down-regulated. We summarize the possible biological function of the signaling pathways mentioned above in the supplementary Figure 1B.

Pathway analysis based on the TRANSFAC database showed similar results to those collected from the KEGG database. Genes under the regulation of transcription factors that are

important for determining cell differentiation, such as Sox5 and Foxo1, are down-regulated by GDNF, while genes under the regulation of Egr3, a transcription factor that is important for cell growth and cell migration, are up-regulated on GDNF treatment. The same goes for Rest, a transcription repressor which is highly expressed in the neuronal progenitor cells and which is known as a negative master regulator of neurogenesis.<sup>53, 54</sup>

## TLRs signaling pathway

TLRs recognize viral and bacterial infections and induce an innate immune response by activating pro-inflammatory cytokines such as TNF $\alpha$ . Furthermore, several studies have shown that TLRs can also induce apoptosis through the activation of Caspases (as reviewed by B. Salaun).<sup>55</sup>

Recently it was shown that TLR4 is expressed in neurons of the myenteric plexus of the human intestine.<sup>36</sup> Tlr3 and Tlr7 are expressed in the ENS and in the dorsal root ganglia (DRG).<sup>35</sup> Our microarray data and our immunostainings show that Tlr2, Tlr4 and Tlr5 are expressed in ENCCs and in the ENS (the progenitors, neurons and glial cells, see Figure 4A-C). The expression of Tlrs in the ENS precursor cells could indicate that these cells play a role in the immunomodulatory mechanism against viral and bacterial infection. This might seem illogical as in the normal situation the ENS is not directly in contact with the luminal contents. However, bacteria and viruses can cross the epithelial barrier and reach the deeper layers of cells, such as the ENS, and thereby induce an immune response in these cells.<sup>56, 57</sup> Moreover, we show that all three Tlrs were down-regulated upon GDNF stimulation, implying that these particular *Tlrs* genes are down-stream effectors of Ret signaling. Based on these data, we hypothesize that RET-GDNF activation will reduce the expression of TLRs and, consequently, will also reduce the expression of cytokines. Therefore, defects in RET signaling, as often seen in HSCR patients, will result in higher expression levels of TLRs, which subsequently lead to an overexpression of cytokines. This condition might well contribute to activating the innate immune response system, which might manifest itself as HSCR-associated enterocolitis. This idea is corroborated by an animal study which demonstrated that mRNA levels of *Tlr2* and *Tlr4* are increased in the intestinal epithelial cells (IEC) of enterocolitis rat.<sup>58</sup> Furthermore, it has also been shown that TLR4 expression is elevated in the intestinal mucosa of humans and that TLR4 plays a critical role in the development of necrotizing enterocolitis.<sup>59</sup>

In summary, there seems to be a general trend in our data that genes encoding proteins that stimulate cell differentiation or that are negative regulators of cell proliferation, and genes that inhibit cell-cell adhesion and apoptosis were generally down-regulated in neural crest cells upon treatment with GDNF. On the other hand, genes that encode proteins that have the

opposite effect, such as genes for an early growth response, those involved in the inhibition of cell differentiation, and those that are important for energy supply and biosynthesis, were up-regulated. Furthermore, in the ENCCs we saw a connection between Ret signaling and the innate immune response, as stimulation of Ret resulted in down-regulation of several TIRs and their pro-inflammatory cytokines. These data provide new insight into the gene networks activated upon RET stimulation by GDNF in ENCCs. They also will serve as a catalog of genes possibly involved in HSCR. This might will prove extremely helpful once we start sequencing exomes or even genomes of HSCR patients and revealing enormous numbers of DNA variants, most of which will not in fact play a role in HSCR.

## Acknowledgements

We thank Jackie Senior for editing the manuscript. This study was supported by grant from Dutch Digestive Foundation (MLDS project WO 09-62).

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## SUPPLEMENTARY DATA

**Supplementary Table 1 - Primers used for qPCR amplification and product length**

Gene	Primers	Product length
TLR2	F: 5' CTCAGAGGATGCTACGAGCTC 3' R: 5' GAATAGAGGTGAAAGACCTGGAGC 3'	147
TLR4	F: 5' GAATCCCTGCATAGAGGTAGTTCC 3' R: 5' TGATCCATGCATTGGTAGGTAATATTA 3'	51
TLR5	F: 5' TCAGCAGGATCATGGCATGTC 3' R: 5' TGAAGATCACACCTATGAGCAAGTCA 3'	51
TNF $\alpha$	F: 5' GACGTGGAAGTGGCAGAAGA 3' R: 5' GCCACAAGCAGGAATGAGAA 3'	101
Il-6	F: 5' CCTCTCTGCAAGAGACTTCCATCCA 3' R: 5' GGCCGTGGTTGTCACCAGCA 3'	64
Il-1b	F: 5' GGCAGGCAGTACTCATT 3' R: 5' AAGGTGCTCATGTCCTCATC 3'	94
P50	F: 5' CATGGTGGTTGGCTTTGCA 3' R: 5' ACACGCCTCTGCATCCGTG 3'	91
IFN $\gamma$	F: 5' ACTGGCAAAGGATGGTGAC 3' R: 5' GCTGATGGCCTGATTGTCTT 3'	98
Gapdh	F: 5' CATCAAGAAGGTGGTGAAGC 3' R: 5' ACCACCCTGTTGCTGTAG 3'	209

**Supplementary Table 2 - Antibodies used in immunohistochemistry**

Gene	Antibodies	Host	Dilution	Supplier
<b>Primary</b>	Ret	Goat	1:500	R&D system, Minneapolis, USA
	Sox10	Mouse	1:2	Kindly provided by Prof.M.Wegner60
	Gfap	Mouse	1:100	Dako, Hamburg, Germany
	Tlr2	Rabbit	1:100	Sigma Aldrich, Saint Louis, USA
	Tlr4	Rabbit	1:100	Sigma Aldrich, Saint Louis, USA
	Tlr5	Rabbit	1:100	Sigma Aldrich, Saint Louis, USA
<b>Secondary</b>	Anti-goat Alexa 488	Donkey	1:500	Invitrogen, Karlsruhe, Germany
	Anti-mouse Alexa 488	Donkey	1:500	Invitrogen, Karlsruhe, Germany
	Anti-mouse Alexa 488	Donkey	1:500	Jackson Immunoresearch, Hamburg, Germany

**Supplementary Table 3 - Single Gene Analysis: Genes were expressed significantly different in ENCCs treated with GDNF compare to untreated ENCCs (Bonferroni :  $p < 0.05$ )**

Rank	Affymetrix Name	Gene Symbol	T	Bonferroni
<b>Up-regulated</b>				
1	1453008_at	Trnp1	-49.711785	1.34E-06
2	1435026_at	Spock2	-43.762722	3.70E-06
3	1452825_at	Tmem59l	-42.657373	4.54E-06
4	1435857_s_at	Aplp1	-40.88871	6.35E-06
5	1437261_at	Mblac2	-35.864703	1.80E-05
6	1436094_at	Vgf	-33.761245	2.92E-05
7	1456379_x_at	Dner	-33.271202	3.28E-05
8	1437292_at	Rlbp1l2	-31.037683	5.69E-05
9	1428333_at	2900062L11Rik	-29.839986	7.78E-05
10	1426604_at	Rnasel	-29.703856	8.07E-05
11	1452653_at	Slc25a22	-29.609467	8.27E-05
12	1437528_x_at	A730017C20Rik	-29.389713	8.78E-05
13	1434427_a_at	Rnf157	-27.830259	0.000135269
14	1417988_at	Resp18	-26.224421	0.000216607
15	1418736_at	B3galnt1	-26.088397	0.000225711
16	1459723_at	Zdhhc22	-25.644417	0.000258556
17	1441662_at	Cyp4x1	-25.349314	0.000283353
18	1449628_s_at	Stard7	-25.219314	0.000295116
19	1443924_at	Wnk3	-25.062297	0.000310062
20	1451679_at	6530401D17Rik	-24.824377	0.000334356
21	1427893_a_at	Pmvk	-24.621329	0.00035679
22	1435756_at	Samd10	-24.571621	0.000362536
23	1421349_x_at	Cend1	-24.564025	0.000363423
24	1426662_at	Cmas	-24.553795	0.000364622
25	1417428_at	Gng3	-24.541655	0.000366051
26	1455260_at	Lcorl	-24.130619	0.000418331
27	1423280_at	Stmn2	-24.044308	0.000430345
28	1418671_at	Capn5	-23.886055	0.000453394
29	1418400_at	Larp6	-23.766463	0.000471734
30	1460262_a_at	1700037H04Rik	-23.762846	0.000472301
31	1455888_at	Lingo2	-23.645762	0.000491093
32	1427482_a_at	Car8	-23.417563	0.000530181
33	1455792_x_at	Ndn	-23.39489	0.000534252
34	1422711_a_at	Pnck	-23.389166	0.000535285
35	1450863_a_at	Dclk1	-23.246707	0.000561739
36	1434754_at	Garnl4	-23.232605	0.000564437
37	1454687_at	Lrfr5	-23.111085	0.000588295
38	1436134_at	Scn2b	-23.073987	0.000595802
39	1440132_s_at	Prkar1b	-22.863039	0.000640594
40	1424624_at	2900011O08Rik	-22.81546	0.000651211
41	1460239_at	Tspan13	-22.703032	0.000677096
42	1438370_x_at	Dos	-22.614644	0.000698258
43	1435105_at	Rnf208	-22.604722	0.000700679



44	1433876_at	Lrrc24	-22.526199	0.00072018
45	1423352_at	Crispld1	-22.47565	0.000733055
46	1436087_at	LOC100047231	-22.311623	0.000776651
47	1422605_at	Ppp1r1a	-22.177671	0.000814419
48	1460292_a_at	Smarca1	-22.159762	0.000819623
49	1424443_at	Tm6sf1	-22.156432	0.000820595
50	1448312_at	Pcsk2	-21.990273	0.000870767
51	1455652_at	Kif3a	-21.95774	0.000880989
52	1433443_a_at	Hmgcs1	-21.884633	0.000904454
53	1416965_at	Pcsk1n	-21.881448	0.000905492
54	1455080_at	Ppp1r16b	-21.802173	0.000931768
55	1450773_at	Kcnd2	-21.669177	0.000977796
56	1436450_at	D11Bwg0517e	-21.570151	0.001013727
57	1452240_at	Brunol4	-21.456076	0.001056969
58	1416935_at	Trpv2	-21.285768	0.001125436
59	1447725_at	C030034E14Rik	-21.204243	0.001159963
60	1447738_s_at	Ankrd13d	-21.110093	0.001201326
61	1434961_at	Asb1	-21.097184	0.001207125
62	1450659_at	Rgs7	-21.007074	0.001248493
63	1424482_at	Arhgef7	-20.930141	0.001285075
64	1437079_at	Slc18a2	-20.809908	0.001344682
65	1436179_a_at	Dnajc5	-20.385681	0.001581199
66	1451092_a_at	Rangap1	-20.242769	0.001671131
67	1434657_at	Gls	-20.023423	0.001820554
68	1420499_at	Gch1	-19.889409	0.001919206
69	1423282_at	Pitpna	-19.852766	0.001947215
70	1459881_at	Fbll1	-19.848639	0.001950398
71	1428370_at	1500011B03Rik	-19.796438	0.001991169
72	1418829_a_at	Eno2	-19.701832	0.002067524
73	1454972_at	Atcay	-19.57182	0.002177875
74	1441197_at	9530059O14Rik	-19.554945	0.002192676
75	1442180_at	Dleu7	-19.524284	0.00221986
76	1448832_a_at	Cplx1	-19.437734	0.002298659
77	1457755_at	Gng8	-19.31441	0.002416422
78	1424017_a_at	Hint1	-19.163534	0.002569807
79	1427229_at	Hmgcr	-19.154594	0.002579232
80	1432464_a_at	2310057J16Rik	-19.053404	0.002688674
81	1441905_x_at	Snrpn /// Snurf	-19.049584	0.002692906
82	1418209_a_at	Pfn2	-18.993873	0.00275549
83	1426615_s_at	Ndrg4	-18.990207	0.002759665
84	1451008_at	St8sia3	-18.922381	0.002838206
85	1423317_at	3110001D03Rik	-18.83419	0.002944109
86	1428765_at	Meg3	-18.818445	0.002963479
87	1451322_at	Cmb1	-18.758269	0.003038838
88	1457092_at	Fam19a1	-18.679781	0.003140383
89	1448595_a_at	Bex1	-18.617769	0.003223309
90	1460223_a_at	Epb4.9	-18.575406	0.003281371
91	1421152_a_at	Gnao1	-18.494733	0.003395217
92	1421493_a_at	Rgs20	-18.48937	0.003402941
93	1433791_at	Rab9b	-18.465285	0.003437872
94	1455014_at	Hint3	-18.252221	0.003765047

95	1453078_at	2610002M06Rik	-18.167393	0.003904918
96	1456392_at	Negr1	-18.164024	0.003910593
97	1429022_at	Adcyap1r1	-18.118995	0.003987329
98	1441944_s_at	Gpr135	-18.100384	0.004019536
99	1448660_at	Arhgdig	-18.070941	0.004071088
100	1436552_at	Jakmip2	-18.063323	0.004084547
101	1418062_at	Eef1a2	-18.011533	0.004177382
102	1435641_at	Mgat4a	-17.985843	0.004224308
103	1451499_at	Cadps2	-17.976442	0.004241627
104	1424308_at	Slc24a3	-17.974323	0.004245543
105	1428089_at	Sliitr1	-17.954827	0.004281753
106	1451394_at	Dpp6	-17.943084	0.004303732
107	1436605_at	Tkt	-17.929896	0.004328565
108	1437234_x_at	Prmt2	-17.917984	0.004351133
109	1437618_x_at	Gpr85	-17.803552	0.004574799
110	1431403_a_at	Mtap7d2	-17.72493	0.004735946
111	1434155_a_at	2310061I04Rik	-17.693038	0.00480312
112	1415671_at	Atp6v0d1	-17.665455	0.004862083
113	1450888_at	Napb	-17.632404	0.004933805
114	1420554_a_at	Rac3	-17.560163	0.005094746
115	1431086_s_at	Pcmt1	-17.539057	0.005142872
116	1428354_at	Foxk2	-17.538998	0.005143007
117	1435630_s_at	Acat2	-17.510206	0.005209486
118	1455313_at	Ablim2	-17.498944	0.00523575
119	1434346_at	Rufy2	-17.491789	0.005252513
120	1450693_at	Rgs17	-17.436449	0.005384226
121	1417997_at	Ngb	-17.427255	0.005406465
122	1423724_at	Zwint	-17.418391	0.005428004
123	1438672_at	Parvb	-17.404948	0.005460853
124	1435014_at	Rab39b	-17.374322	0.005536528
125	1438619_x_at	Zdhhc14	-17.338304	0.005627033
126	1422941_at	Wnt16	-17.293055	0.005743098
127	1416392_a_at	Atp6v0c	-17.225169	0.005922297
128	1440270_at	Fgf12	-17.217381	0.005943254
129	1442884_at	Hgf	-17.201465	0.00598634
130	1425893_a_at	Fhit	-17.112659	0.006233277
131	1449983_a_at	Nqo2	-17.103442	0.006259555
132	1449377_at	Nicn1	-17.072009	0.006350107
133	1436237_at	Ttc9	-17.059961	0.006385204
134	1451503_at	Nol3	-17.025724	0.006486134
135	1455258_at	Kcnc2	-16.969797	0.006654878
136	1428550_at	Ydjc	-16.941611	0.006741787
137	1433987_at	Hpcal4	-16.937396	0.006754889
138	1456108_x_at	Rnf112	-16.917326	0.006817684
139	1449491_at	Card10	-16.881509	0.006931384
140	1452332_at	Ccdc85a	-16.846635	0.007044136
141	1443327_at	D130043K22Rik	-16.840347	0.007064684
142	1434170_at	Decaf12l1	-16.833805	0.007086133
143	1438054_x_at	Ppp2r2c	-16.825827	0.007112391
144	1426249_at	Adrbk1	-16.819502	0.007133283
145	1438667_at	5730410E15Rik	-16.816992	0.007141596

