**The expression patterns of vestigial like family member** [**4 genes in zebrafish embryogenesis**](https://www.ncbi.nlm.nih.gov/pubmed/25354458)

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**Abbreviations and symbols:** dpf, days post fertilization; E, eyes; Ep, epidermis; EpN, epidermis at the border of the neural plate; DFC, dorsal forerunner cells; HB, hindbrain; hpf, hours post fertilization; J, jaw; L, lens; LL, lateral line; LLP, lateral line primordium; MB, midbrain; MDR, midline of diencephalic roof; MHB, midbrain–hindbrain boundary; MTc, midbrain tectum; MTg, midbrain tegmentum; N, neuromasts; OfV, olfactory vesicle; OV, otic vesicle; P, pharynx; PA, pharyngeal arches; PD, pronephric duct; PF, pectoral fin; PP, pharyngeal pouches; Pr, proctodeum; R, retina; r, rhombomeres; r1, rhombomere 1; T, telencephalon; Ve, ventricle; *vgll4*, vestigiallike family member 4; WISH, whole-mount in situ hybridization.

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**Abstract**

Transcriptional cofactor Vestigial-like 4 (VGLL4) was considered to take part in the early stage of development. Different from human, three paralogs of *vgll4* were found in zebrafish, which were *vgll4a*, *vgll4b* and *vgll4l*. However, the expression patterns of the three paralogs during zebrafish development remains unknown. In this study, we used in situ hybridization to elucidate the temporal and spatial expression of zebrafish *vgll4* paralogs during normal embryonic and larval development. Similar expression was shown in certain areas at similar stages for the three paralogs. Expression of *vgll4a*, *vgll4b* and *vgll4l* were all found in pectoral fins and otic vesicles during the early developmental stages. On the other hand, a few differences of the three paralogs expression were found in eyes, pharynx, pharyngeal arches and brain tissues. The expression of *vgll4a* was weak and ubiquitous, while *vgll4b* was obviously expressed in brain tissues and *vgll4l* was clearly restricted to each pair of pharyngeal pouches. What’s more, *vgll4b* and *vgll4l* had unique expression at mature lateral line neuromasts and forerunner cells respectively. Despite the conservativeness of functional domains, the three paralogs of zebrafish *vgll4* shared several similarities and displayed some distinctions in the expression patterns, indicating that they may still have different and exclusive functions, which need to be further explored.

**1. Introduction**

Transcription cofactor vestigial-like (VGLL) proteins, named after *Drosophila* transcriptional co-activator Vestigial (Vg) involved in various biological processes, are found expressed both in invertebrates and vertebrates. There are four members of VGLL identified in mammals namely VGLL1, VGLL2, VGLL3 and VGLL4. Acting as transcriptional cofactors, VGLLs do not contain DNA binding domain, their transcriptional regulation functions are mediated through the interaction between TONDU (TDU) domains and other transcription factors (HH Chen *et al.*, 2004). Unlike VGLL1, VGLL2 and VGLL3 which possess a unique TDU domain, there are two TDU domains in series at the carboxyl-terminus of VGLL4. Therefore, VGLL4 is assumed to have different functions from the other three homologs (Faucheux *et al.*, 2010).

Physiologically, VGLL4 was considered to take part in the early stage of development. Similar to VGLL1 and VGLL2, VGLL4 interacts with the transcription factor myocyte enhancer factor (MEF) 2 (Maeda *et al.*, 2002). The tandem TDU domains of VGLL4 might form a bridge between MEF2 and TEAD, both of which are cardiac and skeletal muscle transcription factors implicated in cardiac muscle differentiation (Pobbati and Hong, 2013). In addition, acetylation of VGLL4 could regulate Hippo-YAP Signaling and postnatal cardiac growth by regulating TEAD stability and YAP-TEAD activity (McNamara *et al.*, 2017). In vitro study also suggested that VGLL4/TEF4(TEAD2)/IRF2BP2 complex could regulate the expression of vascular endothelial growth factor (VEGF) in muscle (Pobbati and Hong, 2013).

Pathologically, downregulation of VGLL4 was frequently found in different types of tumors including lung cancer (Zhang *et al.*, 2014) and colorectal carcinoma (Jiao *et al.*, 2017). Therefore, VGLL4 was considered as a tumor suppressor by interfering with several important transcription factors. For example, VGLL4 was shown directly competing with YAP in binding to TEADs through its two TDU domains, thus negatively regulating the YAP-TEAD transcriptional complex and executing its growth-inhibitory function in lung cancer (Zhang *et al.*, 2014) and gastric cancer (Jiao *et al.*, 2014). It was also reported that VGLL4 interfered a TEAD4-TCF4 complex to regulate Wnt and Hippo signaling in colorectal cancer (Jiao *et al.*, 2017).

Although a series of biological functions of *vgll4* has been discovered, the endogenous expression of *vgll4* gene is not well explored. Northern blot analysis showed that in human tissues *vgll4* expression was highest in heart, kidney, and brain (HH Chen *et al.*, 2004). In *Xenopus* two *vgll4* genes, namely *vgll4* and *vgll4l*, were identified and their expression patterns were described mainly in neural tissues during the very early development stage with several distinctions and coincidence (Barrionuevo *et al.*, 2014). Thus a comprehensive spatio-temporal expression pattern study of *vgll4* will be useful for its physiological function interpretation.

