ABSTRACT

Long noncoding RNAs (lncRNAs) represent a group of regulatory RNAs that play critical roles in numerous cellular events, but their functional importance in development remains largely unexplored. Here, we discovered a series of previously unidentified gene clusters harboring conserved lncRNAs at the nonimprinting regions in brain (CNIBs). Among the seven identified CNIBs, human CNIB1 locus is located at Chr q933.3 and conserved from Danio rerio to Homo sapiens. Chr q933.3-q934.11 microdeletion has previously been linked to human nail-patella syndrome (NPS) which is frequently accompanied by developmental and visual deficiencies. By generating CNIB1 deletion alleles in zebrafish, we demonstrated the requirement of CNIB1 for proper growth and development, and visual activities. Furthermore, we found that the role of CNIB1 on visual activity is mediated through a regulator of ocular development-lmx1bb. Collectively, our study shows that CNIB1 lncRNAs are important for zebrafish development and provides an lncRNA cluster-mediated pathophysiological mechanism for human Chr 9q33.3-9q34.11 microdeletion syndrome.

INTRODUCTION

A gene cluster consists of a group of genes closely spaced on a chromosome that are coordinately expressed or have related functions (1,2). This fascinating system has been a central model for improving our understanding of genomic function and evolution (2–4). However, most of our knowledge about gene clusters comes from protein coding genes. Recently, several types of small noncoding RNA (ncRNA) clusters have been explored. For example, microRNAs (miRNAs) are often found organized in clusters (5–11). The Sfmbt2 miRNA cluster, one of the largest miRNA clusters in mouse, is located in intron 10 of the Sfmbt2 gene and contains 72 miRNA precursors. Both Sfmbt2 and its miRNAs are expressed predominantly from the paternal allele in the placenta. Loss of the entire Sfmbt2 miRNA cluster in Mus. musculus leads to severely impaired placenta development, frequent lethality, and fetal defects (8). Prader-Willi Syndrome (PWS) is a neurodevelopmental disorder associated with the paternal Chr 15q11-q13 deletion (12,13). The imprinted small nuclear ribonucleoprotein N (SNRPN) locus inside this deletion is likely the minimal critical region of PWS and has been found to host at least two small nucleolar RNA (snoRNA) clusters (14–16). Several studies have suggested that a deficiency of the snoRNA clusters confers most, if not all, of the phenotype of PWS (14,17,18), establishing a strong link between misexpression of the snoRNA clusters and central nervous system (CNS) diseases.

Long noncoding RNAs (lncRNAs) represent a large (e.g. ~68% of transcripts in human) and heterogeneous family of RNAs that are over 200 nucleotides in length and regulate various cellular events at the RNA level (19,20). A few independent studies have suggested that lncRNAs may form ‘clusters’ as well (19–21). For instance, we have reported previously that several adjacent lncRNAs derived from the 5’ regulatory region of the CCND1 gene (ncRNA_CCND1) repressed the transcription of the CCND1 gene upon genotoxic stress (20). However, this work did not thoroughly analyze whether these lncRNAs were unique transcripts or processing products from a large single parent lncRNA. Similarly, Tomita S. et al. identified a series of intragenic lncRNAs called Eleonors (ESR1 locus enhancing and activating noncoding RNAs) that play important roles in cis activation of the estrogen receptor during breast cancer adaptation (21).
The human brain, one of the most enriched organs for lncRNAs, has been reported to contain \( \sim 20,000 \) specifically expressed lncRNAs (22), making the number of human brain-expressed lncRNAs comparable to the total number of coding genes in the entire human genome (22). Mercer et al. screened 1328 of Allen Brain Atlas (ABA) probes in *Mus musculus* and discovered 849 brain-specific lncRNAs with precise spatiotemporal expression patterns by *in situ* hybridization (23). Functional studies have suggested that lncRNAs contribute to neuronal cell type identity, plasticity, synaptic transmission, advanced cognitive activity, and brain development (23–25). Although there is a growing body of information and an increasing number of novel identified lncRNAs, the full scope of CNS expressed lncRNAs is only beginning to be understood.

**Danio rerio** (zebrafish) is a well-established vertebrate model system for studying human diseases and has yielded advances in multiple fields including developmental biology, neurobiology, and evolutionary theory of protein encoding genes (26). Recently, several landmark studies have explored the characteristics of zebrafish lncRNAs (27–29). Similar to their mammalian counterparts (22–25,30), zebrafish lncRNAs have been reported to have low-level conservation across species (27,29,31,32). However, it is worth mentioning that although the conserved lncRNAs accounted for <9% of the total lncRNAs in zebrafish, 467 zebrafish lncRNA transcripts had orthologues in both primates and rodents (29). It has been speculated that although the majority of lncRNAs serve as regulators for sophisticated physiological events (33), a small portion of conserved lncRNAs may be indispensable for embryogenesis, viability, and development (31,34). Therefore, by taking advantage of well-established model organisms such as zebrafish, an investigation of the conserved lncRNAs may offer significant advances in understanding the biological roles and molecular consequences of human disease-related lncRNAs.

Here, we report the discovery of a type of novel genomic structure that is composed of lncRNA-based gene clusters and named as *CNIBs* (conserved, nonimprinting lncRNA clusters in the brain). Among the seven identified *CNIBs*, the *CNIB1* locus exhibits the highest conservation score from zebrafish to primate and possesses strong association with human Chr 9q33.3-9q34.11 microdeletion syndrome. The *CNIB1* locus is shown to be required for proper development, locomotor activity, and visual functions in zebrafish. These defects are observed in a portion of Chr 9q33.3-9q34.11 microdeletion patients.

