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Two Nipped-B-Like Protein A (Nipbla) Gametologs in Chinese Tongue Sole (*Cynoglossus semilaevis*): The Identification of Alternative Splicing, Expression Pattern, and Promoter Activity Analysis

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In mammals, the mutation of nipped-B-like protein (nipbl) leads to Cornelia de Lange Syndrome (CdLS), characterized by low birth weight, short stature, and structural abnormalities of the skeleton, heart, and gut. In Chinese tongue sole (*Cynoglossus semilaevis*), a typical marine fish exhibiting sexual size dimorphism, the *nipbl* homolog gene (nipped-B-like protein A (nipbla)) was also screened with female higher expression level by somatotropic and reproductive tissues' transcriptomic analysis. In this study, two nipbla genes, namely, *nipbla-w* and *nipbla-z*, were identified from the W and Z chromosomes of *C. semilaevis*, respectively. Similar to other mammalian and fish species nipbl, the two homolog proteins of *C. semilaevis* contained two conserved domains, namely, cohesion_HEAT and Nipped-B_C. The phylogenetic tree analysis showed that these two nipbla gametolog proteins were first clustered together and then grouped with other fish species. At least two types of alternative splicing sites were observed in exon 12 of the *nipbla-z* gene, which produced *nipbla-z-tv1* and *nipbla-z-tv2*. Also, the sex-biased expression patterns of different *nipbla-w* and *nipbla-z* transcripts in female and male tissues were revealed by quantitative PCR (qPCR). The highest expression level of *nipbla-w* was observed in female gonad. While *nipbla-z-tv1* exhibited relatively high expression in the muscle, liver, gonad, and brain, *nipbla-z-tv2* only showed its expression superiority in the muscle of male individuals. The promoter regions of *nipbla* genes were amplified, and their transcription activity was successfully verified by a dual-luciferase reporter system. After *nipbla-w* and *nipbla-z* knockdown in the brain cell lines by RNA interference, a series of growth-related genes were influenced,

including Bone Morphogenetic Protein 4 (*bmp4*), Wnt Family Member 11 (*wnt11*), and Sprouty Related EVH1 Domain Containing 2 (*spred2*). The prediction of transcription factors suggested that c-Jun, sex-determining region Y (SRY), POU Class 1 Homeobox 1 (POU1F1a), myogenic differentiation antigen (MyoD), signal transducer and activator of transcription 5a (STAT5A), and nuclear factor I C (Nfic) might be the putative upstream regulatory factors for *nipbla*; among them, c-Jun has been verified to effectively regulate the transcriptional activity of *nipbla*. The identification of two *nipbla* genes provided important data for interpreting the sexual size dimorphism in *C. semilaevis.*

Keywords: *nipbla*, sex-biased expression, Chinese tongue sole (*Cynoglossus semilaevis*), sexual size dimorphism, gametolog

INTRODUCTION

Sexual dimorphism in the sizes, shapes, and colors between female and male individuals has been broadly described in mammals, reptiles, birds, and fishes (Parker, 1992; Foellmer and Moya-Larano, 2007; Lindenfors et al., 2007; Székely et al., 2007; Mei and Gui, 2015). The sex-biased gene expression was a common phenomenon in various animals (Ranz et al., 2003; Yang et al., 2006; Mank et al., 2007, 2010) and has been linked to the sexual body size of wild turkey (*Meleagris gallopavo*) (Pointer et al., 2013). Thus, the study of sex-biased gene expression is crucial for understanding the molecular mechanism of sexual dimorphism.

In our previous transcriptomic study, thousands of sex-biased genes have been identified from the somatotropic and reproductive tissues of Chinese tongue sole (*Cynoglossus semilaevis*) (Wang et al., 2018), which is a female heterogamete (ZW/ZZ) fish species with typical female-biased sexual size dimorphism. One female-biased gene, i.e., nipped-B-like protein A (*nipbla*), and also one hub gene derived from the positive-related module (Wang et al., 2018) have caused our interest. Importantly, the mutation or disruption of *nipbl* in mammals leads to transcription dysregulation, which accounts for 60% of Cornelia de Lange syndrome, a disorder characterized by growth delay, limb reduction defects, and structural defects of multiple organs (Krantz et al., 2004; Liu et al., 2009; Mannini et al., 2013; Lindgren et al., 2014). Furthermore, five single-nucleotide polymorphisms (SNPs) of human nipbl based on the GWAS Catalog[1] have been identified to be associated with body height and weight ($p < 1 \times 10^{-5}$) (MacArthur et al., 2017). As the homolog gene of the *Drosophila melanogaster* Nipped-B and fungal Scc2-type sister chromatid cohesion proteins, nipbl in mammals not only plays a vital role in the loading of the cohesin complex onto chromatin by forming a heterodimeric complex with MAU2/SCC4 but also involves in cohesin loading at sites of DNA damage (Muto et al., 2014; Rhodes et al., 2017). In teleost, *nipbl* has been reported to regulate expression of *Hox* cluster genes as an upstream factor; thereinto, the hoxA and D clusters are crucial for limb/fin development (Zakany and Duboule, 2007; Muto et al., 2014). More studies show that *nipbl* deficiency may be the multifactorial origins of heart and gut defects in teleost (Chien et al., 2011). In zebrafish, nipbl is believed to control the cell cycle, which downregulates the canonical Wnt pathway genes observed in patient-specific fibroblasts during neural development (Pistocchi et al., 2013). Regarding embryo development in zebrafish, nipped-B-like protein B (nipblb) loss-of-function has a role of dysregulation in hematological malignancies (Spreafico et al., 2020).

Interestingly, by chromosome location analysis, two allele genes located on chromosomes W and Z of *C. semilaevis* were found and named as *nipbla-w* and *nipbla-z*, respectively. Based on their different expression patterns in RNA-seq, this study aimed to reveal their different chromosome location and genomic structure information, examine their expression in more wide tissues by quantitative PCR (qPCR), survey the transcription activity by promoter region identification and analysis, and assay their knockdown effect by RNA interference.

MATERIALS AND METHODS

Ethics Statement

The sampling and treatment of the *C. semilaevis* in this study were approved by the Animal Care and Use Committee at the Chinese Academy of Fishery Sciences (Approval number: YSFRI−2021024, dated 8 December 2021), and all the experimental procedures were carried out in line with the guidelines for the Care and Use of Laboratory Animals at the Chinese Academy of Fishery Sciences.

Fish and Cells

Ten different tissues including brain, liver, testis, ovary, muscle, intestine, spleen, kidney, heart, and skin were isolated from three female and three male individuals of 3-year-old *C. semilaevis* cultivated in Haiyang Yellow Sea Aquatic Product Co., Ltd (Shandong, China) and stored for subsequent RNA extraction. The gonads, were stored in the alcohol for DNA extraction by TIANamp Marine Animals DNA Kit (Tiangen). The genetic sex identification was performed by the primers sex-F (CCTAAATGATGGATGTAGATTCTGTC) and sex-R (GATCCAGAGAAAATAAACCCAGG) described in the previous study (Liu et al., 2014). Human embryonal

[1] www.ebi.ac.uk/gwas

TABLE 1 | Primers used in this study.

