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Two different *vestigial like 4* genes are differentially expressed during *Xenopus laevis* development

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ABSTRACT The *vestigial* gene (*vg*) was first characterized in *Drosophila* and several homologues were identified in vertebrates and called *vestigial like 1-4* (*vgll1-4*). Vgll proteins interact with the transcription factors TEF-1 and MEF-2 through a conserved region called TONDU (TDU). Vgll4s are characterized by two tandem TDU domains which differentiate them from other members of the vestigial family. In *Xenopus* two genes were identified as *vgll4*. Our bioinformatic analysis demonstrated that these two genes are paralogues and must be named differently. We designated them as *vgll4* and *vgll4l*. *In situ* hybridization analysis revealed that the expression of these two genes is rather different. At gastrula stage, both were expressed in the animal pole. However, at neurula stage, *vgll4* was mainly expressed in the neural plate and neural folds, while *vgll4l* prevailed in the neural folds and epidermis. From the advanced neurula stage onward, expression of both genes was strongly enhanced in neural tissues, anterior neural plate, migrating neural crest, optic and otic vesicles. Nevertheless, there were some differences: *vgll4* presented somite expression and *vgll4l* was localized at the skin and notochord. Our results demonstrate that *Xenopus* has two orthologues of the *vgll4* gene, *vgll4* and *vgll4l* with differential expression in *Xenopus* embryos and they may well have different roles during development.

KEY WORDS: *ectoderm, Vgll4, Vgll4l, TONDU*

Vestigial (*vg*) genes are expressed in invertebrates and vertebrates, and have been shown to be involved in a variety of developmental processes. In *Drosophila*, Vestigial (Vg) interacts with the transcription factor Scalloped (Sd) to regulate myogenesis and wing development (Kim *et al.*, 1996, Simmonds *et al.*, 1998). This binding specifically activates numerous target genes in the cell fate determination process (Halder *et al.*, 1998, Simmonds *et al.*, 1998). In vertebrates, several *vg* homologues have been identified. They are called *vestigial like 1-4* (*vgll*1-4) (Chen *et al.*, 2004, Faucheux *et al.*, 2010, Maeda *et al.*, 2002). In zebrafish, two orthologues of mammalian *vgll2* named as *vgll2a* and *vgll2b* (Johnson *et al.*, 2011, Mann *et al.*, 2007) were described. Recently, were also identified two *vgll4* orthologues designated as *vgll4* and *vgll4l* (Melvin *et al.*, 2013).

The *vgll* genes encode proteins that have a conserved region called TONDU (TDU) motif (Maeda *et al.*, 2002, Vaudin *et al.*, 1999). Vgll proteins were shown to physically interact with Scalloped homologues such as Transcriptional Enhancer Factor-1 (TEF-1 also known as TEAD-1) (Gunther *et al.*, 2004) and Myocyte Enhancer Factor-2 (MEF-2) (della Gaspera *et al.*, 2009, Maeda *et al.*, 2002). Unlike other members of the Vgll family that have a single interaction domain TDU, Vgll4 has two tandem TDU motifs in its carboxyl-terminal domain suggesting that Vgll4 might form a bridge between TEF-1 and MEF-2 transcription factors (Chen *et al.*, 2004, Faucheux *et al.*, 2010).

In *Xenopus* a *vgll4* gene was identified and its expression pattern was described only for late midneurula stage embryos. The expression was localized in the epidermis, olfactory placodes and neural crest cells (Faucheux *et al.*, 2010). However, a detailed and comparative analysis of its early expression pattern during *Xenopus* embryogenesis is still lacking. We identified a new *vgll4* paralogue and found it to be different from the previously described (Faucheux *et al.*, 2010). We called this paralogue as *vgll4l* based on it similarity with zebrafish *vgll4l*. The bioinformatic analysis of both *Xenopus* Vgll4s protein sequences revealed the

Abbreviations used in this paper: vg, vestigial gene; vgll, vestigial-like; vgll4l: vestigial like 4-like; TDU, tondu region; TEF, transcriptional enhancer factor.

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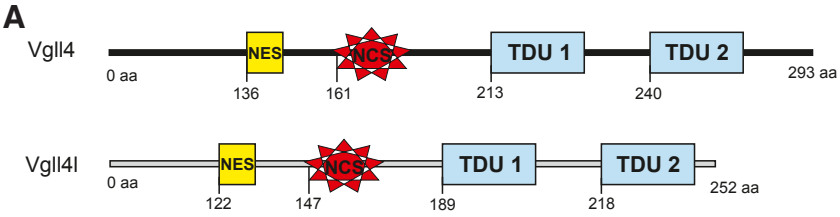
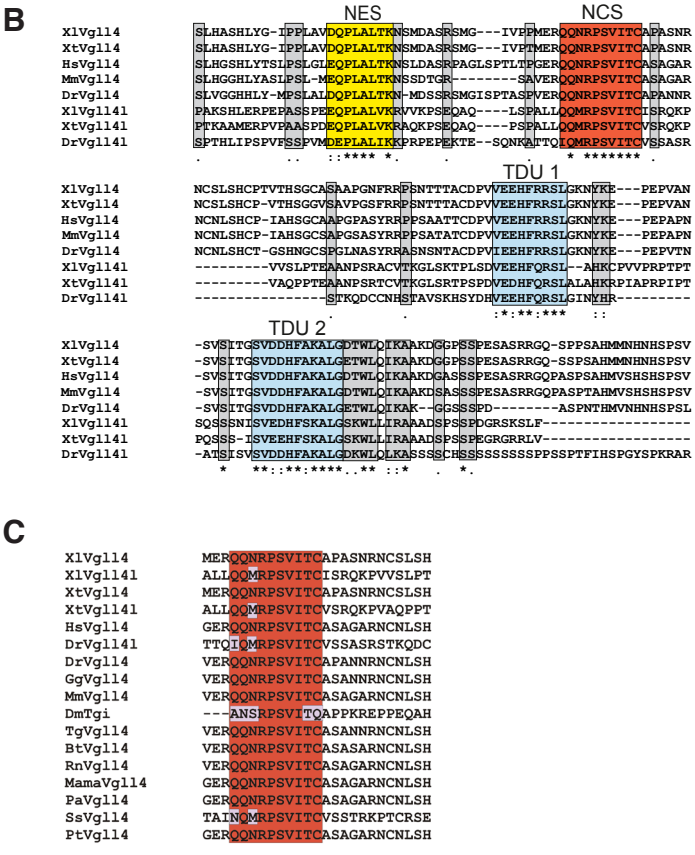
presence of the two TDU motifs (Chen *et al.*, 2004, Faucheux *et al.*, 2010), and new phylogenetic features of the Vgll family. In addition, the comparative analysis of the expression pattern of both genes in different developmental stages of *Xenopus laevis* embryos revealed undescribed and interesting distinct domains of expression for each gene.