**MATERIALS AND METHODS**

**Experimental animals**

Ten-week-old male BALB/c mice (Vital River Laboratories, Beijing, China) were used in this study and were housed in a temperature-controlled room under a 12-h light/12-h dark cycle, with free access to food and water *ad libitum*. The adult mice were euthanized by cervical dislocation, and their brains were carefully dissected using a stereomicroscope to isolate the four experimental brain regions of interest for this experiment (olfactory bulb, hypothalamus, hippocampus, and cerebellum) according to the mouse brain atlas (35).

AB zebrafish (*D. rerio*) were used as wild-type (WT) zebrafish in this study. Zebrafish embryos were maintained on a 14-h light/10-h dark cycle at 28.5 °C in E3 embryo medium. N-Phenylthiourea (PTU, Sigma, USA) was used to prevent larval pigment formation. All zebrafish surgeries were performed after anesthetization with tricaine methane-sulfonate (Sigma, Cat. No. MS-222) treatment. To determine the temporal and spatial expression levels of *CNIB1* lncRNAs, fish tissue from brain, eye, heart, and intestine were dissected from 15, 30, 60, 120 dpf zebrafish according to a previous study (36).

All animal manipulations were conducted in strict accordance with the guidelines and regulations set forth by the University of Science and Technology of China (USTC) Animal Resources Center and University Animal Care and Use Committee. The protocol was approved by the Committee on the Ethics of Animal Experiments of the USTC (Permit Number: PXHG-SXY201510183 for mouse experiments and USTCACUC1103013 for zebrafish experiments).

**Reverse transcription and quantitative PCR (qPCR)**

Total RNAs from a set of three mouse brain tissues, three adult zebrafish, or a set of 20 zebrafish larvae were extracted using TRIzol Reagent (Ambion, Cat. No. 15596) following the manufacturers’ instructions. RNase-free DNase (Promega, Cat. No. M6101) was used to remove residual DNA. Reverse transcription was performed using the commercially available reverse transcription system (Promega, Cat. No. A5001). The real-time qPCR experiment was performed on Bio-Rad CFX96 qPCR system according to the manufacturers’ instructions (Vazyme, Cat. No. Q111-03). Gene expression levels were calculated relative to the reference gene β-actin. Three technical replicates were performed for each RT-qPCR reaction and at least three biological replicates were performed for each condition. The RT-qPCR primer sequences are listed in Supplementary Table S1.

**LncRNA microarray**

Arraystar Mouse LncRNA Microarray V3.0 (Arraystar Inc.) was designed for the global profiling of mouse lncRNAs and protein-coding transcripts. Approximately 32 368 lncRNAs and 23 395 coding transcripts can be detected using this LncRNA Microarray. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology) with minor modifications. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies). After quantile normalization of the raw data, lncRNAs and mRNAs were chosen for further data analysis. Three biological replicates were used per condition for Microarray analysis, and three to ten mice were used for RT-qPCR assay to validate the Microarray results.
Empirical bayes (eBayes) method

The eBayes method is a practical approach for general Microarray experiments with arbitrary numbers of treatments and RNA samples (37). This approach is equivalent to shrinkage of the estimated sample variances towards a pooled estimate, resulting in greatly stabilized inference when the number of arrays is small. The eBayes method was used for screening region-specific lncRNAs/mRNAs in four brain regions in our research. The six key steps of the eBayes approach include constructing gene expression matrix and an experimental design matrix, building a comparison model and simulating log linear models, eBayes testing, and producing testing results. The eBayes approach can be implemented using the limma software package based on the R and Bioconductor platforms. We screened the region-specific lncRNAs according to three requirements: a normalized expression value larger than 8; a fold change >6 times; a P-value lower than 0.01. The normalized value >8 threshold was decided to ensure that all possible bias of false positive expressing transcripts were removed. ‘Fold change >6’ was decided based on comparing different parameter settings from >2, >4, >6 to >8. The normalized value >8 was found to show the strongest correlation between the brain function and the lncRNAs identified.

RNA-Seq data analysis

The published RNA-Seq data of wild type mouse olfactory bulb (OB) tissue (Gene Expression Omnibus (GEO) accession number GSE93061) was downloaded and analyzed for the genome wide distribution of lncRNAs using the TopHat software. The ‘FPKM (fragments per kilobase of transcript per million mapped reads) value >40’ was then used to select the lncRNAs that were highly expressed in the OB in GSE93061. The ‘FPKM > 40’ threshold was used because that was roughly equivalent to the ‘normalized value > 8’ used in Microarray analysis. It is calculated by the expression level of common lncRNAs detected both in our Microarray data (GSE106832) and published RNA-Seq data (GSE93061).

LncRNA capping assay using anti-2,2,7-trimethylguanosine antibody

The lncRNA capping assay has been previously described by Li et al. (38). Briefly, total RNAs from mouse tissues were separated by TRIzol Reagent after being grounded and homogenized. The obtained RNA samples were then added to beads (15 μl per sample) coupled with anti-2,2,7-trimethylguanosine (anti-m2,2,7,G) (Calbiochem, Cat. No. NA02A) or negative control anti-HA (Proteintech, Cat. No. 51064-2-AP) and equilibrated in cap binding buffer (25 mM Tris 7.5, 10 mM MgCl2, 0.5 mM DTT and 1 U/μl SUPERase.IN (Invitrogen, Cat. No. AM2696)). After incubation for 1 h at 4°C, the beads were washed three times with cap binding buffer. The cap-binding RNA was finally extracted and detected by RT-qPCR using the same primers as listed in Supplementary Table S1.