Primers	Sequences	Information
nipbw+s	ACGGAGCTCGGTTCAGGCCAGTTCTGTGACC	Promoter cloning
nipbw-x	ACGCTCGAGCTTCGGATGTTGCGTTCAAGG	Promoter cloning
nipbz+s	ACGGAGCTCCTGCTTAGTCCCAACGATTG	Promoter cloning
nipbz-x	ACGCTCGAGCGGATGTTGCGTTCAAAGG	Promoter cloning
nipbla-F	GCAAAATGTAGCAGCACGGC	Splicing detection
nipbla-R	CGATCTTTGTCTCGGCGTTG	Splicing detection
nipbla-w-F	GCAAACTAACGCCGAAAGAGG	qRT-PCR
nipbla-w-R	CACCAAACCCAACCTCTCCAG	qRT-PCR
nipbla-z-tv1-F	CAGAGATAGGTCGGGTTTCC	qRT-PCR
nipbla-z-tv1-R	CACCACTATGCCCTCCCAG	qRT-PCR
nipbla-z-tv2-F	CAGCGAGACAAGGACAGC	qRT-PCR
nipbla-z-tv2-R	CGGAGGAGCCGCTGAATTTG	qRT-PCR
β-actin-F	TTCCAGCCTTCCTTCCTT	qRT-PCR
β-actin-R	TACCTCCAGACAGCACAG	qRT-PCR
jun+H	ACGAAGCTTATGTATGCCAAGATGGAAACTAC	Vector construction
jun-E	ACGGAATTCTCAGAAAGTCTGGAGCTGC	Vector construction
wp-junMuF	CGCCGATGGAGGAGTGCCCTCCTGAGATC	Vector mutation
wp-junMuR	GATCTCAGGAGGGCACTCCTCCATCGGCG	Vector mutation
zp-junMuF	CGCCGGTGGAGGAGTGCCCTTCTGAGATC	Vector mutation
zp-junMuR	GATCTCAGAAGGGCACTCCTCCACCGGCG	Vector mutation
siRNA-nipbla-w	CAAACAAGACAGTGGGACA	siRNA site
siRNA-nipbla-z	CATCAAACCGTCAAGAACA	siRNA site

kidney (HEK) 293T cells were cultured in DME/F-12 medium (HyClone, UT, United States) supplemented with 10% fetal bovine serum (FBS; Gibco, Australia) and maintained in 5% CO_2 at 37°C. *C. semilaevis* brain cells (CSB) were maintained in L-15 medium supplemented with 10% FBS and 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen, MD, United States) at 24°C (Wang et al., 2015). Before transfection, cells were cultured in 24-well or 12-well plates with 60–80% density. The Lipofectamine 2000™ (Invitrogen) and riboFECT™ CP Transfection Kit (RiboBio) were separately used for plasmids and siRNA transfection.

The Characterization of *Nipbla-w* and *Nipbla-z* in Different Chromosomes

Based on the information of RNA-seq in our previous study, two *nipbla* homolog genes (GenBank number: 103397102 and 103398179) were found in chromosomes W and Z, respectively. To clarify the two genes, we denoted these two genes as *nipbla-w* and *nipbla-z*. The two genes' locus information in chromosomes W and Z were further analyzed based on the genomic data of *C. semilaevis*. Also, the nipbla location information in other fish species including zebrafish (*Danio rerio*), marine medaka (*Oryzias melastigma*), Senegalese sole (*Solea senegalensis*), and turbot (*Scophthalmus maximus*) were exhibited.

The Phylogenetic Analysis of *Nipbla* Proteins

To identify the ortholog of nipbla of *C. semilaevis*, a phylogenetic analysis was carried out using MEGA 7.0 and EvolView. In brief, a list of nipbla or nipbl proteins were screened from UniProt by BLAST tools and submitted to MEGA 7.0 for the construction of a phylogenetic tree by the neighbor-joining (NJ) method (Kumar et al., 2016). Furthermore, the Newick file from MEGA 7.0 was uploaded into the EvolView software[2] for the phylogenetic relationship presentation and domain description of nipbla/nipbl proteins.

Identification of Alternative Splicing and qPCR Analysis of *Nipbla* in Female and Male Individual Tissues

First, two primers nipbla-F and nipbla-R, respectively, located in exon 11 and exon 15, were designed to detect the splicing variants. To measure the expression patterns of *nipbla-w* and *nipbla-z* in different tissues of female and male individuals, primers *nipbla-w*-F/R, *nipbla-z-tv1*-F/R, and *nipbla-z-tv2*-F/R (**Table 1**) were first designed based on the mRNA sequences of *nipbla-w*, *nipbla-z-tv1*, and *nipbla-z-tv2*. The qPCR was further carried out, and β-*actin* was used for the internal reference. In brief, ten tissues, including brain, testis, ovary, heart, intestine, kidney, liver, muscle, skin, and spleen, were isolated from three female and three male individuals of 3-year-old *C. semilaevis*. The total RNAs of these samples were extracted by TRIzol Reagent and used for cDNA preparation with PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Japan). The 20 μl qPCR reactions were conducted by 95^{o}C for 10 s, followed by 40 cycles of 95^{o}C for 5 s and 60^{o}C for 34 s on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, United States). The $2^{-\Delta\Delta Ct}$ method was employed for evaluating

[2]https://www.evolgenius.info/evolview/#login

the relative expression levels of genes (Livak and Schmittgen, 2001). The data obtained from qPCR were performed using the OmicShare tools for the least significant difference (LSD) analysis[3].

The Promoter Activity Analysis for Two Genes

The putative promoter regions of *nipbl-w* and *nipbl-z* were first amplified by the primers (i.e., nipbw+s, nipbw-x, nipbz+s, and nipbz-x in the **Table 1**) and subcloned into pGL3-Basic to construct the recombinant plasmids pGL3-nipbla-wp and pGL3-nipbla-zp by the restriction enzymes *Sac*I and *Xho*I. Then, pGL3-nipbla-wp, pGL3-nipbla-zp, pGL3-control, and pGL3-Basic were respectively transfected into HEK 293T cells by Lipofectamine 2000^{TM} following the protocol with 800 ng/well in 24-well plates. The pRL-TK plasmid was used as the internal reference with 40 ng/well. At 48 h, cells transfected by plasmids were detected by a Dual-Luciferase Reporter Gene Assay Kit (Beyotime, Shanghai, China) with a Varioskan Flash spectral scanning multimode reader (Thermo, Vantaa, Finland) for the activities of firefly and *Renilla* luciferase. Each experiment was performed in triplicate. The abovementioned data were performed using the OmicShare tools for the LSD analysis (see text footnote 3).

Transcription Factor Prediction and Verification

To screen the putative transcription factors, the promoter sequences of *nipbla-w* and *nipbla-z* were submitted to the online tool PROMO[4] and JASPAR[5]. To analyze the binding activity of transcription factors, the coding region of a c-Jun homolog in *C. semilaevis* (XM_025052424.1) was amplified by primers (jun+H and jun-E in **Table 1**) and ligated with pcDNA3.1 by *Hin*dIII and *Eco*RI to construct pcDNA3.1-c-Jun. Also, the mutation vector of pGL3-nipbl-wp (M) and pGL3-nipbla-zp (M) for c-Jun were constructed by the primers (i.e., wp-junMuF, wp-junMuR, zp-junMuF, and zp-junMuR in **Table 1**) according to the protocol of the Fast Site-Directed Mutagenesis Kit (Tiangen, China). The promoter and transcription factor plasmids were co-transfected into 293T cells to examine the luciferase activity.

The DNA Methylation Status of Two Gametologs in Different Sexes

To understand the influence of *nipbla* methylation on gene transcript formation, we analyzed the methylation profiles of the *nipbla* in genomic sequences of female, male, and pseudo-male individuals. Based on our methylome from *C. semilaevis* female, male, and pseudo-male gonad (Wang et al., 2021), the methylation levels of *nipbla-w* and *nipbla-z* in different sexual gonad tissues were demonstrated by R software. The methylation level analysis, which included the *nipbla-w* and *nipbla-z* upstream of 2,000 bp, gene body, and downstream of 2,000 bp regions, was employed for the examination of three sexual genotypes in Chinese tongue sole.

