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 (vgll1-4) (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 domain 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...
presence of the two TDU motifs (Chen et al., 2004, Fauchex 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 Fauchex et al., (2010). In 2006, a microarray screening was performed by Chalmers et al., who identified a gene (NIBB clone X4600005) 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 (Xt) 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 (Ma), 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 synteny regions containing Vgll4 and Vgll4l loci in Xenopus tropicalis (X.t.), zebrafish (D.r.), puffer fish (Takifugu rubripes, T.r.), stickleback (Gasterosteus aculeatus, G.a.), medaka (Oryzias latipes, O.I.), 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 X1460005. 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 X1460005 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 Vgll4 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

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### 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: Vgl4 is more similar than Vgl4l to mammalian Vgl4s (Fig. 1D) and it also shows a high similarity with Xenopus tropicalis Vgl4 (92%). Xenopus laevis Vgl4 has 73% identity to human and 70% to mouse Vgl4 proteins. By contrast, Xenopus laevis Vgl4l is only 31% identical to mouse and 33% to human Vgl4 and Xenopus tropicalis Vgl4l is 42% identical to human Vgl4. Furthermore, Xenopus laevis Vgl4 and Vgl4l are 40% identical while Xenopus tropicalis Vgl4 and Vgl4l are 30% identical. On the other hand, strong Vgl4 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 Vgl4l as a paralogue of Vgl4.

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 vgl4l to perform such analysis. As shown in Fig. 1E, vgl4l and vgl4l 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 vgl4l, 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 vgl4l occurred, no vgl4l paralogue was identified. These findings suggest that the chromosomal regions containing vgl4l and vgl4l genes have been conserved for at least 340-350 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 vgl4l genes are present.

**Analysis of vgl4ls expression in Xenopus embryos**

First, we assessed the temporal expression profile of vgl4ls by reverse transcriptase-polymerase reaction (RT-PCR) (Fig. 2A). Our findings agree with what has been reported for vgl4l (Fauchex et al., 2010). This gene was maternally expressed and its expression remains constant throughout development (Fig. 2A). vgl4l was also maternally expressed and the transcripts were continuously detectable until after the hatching stage (Fig. 2A). In addition, we analyzed vgl4ls expression in adult tissues. vgl4l was observed in all the tissues analyzed with exception of the skin (Fig. 2B). Previous reports showed that vgl4l was expressed at a similar level in all the tissues analyzed (Fauchex et al., 2010). This expression we found for vgl4l is different from vgl4l, 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 vgl4ls is coincident with vgl4l 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).
the epidermis (Chalmers et al., 2006). We performed a detailed analysis of \textit{vgll4} expression at different stages comparing it with different neural and epidermal marker genes. Our results showed that in early gastrula \textit{vgll4} was widely expressed in the animal hemisphere (Fig. 3A). \textit{vgll4} 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 \textit{vgll4} 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 \textit{xk81a} (Fig. 3G, asterisk). Transversal sections confirmed that \textit{vgll4} expression is located in the inner layer of the ectoderm compared with \textit{xk81a}, which is expressed in the external layer (Fig. 3H, O). Besides, we performed a RT-PCR determination of \textit{vgll4} in explants samples dissected from the internal and external layers of the epidermis. This analysis showed a clear \textit{vgll4} expression in the internal but not in the external layer (Fig. 2C) that correlates with the internal layer marker \textit{deltaNp63} expression (Tribulo et al., 2012). Double \textit{in situ} hybridization showed that \textit{vgll4} anterior expression corresponds to the anterior neural fold because it is complementary and does not overlap with the neural plate territory expressing \textit{sox2} marker (Fig. 3I, N, arrowhead). The expression of \textit{vgll4} in the lateral neural folds shows a gap between its expression and \textit{sox2} (Fig. 3J, K, N, brackets) and partially overlaps with the neural crest marker \textit{foxd3} (Fig. 3L, M, N, O, black arrow and brackets), demonstrating that \textit{vgll4} expression is located in the most ventral region of the prospective neural crest. Moreover, \textit{vgll4} expression extends laterally beyond \textit{foxd3} expression into the contiguous territory that corresponds to the pre-placodal ectoderm (Fig 3E, G, I, black arrowhead and black arrow). Two rows of \textit{vgll4} expression that extend surrounding the limit between neural plate and neural crest can also be seen (Fig. 3E, 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. 3H, O, small black arrow).