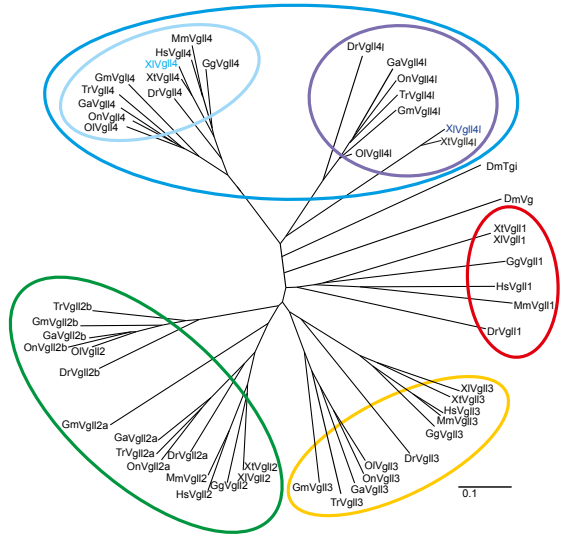
Results and Discussion

Identification and sequence analysis of vgll4 *and* vgll4l *in* Xenopus laevis

Xenopus vestigial like 4 (*vgll4*) gene was first cloned in 2002 by Klein *et al.*, (Accession Number: BC123267) and it was initially characterized as a gene of the *vestigial* family by Faucheux *et al.*, (2010). In 2006, a microarray screening was performed by Chalmers *et al.*, who identified a gene (NIBB clone Xl460o05) expressed in the internal ectoderm layer and named it also as *vestigial like 4*. Our interest in identifying novel players in epidermis development led us to search for genes expressed differentially in this tissue. We







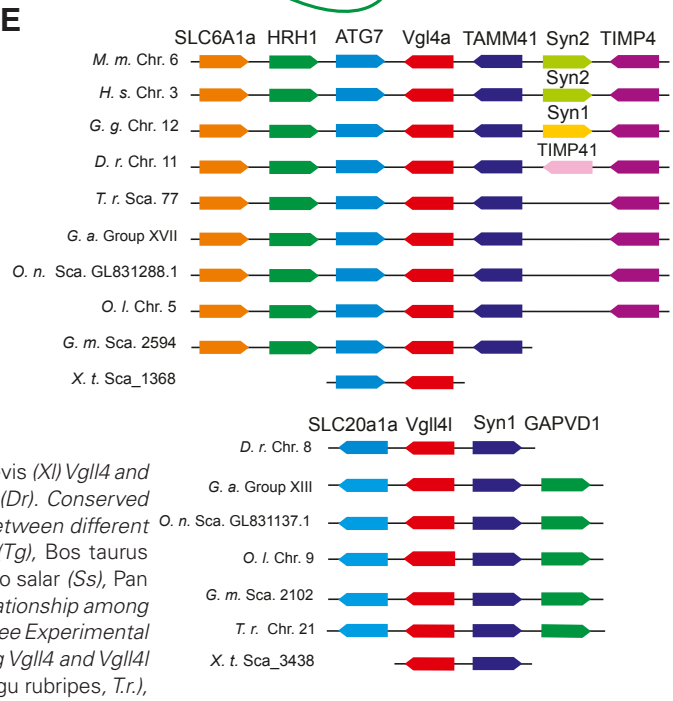


Fig. 1. Protein sequence analysis, structural comparison, family relationships and synteny analysis of Vgll4 and Vgll4l. (A) *Protein structure of Vgll4 and Vgll4l. NES, nuclear export signal; NCS, novel conserved sequence; TDU, TONDU domain.* **(B)** *Protein sequence alignment comparing* Xenopus laevis *(Xl) Vgll4 and Vgll4l with* Xenopus tropicalis *(Xt), human (Hs), mouse (Mm), and zebrafish (Dr). Conserved Amino acids are shown in grey.* **(C)** *A wide comparison of the NCS region between different species. Chicken (Gg),* Drosophila melanogaster *(Dm),* Taeniopygia guttata *(Tg),* Bos taurus *(Bt),* Rattus norvegicus *(Rn),* Macaca mulatta *(Mama),* Pongo abelii *(Pa),* Salmo salar *(Ss),* Pan troglodytes *(Pt).* **(D)** *Unrooted phylogenetic tree showing the evolutionary relationship among different Vgll proteins. The tree was based on amino acid sequence alignment (see Experimental Procedures for details).* **(E)** *Analysis of conserved syntenic regions containing Vgll4 and Vgll4l loci in* Xenopus tropicalis *(X.t.), zebrafish (*Danio rerio, *D.r.), puffer fish (*Takifugu rubripes, *T.r.), stickleback (*Gasterosteus aculeatus, *G.a.), medaka (*Oryzias latipes, *O.l.), tilapia (*Oreochromis niloticus, *O.n.), cod (*Gadus morhua, *G. m.), human (H.s.), mouse (M.m.), and chicken (G.g.) genomes. Genes are represented as boxes and arrows indicate the orientation of the transcription unit. Boxes with the same color indicate orthologue genes. The genomic regions representations are not to scale to avoid complexity.*

were interested in the *vestigial like 4* gene described by Chalmers *et al.,* 2006; NIBB clone Xl460o05). We first sequenced and analyzed this clone *in silico* (Accession number: KF963131) and found it to be different from the sequence named as *vestigial like 4* at the NCBI Nucleotide database (Klein *et al.*, 2002) (Accession number: BC123267). The Xl460o05 clone showed high identity with a sequence called *rexp52* (90% identity) (Acc. Number: DQ096895.1), an uncharacterized gene with differential expression found in a large-scale, semi-automated whole mount *in situ* hybridization screening performed in *Xenopus laevis* (Pollet *et al.*, 2005). Thereby, currently there are two sequences named as *vestigial like 4*. Notwithstanding, our bioinformatics analysis of these sequences demonstrated that they are two different genes so they should have different names. Thus, we propose in *Xenopus laevis* the name *vestigial like 4 (vgll4)* for the first cloned gene (Accession number: BC123267) and *vestigial like 4-like (vgll4l)* for the second (Accession number: KF963131).

In *Xenopus tropicalis* only a sequence corresponding to *vgll4* was found into the Ensembl genomic information. We extended our in silico analysis to multiple sequences databases and this led us to find a sequence that corresponds to *Xenopus tropicalis vgll4l* (Accession number: KJ690263, Fig. 1B). This gene was found in the Gurdon Institute *Xenopus tropicalis* EST Database (Transcript name 1012072090).