RNAi-Mediated Knockdown for Two Nipbla Gametologs in *C. semilaevis*

The *nipbla* small-interfering RNAs (i.e., si-*nipbla-w* and si-*nipbla-z* in **Table 1**) were designed and synthesized by RiboBio Co., Ltd. (Guangzhou, Guangdong Province, China). Even more, a non-specific siRNA negative was executed as a control [negative control (NC)] during the cell transfection process. Three replicates of si-*nipbla-w*, si-*nipbla-z*, and NC groups were conducted at 20 nM. After transfection for 48 h, the total RNA was extracted from the cells, and cDNA was synthesized according to the abovementioned methods. The relative expression levels of the genes related to growths, such as Bone Morphogenetic Protein 4 (*bmp4*), Wnt Family Member 11 (*wnt11*), and Sprouty Related EVH1 Domain Containing 2 (*spred2*), and FAT Atypical Cadherin 4 (*fat4*) were evaluated by qPCR.

RESULTS

Chromosome Locus Information and Gene Structure of *Nipbla-w* and *Nipbla-z*

The *nipbla-w* (GenBank number: 103397102) and *nipbla-z* (GenBank number: 103398179) genes were separately located in the W and Z chromosomes (**Figure 1**). Also, no obvious syntenic arrangement was found in these two chromosomes. While the syntenic arrangement relationship of chromosome Z is similar to *D. rerio*, *O. melastigma*, *S. senegalensis*, and *S. maximus* nipbla. The similarity of mRNA (XM_008335345.2 and XM_017042947.2) and proteins (XP_008333567.1 and XP_016898436.1) derived from NCBI database between *nipbla-w* and *nipbla-z* were 88.4% and 88.7%, respectively.

The Polygenetic Analysis of Nipbl Proteins

The other nipbla or nipbl proteins were screened by searching the UniProt database using two kinds of nipbla proteins in *C. semilaevis*. A total of 32 nipbl or nipbla proteins including fruit fly (*D. melanogaster*), human (*Homo sapiens*), mouse (*Mus musculus*), pig (*Sus scrofa*), chicken (*Gallus gallus*), zebrafish (*D. rerio*), medaka (*Oryzias latipes*), turbot (*S. maximus*), Nile tilapia (*Oreochromis niloticus*), channel catfish (*Ictalurus punetaus*) blind cave fish (*Astyanax mexicanus*), and so on. The amino acid sequences using the NJ method were submitted to the construction of a polygenetic tree by MEGA 7.0. The subsequent phylogenetic relationship presentation and domain description (**Figure 2**) by EvolView software indicate that two nipbla homolog proteins in *C. semilaevis* were first clustered together and then clustered with other fish including medaka and marine medaka. Similar to other mammalian and fish species

[3]http://www.omicshare.com/tools

[4]http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3

[5]http://jaspar.genereg.net/

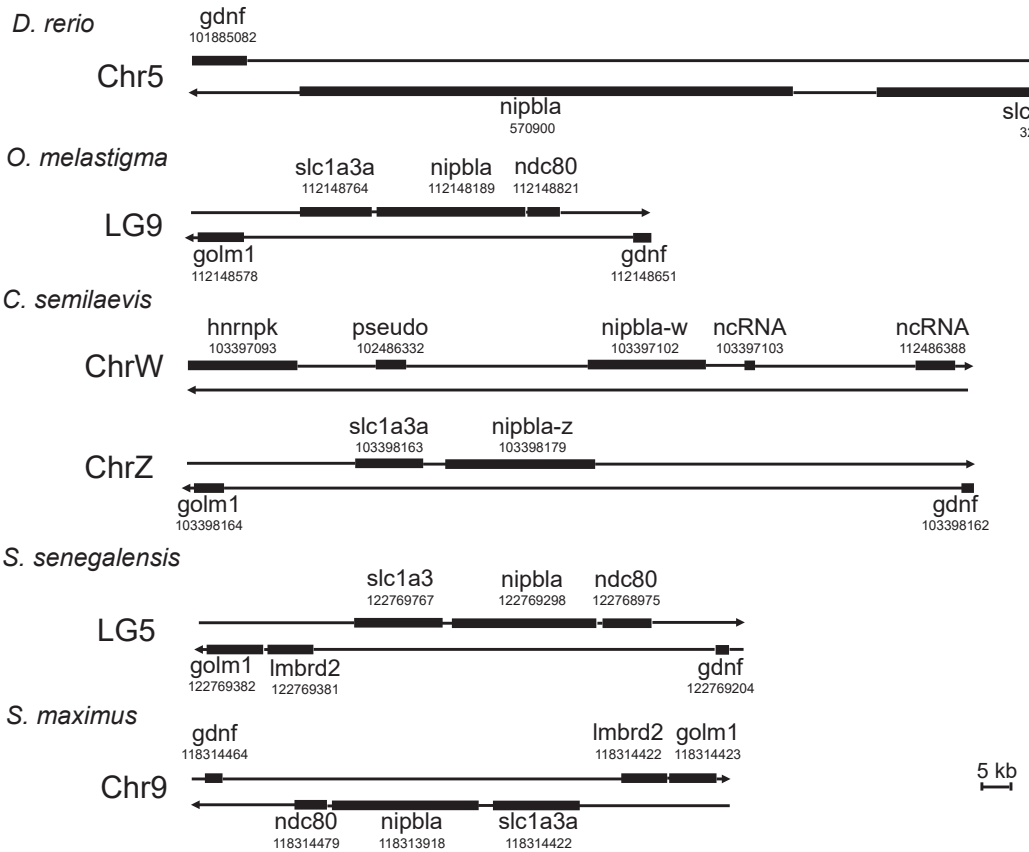

FIGURE 1 | The chromosome location information of *nipbla-w* and *nipbla-z*. *Danio rerio* chromosome 5_7,986,080–8,171,694, *Oryzias melastigma* LG9_15,171,132–15,252,085, *Cynoglossus semilaevis* chromosome w_8,015,650-8,133,838, *C. semilaevis* chromosome z_16,746,071-16,882,207, *Solea senegalensis* LG5_13,731,413–13,824,383, and *Scophthalmus maximus* chromosome 9_15,599,751–15,691,435 were illustrated with nipbla and other genes location. hnrnpk, heterogeneous nuclear ribonucleoprotein K; golm1, golgi membrane protein 1; slc1a3a, solute carrier family 1 member 3a; gdnf, glial cell line-derived neurotrophic factor; ndc80, NDC80 kinetochore complex component; ncRNA, non-coding RNA.

nipbl, the two homolog proteins of *C. semilaevis* contained two conserved domains, namely, cohesion_HEAT and Nipped-B_C.

The Expression Patterns of Two Genes in Female and Male Tissues

By using primers *nipbla*-F and *nipbla*-R, respectively, located in the exon 11 and exon 15, the alternative 5' splicing was detected in the exon 12 of *nipbla-z*, which produced *nipbla-z-tv1* and *nipbla-z-tv2* (**Supplementary Figure 1**). Their sequences are also shown in **Supplementary Figure 1**.

The qPCR analysis revealed that *nipbla-w*, *nipbla-z-tv1*, and *nipbla-z-tv2* exhibited sex-biased expression patterns in female and male individuals (**Figure 3**). In the heart, skin, kidney, intestine, spleen, gonad, liver, brain, and muscle, *nipbla-w* exhibited generally higher expression levels in female than male individuals and showed significantly highest expression level in female gonad. Concretely for different transcripts, *nipbla-z-tv1* and *nipbla-z-tv2* showed slight expression difference, and *nipbla-z-tv1* demonstrated significantly higher expression levels in the muscle, liver, brain, and gonad of male individuals. But *nipbla-z-tv2* exhibited the highest in the muscle of male individuals. The *nipbla-w* transcripts were gradually increased with gonadal development (**Figure 4**). Due to its low expression in testes, *nipbla-w* was not observed in the transcription level of changes during different developmental stages of testes. For the results of expression differences in stages of gonads development, both *nipbla-z-tv1* and *nipbla-z-tv2* showed a prominent expression in male individuals from 40 dph to 1.5 years, respectively. Furthermore, *nipbla-z-tv1* reached a peak transcription level at 6 months, but *nipbla-z-tv2*, at 1.5 years (**Figure 4**).