146	1452711_at	Atl1	-16.798857	0.007201962
147	1417609_at	Ube2a	-16.792496	0.007223273
148	1418178_at	LOC100047943	-16.758641	0.00733789
149	1455796_x_at	Olfm1	-16.736336	0.007414517
150	1455509_at	Nacad	-16.717595	0.007479597
151	1460570_at	Pgbd5	-16.691408	0.007571608
152	1426956_a_at	Trp53bp1	-16.678548	0.007617257
153	1422852_at	Cib2	-16.675134	0.007629427
154	1452800_a_at	Apoo	-16.673311	0.007635937
155	1442786_s_at	Rufy3	-16.627761	0.007800581
156	1422072_a_at	Gstm6	-16.609159	0.007868961
157	1422977_at	Gp1bb	-16.586468	0.007953284
158	1435113_x_at	Stmn3	-16.580414	0.007975952
159	1420880_a_at	Ywhab	-16.576786	0.007989571
160	1415993_at	Sqle	-16.490009	0.008323233
161	1451895_a_at	Dhcr24	-16.453165	0.008469606
162	1435605_at	Actr3b	-16.433418	0.008549245
163	1416098_at	Syngn3	-16.413526	0.008630315
164	1426712_at	Slc6a15	-16.379991	0.008768951
165	1428996_at	Pdp2	-16.323407	0.00900857
166	1435917_at	Ociad2	-16.31635	0.009038965
167	1428578_s_at	Ppfia4	-16.305943	0.009084001
168	1433787_at	Nell1	-16.276958	0.009210769
169	1459890_s_at	1110008P14Rik	-16.227167	0.009433186
170	1455321_at	Ddhd1	-16.208564	0.009517823
171	1420545_a_at	Chn1	-16.197733	0.009567496
172	1437018_at	Pnma2	-16.190394	0.009601318
173	1459980_x_at	Rab3a	-16.187444	0.009614952
174	1417672_at	Slc4a10	-16.177399	0.009661535
175	1450675_at	Smap2	-16.166271	0.009713438
176	1452915_at	Prkar2a	-16.141985	0.009827805
177	1435200_at	Ncma00086	-16.131233	0.009878915
178	1454787_at	Zdhhc9	-16.095105	0.010052862
179	1426255_at	Nefl	-16.074527	0.010153473
180	1423418_at	Fdps	-16.052983	0.010260016
181	1418085_at	Prkcz	-16.000829	0.010523169
182	1448991_a_at	Ina /// LOC100047943	-15.915841	0.010968373
183	1419580_at	Dlg4	-15.895684	0.011077038
184	1425760_a_at	Pitpnm1	-15.797923	0.011621484
185	1417788_at	Sncg	-15.797841	0.011621952
186	1435083_at	Ctnn1	-15.788938	0.011673013
187	1437211_x_at	Elovl5	-15.773696	0.011761007
188	1437147_at	Gabrg2	-15.765563	0.011808265
189	1426345_at	Prepl	-15.718717	0.012084646
190	1460740_at	LOC100046457	-15.699916	0.012197603
191	1451747_a_at	Atg12	-15.694463	0.012230591
192	1417471_s_at	D1Ert622e	-15.692671	0.012241453
193	1417799_at	Atp6v1g2	-15.682707	0.012302034
194	1428664_at	Vip	-15.662437	0.012426332
195	1431076_at	Add2	-15.647016	0.012521837
196	1448663_s_at	Mvd	-15.623501	0.012669052

197	1427045_at	Synpo	-15.611553	0.012744603
198	1433714_at	Sult4a1	-15.595042	0.012849827
199	1434027_at	Rcan3	-15.578334	0.012957308
200	1455269_a_at	Coro1a	-15.536606	0.013230161
201	1433566_at	Rasl10b	-15.530133	0.01327306
202	1418361_at	Gas8	-15.521722	0.013329038
203	1450961_a_at	Tceal6	-15.513995	0.013380698
204	1441317_x_at	Jakmip1	-15.508048	0.013420608
205	1453127_at	Ppm1j	-15.499416	0.013478782
206	1433947_at	Rab37	-15.485122	0.013575728
207	1457587_at	Kcnq5	-15.465515	0.013709985
208	1454782_at	Bai3	-15.455587	0.013776571
209	1418474_at	Fam158a	-15.449029	0.013824022
210	1423328_at	Gdap1	-15.443272	0.013864094
211	1434969_at	Bruno15	-15.397101	0.014190232
212	1422431_at	Magee1	-15.392193	0.014225406
213	1430642_at	2900001G08Rik	-15.387067	0.01426224
214	1417840_at	1500031L02Rik	-15.375277	0.014347378
215	1434951_at	Armc8	-15.360494	0.014454934
216	1436148_at	Tnr	-15.34392	0.014576593
217	1426236_a_at	Glul	-15.343505	0.01457965
218	1437185_s_at	Tmsb10	-15.327694	0.01469681
219	1424182_at	Acat1	-15.307308	0.01484943
220	1420013_s_at	Lss	-15.287392	0.015000248
221	1416023_at	Fabp3	-15.281706	0.015043629
222	1420955_at	Vsnl1	-15.278605	0.015067338
223	1417524_at	LOC100044812	-15.272629	0.015113157
224	1427356_at	Fam89a	-15.226715	0.015470435
225	1448211_at	Atp6v0e2	-15.22373	0.01549399
226	1424069_at	Napg	-15.222628	0.015502694
227	1460576_at	Exoc6	-15.20585	0.01563592
228	1417676_a_at	Ptpro	-15.205287	0.015640412
229	1450466_at	Cdk5r2	-15.190918	0.015755567
230	1450853_at	Tle4	-15.179773	0.015845535
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232	1434455_at	Fbxo44	-15.110964	0.016413938
233	1442023_at	A530030E21Rik	-15.076419	0.016707893
234	1437855_at	Mtap4	-15.067419	0.016785435
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237	1458413_at	Fbxw8	-15.055287	0.016890616
238	1433737_at	LOC677213 /// Uhmk1	-15.042434	0.017002847
239	1436795_at	9630058J23Rik	-15.026237	0.017145479
240	1449259_at	Rab3d	-15.011606	0.017275464
241	1431255_at	Calr3	-14.991252	0.017458146
242	1439770_at	6430598A04Rik	-14.978284	0.017575656
243	1437912_at	lqsec3	-14.968554	0.017664417
244	1417002_at	0610012G03Rik	-14.955263	0.017786472
245	1422692_at	Sub1	-14.924587	0.018071797
246	1449298_a_at	Pde1a	-14.916057	0.018152048
247	1427280_at	Scn2a1	-14.8936	0.018365234

248	1418453_a_at	Atp1b1	-14.886328	0.018434873
249	1425248_a_at	Tyro3	-14.85919	0.018697362
250	1423906_at	Hsbp1	-14.845171	0.018834599
251	1426094_at	Rhbd1l	-14.823518	0.019048801
252	1439031_at	Jph4	-14.760349	0.019689448
253	1453003_at	Sor11	-14.756716	0.01972702
254	1420872_at	Gucy1b3	-14.745665	0.019841815
255	1451257_at	Acs16	-14.731314	0.019991989
256	1424455_at	Gprasp1	-14.730476	0.020000802
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258	1451935_a_at	Spint2	-14.699556	0.020328891
259	1431826_a_at	Brsk2	-14.690931	0.02042148
260	1452779_at	3110006E14Rik	-14.679031	0.020550015
261	1434270_at	Cbx6-Nptxr /// Nptxr	-14.66256	0.020729408
262	1427004_at	Fbxo2	-14.658023	0.020779128
263	1449229_a_at	Cdkl2	-14.642309	0.020952374
264	1428638_at	Efhc2	-14.636313	0.021018912
265	1440531_at	Rbm11	-14.624637	0.021149153
266	1449494_at	Rab3c	-14.586689	0.021578746
267	1435028_at	Wdr7	-14.580738	0.021646998
268	1428387_at	Acs13	-14.57717	0.021688032
269	1456210_at	5430407P10Rik	-14.573942	0.021725238
270	1434641_x_at	Sez6l2	-14.556641	0.02192584
271	1424386_at	Reep2	-14.514886	0.022418612
272	1424145_at	Prr3	-14.511926	0.022454011
273	1423569_at	Gatm	-14.501683	0.022577001
274	1417683_at	Diablo	-14.494192	0.022667423
275	1456708_at	2610028E06Rik	-14.490687	0.022709871
276	1451040_at	Dtd1	-14.481389	0.022822909
277	1424764_at	Sez6l	-14.472233	0.022934832
278	1434640_at	Nrsn2	-14.468432	0.022981479
279	1426272_at	Lmbr1	-14.447456	0.023240822
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282	1420824_at	Sema4d	-14.428484	0.023478205
283	1433607_at	Cbln4	-14.392068	0.023941506
284	1455829_at	Usp14	-14.382448	0.024065607
285	1436099_at	A1836003	-14.377229	0.024133227
286	1424124_at	Mospd2	-14.372671	0.024192468
287	1423642_at	Tubb2c	-14.364696	0.024296502
288	1455481_at	lds	-14.341279	0.024604888
289	1450647_at	Hps3	-14.302782	0.025121437
290	1426167_a_at	Camk4	-14.280286	0.025428918
291	1417374_at	Tuba4a	-14.245118	0.025918074
292	1439971_at	6330439K17Rik	-14.23563	0.026051842
293	1436403_at	Fam171a2	-14.231944	0.026104025
294	1419062_at	Epb4.1l3	-14.186862	0.026751752
295	1455369_at	Apba1	-14.181556	0.026829163
296	1423055_at	Nsg1	-14.173006	0.026954431
297	1436624_at	Dnm3	-14.150986	0.027280081
298	1435123_at	Efr3b	-14.145269	0.027365344

299	1455636_at	Lsamp	-14.144254	0.027380527
300	1428405_at	Hcfc1r1	-14.139908	0.02744558
301	1428209_at	Bex4	-14.121669	0.027720511
302	1424935_at	Gdap111	-14.107253	0.027940012
303	1418263_at	Ddx25	-14.105193	0.027971533
304	1448530_at	Gmpr	-14.103178	0.028002399
305	1451783_a_at	Kifap3	-14.09768	0.02808683
306	1437744_at	Slitrk4	-14.092544	0.02816596
307	1423285_at	Coch	-14.090176	0.028202538
308	1418493_a_at	Snca	-14.075296	0.028433546
309	1448193_at	5730403B10Rik	-14.031732	0.02912215
310	1417626_at	Pde4dip	-14.027722	0.029186469
311	1435389_at	Reps2	-14.006277	0.029533148
312	1416102_at	Ywhaz	-13.949724	0.030469732
313	1455021_at	Gabbr1	-13.938683	0.030656444
314	1426517_at	Gnaz	-13.932864	0.03075536
315	1436674_at	Rundc3a	-13.929905	0.030805795
316	1457045_at	Galnt13	-13.929549	0.030811866
317	1450053_at	Kif2a	-13.90716	0.031196596
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319	1417559_at	Sfxn1	-13.900464	0.031312704
320	1449286_at	Ntng1	-13.870132	0.031844757
321	1416936_at	Aatk	-13.864694	0.031941213
322	1422533_at	Cyp51	-13.857524	0.032068874
323	1422592_at	Ctnnd2	-13.847363	0.032250802
324	1437075_at	Frmf3	-13.791405	0.033273573
325	1422583_at	Rab3b	-13.786418	0.033366474
326	1449423_at	Mast1	-13.786152	0.033371438
327	1437472_at	Unc13a	-13.761468	0.033835643
328	1452451_at	mCG_21548	-13.755737	0.033944448
329	1424988_at	Mylip	-13.751982	0.034015968
330	1434676_at	Mtmr9	-13.747122	0.034108754
331	1418164_at	Stx2	-13.72877	0.034461743
332	1416253_at	Cdkn2d /// Gm4694	-13.72401	0.034553967
333	1417154_at	Slc25a14	-13.721546	0.034601811
334	1428434_at	Zcchc12	-13.719647	0.034638726
335	1420505_a_at	Stxbp1	-13.716675	0.034696601
336	1426068_at	Slc7a4	-13.699416	0.035034879
337	1438164_x_at	Flot2	-13.691769	0.035185919
338	1429723_at	6330409N04Rik	-13.648523	0.036054028
339	1429013_at	Mtap7d2	-13.630973	0.036413156
340	1424691_at	5930434B04Rik	-13.62781	0.036478301
341	1444085_at	Pdss2	-13.626078	0.036514028
342	1428525_at	4930488B01Rik	-13.624238	0.036552022
343	1434398_at	Nkrf	-13.615835	0.03672614
344	1454800_at	Morn2	-13.60874	0.036873865
345	1448995_at	Pf4	-13.580105	0.037476873
346	1451490_at	Lyplal1	-13.566281	0.037771939
347	1455087_at	D7Erd715e	-13.565406	0.037790709
348	1451122_at	Idi1	-13.562816	0.037846303
349	1416256_a_at	Tubb5	-13.537593	0.038392615