As a widely used model organism, zebrafish displays much superiority in the research of development. High conservation of *vgll4* was observed between human and zebrafish from the point view of sequence, synteny, and interactants, making zebrafish a potential model for investigating the functions of *vgll4* during development. Different from human, three *vgll4* paralogs including *vgll4a*, *vgll4b* and *vgll4l* were found in zebrafish. Previous analysis has shown that *vgll4l* is expressed primarily in the endodermally derived pharyngeal pouches (Thisse *et al.*, 2001). However, the expression patterns of the other two members, the possible differences among the three paralogs and potential connection of each paralog to its functions in zebrafish development remain unknown. In this study, temporal and spatial gene expression of *vgll4* paralogs in zebrafish was analyzed in normal embryonic and larval development.

**2. Results**

**2.1 Characterization of the zebrafish *vgll4* genes**

Different from human counterpart, three paralogs of *vgll4* were found in zebrafish, naming *vgll4a* (mapped to chromosomes 23，NCBI Reference Sequence: XM\_005161921), *vgll4b* (mapped to chromosomes 11, NCBI Reference Sequence: NM\_213275) and *vgll4l* (vestigial like 4 like, mapped to chromosomes 8, NCBI Reference Sequence: NM\_001079998). Among these three genes, zebrafish *vgll4b,* locating between *atg7* and *tamm41,* is the potential homolog of human *VGLL4* by synteny analysis (Fig.1A). Although the whole amino acid sequences of zebrafish VGLL4a, VGLL4b and VGLL4l are only 39%, 70% and 32% identical to that of human VGLL4 (blastp, www.ncbi.nlm.nih. gov), the functional domains, such as nuclear conservative signal (NCS) and Tondu (TDU1 and TDU2), show great conservativeness with those of human VGLL4 (Fig.1B).

**2.2 *Vgll4a* expression during the development of zebrafish**

The expression patterns of three zebrafish*vgll4* genes were analyzed by whole-mount in situ hybridization (WISH) using a DIG-labeled anti-sense probe and a sense probe as a negative control for each gene. No positive signals were found in the embryos of various stages incubated with the three sense probes (data not shown).

During the gastrula period and before, hardly could any *vgll4a* expression be detected (data not shown). Faint and ubiquitous expression of *vgll4a* was observed in the embryo trunk at about 14 hpf during the preliminary segmentation period (Fig.2A). At 24 hpf, *vgll4a* was expressed dispersively in pharynx (P), eyes (E), otic vesicles (OV) and brain tissues, especially strong in the telencephalon (T), midline of diencephalic roof (MDR), the midbrain (MB), the hindbrain (HB) and the ventricle (Ve) (Fig.2B-2D). At 36 hpf and 48 hpf, *vgll4a* showed similar expression patterns as at 24 hpf in the brain, with more prominent expression at the midbrain–hindbrain boundary (MHB) and the rhombomere 1 (r1) at 48 hpf. Enhanced *vgll4a* expression could be detected in chondrogenic tissues including pharyngeal arches (PA) and pectoral fins (PF) at these stages (Fig.2E-2H, Fig.2J). All these expression signals were sharply weakened at 72 hpf (Fig.2I) and faded at 5 days post fertilization (dpf) (data not shown).

**2.3 *Vgll4b* expression during the development of zebrafish**

*Vgll4b*, which possesses the highest homology with human *vgll4* among the three zebrafish *vgll4* paralogs, could be detected from a very early stage after fertilization. Ubiquitous expression was displayed in the shield stage at 6 hpf (Fig.3A) and in the whole trunk of the embryo at 12 hpf (Fig.3B). At 24 hpf, *vgll4b* was highly expressed in head mainly including the areas of eyes, otic vesicles, and pharynx (Fig.3C-3E). As the brain tissues concerned, *vgll4b* expressions in telencephalon, midbrain tectum (MTc), midbrain tegmentum (MTg) and hindbrain were most prominent. Additionally, obvious expression was also detected in lateral line primordium (LLP) at this stage (Fig.3C-3E).

From 36 hpf to 72 hpf, similar expression pattern was observed with high *vgll4b* expression in eyes, olfactory vesicles (OfV), otic vesicles, brain tissues and pharyngeal arches and pouches (PA and PP) (Fig.3F-J, L-M). The strong signals of the ubiquitous expression in the brain tissues gradually converted to restricted local expression in midbrain–hindbrain boundary and rhombomeres(r) (Fig.3H, Fig.3J). With the migration and deposition of lateral line primordium cells, *vgll4b* was expressed in multiple neuromasts (N) of lateral line (LL). Faint expression was also found in proctodeum (Pr) and epidermis (Ep) at these stages (Fig.3F-3L). Among all the time points we checked, only at 48 hpf, a notable expression in heart could be detected，but not for the embryos incubating with the other two parolog probes (Fig.3K). By 5 dpf, signals appeared in swim bladder and signals in brain tissues, eyes, pharyngeal arches and pectoral fins were significantly reduced, while those in otic vesicles and neuromasts were retained (Fig.3N).

**2.4 *Vgll4l* expression during the development of zebrafish**

The initial expression of *vgll4l*, located in epidermis and the dorsal forerunner cells (DFC) at 6 hpf, was quite different from the other two paralogs (Fig.4A). Dorsal forerunner cells appear adjacent to the embryonic shield at midgastrulation and then generates an organizer region called Kupffer’s vesicle (KV) by the 4- to 6-somite stages. However, such particular expression of *vgll4l* could no longer be detected in KV at 12 hpf, instead, the expression signals appeared on the epidermis at the border of the neural plate (EpN) (Fig.4B). At 24 hpf, deposition of *vgll4l* could be found in areas of olfactory vesicles, eyes, midbrain, hindbrain, pharyngeal pouches, otic vesicles and proctodeum (Fig.4C-4E). From 36 hpf to 72 hpf, while gradually vanished in eyes, brain and proctodeum, *vgll4l* expression was highly concentrated in pharyngeal pouches, pectoral fins and otic vesicles (Fig.4F-4J). Expression of *vgll4l* also appeared in the lateral line at 36 hpf (Fig.4G) but failed to be detected in multiple neuromasts subsequently at 48 hpf and 72 hpf with the migration and deposition of lateral line primordium cells as *vgll4b* did. *Vgll4l* was also expressed in the jaw (J) at 72 hpf (Fig.4I). Finally, it did not show any staining at 5 dpf (data not shown). Our WISH result of *vgll4l* expression is similar to that of Thisse’s (Thisse *et al.*, 2001).