In vitro transcription

The cDNAs of zebrafish lmx1bb and zebrafish and mouse CNIB1 lncRNAs were cloned into pGEM-T easy vector (General Biosystems, China). The plasmids were linearized to produce DNA templates and RNAs were in vitro transcribed using T7 RNA polymerase (Invitrogen, Cat. No. 18033019) or MAXIscript SP6 Transcription Kit (Invitrogen, Cat. No. AM1330) in combination with normal NTPs (Invitrogen, Cat. No. 18109017). In vitro transcribed RNAs were further used in zebrafish for overexpression, northern blotting, or ISH assays directly or after biotin/digoxigenin modifications, as described below.

Northern blotting

Antisense or sense in vitro transcribed probes for CNIB1 lncRNAs were labeled with biotin RNA labeling mix (Sigma, Cat. No. 11685597910), followed by Northern blotting as described previously (39). Briefly, 10 μg of RNAs from the eyes of 2-month-old mice or zebrafish were separated in a 1% denaturing gel. RNAs were then transferred to Hybond-N membrane and hybridized with the individual biotin-labeled probes in hybridization buffer (Invitrogen, Cat. No. AM8670). After hybridization, blots were washed and detected by chemiluminescent nucleic acid detection module (Thermo Scientific, Cat. No. 89880). The Northern probes sequences are listed in Supplementary Table S2.

In situ hybridization

Whole mount in situ hybridization was performed as previously described (40). Briefly, fixed larvae were incubated in 50% formamide hybridization buffer with digoxigenin-labeled CNIB1, pbx3b or lmx1bb RNA probes, which were synthesized by in vitro transcription as described above. Following probe hybridization to processed samples, the digoxin-labeled probes were recognized using anti-digoxin (Roche, Cat. No. 11093274910, 1:5000 dilution) antibody at 68°C for 16–20 h. This digoxin antibody was reacted with the substrate of alkaline phosphatase (Promega, Cat. No. 0000185009). Approximately 8–15 larvae were used in each ISH experiment. Three independent ISH experiments were conducted with the specific antisense probe, and the stained embryos were imaged with BX-60 and BX-16 microscope (Olympus, Japan), respectively. The ISH probe sequences are listed in Supplementary Table S3.

CRISPR-Cas9 mediated gene modification and identification

The sgRNAs were designed using CRISPR-DO software (41) to target the upstream site of uc.273+ and the downstream site of uc.277+ (one sgRNA for each site) in order to excise the whole genomic region of the five intronic lncRNAs (uc.273+, uc.275+, uc.275−, uc.276+, and uc.277+) in zebrafish. In vitro synthesized Cas9 mRNA obtained with the T7 mMESSAGE mMACHINE Kit (Ambion, Cat. No. AMB13345) and the sgRNAs synthesized with T7 Riboprobe Systems (Promega, Cat. No. P1460) were co-microinjected into one-cell zebrafish embryos. Genomic DNA was extracted from 10 embryos at 24 hours postfertilization (hpf) and used as templates for genotyping to test
the engineering efficiency. Three pairs of specific primers for polymerase chain reaction (PCR) were designed to distinguish wild type, heterozygous, or homozygous knockout (KO) alleles. Each resulting PCR product was confirmed by gel electrophoresis. Separated electrophoresis bands of the PCR products were then purified and sequenced to ensure the genotype of tested zebrafish. The sgRNA and genotype primer sequences are listed in Supplementary Table S4.

The rest of injected embryos (F0) were raised to adulthood and then crossed with wild-type zebrafish to produce F1 embryos. From each cross, fin-clipped DNA of 10 F1 embryos were collected for genotyping. The F1 embryos that carried heterozygous knockout alleles or WT alleles were raised to adulthood and further crossed with their same genetic background siblings to generate F2 fish for downstream experiments.

Knockdown of \textit{lmx1bb} using morpholino oligonucleotides (MO)

\textit{Lmx1bb} MOs were designed as previously described (42) to target the splicing site junction between exon 1 and intron 1. Mismatch \textit{lmx1bb} control morpholino was synthesized as a negative control. The MOs were resuspended in embryo medium. For efficient knockdown, \textit{lmx1bb} MO (4 ng) or mismatch MO (4 ng) was injected per WT embryo. About 300 embryos were injected in each experiment. The data were analyzed from three independent experiments. The morpholino sequences are listed in Supplementary Table S4.

\textbf{RNAs microinjection in zebrafish embryos}

Five \textit{CNIB1} lncRNAs (\textit{uc.273} +, \textit{uc.275} +, \textit{uc.275} −, \textit{uc.276} +, and \textit{uc.277} +), their antisense controls (except for \textit{uc.275} +/−), and \textit{lmx1bb} mRNA were obtained by \textit{in vitro} transcription as described above in the ‘\textit{In vitro} transcription’ section. About 2 ng of \textit{lmx1bb} mRNA, 1 ng of \textit{CNIB1} lncRNAs mix, or 1 ng of antisense lncRNAs mix were injected into WT and/or mutant embryos. The expression levels of \textit{CNIB1} lncRNAs, \textit{lmx1bb} mRNA, and Lmx1bb protein were detected at 48 hpf. Each data point contained expression data for 30 embryos. Three biological repeats were used per condition.

\textbf{Zebrafish locomotor activity analysis}

Zebrafish locomotor activity analysis was performed as described previously with minor modifications (43). At 4 dpf, a single larva was placed in each of the 96 wells of a 96-well plate (32 WT larvae, 32 offspring of \textit{CNIB1}+/− incross larval type 1, and 32 offspring of \textit{CNIB1}+/− incross larval type 2), in a Viewpoint apparatus (France). The locomotor behavior of larvae was monitored for three consecutive days under the previously stated LD cycle using an automated video-tracking system (VideoTrack, Viewpoint), and the movement of each larva was recorded and analyzed using Zebralab 3.10 software (Viewpoint). Inside the View-Point chamber, constant infrared light was provided, and white light was provided from 08:30 A.M. to 10:30 P.M. Temperature controlling devices were placed in the chamber to maintain a constant temperature of 28.5°C. The Video-Track quantization parameters were selected as described previously (44). Data were further analyzed using GraphPad software and Visual Basic macros for Microsoft Excel.

