

Comparative Expression Pattern of Two *Vestigial-Like 2* Genes in Zebrafish

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Abstract The vestigial *Vg* gene, initially characterized in *Drosophila*, encodes a transcription co-factor which is crucial for wings development. *Vg* binds via its Sd interaction domain (SID) to the Scalloped (*Sd*) transcription factor and its vertebrate homolog *Tef1*. Previous studies identified several vertebrate genes sharing high homology with *Drosophila Vg* SID such as *Vgl* (for *Vg*-like), *TONDU* (also known as *Vgl1*) or *Vito-1* (also known as *Vgl2*). In order to investigate the role of vestigial-like 2 (*vgll2*) in zebrafish muscle development, we managed to clone and characterize two zebrafish *Vgll2* homolog genes, *Vgll2a* and *Vgll2b*. Alignment data showed high sequence homology of *Vgll2a* to vertebrates *Vgll2* sequences. *In situ* hybridization showed that the two investigated *Vgll2* genes had a similar expression pattern: they were first detected in adaxial cells (11 hpf), then expanded laterally in somites and at the end of segmentation, both genes were expressed in additional structures including head muscles and fin buds. In addition, different expression patterns of the two genes were observed. *Vgll2a* was expressed in bronchial arches precursor streams, derived gill muscles and hypothalamic precursors. *Vgll2b* was expressed in notochord at 14 hpf and regressed following notochord maturation at 18 hpf. Furthermore, the genetic regulation of *vgll2* genes was analysed using *smu* mutants and data revealed that both genes are regulated via the Hedgehog signaling pathway.

Keywords Zebrafish; *Danio rerio*, *Vgll2*, *Vgl2*, *Vestigial*, Somite, Myogenesis, Skeletal Muscles, Jaws Muscles, Branchial Arches, Gill Muscles, Notochord, Hypothalamus

1. Introduction

Invertebrates and vertebrates vestigial (*vg*) and vestigial-like (*vgl*) genes are involved in embryonic patterning and cell fate determination.

In *Drosophila*, the nuclear protein Vestigial (*Vg*) is a

cofactor of Scalloped (*Sd*) and plays a central role in the development and patterning of wings [1,2]. Several works reported that *Vg* is also involved in the specification of a subset of flight muscle. Indeed, *Vg* is expressed in myoblast that will contribute to indirect flight muscle and controls the expression of *Cut* in the direct flight muscle [3,4]. In vertebrates, the expression of *Tef1*, the homologue of *Sd*, is not restricted to a specific tissue however it controls the expression of several genes during development in skeletal muscle and placenta through binding to a M-CAT element in regulatory regions of these genes [5-8]. This has led to the hypothesis that tissue-specificity of *Tef1* control is due to interaction of *Tef1* with tissue-specific cofactor(s). Four genes have been identified in mouse which contains a sequence motif highly related to the *Sd/Tef1* Interaction Domain (SID) of *Vg*. These genes have been named *vgll1*, *vgll2*, *vgll3* and *vgll4*. *vgll1* and *vgll3* are expressed in placenta [9] while *vgll2*, also known as *VIT01*, is expressed in developing somites and muscle, in branchial arches and in limb buds [6,10,11]. The fourth gene, *vgll4* has been cloned that has a widespread expression but is the only vertebrate *vgll* gene expressed in heart muscle [12]. Functional studies have revealed that *Vgll2* and *Vgll4* interact with *Tef-1* through their SID domain and with Myocyte enhancer factor 2 (*Mef2*) [10-12]. *Vgll4* modulates *Tef1* activity *in vitro* [12] and *Vgll2* activates muscular differentiation and is translocated in the nucleus during this process *in vitro* [10,13]. Thus *Vgll2* appears to be the skeletal muscle specific cofactor of *Tef-1* in vertebrates. In addition, a recent report has described the expression of *Vgll2* in the skeletal myogenic lineages of the chicken embryo under the control of myogenic factors [14].

