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ORIGINAL ARTICLE

Basic Study

Depletion of the *IKBKAP* ortholog in zebrafish leads to hirschsprung disease-like phenotype

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Abstract

AIM: To investigate the role of *IKBKAP* (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein) in the development of enteric nervous system (ENS) and Hirschsprung disease (HSCR).

METHODS: In this study, we injected a morpholino that blocked the translation of *ikbkap* protein to 1-cell stage zebrafish embryos. The phenotype in the ENS was analysed by antibody staining of the pan-neuronal marker HuC/D followed by enteric neuron counting. The mean numbers of enteric neurons were compared between the morphant and the control. We also studied the expressions of *ret* and *phox2bb*, which are involved in ENS development, in the *ikbkap* morpholino injected embryos by quantitative reverse transcriptase polymerase chain reaction and compared them with the control.

RESULTS: We observed aganglionosis (χ^2 , $P < 0.01$) and a reduced number of enteric neurons (38.8 ± 9.9 vs 50.2 ± 17.3 , $P < 0.05$) in the zebrafish embryos injected with *ikbkap* translation-blocking morpholino (morphant) when compared with the control embryos. Specificity of the morpholino was confirmed by similar results obtained using a second non-overlapping morpholino that blocked the translation of *ikbkap*. We further studied the morphant by analysing the expression levels of genes involved in ENS development such as *ret*, *phox2bb* and *sox10*, and found that *phox2bb*, the ortholog of human *PHOX2B*, was significantly down-regulated (0.51 ± 0.15 vs 1.00 ± 0 , $P < 0.05$). Although we also observed a reduction in the

expression of *ret*, the difference was not significant.

CONCLUSION: Loss of *IKBKAP* contributed to HSCR as demonstrated by functional analysis in zebrafish embryos.

Key words: Hirschsprung disease; Enteric nervous system; *IKBKAP*; Zebrafish; Morpholinos

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Core tip: To investigate the functional role of *IKBKAP* in enteric nervous system (ENS) development, we knocked down the zebrafish ortholog *ikbkap* using a translation blocking antisense morpholino. Loss of *ikbkap* caused aganglionosis and a reduced number of enteric neurons, indicating that *IKBKAP* is important for proper ENS development.

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INTRODUCTION

The enteric nervous system (ENS) is an intrinsic, autonomic nervous system in the intestine of vertebrates and is responsible for regulating peristalsis, transmucosal movement of fluids and local blood flow. Neurons and glia in the ENS are organised into ganglia, which form the myenteric (between the intestinal longitudinal and circular muscles) and the submucosal (underneath the submucosal) plexuses. Enteric neurons and glia are originated from vagal neural crest cells (NCCs) during embryonic development. Vagal NCCs migrate from the dorsal neural tube and colonise the intestine, and differentiate into mature enteric neurons and glia^[1]. In humans, defective ENS development results in hirschsprung disease (HSCR, MIM142623).

HSCR is a congenital neuropathy, characterised by the absence of enteric ganglion (aganglionosis) and impaired peristaltic movement along variable lengths of distal intestine. HSCR patients develop constipation, diarrhoea, vomiting and sometimes life-threatening colon complications such as enterocolitis. Currently the treatment of HSCR is by surgery in which the aganglionic, non-functioning distal intestine is resected and the normal proximal intestine is reconnected to the anus. HSCR is a complex multifactorial disease, which most commonly presents sporadically (80%-95%) although it can be familial (5%-20%). It displays high heritability, large sex bias (male to female 4:1), high sibling recurrence risk, non-Mendelian inheritance in

families and higher frequency in Asian populations (28 per 100000 live births). Coding sequence mutations of *RET*, which encodes a receptor tyrosine kinase, account for up to 50% of the familial and between 7% and 35% of the sporadic HSCR cases. *RET* is important for ENS development, as mice without *Ret* exhibit an HSCR-like phenotype. Mutations in other genes that are important for ENS development, such as endothelin receptor B, transcription factors SRY-box 10 (*SOX10*) and paired-like homeobox 2b (*PHOX2B*), are subsequently found to cause HSCR. However, all these known HSCR-causing mutations together only explain a fraction of all HSCR cases, suggesting unknown mutations are yet to be discovered^[2]. To uncover new HSCR-susceptibility loci, we fine mapped the chromosomal region 9q31^[3], which was previously shown to segregate in families bearing either hypomorphic or non-coding *RET* mutations^[4]. We narrowed this region down to the gene *IKBKAP* (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein) and confirmed the association of this gene to Chinese HSCR patients^[3].

