MicroRNA-939 inhibits cell proliferation via targeting LRSAM1 in Hirschsprung’s disease

Guanglin Chen1,3,*, Chunxia Du1,3,* , Ziyang Shen1,3,4,* , Lei Peng1,3 , Hua Xie1,3 , Rujin Zang1,3 , Hongxing Li1,3 , Yankai Xia1,2 , Weibing Tang1,3

1State Key Laboratory of Reproductive Medicine, Institute of Toxicology, School of Public Health, Nanjing Medical University, Nanjing 211166, China
2Key Laboratory of Modern Toxicology (Nanjing Medical University), Ministry of Education, China
3Department of Pediatric Surgery, Children’s Hospital of Nanjing Medical University, Nanjing 210008, China
4Department of Endocrinology and Metabolism, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China
*Equal contribution

Correspondence to: Weibing Tang; email: twbcn@163.com
Keywords: Hirschsprung’s disease, microRNA (miRNA), proliferation, migration, autophagy
Received: July 10, 2017 Accepted: November 26, 2017 Published: December 18, 2017
Correction: This article has been corrected. See Aging 2018; 10: https://doi.org/10.18632/aging.101650

Copyright: Chen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY 3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

Background: Hirschsprung’s disease (HSCR) is a common digestive disease caused by impaired development of neural crest cells. Some studies have revealed the roles of microRNA (miRNA) in various diseases. But the function of miRNA in HSCR needs further investigation.

Methods: We adopted qRT-PCR and immunoblot analyses to explore the relative expression of miR-939 and LRSAM1 in 80 HSCR bowel tissues and 80 normal bowel tissues. CCK-8 assay, transwell assay and flow cytometry were used to evaluate the function of miR-939 by overexpression of miR-939 in 293T, SK-N-BE(2), SH-SY5Y cell lines. The direct connection between miR-939 and LRSAM1 was validated by dual-luciferase reporter assay. We also investigated the autophagy level via immunoblot analyses.

Results: Mir-939 was significantly upregulated in HSCR tissues with decreased expression of LRSAM1. Overexpression of miR-939 suppressed cell proliferation without affecting cell apoptosis, cell cycle or cell migration. And LRSAM1 exerted similar function. Autophagy was impaired in HSCR tissues compared with control samples. Mir-939 did not inhibit the autophagy although it decreased the expression of LRSAM1.

Conclusions: Our study shows the potential function of mir-939 through regulating LRSAM1 in HSCR and infers that autophagy may also confer the risk of HSCR.

INTRODUCTION

Hirschsprung's disease (HSCR) characterized by the absence of intestinal ganglion cells in myenteric and submucosal plexuses is a developmental malformation of enteric nervous system which affects 1:5000 live births and has a male preponderance of 4:1 [1, 2]. HSCR is a complex disease caused by multiple factors including genetic and environmental factors that regulate enteric neural crest cells (ENCCs) proliferation, differentiation and migration in the embryonic stage [3, 4].

MicroRNAs (miRNAs) are endogenous small non-coding RNAs ranging in size from 19 to 25 nucleotides and play important roles in multiple cellular processes such as proliferation, apoptosis, development, and differentiation [5, 6]. They mainly regulate gene
expression by targeting to the complementary mRNA and resulting in mRNA cleavage and/or translation repression [7]. Dysregulation of miRNAs has been shown to be associated with HSCR such as miR-206, miR-192/215 by suppressed cell proliferation [8, 9]. Our previous study through miRNA microarray has found numerous dysregulated miRNA in HSCR samples. MiR-939 was significantly upregulated in HSCR cases compared with matched controls (data not shown). Recent studies have demonstrated that miR-939 is responsible for ovarian cancer due to its impact on cell proliferation and regulates human inducible nitric oxide synthase gene expression [10, 11]. However, the role of miR-939 in the pathological process of HSCR has not been reported until now.

Autophagy is a highly regulated biological mechanism involved in recycling cellular constituents and intracellular degradation of damaged organelles and protein aggregates [12, 13]. In addition to housekeeping and homeostatic functions at basal level, autophagy is initiated under stress conditions such as nutrient or growth factor deprivation, hypoxia and inflammation [14-16]. Autophagy has participated in various diseases including cancer and neurodegenerative disorders [17, 18]. According to available miRNA databases, LRSAM1 is a potential target of miR-939, which is associated with autophagy induction and motor neuropathies. However, whether LRSAM1 participates in the development of HSCR remains to be addressed.