350	1421396_at	Pcsk1	-13.529433	0.038571235
351	1438176_x_at	Snap47	-13.508043	0.039043896
352	1431046_at	Ppfia3	-13.50241	0.039169451
353	1429266_at	Tmhs	-13.502068	0.039177096
354	1418746_at	Pnkd	-13.490861	0.039428275
355	1430034_at	Cct4	-13.469681	0.039907937
356	1434083_a_at	Elmod1	-13.440734	0.040574116
357	1416022_at	Fabp5	-13.436336	0.040676421
358	1424072_at	2010107G23Rik	-13.435663	0.040692096
359	1418444_a_at	Gde1	-13.422497	0.041000217
360	1452445_at	Slc41a2	-13.411501	0.04125955
361	1449381_a_at	Pacsin1	-13.410627	0.041280234
362	1427347_s_at	Tubb2a	-13.378011	0.042060757
363	1420048_at	C78859	-13.361665	0.042458149
364	1438396_at	Ocr1	-13.352984	0.042670904
365	1440928_at	D630037F22Rik	-13.352895	0.042673085
366	1418588_at	Nrsn1	-13.342067	0.042940144
367	1458622_at	Ntrk2	-13.332992	0.043165406
368	1435968_at	Ptpn2	-13.330754	0.043221154
369	1448812_at	Hpcal1	-13.325271	0.043358091
370	1456633_at	Trpm3	-13.313853	0.043644829
371	1452473_at	Prr15	-13.309305	0.043759635
372	1456523_at	C77713	-13.293546	0.044160059
373	1434535_at	Krit222	-13.285202	0.044373736
374	1415845_at	Syt4	-13.27695	0.044586181
375	1435783_at	Fam169a	-13.272256	0.044707546
376	1455882_x_at	Vwc2	-13.272005	0.044714039
377	1420679_a_at	Aig1	-13.254862	0.045160473
378	1438305_at	Rims1	-13.221414	0.046045986
379	1419546_at	Atp6v1c1	-13.218557	0.046122538
380	1430780_a_at	Pmm1	-13.215341	0.046208845
381	1434315_at	Nipal3	-13.21243	0.046287131
382	1428049_a_at	Nudt16l1	-13.211124	0.046322306
383	1426448_at	Pja1	-13.197898	0.046680205
384	1439459_x_at	Acly	-13.17696	0.047253141
385	1448600_s_at	Vav3	-13.173794	0.047340438
386	1426887_at	Nudt11	-13.157439	0.04779438
387	1424029_at	Tspyl4	-13.157206	0.047800892
388	1435852_at	Spre3	-13.140179	0.048278733
389	1455144_s_at	AU040829	-13.129505	0.048581007
390	1434105_at	Epm2aip1	-13.105586	0.049266118
391	1423221_at	Tubb4	-13.099685	0.049436807
392	1453065_at	Aldh5a1	-13.090774	0.049695791
393	1453120_at	Tmx4	-13.086395	0.049823632
394	1451033_a_at	Trpc4	-13.076864	0.050103114
395	1452323_at	Spryd3	-13.076565	0.05011191
396	1436448_a_at	Ptgs1	-13.066511	0.050408738
397	1433596_at	Dnajc6	-13.065176	0.050448271
398	1455148_at	Tmem130	-13.064905	0.050456309
399	1451229_at	Hdac11	-13.058079	0.050659175

## Down-Regulated

1	1426048_s_at	Tcfap2a	18.8011969	0.002984862
2	1426812_a_at	Fam129b	18.6740158	0.00314799
3	1450994_at	Rock1	17.4197496	0.005424697
4	1442063_at	Adamtsl1	16.7292229	0.007439144
5	1436840_x_at	Rpl35	16.4723071	0.008393202
6	1456135_s_at	Pxn	16.4592421	0.008445266
7	1423771_at	Prkcdbp	16.0717801	0.010166986
8	1430978_at	Rps25	16.029682	0.010376663
9	1423425_at	Plbd2	15.4008967	0.014163098
10	1449007_at	Btg3 /// Gm7334	15.3044709	0.014870813
11	1428113_at	Tmtc4	15.2629261	0.015187883
12	1424704_at	Runx2	15.0035771	0.017347267
13	1460036_at	Ap1s2	14.9437791	0.017892688
14	1449484_at	Stc2	14.723464	0.020074682
15	1439439_x_at	Eef1d	14.7222485	0.02008752
16	1423536_at	Strn3	14.7105706	0.020211329
17	1448785_at	Runx1t1	14.6854947	0.020480086
18	1436325_at	Rora	14.5638037	0.021842541
19	1435972_at	Cast	14.5157617	0.022408155
20	1418135_at	Aff1	14.3268176	0.02479752
21	1448347_a_at	Caprin1	14.3194122	0.024896819
22	1449099_at	Lrba	14.2400722	0.025989119
23	1421043_s_at	Arhgef2	14.1050282	0.027974052
24	1460644_at	Bckdk	13.9108203	0.031133331
25	1423753_at	Bambi	13.8244339	0.032665566
26	1460211_a_at	Kdelr1	13.7376391	0.034290641
27	1435777_at	Itpril2	13.6238068	0.036560938
28	1416133_at	Efr3a	13.619167	0.036656982
29	1418564_s_at	Serbp1	13.1277282	0.048631537

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T (-) : Up-regulated in ENCCs with GDNF

T (+): Down-regulated in ENCCs without GDNF

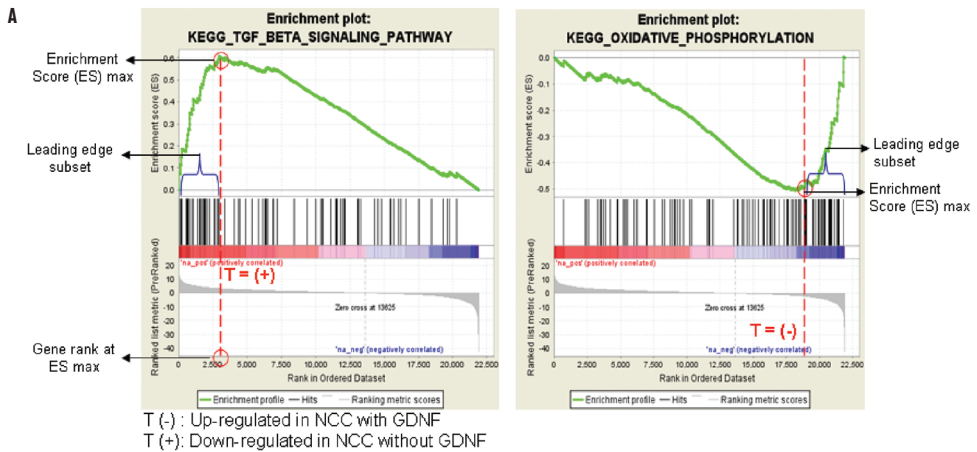


**Supplementary Table 4** - Single Gene Analysis: Genes with higher expression in ENCCs than in the gut and further up-regulated in ENCCs (+ GDNF) (Bonferroni :  $p < 0.05$ )

No	Gene Symbol	Gene Name	Human Chromosome
1	Aplp1	amyloid beta (A4) precursor-like protein 1	19q13.12
2	Napg	N-ethylmaleimide-sensitive factor attachment protein, gamma	18p11.22
3	Mtap4	microtubule-associated protein 4	3p21.31
4	Dpp10	Dipeptidyl-peptidase 10	2q14.1
5	Brsk2	BR serine/threonine kinase 2	11p15.5
6	Sez6l2	Seizure related 6 homolog (mouse)-like 2	16p11.2
7	hcfc1r1	Host cell factor C1 regulator 1 (XPO1 dependent)	16p13.3
8	Snca	Synuclein, alpha	4q22.1
9	Gabbr1	gamma-aminobutyric acid (GABA) B receptor, 1	6p22.1
10	Galnt13	UDP-N-acetyl-alpha-D-galactosamine:polypeptide	2q23.3
11	Cend1	cell cycle exit and neuronal differentiation 1	11p15.5
12	Stmn2/Scg10	stathmin-like 2	8q21.13
13	Pcsk1n	proprotein convertase subtilisin/kexin type 1 inhibitor	Xp11.23
14	Brunol4	Bruno-like protein 4	18q12.2
15	Napb	N-ethylmaleimide-sensitive factor attachment protein, beta	20p11.21
16	Kcnq5	potassium voltage-gated channel, KQT-like subfamily, member 5	6q13
17	Gdap1	ganglioside-induced differentiation-associated protein 1	8q21.11
18	Asb1	ankyrin repeat and SOCS box-containing 1	2q37.3
19	Arhgef7	Rho guanine nucleotide exchange factor (GEF) 7	13q34
20	Dnajc5	DnaJ (Hsp40) homolog, subfamily C, member 5	20q13.33
21	Ndrp4	NDRG family member 4	16q21
22	Negr1	neuronal growth regulator 1	1p31.1
23	Rufy2	RUN and FYVE domain containing 2	10q21.3
24	Rgs17	Regulator of G-protein signaling 17	6q25.2
25	Rab39b	RAB39B, member RAS oncogene family	Xq28
26	Kcnc2	Potassium voltage gated channel, Shaw-related subfamily, member 2	12q21.1
27	Ppp2r2c	protein phosphatase 2, regulatory subunit B (PR 52), gamma isoform	4p16.1
28	Atl1	atlastin GTPase 1	14q22.1
29	slc6a15	solute carrier family 6 (neurotransmitter transporter), member 15	12q21.31
30	Frmf3	FERM domain containing 3	9q21.32
31	mtmr9	myotubularin related protein 9	8p23.1
32	stxbp1	syntaxin binding protein 1	9q34.11
33	Pcsk1	proprotein convertase subtilisin/kexin type 1	5q15
34	Pmm1	brain glucose-1,6-bisphosphatase	22q13.2
35	6330439K17Rik /C20orf12	RIKEN cDNA 6330439K17 gene/ chromosome 20 open reading frame 12	20p11.23
36	Atp6v0e2	ATPase, H+ transporting, lysosomal V0 subunit E2	7q36.1
37	Cdkl2	cyclin-dependent kinase-like 2	4q21.21
38	Slc41a2	solute carrier family 41, member 2	12q24.11
39	Tmx4	thioredoxin-related transmembrane protein 4	t20p12

**Supplementary Table 5** - Genes with higher expression in ENCCs than in the gut and down-regulated in ENCCs (+ GDNF) (Bonferroni :  $p < 0.05$ )

No	Gene	Gene Name	Human Chromosome
1	Aff1	AF4/FMR2 family, member 1	4q21.3
2	Cast	Calpastatin	5q15
3	Tmtc4	Transmembrane and tetratricopeptide repeat containing 4	13q32.3
4	Btg3	B-cell translocation gene 3	21q21.1
5	Prkcdpb	protein kinase C, delta binding protein	11p15.4
6	Adamts1	ADAMTS-like 1	9p22.2
7	Fam129b	family with sequence similarity 129, member B	9q34.11
8	Tcfap2a	transcription factor AP-2 alpha	6p24.3
9	Rora	RAR-related orphan receptor A	15q22.2
10	arhgef2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	1q22



**B**

		Biosynthesis	Differentiation	Migration	Proliferation	Apoptosis	Miscellaneous
<b>Up-regulated</b> by GDNF in ENCCs	Parkinson's disease						■
	Citrate Cycle	■					
	Oxidative phosphorylation	■					
	Peroxisome	■					
	Steroid Biosynthesis	■					
	Valin-Leucin-Isoleucin	■					
	PPAR	■					
	Butanoate metabolism	■					
	Propanoate metabolism	■					
<b>Down-regulated</b> by GDNF in ENCCs	N-Glycan Biosynthesis	■					
	Notch		■				
	Wnt		■				
	TGFβ/BMP		■				
	Axon guidance		■				
	Adherens junction			■	■		
	Tight junction			■	■		
	Focal adhesion			■	■		
	Cell cycle			■	■		
	p53				■	■	
	TLRs					■	
	Pathways in Cancer						■
	Spliceosome						■
	Ribosome						■
	Chronic Myeloid						■

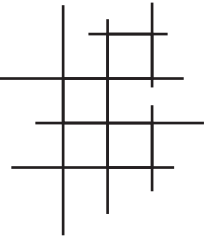
**Supplementary Figure 1 - A)** An example of GSEA enrichment profile of the down-regulated (Tfb-β signaling pathway) and the up-regulated (Oxidative Phosphorylation signaling pathway) in ENCCs treated with GDNF. **B)** Summary of GSEA enriched signaling pathways as extracted from the KEGG database (when comparing ENCCs treated with GDNF to untreated cells). The possible biological function in ENCCs during ENS development is given.





# 6

## General Discussion and Future Prespective



HSCR is a congenital disease characterized by the absence of ganglia in the myenteric and submucosal plexus of the gastrointestinal (GI) tract.<sup>1</sup> So far, 12 genes and five loci have been found associated with disease development. Of these genes *RET* has proved to be the major risk factor for HSCR, as 50% of the familial cases and 15-35% of the sporadic cases have *RET* coding sequence (CDS) mutations.<sup>2</sup> Mutations in the other 11 genes (*EDNRB*<sup>3</sup>, *EDN3*<sup>4,5</sup>, *GDNF*<sup>6,7</sup>, *NTN*<sup>8</sup>, *SOX10*<sup>9</sup>, *PHOX2B*<sup>10</sup>, *ECE1*<sup>11</sup>, *KIAA1279/KBP*<sup>12</sup>, *ZFHX1B*<sup>13,14</sup>, *NRG1*<sup>15</sup> and *TTF1*<sup>16</sup>) occur in no more than 20% of the remaining cases, most of which are syndromic-HSCR cases. The aims of the studies described in this thesis were first: to identify new HSCR-genes in associated loci that have been reported in previous studies, in particular in 9q31 and 4q31.1-q32.2.<sup>17</sup> <sup>18</sup> Second, we investigated whether the non-coding *RET* SNP rs2506004 (C>A), which we had previously identified as a candidate disease-associated mutation, was indeed pathogenic.<sup>19</sup> For this, we performed functional assays and we revealed the possible molecular mechanism by which the mutation pinpointed by this SNP contributes to disease development. As *RET* seems to be the central factor for HSCR- and enteric nervous system (ENS) development, we studied the *RET* networks in detail by performing gene expression profiling studies of mouse embryonic enteric neural crest cells (ENCCs), the progenitor cells of the ENS, untreated or treated with GDNF, the ligand of *RET*.