**2.5 The quantitative** **abundance of the three zebrafish vgll4 genes**

After WISH analysis of the three zebrafish vgll4 paralogs, real time quantitative polymerase chain reaction (RT-qPCR) was utilized to analyze the expression level of the three *vgll4* paralogs at 6 hpf, 12 hpf, 24 hpf, 36 hpf, 48 hpf, 3 dpf and 5 dpf.

Embryos of all the seven stages we detected displayed a comparative expression level of the three genes. From 6 hpf to 5 dpf, the expression level of *vgll4a* gradually descended to the lowest point at 24 hpf and ascended afterwards. Expression tendency of *vgll4l* was somehow similar to that of *vgll4a*. Different from the other two paralogs, low level of *vgll4b* expression was detected at the early stage but it was continually up-regulated during the embryo development until the highest level at the 5 dpf.

**3.Discussion**

By comparing the expression patterns of the three *vgll4* paralogs detected by WISH, it could be noticed that similar expression was shown in certain areas at similar stages. Significantly, during the early developmental process of zebrafish, in addition to the expression in pectoral fins and otic vesicles, varying degrees of expression of the three paralogs were found in eyes, brain tissues, pharynx and pharyngeal arches and pouches. Nevertheless, a few differences existed among the three expression patterns. For the brain tissues, signals of *vgll4b* expression were strong and specific while those of *vgll4a* were weak and ubiquitous. Notable expression was still observed in rhombomeres even by 72 hpf for *vgll4b,* in contrast, expression of *vgll4l* in the hindbrain had already faded by 48 hpf. Moreover, for the pharynx, the three paralogs all showed enhanced expression. The subtle difference was that *vgll4a* and *vgll4b* were extensively spread in pharynx, pharyngeal arches and pharyngeal pouches, whereas *vgll4l* was mainly restricted to each pair of pharyngeal pouches.

Despite several similar expression regions and high conservative functional domains, different expression patterns of the three paralogs in these particular tissues or organs suggested that functions of the three paralogs may not be redundant. For example, the pronounced expression of *vgll4l* in pharyngeal pounches indicated that it may play an important role in craniofacial development. It was reported by Melvin *et al’* study that *vgll4b* morphants displayed only a minor craniofacial phenotype at the highest morpholino (MO) doses, while *vgll4l* morphants displayed a distinct impairment. Severe *vgll4l* morphants even displayed complete loss of all viscerocranial cartilages and anterior neurocranial cartilages were further reduced or completely absent (Melvin *et al.*, 2013).

Furthermore, the distinct expression in certain regions of the three *vgll4* paralogs demonstrated their unique roles in these tissues. For instance, *vgll4b* was proved to be high expressed in each mature lateral line neuromast, while the expression of *vgll4l* in lateral line didn’t retained with the generation of neuromasts. The lateral line is a mechanoreceptive system of zebrafish. The neuromasts were originated from the anterior and posterior lateral line, and the terminal proneuromasts were formed by the migration and deposition of the lateral line primordium by 48 hpf (Tingaud-Sequeira *et al.*, 2004). This progress was accompanied by the strong and specific expression of *vgll4b*, indicating that it may take part in the course of proliferation, migration and deposition. Similar expression in the neuromasts has been found in a number of other genes like *negaly6* (Ji *et al.*, 2015), *esr1*, *esr2a* and *esr2b* (Tingaud-Sequeira *et al.*, 2004). In addition, at 6 hpf, distinct expression of *vgll4l* was detected in dorsal forerunner cells. These cells are produced from dorsal surface epithelial cells at mid-gastrulation stage, gathering and migrating to the vegetal pole to generate Kupffer’s vesicle (KV) later in the early somitogenesis stage. KV subsequently transfers left–right asymmetry signals such as asymmetric nodal-related genes expressions to the lateral plate mesoderm (LPM) and is very essential for the establishment of zebrafish left-right asymmetric patterning, namely the asymmetry of organs and tissues (Matsui and Bessho, 2012). A number of other genes have been reported to express in this region which all have been confirmed to have significant impact on the zebrafish left-right asymmetry determination, such as *atp6ap1b* (Gokey *et al.*, 2015), β-catenin 1 and β-catenin 2 (Zhang *et al.*, 2012), *cnpy1* (Matsui *et al.*, 2011), chordin (Aamar and Dawid, 2010). Asymmetric development of certain organs, e.g. heart, might be affected by the deficiency of *vgll4l*.

The low expression levels at late developmental stages shown by WISH might be due to the limit of unequal accessibility to in situ probes, while RT-qPCR analysis displayed the real abundances of *vgll4* paralogs at different stages. The different trend of the expression abundance might indicate that the three paralogs of *vgll4* may have different functions during the different development stages of zebrafish embryos.

Previous research has provided us with *vgll4* expression patterns of another aquatic animal *Xenopus laevis* with two *vgll4* paralogs, *vgll4* and *vgll4l*, which are the orthologs of zebrafish *vgll4b* and *vgll4l* respectively (Faucheux *et al.*, 2010). Similar to zebrafish counterparts, both of *Xenopus vgll4* and *vgll4l* are expressed in central nervous system, otic vesicles and branchial arches (Barrionuevo *et al.*, 2014). Furthermore, by searching the Eurexpress database (http://www.eurexpress.org/ee/), we found the expression patterns of murine *vgll4* in developing embryos (Embryo Age: 14.5 dpc, Assay ID: euxassay\_000238) is quite similar to that of zebrafish and *Xenopus*. High *vgll4* expression could also be seen at central nerve system, lateral ventricles, cerebellum, as well as eyes, skin and alimentary system. Such high consistency indicates that *vgll4* orthologs may preserve conservative functions in different species.