\textbf{Measurement of the zebrafish larval optokinetic response (OKR)}

WT or offspring of \textit{CNIB1}+/− incross larval zebrafish at 5 dpf were conducted for OKR as described previously (45). A customized program was developed for sine-wave grating generation and real-time recording of eye movements based on LabVIEW 7.1 (National Instruments, USA) and NI-IAQ 3.7 (National Instruments, USA). The 5 dpf larvae were placed dorsal side up in 5% methylcellulose in the center of a transparent petri dish located under a cone-shaped white paper screen to immobilize them during the experiments (46). As rotating grating patterns were presented around eyes of the larvae, the elicited eye movement was recorded in real time by an infrared-sensitive charge-coupled device (CCD camera) (TCA-1.3BW, China). All tests were conducted at 05:00 P.M. The gain value, i.e. the ratio of eye velocity to the angular velocity of the grating, was calculated using the software as previously described (46).

\textbf{Histology and immunohistochemistry}

WT or offspring of \textit{CNIB1}+/− incross larval zebrafish were raised to 5 dpf and used for tyrosine hydroxylase (Th) immunofluorescence staining. The larvae were fully dark adapted for ~30 min in order to separate the pigment epithelium from the retina, and they were then fixed with 4% PFA in PBS. The fixed larvae were rinsed with PBS. The eyes were dissected out, and both the crystalline lens and the pigment epithelium were removed. The fixed retinas were incubated in blocking solution at 4°C overnight and then incubated in Th primary antibody (Millipore, Cat. No. MAB318, 1:1000) at 4°C overnight. The following day, the samples were rinsed with PBS, incubated with secondary antibody (Alexa 488-conjugated anti-mouse, Invitrogen, Cat. No. A-21202, 1:1000) for 3 h, and then rinsed with PBS. All the specimens were mounted on slides for fluorescence microscopy (BX60, Olympus).

\textbf{Statistical analysis}

All experiments were independently repeated at least three times. The data were analyzed with an unpaired, two-tailed t-test or one-way ANOVA using GraphPad Prism. The results are shown as the mean ± SEM. The level of significance was set to \( P < 0.05 \). * * * represented \( P < 0.01 \), and \( P < 0.001 \), respectively.

\textbf{RESULTS}

\textbf{Analysis of lncRNAs that are highly expressed in the brain}

To decipher the spatial expression landscape of well-annotated lncRNAs in various brain regions, we dissected adult male mouse brain tissues from the olfactory bulb (OB), the hypothalamus (Hypo), the hippocampus (Hippo),...
Identification of lncRNA clusters that are conserved, located in nonimprinting regions and expressed in the brain (CNIBs)

In analyzing the brain highly expressed lncRNAs, we noticed that 34 lncRNA loci were localized close together, forming nine clusters on seven chromosomes, with a minimum number of three lncRNAs for each cluster (Table 1 and data not shown). The expression of these 34 lncRNAs was further confirmed by data mining of published RNA-Seq data generated from wild type (WT) mouse tissues by Li et al. (47) (data not shown). Due to the high conservation of various types of gene clusters that have been previously reported, we performed conservation analysis of the identified 9 lncRNA clusters using the phastCons method (Supplementary Figure S2). Among these clusters, seven were considered to be conserved clusters because the involved lncRNAs were derived from either ultraconserved elements (UCRs) discovered by Bejerano et al. (48) or from shared conserved patches (SCR; ~71–1066 bp, 65–100% identity; Table 1, Figure 1A, Supplementary Figure S3). All seven conserved clusters were located in nonimprinting regions according to the Geneimprint database (http://www.geneimprint.com/site/home; Supplementary Figure S2). We thus named these seven lncRNA clusters as CNIB lncRNA clusters, referring to conserved, nonimprinting lncRNA clusters in the brain. A flowchart of the analytical process is shown in Supplementary Figure S2.

Characterization of CNIB1 lncRNAs

Among the seven CNIBs, CNIB1 was particularly interesting because it consisted of the largest number (8) of lncRNAs. Moreover, the 8 lncRNAs in CNIB1 included seven UCRs (uc.273+, uc.275+, uc.276+, uc.275−, uc.274+, and uc.273+) and 1 SCR (ENSMUST00000155423). Except for uc.274+ (originating from G. gallus), the remaining six UCR and one SCR loci appeared since zebrafish (Danio rerio) (Figure 1A). At the level of DNA sequences, uc.280+ showed 76% identity throughout evolution, while the remaining 7 CNIB1 lncRNAs contained hundreds of nucleotide sequences that were 87%–100% identical during evolution. In addition, these CNIB1 lncRNAs were conserved at their genomic location (derived from the transcriptional factor pbx3 locus) (Figure 1B) and overall organization (same synthetic order and strand) once they appeared.