In zebrafish two sequences of 282 (Accession number: NP998440) and 266 amino acids (Accession number: NP001073467) were identified and named as *vgll4* and *vgll4l,* respectively (Melvin *et al.*, 2013). This finding could be extended to other bony fishes such as puffer fish, stickleback, tilapia and cod. According to their phylogenetic relationship (Fig. 1D) and sequence similarity (Fig. 1B) *Xenopus vgll4* is the orthologue of fish *vgll4* and *Xenopus vgll4l* is the orthologue of fish *vgll4l.*

The protein sequence analysis showed that *Xenopus laevis* Vgll4 has the same sequence that the previously described Vgll4 (Klein *et al.,* 2002, Faucheux *et al.*, 2010). The bioinformatic analysis of *Xenopus laevis* and *Xenopus tropicalis* Vgll4l protein sequence allowed us to identify the two TDU motifs that characterize Vgll4 co-factors (Fig. 1A,B), and the putative NES (nuclear export signal) motif. When we compared Vgll4 and Vgll4l with Vgll4s from other species, we found an undescribed conserved region that we identified as NCS (novel conserved sequence) (Fig. 1 A,C). This region is unique in vgll4 genes and probably can act as a feature that differentiates Vgll4s from other members of the Vgll family and could interact with other protein components. A phylogenetic tree based in amino acid sequences indicate that Vgll genes of different species can be organized into four different groups, Vgll1, 2, 3 and 4 (Fig. 1D). This phylogenetic analysis is in agreement with Faucheux *et al.,* (2010) and Koontz *et al.,* (2013), who demonstrated that Vgll1-3 are related to *Drosophila* Vg, while all Vgll4s are related to the recently identified *Drosophila* orthologue, Tgi. A divergence between Vgll4 and Vgll4l is reflected in the extent of conservation of the

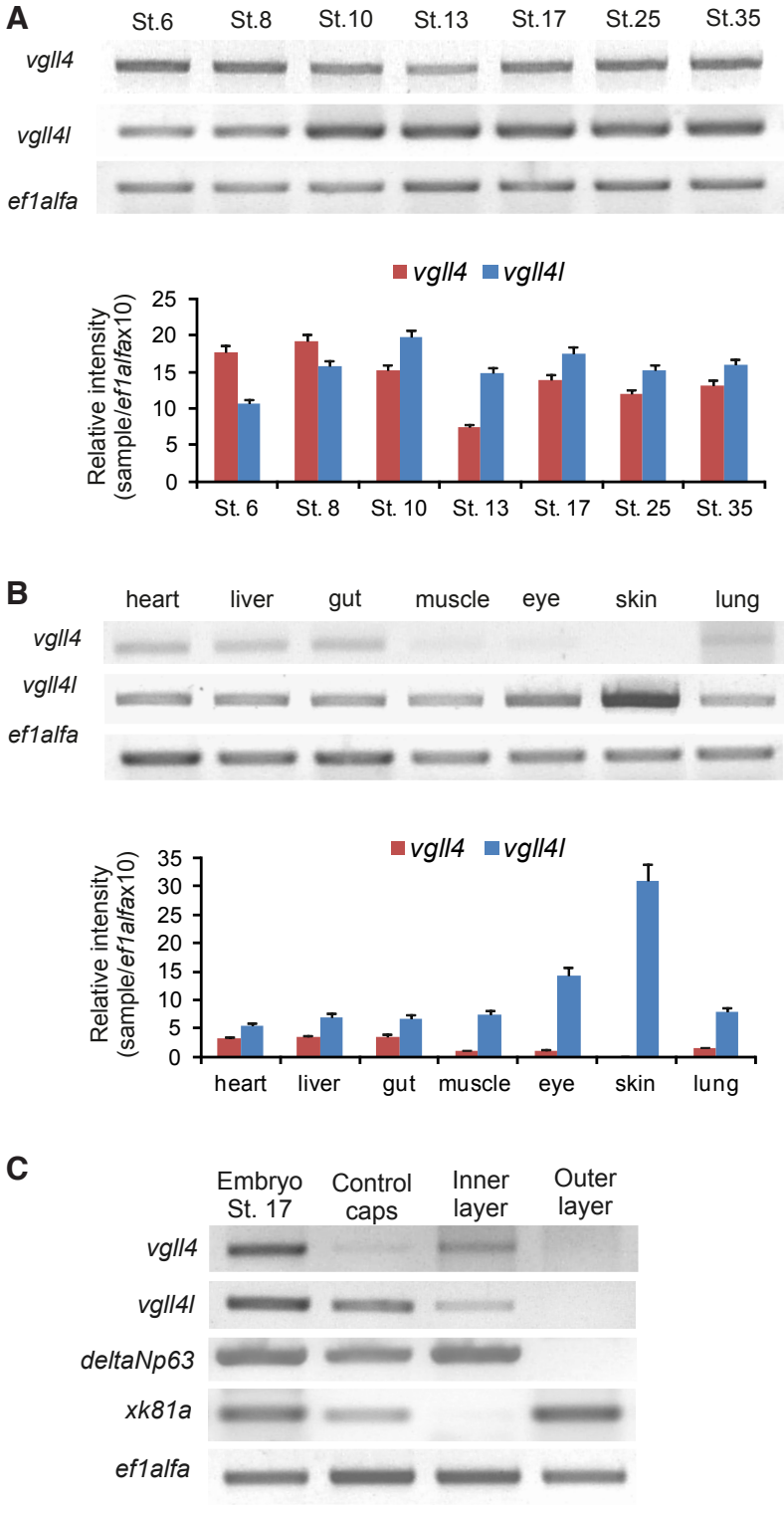


Fig. 2. RT-PCR analysis of *vgll4* gene expression in embryos and adult tissues. *RT-PCR was performed on total RNA extracted from embryos at different embryonic stages, adult tissues and internal and external layers of animal caps.* ef1alfa *was used as loading control.* **(A)** *Temporal expression of* vgll4 *and* vgll4l *throughout development.* **(B)** *Analysis of* vgll4 *and* vgll4l *expression in adult tissues.* **(A,B)** *Quantifications of gel are shown; the results are expressed as Relative Intensity (sample/*ef1alfa *X 10).* **(C)** vgll4 *and* vgll4l *expression in isolated layers of the animal cap ectoderm.*

protein sequence: Vgll4 is more similar than Vgll4l to mammalian Vgll4s (Fig. 1D) and it also shows a high similarity with *Xenopus tropicalis* Vgll4 (92%). *Xenopus laevis* Vgll4 has 73% identity to human and 70% to mouse Vgll4 proteins. By contrast, *Xenopus laevis* Vgll4l is only 31% identical to mouse and 33% to human Vgll4 and *Xenopus tropicalis* Vgll4l is 42% identical to human Vgll4. Furthermore, *Xenopus laevis* Vgll4 and Vgll4l are 40% identical while *Xenopus tropicalis* Vgll4 and Vgll4l are 30% identical. On the other hand, strong Vgll4 homology was observed in TDU domains, which are completely conserved between human, mouse, zebrafish, *Xenopus tropicalis* and *Xenopus laevis* (Fig. 1B), as well as for NES and NCS regions (Fig. 1C). This high conservation in specific regions allows us to designate Vgll4l as a paralogue of Vgll4.