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

At advanced neurula (Stage 19), \textit{vgll4} continued to be expressed at the neural crest and epidermis (Fig. 5A). At tailbud stage, \textit{vgll4} expression was located at the migrating cephalic neural crest (Fig. 5B), principally in the mandibular stream (Fig. 5B, D; arrowhead) and continued to be located in the internal layer of the epidermis (Fig. 5A-C; asterisk and black arrow). When the development progresses, the main expression of \textit{vgll4} is located in tailbud skin

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**Fig. 4. Spatio-temporal expression pattern of \textit{vgll4} during early development by \textit{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 \textit{in situ} hybridization for \textit{vgll4} (purple) and \textit{sox2} (turquoise). (F) Double \textit{in situ} hybridization for \textit{vgll4} (purple) and \textit{xk81a} (turquoise). (B-D) Arrowheads, anterior and posterior \textit{vgll4} expression in the neural plate. Brackets, lack of expression in the middle neural plate. (F, H) Asterisk, weak \textit{vgll4} expression in the prospective epidermis. Arrow, neural folds \textit{vgll4} expression. Arrowhead, neural plate expression. (J, K) Schematic diagrams summarizing the expression of \textit{vgll4} and neural plate markers. (J) Dorsal view. (K) Transversal section. References: b.l., blastopore lip; e.l., external layer; e.n., endoderm; i.l.: internal layer; n, notochord; s, somites.
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. 5K, L). At later stages, the expression is located in the eye primordium and branchial arches and persists at somites and brain (Fig. 5M, 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 vgll4 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.

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 chdRNA revealed an up-regulation of sox2 consistent with the neuralization effect and a decrease in the epidermal marker kk81a. 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 vgll4 is involved in the craniofacial development of zebrafish embryos. Morpholino knock-down of vgll4 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.

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 (Tajoner 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

Phylogenetic analyses were performed with the following sequences: *Xenopus laevis*: XrVgll1 NP_001182314, XrVgll2 NP_001080827, XrVgll3 BP689609, XrVgll4 AA123268 (BC132367), XrVgll4l (KF963131); *Xenopus tropicalis*: XrVgll1 XP_002932640, XrVgll2 NP_989178, XrVgll3 NP_001072251, XrVgll4 NP_001072615, XrVgll4l KJ690263; *Homo sapiens*: HsVgll1 AA03362, HsVgll2 NP_872586, HsVgll3 EAW88870, HsVgll4 NP_001121691; *Mus musculus*: MmVgll1 EDL42164.1, MmVgll2 EDL5072.1, MmVgll3 NP_082848.1, MmVgll4 EDK99520; *Danio rerio*: DrVgll1 XM_681743, DrVgll2a XM_001025486, DrVgll2b NP_001028267, DrVgll3 XP_002683398, DrVgll4 NP_998440, DrVgll4l NP_001073467; *Salmo salar*: SaVgll1 EDL05072.1, SaVgll2 EDL05072.1, SaVgll3 NP_082848.1, SaVgll4 EDK99520; *Taeniopygia guttata*: TgVgll1 XP_002187401, TgVgll2a ENSOIG0000015743, TgVgll2b ENSOIG0000015743, TgVgll3 ENSOIG0000015743, TgVgll4 ENSOIG0000015743; *Oreochromis niloticus*: OnVgll2a ENSONIG0000015743, OnVgll2b ENSONIG0000015743, OnVgll3 ENSONIG0000015743, OnVgll4 ENSONIG0000015743; *Acipenser baerii*: AcBvgl2 NP_001181777; *Acipenser ruthenus*: AcBrvgl2a ENSORLT0000001568, AcBrvgl2b ENSORLT0000001568, AcBrvgl3 ENSORLT0000001568, AcBrvgl4 ENSORLT0000001568; *Acipenser schrenckii*: AcAsvgl2a ENSORLT0000001568, AcAsvgl2b ENSORLT0000001568, AcAsvgl3 ENSORLT0000001568, AcAsvgl4 ENSORLT0000001568.

### 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

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**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 9 embryos and treated and control caps and the expression of vgll4 and vgll4l was analyzed by RT-PCR. ef1alpha was used as loading control. Quantification of gels is shown; the results are expressed as Relative Intensity (sample/ef1alpha)10. Differences were considered statistically significant at P < 0.001(**).*
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

The primers used were:

- vglil 5'-CAACACATCGTATTCTCTGTC-3' and 5'-TTCTTGAGGTCTTTTCAAGAG-3'
- vglil 5'-TAAGAAATAGATGGCCGCTCT-3' and 5'-AGAGGAAGACACCTGCTGT-3'
- deltaNp63 5'-AGGATGAGAAGGCTGAGGAG-3'
- xk81a 5'-CACCGAGACACAGATTAC-3'
- sox2 5'-GACAGTGACCTGACAAGC-3'
- ef1alfa 5'-CAGATGGTGCTGATGTCG-3'
- ef1alfa 5'-CTGCCCTGTAGACTCTCAG-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 perform 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

vglil4 CDNA was obtained from Open Biosistems, clone MXL1736-99822090, BC123267. Vestigial like 4 and vglil4 was obtained from NIBB, clone X460065. Anti-sense probes containing digoxigenin-11-UTP or fluorescein-12-UTP were prepared, hybridized and stained for vglil4 (Faucheux et al., 2010), vglil4 (Chalmers et al., 2006), sox2, xk81a, fox3 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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