To further investigate the characterization of CNIB1 lncRNAs, we collected Encyclopedia of DNA Elements (ENCODE) chromatin immunoprecipitation sequencing (ChIP-Seq) data for the distribution of peaks of promoter marker (H3K4me3), enhancer markers (H3K27ac and H3K4me1), insulator marker CTCF, and RNA polymerase II binding sites in murine CNIB1 genomic region. The ChIP-Seq data showed that each lncRNA had a unique transcriptional start site and was transcribed independently (Figure 1B). In addition, using the anti-methyltransferase antibody, we showed that murine CNIB1 lncRNAs were capped at their 5′ ends (Figure 1C). According to our northern blotting results (Figure 1D and E), most of the CNIB1 lncRNAs seemed to have multiple variants both in mouse and zebrafish. RT-qPCR (Figure 1F and G) experiments showed that all the five detectable CNIB1 lncRNAs (uc.273+, uc.275+, uc.275−, uc.276+, uc.277+) exhibited similar temporal and spatial expression patterns in both mouse and zebrafish. It is worth noting that many CNIB1 lncRNAs showed elevated expression levels in brain and eye of both mice and zebrafish (Figure 1F and G), which was further confirmed by whole-mount ISH performed on zebrafish larvae (Figure 1H and I). Taken together, the similar characterization and expression pattern of CNIB1 lncRNAs in mouse and zebrafish indicated a conserved function for CNIB1 during evolution.

Microdeletion of Chr 9q33.3–9q34.11, encompassing the CNIB1 locus, is associated with the clinical spectrum of developmental retardation and visual defects

As illustrated in Figure 2A, the human homologous sequence of CNIB1 was located on Chr 9q33.3. In human, microdeletion of Chr 9q33.3–9q34.11 is associated with nail-patella syndrome (NPS) (49–53). NPS is an autosomal dominant disease, hallmarkd by nail dysplasia and patellar aplasia or hypoplasia. Due to the complex genetic background, individuals with NPS display varying degrees of severity. Other areas of the body are often affected among NPS patients, including kidneys and the optical system. LMX1B, located on Chr 9q33.3, is a member of the LIM homeobox transcription factor and currently thought to be associated with NPS manifestations (54, 55). However, it remains unclear whether the CNIB1 region is also involved in the development of NPS (49, 56).

To date, 10 NPS patients with relatively full clinical and genetic characterization have been reported (49–53) (Figure 2B). A summary of the clinical symptoms of each patient is listed in Supplementary Table S6. The common deletion region of Lmx1b is marked as Region b in Figure 2B. Among these 10 patients, 3 patients (patients 3, 5, and 6) had CNIB1 deletion (Region a in Figure 2B). All three CNIB1 deletion patients displayed severe developmental retardation and neurological manifestations. Moreover, two of these patients exhibited optical deficiencies (Supplementary Table S6). This information suggested that the visual
Figure 1. Characteristics of CNIB1. (A) Phylogenetic tree of CNIB1 genomic locus in the 14 indicated species. (B) Comprehensive view of multiple peak distributions of promoter marker (H3K4me3), enhancer markers (H3K27ac and H3K4me1), insulator marker CTCF and Pol2 binding sites on CNIB1 locus suggest these lncRNAs in CNIB1 are transcribed as independent lncRNAs. The three regions highlighted in turquoise were predicted to be the transcription start sites for CNIB1 lncRNAs. Scale bar: 10kb. (C) Cap assay of indicated CNIB1 lncRNAs. Immunoprecipitation was performed using anti-m2,2,7G antibody or control anti-HA, followed by RT-qPCR detection. Bar plots represent relative enrichment of RNAs immunoprecipitated by the antibody and normalized to GAPDH. (D, E) Northern blotting of CNIB1 lncRNAs in the eyes of mouse (D) and of zebrafish (E) both at 2 months. (F, G) Heatmap of the expression level of CNIB1 lncRNAs. qPCR assays were performed using the brain, eye, heart, and intestine tissues from three 10-week old male mice (F) and six zebrafish (G) at the indicated developmental time points (15, 30, 60 and 120 dpf, respectively), normalized to β-actin. (H) Schematic representation of zebrafish. (I) Whole-mount ISH of five indicated CNIB1 lncRNAs (uc.273+, uc.275+, uc.275-, uc.276+, uc.277+) and pbx3b using corresponding antisense digoxigenin-labeled probes in larval zebrafish (n = 8). The yellow dotted oval marks the area where the eye of zebrafish is located.

disorders and other symptoms of NPS might be attributed to the CNIB1 region.

Interestingly, besides the association between CNIB1 locus and NPS syndrome, we found that three other CNIB (CNIB2, CNIB3, and CNIB4) loci were also linked to human CNS defects (Supplementary Figures S4 and S5 and Supplementary Tables S7–S9). CNIB2 was transcribed from the Meis2 gene. Among the 17 patients with CNS developmental defects and carrying a Chr 15q14 microdeletion, the region encompassing CNIB2 and its nearby Meis2 was the minimal common key fragment (Supplementary Figure S4A–C). CNIB3 region was also the smallest shared part in over half of patients with Chr 13q32.2-33.2 deletion, which manifested as severe neural tube defects, abnormalities of eye/hand/foot, and facial dysmorphisms (Supplementary Figure S5A–C) (63). Dlx-5 and Dlx-6, the host genes of CNIB4, have been reported as critical regulators of neuronal-glial fate specification and oligodendrocyte lineage maturation (64). NR_015388 and ENSMUST00000159827 (also known as Evf2) are within the CNIB4 locus. These two lncRNAs are able to interact with BRG1 to mediate chromatin remodeling events (65). Although genome mutations were not found for CNIB4 lncRNAs, we discovered that the mutation of the RNA binding domain of BRG1 protein was associated with Coffin-Siris syndrome, a neurodevelopmental disorder (Supplementary Tables S8 and S9) (65).
To investigate the locomotor activity, and visual abnormalities $CNIB1$ knockout fish. Two independent deletion alleles were obtained in F1 generation by fin-DNA genotyping; $CNIB1^{+/−}$ 1 was the result of a 48 247 bp deletion and $CNIB1^{+/−}$ 2 had a 48 221 bp deletion (Figure 3B). These F1 heterozygous fish were incrossed to generate F2 offspring. Interestingly, we could not obtain any homozygous offspring and the ratio between wild-type (WT) and heterozygous offspring was about 1:2 (Figure 3C). Further investigation showed that the number of surviving eggs from heterozygous incross was reduced to three quarters of that of WT incross at 24 hpf, although both groups had almost identical numbers of laid and living eggs at 4 and 12 hpf (Figure 3D). These results suggested that the $CNIB1^{−/−}$ zebrafish was embryonic lethal and the mortality might begin during segmentation.