In this study, we demonstrate that how miR-939 interacts with its target gene LRSAM1 and explore the autophagy level in HSCR.

**RESULTS**

**Clinical specimens**

The Table 1 summarized the clinical information obtained from all participants including age, gender (male/female) and body weight. There were no statistically significant differences in terms of age and body weight. The male/female ratio of HSCR cases was 3.7:1, which was consistent with the gender rate of this disease. In total, 80 HSCR cases and 80 matched controls were enrolled in this study.

**Overexpression of miR-939 in HSCR**

qRT-PCR was used to examine the expression level of miR-939 in all colon tissues, and showed that the expression level of miR-939 was significantly higher in HSCR cases compared with matched controls (Fig. 1A). Similar results could be found in ganglionic and aganglionic intestine of the same HSCR participants (Supplemental Fig. 1A). The results indicated that miR-939 might play an important role in the pathological development of HSCR.

**MiR-939 mimics decreased cell proliferation**

In order to confirm the effect of miR-939 on cell function in vitro, cell migration, cell proliferation, cell cycle and apoptosis were examined by transfecting the 293T, SH-SY5Y and SK-N-BE(2) cell lines with miR-939 mimics. Overexpression of miR-939 significantly reduced the number of proliferating cells (Fig. 1B). Flow cytometry analysis was conducted to investigate the role of miR-939 in cell cycle and apoptosis. However, no statistical differences in the percentage of migrating cells, apoptotic cells or cell cycle process were detected between cells treated with miR-939 mimics and the negative control (Fig. 1C-E).

**Target prediction of miR-939**

To investigate the molecular mechanism by which miR-939 affects cell function, we used three miRNA databases (Targetscan, DIANA LAB and miRDB) to predict the target genes. After the prediction and functional analysis, three potential target genes LRSAM1, LNX2 and UBE2D3 were selected. LRSAM1 plays key roles in autophagy induction and is involved in motor neuropathies [19, 20]. LNX2 is a member of LNX family expressed in nervous system and promotes neuronal cell proliferation and differen-

---

**Table 1. Demographic clinical features of study subjects.**

| Variable          | Control(n=80) | HSCR(n=80) | P     |
|-------------------|---------------|------------|-------|
| Age(days,mean,SE) | 127.91(7.65)  | 115.42(6.67)| 0.22  |
| Weight(kg,mean,SE)| 5.53(0.15)    | 5.32(0.13) | 0.30  |
| Sex(%)            |               |            |       |
| Male              | 56(70.00)     | 63(78.75)  | 0.11  |
| Female            | 24(30.00)     | 17(21.25)  |       |
UBE2D3 is a member of the E2 family and regulates basic cellular activities including cell cycle, DNA damage response and apoptosis [23].

**LRSAM1 was down-regulated in HSCR tissues**

qRT-PCR was adopted to analyze the mRNA level of all the three predicted targeted genes in 80 cases of HSCR and 80 matched controls. LRSAM1 was the only candidate gene which was down-regulated in cases compared with controls. However, LNX2 and UBE2D3 showed no difference in expression level between cases and controls (Fig. 2A). Furthermore, the protein expression level of LRSAM1 was consistent with mRNA levels by western blot (Fig. 2B). Decreased expression of LRSAM1 could also be found in stenotic segment and matched dilated segment of the same HSCR cases (Supplemental Fig. 1B, C).

**LRSAM1 was target gene for miR-939**

To confirm the miRNA-target interactions, 293T, SH-SY5Y and SK-N-BE(2) cell lines were transfected with miR-939 mimics. The results showed ectopic expression miR-939 significantly suppressed the mRNA and protein expression of LRSAM1 in three cell lines (Fig. 3A). In addition, miR-939 decreased the luciferase activity of the wild-type pGL3-LRSAM1 but not the mutant reporter gene GL3-LRSAM1-mut, indicating that miR-939 suppressed LRSAM1 by binding to the 3′UTR region (Fig. 3B). Next, we wondered whether suppression of LRSAM1 expression by siRNA has a similar function like miR-939. As shown in Supplemental Fig. 2, suppression of LRSAM1 decreased cell proliferation. The results suggested that miR-939 suppressed cell proliferation directly by regulating LRSAM1.