The Amplification and Activity Detection of the Putative Promoters

By designing the primers based on the *C. semilaevis* genome, the putative 1,636 bp and 1,724 bp promoter regions were amplified from the upstream of *nipbla-w* and *nipbla-z* genes, respectively, and their GenBank numbers are MW349831 and MW349832, respectively. Their sequences shared 67.6% similarity, and their structure information were illustrated in **Supplementary Figure 2**. Analyzed by PROMO and

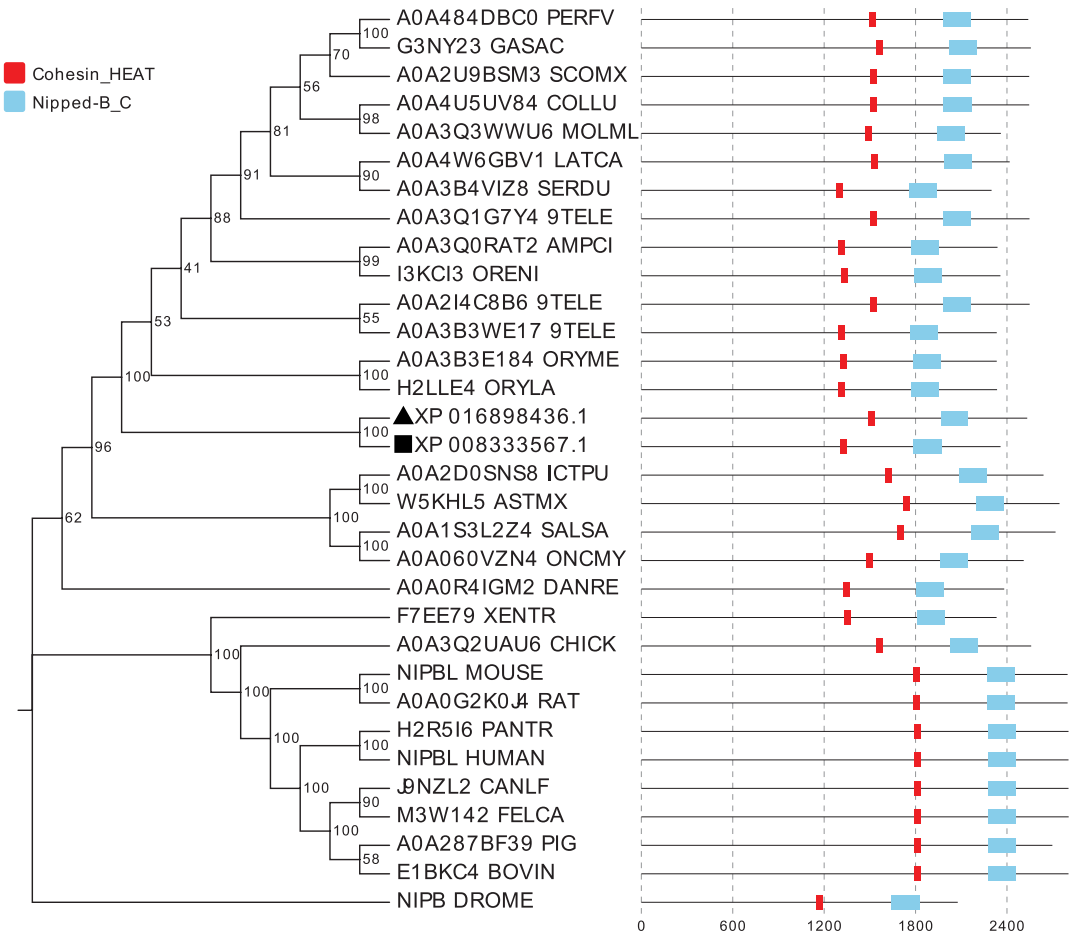

FIGURE 2 | The evolutionary relationships of nipbl proteins in different species. A total of 32 nipbl proteins were used for the phylogenetic tree construction using the neighbor-joining method in MEGA 7.0. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Notably, 32 nipbl proteins are listed as follows: A0A4W6GBV1_LATCA (*Lates calcarifer*, barramundi perch), A0A3B4VIZ8_SERDU (*Seriola dumerili*, greater amberjack), A0A3Q0RAT2_AMPCI (*Amphilophus citrinellus*, midas cichlid), I3KCI3_ORENI (*Oreochromis niloticus*, Nile tilapia), A0A3Q1G7Y4_9TELE (*Acanthochromis polyacanthus*, spiny chromis), A0A4U5UV84_COLLU (*Collichthys lucidus*, big head croaker), A0A3Q3WWU6_MOLML (*Mola mola*, ocean sunfish), A0A2U9BSM3_SCOMX (*S. maximus*, turbot), A0A484DBC0_PERFV (*Perca flavescens*, American yellow perch), G3NY23_GASAC (*Gasterosteus aculeatus*, three-spined stickleback), XP_008333567.1 (*nipbla-w* in *C. semilaevis*), XP_016898436.1 (*nipbla-z* in *C. semilaevis*), A0A2I4C8B6_9TELE (*Austrofundulus limnaeus*, annual killifish), H2LLE4_ORYLA (*Oryzias latipes*, medaka), A0A3B3WE17_9TELE (*Poecilia mexicana*, Atlantic molly), A0A2D0SNS8_ICTPU (*Ictalurus punctatus*, Channel catfish), A0A1S3L2Z4_SALSA (*Salmo salar*, Atlantic salmon), NIPBL_MOUSE (*Mus musculus*, mouse), J9NZL2_CANLF (*Canis lupus familiaris*, Dog), M3W142_FELCA (*Felis catus*, cat), H2R5I6_PANTR (*Pan troglodytes*, chimpanzee), NIPBL_HUMAN (*Homo sapiens*, human), A0A060VZN4_ONCMY (*Oncorhynchus mykiss*, rainbow trout), A0A3Q2UAU6_CHICK (*Gallus gallus*, chicken), F7EE79_XENTR (*Xenopus tropicalis*, western clawed frog), W5KHL5_ASTMX (*Astyanax mexicanus*, blind cave fish), A0A287BF39_PIG (*Sus scrofa*, Pig), E1BKC4_BOVIN (*Bos taurus*, bovine), A0A0G2K0J4_RAT (*Rattus norvegicus*, rat), NIPB_DROME (*Drosophila melanogaster*, fruit fly), A0A0R4IGM2_DANRE (*D. rerio*, zebrafish), and A0A3B3E184_ORYME (*O. melastigma*, marine medaka). Black square and triangle represent *nipbla-w* and *nipbla-z* proteins, respectively.

JASPAR, several transcription factors including c-Jun, sex-determining region Y (SRY), POU Class 1 Homeobox 1 (POU1F1a), myogenic differentiation antigen (MyoD), signal transducer and activator of transcription 5a (STAT5A), and nuclear factor I C (Nfic) were predicted, and their binding sites were indicated in **Supplementary Figure 2**. The recombinant plasmids based on these putative promoters were constructed and named as pGL3-nipbla-wp and pGL3-nipbla-zp, respectively. After the transfection with pRL-TK and the detection of dual-luciferase activities (**Figure 5A**), the pGL3-nipbla-wp and pGL3-nipbla-zp transfected cells manifested significantly higher firefly/*Renilla* luciferase activity than cells transfected with pGL3-Basic and no transfection cells. This suggested that nipbla-wp and nipbla-zp could successfully drive the expression of luciferase in 293T cells. Also, the activity of nipbla-zp was significantly higher than nipbla-wp.

