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# Nucleolar protein 4-like has a complex expression pattern in zebrafish embryos

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**ABSTRACT The *nucleolar protein 4-like* (*NOL4L*) gene is present on chromosome 20 (20q11.21) in humans. Parts of this gene have been shown to fuse with *RUNX1* and *PAX5* in acute myeloid leukemia and acute lymphoblastic leukemia, respectively. The normal function of NOL4L in humans and other organisms is not well understood. The expression patterns and functions of NOL4L homologs during vertebrate development have not been reported. We sought to address these questions by studying the expression pattern of zebrafish *nol4l* during embryogenesis. Our data show that *Znol4l* mRNA is expressed in multiple organs in zebrafish embryos. The sites of expression include parts of the brain, spinal cord, pronephros, hematopoietic cells and gut.**

KEY WORDS: *diencephalon, telencephalon, trigeminal ganglia, spinal cord neuron, pronephros*

## NOL4L proteins are highly conserved among vertebrates

*NOL4L* (also known as *C20ORF112*) was reported as a gene that fuses with *PAX5* and *RUNX1* due to chromosomal translocation in humans. These fusion proteins have been detected in acute lymphoblastic leukemia and acute myeloid leukemia patients (An *et al*., 2008; Guastadisegni *et al*., 2010; Kawamata *et al*., 2008). There are multiple isoforms of NOL4L. Delta blast was carried out with the longest isoform to identify the orthologs of this protein in other organisms. NOL4L orthologs are present in most vertebrates and are well conserved in human, mouse, chicken, *Xenopus* and zebrafish. *Znol4la* located on chromosome 8 and *Znol4lb* located on chromosome 23 may be paralogs. Sequence alignment of longest form of NOL4L proteins from above five species shows that, there is a high degree of conservation among these proteins (Fig. 1). We have focused on Znol4lb in this study, which shows slightly higher identity with human NOL4L.

## Temporal expression pattern of *Znol4lb*

Two different primer pairs were used to study the temporal expression pattern of *Znol4lb* mRNA during zebrafish development (Fig. 2A). Primer pair-1 can recognize all the transcripts of *Znol4lb*, whereas primer pair-2 does not recognize the shortest transcript (NM_001080652.1). The PCR amplified products with these primers showed near identical temporal expression pattern (Fig. 2B). The maternal expression of *Znol4lb* seen with primer pair-1 indicates the presence of the shortest transcript at this stage, as the primer pair-2 recognizing all other transcripts do not show maternal expression. Low level of *Znol4lb* expression can be detected up to 18 hours post fertilization (hpf). Increased expression of *Znol4lb* can be seen from 24 hpf onwards and remains constant until 72 hpf (Fig. 2B).

## Spatial expression pattern of *Znol4lb*

Whole embryo mRNA *in situ* hybridization (WISH) was performed on different developmental stages of zebrafish embryos to study the spatial expression pattern of *Znol4lb*. Localized mRNA expression was detected from 12 hpf onwards. *Znol4lb* mRNA is localized to the intermediate mesoderm in 12 and 14 hpf embryos (Fig. 3 B,C). Sixteen hpf embryos show strong expression in pronephric mesoderm (Gerlach and Wingert, 2013), the spinal cord (Lewis *et al*., 2003), trigeminal ganglia (Canger *et al*., 1998) and hind brain (Moens and Prince, 2002). Its expression in diencephalon and telencephalon also becomes visible (Fig. 3D). *Znol4lb* is expressed in parts of fore brain such as diencephalon, telencephalon and epiphysis in 22 hpf embryos. Its expression can be seen in tegmentum (Fig. 3E). It is also expressed in hind brain and the spinal cord. *Znol4lb* is expressed in the pronephric tubule, pronephric duct and proctodeum (Fig. 3E). The expression of *Znol4lb* in pronephric tubule and duct was confirmed by

*Abbreviations used in this paper:* NOL4L, nucleolar protein 4-like.

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analyzing the expression of pronephric marker *Zcdh17* (Fig. 3 F,F') (Wingert *et al.*, 2007). Its expression in the ventral mesenchyme may represent the hematopoietic tissue (Fig. 3F').