In order to characterise genes involved in the control of muscle development, we have cloned *vgll2* in zebrafish. Here, we report the cloning and expression of two zebrafish *vgll2* genes. Both genes share high homology with mammalian *Vgll2* and are expressed in body, head and fin bud muscles during embryonic development. In addition to this expression in muscle, *vgll2a* is also expressed in

hypothalamic precursors and *vgll2b* is transiently expressed in notochord. Finally, we show that both genes are regulated by Hh signalling.

2. Material & Methods

2.1. Zebrafish Breeding

Fish were obtained from a local pet shop and were bred following classical condition with a 14h/10h day/night period. Embryos were obtained from pair mating and developmental stages determined according to Kimmel [27].

2.2. Molecular Biology

PCR primers were deduced from sequence of zebrafish ESTs and genomic fragments sharing homology with mouse *Vgll2* identified in databases Ensembl[28] and WashU-Zebrafish Genome Resources Project [29]. mRNA were extracted from embryos of mixed developmental stages in the segmentation period using and Trizol reagent (Life Technologies). RT-PCR were performed with 1µg of total RNA using ExpandRT (Roche) followed by PCR reaction (Qbiogene). PCR fragments were cloned in pGEM-T vector (Promega). Sequences were performed using BigDye v1.1 fluorescent sequencing kit from Applied Biosystems.

Alignments were performed with the ClustalX program [30] with default settings. Alignment was decorated with Boxshade software [31]. *Vgll2a* and *vgll2b* names have been approved by Zebrafish gene name nomenclature committee.

2.3. In Situ Hybridisation

Antisense DIG probes were synthesised with appropriate RNA polymerase (Roche). In situ hybridisations were performed as previously described [24] and signal revealed with NBT/BCIP (Roche).

Gene's expression in wild-type and *smu* mutants has been quantified using ImageJ. Image was converted to greyscale after background subtraction and then expression intensity quantified in somites using the Fire LUT (Schröter et al., 2012). Statistical significance of relative expression was evaluated using a Mann-Whitney non-parametric test (Prism 6.0, Graphpad) [32].

3. Results and Discussion

3.1. Molecular Characterisation of Two *Vgll2* Genes in Zebrafish

We identified two putative *vgll2*-related sequences in Ensembl and ESTs WashU databases. We deduced primers from these sequences and used them to clone corresponding cDNAs by RT-PCR. Both cDNAs encode a protein containing a SID and sharing high homology with vertebrates *Vgll2* (Fig. 1A). The two zebrafish *Vgll2*s share a

rather weak identity (39 %). One deduced peptide appears to be much closer to vertebrate *Vgll2* and was named *Vgll2a*. *Vgll2a* shares 64 to 72 % identity with vertebrates *Vgll2*. The second gene was named *vgll2b*, the deduced peptide (*Vgll2b*) shares 39 to 43 % identity with vertebrate *Vgll2*. When comparison is restricted to the putative SID domain, conservation of zebrafish *Vgll2* is much higher with vertebrate *Vgll* (92 to 100%) and with *Drosophila* *Vg*, 67% and 71% for *Vgll2a* and *Vgll2b* respectively (Fig. 1B). *Vgll2a* and *Vgll2b* SIDs share 96% identity. Sequence homology with other *Vgll* is limited to the SID (not shown).