We analyzed the evolution of the vestigial-like genes in vertebrate genomes by synteny analysis. The genomic databases (i.e. Ensembl) provided us with sufficient information on the chromosome regions containing the orthologues of *X. laevis vgll4* to perform such analysis. As shown in Fig. 1E, *vgll4* and *vgll4l* are remarkably conserved between *X. tropicalis*, zebrafish (*D. rerio*), puffer fish (*T. rubripes*), medaka (*O. latipes*), stickleback (*G. aculeatus*), tilapia (*O. niloticus*) and cod (*G. morhua*). In the case of *X. tropicalis vgll4l* the genomic information available is limited but shows that this gene is syntenic with other species, since its neighbor *synapsin-1* (*syn-1*) could be identified (Fig. 1E). Curiously, in chicken, mouse, and human genomes only the *vgll4* occurred, no *vgll4l* paralogue was identified. These finding suggest that the chromosomal regions containing *vgll4* and *vgll4l* genes have been conserved for at least 340-390 million years, the expected divergence time for amphibian and amniota lineages (Blair and Hedges, 2005). Our results from phylogenetic and syntenic analyses support the hypothesis that in fishes and amphibians two different *vgll4* genes are present.

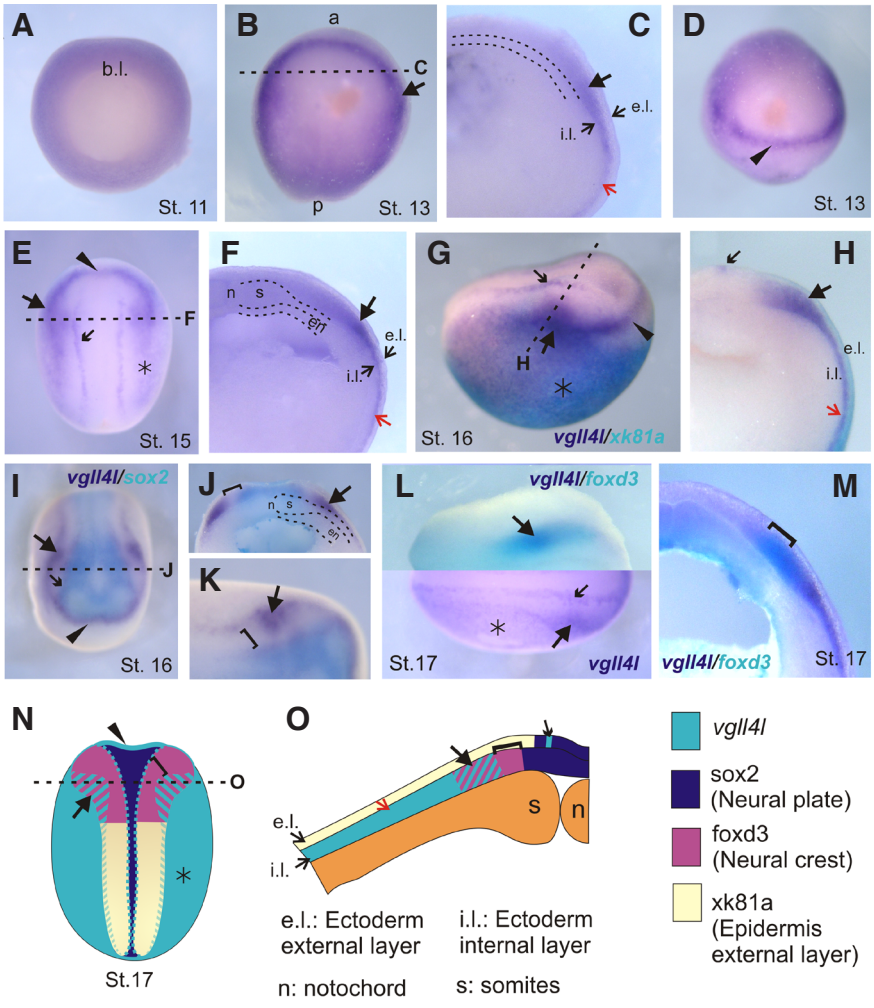


Fig. 3. Spatio-temporal expression pattern of *vgll4l* during early development by *in situ* hybridization. (A) *Vegetative view.* **(B, E)** *Dorsal view, anterior to the top.* **(D, I)** *Anterior view, dorsal to the top, anterior to the front.* **(G)** *Lateral view anterior to the right.* **(L)** *Dorsal view, anterior to the right.* **(C, F, H, J, M)** *Transversal sections.* **(K)** *Higher magnification view of the anterior region of the embryo shown in I. Black dashed lines in B, E, G, I indicate the positions of the cuts made to give transversal-sections in C, F, H, J.* **(G, H)** *Double* in situ *hybridization for* vgll4l *(purple) and* xk81a *(turquoise).* **(I-K)** *Double* in situ *hybridization for* vgll4l *(purple) and* sox2 *(turquoise).* **(L)** *(upper half) Double* in situ *hybridization for* vgll4l *(purple) and* foxd3 *(turquoise),* **(M)** *cross- sectioned embryo labeled by double* in situ *hybridization for* vgll4l *(purple) and* foxd3 *(turquoise).* **(E, G, L)** *Asterisk,* vgll4l *expression in the prospective epidermis.* **(B, C, E, F, H-L)** *Large black arrow,* vgll4l *neural folds expression.* **(C, F, H)** *Red arrow,* vgll4l *expression in the internal layer of the ectoderm.* **(E, G, H, I, L)** *Small black arrow,* vgll4l *row expression surrounding neural plate.* **(J, K, M, N)** *Brackets,* vgll4l *neural folds gap of expression.* **(N, O)** *Schematic diagrams summarizing the expression of* vgll4l *and neural plate (*sox2*), neural crest (*foxd3*) and epidermal (*xk81a*) markers.* **(N)** *Dorsal view.* **(O)** *Transversal section. References: a, anterior. b.l., blastopore lip. e.l., external layer. en, endoderm. i.l., internal layer. n, notochord. p, posterior. s, somites.*

Analysis of vgll4s *expression in* Xenopus *embryos*

First, we assessed the temporal expression profile of *vgll4s* by reverse transcriptase-polymerase reaction (RT-PCR) (Fig. 2A). Our findings agree with what has been reported for *vgll4* (Faucheux *et al.*, 2010). This gene was maternally expressed and its expression remains constant throughout development (Fig. 2A). *vgll4l* was also maternally expressed and the transcripts were continuously detectable until after the hatching stage (Fig. 2A). In addition, we analyzed *vgll4s* expression in adult tissues. *vgll4* was observed in all the tissues analyzed with exception of the skin (Fig. 2B). Previous reports showed that *vgll4* was expressed at a similar level in all the tissues analyzed (Faucheux *et al.*, 2010). The expression we found for *vgll4* is different from *vgll4l,* which was expressed at a roughly similar level in all tissues analyzed but showed a higher expression in skin (Fig. 2B). This not tissue-restricted expression of *vgll4s* is coincident with *vgll4* expression in human that was detected in the heart, kidney and brain as well as in other tissues at lower levels (Chen *et al.*, 2004).