In $CNIB1$ heterozygous fish, all 5 involved lncRNAs were significantly downregulated when compared with their WT siblings (Figure 3E). In contrast, gapvd1 and mwb12wb, the two genes neighboring $CNIB1$ and $pbx3b$, showed no significant changes (Figure 3E). In order to eliminate the possibility that $CNIB1^{−/−}$ affected splicing of the $pbx3b$ gene, we next sequenced the exon 2–exon 3 junction (Figure 3F) and the tested alternative splicing events of the $pbx3b$ gene, and designed multiple PCR primer sets for alternative splicing events of the $pbx3b$ gene. Our data showed that exon 2–exon 3 junction (Figure 3F) and the tested alternative splicing events of the $pbx3b$ gene (Figure 3G and Supplementary Figure S6) remained the same under both WT and $CNIB1^{+/−}$ backgrounds. These data together indicated that $CNIB1^{+/−}$ had no effect on the splicing of host gene $pbx3b$.

Furthermore, we found that F2 $CNIB1^{+/−}$ zebrafish displayed developmental delay, decreased locomotor activity and visual abnormalities when compared with offspring generated from WT incross. First, at 48 hpf, more than 75% of the eggs laid by the WT incross group hatched, while the hatching rate of $CNIB1^{+/−}$ 1 or $CNIB1^{+/−}$ 2 incross...
Figure 2. Schematic view of the genetic background of Chr 9q33.3–9q34.11 microdeletion patients. (A) Schematic representation of the Chr 9q33.3–9q34.11 region encompassing the CNIB1 locus (ENSMUST00000155423, uc.273+, uc.275+, uc.275-, uc.276+, and uc.277+, pbx3, and lmx1b. (B) Schematic view of the genetic background of ten Chr 9q33.3–9q34.11 microdeletion patients as reported in published articles (see Supplementary Table S6). Information of patients 1-4 was adopted from the published article (85).

was less than 20% (Figure 4A and B). Second, the body lengths of larval at 72 hpf (Figure 4C and D) and adult fish at 4 months of age (Figure 4E and F) were significantly shorter in offspring from $CNIB1^{+/−}$ or $CNIB1^{+/−}$ 2 incross. Similar results were observed in the F1 generation (Supplementary Figure S7A and B). Third, the locomotor activity assay showed that the average daytime and nighttime speeds of offspring from $CNIB1^{+/−}$ or $CNIB1^{+/−}$ 2 incross were significantly decreased (Figure 4G–J). Finally, offspring from $CNIB1^{+/−}$ or $CNIB1^{+/−}$ 2 incross exhibited smaller eyes (Figure 5A and B) and impaired eye velocity based on the decreased gain value in the optokinetic response (OKR) assay (66) (Figure 5C and D). To further study the effect of $CNIB1$ on eye development and function, we tested whether the structure of the visual system was affected in the $CNIB1$ knockout fish. By performing DAPI staining on the eyes, our results showed that there was no gross morphological difference in the retinas between $CNIB1^{+/−}$ 2 and WT zebrafish (Supplementary Figure S8). These results suggested the defect in $CNIB1^{+/−}$ 2 is not structural. Retinal dopamine, synthesized by the action of tyrosine hydroxylase (TH) in dopamine (DA) neurons, plays multiple trophic roles in light adaptation, cell survival, and eye growth (67–69). TH is a widely used marker of DA neurons (70). Therefore, we tested the number of retinal DA neuron and the expression level of $th$. Our results showed a decreased number of TH-labeled DA neurons in isolated $CNIB1^{+/−}$ fish retinas (5 dpf) (Figure 5E and F) and a significant reduction of $th$ mRNA level in $CNIB1^{+/−}$ 2 zebrafish (Figure 5G) when compared to their WT siblings. Taken together, our data indicated that the $CNIB1$ locus was essential for the proper development, locomotor activity, and visual function in zebrafish.