## 1. Identification of disease-causing genes in HSCR-associated loci

Mutations in the coding regions of the 12 genes that have been identified so far only explain about 15-35% of the sporadic HSCR cases and slightly more than 50% of the familial HSCR cases. Furthermore, reduced penetrance of the coding sequence (CDS) mutations of the 12 genes and the phenotypic variability suggests the involvement of other modifier genes or factors in the disease development. Bolk and colleagues identified a *RET*-dependent modifier locus on 9q31 in families without *RET* CDS mutations but who are linked to the *RET* locus (with the exception of one family). In Chapter 2 we tried to fine map this locus and to identify the disease-causing gene by genotyping 301 tag-SNPs spanning 7 Mb of the 9q31 region. As the 9q31 locus was identified in patients of European descent, we performed this experiment on 137 HSCR Dutch trios. We grouped the probands based on their *RET* CSD mutations status (121 probands without the *RET* CDS mutation and 16 probands with it). We identified the SNPs rs10816998 and rs7038415, located within *SVEP1*, as strongly associated with HSCR in the patients who do not carry a *RET* CDS mutation. This result is in line with the finding of Bolk and colleagues.<sup>17</sup> However, we could not replicate this result in the independent

case-control study (again using Dutch patients and controls). This might be due to population stratification issues, but it might also mean that this locus, in the end, does not contain a common disease variant for the Dutch HSCR patients. If such a common mutation does exist, the sample size we used was simply not large enough to detect significant association. We also replicated our study in 173 Chinese patients and 436 controls and we identified associated SNPs in different locations than those initially found in the Dutch population. These SNPs are located in the intronic and exonic regions of *IKBKAP*. This result was replicated in a second independent patient cohort, also from China. In contrast to the previous finding by Bolk, this association was stronger in patients with *RET* CDS mutations. We checked whether there were major linkage disequilibrium (LD) differences between the CEU and CHB HapMap populations in this locus. This was not the case, so we concluded that the differences we found were not due to differences in LD structure but due to the different ethnic origins of the patient cohorts. In view of these results, we concluded that in the Chinese population, the 9q31 locus, which was found associated with HSCR in families bearing *RET* CDS mutations, might at least contain a common disease-associated variant in the Chinese patient population. As this Chinese locus was not identified in the Dutch patient population, suggesting population specificity. The unknown variant in the Chinese patient population is likely present in the *IKBKAP* gene.

Brooks and colleagues identified a linkage region on 4q31.1-q32.2 in a multi-generational Dutch family affected by HSCR disease.<sup>18</sup> This region contains 57 genes. Using an exome sequencing approach on two out of five affected family members (**Chapter 3**), we tried to identify the disease-causing gene in this locus. We found only one possible disease-causing variant in the linkage region, in exon 20 of the *LRBA* gene. This variant was present in all five affected family members. However, we looked for the presence of this variant in a control population and unfortunately found it was present in heterozygously 4 of the 220 controls (0.9%, 4 out of 440 chromosomes). This makes it hard to conclude that it is the disease-associated mutation. As our screen was genome-wide, we were also able to look for disease modifiers not present on chromosome 4. A possible modifier present in three out of the five affected family members was found on chromosome 2, in exon 19 of *ARMC9*. Again however, we found this variant in 2% of the control population (6 out of 289 chromosomes). These results showed that these variants are unlikely to be HSCR-predisposing mutations.

Why did we not find any clear mutation on chromosome 4 although linkage was identified? Did we miss the mutation? It seems we did. The enrichment method we used only covered around 81.33% of all human exons, meaning that we lost 18.67% data to begin with. Furthermore, only 62.8% and 66.70% of the data for the two patients had a minimum coverage



of 10, the criteria we set for SNP calling. In total, we were unable to look at coding mutations in more than 40% of the exome. This we think is probably the best explanation for not finding the real causative-variant in the linkage region.

This finding also directly points to the difficulties we, as researchers, will meet when performing exome sequencing. Of course, we might find a mutation (and would be happy), but if unsuccessful, the question remains of what should we do next? Wait for better enrichment kits or develop locus-specific exome enrichment kits? This seems like a good idea and might solve most of the problems. However, we should not totally exclude the possibility that the mutation is present in an intronic or regulatory sequence. For the *LRBA* gene, we speculate in Chapter 3 that there may indeed be non-coding mutations that influence not the *LRBA* gene but the *MAB21L2* gene, which is present within the *LRBA* locus (for an extended discussion on this, see Chapter 3). If so, the enrichment kits might just not cover the mutation. We could of course go back to specific genes in the linkage area and focus on those, and think of making enrichment kits for these candidates (both coding and non-coding). But, as shown in Chapter 3, we did go back to the best candidate gene, *MAB21L2*, and sequence the entire genome region, including the regulatory sequences, but we still did not find any candidate disease-causing mutation. So focusing on a candidate gene is not always the answer to the problem. In the case of *MAB21L2*, we might extend the sequence region to the entire *LRBA* gene (see above) but even this might not prove successful. These difficulties are also nicely illustrated by a previous study we performed on a family with Goldberg-Shprintzen syndrome.<sup>12</sup> None of the candidate genes in the linkage area proved to be mutated, and in fact it was the last gene on a list of 35 that finally proved to be mutated. In this specific family, the unbiased approach of exome sequencing might have been helpful.

Another problem that became clear in this study is that our search for modifiers will be extremely difficult. Screening exomes results in hundreds of variants, while screening large numbers of patients and controls will be necessary to prove any possible involvement. Furthermore, the variants we found were not present in the larger databases such as dbSNP and 1000 Genomes, but by screening population-specific controls, we confirmed their presence in our population. This last finding shows that we really need to build population-specific databases.

Clearly, exome sequencing is a great tool that will help us in an unbiased way to find mutations, but equally clearly, this approach will not always be successful and the results obtained should be examined with great care.

## 2. Functional studies on HSCR-associated variant SNP rs2506004 (C>A)

As mentioned above, mutations in coding sequences of the known HSCR genes only explain some of the HSCR cases, hence it is predicted that there are more genes involved in the disease development. However, besides other genes, non-coding mutations in the known genes might also contribute to disease development. Indeed, in the past six years, substantial evidence was presented that *RET* non-coding sequence mutations play an important role in disease development, especially in sporadic S-HSCR cases. At least three *RET* non-coding variants have been identified and proven to be functional variants, as the mutant allele of these variants decreased reporter gene expression in luciferase assays. Two out of the three variants are located in the *RET* promoter region (rs10900296, G>A; rs10900297, A>C) and one is located in the multi-species conserved region (MCS+9.7) in intron 1 (rs2435357, C>T) of *RET*. We identified another associated-variant (SNP rs2506004, C>A) which is located 217 bp downstream of SNP rs2435357, also in MCS+9.7.<sup>19</sup> We considered this variant a good candidate since it is highly associated with HSCR disease and conserved not only in mammals but also in avian species (chicken). Interestingly, when Emison and colleagues performed luciferase assays for the region containing SNP rs2435357 (named Enh1 from now onward), they also included SNP rs2506004 (named Enh2 from now onward). In their *in vitro* studies they showed that the T- (disease-associated) allele of Enh1 reduced the luciferase expression and disturbed the binding site of SOX10, the transcription factor of *RET*. However, it has not yet been proven that Enh2, which was present in the construct used in these studies, did not contribute to the disease development. We therefore decided to perform functional assays for Enh2 (**Chapter 4**). Luciferase assays showed that both of the disease-associated alleles (Enh1-T and Enh2-A) reduced the luciferase expression independently under regulation of the *RET*-promoter.

Using *in silico* analysis we identified Enh2-C, the non-disease allele, and its surrounding sequence to be present in the -ACGTG- sequence, also known as the Central Nervous System Midline Element (CME), is a binding site of Nxf/Arnt2 and the Sim2/Arnt2 heterodimers. By electrophoresis mobility shift assay (EMSA) we showed that Nxf/Arnt2 has less binding affinity to CME containing the Enh2-T (disease associated) allele compared to CME containing the Enh2-C (non-disease) allele, while Sim2/Arnt2 has the same binding affinity to both disease and non-disease associated alleles. Interestingly, we identified six CME regions in the *RET* promoter. This led us to hypothesize that NXF/ARNT2 and SIM2/ARNT2 regulated *RET* expression not only by binding to Enh2 but also by binding to the *RET* promoter. We proved this hypothesis by co-transfection of constructs containing *Nxf/Arnt2* or *Sim2/Arnt2* with the *RET* promoter coupled to *luciferase* into a mouse neuroblastoma cell line (Neuro2A). The re-

sults showed that *Nxf/Arnt2* and *Sim2/Arnt2* increase and decrease the luciferase expression, respectively. Furthermore, the transfection of *Nxf/Arnt2* or *Sim2/Arnt2* into mouse neuroblastoma cells line (Neuro2A) showed increased and decreased endogenous RET expression. This strengthens the idea that *NXF/ARNT2* and *SIM2/ARNT2* are transcription regulators for RET expression. It is interesting to mention that *SIM2* is located in the critical region of Down's syndrome (DS) in chromosome 21. It has been hypothesized that overexpression of *SIM2* in DS patients reduces the expression of Drebrin (*DBN1*), a protein that is important for synaptic plasticity. In this study we showed that overexpression of *Sim2* leads to down-regulation of *Ret* expression. This could explain why DS patients have a 40-fold higher risk of having HSCR disease. In conclusion, in this study we showed that *Enh1* and *Enh2* are both functional variants and the disease-associated alleles of both variants decrease *luciferase* expression. We also showed that *Nxf/Arnt2* and *Sim2/Arnt2* are expressed in the enteric nervous system (ENS) progenitor cells and these heterodimers are a transcription activator and repressor, respectively, for *RET* expression.

Besides the fact that we could confirm the probable involvement of the SNP we had previously identified, we had to conclude that multiple SNPs present on the same disease-associated haplotype may play a role in the disease, likely at different stages of development, or are involved in different processes. As mentioned above, previous studies showed evidence for the involvement of at least two additional SNPs on the same haplotype. These SNPs are located at -5 and -1 from the start site of the *RET* gene (Figure 1 in Chapter 4). It was also shown that the TTF-1 protein binds to this region and that TTF-1 can activate the *RET* promoter<sup>16, 20, 21</sup>. Therefore, previously published data and our new data hold an important message for the study of complex diseases, namely that more than one SNP on an associated haplotype might influence disease development. So far, almost all studies have stopped if they found a SNP with a proven functional effect. We have shown that even when one such SNP has been found, there may still be more involved in the disease development. This means that polygenic diseases are even more complex than originally thought.

As we have identified new transcription regulators for *Ret*, there is the question of whether variants/mutations in those transcription regulators might also contribute to disease development. So far, association to 11q13 (where *NXF* is located) or 15q25.1 (where *ARNT2* is located) has not been reported. Rare coding mutations have not yet been looked for, but whether they exist in these transcription factors and cause or contribute to HSCR can, however, be doubted. These transcription factors are involved in many developmental processes and if any mutation were present, it is most likely it would result in a complex clinical phenotype. It is more likely that the sequences in HSCR genes to which these transcription fac-

tors bind (the CME regions) might contain SNPs that disturb proper binding, as shown for SNP rs2506004 in MCS+9.7. We have already performed one study to test this hypothesis. We checked the six CME regions within the *RET* promoter for mutations in 107 HSCR patients (patients who do not carry *RET* CDS) but did not find any mutation (unpublished data).

### 3. RET mediated gene expression in ENS precursors

The 12 HSCR genes that have been identified so far can be roughly classified into three groups: those involved in the RET pathways (*RET*, *GDNF*, *NTN*), those involved in EDNRB pathways (*EDNRB*, *EDN3*, *ECE-1*), and transcription factors that can affect both RET and/or EDNRB pathways (*SOX10*, *ZFXH1B*, *PHOX2B*). These three protein groups/pathways, however, are not totally independent. For instance, a genome-wide association study using 43 trios from a genetically isolated Mennonite population reported statistically significant joint transmission of RET and EDNRB alleles.<sup>22,23</sup> Furthermore, mice homozygous for the recessive hypomorphic allele of *Ednrb* (*Ednrb*S or Piebald) and heterozygous for a *Ret* null mutation (*Ret*<sup>+/-</sup>) showed high frequencies of aganglionosis. These findings suggest epistasis between EDNRB and RET, and this idea was further supported by *Ret/Ednrb* mouse crosses.<sup>24</sup> Similar studies showed that, whereas *Ret*<sup>51/51</sup> and *Edn3*<sup>ls/ls</sup> (lethal spotting) mice display colonic aganglionosis, combinations of these mutant alleles lead to almost complete intestinal aganglionosis.<sup>25</sup> Moreover, the transcription factor SOX10 is involved in regulating both *RET* and *EDNRB* expression.<sup>26-28</sup> PHOX2B is also a transcription factor that is expressed in several classes of differentiating neurons of both the peripheral and central nervous systems. Mice with a homozygous disruption of *Phox2b* show no enteric ganglia and no *Ret* expression, which suggests that *Phox2b* might play a regulatory role in *Ret* expression.<sup>29</sup>

These data together indicate that all the known HSCR genes seem to be connected, physically or indirectly, and they point to a central role for RET in the development of both HSCR and the enteric nervous system (ENS) development. Therefore, understanding the protein networks in which the HSCR associated proteins are involved, in particular the RET associated pathways, might help us not only in understanding ENS development, but also in identifying the missing HSCR genes.

However, these pathways are still largely unknown, in particular the signaling pathways regulated by RET in ENCCs. To gain more insight into the RET protein network, we performed gene expression profiling studies on RNA isolated from mouse ENCCs, untreated or treated with GDNF, the ligand for the RET receptor (**Chapter 5**). We compared these data to

each other and also to the expression profile from the whole mouse embryonic gut (all E14.5). The data were used to perform pathway analysis using GSEA and for single gene analysis.

With single gene analysis we identified genes that were differentially expressed in ENCCs with GDNF compared to those in untreated ENCCs. Moreover, by having gene expression profiling from the mouse gut, we also identified genes that were differentially expressed in ENCCs compared to those in the gut. This group of genes could be considered as the ENS marker candidate resource, as Sox2 that was recently identified as a maker of ENS progenitor cells by Heanue et al. was listed in this group.<sup>30</sup> Two HSCR-associated genes, *Phox2b* and *Ece1*, were up- and down-regulated respectively in ENCCs compared to those in the gut. By comparing the gene expression profiling of ENCCs treated with GDNF to those in the gut, we were able to identify more HSCR-associated genes, including *Ret* itself, *Phox2b*, *Edn3*, *Nrg1*, *Gfra1* and *Nrtn*. This led to the question whether these genes, including *RET*, are down-effectors of the RET signaling pathway and hence RET signaling would have a positive feedback loop system. As it was known from a previous study that PHOX2B is a transcription activator for RET expression, the up-regulation of PHOX2B upon GDNF stimulation, as shown in this study, would be a possible mechanism of the RET positive feedback loop system. We also identified *Snca* ( $\alpha$ -Synuclein), a gene known to be involved in Parkinson's disease, as being up-regulated in ENCCs and further up-regulated upon GDNF stimulation. Our data suggest that defects in RET signaling would most likely reduce the expression of  $\alpha$ -synuclein. Interestingly, in Parkinson's patients, the presence of Lewy bodies (aggregation of  $\alpha$ -synuclein) in their gut is associated with chronic constipation. These findings suggest that  $\alpha$ -synuclein might play an important role in ENS functioning. Recently, it has been proposed that detecting Lewy bodies in the gut could be a method for the early detection of Parkinson's disease.