This expression pattern of *Znol4lb* is maintained in 24 hpf embryos (Fig. 3 G-M"). The hind brain expression of *Znol4lb* starts at the midbrain hind brain boundary (MHB) region (Rhinn and Brand, 2001), but excluded from the boundary itself as concluded from two colour WISH analysis of MHB markers *Zpax2a* and *Zpea3* (Fig. 3 H,I) (Pfeffer *et al.*, 1998; Munchberg *et al.*, 1999). A coronal section through the forebrain presents a better visualization of its expression (Fig. 3J). A transverse section through the trunk region provides clear visualization of *Znol4lb* in pronephros, gut and the spinal cord neurons (Fig. 3K). *Znol4lb* may also be expressed in the Rohon-Beard neurons as seen by its co-expression with *Zpea3* (Fig. 3L) (Munchberg *et al.*, 1999).

The dorsal view of a 24 hpf embryo shows that, *Znol4lb* is strongly expressed in the hind brain and the spinal cord (Fig. 3M). A closer view confirms that *Znol4lb* is localized to the trigeminal ganglia (Fig. 3 M',M").

Both 27 hpf and 30 hpf embryos show strong expression of *Znol4lb* in diencephalon, telencephalon, epiphysis, tegmentum, trigeminal ganglia, hind brain and the spinal cord neurons (Fig. 3 N,O). Expression of *Znol4lb* is also visible in pronephros, proctodeum, the gut (Ober *et al.*, 2003) and ventral mesenchyme, representing

**A**

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Hsa             -MPKPTLLLRGGWERERSPGDSELGRQFRDWCLRTYGDSAKTKTVTRSKYQRIAEVLQGGGGTGAGSGPAAGEKGKFQFWVRSKGFRLGSGRE-------PKMGQVVYVPVKTGSGADGL 112
Mmu             -MPKPTLLLRGGWERERSPGDSELGRQFRDWCLRTYGDSAKTKTVTRSKYQRIAEVLQGGGGTGAGSGPAAGEKGKFQFWVRSKGFRLGSGRE-------PKMGQVVYVPVKTGSGADGL 112
Dre_nol4lb      MSTKK-RVDLTRRSRSPAVSEAEMFGDFQEWCLRTYGDSGKTKTVTRKKYNKILQTLLQSDDSEGVIIESNHINAKFKFWVKSKGFQVGRSES--ENENGAADRPVLYVPIKA-TCVDGV 116
Dre_nol4la      MATKKPCADLTKRSRSPVVLEAEMFSEFQDWCLRTYGDSGKTKTVTRKKYNKIMQTLLQNEESDGVYVDNSHINAKFKFWVKSKGFQVGSNILGEHNKKESSGKPVLYVPVKS-TCSDGG 119
Gga             ----------------------------------------------------------------------------------------------------MIDGV 5
Xtr             ----------------------------------------------------------------------------------------------------

Hsa             SEPEGISLKRVAVVEDFFDIIYSMHVESSAEPGKAPKHAGQKKTYRAIAETYAFLPREAVTRFLMSCTECQKRMHFNSNGLEPKENEPPSPLVSGIIDYNMPLTSTYLKQMKLRVMNSQ- 231
Mmu             SEPEGISLKRVAVVEDFFDIIYSMHVESSAEPGKAPKHAGQKKTYRAIAETYAFLPREAVTRFLMSCTECQKRMHFNSSGPEPKENEPPSPLVSGIIDYNMPLTSTYLKQMKLRVMNSQ- 231
Dre_nol4lb      LGPDGSSLKRVAVVEDFYDIIYAMHVEISSDPAKAPKHAGQKKTYKAIAETYAFLPREAVTRFLMSCGECQKRMHISPSGAEFKENNQPSSLAADLIDYNMPLTTTYLKQKKLQCMNNN- 235
Dre_nol4la      TAQD-SSLKRVAVVEDFFDIIYAMHVEMGADPGRAPKHAGQKKTYKAIAETYAFLPREAVTRFLMSCGECQKRMHINPSTAEFKENDRPTSLVPDLIDYNMPLTATYLKQMKLQCMNTN- 237
Gga             GVDEKLSLRRVAVVEDFFDIIYSMHVETGPNGEQIRKHAGQKRTYKAISETYAFLPREAVTRFLMSCSECQKRMHLNPDGADHKDNGKPPTLVTSMIDYNMPITMAYMKHMKLQLLNSQQ 125
Xtr             ----------------------MHVETGPNGEQIRKHAGQKRTYKAISETYAFLPREAVTRFLMSCSECQKRMHLNPDGGEHKDNGKPPTLVTSMIDYNMPITMAYMKHMKLQLLNSQQ 97
                                      **** ..:  :  ******:**:**:********** ******* *******:...  : *:*  *..*...:******:* :*:*: **: :*.:

Hsa             EQDETSVSSEDFDMSDSTWMSADP--HLASSLSPSQ--DERMRSP-QNLHSQEDDDSSSESGS-GNGSSTLNPST--------SSSTQGDPAFPEMNGNGAVAPMDFTTAAE------DQ 331
Mmu             EQDETSVSSEDFDMNDSAWMSADP--HLASSLSPSQ--EERMRSP-QNLHSQEDDDSSSESGS-GNGSSTLNPST--------SSSTQGDPAFPEMNGNGAAAPMDFTATAE------DQ 331
Dre_nol4lb      EQDECSVSSEEFDMSDPTWISAERHGTLSDLNHPSS--ADRMHSP-QNAHKEEDDDSSSESGS-GNGIRTLTPPSVGAAGVN-DSTGPGSGSYAEVNGNSTAAPLDFSTTSSSSSSEDQQ 350
Dre_nol4la      EQDESSVSSEDLDMAEPAWVSTER-APAADPSSPAAPYTERMPTPPQPNIKEEEDDSSSENGSTANGLPALTPPGGAAMATGGVPASEGVVPYGEVNGNGAAAPLDFSTSSSSSSQEGQQ 356
Gga             DEDESSIESDEFDMSDSTRMSAVN-------SDLSSNLEERMQSP-QNLQSQQDDDSAAESFN---GNETVGHSS----------NASGGTHCREMAETNS-NGKTNLEQDE-------Q 216
Xtr             DEDESSIESDEFDMSDSTRMSAVN-------SDLSSNLEERMQSP-QNLQGQQDDDSAAESYT---CNETVGQAS----------NSTGATHNREMMEVITTNGNAISEQEE-------Q 189
                ::** *:.*:::** :.: :*:           :    :** :* *    :::***:::*. .      ::  .              *     *:   :          .       *

Hsa             PINLCDKLPPATALGTAS-------YPSDGCG--ADGLRSRVKYG---VKTTPESPPYSSGSYDSIKTEVSG-CPEDLTVGRAPTADDDDDDHDDHEDNDKMNDSEGMDPERLKAFNMFV 438
Mmu             PINLCEKLPPGTALGTPS-------YPSDGCG--TDGLRSRVKYG---VKNTPESPPYSSGSYDSIKTEVSG-CPEDLTVGRAPAADDDDDDHDDHEDNDKMNDSEGMDPERLKAFNMFV 438
Dre_nol4lb      PVNLSDRLSQALPTAAAMLATFTPEHTEELRR--KYPKSTELRLDRDIRDYNNKSPQYSSGSYDSIKMEI---CAEDLTVGRSQAADDDDDDHDDHDDSDKINDVEGVDPERLKAFNMFV 465
Dre_nol4la      PVNLSDRLLPGVTLGS---------YASDPSR--KFPVKT---------EYGNKSPQYSSGSYDSVKTELST-CGEDMTPGRSQVIDDDDDDHDDHDDSDKINDAEGLDPERLKAFNMFV 455
Gga             PLNLSDSPLSAQLTSEYR----LDDHSSNGKNKYKTLLISDLKMERERARENGSKSPAHSYSSYDSGKNESVDRGAEDLSLNRG---DEDEDDHDEQEDPEKVNESDGVEAERLKAFNMFV 329
Xtr             PLNLSDCPVSSQLNPEYR----IDDHSSNGKHPYKNLLIGDLKMDRERARENGNKSPAHSYSSYDSGKNESVGGCGEDLSMTRG---DEDEDDHDDHEDAEKAAESDGVEAERLKAFNMFV 302
                *:**.:    .               :..:                    .   :** :* .**** * *      **::  *.   *:*:****:::* :*  : :*::.**********