IKBKAP encodes the protein IkappaB kinase complex associated protein, also known as elongator complex protein 1 (ELP1). It is a scaffold protein that forms the elongator complex with ELP2, 3, 4, 5 and 6. Previous studies on *IKBKAP* and the elongator complex suggested they performed diverse functions. The elongator complex was found to be associated with RNA polymerase II to regulate transcription^[5]. In addition, *IKBKAP* has also been suggested to be responsible for DNA demethylation^[6], JNK activation^[7], exocytosis^[8], tRNA modification^[9], α -tubulin acetylation^[10,11], actin organisation^[12,13], and cell migration^[14] and survival^[15]. Knocking down *IKBKAP in vitro* in human neuroblastoma cells resulted in the down regulation of *RET*^[16], suggesting *IKBKAP* might regulate *RET* expression and ENS development. Mutations in human *IKBKAP* lead to familial dysautonomia (FD, MIM223900), a neuropathy of the autonomic nervous system characterised by abnormal or incomplete neuronal development and progressive neuronal degeneration^[17], with more than 60% of patients also being affected with gastrointestinal dysfunction^[18]. Interestingly, FD patients were shown to have reduced ganglia and neurons density in the ENS^[19] and a co-occurrence of HSCR and FD has been reported^[20]. These findings suggested that HSCR and FD might share a common etiology. A number of murine models were established to study the function of *IKBKAP* in development^[21-26]. However, these studies primarily focused on analysing FD-related phenotypes in the central nervous system, dorsal root ganglia, behavioural changes, *etc.*, without thoroughly investigating the ENS. No prior study exists on the role of *IKBKAP* in the development of ENS and the pathogenesis of HSCR.

Prompted by the above information, we set out

to investigate whether *IKBKAP* plays a role in ENS development. Thus in this study, we knocked down *ikbkap* in zebrafish embryos using antisense morpholino. We found that *ikbkap* knock down resulted in aganglionosis and a reduced neuron number in the ENS of zebrafish embryos, and reduced expression of *phox2bb* (ortholog of human *PHOX2B*).

MATERIALS AND METHODS

Animals

Wild-type adult zebrafish (*Danio rerio*) were purchased from local aquariums and embryos were obtained from natural spawning. The maintenance of the zebrafish and the culture of the embryos were carried out as described previously^[27]. Embryos were staged by hours post-fertilisation or days post-fertilisation (dpf) at 28.5 °C^[28].

Morpholino microinjection

Two antisense morpholinos (Gene Tools LLC) specifically blocking *ikbkap* translation (5'-TCAGCAG-ACTGAGGTTTCTCATTGT-3', 5'-GGTTTATGTTTTCTCAAGATGAGA-3') and the standard control morpholino (5'-CCTCTTACCTCAG TTACAATTTATA -3') were used in the study. The preparation of the morpholinos and the procedure for zebrafish microinjection were carried out as described previously^[29]. Morpholino injected embryos were cultured at 28.5 °C to later stages for analysis.

Immunohistochemistry and neuron counting

Morpholino injected embryos were cultured to 5 dpf and immunohistochemistry of the neuronal marker HuC/D was conducted as described^[30]. The following antibodies and dilutions were used: anti-HuC/D (16A11, Molecular Probes A-21271) 1 in 1000, anti-*IKBKAP* (Santa Cruz sc-8336) 1 in 200, and goat-anti-mouse HRP-conjugated secondary antibody (Abcam ab6789) 1 in 1000. DAB+ (Dako K346811) was used for signal development. Images were taken by a standard upright microscope. For neuron counting, HuC/D positive cells in the 30 µm length of distal-most intestine were counted. Results were analysed by the χ^2 test or the Student's *t* test.