Despite many interesting new findings in single gene analysis, it is still rather difficult to gain a bigger picture and conclude what actually happens when we stimulate RET with GDNF in ENCCs. RET is believed to be important for ENCCs proliferation, differentiation, migration and survival during ENS development, but it has not yet been reported in detail which signaling pathways triggered by RET play these roles. To get more insight into the RET signaling pathway in ENCCs, we therefore decided to analyze our data by pathway analysis, using the gene set enrichment analysis (GSEA) method.<sup>31</sup> The results showed that some pathways previously implicated in ENS, as well as in embryonic development, such as Tgf $\beta$ /Bmp, Notch and Wnt were down-regulated in ENCCs upon GDNF stimulation. These pathways are known to be important for cell differentiation and neurogenesis. It has been reported earlier that GDNF stimulation inhibits cell apoptosis of ENCCs.<sup>32</sup> In this study, we showed that the signaling pathways important for inducing cell apoptosis, e.g. p53 and TOLL-like

receptors (TLRs), were down-regulated, and the set of genes that induce cell cycle arrest in the cell cycle pathway (p53, mdm2, p21) were also down-regulated. Our study showed that to induce cell proliferation, instead of activating cell division genes, the RET signaling pathway represses the negative regulators of cell cycle genes. GDNF is known to be a chemo-attractant for ENCCs and to stimulate their migration and we showed that GDNF down-regulated adherens and tight junction pathways in ENCCs. As a consequence the cell-cell contact might loosen and thereby stimulate cell migration. This process is a possible mechanism for how GDNF encourages ENCCs to migrate during ENS development. Altogether, there is a general trend in our data that genes encoding proteins that stimulate cell differentiation, negative regulators of cell proliferation, and those important for cell-cell adhesion and apoptosis are down-regulated in ENCCs upon treatment with GDNF. On the other hand, genes that encode proteins that have the opposite effect, such as genes for early growth response, those involved in inhibiting cell differentiation, and those important for energy supply and biosynthesis, are up-regulated.

It is known that the TLRs signaling pathway can induce cell apoptosis and innate immune response by stimulating pro-inflammatory cytokine expression. Down-regulation of TLRs expression upon GDNF stimulation in ENCCs might repress the expression of pro-inflammatory cytokines. Furthermore, to our surprise, the JAK-STAT pathway that is known to be activated by RET-GDNF was down-regulated in our data set.<sup>33</sup> We went through the genes listed in the leading subset of genes from GSEA analysis of the JAK-STAT signaling pathway and found many of them are genes encoding cytokines and cytokine receptors. Based on data of these two pathways, it seems that RET is involved in regulating the innate immune response and GDNF activation down-regulates this response in ENCCs. We confirmed Tlrs expression in mouse gut (E14.5) by immunostaining and showed that Tlr2, Tlr4 and Tlr5 are indeed expressed in neuron, glial and ENS progenitor cells in the myenteric plexus. We also confirmed by qRT-PCR that GDNF down-regulates the expression of *Tlr2*, *Tlr4* and *Tlr5* and some pro-inflammatory cytokines, such as *Tnfa* and *Il-6*. These data showed that ENCCs have an immunomodulatory capacity and that the RET signaling pathway is involved in regulating the innate immune response. Defects in RET signaling, as often seen in HSCR patients, will induce TLRs expression and subsequently pro-inflammatory cytokine expression, and activate the innate immune response. Overexpression of pro-inflammatory cytokines in the gut might lead to HSCR-associated enterocolitis. However, the level of *Tnfa* expression in ENCCs was very low so that we could not detect protein levels by ELISA. On the other hand, for the *Il-6*, even though we were able to detect its protein level by ELISA, we were not able to detect the changes in protein level in ENCCs, untreated or treated with GDNF. We think that even

though ENCCs may play a role in the immune response, their response is not as fast as that of immune cells and that it therefore takes much longer for them to produce pro-inflammatory cytokines. The immune response is a very complex process, where the cross-talk between cytokines produced by different cells is needed to modulate the response. The *in vitro* study that we performed might not be a perfect model for studying such a complex process. A better and more straightforward method to prove the involvement of RET-GDNF signaling in the immune response would be an *in vivo* study using transgenic mouse bearing RET<sup>(+/+)</sup> and RET<sup>(-/-)</sup>, and wild-type mouse, to sensitize them with bacteria and virus infections in their gut and compare their sensitivity in developing enterocolitis. We could also perform quantitative immunostaining for TLRs and RET in myenteric plexus and check for its association with enterocolitis development in these mice. This study could be combined with *in vitro* studies on ENCCs isolated from the transgenic and wild-type mice mentioned above. We could then check the expression levels of the pro-inflammatory cytokines upon stimulation with various pathogens recognized by TLRs (e.g. LPS, LPA, Flagellin).

## 4. Overall conclusion

In this thesis we describe both genetic and functional studies performed to identify and characterize HSCR-associated mutations and genes. Our work clearly shows the many difficulties encountered when working on a complex disease.

Identification of a gene in a susceptibility locus proved difficult. Both our attempts on chromosomes 4 and 9 were largely unsuccessful (**Chapters 2 and 3**). Larger patient cohorts for the association and exome sequence studies and better enrichment kits for the exome sequencing are necessary to be more successful.

Moreover, we should not forget that non-coding sequence variants can also play an important role in complex diseases (**Chapters 4**). This should certainly be taken into consideration if exome sequencing proves unsuccessful (**Chapter 3**). Importantly, we showed that multiple SNPs on a haplotype can be involved in disease development.

Finally, we gained insight into the signaling properties of RET in ENCCs (**Chapter 5**). Not only does this study offer new insight into ENS-related processes and a long list of candidate HSCR genes, it also made us hypothesize that RET could be a factor in the occurrence of enterocolitis in HSCR patients.



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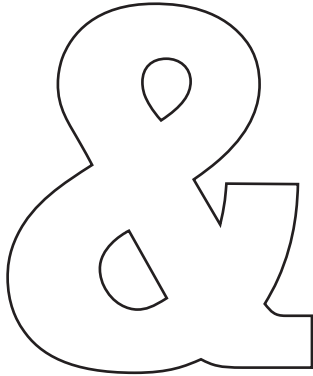
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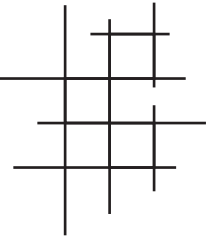
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**Summary**  
**Samenvatting**  
**Ringkasan**  
**List of Abbreviation**  
**Acknowledgments**



## Summary

In **Chapter 1**, we review the genetics, the developmental background, the clinical characteristics, and the diagnosis and treatment of Hirschsprung disease (HSCR). HSCR is a congenital disease characterized by the absence of ganglia in the myenteric and submucosal plexus of the gastrointestinal (GI) tract. The incidence of HSCR is 1 in 5000 live births and most HSCR patients present as sporadic cases, although in about 5-15% of the cases the disease is familial. In about 70% of the cases, HSCR occurs as an isolated trait, while the rest are associated with other diseases or syndromes. So far at least 12 genes and five loci (9q31, 4q31-q32, 3p21, 19q21 and 16q23) have been found associated with HSCR development. *RET* is a major genetic risk factor for HSCR as *RET* coding sequence (CDS) mutations are identified in 50% of the familial cases and in 15-35% of the sporadic cases. Mutations in 11 other genes (*EDNRB*, *EDN3*, *GDNF*, *NTN*, *SOX10*, *PHOX2B*, *ECE1*, *KIAA1279/KBP*, *ZFHX1B*, *NRG1* and *TTF1*) occur in around 20% of the cases, most of which prove to be syndromic. This leads to our hypothesis that there are probably several other disease-causing genes.

Bolk and colleagues identified linkage to 9q31 in five large HSCR families, all of which also showed linkage to *RET* (10q11.2), but none of them had a clear *RET* coding mutation and in one family that was not linked to *RET* and obviously did not have a *RET* coding mutation. In **Chapter 2** of this thesis, we describe the fine mapping of this locus, to identify the disease-contributing gene. We genotyped 301 tag-SNPs spanning 7 Mb on 9q31 region in 137 HSCR Dutch trios, of whom 121 probands did not carry a *RET* CDS mutation while 16 probands did. We identified two HSCR-associated SNPs in the gene *SVEP1* (rs10816998 and rs7038415) in patients not having a *RET* CDS mutation confirming Bolk's data. However, this result was not replicated in 107 independent HSCR Dutch patients without a *RET* CDS mutation. In a Chinese HSCR population, HSCR was found associated with SNPs in *IKBKAP* but not in *SVEP1*. This association was stronger in patients with *RET* CDS mutations than those without *RET* CDS mutations. The data was confirmed in an independent Chinese HSCR cohort. We concluded that association with chromosome 9 is population-specific and that in Chinese HSCR patients the gene likely involved is *IKBKAP*. The data, as found in the Chinese patients, further implies that Chinese *RET* mutation carriers may have an additional risk through this associated *IKBKAP* variant.

In a previous linkage study performed by Brooks and colleagues on a Dutch multi-generational HSCR family, linkage was identified for a region on 4q31-q32. In **Chapter 3** we tried to identify the disease-causing mutation in this linkage region. All of this was done by exome sequencing. We identified only one variant in the linkage region, in exon 20 of the *LRBA* gene.

The *LRBA* gene harbors *MAB21L2* and *MAB21L2* plays a role in ENCCs migration during ENS development. It was therefore postulated that this variant might influence not *LRBA* but *MAB21L2*. However, although this variant was present in all five affected family members, it was also identified in 0.9% of healthy controls (4/440). It is thus unlikely that this variant is the disease-causing variant. But as this was the only variant we identified in the linkage region, we assume that we must have missed the causative mutation, possibly because the enrichment method and criteria we used was only able to analyze 65% of the target region properly. We will therefore repeat this experiment with a better enrichment method which covers all the exons in the genome.

Previous studies showed that, regardless of the *RET* CDS mutation status, almost all familial cases of HSCR are linked to the *RET* locus. Furthermore, association studies performed by several groups in Caucasian and Asian populations revealed a strongly associated *RET* haplotype. This haplotype covers 27 kb, starting 4 kb upstream of *RET* through intron 1 to the beginning of exon 2. Some of the variants present on this haplotype have been reported or suggested as HSCR-causative variants. However, for some associated variants, in particular SNP rs2506004 (C>A), which is present in an enhancer region for *RET*, the pathogenic nature has not yet been examined. In **Chapter 4**, we describe functional studies performed on this SNP (rs2506004, C>A). By *in silico* analysis we found that the C-allele (wild type) is located in a Central Midline Element (CME) sequence element, which is a binding site for Nxf/Arnt2 and Sim2/Arnt2, transcription activators and repressors, respectively. Luciferase assays showed that the enhancer element containing the A- (disease-associated) allele, when coupled to the *RET* promoter, reduced Luciferase expression compared to that measured for the same construct having the C- (non-disease associated) allele, or to the construct having the *RET* promoter only. We checked the binding affinity of Nxf/Arnt2 and Sim2/Arnt2 heterodimers to the oligos containing the C-allele or A-allele by EMSA and a supershift assay. We confirmed that Nxf/Arnt2 binds to the C-allele much more strongly than to the A-allele, while Sim2/Arnt2 has an equal binding capacity to the A- and C-alleles. The effects of Nxf/Arnt2 and Sim2/Arnt2 on the expression of Ret were further confirmed by co-transfection of constructs containing *Nxf/Arnt2* or *Sim2/Arnt2* into a mouse neuroblastoma (Neuro2A) cells line. We checked the endogenous Ret expression on protein level by Western blot and could show that Nxf/Arnt2 and Sim2/Arnt2 increased and decreased the endogenous expression of Ret, respectively. SIM2 is located in the Down syndrome's (DS) critical region in chromosome 21, and interestingly, 77% of DS patients have gastrointestinal (GI) abnormality, in which 2-15% of the cases have HSCR disease. As we proved that Sim2 is a transcription repressor of Ret, this might partly explain the high incidence of HSCR disease in DS patients.

All the published data indicate that the known HSCR genes identified so far seem to be connected, physically or indirectly. Moreover, they point to a central role for RET in the development of both HSCR and the enteric nervous system (ENS). Therefore, understanding the protein networks in which the HSCR-associated proteins are involved, in particular the RET-associated pathways, might help us to not only in understanding better ENS development, but also to identify candidate HSCR genes. However, these pathways are still largely unknown, in particular the signaling pathways that are regulated by RET in neural crest cells (ENCCs). To gain more insight into the RET protein network, we performed gene expression profiling studies on RNA isolated from mouse ENCCs, untreated or treated with GDNF, the ligand for the RET receptor (**Chapter 5**). We compared these data to each other and also to expression profiles from the whole mouse embryonic gut (all E14.5). The data were used to perform pathway analysis using gene set enrichment analysis (GSEA) and for a single gene analysis. The results of the pathway analysis showed a general trend in ENCCs treated with GDNF, towards down-regulation of genes encoding proteins that stimulate cell differentiation, negative regulators of cell proliferation, and proteins important for cell-cell adhesion and apoptosis. On the other hand, genes that encoded proteins that have the opposite effect, such as genes for early growth response, those involved in the inhibition of cell differentiation, and genes that are important for energy supply and biosynthesis, were up-regulated.

The Tlr5 signaling pathway which plays a role in cell apoptosis and the innate immune response was also down-regulated by GDNF stimulation. By immunostaining we confirmed that Tlr2, Tlr4 and Tlr5 were expressed in the neuron, glial and ENS progenitor cells in the myenteric plexus of mouse gut E14.5. We also confirmed by qPCR that down-regulation of Tlr5 in ENCCs by GDNF stimulation reduced the mRNA level of the pro-inflammatory cytokines *Tnfa* and *Il-6* significantly. This result showed, for the first time, the connection between RET signaling and the innate immune response of ENCCs in the gut. This suggests that the inactivation of RET (e.g. as in RET CDS mutations in HSCR patients) could lead to the activation of the innate immune response (i.e hypersensitivity of immune response), which might manifest itself as HSCR-associated enterocolitis.