Temporal and spatial gene expression of *vgll4* paralogs was elucidated and similarities and differences among the three paralogs were discussed in this study, however, further investigations are still needed to explore the mechanism of these phenomena.

**4.Experimental Procedures**

**4.1 Zebrafish maintenance**

Zebrafish wild type Tubingen (Tu) strain was maintained and the stages of embryos were defined as previously described by Kimmel et al(Kimmel *et al.*, 1995). Embryos were obtained through natural mating and were treated with 0.0045% 1-phenyl-2-thiourea (Sigma-Aldrich, St. Louis, MO, USA) to inhibit the pigments.

**4.2 Synthesis of RNA probes**

Coding sequences of *vgll4a*, *vgll4b* and *vgll4l* were amplified from the cDNA library of 36 hpf and 48 hpf zebrafish embryos by PCR with the primers as follows: *vgll4a* forward primer: 5’-CCGAATTCATGGATATTTTGATCAATGAAATG-3’, *vgll4a* reverse primer: 5’-TTCTCGAGTTAAGACTGACCAACATGATTG-3’; *vgll4b* forward primer: 5’-CCGAATTCATGCTTTTTACCAAAATGGACCTGTTGAACTACC -3’, *vgll4b* reverse primer: 5’- TTCTCGAGTCAAGACACCAGGGACGGGGAGTGATTGTGGTTC -3’; *vgll4l* forward primer: 5’- CCGAATTCATGGCGGTCACTAATTTCCACTAC-3’, *vgll4l* reverse primer: 5’-TTCTCGAGTCATTTATCACCAGAAGTTTGGTG-3’. The resulting amplicons were inserted into PCS II vectors using T4 DNA Ligase (promega). Anti-sense and sense DIG-labeled RNA probes were synthesized from the linearized plasmids containing sequences of relevant genes, using the DIG RNA Labeling Kit (SP6/T7) (Roche) under the guidance of the manufacturer’s protocol.

**4.3 Whole mount RNA in situ hybridization**

Embryos were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C, washed with PBST, dehydrated in ethanol and stored at −20 °C until use. Whole mount RNA in situ hybridization was performed as described previously by Thisse (Thisse and Thisse, 2008) and the DIG-labeled probes were visualized by BCIP/NBT staining (Vector Laboratories) using alkaline phosphatase-coupled anti-digoxigenin Fab fragment antibody (Roche).

**4.4 RT-qPCR analysis**

At different developmental stages, total RNA was isolated from the embryos using Trizol reagent (Invitrogen). After determining the concentration of total RNA, retro-transcription was performed using GoScript™ Reverse Transcriptase Mix, Oligo （dT） (Promega) to synthesize the cDNA following the manufacture’s protocol. Real-time quantitative polymerase chain reaction was performed using SYBR green detection method with ABI ViiA7. The SYBR green reagent was provided by Toyobo. The reaction system and amplification program were set according to the recommended protocol of the product. A negative control without cDNA template was run in each assay. Relative mRNA levels were calculated by the comparative CT method (Livak and Schmittgen, 2001).*β-actin* was utilized as the internal reference. The expression levels of the three paralogs of *vgll4* were normalized to the *β-actin* expression level at each specific stage. Primer pairs used in qPCR were as follows: *vgll4a* forward primer: 5’-GAATCAACAGTTAGCGTGCTTC-3’, *vgll4a* reverse primer: 5’-GATGCTTCTGAACCATCTTCGC-3’, *vgll4b* forward primer: 5’-GCGATTAAAAGTCCATAAATGC-3’, *vgll4b* reverse primer: 5’-GACGACAGAGACTGCATTCTGG-3’, *vgll4l* forward primer: 5’-GTGCGTTGAAGATTCAGGAATG-3’, *vgll4l* reverse primer: 5’-CACTCTGTCGCTGGTCATGTGC-3’, *β-actin* forward primer: 5’- TGCTGTTTTCCCCTCCATTG-3’, *β-actin* reverse primer:5’- TTCTGTCCCATGCCAACCA-3’.

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**References**

AAMAR E, DAWID IB (2010). Sox17 and chordin are required for formation of Kupffer’s vesicle and left-right asymmetry determination in zebrafish. *Dev Dyn* 239: 2980–2988.

BARRIONUEVO MG, AYBAR MJ, TRÍBULO C (2014). Two different vestigial like 4 genes are differentially expressed during Xenopus laevis development. *Int J Dev Biol* 58: 369–377.

CHEN H-H, MULLETT SJ, STEWART AFR (2004). Vgl-4, a novel member of the vestigial-like family of transcription cofactors, regulates alpha1-adrenergic activation of gene expression in cardiac myocytes. *J Biol Chem* 279: 30800–30806.

FAUCHEUX C, NAYE F, TRÉGUER K, FÉDOU S, THIÉBAUD P, THÉZÉ N (2010). Vestigial like gene family expression in Xenopus: Common and divergent features with other vertebrates. *Int J Dev Biol* 54: 1375–1382.

GOKEY JJ, DASGUPTA A, AMACK JD (2015). The V-ATPase accessory protein Atp6ap1b mediates dorsal forerunner cell proliferation and left-right asymmetry in zebrafish. *Dev Biol* 407: 115–130.