Hsa             RLFVDENLDRMVPISKQPKEKIQAIIESCSRQFPEFQERARKRIRTYLK3CRRMKKNGMEMTRPTPPHLTSAMAENILAAACESETRKAAKRMRLEIYQSSQDEPIALDKQHSRDSAAIT 558
Mmu             RLFVDENLDRMVPISKQPKEKIQAIIESCSRQFPEFQERARKRIRTYLKSCRRMKKNGMEMTRPTPPHLTSAMAENILAAACESETRKAAKRMRLEIYQSSQEEPIALDKQHSRDSTAIT 558
Dre_nol4lb      RLFVDENLDRMVPISKQPKEKIQAIIESCSRQFPEFQERARKRIRTYLKSCRRMKKNGMEQTRPTPPHLTSAMAENILAAACESESRKAAKRMRMDVYQ-AHEEPVSLDKAVSREPVSLT 584
Dre_nol4la      RLFVDENLDRMVPISKQPKEKIQAIIESCSRQFPEFQERSRKRIRTYLKSCRRMKKGGFE-SRPTPPHLTSAMAENILAAACESETRNAAKRMRLDIYQ-AHDESTTADKPNSRDPAALA 573
Gga             RLFVDENLDRMVPISKQPKEKIQAIIDSCRRQFPEYQERARKRIRTYLKSCRRMKRSGFEMSRPIPSHLTSAVAESILASACESESRNAAKRMRLDRQQ---DEATPADKQYKQEPAQVT 446
Xtr             RLFVDENLDRMVPISKQPKEKIQAIIDSCRRQFPEYQERARKRIRTYLKSCRRMKRSGFEMSRPIPSHLTSAVAETILASACESESRNAAKRMRLDRQ----DETPPADKSHKQDTAQVT 418
                ***************************:** *****:***:***************:.*:* :** *.*****:**.***:*****:*:******::      :*. . **  .:::. ::

Hsa             HSTYSLPASSYSQDPVYANGGLNYSYRGYGALSSNLQPPASLQTGNHS----------------------NGPTDLSMKGGASTTSTTPTPTPSSTSTSRPVPTAQLSPTEISAVRQLI 655
Mmu             HSTYSLPASAYSQDPVYVNGGLNYSYRGYGSLSSNLQPSASLQTGNHS----------------------NGPTDLSMKGGASTP-TPPTPTPSSNSTSRTMPTAQLSPTEISAVRQLI 654
Dre_nol4lb      HSAYSLAASAYSQDQLYINGGLNYSYRGYGGLCASMQHPVTLNTATAQT----------------------NGPTDLSMKSLASNSSSSSSSS---LGRNGGGASTQLSHTEVAAVRQLI 679
Dre_nol4la      HSGFTLAASAYSQDQLYTNGGLNYSFRGYGTLGNNLQ-----NNGTSQT----------------------NGPTDLSMKSLASNSTSSSSSNSHGQGGGGGGASAQLSPPEVTAVRQLI 666
Gga             YSTSAVSS---TQEVLYINGNGTYSYHSYRGLGGGLLNLNDASSSDLQPTSTTATTQHCIPGSTNVQSPSNSGPTDLSMKRQLASSSGSSSSS----------SSRPQLSPTEINAVRQLV 554
Xtr             YSTSAVSS---TQDVLYINGNGTYSYHTYRGLGGGLLNLSDTS----------------------------NSGPTDLSMKRPLATSSTSPSNT----------SNRTQLNPTEINAVRQLV 499
                :*  ::.:   :*: :* **. .**:: *  *  .:       .                            .********   :..  ..: .           .**. .*: *****:

Hsa             AGYRESAAFLLRSADELENLILQQN 680
Mmu             AGYRESAAFLLRSADELENLILQQN 679
Dre_nol4lb      AGYRESAAFLLRSADELENLILQQN 704
Dre_nol4la      AGYRESAAFLLRSADELENLILQQN 691
Gga             AGYRESAAFLLRSADELENLILQQN 579
Xtr             AGYRESAAFLLRSADELENLILQQN 524
```

**B**

| | Hsa | Mmu | Dre nol4lb | Dre nol4la | Gga | Xtr |
|---|---|---|---|---|---|---|
| Hsa-NP_001243727.1 | 100.00 | 96.32 | 62.95 | 62.59 | 57.17 | 57.73 |
| Mmu-NP_001127772.1 | 96.32 | 100.00 | 62.74 | 62.84 | 56.35 | 57.06 |
| Dre_nol4lb-XP_009295020.1 | 62.95 | 62.74 | 100.00 | 74.19 | 56.47 | 56.12 |
| Dre_nol4la-XP_001922545.4 | 62.59 | 62.84 | 74.19 | 100.00 | 56.02 | 56.11 |
| Gga-XP_004939884.1 | 57.17 | 56.35 | 56.47 | 56.02 | 100.00 | 87.76 |
| Xtr-XP_002934174.2 | 57.73 | 57.06 | 56.12 | 56.11 | 87.76 | 100.00 |

**C**

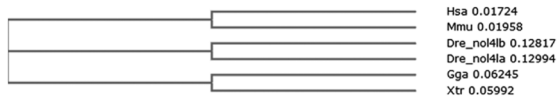


**Fig. 1. Alignment of NOL4L protein sequences. (A)** *The longest form of the protein from* Homo sapiens *(Hsa),* Mus musculus *(Mmu),* Gallus gallus *(Gga),* Xenopus tropicalis *(Xtr) and* Danio rerio *(Dre) were aligned using ClustalW2.* **(B)** *Percent identity matrix showing high degree of conservation of NOL4L among these species.* **(C)** *The phylogenetic tree.*

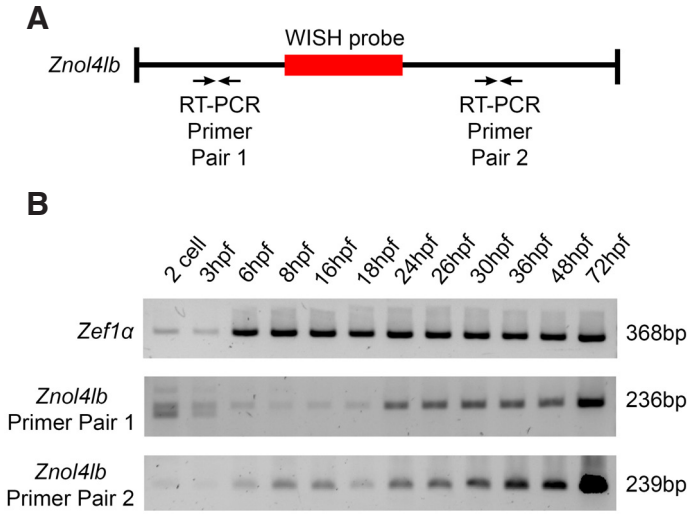


**Fig. 2. Temporal expression of zebrafish *nol4lb (Znol4lb)* mRNA. (A)** *Schematic representation of* Znol4lb *and position of two sets of primers used in RT-PCR and the probe used for WISH.* **(B)** *Expression of* Znol4lb *mRNA during zebrafish development.* Zef1α *is used as a loading control.*

caudal hematopoietic tissue (Fig. 3N) (Jing and Zon, 2011). Two colour WISH of *Znol4lb* and *Zcdh17* confirms the expression of this gene in pronephric tubule and duct. *Znol4lb* is expressed in the gut, which lies ventral to the *Zcdh17* expression domain (Fig. 3 O,O'). Similarly, *Znol4lb* is seen outside the *Zcdh17* expression domain on the dorsal side, presumably in the hematopoietic territory (Fig. 3O'). A higher magnification picture of the trunk region of a 72 hpf embryo shows that, *Znol4lb* is expressed both in the pronephric tubule, pronephric duct and the gut (Fig. 3P).

Here, we have described the temporal and spatial expression pattern of *Znol4lb* during zebrafish embryogenesis. It is expressed in the central nervous system, pronephric tubule, pronephric duct,