Real-time-polymerase chain reaction and quantitative polymerase chain reaction

Morpholino-injected embryos were cultured to 1 dpf. 30 embryos from each treatment were pooled, dechorionated, and homogenised, and RNA was extracted by TRIzol[®] Reagent (Life Technologies 15596-026) according to the manufacturer's protocol. 1 µg of RNA was used for cDNA synthesis by the Reverse Transcription System (Promega A3500) using oligo-dT primer. For qualitative real-time-polymerase chain reaction (RT-PCR), standard PCR was performed using AmpliTaq Gold[®] (Applied

Biosystems) and the products were resolved in 0.8% agarose gel. The following primer pairs were used: *ret*: 5'-GTCCTGTTACTGGGCGGTTA-3', 5'-GTGGCTCATCCGTTTTTCAGT-3'; *phox2bb*: 5'-AGGAGCTCGCGCTTAAGATT3', 5'-TTGCCTCTTTGCTGTCC TCT-3'; *sox10*: 5'-CAGCCAATCGCATTACAAGA-3', 5'-GGTGGGAGATACTGGTTCGAA-3'; *elfa1*: 5'-CTTCTCAGGCTGACTGTGC-3', 5'-CCGCTAGCATTACCCTCC-3'. Quantitative polymerase chain reaction (q-PCR) was conducted as described previously^[31] using TaqMan[®] Gene Expression Assays (Applied Biosystems) for *ret* (Dr03119148_g1), *phox2bb* (Dr03423610_g1) and *beta-actin* (Dr03432610_m1). Relative gene expression was determined by the Livak method^[32].

RESULTS

Loss of *ikbkap* resulted in aganglionosis and a reduced number of enteric neurons

We injected an antisense morpholino that blocked the translation of *ikbkap* protein into 1 to 4-cell stage zebrafish embryos to study the effect of *ikbkap* loss-of-function in ENS development. The morpholino successfully blocked the translation of *ikbkap* protein, as shown in the *ikbkap* antibody staining of 3 dpf embryos (Figure 1). In the control morpholino injected embryo (control), *ikbkap* was expressed in the intestinal epithelium and surrounding tissues while the expression was absent in the *ikbkap* morpholino injected embryos (morphants). At 5 dpf, HuC/D positive enteric neurons were found along the entire intestine as shown in the control (Figure 2A). Aganglionosis in the distal intestine (Figure 2B) was observed in 6.4% (7 in 110, Figure 2D) of the morphants that mimicked HSCR in humans. For the rest of the morphants, the number of enteric neurons seemed to be reduced (Figure 2C) and this was confirmed by enteric neuron counting at the 30 µm-length region in distal-most intestine (Figure 2E). On average the morphants had fewer enteric neurons at the distal intestine than the control and the difference was statistically significant ($P < 0.05$). The experiment was repeated with a second non-overlapping morpholino blocking *ikbkap* translation and similar results were observed (data not shown).

Loss of *ikbkap* down regulated *phox2bb* expression

It was reported that knocking down *IKBKAP* in human neuroblastoma cells resulted in reduced *RET* expression^[16]. We speculated that a similar phenomenon would happen in our embryos injected with *ikbkap* morpholino, which could account for the reduced enteric neuron number and aganglionosis observed. To test this hypotheses we extracted total RNA from *ikbkap* morphants and control embryos and checked the expression of *ret*, *phox2bb* and *sox10* by qualitative RT-PCR. Expression of *phox2bb* was markedly reduced, while there was a marginal

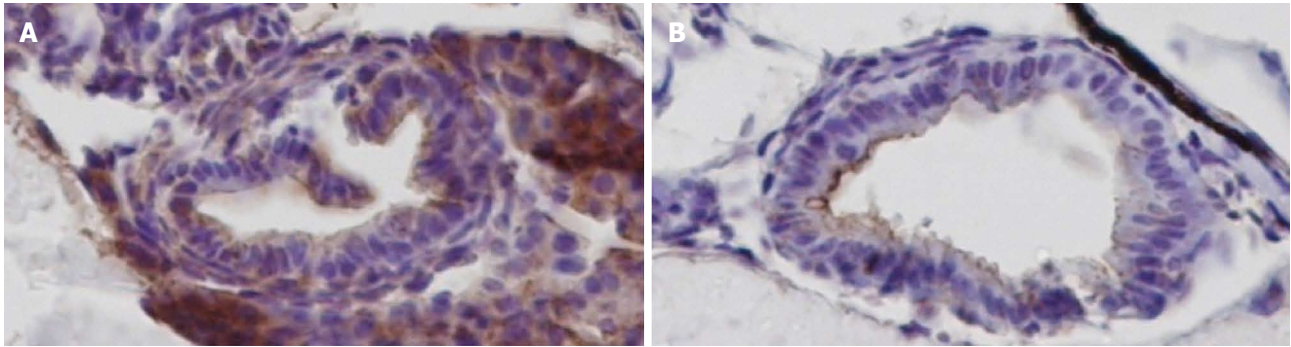


Figure 1 *Ikbkap* depletion by morpholino. A: In 3 dpf control embryo *ikbkap* protein was detected by antibody staining (reddish-brown, $\times 400$); B: 3 dpf *ikbkap* morphant embryo. The level of *ikbkap* was greatly reduced (magnification $\times 400$).