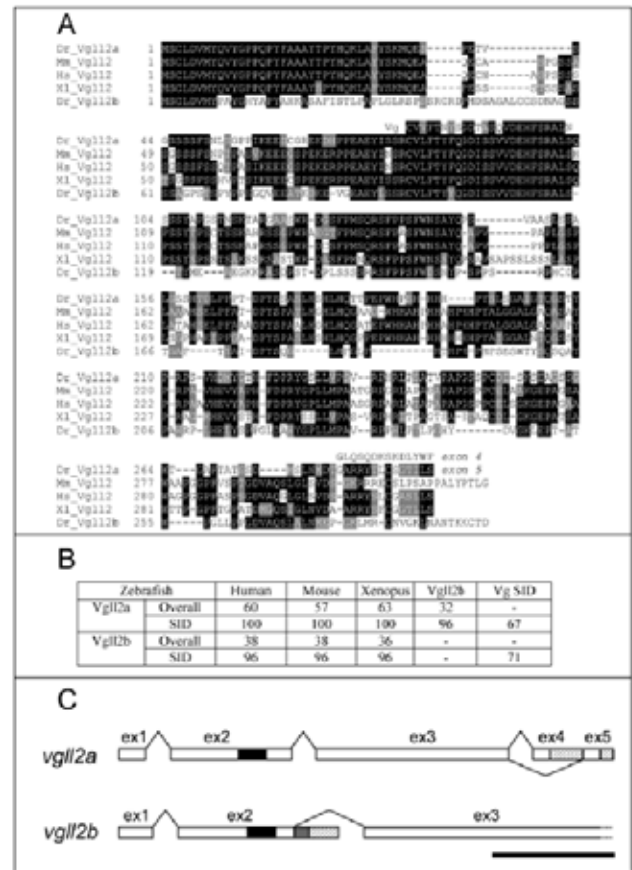


Figure 1. Characterisation of zebrafish *Vgll2* and *vgll2* genes organisation.

A) Alignment of zebrafish *Vgll2* with their vertebrates counterparts and with *Drosophila* *Vg* SID (Dr: *Danio rerio*; Mm: *Mus musculus*; Hs: *Homo sapiens*; Xl: *Xenopus laevis*). Identical residues in all sequences appear black boxed. Grey shading marks similar residues. All vertebrate *Vgll2* display almost strict conservation at the level of the SID and strong identity with *Drosophila* *Vg* SID. *Vgll2a* alternate peptide encoded by exon 4 is presented above peptide deduced from exon 5 which is closely related to human and *Xenopus* C-terminus. B) Comparison of vertebrate *Vgll2* sequences and identity at the level of SID. Percentage of identity was deduced from the alignment shown in A. Homology is even stronger in the functional SID domain including *Drosophila* *Vg* SID domain. This comparison clearly assigns *Vgll2a* as a closer relative to vertebrates *Vgll2* than *Vgll2b*. C) Genomic structure of zebrafish *vgll2* genes. SID is indicated in exon 2 of both genes as a black box. 3' untranslated regions are indicated as hatched boxes. *vgll2a* gene contains 5 exons. Exons 4 and 5 are alternatively used. In the cDNA containing exon 4, exon 5 is included in the 3' untranslated region. Exon 5 encodes a C-terminus peptide highly homologous with *Xenopus* and human *Vgll2* C-terminus (see A). *vgll2b* contains at least 3 exons. Exon 3 incompleteness is indicated as a dotted open box in 3'. The grey box after *vgll2b* exon 2 corresponds to the readthrough alternate transcript. The significance of this readthrough cDNA is not clear (see text). The black bar under gene schemes represent 100 base pair length.

From RT-PCR experiments, we obtained two cDNAs for *vgll2a* differing only by their 3' extremities. Comparison with genomic sequence information from Ensembl database reveals that they are obtained after alternate splicing of exon 4 and 5 (Fig 1C). The peptide deduced from exon 5 shows high homology with the C-terminus of *Xenopus* and human Vgll2 (Fig. 1A). When exon 4 is used, exon 5 is included in the 3'untranslated region and both cDNAs use the same polyadenylation site. *Vgll2b* gene structure is simpler with only 3 exons, however, no stop codon has been found within the open reading frame. Comparison of cDNA and genomic sequences suggests that the oligo-dT oligonucleotide used for reverse transcription has primed in a A-rich region of the cDNA. Presented sequence is thus partial. The sequence of a *vgll2b* EST suggests that an alternate transcript with a readthrough of genomic sequence located after exon 2 may exist. We searched for such an alternate splicing by 3' RACE strategy and identified such a cDNA. *In situ* hybridizations performed with probes covering *vgll2b* exon 1 and 2, *vgll2b* exon 3 and all three exons (see Fig. 1C) reveal the same expression pattern indicating that if this readthrough transcript has a physiological significance, it is not associated with a specific tissue expression (not shown). It is to note that even if gene organization of *vgll2a* and *vgll2b* is similar, a first short exon and a second exon containing the SID, splicing sites are not conserved between these genes (Fig. 1C).