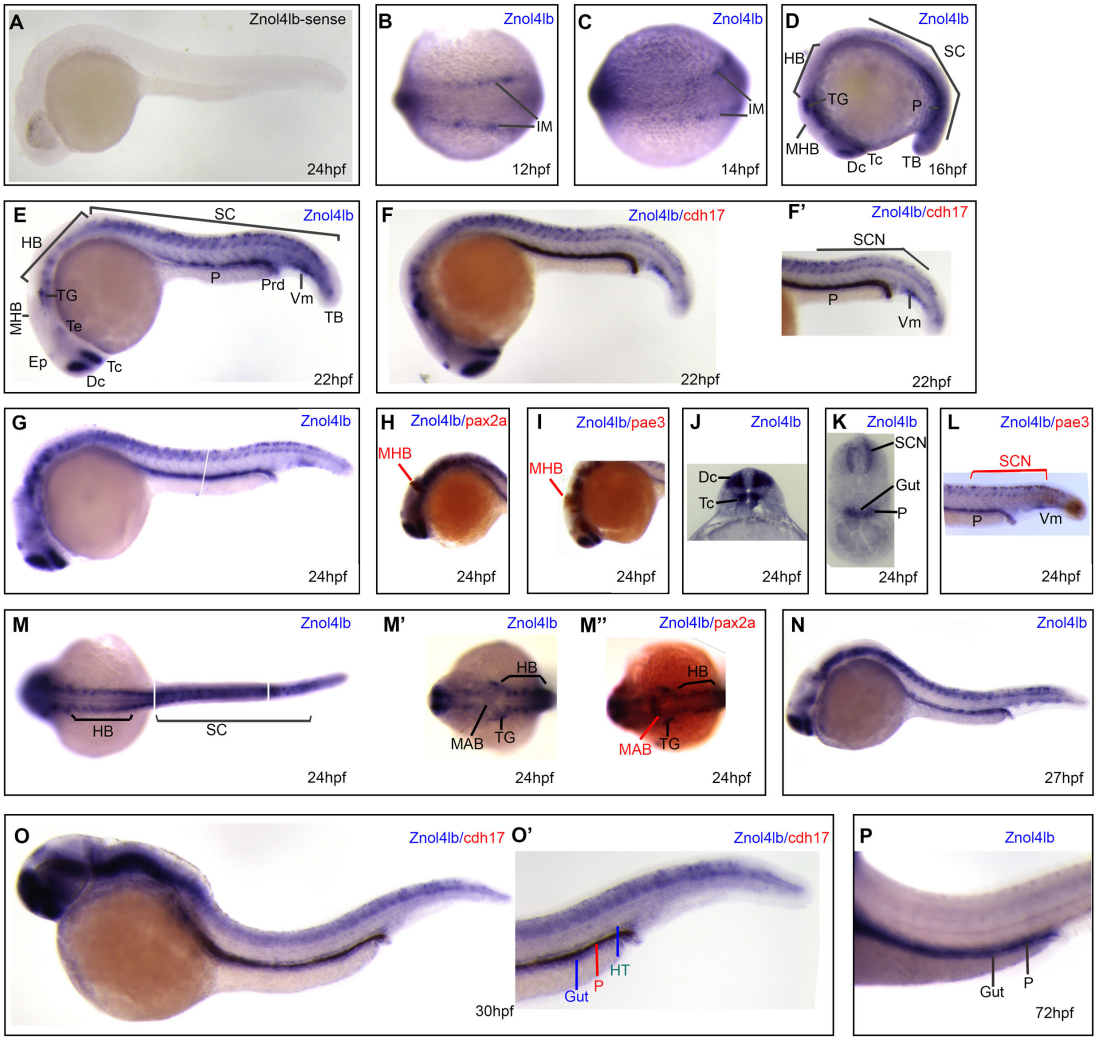


**Fig. 3. Spatial expression pattern on *Znol4lb* mRNA.** *Whole embryo mRNA* in situ *hybridization (WISH) on wild type embryos was carried out from 12 hpf to 72 hpf.* **(A)** *DIG-labeled sense probe shows no background staining.* Znol4lb *expression in intermediate mesoderm at 12* **(B)** *and 14 hpf* **(C)**. **(D)** *Expression of* Znol4lb *in telencephalon (Tc), diencephalon (Dc), trigeminal ganglia (TG), hind brain (HB), spinal cord (SC), pronephric mesoderm (P) and tail bud (TB) at 16 hpf.* **(E)** *At 22 hpf,* Znol4lb *is expressed in telencephalon, diencephalon, epiphysis (Ep), Tegmentum (Te), trigeminal ganglia, hind brain, spinal cord, pronephric tubule and duct (P), proctodeum (Prd) and ventral mesenchyme (Vm).* **(F,F')** *Coexpression of* Znol4lb *and* Zcdh17 *in the pronephric tubule and duct.* **(G)** *24 hpf embryo showing expression domains of* Znol4lb. *Two colour WISH showing expression of* Znol4lb *and* Zpax2a **(H)** *and* Zpea3 **(I)** *in the head.* **(J)** *Section through forebrain.* **(K)** *Section through the trunk showing* Znol4lb *expression in pronephros (P), gut and spinal cord neurons (SCN).* **(L)** Znol4lb *is expressed with* Zpea3 *in SCN.* **(M)** *Dorsal view of a 24 hpf zebrafish embryo showing* Znol4lb *expression in the hind brain and spinal cord.* **(M')** *Higher magnification picture showing* Znol4lb *expression in the trigeminal ganglia.* **(M")** *Two colour WISH with* Zpax2a *shows that,* Znol4lb *is not expressed in the midbrain-hind brain boundary (MHB).* **(N)** *Expression of* Znol4lb *at 27 hpf.* **(O)** *30 hpf embryo showing overlapping expression of* Znol4lb *and* Zcdh17 *in pronephric tubule and duct.* **(O')** *Higher magnification image of trunk region shows that,* Znol4lb *is expressed in pronephros (P), the gut and the hematopoietic territory (HT).* **(P)** Znol4lb *is expressed in pronephros and the gut at 72 hpf.*

and the gut and at low levels in the hematopoietic cells. This complex expression pattern underpins the important functions this gene may have during zebrafish development. The human ortholog of this gene is implicated in leukemia. It would be interesting to study the function of this gene in the embryonic and adult hematopoietic system in zebrafish. Genetic and functional characterization of *Znol4lb* will clarify its function during zebrafish development and would provide clues for understanding its function in other vertebrates including humans.