$CNIB1$ intronic lncRNAs mediate visual function through $lmx1b$ in zebrafish

In order to determine if the $CNIB1$ lncRNAs contributed to the visual defects observed in offspring generated from heterozygous incross, we microinjected a pool of in vitro transcripts of the 5 intronic $CNIB1$ lncRNAs (uc.273+, uc.275+, uc.275-, uc.276+, and uc.277+) into the embryos of either WT or $CNIB1^{+/−}$ 2 incross. Beside the sense $CNIB1$ lncRNAs ($CNIB1_S$) injections, several parallel injections were performed to the same batch of fish siblings serving as negative controls, including sham operation without RNA (Control-Injected), and a pool of antisense transcripts from single stranded uc.273+, uc.276+, and uc.277+ ($CNIB1_AS$). Our RT-qPCR assay showed that all RNAs were overexpressed in 48 hpf embryos (Supplementary Figure S9A, B, C, and D). Further investigation showed that, compared to the ‘Control-Injected’ or ‘$CNIB1_AS$’ controls, those F2 fish growing from $CNIB1^{+/−}$ 2 incrossed em-
Figure 3. Generation of CNIB1 knockout by CRISPR-Cas9 in zebrafish. (A) Schematic representation of the knockout strategy using CRISPR-Cas9. Scale bar: 10 kb. (B) The genotype sequencing results of CNIB1+/+ and CNIB1/−/− zebrafish confirmed by fin-DNA genotyping (n = 6). (C) The number and the percent of CNIB1 zebrafish genotype (n = 24) from offspring of CNIB1+/+ incross confirmed by embryo-DNA genotyping. (D) The average number of surviving embryos in offspring of both CNIB1+/+ incross (n = 289 at 4 hpf, n = 250 at 12 hpf, n = 207 at 24 hpf) and CNIB1/−/− zebrafish incross (n = 277 at 4 hpf, n = 244 at 12 hpf, n = 161 at 24 hpf) at 4 hpf, 12 hpf, and 24 hpf, respectively (n = 4). (E) The expression levels of CNIB1 lncRNAs and two nearby genes (gapvd1 and mhb12bb) in 30 CNIB1+/+ and 30 offspring of CNIB1/−/− zebrafish, normalized to β-actin. (F) Detection of the exon 2–exon 3 junction of pbx3b mRNA by cDNA sequencing in CNIB1+/+ and CNIB1/−/− zebrafish confirmed by fin-DNA genotyping (n = 6). (G) Alternative splicing events of pbx3b in CNIB1+/+ and CNIB1/−/− zebrafish confirmed by fin-DNA genotyping (n = 6).
Figure 4. CNIB1\textsuperscript{+/-} zebrafish exhibit developmental retardation and impaired locomotor activity. (A) Representative hatching status of the zebrafish offspring at 48 hpf from CNIB1\textsuperscript{+/-} (n = 130), CNIB1\textsuperscript{+/-} 1 (n = 130) and CNIB1\textsuperscript{+/-} 2 (n = 130) incross. Scale bars: 2 mm. (B) Schematic representation of A. (C, D) Representative images and the schematic representation of the fork lengths of the zebrafish offspring at 5 dpf from CNIB1\textsuperscript{+/-} (n = 42), CNIB1\textsuperscript{+/-} 1 (n = 27) and CNIB1\textsuperscript{+/-} 2 (n = 15) incross. Scale bars: 500 μm. (E, F) Representative images and the schematic representation of the fork lengths of CNIB1\textsuperscript{+/-} (n = 11), CNIB1\textsuperscript{+/-} 1 (n = 8) and CNIB1\textsuperscript{+/-} 2 (n = 5) adult zebrafish at 4 months. Scale bars: 4500 μm. (G, H) Activity records of the zebrafish offspring at 5 dpf from CNIB1\textsuperscript{+/-} (n = 16), CNIB1\textsuperscript{+/-} 1 (n = 16) and CNIB1\textsuperscript{+/-} 2 (n = 16) incross at day (G) or night (H). (I, J) The schematic representation of the average locomotion speed gathered from figures G and H, respectively.

bryos that were injected with CNIB1\_S exhibited reduced mortality rates (Supplementary Figure S10) and exhibited significant improvement in ameliorated gain value assayed by OKR (Figure 5H). Our results indicate that CNIB1 intronic RNAs play critical roles in ocular function. However, a possible involvement of CNIB1 locus is not ruled out.

Lmx1b, known as lmx1bb or lmx1b.1 in zebrafish, the proposed causative gene of Chr 9q33.3-9q34.11 microdeletion syndrome (54,55), has also been reported to influence fgf-mediated retinal patterning during zebrafish eye development (42). To investigate the potential involvement of lmx1bb in CNIB1-mediated visual regulation, we first examined whether CNIB1 regulated the distribution and expression of lmx1bb byISH. Our results showed that the expression levels of lmx1bb in the eye and brain at 48 hpf were significantly reduced in CNIB1\textsuperscript{+/-} zebrafish when compared with WT zebrafish (Figure 6A, B). Moreover, this reduction could be completely restored to the normal level after over-expression of CNIB1 incRNAs (CNIB1\_S) (Figure 6B).

It has been reported that both lmx1bb-mutant zebrafish (71) and lmx1bb morphants exhibited visual impairment, which could be rescued by co-injection of lmx1bb mRNA (Figure 6C–E). Although overexpression of many transcription factors can cause abnormalities in WT embryos, it has been reported that overexpression of lmx1bb did not cause obvious developmental or visual abnormalities in frog (77), chicken, and mouse (78). Consistent with reported observations, when we overexpressed lmx1bb in WT zebrafish embryos, the fish embryos showed similar developmental processes and normal visual function when compared to the control injected fish (Supplementary Figure S11 and Figure 6H). Furthermore, overexpression of lmx1bb in offspring generated from CNIB1\textsuperscript{+/-} 2 incross (Supplementary Figure S12 and Figure 6F and G) significantly promoted visual function compared with offspring generated from CNIB1\textsuperscript{+/-} 2 zebrafish (Supplementary Figure S13). However, only the expression level of foxc1a was shown to be reverted in lmx1bb overexpressed zebrafish. Taken together, our data suggests that the activity of CNIB1 is achieved, at least partially, through CNIB1 intronic IncRNAs-lmx1bb-foxc1a.
A notable characteristic of lncRNAs is their low-level of conservation across evolution. Because of this outstanding feature for the majority of lncRNAs, lower organism such as Danio rerio is not considered a good model system for lncRNA-related human disease studies. Here, we discovered a previously unidentified genomic structure, designated as CNIBs, which are formed by highly conserved lncRNAs and appear as gene clusters. We further used CNIB1 as an example to demonstrate that Danio rerio is an applicable model system to investigate the biological consequences of human disease-related lncRNAs which have orthologues in zebrafish.