3.2. Expression during Development

We have analysed spatial and temporal expression pattern of zebrafish *vgll2a* and *vgll2b* by whole mount *in situ* hybridisation during embryonic development up to 72 hpf using labelled antisense riboprobes. Both *vgll2* genes are first detectable at the 4-somite stage (~11.5 hpf) in somites, in adaxial cells lining the notochord (Fig. 2A, B). This expression pattern extends as new somites are formed (Fig. 2C, D) and is similar to that of myogenin and of all three *mef2* genes identified in zebrafish albeit *vgll2* genes expression timing is closer to *mef2D* [15,16]. Expression of *vgll2* in cardiac precursors has never been detected. This observation is similar to the one made in developing mouse embryos and contrast with *mef2A* and *mef2C* expression which are detectable in this structure by 14 hpf [15]. As somites mature, expression extends laterally in somites from 15 hpf (Fig. 2E -H). Expression in whole somite is still observed at 24 hpf and decreases by 36 hpf. By 72 hpf both *vgll2* genes are expressed in fin buds mesenchyme (Fig. 2K, L).

In somites, *vgll2* genes expression pattern is similar to the one of *myogenin* and *mef2* myogenic factors [17,18]. This is in agreement with the involvement of Vgll2 in the control of muscle differentiation program suggested in mouse [10]. This expression is also similar to the recent study in *xenopus* embryos, where Vgll2 is expressed in the skeletal muscle lineage downstream of myogenic factors [19].

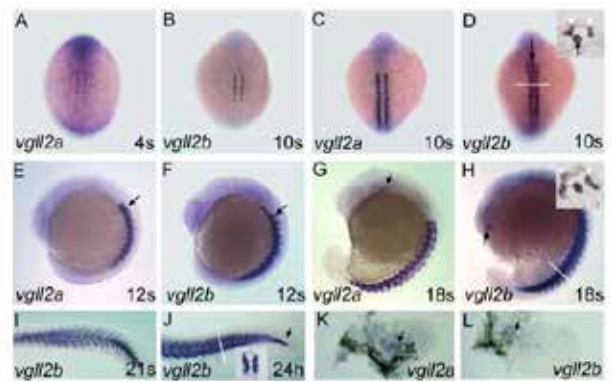


Figure 2. Early expression pattern of zebrafish *vgll2* genes.

vgll2a (A) and *vgll2b* (B) are first detectable at the 4-somite stage in adaxial cells. (C, D) *vgll2* expression extends to new somites as they are formed. At 10-somite stage *vgll2b* is expressed in the anterior aspect of notochord (arrowhead in D; and insert: section at the level of the white line). (E, F) At the 12-somite stage, *vgll2* genes expression enlarges in lateral somites from anterior to posterior somites. *Vgll2b* is now expressed in whole notochord (F). (G, H) At 18-somite stage, *vgll2* expression is detected in whole somites (arrows). From this stage, *vgll2a* is also expressed in branchial arches precursor's streams (arrow in G) and *vgll2b* is expressed in an anterior bilateral cluster (arrow in H). Insert in 2H: section performed at the level of the white line showing expression in somites (white arrowhead) and notochord. (I, J) Lateral view reveals at 21-somite stage that expression of *vgll2b* regresses in anterior notochord (I). At 24 hpf expression in notochord remains only in the tip of the tail (arrow in J). Insert in J, section at the level of yolk extension (white line in J) reveals expression in somites and the absence of expression in notochord. (K, L) Expression of *vgll2a* and *vgll2b* can be seen in mesenchyme of developing fin buds dissected out from 72 hpf embryos. The shown phenotype is representative of 95% of the embryos, n = 70

At the 10-somite stage (14 hpf) *vgll2b* begins to be expressed in anterior part of the notochord (Fig. 2D) and rapidly in the entire notochord (Fig. 2F,H). From 18hpf, expression regresses in the anterior aspect of notochord (Fig. 2I). This expression remains in the tip of the tail until 24 hpf (Fig. 2J). This dynamic expression appears to be a novel function acquired by *vgll2b* since *Vgll2* expression has never been detected in axial structure in mouse during development. We have never detected *vgll2a* expression in notochord.