To further analyze the activity of key transcription factors, the transfection in 293T cells and the dual-luciferase detection assay were observed that c-Jun could make a significant effect on the transcriptional activity of nipbla-zp, but for nipbla-wp, there was no discernible effect (**Figure 5B**).

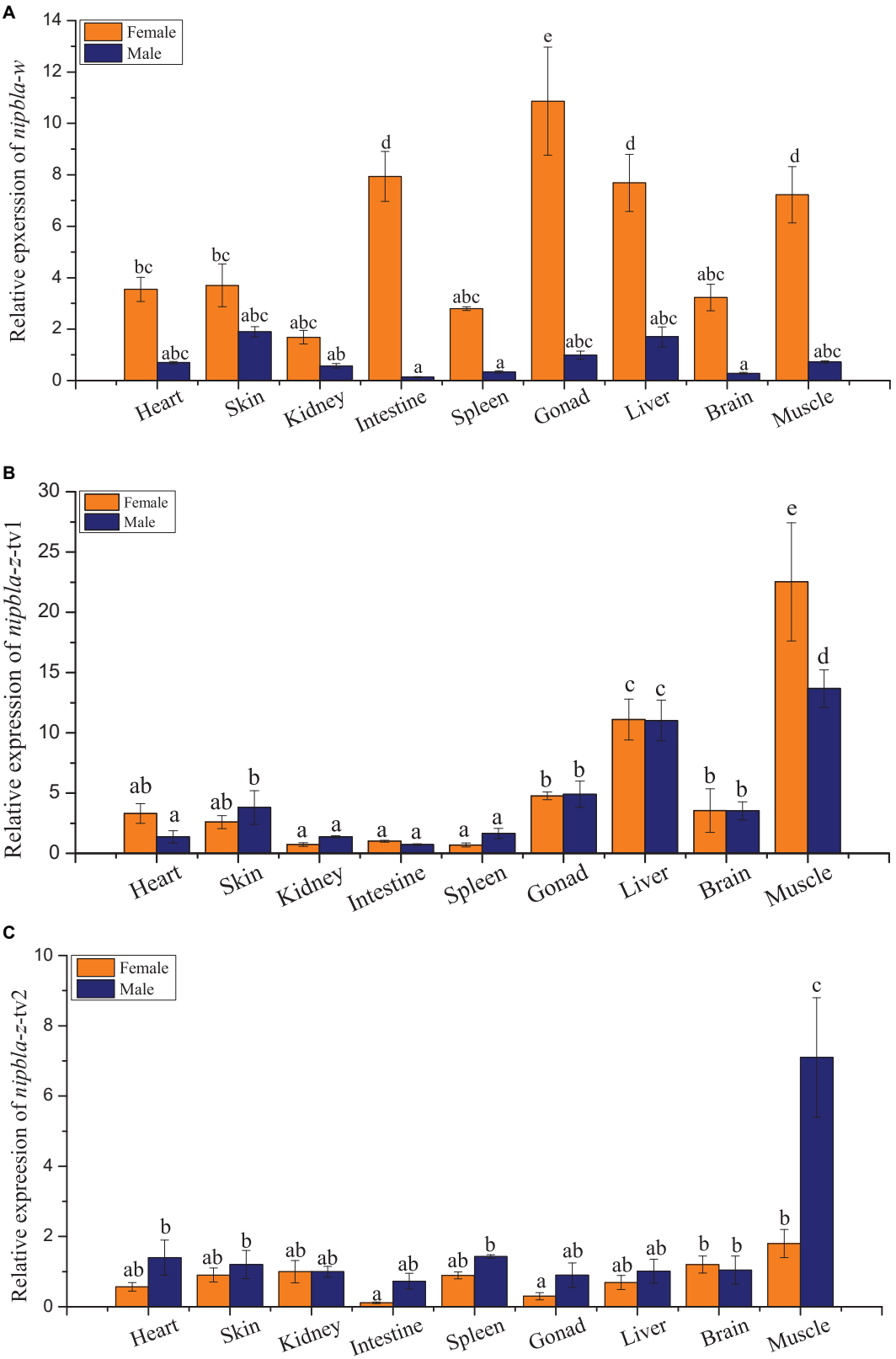

FIGURE 3 | The relative expression patterns of *nipbla-w* and *nipbla-z* in female and male tissues. The relative repression levels of *nipbla-w* and *nipbla-z* in brain, gonad, spleen, heart, intestine, kidney, liver, muscle, skin, and spleen and of female and male individuals are shown in parts **(A–C)**, respectively. Different letters represent significant differences.

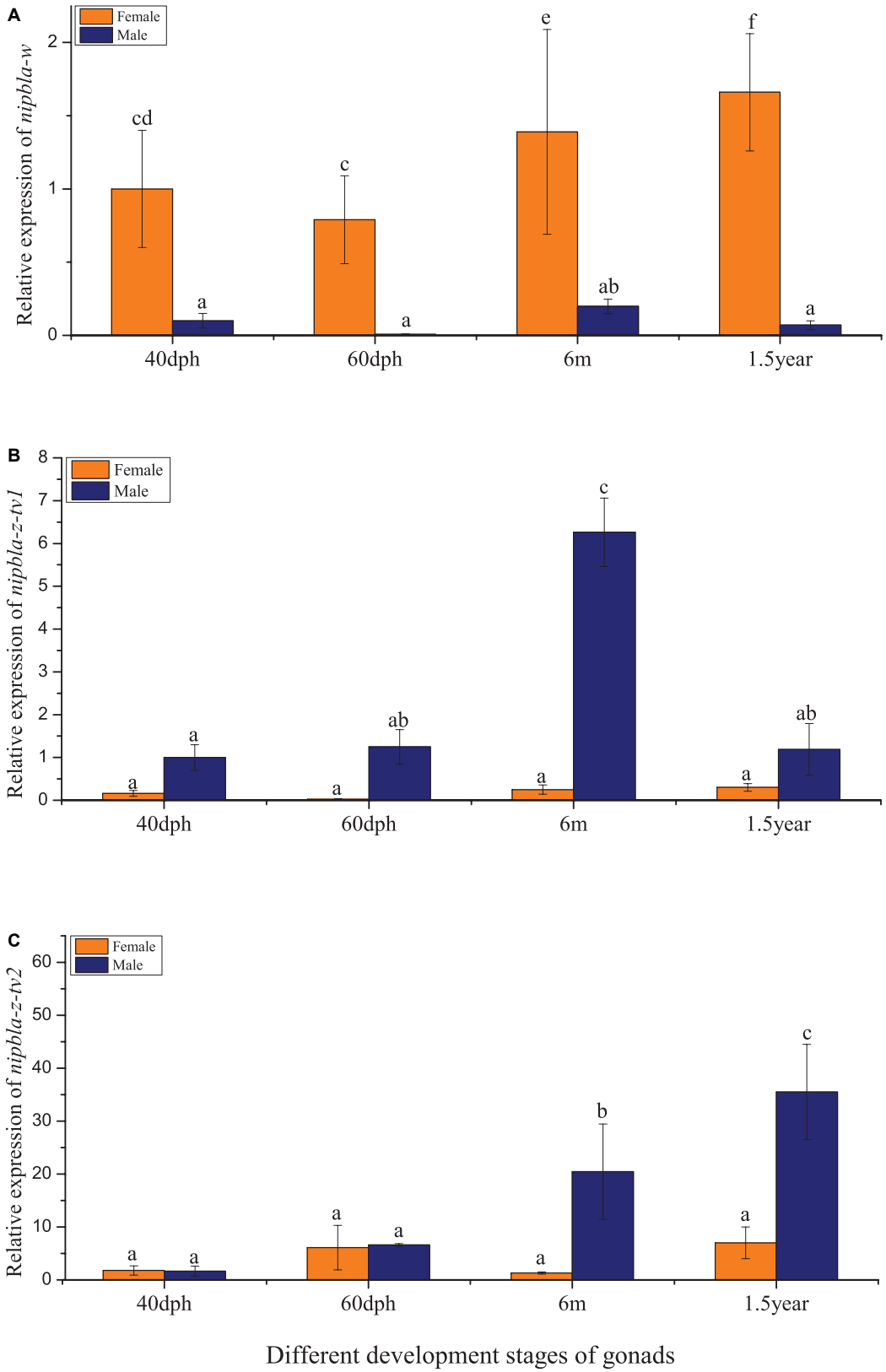

FIGURE 4 | The relative expression patterns of *nipbla-w* and *nipbla-z* in female and male individuals in the development of gonads. **(A)** The *nipbla-w* transcription in the gonads at different developmental stages. **(B)** The *nipbla-z-tv1* transcription in the gonads at different developmental stages. **(C)** The *nipbla-z-tv2* transcription in the gonads at different developmental stages. Different letters represent significant differences.