---

**Figure 1. Mir-939 was upregulated in HSCR tissues and cytobiology change after treating cells with its mimics.** (A) Mir-939 was significantly overexpressed in HSCR (n=80) tissues compared with control samples (n=80). Human 293T, SK-N-BE(2), SH-SY5Y cell lines were transfected with miR-939 mimics, upregulated mir-939 suppressed cell proliferation indicated by the CCK-8 assay (B) without impact on cell migration (C), cell cycle (D) and cell apoptosis (E). *indicates significant difference compared with control group, P<0.05.
Autophagy was suppressed in HSCR

Given that LRSAM1 was an important gene involved in autophagy, we investigated the autophagy level in HSCR [20]. We detected the conversion of LC3-I to LC3-II by western bolt and immunohistochemical staining. As shown in Fig. 4 A-B, the level of autophagy was significantly lower in HSCR cases. We then
wondered if miR-939 inhibited the autophagy in vitro. However, we found no difference between groups of miR-939 mimics and control ones. Results above hinted that autophagy was impaired in HSCR and miR-939 did not participate in this biological process.

**DISCUSSION**

HSCR is one of the most common digestive diseases with the symptoms of constipation, vomiting and abdominal distension in the new born. Nowadays, the main treatment of HSCR is to remove the aganglionic bowels. However, the long term outcome is not always satisfactory especially when patients with enterocolitis [24, 25]. The cost for initial hospitalization and time of hospital stay is $100,000 and a month on average respectively [26]. Therefore, it is essential to elucidate the exact pathogenesis underlying HSCR which can offer a better understanding in treatment and early diagnosis. In our study, we found potential targeted genes of miR-939 using Targetscan, DIANA LAB and miRDB. LRSAM1 was significantly differentially expressed between HSCR cases and matched controls. Then, we used dual-luciferase report assay to validate that LRSAM1 is directly regulated by miR-939. In vitro results showed that up-regulation of miR-939 suppressed cell proliferation without influencing cell migration, cell cycle and cell apoptosis. Meanwhile, the similar results generated by LRSAM1 siRNA hinted that miR-939 performed its function by regulating LRSAM1.

LRSAM1 (leucine-rich repeat and sterile alpha motif-containing protein 1) is a highly conserved ubiquitin-protein ligase, which is expressed in the peripheral neural system as well as the central neuron system [27, 28]. Previous studies showed that mutation of LRSAM1 is associated with neurological disorders such as Charcot-Marie-Tooth and Huntington’s disease [19, 29]. Meanwhile, LRSAM1 also participates in WNT signaling which is related to HSCR [30]. Thus, we speculate that miR-939 could contribute to the pathogenesis of HSCR through negative regulation of LRSAM1. Moreover, LRSAM1 has been taken as important regulators of autophagy against bacteria via its LRR domain and RING domain [31]. Therefore, we detect the autophagy level in HSCR and the result shows higher level in matched controls. But LC3 II/LC3 I do not show significant difference between miR-939 mimics and control ones according to in vitro results. It is well known that one miRNA always has not only one targeted gene. So miR-939 may also affect other genes related to autophagy. But further research is needed to explore the meaningless effect of miR-939 on autophagy.

In conclusion, our data reveal that miR-939 is related to pathogenesis of HSCR via regulation of LRSAM1 and autophagy level is impaired in HSCR. Our research may shed new light in understanding the relationship between HSCR and autophagy.

![Figure 4. Downregulated level of autophagy in HSCR.](https://www.aging-us.com) The autophagy was significantly suppressed in HSCR via detecting the expression of LC3 through IHC (A) and WB (B). (C) Autophagy level was not changed when mir-939 was upregulated by mimics. *indicates significant difference compared with control group, P<0.05.
MATERIALS AND METHODS

Tissues collection

All study subjects including 80 HSCR case samples and 80 matched controls were derived from patients undergoing surgical procedures at Nanjing Children’s Hospital Affiliated to Nanjing Medical University from October 2009 to May 2014. The diagnosis of HSCR was confirmed by pathological analysis after surgery. The matched controls were collected from patients’ intestinal without the ischemia or necrosis parts, which were proven to be without HSCR or other congenital malformations. All tissue samples were stored at -80°C immediately after surgery. Written informed consent was obtained from patients’ guardians according to the legal institutional guidelines and ethical permission.