JI D, LI L, ZHANG S, LI H (2015). Identification of a Ly-6 superfamily gene expressed in lateral line neuromasts in zebrafish. *Dev Genes Evol* 225: 47–53.

JIAO S, LI C, HAO Q, MIAO H, ZHANG L, LI L, ZHOU Z (2017). VGLL4 targets a TCF4–TEAD4 complex to coregulate Wnt and Hippo signalling in colorectal cancer. *Nat Commun* 8: 14058.

JIAO S, WANG H, SHI Z, DONG A, ZHANG W, SONG X, HE F, WANG Y, ZHANG Z, WANG W, WANG X, GUO T, LI P, ZHAO Y, JI H, ZHANG L, ZHOU Z (2014). A Peptide Mimicking VGLL4 Function Acts as a YAP Antagonist Therapy against Gastric Cancer. *Cancer Cell* 25: 166–180.

KIMMEL CB, BALLARD WW, KIMMEL SR, ULLMANN B, SCHILLING TF (1995). Stages of embryonic development of the zebrafish. *Dev Dyn an Off public* 203: 253–310.

LIVAK KJ, SCHMITTGEN TD (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* 25: 402–408.

MAEDA T, CHAPMAN DL, STEWART AFR (2002). Mammalian vestigial-like 2, a cofactor of TEF-1 and MEF2 transcription factors that promotes skeletal muscle differentiation. *J Biol Chem* 277: 48889–48898.

MATSUI T, BESSHO Y (2012). Left-right asymmetry in zebrafish. *Cell Mol Life Sci* 69: 3069–3077.

MATSUI T, THITAMADEE S, MURATA T, KAKINUMA H, NABETANI T, HIRABAYASHI Y, HIRATE Y, OKAMOTO H, BESSHO Y (2011). Canopy1, a positive feedback regulator of FGF signaling, controls progenitor cell clustering during Kupffer’s vesicle organogenesis. *Proc Natl Acad Sci* 108: 9881–9886. 1017248108.

MCNAMARA JW, LI A, LAL S, BOS JM, HARRIS SP, VAN DER VELDEN J, ACKERMAN MJ, COOKE R, DOS REMEDIOS CG (2017). MYBPC3 mutations are associated with a reduced super-relaxed state in patients with hypertrophic cardiomyopathy. *PLoS One* 12.

MELVIN VS, FENG W, HERNANDEZ-LAGUNAS L, ARTINGER KB, WILLIAMS T (2013). A morpholino-based screen to identify novel genes involved in craniofacial morphogenesis. *Dev Dyn* 242: 817–31.

POBBATI A V., HONG W (2013). Emerging roles of TEAD transcription factors and its coactivators in cancers. *Cancer Biol Ther* 14: 390–398.

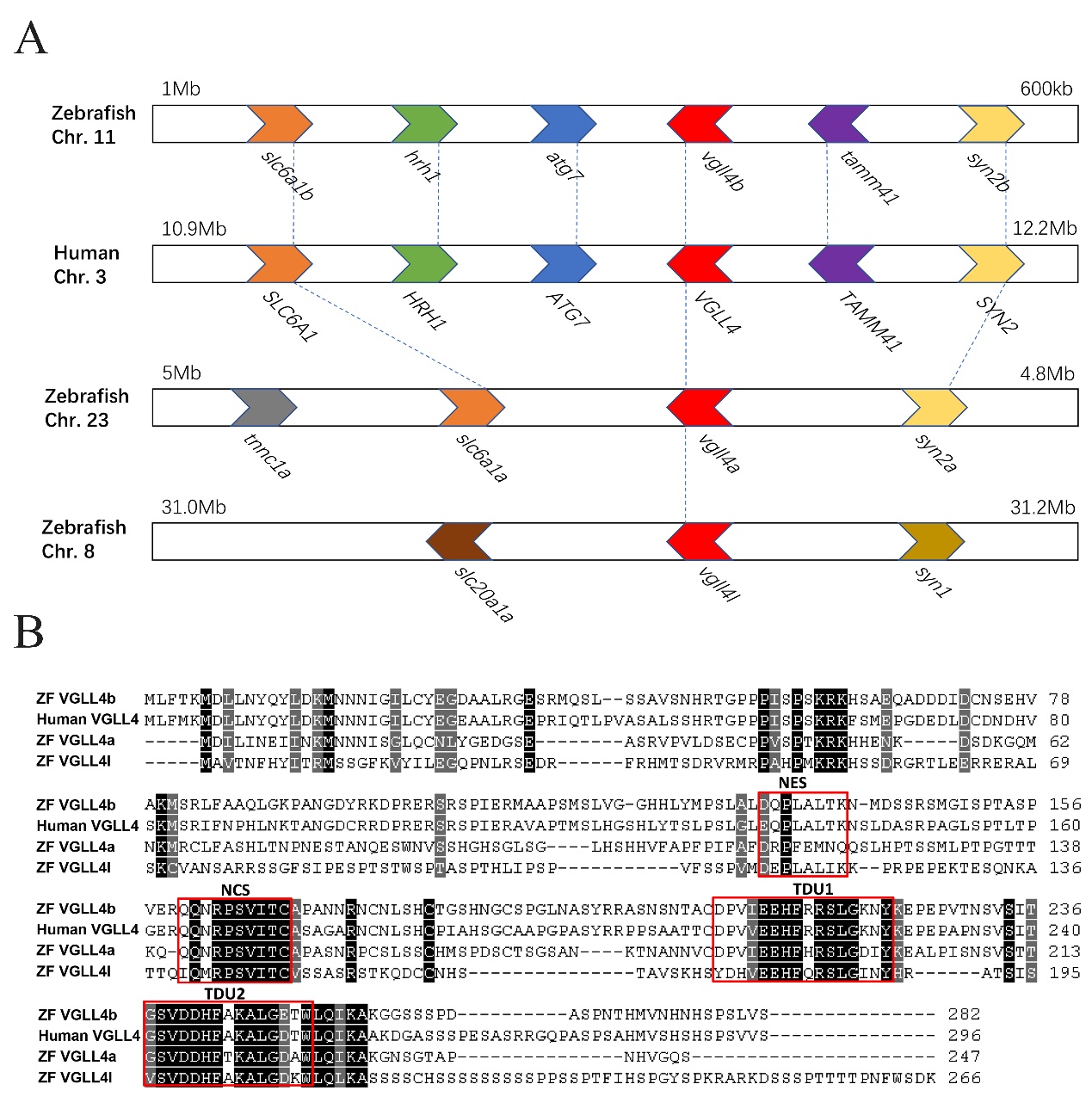
THISSE B, PFLUMIO S, FÜRTHAUER M, LOPPIN B, HEYER V, DEGRAVE A, WOEHL R, LUX A, STEFFAN T, CHARBONNIER XQ, THISSE C (2001). Expression of the zebrafish genome during embryogenesis. *ZFIN Direct Data Submiss*: https://zfin.org/ZDB-PUB-010810-1.