Then, we analyzed the spatiotemporal expression of *vgll4* and *vgll4l.* Previous results showed that *vgll4l* (named as *vgll4*) expression analyzed by *in situ* hybridization begins at gastrula stage and in stage 14 is located in the inner layer of

the epidermis (Chalmers *et al.*, 2006). We performed a detailed analysis of *vgll4l* expression at different stages comparing it with different neural and epidermal marker genes. Our results showed that in early gastrula *vgll4l* was widely expressed in the animal hemisphere (Fig. 3A). *vgll4l* expression was decreased in the dorsal and posterior ectoderm during gastrulation. In neurula, stage 13 onwards, it was expressed surrounding the neural plate anteriorly and laterally (Fig. 3B, black arrow; Fig. 3D, black arrowhead). Transversal sections revealed that *vgll4l* expression is restricted to the internal layer of the ectoderm (Fig. 3C, red arrow). At neurula stage the expression is restricted to the anterior and lateral neural folds (Fig. 3E, arrowhead and black arrow) and displays an expression pattern that in the ectodermal domain overlaps with the epidermal marker *xk81a* (Fig. 3G, asterisk). Transversal sections confirmed that *vgll4l* expression is located in the inner layer of the ectoderm compared with *xk81a*, which is expressed in the external layer (Fig. 3 H,O). Besides, we performed a RT-PCR determination of *vgll4l* in explants samples dissected from the internal and external layers of the epidermis. This analysis showed a clear *vgll4l* expression in the internal but not in the external layer (Fig. 2C) that correlates with the internal layer marker *deltaNp63* expression (Tribulo *et al.*, 2012). Double *in situ* hybridization showed that *vgll4l* anterior expression corresponds to the anterior neural fold because it is complementary and does not overlap with the neural plate territory expressing *sox2* marker (Fig. 3 I,N, arrowhead). The expression of *vgll4l* in the lateral neural folds shows a gap between its expression and *sox2* (Fig. 3 J,K,N, brackets) and partially overlaps with the neural crest marker *foxd3* (Fig. 3 L,M,N,O, black arrow and brackets), demonstrating that *vgll4l* expression is located in the most ventral region of the prospective neural crest. Moreover, *vgll4l* expression extends laterally beyond *foxd3* expression into the contiguous territory that corresponds to the pre-placodal ectoderm (Fig 3 E,G,I, black arrowhead and black arrow). Two rows of *vgll4l* expression that extend surrounding the limit between neural plate and neural crest can also be seen (Fig. 3 E,G,H,L,N,O, small black arrow). Transversal sectioning demonstrated that this expression is the only one located in the external layer of the ectoderm (Fig. 3 H,O, small black arrow).

Our comparison between *vgll4l* and *vgll4* revealed significant differences between their expressions. At gastrula stage both genes are expressed in the animal pole but *vgll4* is more strongly expressed in the dorsal region of the embryo (Fig. 4A). At early neurula *vgll4* is expressed in the anterior and posterior region of the neural plate but not in the middle region (Fig. 4 B,C, black arrowhead). The comparison between *vgll4* and *sox2* demonstrate that at stage 13 there is not *vgll4* in the middle region of the neural plate (Fig. 4D). Since stage 16 *vgll4* expression is located in the neural plate (small black arrowhead) and in the neural folds (arrow) (Fig. 4 E,G,J). It also presents a weak expression in the epidermis (Fig. 4 F,H,J, asterisk). Transversal sections confirmed that *vgll4* is expressed in the neural plate, neural folds and epidermis of midneurula embryos (stage 17, Fig. 4 H,I,K) and also showed a faint expression in the mesoderm (Fig. 4I). Similarly to *vgll4l*, this gene presents expression in the internal layer of the ectoderm (Fig. 2C, 4I). The onset of *vgll4* expression was detected earlier than in Faucheux *et al.*, (2010) that have shown its expression since stage 16 onward. Our results extend the initial findings that reported expression of *vgll4* in epidermis and neural crest (Facheux *et al.*, 2010) but not in the neural plate.

At advanced neurula (Stage 19), *vgll4l* continued to be expressed at the neural crest and epidermis (Fig. 5A). At tailbud stage, *vgll4l* expression was located at the migrating cephalic neural crest (Fig. 5B), principally in the mandibular stream (Fig. 5 B,D; arrowhead) and continued to be located in the internal layer of the epidermis (Fig. 5 A-C; asterisk and black arrow). When the development progresses, the main expression of *vgll4l* is located in tailbud skin