Together, our studies led to the identification of a candidate HSCR gene in the 9q31 locus, specifically for Chinese HSCR patients. We revealed a possible mechanism for SNP rs2506004 in regulating the expression of *RET* and hence revealed how the disease-associated allele contributes to disease development. Furthermore, we studied the RET signaling pathway in ENCCs in more detail. Our studies have led to more insight into HSCR- and ENS development, the genes and pathways involved, and how HSCR might develop.

## Samenvatting

In **hoofdstuk 1** bespreken we de genetica, de embryologische ontwikkeling, de klinische kenmerken en de diagnose en behandeling van de ziekte van Hirschsprung (HSCR). HSCR is een aangeboren ziekte die wordt gekenmerkt door de afwezigheid van ganglia in de myenteric en submucosale plexi van het maag-darmkanaal. De incidentie van HSCR is 1 op de 5000 levend geboren. De meeste HSCR patiënten zijn sporadisch (zijn de enige in de familie) maar familiale HSCR komt wel degelijk voor. We zien dit in ongeveer 5-15% van alle gevallen. In 70% van alle gevallen hebben de patiënten alleen HSCR, terwijl de overige 30% van de patiënten naast HSCR ook een andere ziekte of een syndroom heeft. Tot nu toe zijn 12 genen en vier chromosomale gebieden (dit noemen we loci) gevonden die geassocieerd zijn met de ontwikkeling van HSCR. *RET* is de belangrijkste erfelijke risicofactor voor HSCR, omdat in 50% van de familiale gevallen een *RET* mutatie wordt gevonden. Dit getal is lager in de niet-familiaire gevallen (15-35%). Mutaties in de 11 andere genen (*EDNRB*, *EDN3*, *GDNF*, *NTN*, *SOX10*, *PHOX2B*, *ECE1*, *KIAA1279/KBP*, *ZFH1B*, *NRG1* en *TTF1*) verklaren ongeveer 20% van de rest van de gevallen. De meeste patiënten met mutaties in de 11 andere genen blijken een syndroom te hebben waar HSCR een onderdeel van is. Omdat een groot deel van de patiënten geen mutatie heeft in de 12 genen is onze hypothese dat er waarschijnlijk nog verscheidene andere ziekte veroorzakende genen moeten zijn.

Bolk en collega's identificeerden een koppeling van de ziekte aan een stukje van chromosoom 9 (9q31). Ze vonden dit in vijf grote HSCR families. Vier van deze families bleken echter ook te koppelen met het *RET* gen (10q11.2), maar in geen van deze families was een duidelijke *RET* mutatie gevonden. De vijfde familie, die niet gekoppeld kon worden aan het *RET* gen, had logischerwijs ook geen *RET* mutatie. In **hoofdstuk 2** van dit proefschrift beschrijven we de zoektocht naar de genmutatie in dit gebied op chromosoom 9. We genotypeerden 301 tag-SNPs (Single Nucleotide Polymorphism (SNP)) verspreid over het gekoppelde gebied in 137 Nederlandse HSCR patiënten en hun ouders (121 hadden geen *RET* mutatie, 16 hadden dat wel). We identificeerden 2 HSCR geassocieerde SNPs in een gen genaamd *SVEP1* (rs10816998 en rs7038415). We vonden de associatie in patiënten die geen *RET* mutatie hebben. Dit bevestigde de data van Bolk. Echter, dit resultaat herhaalde zich niet in 107 onafhankelijke Nederlandse HSCR patiënten zonder een *RET* mutatie. In een Chinese HSCR populatie, bleek ook een associatie aanwezig te zijn maar met een ander gen, genaamd *IKBKAP*. Deze associatie was sterker in de groep patiënten met een *RET* mutatie dan in de groep zonder *RET* mutatie. De Chinese data kon wel worden bevestigd in een onafhankelijke groep Chinese HSCR patiënten. We concludeerden daarom dat de associatie met chromosoom 9 populatie specifiek



is en dat in de Chinese HSCR patiënten waarschijnlijk het gen *IKBKAP* betrokken is bij de ontwikkeling van de ziekte.

In een studie, beschreven door Alice Brooks en haar collega's werd in een grote Nederlandse HSCR familie koppeling gevonden tussen de ziekte en een regio op chromosoom 4 (4q31-q32). In **hoofdstuk 3** hebben we een poging gedaan het gen en de genmutatie te vinden die de ziekte in deze familie veroorzaakt. Om dit te doen hebben we alle genen in het genoom geanalyseerd ('Exoom sequencing'). We vonden één variant in het gekoppelde gebied, in exon 20 van het *LRBA* gen. In het *LRBA* gen ligt ook een ander gen, te weten *MAB21L2*. Van dit gen is bekend dat het een rol speelt in de migratie van NCCs en dus betrokken is bij de ontwikkeling van het zenuwstelsel van de darm. Mogelijk zou een variant in *LRBA* een invloed kunnen hebben op *MAB21L2* en dus indirect HSCR kunnen veroorzaken. Toen we echter controles gingen analyseren op aanwezigheid van de variant bleek deze ook in de normale populatie voor te komen (4/440), wat het zeer onwaarschijnlijk maakt dat de variant de ziekte kan veroorzaken. Waarschijnlijk hebben we dus de mutatie gemist wat mogelijk komt doordat we niet alle genen in het koppelingsgebied helemaal hebben kunnen bekijken. We hebben kunnen berekenen dat we slechts 65% van alle genen hebben bekeken, dus mogelijk zit er een mutatie in het missende deel. Daarom gaan we dit experiment herhalen.

Eerdere studies lieten zien dat, ongeacht het hebben van een mutatie in het *RET* gen, bijna alle familiale gevallen aan *RET* zijn gekoppeld. Dit blijkt echter niet alleen het geval te zijn voor de familiale gevallen, het is ook zo voor de sporadische patiënten. Associatie studies, uitgevoerd op patiënten groepen uit Europa en Azië, lieten sterke associatie zien met één specifiek *RET* haplotype. Dit haplotype spreidt zich uit over een gebied van 27.000 bp, startende 4000 bp upstream van de *RET* startsite tot en met het einde van exon 2. Een aantal aanwezige varianten in dit gebied is gemeld of gesuggereerd als HSCR-veroorzakende varianten. Echter, niet voor alle geassocieerde varianten, in het bijzonder SNP rs2506004 (C>A) is de pathogeniciteit ook daadwerkelijk onderzocht. In **hoofdstuk 4** beschrijven we functionele studies die voor deze SNP (rs2506004, C>A) zijn uitgevoerd. Met behulp van *in silico* analyse hebben we gevonden dat het C-allel (wild type) gelokaliseerd is in een Central Midline Element (CME) sequentie element, dat een bindingsplaats is voor de eiwitcomplexen Nxf/Arnt2 en Sim2/Arnt2, respectievelijk transcriptie activatoren en repressors. Luciferase assays lieten zien dat de sequentie met het ziekte geassocieerde allel, wanneer zij gekoppeld is aan de *RET* promotor, verminderde expressie liet zien als we die vergeleken met het niet ziekte geassocieerde allel. We controleerden de bindingsaffiniteit van de Nxf/Arnt2 en Sim2/Arnt2 heterodimeren aan DNA oligos met het C-allel of het A-allel met behulp van EMSA (elektroforetische mobiliteit shift assay) en een supershift assay. We bevestigen dat Nxf/Arnt2 veel sterker

bindt aan het C-allel dan aan het A-allel, terwijl Sim2/Arnt2 een gelijke bindingscapaciteit heeft voor de A- en C-allelen. De effecten van Nxf/Arnt2 en Sim2/Arnt2 op de expressie van *RET* werden verder bevestigd door co-transfecties met constructen die Nxf/Arnt2 of Sim2/Arnt2 tot expressie brachten in een muize neuroblastoma (Neuro2A) cellijn. We controleerden de endogene *RET* expressie op eiwit niveau met behulp van Western blotting en konden laten zien dat Nxf/Arnt2 en Sim2/Arnt2, respectievelijk de endogene expressie van *RET* verhogen en lieten afnemen. We hebben hiermee aangetoond dat de SNP daadwerkelijk bij de ziekte is betrokken. Sim2 bevindt zich in het kritische gebied voor Down syndroom op chromosoom 21. 77% van de patiënten met het syndroom van Down hebben maagdarm (GI) afwijkingen, waarvan 2-15% van de gevallen HSCR hebben. Omdat Sim2 een transcriptie repressor voor *RET* is, zou overexpressie van SIM2 veroorzaakt door een extra kopie van het gen, de hoge incidentie van HSCR in Down syndroom patiënten mogelijk kunnen verklaren.

Alle gepubliceerde data geven aan dat de tot nu toe geïdentificeerde HSCR genen direct of indirect met elkaar verbonden zijn. Bovendien wijst alle data op een centrale rol voor *RET* in de ontwikkeling van zowel HSCR als het zenuwstelsel van de darm. Het ontrafelen van het *RET* geassocieerde eiwitnetwerk zou ons daarom niet alleen inzicht geven in hoe het zenuwstelsel in de darm zich ontwikkelt, maar ook zou het helpen in het identificeren van kandidaat HSCR genen. Deze kennis, en dan met name de eiwitnetwerken rond *RET* in de voorloper celen van het darm zenuwstelsel enteric neurale-lijst-cellen (ENCCs), ontbreekt helaas nog. Om hier inzicht in te krijgen hebben we genexpressie studies uitgevoerd op NCCS geïsoleerde uit de darm van de muis. In deze cellen hebben we *RET* geactiveerd of juist niet door ze te behandelen met GDNF, de activator (ligand) voor de *RET* receptor (**hoofdstuk 5**). De genexpressie data van de ENCCs hebben we met elkaar vergeleken (wel of niet gestimuleerd met GDNF) en vergeleken met het expressieprofiel van de gehele (embryonale) muizendarm. De data hebben we geanalyseerd met behulp van een programma dat kijkt naar signaalroutes en gennetwerken die verrijkt zijn in de dataset waarin de onderzoeker geïnteresseerd is ('gene set enrichment analysis, GSEA') en we hebben de data geanalyseerd per gen (zogenaamde 'single gene analysis'). Uit de resultaten die gegenereerd waren met behulp van het GSEA programma bleek dat, over het algemeen, een activatie van *RET* resulteert in een lagere expressie van genen die coderen voor eiwitten die betrokken zijn bij celdifferentiatie, eiwitten die celdeling negatief beïnvloeden en eiwitten die belangrijk zijn voor cel-cel interacties en apoptose. De expressie gaat echter juist omhoog voor genen die coderen voor eiwitten die celdifferentiatie tegengaan en eiwitten die een rol spelen in de energie bevoorrading en biosynthese.

Een eiwitnetwerk dat ook als geheel een lagere expressie liet zien was de Toll like recep-

tor (Tlr) signaalroute, een route betrokken bij apoptose en bij de aangeboren (aspecifieke) immuunrespons. We hebben met behulp van immuno-kleuringen kunnen bevestigen dat Tlr2, Tlr4 en Tlr5 inderdaad tot expressie komen in de neuronen, de glia en de zenuwcel voorlopercellen (ENCCs) in de darmwand van de muis. We konden ook laten zien dat GDNF stimulatie van RET leidt tot een lagere expressie van de eiwitten die door Tlrs worden geactiveerd, Tnfalpha, and IL-6. We laten hiermee voor het eerst zien dat RET activiteit een effect heeft op de de aangeboren immuunrespons van de ENCCs die zich in de darm bevinden. HSCR patiënten hebben vaak RET mutaties die resulteren in een verlaging van de activiteit van RET en dit zal daarom zorgen voor een hogere immuunrespons. HSCR patiënten hebben vaak last van chronische ontstekingen aan de darm (HSCR-associated enterocolitis). Onze bevindingen zouden dit mogelijk kunnen verklaren.

Concluderend kunnen we zeggen dat onze studies hebben geresulteerd in de identificatie van een HSCR gen in het 9q31 gebied, specifiek voor Chinese HSCR patiënten. Verder hebben we kunnen aantonen dat SNP rs2506004 betrokken is bij het ontstaan van HSCR en hebben we het mechanisme achter de mutatie opgehelderd. Ook hebben we ons inzicht vergroot met betrekking tot het RET eiwit netwerk in de ENCC. Onze resultaten geven meer duidelijkheid over het ontstaan van het zenuwstelsel van de darm, over de genen betrokken bij HSCR en leveren een lijst van goede kandidaatgenen voor HSCR op.

## RINGKASAN

**Bab 1** mengulas genetika, biologi perkembangan, karakteristik klinis, diagnosis dan pengobatan penyakit Hirschsprung (HSCR). HSCR adalah penyakit bawaan yang ditandai oleh ketidakadaanya ganglia dalam pleksus submukosa dan *myenteric* di saluran pencernaan. Insiden penyakit HSCR adalah 1 dalam setiap 5000 kelahiran, yang mana mayoritas merupakan kasus sporadis dan sekitar 5-15% merupakan kasus turunan. Sekitar 70% kasus merupakan kasus HSCR terisolasi sedangkan sisanya merupakan kasus sindromik, yaitu HSCR yang disertai sindrom lainnya. Sampai saat ini, 12 gen dan lima lokus (9q31, 4q31-P32, 3p21, 19q21 dan 16q23) telah teridentifikasi berasosiasi dengan perkembangan penyakit HSCR. Gen *RET* adalah faktor genetik utama penyakit HSCR dan mutasi pada gen *RET* (*CDS*) teramati pada sekitar 50% kasus turunan dan pada 15-35% kasus sporadis. Mutasi pada 11 gen lainnya (*EDNRB*, *EDN3*, *GDNF*, *NTN*, *SOX10*, *PHOX2B*, *ECE1*, *KIAA1279/KBP*, *ZFHX1B*, *NRG1* dan *TTF1*) hanya terjadi pada sekitar 20% kasus, yang sebagian besar ditemukan pada kasus sindromik. Hal ini mengarah pada hipotesis bahwa terdapat gen-gen lain yang berkontribusi kepada perkembangan penyakit HSCR.

Bolk dan kolega mengidentifikasi lokus 9q31 berasosiasi dengan HSCR pada enam keluarga. Lima diantaranya menunjukkan asosiasi dengan lokus 10q11.2, tempat gen *RET* berada.