THISSE C, THISSE B (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* 3: 59–69.

TINGAUD-SEQUEIRA A, ANDRÉ M, FORGUE J, BARTHE C, BABIN PJ (2004). Expression patterns of three estrogen receptor genes during zebrafish (Danio rerio) development: evidence for high expression in neuromasts. *Gene Expr Patterns* 4: 561–8.

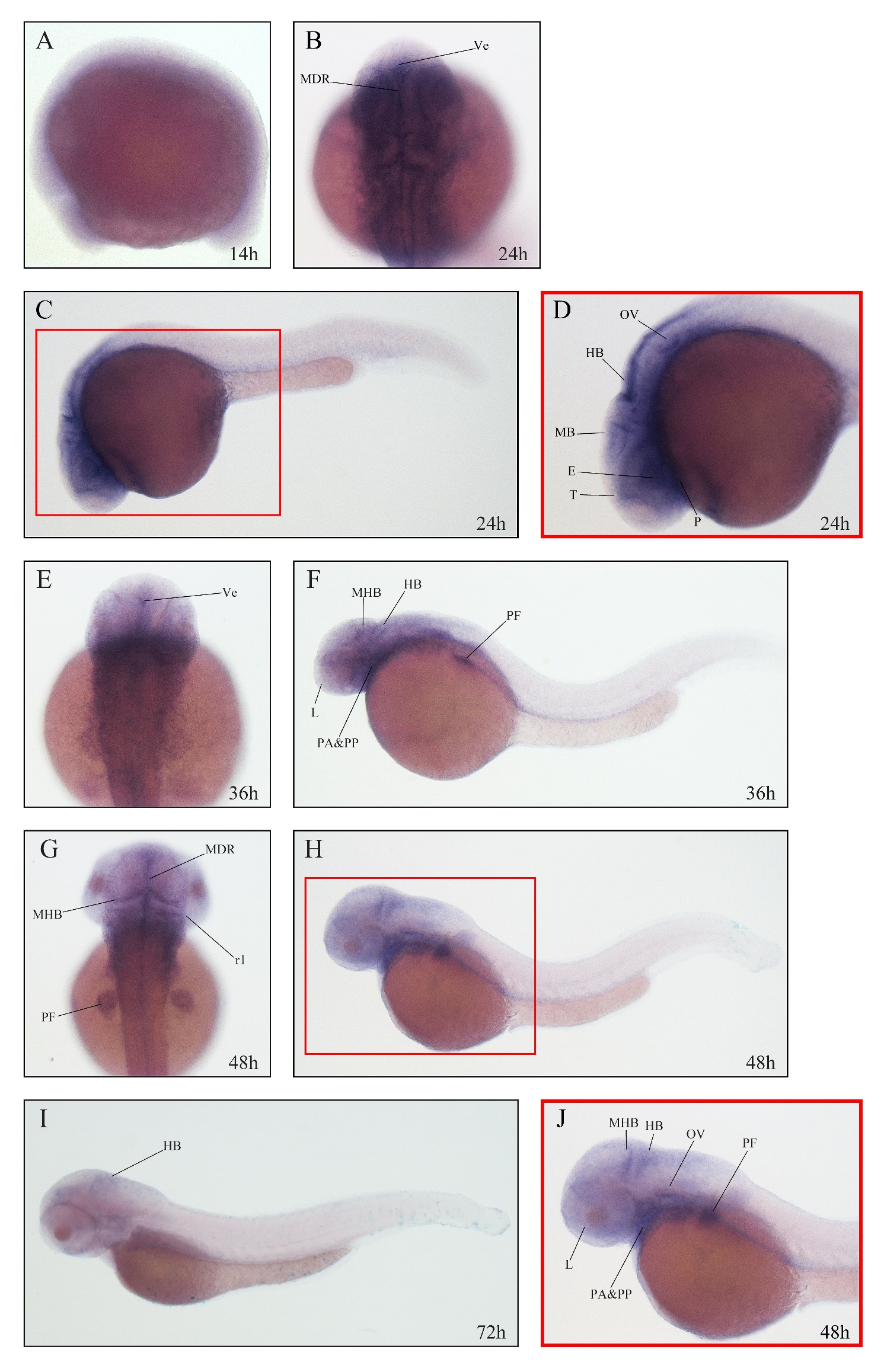
ZHANG M, ZHANG J, LIN S-C, MENG A (2012). β-Catenin 1 and β-catenin 2 play similar and distinct roles in left-right asymmetric development of zebrafish embryos. *Development* 139: 2009–2019.