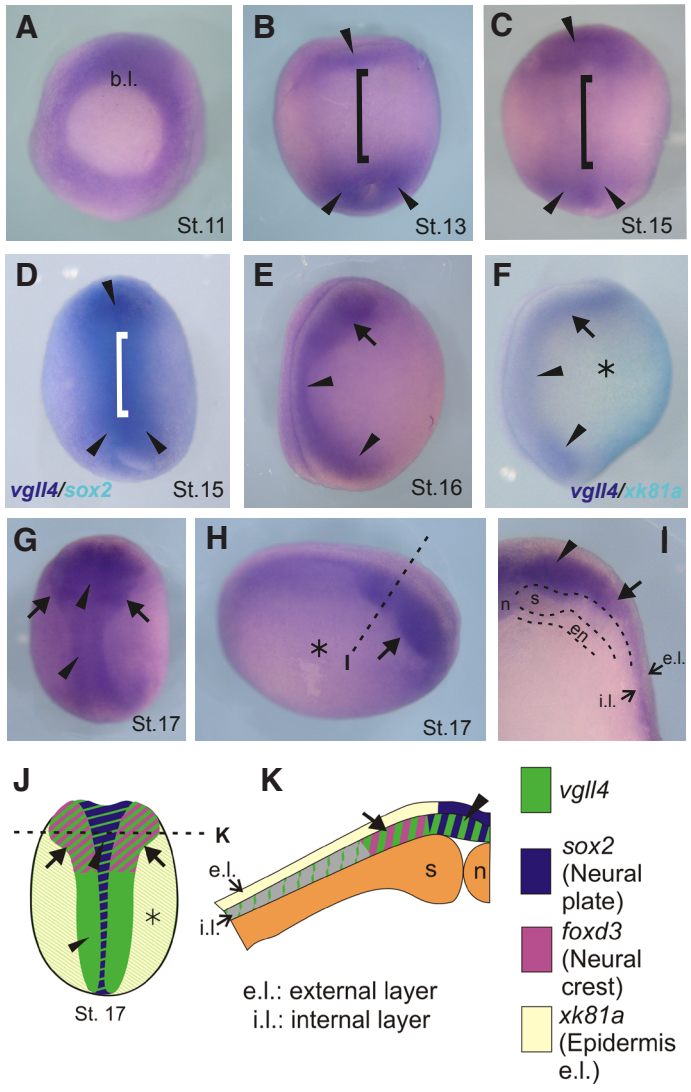


Fig. 4. Spatio-temporal expression pattern of *vgll4* during early development by *in situ* hybridization. (A) *Vegetative view.* **(B-D, G)** *Dorsal view, anterior to the top.* **(E, F)** *Lateral view, dorsal to the left, anterior to the top.* **(H)** *Lateral view, dorsal to the top, anterior to the right.* **(I)** *Transversal section. Black dashed line in H indicates the position of the cut made to give cross-section in I.* **(D)** *Double* in situ *hybridization for* vgll4 *(purple) and* sox2 *(turquoise).* **(F)** *Double* in situ *hybridization for* vgll4 *(purple) and* xk81a *(turquoise).* **(B-D)** *Arrowheads, anterior and posterior* vgll4 *expression in the neural plate. Brackets, lack of expression in the middle neural plate.* **(F, H)** *Asterisk, weak* vgll4 *expression in the prospective epidermis. Arrow, neural folds* vgll4 *expression. Arrowhead, neural plate expression.* **(J, K)** *Schematic diagrams summarizing the expression of* vgll4l *and neural plate (*sox2*), neural crest (*foxd3*) and epidermal (*xk81a*) markers.* **(J)** *Dorsal view.* **(K)** *Transversal section. References: b.l., blastopore lip. e.l., external layer. en, endoderm. i. l.: internal layer. n, notochord. s, somites.*

(Fig. 5 D,G; black arrow) and branchial arches (Fig. 5 E,G,I; black arrowheads). It is also expressed in the notochord (red arrow) and otic vesicle (Fig. 5 E,G,H). The expression of *vgll4* in these stages was consistent with previous reports (Faucheux *et al.*, 2010). At stage 19 it can be observed at the neural plate, preferentially in the anterior region and in the neural crest that is starting to migrate (Fig. 5J). At stage 24 *vgll4* is observed at migrating neural crest (red arrowheads), prospective brain (black small arrow) and somites (white arrowhead) (Fig. 5 K,L). At later stages, the expression is located in the eye primordium and branchial arches and persists at somites and brain (Fig. 5 M,N). Although *vgll4* and *vgll4l* expression at these stages have some coincidences they are not expressed in exactly the same territories. *vgll4l* remains expressed in the epidermis and later in the derived skin while *vgll4* do not present expression in this tissues. Nevertheless, both are expressed in the branchial arches and central nervous system. To the best of our knowledge, these are the first results that differentiate *vgll4* from *vgll4l* gene expression in *Xenopus*.

In zebrafish *vgll4l* is expressed in the epidermis since early development. During somitogenesis the expression is located in the epidermis and in the neural plate border. At later stages (24-48 hpf) *vgll4l* remains located at the epidermis and also is expressed in the nose, otic vesicle, pharyngeal pouches and lateral line (Thisse *et al.,* 2001). *vgll4* in chicken is expressed in the caudal notochord and in the migratory neural crest cells (Rabadan *et al.*, 2013). In mammals *vgll4* expression was assessed only in adult tissues and its expression was high in the heart, kidney and brain (Chen *et al.*, 2004). However, there are not reports about *vgll4* expression in early mammalian development.

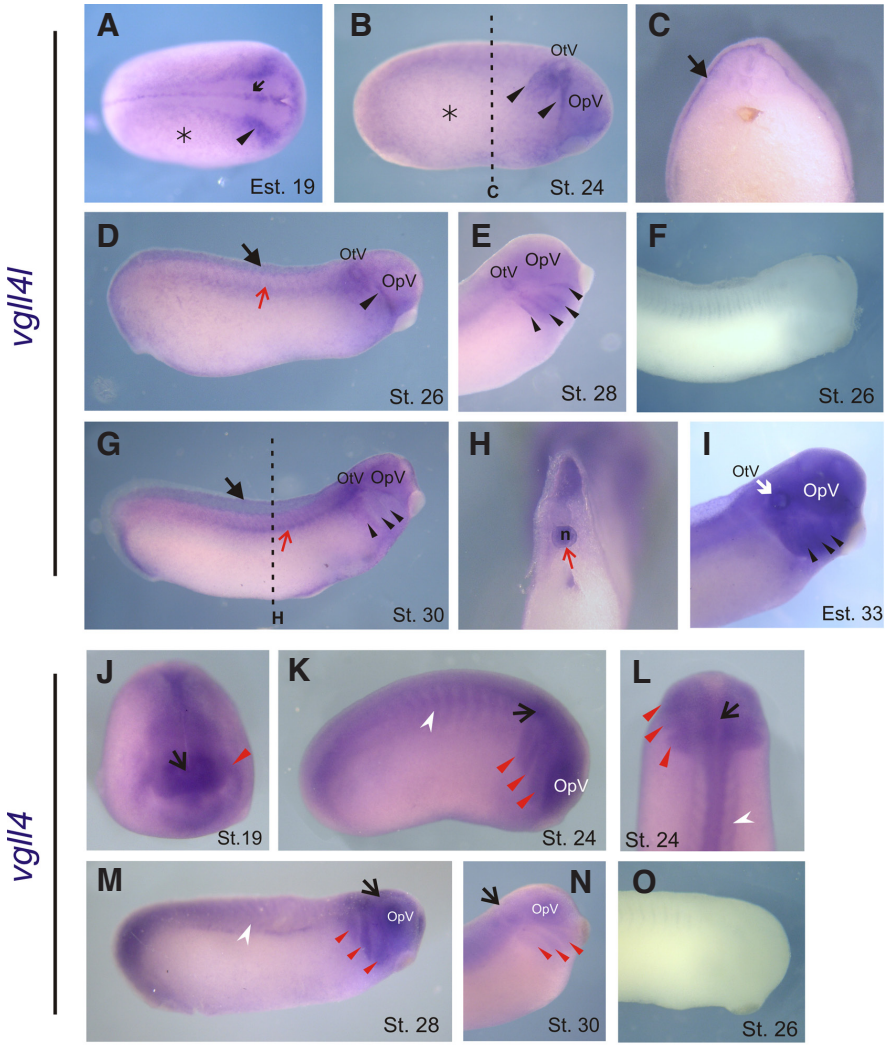


Fig. 5. Expression of *vgll4l* and *vgll4* at advanced neurula and tailbud stages. (A-I) vgll4l *expression.* **(J-O)** vgll4 *expression.* **(A)** *Dorsal view, anterior to the right.* **(B, D, F, G, K, M, O)** *lateral view, anterior to the right.* **(C, H)** *Transversal section. Black dashed lines in B and G indicate the positions of the cuts made to give cross-section in C and H.* **(E, I, N)** *Higher-magnification views of head region.* **(J)** *Anterior view, dorsal to the top.* **(L)** *Dorsal view, anterior to the top.* **(F)** *Negative control,* vgll4l *sense probe.* **(A-B)** *Asterisk,* vgll4l *epidermal expression.* **(A)** *Arrowhead:* vgll4l *neural folds expression.* **(B, D)** *Arrowhead,* vgll4l *expression in the migrating neural crest.* **E, G, I:** *Arrowhead,* vgll4l *branchial arches expression.* **(C)** *Black arrow,* vgll4l *expression in the internal layer of the ectoderm.* **(D, G)** *Black arrow,* vgll4l *skin expression.* **(D, G, H)** *Red arrow,* vgll4l *notochord expression.* **(O)** *Negative control,* vgll4 *sense probe.* **(J-N)** *Arrow,* vgll4 *expression in the prospective brain.* **(J-L)** *Red arrowhead,* vgll4 *expression in the migrating neural crest.* **(M-N)** *Red arrowheads,* vgll4 *branchial arches expression.* **(K, L, M)** *White arrowhead,* vgll4 *somites expression. n, notochord. OpV, optic vesicle. OtV, otic vesicle.*