From the 18-somite stage, *vgll2* genes are also expressed in several developing head structures. *Vgll2a* is expressed in branchial arches precursor's streams from 18-somite stage (Fig. 2G) and in eye muscle at later stages (Fig. 3C-F). From 24 hpf, *vgll2a* is also expressed in hypothalamus precursors (Fig. 3A). By 36 hpf, *vgll2a* expression in hypothalamus is more precisely seen in pre-optic hypothalamus and in migrating neuro-hypophysis precursors (Fig. 3C). This pattern is similar to the one of *xenopus* Vgll2 where it occurs in the branchial arches and the stomodeal-hypophyseal anlage [19]. Expression in these territories persists until 72 hpf (Fig. 3D-F). This striking expression reminds the expression of another myogenic factor, *myf5*, in mouse hypothalamus [20]. From the 18-somite stage, *vgll2b* is expressed in bilateral clusters in the head (Fig. 2H, 3G). At 48 hpf, expression is detected in several head muscle precursors (Fig. 3H). At 72 hpf, these muscles can clearly be identified as jaw muscles and eye muscles (Fig. 3I). It is to note that in head, *vgll2* genes are expressed in mutually

exclusive subsets of muscle suggesting they acquired specialized functions. A recent report has detected zebrafish *vgll2a* expression in the pharyngeal endoderm and ectoderm surrounding the neural crest derived mesenchyme of the pharyngeal arches [21].

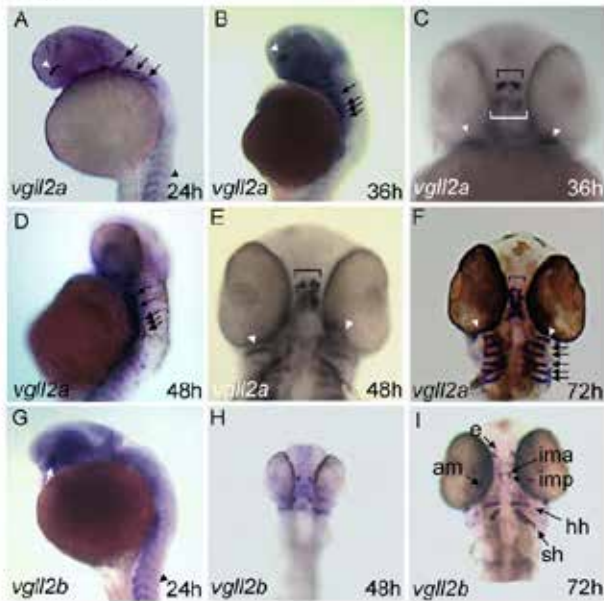


Figure 3. Expression pattern of zebrafish *vgll2* genes in the head.

(A) Lateral view of 24 hpf embryos showing *vgll2a* expression in the head. At this stage, *vgll2a* is expressed in migrating branchial arches streams (white arrows) and is still weakly expressed in somites (arrow). A new domain of expression appears in the head in hypothalamus territory (white arrowhead). (B) Expression is similar at 36 hpf. (C) Frontal view of a 36 hpf embryo showing expression in eye muscle (white arrows) and hypothalamic precursors (brackets). The anterior cluster of cells (black bracket) constitute the pre-optic component of the hypothalamus, while the posterior one (white bracket) represents the precursors of developing neuro-epiphysis. (D-F) At 48 and 72 hpf, *vgll2a* is expressed in developing gill muscles (white arrows) and eye muscles (white arrowheads) and hypothalamus (brackets). (G) At 24 hpf, *vgll2b* is expressed in head muscle precursors and is still visible in somites. (H) Frontal view of a 48 hpf embryo reveals expression of *vgll2b* in jaw and eye muscle precursors. (I) In this frontal view of a 72 hpf embryo, *vgll2b* expression can be seen in several jaw and eye muscles. am, adductor mandibulae; e, eye; hh, hyohyoideus; ih, inter hyoideus; ima, inter mandibularis anterior; imp, inter mandibularis posterior; io, inferior oblique; ir, inferior rectus; mr, medial rectus. The shown phenotype is representative of 96% of the embryos, n = 76