QRT-PCR

Total RNA, including miRNA, was extracted using Trizol reagent (Life technologies, CA, US). For microRNA analysis, we used TaqMan® MicroRNA Assays (Applied Biosystems, CA, US) as the probe for hsa-miR-939 and hsa-U6 which served as an internal control. For the mRNA detection, the mRNA was measured by ABI 7900HT along with the GAPDH as the endogenous control. Primer sequences were listed in Supplemental Table 1.

Antibodies and Western blot (WB)

The tissue samples and cells were lysed using a RIPA buffer (Beyotime, Nantong, China). The membranes with the target protein were incubated with the primary antibody at 4 °C overnight and then incubated with the secondary antibody (Beyotime, Nantong, China) for 1 hour at room temperature. The primary antibodies used were LC3A/B (12741, cell signaling), GAPDH (sc-25778, Santa Cruz) and LRSAM1(ab171556, abcam). Image J software was used to detect and quantify the protein level in the Western Blot.

Cell culture and transfection

The 293T, SK-N-BE(2) and SH-SY5Y cell lines were obtained from American Type Culture Collection (ATCC, Manassas VA, USA) and cultured in DMEM medium (Hyclone, UT, US) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100U/ml) under the condition of 37°C and 5% CO2. The miRNA precursor molecules of miR-939, siRNA of LRSAM1 and negative control (GenePharma, Shanghai, China) were used in transfection experiments with Lipofectamine 2000 Reagent (Invitrogen,CA, US) according to manufacturer’s instructions.

Cell transwell assays

Transwell migration chambers (8 um pore size, Millipore Corporation, Billerica, MA) were used to evaluate the capacity of cell migration after transfection of cells with miR-939 mimics and negative control. About 100ul cell suspension with serum-free medium were seeded in the upper chamber (1×10⁶ cells/ml) with the 600ul medium containing 10% fetal bovine serum in the lower chamber. After 48h transfection, cells were stained with crystal violet staining solution (Beyotime, Nantong, China), counted and photographed under 40 × magnification (five views per well). The number of migrated cells was counted using Iamge-pro Plus 6.0 while the amout of control cells was normalized to 1.

Cell proliferation assays

CCK-8 assay (Beyotime, Nantong, China) was conducted to evaluate the cell proliferation after 24h transfection. We used TECAN infinite M200 Multi-mode microplate reader (Tecan, Mechelen, Belgium) to measure the absorbance at 450nm.

Cell cycle and apoptosis analysis

For the cell cycle assay, cells transfected with miR-939 mimics were collected and detected by BD Biosciences FACScalibur Flow Cytometry (BD Biosciences, NJ, US). For detection of apoptosis, cells were harvested and stained with the Annexin V-FITC/Propidium Iodide Kit (KeyGen Biotech, Nanjing, China) according to the manufacturer’s instructions. Data were analyzed with FlowJo V7 software (Tree Star, Ashland, OR, US).

Dual-luciferase reporter assay

The wild-type and mutated 3’-UTR sequence of LRSAM1 mRNA named pGL3-LRSAM1 and pGL3-LRSAM1-mut respectively were inserted into the KpnI and Sacl sites of pGL3 promoter vector (Genescript, Nanjing, China). After transfection with negative control, miR-939 mimics, pGL3-LRSAM1 and pGL3-LRSAM1-mut for 48h following the manufacturer’s protocols, cells were collected and measured using the Dual Luciferase Assay (Promega, Madison, WI).