ZHANG W, GAO Y, LI P, SHI Z, GUO T, LI F, HAN X, FENG Y, ZHENG C, WANG Z, LI F, CHEN H, ZHOU Z, ZHANG L, JI H (2014). VGLL4 functions as a new tumor suppressor in lung cancer by negatively regulating the YAP-TEAD transcriptional complex. *Cell Res* 24: 331–43.



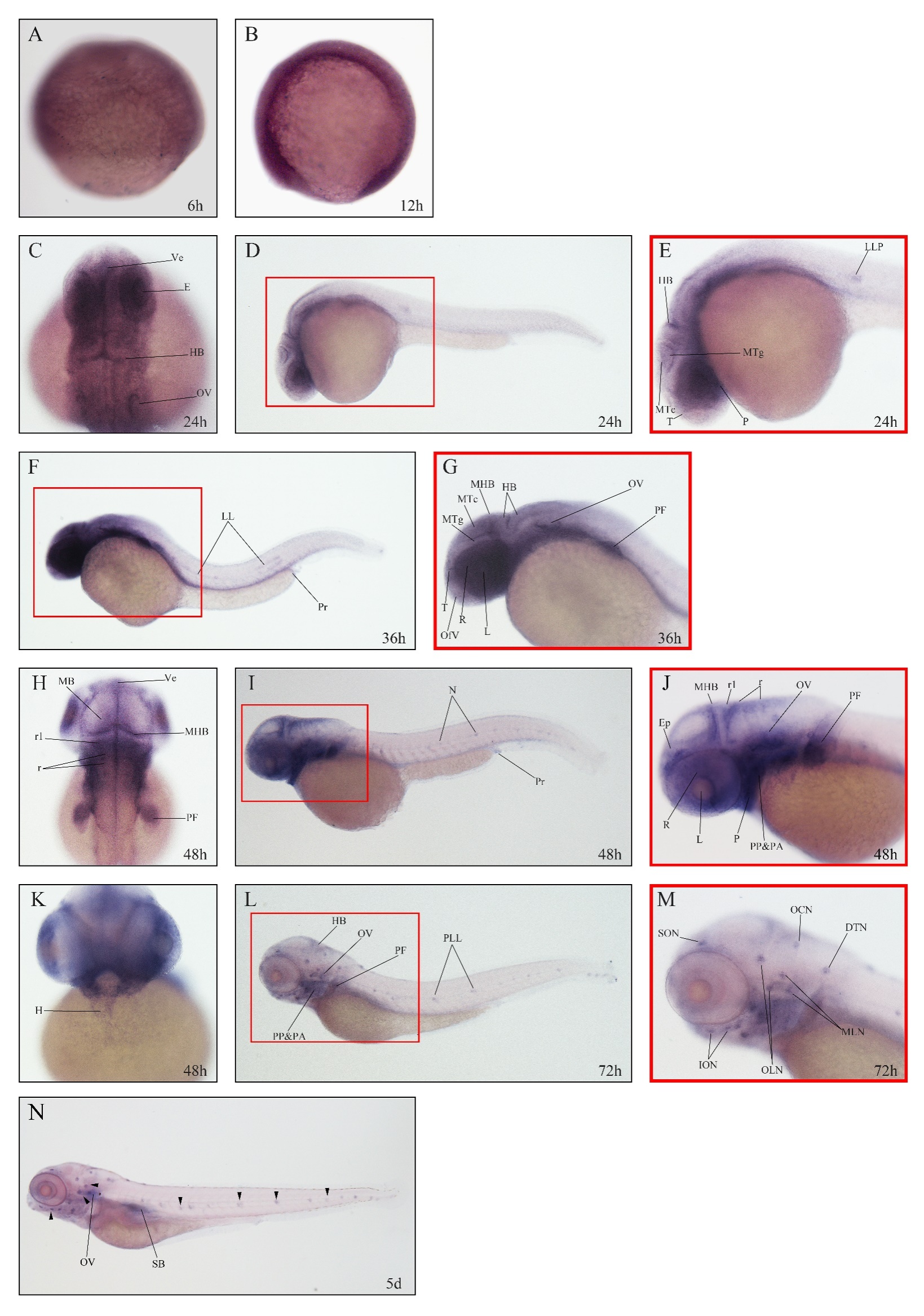
**Fig.1. Amino acid sequences and** **synteny analysis of zebrafish VGLL4a, VGLL4b and VGLL4l and human VGLL4**

**(A)** Synteny analysis of zebrafish *vgll4a*, *vgll4b*, *vgll4l* and human *VGLL4.* Location information of each gene was obtained from the Ensembl database (http://www.ensembl.org/index.html). Genes of a relationship of paralogs or orthologs shared the same color and were linked by dotted line. The directions of the arrowheads represent for the transcription direction of each gene. **(B)** The amino acid sequences of VGLL4a, VGLL4b and VGLL4l and human VGLL4*.* The protein ID are as follows: human-VGLL4 (NP\_001121691.1), ZF-VGLL4a (XP\_005161978.1), ZF-VGLL4b (NP\_998440.1), ZF-VGLL4l (NP\_001073467.1). Functional domains as NES, NCS, TDU1 and TDU2 are marked in boxed areas. Identical amino acids were shown in black background and amino acids with high similarities of these four sequences were shown in grey background.