3.3. Genetic Control of *vgll2* Expression

To identify genetic pathways that could be involved in the control of *vgll2* genes expression. We analyzed the expression of *vgll2* genes in *acerebellar(ace)* and *slow muscle omitted (smu)* mutants. *acemutation* results in the production of an inactive Fgf8 [22] and an altered fast myogenesis [23,24]. Expression of *vgll2* genes appears unchanged in *ace* mutants indicating they are not regulated by Fgf8 (not shown). Smoothed is part of the Hedgehog (Hh) receptor complex and is mutated in *smu* mutant [25,26], this mutation produces a blockade of all Hh signalling and, among other phenotypes, in a suppression of *myoD* expression in adaxial cells and then of slow muscle development [25]. We analyzed the expression of *vgll2* genes at the 15-somite stage, when these genes are both

expressed in adaxial and somitic muscle progenitors. In both cases, expression in adaxial cells is suppressed in *smu* embryos (Fig 4). Strikingly, *vgll2b* is also suppressed in somites (Fig. 4D). The mechanism underlying this regulation of *vgll2b* in fast muscle precursors by Hh signalling remains unknown. Expression of *vgll2b* is not affected in notochord of *smu* embryos (Fig. 4D). Relative expression of *vgll2a* and *vgll2b* has been assessed in somites of *smu* mutants. Expression of *vgll2a* in *smu* embryos and wild-type were similar (relative expression 91.7 ± 26.3 %; $p=0.4$; $n=10$). Expression of *vgll2b* in somites of *smu* mutants was significantly lower compared to expression in wild-type embryos (relative expression 27.9 ± 17.1 %; $p<0.01$; $n=10$).

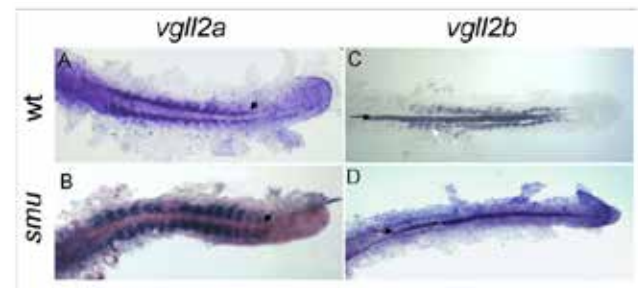


Figure 4. Expression of zebrafish *vgll2* genes in Hh signalling devoid embryos.

Flat-mount embryos at 15-somite stage. (A, B) *vgll2a* expression is suppressed in adaxial cells of *smu* embryos. (C, D) *vgll2b* is also absent in adaxial cells of *smu* embryos, but more surprisingly, it is also absent in somites. Expression in notochord is not affected by absence of Hh signalling.

4. Conclusion

In summary, we have identified two zebrafish *vgll2* orthologous. These genes are expressed in trunk and head muscles precursors during development. Expression in trunk muscle follows *myogenin* and *mef2* genes expression timing. In the head, *vgll2* genes are expressed in exclusive muscle, *vgll2a* being expressed in gill and a subset of eye muscles while *vgll2b* is expressed in jaw and other eye muscles. In addition, *vgll2* genes are expressed in non-muscle structures, *vgll2a* in ventral hypothalamus and *vgll2b* in notochord. Expression in such structures has never been described in mouse and reveals new functions acquired by these factors during evolution which deserve additional investigation. Finally, we have shown that in trunk muscle both *vgll2* genes are regulated by Hh signalling, but again differentially. *vgll2a* is controlled by Hh in adaxial cells as are *myoD* and *myogenin*, while *vgll2b* requires Hh signal to be expressed in both adaxial cells and somites.

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