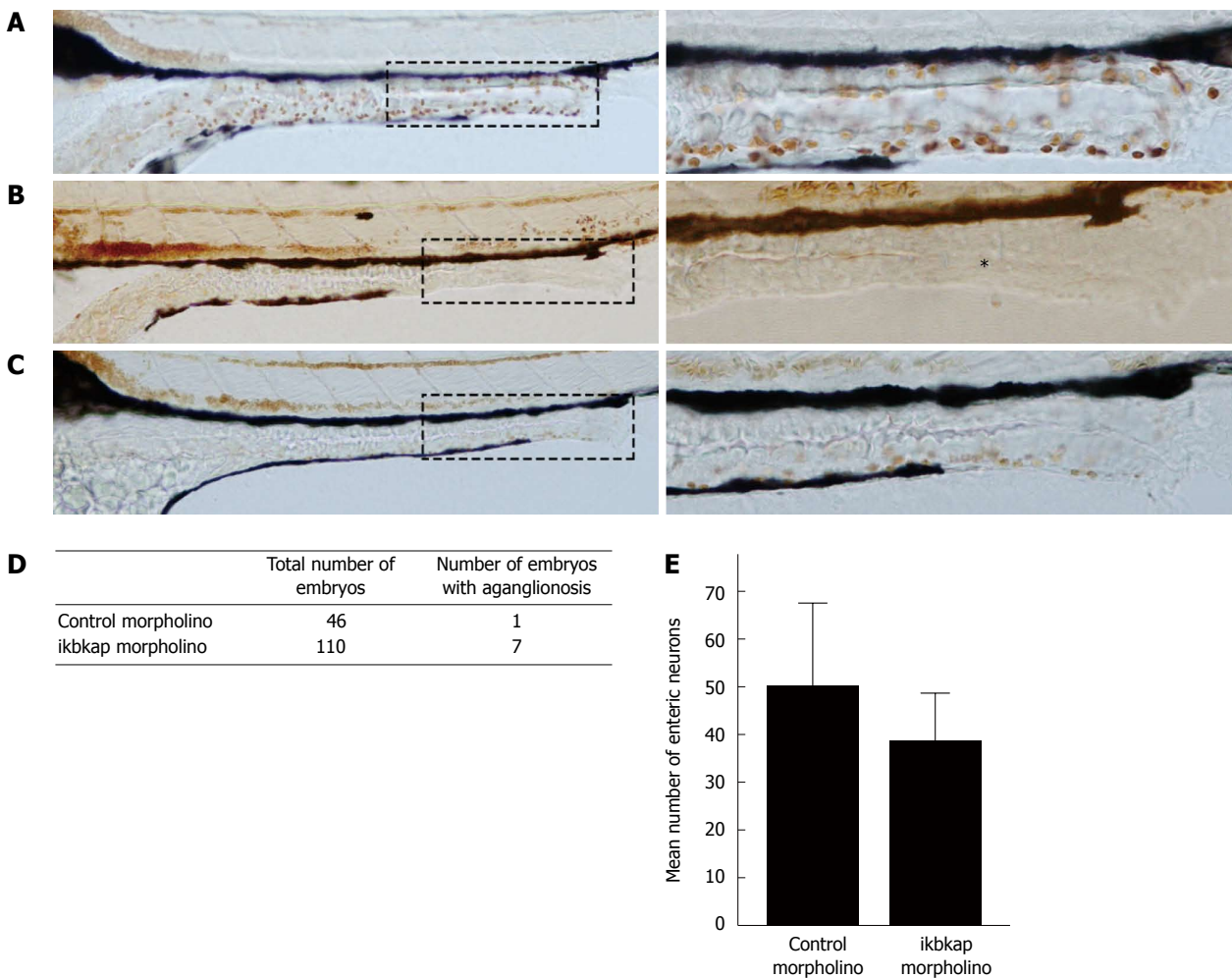


Figure 2 Loss of *ikbkap* resulted in Hirschsprung disease-like phenotype. A: Control embryo showed normal enteric nervous system (ENS) development with enteric neurons present along the whole intestine (reddish-brown); B: Morphant displayed complete absence of enteric neuron in distal intestine (asterisk); C: Morphant showed milder phenotype of reduced number of enteric neurons. Magnification $\times 200$ and $\times 400$; D: Summary of aganglionosis observed in morphants ($n = 110$) and control ($n = 46$). The difference was statistically significant ($P < 0.01$) by χ^2 test; E: Summary on the number of enteric neurons in morphants ($n = 17$) and control ($n = 17$). The difference was statistically significant (38.8 ± 9.9 vs 50.2 ± 17.3 , $P < 0.05$) by Student's *t* test.

down regulation of *ret* with *sox10* expression unaffected (Figure 3A). We verified the results by q-PCR (Figure 3B). Expression of *phox2bb* was significantly reduced ($P < 0.05$). Although an apparent reduction in the level of *ret* transcript was observed, the