**Bab 2** memaparkan pemetaan lokus 9q31 dalam mengidentifikasi gen yang berkontribusi terhadap perkembangan penyakit HSCR. Kami memeriksa genotip 301 tag-SNP (*single nucleotide polymorphism*) yang mencakup 7 Mb (juta pasang basa) pada lokus 9q31 dari sampel DNA 137 penderita penyakit HSCR beretnis Belanda beserta DNA orang tuanya (data trios). Sebanyak 121 diantara penderita tersebut tidak memiliki mutasi pada gen *RET*, sementara 16 lainnya memiliki mutasi pada gen *RET*. Kami mengidentifikasi dua SNP pada gen *SVEP1* (rs10816998 dan rs7038415) berasosiasi dengan HSCR pada pasien yang tidak memiliki mutasi gen *RET*. Akan tetapi, hasil ini tidak dapat dikonfirmasi ulang pada *cohort* pasien lain dari etnis yang sama (107 pasien etnis Belanda yang tidak memiliki mutasi *RET*). Percobaan serupa dilakukan pula dengan sampel DNA yang diperoleh dari penderita penyakit HSCR beretnis Cina. Pada penelitian yang menggunakan pendekatan *case-control study* ini, teridentifikasi beberapa SNP yang terletak pada gen *IKBKAP* berasosiasi dengan HSCR. Asosiasi ini lebih kuat pada pasien dengan mutasi *RET* dibandingkan pasien tanpa mutasi *RET*. Data ini dapat dikonfirmasi ulang pada *cohort* HSCR pasien etnis Cina dan dapat disimpulkan bahwa asosiasi kromosom 9 dengan HSCR adalah populasi spesifik dan pada pasien HSCR etnis Cina terdapat kemungkinan keterlibatan gen *IKBKAP*. Data yang diperoleh menunjukkan bahwa pembawa mutasi *RET* pada etnis Cina dapat memiliki tambahan resiko menderita

penyakit HSCR jika memiliki varian gen *IKBKAP*.

Melalui metoda *Linkage analysis*, Brooks dan kolega mengidentifikasi locus 4q31-32 berasosiasi dengan HSCR pada keluarga multi-generasi etnis Belanda. **Bab 3** memaparkan penelitian guna mengidentifikasi gen pada lokus 4q31-32 yang bertanggung jawab atas perkembangan penyakit HSCR pada keluarga multi-generasi tersebut. Variasi pada exon 20 gen *LRBA* dapat diidentifikasi dengan menggunakan teknik *exome sequencing*. Pada untai DNA *anti-sense* gen *LRBA* terdapat gen *MAB21L2* yang berperan penting pada proses migrasi ENCCs saat perkembangan ENS. Penelitian telah menunjukkan bahwa beberapa daerah gen *LRBA* adalah pengatur ekspresi gen *MAB21L2*. Hal ini menimbulkan spekulasi bahwa varian yang ditemukan pada exon 20 gen *LRBA* adalah bagian dari daerah pengatur ekspresi *MAB21L2*. Varian gen *LRBA* ini terbukti dimiliki oleh seluruh penderita HSCR pada keluarga tersebut di atas. Namun demikian, varian ini juga ditemukan pada 4 dari 440 kromosom kontrol yang diperiksa (0.9% dari populasi). Oleh karena kemungkinan varian tersebut adalah varian yang bertanggung jawab terhadap perkembangan penyakit HSCR adalah kecil. Akan tetapi, karena varian pada exon 20 gen *LRBA* adalah satu-satunya varian yang teridentifikasi pada lokus 4q31-32, maka besar kemungkinan varian penyebab HSCR yang sesungguhnya belum teridentifikasi. Hal ini bisa disebabkan karena metoda pengayaan (*enrichment method*) dan analisis *exome sequencing* yang digunakan hanya mampu menganalisis sekitar 65% exon genom manusia. Oleh karena itu, penelitian selanjutnya akan menggunakan *exome sequencing* dengan metoda pengayaan yang mencakup seluruh exon genome manusia.

Penelitian sebelumnya menunjukkan bahwa, terlepas dari status mutasi gen *RET*, hampir seluruh kasus HSCR turunan berasosiasi dengan lokus *RET*. *Association study* yang telah dilakukan oleh beberapa kelompok peneliti atas populasi Kaukasia dan Asia mengidentifikasi adanya *general RET haplotype* yang berasosiasi dengan penyakit HSCR. *Haplotype* ini mencakup 27 kb (ribu pasang basa), mulai dari 4 kb di bagian hulu gen *RET*, intron 1 dan sampai ke bagian awal ekson 2. Beberapa varian yang diidentifikasi pada *haplotype* ini telah dilaporkan dan diusulkan sebagai varian yang terlibat dalam perkembangan penyakit HSCR. Namun pengujian fungsi beberapa varian seperti halnya SNP rs2506004 (C> A) yang terletak di intron 1 gen *RET* belum pernah dilakukan. **Bab 4** memaparkan hasil uji fungsi SNP rs2506004 (C> A). Melalui analisis *in silico* teridentifikasi bahwa C-alel (*wild type*) SNP rs2506004 terletak pada elemen *Central Nervous System Midline Enhancer* (CME). CME merupakan urutan DNA tempat menempelnya Nxf/Arnt2 dan Sim2/Arnt2 yang masing-masing merupakan aktivator dan repressor transkripsi. *Luciferase assay* menunjukkan penurunan ekspresi gen *Luciferase* jika elemen *enhancer* yang memiliki A-alel (alel yang berasosiasi dengan HSCR) digabungkan dengan *promoter RET*; dibandingkan dengan elemen *enhancer* yang

memiliki C-alel atau dibandingkan dengan *promoter RET* saja (kontrol). Afinitas Nxf/Arnt2 dan Sim2/Arnt2 *heterodimer* terhadap *oligonucleotide* pembawa C-alel atau A-alel kami uji dengan menggunakan metoda EMSA (*Electrophoretic Mobility Shift Assay*) dan *supershift assays*. Hasil pengujian menunjukkan Nxf/Amt2 memiliki afinitas yang lebih tinggi terhadap oligonukleotida pembawa C-alel, sementara Sim2/Amt2 memiliki afinitas yang tidak berbeda terhadap A-alel maupun C-alel. Efek Nxf/Arnt2 dan Sim2/Arnt2 terhadap ekspresi gen *RET* dibuktikan lebih lanjut dengan pemeriksaan level ekspresi protein *RET endogenous* pada sel Neuro2A saat ditransfeksi dengan plasmid pengkode *Nxf/Arnt2* atau *Sim2/Arnt2* melalui metoda *Western blot*. Hasilnya menunjukkan bahwa Nxf/Arnt2 dan Sim2/Arnt2 masing-masing meningkatkan dan menurunkan ekspresi *RET* protein pada sel Neuro2A. Salah satu hal yang menarik adalah, gen *SIM2* terletak pada “*critical region of Down Syndrome (DS)*” pada kromosom 21, yang mana 77% pasien DS mengalami gangguan saluran pencernaan dan 2-15% kasus tersebut adalah HSCR. Penelitian ini telah menunjukkan bahwa *SIM2* adalah repressor transkripsi *RET*. Hal ini dapat menjelaskan tingginya insiden penyakit HSCR pada pasien DS.

Sejauh ini, data menunjukkan gen-gen penyebab penyakit HSCR saling terkait, baik secara langsung maupun tidak, dengan gen *RET*. Data-data tersebut mengarah pada peran sentral gen *RET* pada perkembangan sistem syaraf *enteric* (ENS) dan penyakit HSCR. Oleh karena itu, dengan mempelajari jaringan protein *RET* pada *enteric neural crest cells* (ENCCs) yang merupakan *progenitor* sel neuron dan glial pada ENS dapat membantu memahami proses perkembangan ENS dan mengidentifikasi kandidat gen yang terlibat pada perkembangan penyakit HSCR. Untuk mempelajari jaringan protein *RET* pada ENCCs kami mengisolasi RNA ENCCs mencit yang distimulasi dan tidak distimulasi dengan GDNF. Dengan menggunakan *microarray*, RNA tersebut digunakan untuk memperoleh data profil ekspresi gen (Bab 5). Hasil *microarray* juga dibandingkan dengan profil ekspresi gen dari usus mencit. Hasil analisis dengan menggunakan analisis gen tunggal dan analisis jaringan protein (GSEA) menunjukkan bahwa pada sel yang diberi perlakuan GDNF terdapat kecenderungan sebagai berikut, adanya represi ekspresi gen-gen yang menstimulasi diferensiasi sel, *negative regulator* sel proliferasi dan gen yang penting untuk kontak antar sel serta *apoptosis*. Di sisi lain, terdapat peningkatan ekspresi gen-gen untuk respon awal pertumbuhan, yaitu yang terlibat dalam penghambatan diferensiasi sel, dan gen yang penting dalam pengaturan pasokan energi dan biosintesis.

Selain represi di atas, stimulasi GDNF pun merepresi alur sinyal *Toll-like receptors* (TLRs) yang berperan dalam *apoptosis* dan respon imun bawaan. Kami menunjukkan bahwa Tlr2, Tlr4 dan Tlr5 diekspresikan pada neuron, glial dan progenitor sistem syaraf *enteric* saluran

pencernaan menciit dengan menggunakan teknik *immunostaining*. qRT-PCR mengkonfirmasi bahwa GDNF dapat menurunkan secara signifikan ekspresi gen *TLRs* dan juga beberapa *cytokines* pencetus reaksi inflamasi seperti IL-6 dan TNF $\alpha$ . Untuk pertamakalinya data yang diperoleh menunjukkan bahwa gen *RET* terlibat dalam pengaturan respon imun bawaan. Hal ini mengarahkan pada hipotesa bahwa inaktivasi gen *RET* (seperti halnya mutasi *RET* CDS pada penderita HSCR) akan mengakibatkan aktivasi reaksi imunitas bawaan (*hypersensitivitas*) yang bisa mengarah pada perkembangan penyakit *enterocolitis*.

Hasil penelitian yang telah kami lakukan mengarah pada identifikasi kandidat gen HSCR pada lokus 9q31 penderita HSCR etnis Cina. Kami juga berhasil mengungkapkan mekanisme keterlibatan SNP rs2506004 (C>A) pada pengaturan ekspresi gen *RET* sehingga dapat menjelaskan bagaimana A-alel pada SNP tersebut berkontribusi terhadap perkembangan penyakit HSCR. Selain itu, kami mempelajari jaringan protein *RET* pada ENCCs dengan lebih terperinci. Secara umum hasil penelitian kami memberikan informasi mengenai gen-gen dan jaringan protein yang terlibat pada perkembangan ENS dan bagaimana mekanisme perkembangan penyakit HSCR.

## List of Abbreviations

ARMC9	Armadillo repeat containing 9
ARNT2	Aryl-hydrocarbon receptor translocator 2
ARTN	Artemin
BMPs	Bone morphogenetic proteins
bp	Base pair
BRCA2	Breast cancer 2, early onset
CCHS	Congenital central hypoventilation
CDS	Coding sequences
CME	Central midline element
CNS	Central nervous system
Cp-Ns	Competitive non-specific
Cp-Sp	Competitive specific
CTNNA1	Catenin (adherin-associated protein), alpha-like 1
DAPI	4',6-diamidino-2-phenylindole
dbSNP	Database single nucleotide polymorphism
DIP	Deletion-insertion polymorphism
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DS	Down's syndrome
E14.5	Embryonic day 14.5
ECE1	Endothelin converting enzyme
EDN3	Endothelin 3
EDNRB	Endothelin receptor type B
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimal essential medium
EMSA	Electrophoretic mobility shift assay
ENCCs	Enteric neural crest cells
Enh	Enhancer
ENS	Enteric nervous system
FD	Familial dysautonomia
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
FOX	Forkhead box
GALNACT-2	Chondroitin sulfate N-acetylgalactosaminyltransferase 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATK	Genome analysis toolkit
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFRA1	Glycosyl-phosphatidylinositol-anchored co-receptor of GDNF-1
GI	Gastrointestinal
GOSH	Goldberg-Shprintzen
GSEA	Gene set enrichment analysis
GTP	Guanosine-5'- triphosphate
GWAS	Genome association study
HAEC	Hirschsprung-associated enterocolitis
HOX	Homebox
HSCR	Hirschsprung
IEC	Intestinal epithelial cells
IFN $\gamma$	Interferon gamma
IKBKAP	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein
IL-6	Interleukin 6



JAK	Janus tyrosine kinase
KBP	Kinesin binding protein
KBP	Kilo base
KEGG	Kyoto encyclopedia of genes and genomes
LD	Linkage disequilibrium
L-HSCR	Long-segment Hirschsprung
LOD	Logarithm of the odds
LPS	Lipopolysaccharide
LRBA	LPS-responsive vesicle trafficking, beach and anchor containing
MAB21L2	Mab-21( <i>C.elegans</i> )-like 2
MAF	Minor allele frequency
MAP3K1	Mitogen-activated protein kinase kinase kinase 1
Mb	Mega base pairs
MCS	Multiple-species conserved sequences
MUSK	Muscle, skeletal, receptor tyrosine kinase
NCAM	Neural cell adhesion molecule
NCSCs	Neural crest stem cells
NEC	Necrotizing enterocolitis
NLBs	Neurosphere-like bodies
NPE	Nuclear protein extract
NRG1	Neuregulin 1
NTN	Neurturin
NXF (NPAS4)	Neuronal PAS domain protein 4
OR	Odd ratio
P53	Tumor protein 53
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PHOX2B	Paired-like homeobox 2B
pNEs	Paralogous, non-coding elements
PPARs	Peroxisome proliferators-activated receptors
PSPN	Persephine
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
RASGEF1A	RasGEF domain family, member 1A
RET	Rearrange during transfection
RETprom	RET promoter
RNA	Ribonucleic acid
RTK	Tyrosine kinase receptor
SD	Standard deviation
S-HSCR	Short-segment Hirschsprung
SIM2	Single-minded homolog 2
SNP	Single nucleotide polymorphism
SOX10	SRY (sex determining region Y)-box 10
STAT	Signal transducer and activator of transcription
SV40	Simian vacuolating virus 40
SVEP1	Sushi, von Willebrand factor A, EGF and pentraxin domain
TCA	Total colonic aganglionosis
TDT	Transmission disequilibrium test
TGFb	Transforming growth factor, beta 1
TIA	Total intestinal aganglionosis
TLRs	TOLL-like receptors
TRANSFAC	Transcription factor binding predictions
TTF1	Thyroid transcription factor 1
WNT	Wingless-type MMTV integration site family, member 1
WS4	Shah-Waardenburg syndrome type 4
YFP	Yellow fluorescent protein
ZFXH1B	Zinc finger homeobox protein 1B

## Acknowledgments

This last five years have been a wonderful and interesting journey for me, I have learned many new things and met a lot of new people. Finally, I can finish what I started and feel grateful that I have had so many people helping and encouraging me to get through this PhD period. It is now time for me to express my gratitude to all the people with whom I worked together or who contributed their help and friendship during my PhD program.