Regulation of vgll4s *expression*

It was determined in *Xenopus* that early ectodermal cell pattern is regulated by a BMP4 signaling pathway. BMP4 is essential for epidermal specification while low or absent BMP4 activity results in neural specification (Wilson and Hemmati-Brivanlou, 1995). Due to the complex *vgll4* and *vgll4l* expression pattern in the ectoderm we analyzed whether BMP4 was necessary for *vgll4s* expression. To assess that, we decreased the levels of BMP4 expressing a dominant-negative truncated BMP4 (*CM-BMP4*) or *chordin (chd)* (Montero-Balaguer *et al.*), both antagonist of BMP4 signaling. RT-PCR analysis of explanted animal caps injected with *CM-BMP4* or *chd* mRNA revealed an up-regulation of *sox2* consistent with the neuralization effect and a decrease in the epidermal marker *xk81a*. In this context we observed an increase in *vgll4* expression and a decrease in *vgll4l* (Fig. 6A). Previous results demonstrated that *vgll4* expression was stimulated in a dose dependent manner by activin, but not by FGF or BMP4 signals (Faucheux *et al.*, 2010).

We also analyzed the effect of *deltaNp63*, a gene that is regulated in a BMP4-dependent manner and that is required during the development of early epidermis (Tribulo *et al.*, 2012). We carried out a loss of function *in vitro* experiment by microinjection of an antisense morpholino oligonucleotide *MOdeltaNp63*. RT-PCR performed on animal caps showed that the inhibition of *deltaNp63* increased the level of *vgll4* and down regulated significantly the expression of *vgll4l* (Fig. 6B). These results together with the expression analysis of *vgll4* and *vgll4l* suggest that these genes participate in the development of different ectoderm derived tissues.

vgll4 could be participating during neural development and *vgll4l* during epidermis and neural crest development.

Recent studies demonstrated that *vgll4l* is involved in the craniofacial development of zebrafish embryos. Morpholino knockdown of *vgll4l* produced a loss of neural crest derived cartilages suggesting an important role of this gene during zebrafish neural crest specification and survival (Melvin *et al.*, 2013). According to *vgll4l* expression pattern and preliminary functional analysis in *Xenopus* there is a strong possibility that this gene also participates in neural crest development. However, further studies are needed to fully understand the role of this gene.

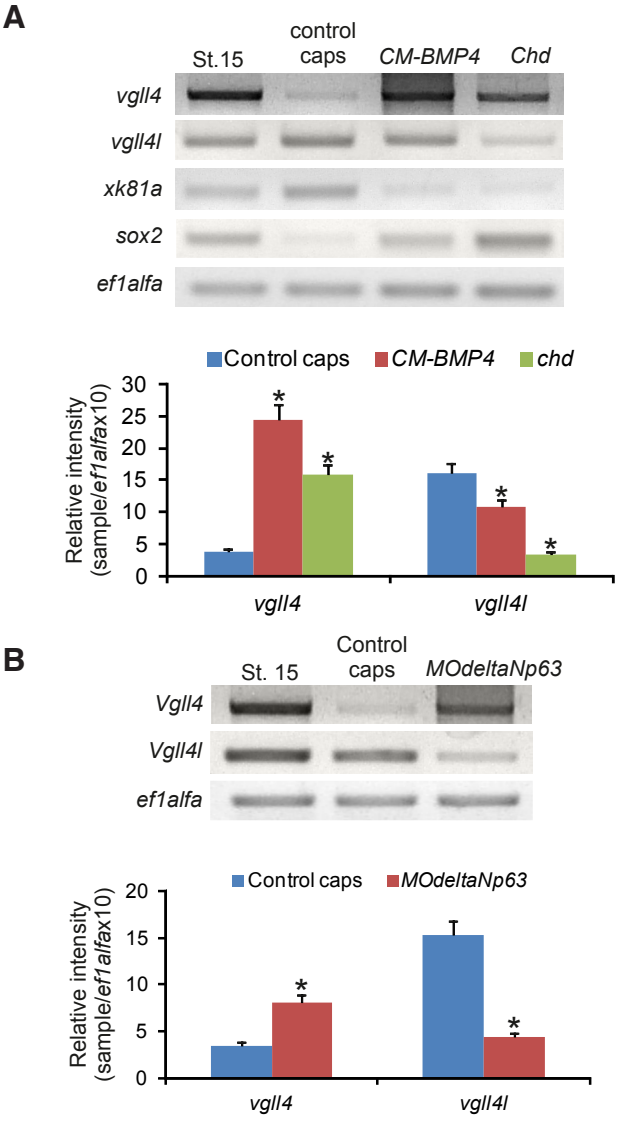


Fig. 6. Regulation of *vgll4* and *vgll4l* expression in the ectoderm. *One-cell stage embryos were injected with* CM-BMP4 *or* chd *mRNA* **(A)** *or* MOdeltaNp63 **(B)**. *Stage 15- control embryos were not injected. At stage 9, animal caps were dissected. Total RNA was isolated from stage 15 embryos and treated and control caps and the expression of* vgll4 *and* vgll4l *was analyzed by RT-PCR.* ef1alfa *was used as loading control. Quantification of gels is shown; the results are expressed as Relative Intensity (sample/*ef1alfa*X10). Differences were considered statistically significant at P < 0.001(*).*

It was demonstrated in mammals that *vgll4* participates in cardiac myocytes differentiation (Chen *et al.*, 2004). Besides, biochemical studies demonstrated that *vgll4* is able to bind to inhibitor of apoptosis proteins (IAPs) playing a role in the apoptotic pathway as an apoptotic promoter (Jin *et al.*, 2011). Nevertheless, in human embryonic stem cells (hESCs) *vgll4* was identified as a positive regulator of survival. It was shown that the overexpression of *vgll4* in hESCs decreases death and enhances colony formation (Tajonar *et al.*, 2013).

Recently, an orthologue of the mammalian Vgll4 that was called Tgi/SdBP and has the two TDU domains was identified in *Drosophila*. In this model Tgi/SdBP suppresses tissue growth participating in the Hippo pathway by interfering with targets transcription (Guo *et al.*, 2013, Koontz *et al.*, 2013).