Immunohistochemical staining (IHC)

For immunohistochemistry, sections of fixed and paraffin-embedded tissue samples were processed in Pathology Department of Nanjing Children’s Hospital. After deparaffinization and hydration, the sections were...
autoclaved in 0.01M trisodium citrate buffer for antigen retrieval and blocked by normal goat serum. Then specimens were incubated with anti-LC3A/B (12741, cell signaling, 1:2000 dilution) overnight at 4 °C, and then stained using biotinylated goat anti-rabbit IgG secondary for 1h, followed by incubating with 3,3'-diaminobenzidine (Beyotime, Nantong, China). Intensity of staining was graded as follows: negative(-); low staining(+); intensive staining(++).

Statistical analysis

Data obtained from tissue samples are analyzed using Wilcoxon rank-sum test while the the data of cell samples are presented as mean±SEM by double-sided Student’s t-test. All statistical analysis were performed using STATA9.2 (StataCorp, College Station, TX, US), and a p value of <0.05 was considered to be statistically significant. Qualitative data were representative of three independent experiments, with each performed in triplicate.

Abbreviations

HSCR: Hirschsprung's disease; miRNA: microRNA; LRSAM1: Leucine-rich repeat and sterile alpha motif-containing protein 1; ENCCs: Enteric neural crest cells; WB: Western blot; IHC: Immunohistochemical staining.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Weibing Tang, Yankai Xia, Xiaouen Xu, Guanglin Chen; Performed the experiments: Guanglin Chen, Chunxia Du, Hua Xie; Analyzed the data: Hua Xie, Hongxing Li, Chunxia Du, Ziyang Shen; Wrote the paper: Guanglin Chen, Ziyang Shen. All authors discussed the results and commented on the manuscript.

ACKNOWLEDGEMENTS

We thank Dr. Jie Zhang, Huan Chen and Changgui Lu (Children’s Hospital of Nanjing Medical University) for sample collection.

CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

FUNDING

This work was supported by Natural Science Foundation of China (NSFC 81370473, NSFC 81400574, NSFC 81570467), and Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

REFERENCES

1. Emison ES, McCallion AS, Kashuk CS, Bush RT, Grice E, Lin S, Portnoy ME, Cutler DJ, Green ED, Chakravarti A. A common sex-dependent mutation in a RET enhancer underlies Hirschsprung disease risk. Nature. 2005; 434:857–63. https://doi.org/10.1038/nature03467

2. Burzynski G, Shepherd IT, Enomoto H. Genetic model system studies of the development of the enteric nervous system, gut motility and Hirschsprung’s disease. Neurogastroenterol Motil. 2009; 21:113–27. https://doi.org/10.1111/j.1365-2982.2008.01256.x

3. Heanue TA, Pachnis V. Enteric nervous system development and Hirschsprung’s disease: advances in genetic and stem cell studies. Nat Rev Neurosci. 2007; 8:466–79. https://doi.org/10.1038/nrn2137

4. Amiel J, Sproat-Emison E, Garcia-Barcelo M, Lantieri F, Burzynski G, Borrego S, Pelet A, Arnold S, Miao X, Griseri P, Brooks AS, Antinolo G, de Pontual L, et al, and Hirschsprung Disease Consortium. Hirschsprung disease, associated syndromes and genetics: a review. J Med Genet. 2008; 45:1–14. https://doi.org/10.1136/jmg.2007.053959

5. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116:281–97. https://doi.org/10.1016/S0092-8674(04)00045-5

6. Di Leva G, Calin GA, Croce CM. MicroRNAs: fundamental facts and involvement in human diseases. Birth Defects Res C Embryo Today. 2006; 78:180–89. https://doi.org/10.1002/bdrc.20073

7. Ambros V. The functions of animal microRNAs. Nature. 2004; 431:350–55. https://doi.org/10.1038/nature02871

8. Sharan A, Zhu H, Xie H, Li H, Tang J, Tang W, Zhang H, Xia Y. Down-regulation of miR-206 is associated with Hirschsprung disease and suppresses cell migration and proliferation in cell models. Sci Rep. 2015; 5:9302. https://doi.org/10.1038/srep09302

9. Zhu D, Xie H, Li H, Cai P, Zhu H, Xu C, Chen P, Sharan A, Xia Y, Tang W. Nidogen-1 is a common target of microRNAs MiR-192/215 in the pathogenesis of Hirschsprung’s disease. J Neurochem. 2015; 134:39–46. https://doi.org/10.1111/jnc.13118