## Materials and Methods

### *Cloning of* Znol4lb

Total RNA was extracted from 24 hpf zebrafish embryos using TriZol reagent (Invitrogen) following manufacturer's instructions. cDNA was synthesized using SuperScript-III cDNA synthesis kit (Invitrogen). The *Znol4lb* was amplified by PCR (forward 5'-CAATCTCTAAACAGCCTAAAGAGAAGA-3', reverse 5'-TAGCTAAAAACAAAGAACATGAGGAAT-3', Tm 60°C, 35 cycles). The amplified fragment was cloned into pCR-BluntII-TOPO vector (Invitrogen) and sequenced.

### *RT-PCR*

Total RNA was extracted from zebrafish embryos of various developmental stages as described above and cDNA was prepared using equal amounts of RNA. Two pair of PCR primers was designed using Primer-3 for checking the expression of *Znol4lb*. The primers and PCR conditions were as follows: Primer pair-1 (forward 5'-GTGTTCAGTGAGTAGCGAGGAGT-3', reverse 5'-GTCCAGTACTGTCATTCACACCA-3', Tm 55ºC, 30 cycles) and primer pair-2 (forward 5'-TTCGCAGATGCCAACACTCT-3', reverse 5' –TGGTTGTGTTTGGGAGGCTT3', Tm 55ºC, 30 cycles). Zebrafish *ef1α* (forward 5'-CTTCTCAGGCTGACTGTGC-3', reverse 5'-CCGCTAGCATTACCCTCC -3', Tm 55ºC, 22 cycles) was used as a loading control.

### *Zebrafish whole embryo* in situ *hybridization (WISH) and histology*

Wild type zebrafish strains, Tübingen and Albino were used in all experiments. The embryos were staged according to Kimmel *et al.*, 1995. WISH experiments were carried out following previously described protocols (Thisse and Thisse, 2008). Digoxigenin or Fluorescein labeled antisense probes was synthesized by linearizing the plasmids and transcribing with T7 polymerases (*Znol4lb* linearized with KpnI, *Zpax2a* linearized with SacII, *Zcdh17* linearized with BamHI, *Zpea3* linearized with NotI).The sense probe for *Znol4lb* was synthesized by linearizing the plasmid with XbaI and transcribing with SP6 polymerase. BM-purple and INTBCIP (Roche) were used as chromogenic substrates in WISH. Pictures were taken using Leica MZ16 stereo microscope. Vibratom sections were prepared from gelatin embedded embryos after WISH.

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