First of all I would like to thank my supervisor Prof. Hofstra. Dear Robert, It was at the end of 2004 when I first heard your name from my boss Dr. Joseph Hilgers. He suggested that I go to your lab and do a research project on HNPCC, as this was the project I was working on back then in Indonesia. But it was not until the end of 2005 that I sent you an email and made an appointment to talk about an internship for my MSc degree. Instead of working on the HNPCC project, I asked you to give me another project so I could learn something different. You gave me a project on Hirschsprung (HSCR) disease, a disease that I had never heard of (despite finding out later that my older sister, whom I never met, had passed away because of this disease). Exciting project! I had a lot to learn since my background was not in genetics, but I enjoyed working on this project very much together with Greg Burzynski. This 9-month project was extended for another 5 years with me as your PhD student. You've always been supportive of all my ideas and gave me so much freedom to explore all the things I wanted to learn and do. I learned to be more independent, to trust my own judgment and to be more creative than ever. I am grateful to have met you and I sincerely thank you for all the opportunities, for your trust, your guidance and all the support you have given to me. Thank you for always being there and for being someone that I can talk to in difficult times, this really means a lot to me.

I would like to thank the members of my reading committee, Prof. Isabella Cechirinni, Prof. U.L.M. Eisel and Prof. H.W.G.M. Boddeke, for their time and remarks on my thesis.

I would like to thank the head of Genetics Department (past and present). Dear Prof. Buys, dear Charles, thank you for welcoming me for the very first time when I came to work in this department, thank you for all the good words you always had for me. Dear Prof. Wijmenga, dear Cisca, thank you for all your input and critical comments on my presentations during the Tuesday meetings and for always encouraging the PhD students to broaden their knowledge and learn about other things than only our own research subject.

I would like to thank Jackie Senior for all her time and effort in editing my thesis. Despite the fact that sometimes I wasn't on time handing in my manuscripts, you were always willing to help me. Thank you also for always giving me feedback during my presentations and help-

ing me to improve my English.

I would like to thank Intan Taufiq, Wangsa Tirya Ismaya and Neng Fisheri who helped me by editing the summary in bahasa Indonesia and also to Helga and Bote for helping me with the Dutch summary. Special thanks to Ella Elviana for the cover illustration and to Briyan Hendro for the cover and book layout.

I am grateful to all my collaborators for their contributions to the work presented in this thesis, especially Dr. Marcia Barcelo and Clara, our collaborators in Hong Kong. It was good to work with you on the chromosome 9 paper, thank you for everything and it is always nice to meet and to share experiences at HSCR consortium meetings. Dr. N. Thapar and Dr. A.J. Burn, dear Nikhil and Alan, thank you for welcoming me in your lab and allowing me to work there for five months. It was good experience and I learned a lot during my stay in London. Dr. M. Metzger, dear Marco, thank you for teaching me how to isolate ENCCs from mouse embryos and how to culture them. I enjoyed working with you, Nikhil and Alan on the RET non-coding variants and expression papers and thank you for helping with my experiments and answering questions.

Dear Rudolf, it was nice to work with you on the expression paper. Thank you for helping me analyze the microarray data. The GSEA data turned out to be better than I expected and have led to an interesting hypothesis. Thank you and good luck with your training in oncology.

Dear Wanda, it's nice to know you and it's always been good to talk to you. Thank you for trying your best to get mouse embryos for my experiments, you were really my savior! Dear Evelyn, thank you so much for teaching me the qRT-PCR, all the tips and tricks, from designing the primers to analyzing the data. Dear Lydia and Hans from the Department of Pathology, thank you for helping with the Luciferase assay and LacZ assays experiments.

Dear Alice Brooks, Morris, Pieter, Cleo and Wil, Ian Sheperd, it was great to work with you all on the exome paper and thank you for all your help. Hopefully, next time, we will have more luck and really find the "true" disease-causing variant in this linkage region. Dear Pieter and Cleo, thank you for the CLC-Bio tutorial, you've been a great help. Thanks also to Martijn and Freerk for helping with the pipeline data over the last few months.

Thank you to my Italian friends Isa, Marta and Ivana, for sharing your scientific results on RET regulating the immune response. Thank you also to all the members of the HSCR consortium, it was always great to meet you all and share our latest scientific results.

To my partners in crime in the HSCR/RET group: Greg Burzynski, Maria, Christine, Hans Verbeek, Thomas, Duco and Rajendra. It's been nice to meet you all, share experiences, share bench work and share an office, thank you for everything and for being such good col-

leagues. Dear Greg, it was nice to work with you on my MSc project, and although you are now far away, you've always been willing to help me during my PhD period. Thanks a lot Greg! Dear Maria, we started nearly everything together, and although we come from two different cultures, I am glad we get along so well together and can share our worries, dreams and going through the good and bad times of PhD life. Thank you for everything and I wish you all the best in the future. Dear Christine, life is not the same without you here, I'm less talkative! I really used to talk a lot to you and about almost everything. It doesn't matter which continent you're on, you always have time to chat with me and show concern, not only for my work but for me as your friend. Thank you also to your family who always invited me to share Christmas dinner. It was a good and warm feeling to be part of your family. Good luck with your MD/PhD program and in the future as a medical doctor, I'm sure you will become a great one. Dear Thomas, it was nice to have you here in the department even though it was only for a short period, thank you for your friendship. Dear Duco, it was great to work with you on the expression paper. Dear Rajendra thank you for helping me on the exome paper, both of you were a great help in the last part of my PhD work. I wish you both good luck with the rest of your own PhDs. Dear Amanda and Nina, my students, thank you for all your help in my research project, I wish you all the best with your future plans!

Dear Jan (Osinga), you were the first person in this department who helped me with all my experiments and taught me some lab techniques. It's always nice to work with you, a lot of laughing, sharing a lot of stories and, most of all, you were the person who persistently talked to me in Dutch. :-D It helped improve my Dutch, too bad that we are no longer office mates because my Dutch is disappearing. Thank you also for being one of my *paranimfen*. I wish you good health and happiness always.

I would like to thank to Joke Verheij for helping and provide me with all the information I needed related to HSCR patients. I thank Bart Eggen for always being willing to spend time discussing my research. It's been a great help and you always have an answer to all my questions and gave me so many ideas on how to solve the problems. Dear Conny, Peter, Jorieke, Nicole, Siobhan, thank for your comments and suggestions and sharing your experiences during the Friday meetings.

Dear Lude and Gerard, thank you for helping me with the eQTL data. It was interesting to discuss things with you both together, I felt like I was in the middle of nowhere but "in a good way", since it stimulated my brain to work hard, to the limit. :-D Dear Gerard, your two years in Indonesia meant you could more or less understand my point of view as an Indonesian living in the Netherlands and so that I could talk to you about everything related to cultures, religion and even politics. :-D However, when it comes to statistics, I almost always give

up... This is really not my 'thing' but thank you for helping me. Dear Vinod and Sebo, thank you for your suggestions and helping me check the HSCR genes network using GeneGo.

Dear Ellen and Dineke, thank you for always being willing to answer all my questions. Dear Ellen, I'm so grateful to have you here in this department, you were always enthusiastic about my work and gave me a lot of ideas on how to answer my research question. The Tuesday meetings were much more interesting and fun when you were there with all your questions and comments. Dear Dineke, thank you for being so kind and discussing in detail so many things related to my experiments, like my EMSA results. Thank you too for tricks and tips on all the cloning stuff.

Dear Mentje, Ria, Bote, Joke and Helena, our secretaries, thank you very much for all your help (how will I survive without you all? :-D). I would also like to thank Mathieu for helping me solve the problems with the *SPOED* *bestellingen*. Dear Yvonne, Jan Jongbloed, Krista Bos, Krista Koi, Bea, Bart Mol, Josh and all the people in the DNA diagnostic lab, thank you for always being willing to help me with DNA samples and all the things related to patient data. Dear Marina, thank you for taking care of the finances, and Edwin, thank you for solving all the computer-related problems. Dear Karen, Jantine, Michiel, Ludolf and all the people in the research lab, thank you for everything, it's been nice working with you all and sharing experiences in the lab.

Dear Helga, Renee, Bahram and Ana Ferreira, the HNPCC people, thanks for all the nice lunches, BBQs in the summer, and dinners. We always found good topics to talk and laugh about. Dear Ana, it was nice to talk to you and share stories; to my surprise we almost always had the same opinion about a lot of things.

To all my officemates and ex-officemates: Eva, Karin, Suzanne, Noortje, Mitja, Rodrigo, Omid, Celine, Olga, Senapati and Asia (or shall I call you Jan Manhattan? :D), I enjoyed sharing this room with you all, despite sometimes feeling the room was too small for all of us. :-D I sincerely apologize if sometimes I sang too loudly and disturbed you, from now on you will be free of my noise! :-D

To all my friends and colleagues in the Department of Genetics, past and present, Jianghua, Tjako, Renee, Annemieke, Mats, Gerben, Anna Posafalvi, Mahdi, Justyna, Jihane, Gosia, Agata, Isis, Paul, Barbara and Javier thank you for all the great times and I wish you all the best.

Home is where the heart is, Groningen is like my second home, and part of the reason is because I have made such great friends here. I would like to thank Asal Hamarneh, Ramune Nedveckyte and Adriana Mantos, for their friendship and support especially for the first two years I lived in Groningen. Thank you for always being there for me. I would like

to thank all my WB housemates, my very best friend mbak Ponky, Mbak Diana, Dini, mbak Puri, Mira, mbak Ike, mas Itob, mas Wangsa, Pak Agus, Ican, Adit, Indra, Senaz, Insanu, Bu Heni and Mas Amal, how dull my life would be in Groningen without you all, thank you for the great times we spent together in Westerbadstraat, sharing stories, worries, dreams, hopes and of course delicious Indonesian cooking, these are unforgettable memories. To my dear housemates in Bezettingslaan, Neng Fisheri, Mbak Ari and Wiwin, thank you for being such wonderful housemates, we started our PhD programs almost at the same time and now we come to the end. I am grateful for having you all to share the good and bad times during these last four years. Good luck with your own theses. :-D Dear mbak Puri and mbak Ari, to have you both like I have my older sisters, felt safe and warm, no matter what problems I had, you always had a shoulder for me to cry on. Dear Neng and Mira, I often wondered how it feels to have little sisters, now I have at least two :-D, and I feel so grateful and blessed to know you. Dear Neng, thank you for being willing to be one of my *paranimfen*, it means a lot to me.

I would also like to thank the families of pak Elfami Yaman and Uni Yulia, and of Guntur and Intan for welcoming me into your home, and of Mas Eko and mbak Inne, Mas Ismail and mbak Agnes, Kang Nandang and teh Nisa. It's been nice to meet you all, share many good moments and trips to places in Europe. E-travel will not be forgotten and hopefully we will keep in contact no matter where we are. Thank you also to the family of Pak Tri and mbak Indah, Wak Aisyah and Om Meno, Mas Chalid and mbak Nona, Om Basuki and Tante Pantja, Mas Yayok and Mbak Lia, Mbak Tina, Mbak Poppy, Mas Donny and Nike, Kang Intan and the Enci, Bayu and Indres, Mas Adi and mbak Sista for the wonderful times we had here in Groningen.

Dear Dini, Dowty, Helmy and Hafidz, how grateful I am to have best friends like you. Thank you for being good listeners, good companions, you were always there during the hardest times of my stay in Netherlands. Although now almost all of us live in different countries, I hope we can keep our friendship forever.

Dear Robby, Lia Atwa, Rachma, Eryth, Iqbal, Astri, Faizah, Alya, Nizar, Irfan, Iging, Desti, Arramel and Puti how wonderful life in Groningen is because of all of you, thank you for everything, for all the wonderful gatherings we had. I wish you all the best for the future and good luck with the remaining PhD work. :-D Dear Mbak Roga, bu Mariana and Okta, sharing stories of PhD life with you made me feel like I was not alone, we went through similar good and bad times. Mbak Roga and bu Maria, good luck with your theses, and Okta, congratulations on being the first of us to reach the finish line, I am happy for you and good luck with your next journey! I would also like to thank to Teguh, kang Hendi, Mas Titah, Seno, Kadek, Wahyu, Tita, Cicin, Wisnu, Yuli, Mutia, mbak Yuni, Kenzie and all friends in deGromiest

(Indonesian Muslims' association in Groningen) and PPIG (Indonesian students' association in Groningen) for our great friendship during my stay in Groningen.

My special thanks also to Dr. Debbie Retnoningrum. Dear bu Debbie, I could not imagine how my life would have been if I hadn't met you. You are the one who introduced me to how fascinating research life is. Thank you very much for the unconditional support you've given me since I was in the BSc program up to now, the end of my PhD. Dr. Joseph Hilgers, dear Jo, my first boss, who became more like a father to me, how I wish you were here with me at the end of my PhD work, but I am sure you are watching from above and will be proud of me. Dear Jo, indeed you were right, this world is not as big as I thought. I am happy that I listened to you and dared to make one of the biggest decisions in my life, to go abroad and experience the differences. Although you will not read this, I sincerely want to thank you for all your love and support.

To my beloved family, Kepada Ibu dan bapak tercinta, tidak ada kata-kata lain yang bisa Uyung ungkapkan selain terimakasih yang tidak terhingga atas semua kasih sayang dan pengorbanan Ibu dan bapak selama ini. Walaupun bapak sudah tidak bersama kita lagi, semoga beliau bisa ikut merasakan kebahagiaan Uyung hari ini. Semoga apa yang Uyung capai selama ini bisa membahagiakan dan membuat ibu bangga. Terimakasih untuk selalu mendukung semua keputusan yang Uyung pilih dan percaya sepenuhnya bahwa Uyung bisa menyelesaikan semua dengan baik. Terimakasih untuk kakak-kakaku, untuk teh Dewi, teh Lilis, teh Kinkin, Aa Dodo, Aa Budi atas semua dukungannya selama ini. Terimakasih untuk keluarga Wa Maman Tistajaya atas dukungan dan juga bantuannya merawat ibu selama ini. Terimakasih untuk de An-an yang sudah bersedia untuk datang dan menemani bi Uyung di hari penting ini. How I miss you all and wish that you were here with me today. Semoga Allah selalu melimpahkan kasih sayang-Nya dan selalu memberkahi kita sekeluarga.

A handwritten signature in black ink, reading "Yulia". The signature is written in a cursive, flowing style with a long horizontal stroke at the end.