Many lncRNAs regulate their neighboring or embedded protein encoding genes in cis (79). However, the intronic CNIB1 lncRNAs we focused on, seem to be functionally independent of their host gene pbx3 (b). First, the expression patterns of pbx3b and CNIB1 lncRNAs were different. While CNIB1 lncRNAs showed strong signals in mouse and zebrafish eyes, pbx3b was not found to be localized in zebrafish eyes in this study or the study by Arment et al. (80). Second, the phenotypes of pbx3b and CNIB1 KO zebrafish were not similar. Zewdu et al. generated pbx3bΔsm8 mutant zebrafish by creating an early stop codon at exon 3 of pbx3b (81). They found that pbx3bΔsm8/sm8 larvae were viable and normal during development. In contrast, CNIB1Δ−/− larvae showed to be embryonic lethal. The pbx3bΔsm8/sm8 zebrafish (5 dpf) did not exhibit any obvious eye defects, while the CNIB1Δ−/− zebrafish exhibited smaller eyes and visual abnormalities at the same developmental stage. It is worth mentioning that pbx3bΔsm8/sm8 fish have intact expression levels of CNIB1 lncRNAs in theory, but a loss of DNA binding activity which is located at its exon 6. Therefore, the discrepancy in phenotypes between pbx3bΔsm8/sm8 and CNIB1 KO fish strongly indicated that the biological function of pbx3b, at least as a transcriptional factor through DNA binding activity, is not associated with CNIB1.

During the review process for this manuscript, Parenteau et al. and Morgan et al. showed that intron RNAs can help cultured yeast cells to adapt to environmental changes associated with nutrient depletion. Similarly to that of CNIB1 lncRNAs, these intron RNAs play different roles from their host protein encoded genes (82,83). The functional RNAs investigated by Parenteau et al. and Morgan et al. were un-spliced pre-mRNAs or excised/debranched introns. In contrast, CNIB1 intrinsic RNAs identified in the current study...
belonged to the lncRNA family. The evolutionary importance of these intron-derived RNAs being independent of their protein gene host requires further investigation.

From zebrafish to human, the CNIB1 cluster is without exception located on the same chromosome as the lmx1b gene. In human, a large genetic deletion encompassing CNIB1 and LMX1B on Chr 9q33.3-9q34.11 has been linked to osteo-onychodysplasia, growth retardation, glomerulopathy, and optical anomalies, which produce NPS (49–53). Genetic studies in both mouse and zebrafish have demonstrated that Lmx1b is an essential regulator of eye development and optical function (42,73–76,84), suggesting a conserved role for Lmx1b in the optical system. Similar to CNIB1−/− zebrafish, Lmx1b−/− mice are embryonic lethal. In zebrafish, lmx1b has been reported to promote the survival of periocular mesenchymal cells by targeting foxc1a and eya2 and to influence fgf-mediated retinal patterning (42). We found that the CNIB1 locus acts as an upstream regulatory signal for Lmx1b, injection with in vitro transcribed lmx1b mRNA. (G) Quantified expression levels of Lmx1b protein (normalized to β-actin) in the zebrafish shown in F, n = 20. (H) Gain value of the OKR assay in the zebrafish shown in F. Control-injected CNIB1+/+, n = 12; lmx1b CNIB1+/+, n = 12; Control-injected CNIB1−/−, n = 9; lmx1b CNIB1−/−, n = 10.

Figure 6. CNIB1 mediates visual function via lmx1b in zebrafish. (A) In situ hybridization of lmx1b (indicated by arrowheads) in zebrafish offspring of CNIB1+/+ and CNIB1−/− 2 incross at 48 hpf. Scale bars: 400 μm. (B) Relative expression level of lmx1b mRNA detected by RT-qPCR in the indicated zebrafish offspring (CNIB1+/+ or CNIB1−/− 2) that were either Control-injected or injected with in vitro transcribed CNIB1 sense (CNIB1_S) or antisense RNAs (CNIB1_AS). The data were normalized to β-actin. (C) Representative images of Western blot for Lmx1b and β-actin in CNIB1+/+ zebrafish with the indicated modifications. Control MO, mismatch lmx1b MO; lmx1b MO, lmx1b morphants; lmx1b MO+lmx1b, co-injection of lmx1b morphants and in vitro transcribed lmx1b mRNA. (D) Quantified expression level of Lmx1b protein (normalized to β-actin) in the zebrafish shown in C, n is 30 in each group. (E) Gain value of the OKR assay in the zebrafish shown in C. Control-injected, n = 6; Control MO, n = 6; lmx1b MO, n = 6; lmx1b MO + lmx1b, n = 6. (F) Representative images of Western blot for Lmx1b and β-actin in zebrafish offspring from either CNIB1+/+ or CNIB1−/− 2 incross with the indicated modifications. lmx1b, injection with in vitro transcribed lmx1b mRNA. (G) Quantified expression levels of Lmx1b protein (normalized to β-actin) in the zebrafish shown in F, n = 20. (H) Gain value of the OKR assay in the zebrafish shown in F. Control-injected CNIB1+/+, n = 12; lmx1b CNIB1+/+, n = 12; Control-injected CNIB1−/−, n = 9; lmx1b CNIB1−/−, n = 10.
CNIBs to human CNS defects as well. The CNIBs identified in this study are unique because of their special genomic structure, location, conservation, and association with human CNS diseases. LncRNA-based gene clusters are likely to be existed in other systems. These clusters may serve as valuable resources to deepen our understanding of IncRNAs during evolution and human diseases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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