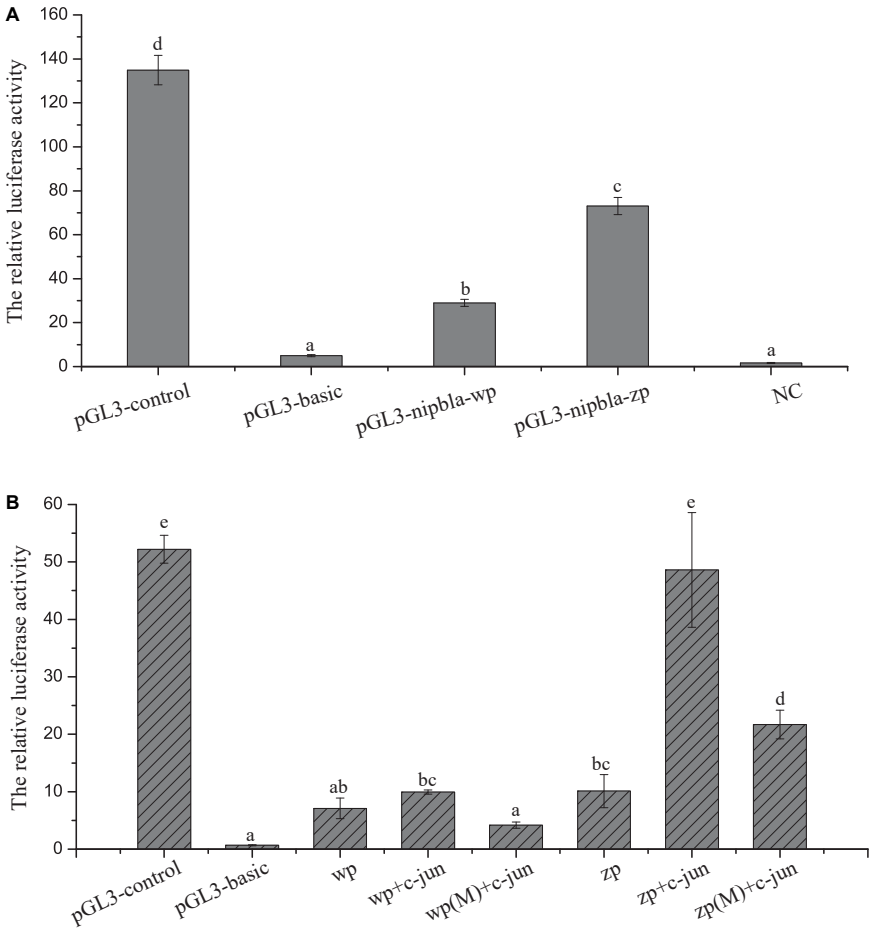

FIGURE 5 | The analysis of *nipbla-w* and *nipbla-z* transcriptional activity and the effect of c-Jun on transcriptional activity of *nipbla-w* and *nipbla-z*. **(A)** The analysis of *nipbla-w* and *nipbla-z* transcriptional activity. **(B)** The effect of c-Jun on transcriptional activity of *nipbla-w* and *nipbla-z* promoter. Different letters represent significant differences.

The Epigenetic Modification of Two Genes and the Relationship Between DNA Methylation and Transcription Activity

The analysis of approximately 2 kb up- and downstream flanking regions of the *nipbla-w* and *nipbla-z* genomic sequence from the female ovary, male and pseudo-male testes are shown in **Figure 6**. Interestingly, as shown, there was a clear difference in the gene body, especially located at first exon region, which exhibited difference between female and pseudo-male individuals in *nipbla-w* and also exhibited difference between female, male, and pseudo-male individuals in *nipbla-z*.

RNAi of *Nipbla* and *in vitro* Growth-Related Gene Expression

The RNAi-mediated knockdown *in vitro* was assisted for *nipbla* in the Chinese tongue sole brain cell line (i.e., CSB). The silencing effects of RNAi between *nipbla-w* and *nipbla-z* expression in CSB were detected by qPCR after siRNA was transfected into cells. The results revealed that the silencing efficiencies of *nipbla-w* and *nipbla-z* were approximately 46% and 55.1%, respectively. Compared with the control, the mRNA levels of neurogenesis, vascular development, and osteogenesis genes, i.e., *bmp4*, Wnt pathway gene *wnt11*, *fat4*, and *spred2*, were detected after RNAi. As shown in **Figure 7**, the expression levels of *bmp4*, *wnt11*, and *spred2* showed strongly alterative after *nipbla-w* and *nipbla-z* RNAi, including the upward trend of *spred2* and the downregulation expression trends of *bmp4* and *wnt11*.

DISCUSSION

The phenomenon of sexual size dimorphism that existed in many fish species has severely hindered the sustainable development of aquaculture. In *C. semilaevis*, a series of genes located in the W and Z chromosomes cause our interest due to their opposite expression pattern. The nipbl loads cohesin onto chromatin, and cohesin mediates sister chromatid cohesion important for mitosis (Dorsett, 2011). Nipbl