difference was not statistically significant.

DISCUSSION

The association of *IKBKAP* to Chinese HSCR patients

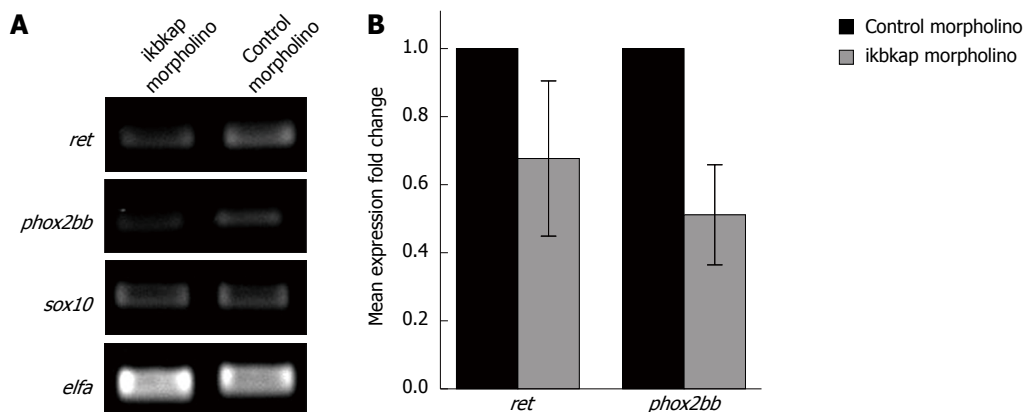


Figure 3 Loss of *ikbkap* caused *phox2bb* down-regulation. A: Qualitative real-time polymerase chain reaction for the expressions *ret*, *phox2bb* and *sox10*. *elfa* served as internal control; B: Quantitative-polymerase chain reaction for *ret* and *phox2bb*. Down-regulation of *phox2bb* was statistically significant (0.51 ± 0.15 vs 1.00 ± 0 , $P < 0.05$) by Student's *t* test.

led to the hypothesis that *IKBKAP* could contribute to the pathogenesis of HSCR. As shown in the current study, knocking down *ikbkap* in zebrafish embryos resulted in aganglionosis and a reduced number of enteric neurons, a phenocopy of HSCR in humans. This result indicated that *ikbkap* is involved in ENS development, and the disruption of *ikbkap* function results in an HSCR-like phenotype in zebrafish.