10. Guo Z, Shao L, Zheng L, Du Q, Li P, John B, Geller DA. miRNA-939 regulates human inducible nitric oxide synthase posttranscriptional gene expression in human hepatocytes. Proc Natl Acad Sci USA. 2012;
20. 109:5826–31. https://doi.org/10.1073/pnas.1118118109

11. Ying X, Li-ya Q, Feng Z, Yin W, Ji-hong L. MiR-939 promotes the proliferation of human ovarian cancer cells by repressing APC2 expression. Biomed Pharmacother. 2015; 71:64–69. https://doi.org/10.1016/j.biopharm.2015.02.020

12. He C, Klionsky DJ. Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet. 2009; 43:67–93. https://doi.org/10.1146/annurev-genet-102808-114910

13. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008; 132:27–42. https://doi.org/10.1016/j.cell.2007.12.018

14. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008; 451:1069–75. https://doi.org/10.1038/nature06639

15. Portt L, Norman G, Clapp C, Greenwood M, Greenwood MT. Anti-apoptosis and cell survival: a review. Biochim Biophys Acta. 2011; 1813:238–59. https://doi.org/10.1016/j.bbamcr.2010.10.010

16. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell. 2011; 147:728–41. https://doi.org/10.1016/j.cell.2011.07.026

17. Nixon RA. The role of autophagy in neurodegenerative disease. Nat Med. 2013; 19:983–97. https://doi.org/10.1038/nmn.3232

18. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell’antonio G, Mautner J, Tonon G, Haigis M, et al. Pancreatic cancers require autophagy for tumor growth. Genes Dev. 2011; 25:717–29. https://doi.org/10.1101/gad.2016111

19. Engeholm M, Sekler J, Schöndorf DC, Arora V, Schittenhelm J, Biskup S, Schell C, Gasser T. A novel mutation in LRSAM1 causes axonal Charcot-Marie-Tooth disease with dominant inheritance. BMC Neuro. 2014; 14:118. https://doi.org/10.1186/1471-2377-14-11

20. Wang Z, Hu J, Li G, Qu L, He Q, Lou Y, Song Q, Ma D, Chen Y. PHF23 (plant homeodomain finger protein 23) negatively regulates cell autophagy by promoting ubiquitination and degradation of E3 ligase LRSAM1. Autophagy. 2014; 10:2158–70. https://doi.org/10.4161/auto.36439

21. Yin FT, Futagawa T, Li D, Ma XY, Lu MH, Lu L, Li S, Chen Y, Cao YJ, Yang ZZ, Oiso S, Nishida K, Kuchiwa S, et al. Caspr4 interaction with LNX2 modulates the proliferation and neuronal differentiation of mouse neural progenitor cells. Stem Cells Dev. 2015; 24:640–52. https://doi.org/10.1089/scd.2014.0261

22. Lenihan JA, Saha O, Mansfield LM, Young PW. Tight, cell type-specific control of LNX expression in the nervous system, at the level of transcription, translation and protein stability. Gene. 2014; 552:39–50. https://doi.org/10.1016/j.gene.2014.09.011

23. Guan GG, Wang WB, Lei BX, Wang QL, Wu L, Fu ZM, Zhou FX, Zhou YF. UBE2D3 is a positive prognostic factor and is negatively correlated with hTERT expression in esophageal cancer. Oncol Lett. 2015; 9:1567–74. https://doi.org/10.3892/ol.2015.2926

24. Tang W, Li H, Tang J, Wu W, Qin J, Lei H, Cai P, Huo W, Li B, Rehan V, Xu X, Geng Q, Zhang H, Xia Y. Specific serum microRNA profile in the molecular diagnosis of Hirschsprung’s disease. J Cell Mol Med. 2014; 18:1580–87. https://doi.org/10.1111/jcmm.12348

25. Suita S, Taguchi T, Ieiri S, Nakatsuji T. Hirschsprung’s disease in Japan: analysis of 3852 patients based on a nationwide survey in 30 years. J Pediatr Surg. 2005; 40:197–201. https://doi.org/10.1016/j.jpedsurg.2004.09.052