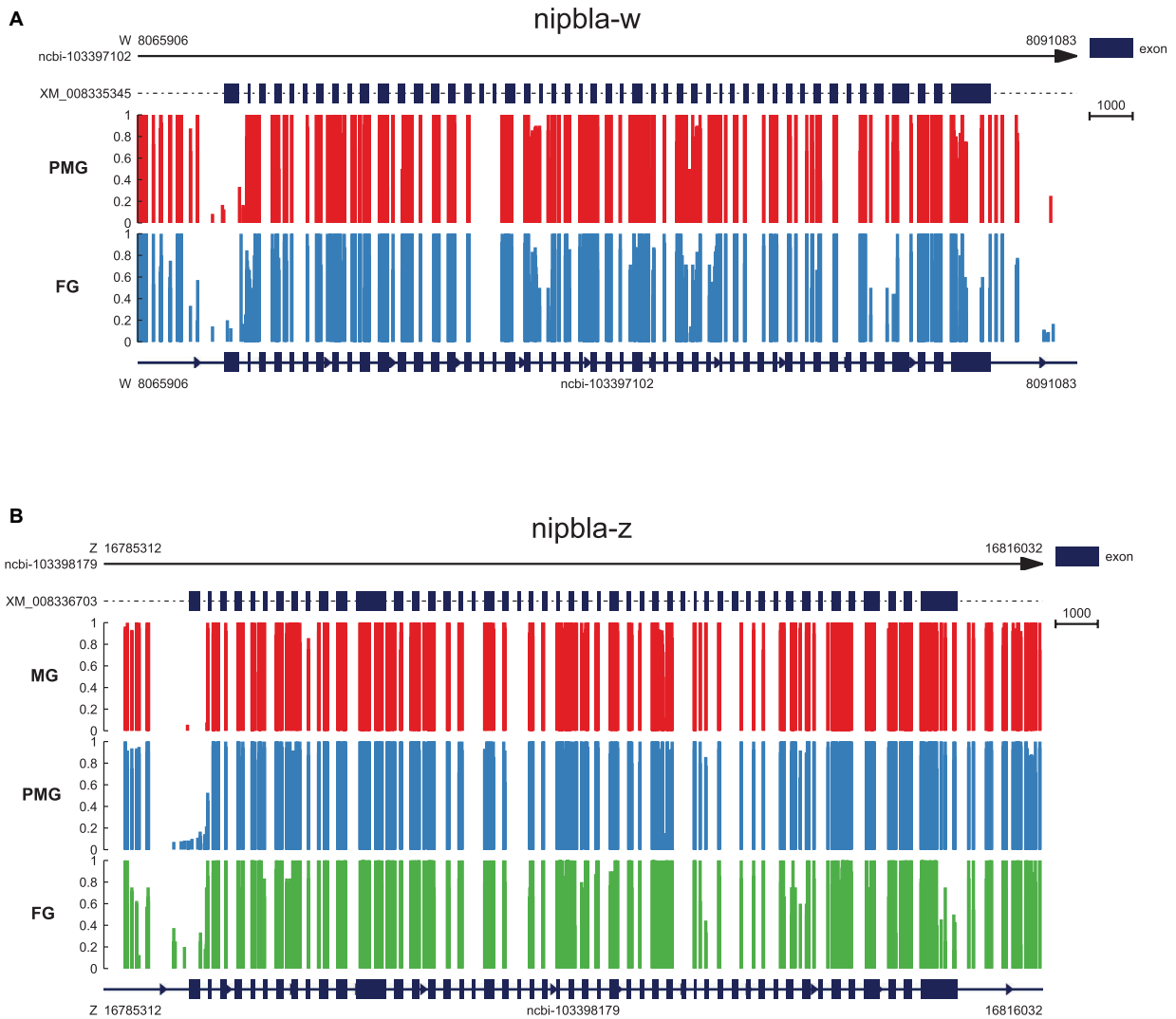

FIGURE 6 | The epigenetic regulation of *nipbla-w* and *nipbla-z* in the gonad. **(A)** The *nipbla-w* genomic sequence with gene body, 2kb upstream and downstream regions; the methylation level of cytosine is shown with red vertical lines in pseudo-male individuals and blue in male individuals. **(B)** The *nipbla-z* genomic sequence with gene body, 2kb upstream and downstream regions; the methylation level of cytosine is shown with red vertical lines in male individuals, blue in pseudo-male individuals, and green in female individuals, respectively.

also involved in specific enhancer-promoter communication (Strachan, 2005). Some reports remind that nipbl co-localizes with the mediator complex at promoters/enhancers of actively transcribed genes in mouse embryonic stem cells (Kagey et al., 2010). The functional range of the nipbl mutations in this gene results in Cornelia de Lange syndrome, a disorder characterized by dysmorphic facial features, growth delay, limb reduction defects, and cognitive disability in most mammals (Garcia et al., 2021). Similarly, *nipbl*-deficient individuals of teleost also produce observed developmental defects in heat, gut, and limb (Chien et al., 2011; Muto et al., 2014). In fish, nipbl and mediator cooperatively regulate gene (e.g., *fgfs*, *shha*, *hand2*, and *Hox*) expression to control limb development which led to size reductions and patterning defects (Muto et al., 2014). Thus, we speculated that *nipbla* was a typical candidate gene for the transcriptional regulation pathway involved in explaining the sexual size dimorphism in *C. semilaevis*. But the effect of *nipbl* gene on growth differences caused by sex dimorphism has not been reported in teleost.

Interestingly, two different *nipbla*, namely, *nipbla-w*, and *nipbla-z*, were identified in the *C. semilaevis* genome of the W and Z chromosomes, respectively, which were the two kinds of homologs of *nipbla* from our previous transcriptomic study. As shown in **Figure 1**, the chromosome syntenic locus analysis of *nipbla* on genome location in *C. semilaevis*, which compared *O. melastigma*, *S. senegalensis*, *D. rerio*, and *S. maximus*, obviously reveals that the syntenic arrangement of *nipbla* in chromosome Z of *C. semilaevis* was more identical to other teleost species of *nipbla*. Obviously, the distribution

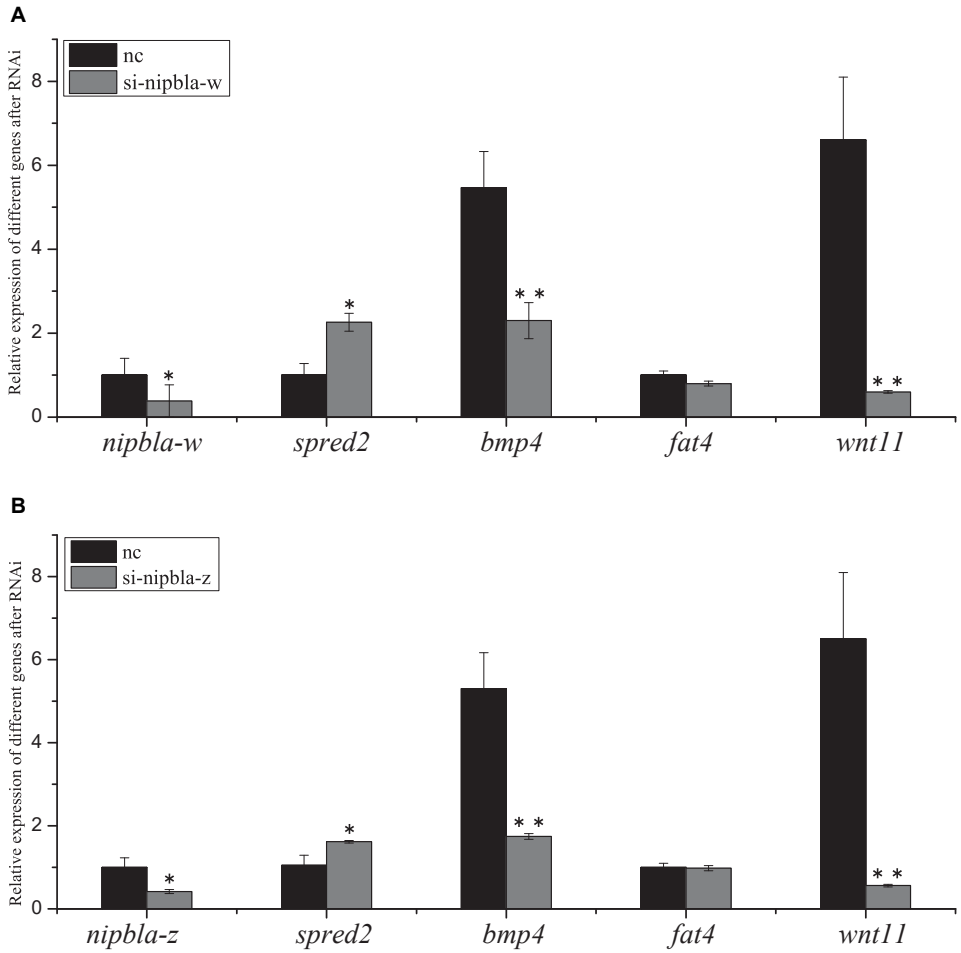

FIGURE 7 | The siRNA effects of *nipbla* in *C. semilaevis* brain cells. The analysis of *nipbla-w*, *nipbla-z* (tv1 and tv2), Sprouty Related EVH1 Domain Containing 2 (*spred2*), Bone Morphogenetic Protein 4 (*bmp4*), FAT Atypical Cadherin 4 (*fat4*), and *wnt11* expression in cultured testis cells after RNAi. **(A)** Expression of *nipbla-w*, *spred2*, *bmp4*, *fat4*, and *wnt11* after transfection of si-*nipbla-w* RNA at 48 h. **(B)** Expression of *nipbla-z-tv1*, *spred2*, *bmp4*, *fat4*, and *wnt11* after transfection of si-*nipbla-z* siRNA at 48 h. NC indicates the testis cells transfected with the siRNA of the negative control. Asterisks indicate significant differences ($p < 0.05$) between the treated group and the control group.