The *ikbkap* morphants showed a down regulation of *phox2bb*, the ortholog of human *PHOX2B*. *PHOX2B* is expressed in NCCs that eventually give rise to mature enteric neurons and glia^[33]. It has also been shown that *PHOX2B* together with *SOX10* and *NKX2-1* regulate *RET* expression^[34]. Together these pieces of evidence suggest *PHOX2B* is essential for proper ENS development. Indeed, *Phox2b* knockout mice or zebrafish display a phenotype reminiscent of that seen in human HSCR patients^[33,35]. In humans, *PHOX2B* mutations are responsible for several neurocristopathies, including neuroblastoma and congenital central hypoventilation syndrome, both of which can have HSCR as part of their phenotypic spectrum^[36-38]. The finding of *phox2bb* down-regulation in our study suggests that *ikbkap* was acting upstream of *phox2bb*. What remains unclear is the mechanism by which *ikbkap* regulates *phox2bb* expression. *ikbkap* protein is one of the subunits in the highly-conserved elongator complex that is associated with RNA polymerase type II. The elongator complex is made up of six subunits, and *ikbkap* acts as a scaffolding protein that holds the complex in its proper configuration^[39]. Knocking down *ikbkap* might disrupt the integrity of the complex and hence the elongation of the target transcripts including *phox2bb*, thereby reducing their expressions. In addition, increasing evidence shows that the elongator complex is also involved in regulating t-RNA modification and translational control, independently of the proposed regulatory function on transcript elongation. Inactivation of the elongator complex resulted in defective t-RNA modification and reduced expression of target proteins^[9,40]. It is

therefore plausible that depletion of *ikbkap* disturbs the translation of target proteins that are involved in regulating *phox2bb* transcription. Lastly, a report demonstrated that the elongator complex is responsible for genome demethylation during embryogenesis^[6]. As DNA demethylation is an important epigenetic control to activate gene transcriptions, failure in the process due to the loss of *ikbkap* might cause the *phox2bb* locus to remain methylated and hence reduce its expression. Further research is required to delineate the molecular connection between *ikbkap* and *phox2bb*.

It was reported that knocking down *IKBKAP* in human neuroblastoma cell line SHSY5Y by shRNA caused a significant reduction in the expression of *RET*^[16]. In our study we have partially replicated that result. The level of *ret* transcript is apparently reduced in the *ikbkap* morphant, by both qualitative RT-PCR and q-PCR. However, the reduction is not statistically significant. A hypothesis to explain this difference would be that in the *in vivo* zebrafish model, a feedback mechanism attenuated the effect of *ikbkap* knock down on *ret* expression, while such a mechanism might not exist in the *in vitro* cell line model. We also noted that in the same study, the authors did not report any change in the expression of *PHOX2B*. This discrepancy has two possible explanations. First, unlike wild type zebrafish, SHSY5Y bore a 20-nt deletion mutation in the exon3 of *PHOX2B*^[41]. This mutated *PHOX2B* might be more resistant to the effect induced by the loss of *IKBKAP* and hence its expression would not be altered. Second, as a cell line derived from human neuroblastoma, there might be other genetic changes such as mutations and chromosome rearrangements in the genome that could counteract the knock down of *IKBKAP*.

In conclusion, our functional analysis in zebrafish embryos confirmed the role of *IKBKAP* in ENS development.

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COMMENTS

Background

Hirschsprung disease (HSCR), caused by genetic mutations, is a congenital neuropathy characterized by the absence of enteric ganglion along variable length of distal large intestine and results in chronic constipation. Over the years a number of disease-causing mutations have been found but they only account for a portion of HSCR cases, suggesting there are more to be identified.

Research frontiers

To uncover the unknown HSCR-causing mutations, their laboratory fine mapped chromosome region 9q31, which has been reported to be associated with HSCR, and narrowed it down to the gene *IKBKAP* and confirmed its association with Chinese HSCR patients. *IKBKAP* encodes a protein that acts a scaffold in the elongator complex, but it is unclear whether this gene is important for enteric nervous system (ENS) development and HSCR.

Innovations and breakthroughs

To discover if *IKBKAP* is essential for ENS development and contributes to HSCR, authors depleted the zebrafish *IKBKAP* orthologs by morpholino-mediated gene knockdown. They found out that depletion of *ikbkap* resulted in HSCR-like phenotype, along with down regulation of *phox2bb*. Their report is the first to demonstrate the importance of *IKBKAP* in ENS development.

Applications

This report enhances the understanding on the cause of HSCR and puts *IKBKAP* a disease-causing gene, which will be useful in the future in developing novel therapeutic strategies and genetic counselling.

Terminology

Hirschsprung disease: A congenital neuropathy characterized by the absence of enteric ganglion, causing chronic constipation; *IKBKAP*: A human gene encoding a scaffold protein in the elongator complex; morpholino: An antisense oligomers that can mediate knockdown of target gene by blocking either protein translation or mRNA splicing.

Peer-review

The manuscript is well organized and clear, represented with graphs and microscopic photos. The article in general was well made with a sound methodology and a good discussion.

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