26. Shinall MC Jr, Koehler E, Shyr Y, Lovvorn HN 3rd. Comparing cost and complications of primary and staged surgical repair of neonatally diagnosed Hirschsprung’s disease. J Pediatr Surg. 2008; 43:2220–25. https://doi.org/10.1016/j.jpedsurg.2008.08.048

27. Wetterman MA, Sorrentino V, Kasher PR, Jakobs ME, van Engelen BG, Sluiter K, de Wissel MB, Sizarov A, Nürnberg G, Nürnberg P, Zelcer N, Schelhasz HJ, Baas F. A frameshift mutation in LRSAM1 is responsible for a dominant hereditary polyneuropathy. Hum Mol Genet. 2012; 21:358–70. https://doi.org/10.1093/hmg/ddr471

28. Bogdanik LP, Sleight JN, Tian C, Samuels ME, Bedard K, Seburn KL, Burgess RW. Loss of the E3 ubiquitin ligase LRSAM1 sensitizes peripheral axons to degeneration in a mouse model of Charcot-Marie-Tooth disease. Dis Model Mech. 2013; 6:780–92. https://doi.org/10.1242/dmm.010942

29. Tang B, Seredenina T, Coppola G, Kuhn A, Geschwind DH, Luthi-Carter R, Thomas EA. Gene expression profiling of R6/2 transgenic mice with different CAG repeat lengths reveals genes associated with disease onset and progression in Huntington’s disease. Neurobiol Dis. 2011; 42:459–67. https://doi.org/10.1016/j.nbd.2011.02.008

30. Chen D, Mi J, Liu X, Zhang J, Wang W, Gao H. WNT3A gene expression is associated with isolated Hirschsprung disease polymorphism and disease status. Int J Clin Exp Pathol. 2014; 7:1359–68.

31. Celli J. LRSAM1, an E3 Ubiquitin ligase with a sense for bacteria. Cell Host Microbe. 2012; 12:375–36. https://doi.org/10.1016/j.chom.2012.11.007
### Table S1. Primer sequence used for qRT-PCR and miR-939, siRNA related sequence.

|                | Primer sequence                                      | Sense          | Antisense       |
|----------------|------------------------------------------------------|----------------|-----------------|
| LRSAM1         | F: AAGGTGCTGATCGTCACGAC                              |                |                 |
|                | R: TGCCCCAGATCAGCTCAGGA                              |                |                 |
| UBE2D3         | F: CCATATCAAACGCGTGTATTCTT                           |                |                 |
|                | R: GCGGACCACAGTCGATCTTGA                             |                |                 |
| LNX2           | F: GGAACAACAAGAGGATGAGATG                            |                |                 |
|                | R: CACAGGGTGAGTCTAGTGAG                              |                |                 |
| GAPDH          | F: GCACCGTCAAGCTGAGAAC                               |                |                 |
|                | R: GGATCTCGTCCTTGAGAAGAT                             |                |                 |
| LRSAM1 siRNA   | Sense: GCUCUCAGAGAUUCCAUUUTT                         |                |                 |
|                | Antisense: AAAUGGAAUCUCUGACGCTT                     |                |                 |
| Negative control| Sense: UUCUCCGAACUGUGUCACGUTT                       |                |                 |
|                | Antisense: ACGUGACACGUUCGGAGAATT                     |                |                 |
| Hsa-miR-939 mimics | Sense: UGGGGGAGCGUGAGCGUCUGGGGUG                    |                |                 |
|                | Antisense: CCCCCAGAGGCCUCAGCUCCCCAUU                 |                |                 |
| Negative control| Sense: UUCUCCGAACUGUGUCACGUTT                       |                |                 |
|                | Antisense: ACGUGACACGUUCGGAGAATT                     |                |                 |

**Figure S1.** Expression of miR-939 and LRSAM1 in different segments of HSCR bowels. (A) Mir-939 was significantly overexpressed in stenotic segment compared with dilated segment of HSCR (n=80). (B, C) Expression of LRSAM1 significantly increased in dilated segment of HSCR in mRNA (B) and protein (C) (n=80). *indicates significant difference compared with control group, P<0.05.

**Figure S2.** Knockdown of LRSAM1 suppressed cell proliferation. All cells transfected with LRSAM1 siRNA showed impaired cell proliferation compared with control. *indicates significant difference compared with control group, P<0.05.