of pseudogenes or non-coding RNA (ncRNA) in chromosome W leads to the inconsistent gene syntenic arrangement, which further confirms the higher gene degeneration in chromosome W than in chromosome Z over a period of evolutionary times (Moghadam et al., 2012; Chen et al., 2014). Interestingly, these relatively conservative linear genes have shown a similar function to *nipbla* and have given concrete examples about it: *slc1a3a* and *gdnf* participate in the regulation of neuronal differentiation and development in teleost (Feng, 2009; Wong et al., 2021). Since *nipbl* is required for embryonic growth in both fish and mice (Kawauchi et al., 2009; Muto et al., 2011), even more, *nipbl* is one of the important regulatory factors in the maintenance of 3D genome organization and genome-controlled gene expression through interacts with cohesion (Gao et al., 2019). Interestingly, the alternative splicing sites in exon 12 of *nipbla-z* in tongue sole attribute to alternative 5' splicing, which produced two transcript genes *nipbla-z-tv1* and *nipbla-z-tv2*. Similar events related to the sex-specific alternative splicing, which is also discovered in sex differential genes, e.g., *dmrt1* and *cyp19a1* in *Lates calcarifer* and factor in the germ line alpha (*figla*) in *C. semilaevis* (Shao et al., 2014; Domingos et al., 2018). Regarding growth traits, there is also myostatin (*mstn*) gene concerned with the alternative splicing phenomenon (Huang et al., 2011). Also, our RNA-seq analysis found that more phenomena of alternative splicing were observed in the male *C. semilaevis* (unpublished data). Undoubtedly, *nipbla* as potential gametologs in the W and Z chromosomes, were involved in the growth of sexual dimorphism in the Chinese tongue sole.

The expression analysis revealed that *nipbla-w* and *nipbla-z* showed significant expression differences of most tissues between female and male individuals, where *nipbla-w* occupied a dominant in female individuals, compared with *nipbla-z* in male individuals. Particularly, even *nipbla-z* showed superiority in male individuals, especially at different developmental

stages, the two transcripts of *nipbla-z* (i.e., *nipbla-z-tv1* and *nipbla-z-tv2*) showed a consistent expression trend. While, in the 3-year matured male individuals, they revealed the differential expression with emphasis, e.g., *nipbla-z-tv1* observed in the muscle, liver, gonad, and brain, but *nipbla-z-tv2* preferentially existed in the muscle. It reveals that *nipbla-w and nipbla-z* play a role in the growth of female and male gonads, respectively, but different transcripts of *nipbla-z* (*nipbla-z-tv1* and *nipbla-z-tv2*) have expression bias in the muscle, even related to gonad and liver associated with *nipbla-z-tv2*.

After the *in vitro* knockdown of *nipbla-w* and *nipbla-z* in the cultured brain cells, qPCR showed the mRNA levels of the growth-related genes, e.g., *bmp4*, *wnt11*, and *spred2*, including the upward trend of *spred2* and the downregulation expression trends of *bmp4* and *wnt11*. Meanwhile, *spred2*, which inhibits FGF, probably influences FGF-mediated phosphorylation, and *wnt11* all depends on the Wnt signaling pathway (Katoh and Katoh, 2006). Even more, *spred2* plays a critical role in cell proliferation and differentiation of the zebrafish brain (Lim et al., 2017). The *bmp4* is also influenced after *nipbla* RNAi; although the mechanisms remain elusive, interestingly, it is reported as a key element to activate the Wnt pathway to promote primordial germ cell formation in chicken (Zuo et al., 2021). Even more, the knockdown of *nipbl* expression led to size reductions and patterning defects that were observed by dysregulated expression of key early limb development genes, including *fgfs*, *shha*, *hand2*, and multiple *Hox* genes (Muto et al., 2014). Therefore, we speculated that *nipbla* may be involved in the growth of sexual dimorphism through the Wnt signaling pathway. Comparatively, nipblb loss-of-function affects DNA replication, ribosome biogenesis, cell cycle, and results involved in pathways related to an overall activation of the Wnt canonical pathway in the early developmental stages of zebrafish embryos (Spreafico et al., 2020). Otherwise, in addition to the Wnt pathway, nipbl can take function through other pathways, and concretely, it influenced the expression of the gene involved in endodermal differentiation (*sox32*, *sox17*, *foxa2*, and *gata5*) and left-right patterning (*spaw*, *lefty2*, and *dnah9*), which is altered in early embryonic development (Muto et al., 2011). It suggested that *nipbl* may fulfill functions that are mainly involved in the Wnt signaling pathway and play a role in gene regulation in fish development, while the mechanisms about it need further identification analysis.

The promoter transcription activities of two *nipbla* genes in *C. semilaevis* were both successfully verified by the dual-luciferase reporter system in HEK 293T cells. The results reveal that the activity of nipbla-zp was much stronger than that of nipbla-wp. The prediction of several transcription factors including SRY, POU1F1a, MyoD, c-Jun, STAT5A, and Nfic from the promoters of nipbla has provided insight into the possible transcription regulation of nipbla. The transcription factors mentioned above are known to bind with growth-related genes to positively regulate gene expression and accelerate growth rate in mammals and teleosts (Li et al., 1990; Yu et al., 2001; Zhu et al., 2019). Further, we focused on c-Jun because it is reported to involve in cell proliferation and differentiation at the cellular level (Hilberg et al., 1993), and it is also proved as a crucial effector to induce normal growth (Castellazzi et al., 1991). There is a common sense among mammals and fish that c-Jun requires its DNA-binding domain and interacts with estrogen receptor α (ERα) and pituitary homeobox-1 (Pitx1) and then mediates GnRH to effect on early growth (Melamed et al., 2006). In *C. semilaevis*, c-Jun transcripts are both downregulated in the liver and gonad based on the transcriptomic analysis (Wang et al., 2018), and it effectively affected the transcriptional activity of the promoter of *nipbla-z* in this study. According to the abovementioned analysis, it is not difficult to infer that *nipbla* make potential role in growth process regulated by the putative transcription factors, especially c-Jun still needs more evidence to exact.

Furthermore, our study revealed that *nipbla-w* and *nipbla-z* exhibited a difference in methylation level in gene body associated with three sexual genotypes in the gonad. There is proof that the alternative splicing of mRNA is linked to the DNA methylation regulation (Shukla et al., 2011). Whether this epigenetic modification would have an effect on the different types of alternative splicing needed to be further investigated. The genome-wide DNA methylation of the liver reveals that nipbl promoter and gene body could be the important regulators for the long-term effects of EE2 in the liver of adult fish (Voisin et al., 2021). This provides that the expression of *nipbla* may be influenced by methylation patterns.

In summary, two *nipbla* gametolog genes were separately identified from *C. semilaevis* chromosomes W and Z. These two genes were found to demonstrate the sex-biased expression pattern in female and male tissues or even involved in different developmental stages of the gonads. The identification, activity verification, and prediction of transcription factors for *nipbla* promoters provide clues for the transcription regulation of *nipbla* in *C. semilaevis*. The identification of two *nipbla* genes provided important data for interpreting the sexual size dimorphism in *C. semilaevis*.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee at the Chinese Academy of Fishery Sciences.

AUTHOR CONTRIBUTIONS

NW conceived and designed the experiments, and carried out the promoter activity experiment. YZ, QY, and RS performed

fish tissue sampling. YZ, QY, RS, and MZ carried out RNAi interference and the qPCR experiment. YZ and NW analyzed the data and wrote the manuscript. SC provided valuable suggestions to the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.833070/full#supplementary-material

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