**Fig.2. Expression of *vgll4a* in zebrafish embryos analyzed by WISH**

Embryos are shown in lateral view with anterior to the left (A, C, D, F, H, I and J) or dorsal view with anterior to the top (B, E, G). Box area in (C) and (H) was shown enlarged in (D) and (J) respectively. **(A)** Faint and ubiquitous expression of *vgll4a* at 14 hpf. **(B, C, D)** High expression in ventricle (Ve), midline of diencephalic roof (MDR), telencephalon (T), eyes (E), the midbrain (MB), hindbrain (HB), otic vesicles (OV) and pharynx (P) at 24 hpf. **(E, F)** Expression of *vgll4a* detected dispersively in ventricle (Ve), midbrain–hindbrain boundary (MHB), hindbrain (HB), lens (L), pharyngeal arches (PA), pharyngeal pouches (PP) and pectoral fin (PF) at 36 hpf. **(G, H, J)** Similar *vgll4a* expression to which at 36 hpf in the brain tissues, including MDR, MHB, HB and rhombomeres1 (r1), L, PA, PP, PF and OV at 48 hpf. **(I)** Sharply decreased expression of *vgll4a* at 72 hpf.



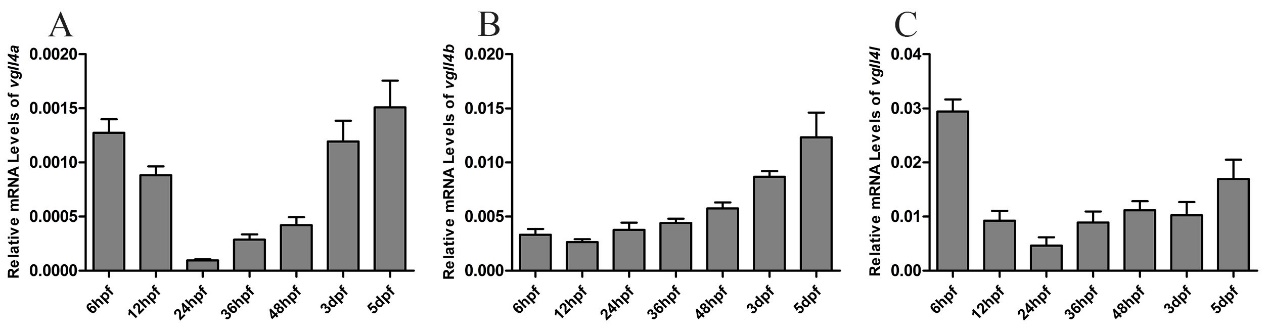
**Fig.3. Expression of *vgll4b* in zebrafish embryos analyzed by WISH**

Embryos are shown in lateral view with anterior to the left (B, D, E, F, G, I, J, L and M), dorsal view with anterior to the top (C, H) or ventral view with anterior to the top (K). Box areas in (D), (F), (I) and (L) were shown enlarged in (E), (G), (J) and (M) respectively. **(A, B)** Ubiquitous expression of *vgll4b* at 6 hpf and in the whole trunk of the embryo at 12 hpf. **(C, D, E)** Expression of *vgll4b* in ventricle (Ve), eyes (E), hindbrain (HB), otic vesicles (OV), pharynx (P), telencephalon (T), midbrain tectum (MTc), midbrain tegmentum (MTg), and lateral line primordium (LLP) at 24 hpf. **(F, G)** High expression of *vgll4b* detected in lens (L), retina(R), olfactory vesicles (OfV), OV, pectoral fin (PF), lateral line (LL), proctodeum (Pr), and the brain tissues including T, MTc, MTg, midbrain–hindbrain boundary(MHB) and HB at 36 hpf. **(H, I, J, L)** Similar expression pattern detected at 48 hpf and 72 hpf. Rhombomeres (r), rhombomeres1 (r1), epidermis (Ep), neuromasts (N), posterior lateral line (PLL). **(K)** *Vgll4b* expression in heart (H) at 48 hpf. **(M)** Specific neuromasts of anterior lateral line system were illustrated: supraorbital neuromast (SON), infraorbital neuromasts (ION), otic lateral neuromasts (OLN), middle line neuromasts (MLN), dorsal trunk neuromast (DTN), occipital neuromasts (OCN). **(N)** Neuromasts pointed with arrows at 5 dpf. Swim bladder (SB).



**Fig.4. Expression of *vgll4l* in zebrafish embryos analyzed by WISH**

Embryos are shown in lateral view with anterior to the left (D, E, G, I and J) or dorsal view with anterior to the top (C, F, H). Box areas in (D) was shown enlarged in (E). **(A)** Expression of *vgll4l* in dorsal forerunner cells (DFC) and epidermis (Ep) at 6 hpf. **(B)** Distinguishing signal detected on the epidermis at the border of the neural plate (EpN) at 12 hpf. **(C, D, E)** Deposition of *vgll4l* in areas of ventricle (Ve), hindbrain (HB), olfactory vesicles (OfV), telencephalon (T), retina (R), pharyngeal pouches (PP), otic vesicles (OV), lateral line primordium (LLP) and proctodeum (Pr) at 24 hpf. **(F, G)** *Vgll4l* concentrated in OfV, HB, pharynx (P), PP, OV, pectoral fin (PF), pronephric duct (PD), Pr, Ep and lateral line (LL) at 36 hpf. **(G, H)** Vanished *vgll4l* in eyes, Ep, brain and Pr and concentration found in PP, PF and OV at 48 hpf. **(I)** Remaining expression of *vgll4l* in PP, OV, jaw (J) and PF at 72 hpf.



**Fig.5. Quantitative gene expression analysed by RT-qPCR**.

Quantitative gene expression of *vgll4a* **(A)**, *vgll4b* **(B)** and *vgll4l* **(C)** during early embryonic development stages. The expression levels of the three *vgll4* paralogs were normalized with actin as the reference. Results were presented as relative expression levels(2-ΔCt) on the y axis versus developmental stages on the x axis. Error bars represented the standard error of means.