Considering the complex functions observed for *vgll4s* genes in other model organisms, it could be relevant to explore the differential functions that *Xenopus vgll4* and *vgll4l* could have during development and organogenesis.

Materials and Methods

Phylogenetic analysis

Sequences were extracted from NCBI and aligned with the Clustal W program. Phylogenetic analyses were performed with the following sequences: *Xenopus laevis*: XlVgll1 NP_001182314, XlVgll2 NP_001080827, XlVgll3 BP689609, XlVgll4 AAI23268 (BC123267), XlVgll4l (KF963131); *Xenopus tropicalis*: XtVgll1 XP_002932640, XtVgll2 NP_989178, XtVgll3 NP_001072251, XtVgll4 NP_001072615, XtVgll4l KJ690263; *Homo sapiens*: HsVgll1 AAH03362; HsVgll2 NP_872586, HsVgll3 EAW68870, HsVgll4 NP_001121691; *Mus musculus*: MmVgll1 EDL42164.1, MmVgll2 EDL05072.1, MmVgll3 NP_082848.1, MmVgll4 EDK99520; *Danio rerio*: DrVgll1 XM_681743, DrVgll2a NM_001025486, DrVgll2b NP_001028267, DrVgll3 XP_002663398, DrVgll4 NP_998440, DrVgll4l NP_001073467; *Gallus gallus*: GgVgll1 XP_001234166.1, GgVgll2 ACN54257, GgVgll3 XP_416671, GgVgll4 NP_001025764; *Oryzias latipes*: OlVgll2 ENSORLP00000015665, OlVgll3 ENSORLG00000015881, OlVgll4 ENSORLT00000005638, OlVgll4l ENSORLT00000016519; *Oreochronis niloticus*: OnVgll2a ENSONIG00000015743, OnVgll2b ENSONIP00000004388, OnVgll3 ENSONIG00000004268, OnVgll4 ENSONIP00000018031, OnVgll4l ENSONIP00000013686; *Takifugu rubripes*: TrVgll2a ENSTRUG00000008567, TrVgll2b ENSTRUP00000030470, Trvgll3 ENSTRUG00000004178, TrVgll4 ENSTRUP00000006707, TrVgll4l ENSTRUP00000019716; *Gasterosteus aculeatus*: GaVgll2a ENSGACP00000007733, GaVgll2b ENSGACG00000009615, GaVgll3 ENSGACG00000006678, GaVgll4 ENSGACT00000009758, GaVgll4l ENSGACT00000015849; *Gadus morhua*: GmVgll2a ENSGMOP00000012209, GmVgll2b ENSGMOP00000020185, GmVgll3 ENSGMOP00000000452, GmVgll4 ENSGMOT00000011483, GmVgll4l ENSGMOT00000006944; *Drosophila melanogaster*: DmVg AAF58444, DmTgi CG10741 (SdPB NP_648658); *Taeniopygia guttata*: TgVgll4 XP_002187401; *Bos taurus*: BtVgll4 DAA16844; *Rattus norvegicus*: RnVgll4 EDM02166; *Macaca mulatta*: MamaVgll4 NP_001181777; *Pongo abelii*: PaVgll4 NP_001125047, *Salmo salar*: SsVgll4 NP_001134829; *Pan troglodytes*: PtVgll4 JAA19895.

The phylogenetic tree was drawn using the Phylodendron application (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). Syntenic analysis was performed using Blast tools in Ensembl genome databases. *X. laevis vgll4* and *vgll4l* were used as queries.

Embryo collection

Xenopus laevis embryos were obtained by stimulating adult male and female specimens with 400 IU and 800 IU of chorionic gonadotropin (HCG, Elea Lab., Argentina), respectively. Fertilized eggs were obtained after natural single-pair mating and were staged according to the Nieuwkoop

and Faber developmental table (1967).

RNA isolation and RT-PCR expression analysis

Total RNA was isolated from whole embryos, adult tissues and animal caps using Trizol reagent (Invitrogen) according to manufacturer's instructions. cDNAs were synthesized by M-MLV reverse transcriptase (Promega, USA) with oligo dT_{15} priming from 3 μg total RNA extracted from embryos at different stages. PCRs were performed with Taq Pegasus (PB-L, Argentina) and *ef1alfa* was used as a loading control. The primers used were:

vgll4 5´-CAACAACATCGCTATTCTCTGC-3´ and
5´-TTTTCGGAGGTCTCTTTAGGAG-3´
vgll4l 5´-TAGAGCAATAGTATGGCCGTCT-3´ and
5´-AGGAAGAGAGACCACTGGCTTT-3´;
deltaNp63, 5'-ATGTTGTATCTGGAAAACAATGCTCAG-3' and
5'- GACAACGCTTCACAACCTCTG-3';
xk81a, 5'-CACCAGAACACAGAGTAC-3' and
5'-CAACCTTCCATCAACCA-3';
sox2, 5-GAGGATGGACACTTATGCCCAC-3' and
5'-GGACATGCTGTAGGTAGGCGA-3'
ef1alfa 5´-CAGATTGGTGCTGGATATGC-3´ and
5´-CTGCCTTGATGACTCCTAG-3´.

PCR amplification, DNA contamination controls, and quantification of gels were performed as previously described (Tribulo *et al.,* 2012). RT-PCR was performed twice with a pool of 20 embryos each one. Quantitation of PCR bands was performed using ImageJ software (NIH, USA) on 8-bit greyscale JPG files. Measures were made six different times and an average was taken to performer the graphics. Values were normalized to the *ef1alfa* levels from the same sample and expressed for comparison as relative intensities (sample/ef1alfaX10).

In situ *hybridization*

vgll4 cDNA was obtained from Open Biosistems, clone MXL1736-99822090, BC123267=Vestigial like 4 and *vgll4l* was obtained from NIBB, clone Xl460o05. Antisense probes containing digoxigenin-11-UTP or fluorescein-12-UTP were prepared, hybridized and stained for *vgll4* (Faucheaux *et al.,* 2010), *vgll4l* (Chalmers *et al*., 2006), *sox2*, *xk81a*, *foxd3* by *in vitro* transcription. Specimens were prepared, hybridized and stained as described (Tribulo *et al*., 2012). For transversal sections embryos were cut using eyebrow knives or a scalpel.

Microinjection and animal cap assay

Xenopus laevis deltaNp63 morpholino antisense oligonucleotide (*deltaNp63MO*) was synthesized as described (Tribulo *et al*., 2012). *CM-BMP4* and *Chordin* (*chd*) were donated by Dr. K. W. Cho (Hawley *et al*., 1995) and cDNAs were linearized and transcribed as indicated in Tribulo *et al*., 2004. Embryos were microinjected with deltaNp63MO, CM-BMP4 or chd mRNA and animal caps were dissected out from them using eyebrow knives as described (Aguero et al., 2012, Tribulo et al., 2004).

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