RESEARCH ARTICLE

Vestigial-like 3 is a novel Ets1 interacting partner and regulates trigeminal nerve formation and cranial neural crest migration

Emilie Simon, Nadine Thézé, Sandrine Fédou, Pierre Thiébaud* and Corinne Faucheux*‡

ABSTRACT

Drosophila Vestigial is the founding member of a protein family containing a highly conserved domain, called Tondu, which mediates their interaction with members of the TEAD family of transcription factors (Scalloped in Drosophila). In Drosophila, the Vestigial/Scalloped complex controls wing development by regulating the expression of target genes through binding to MCAT sequences. In vertebrates, there are four Vestigial-like genes, the functions of which are still not well understood. Here, we describe the regulation and function of vestigial-like 3 (vgll3) during Xenopus early development. A combination of signals, including FGF8, WntB8, Hoxa2, Hoxb2 and retinoic acid, limits vgll3 expression to hindbrain rhombomere 2. We show that vgll3 regulates trigeminal placode and nerve formation and is required for normal neural crest development by affecting their migration and adhesion properties. At the molecular level, vgll3 is a potent activator of pax3, zic1, Wnt and FGF, which are important for brain patterning and neural crest cell formation. Vgll3 interacts in the embryo with Tead proteins but unexpectedly with Ets1, with which it is able to stimulate a MCAT driven luciferase reporter gene. Our findings highlight a critical function for vgll3 in vertebrate early development.

KEY WORDS: Vestigial-like, Ets1, Xenopus, Cranial neural crest, Trigeminal nerve, Wnt-FGF

INTRODUCTION

The vestigial-like (VGLL) family of proteins takes its name from the Drosophila Vestigial (Vg), which is required for wing formation (Halder et al., 1998; Kim et al., 1996). Vestigial forms a co-transcriptional activator complex with the protein Scalloped (Sd), a member of the TEAD family of transcription factors, which activates genes involved in wing morphogenesis (Guss et al., 2001). Several Vestigial-like genes have been identified in vertebrates; all encode proteins with a Tondu domain that mediates interaction with TEADs (Bonnet et al., 2010; Chen et al., 2004; Faucheux et al., 2010; Maeda et al., 2002; Mielcarek et al., 2002, 2009; Simon et al., 2016).

Although the Vestigial function in Drosophila is well known, the roles played by vertebrate orthologs have not been fully explored to date. Mammalian VGLL2 is an essential cofactor of TEAD, able to stimulate muscle differentiation, and in zebrafish embryo it is involved in the development of the neural crest (NC) cell-derived craniofacial skeleton (Gunther et al., 2004; Johnson et al., 2011; Maeda et al., 2002). Mammalian VGLL4 acts, like its Drosophila homolog Tgi, as a repressor of the Hippo pathway (Chen et al., 2004; Guo et al., 2013; Koontz et al., 2013).

Vgll3 has received less attention, although the gene is the best conserved in the family in terms of structure and expression in the brain and nervous system (Simon et al., 2016). One peculiarity of vertebrate Vgll3 is the presence of a histidine repeat (six or more residues), a relatively uncommon feature with unknown function that is found in only 86 human proteins (Salichs et al., 2009). Several antagonist functions have been speculated for VGLL3 in human deduced from clinical observations. VGLL3 displays both roles in the tumor suppression pathway (Cody et al., 2009; Gambaro et al., 2013) or has oncogenic properties (Antonescu et al., 2011; Hallor et al., 2009; Helias-Rodzewicz et al., 2010). Very recently, VGLL3 has been identified as a regulator of a gene network that promotes female-biased autoimmunity (Liang et al., 2017).

We have described the expression pattern of the vgll family during Xenopus development, and shown that vgll3 expression is tightly regulated in the embryo and restricted to rhombomere 2 (r2) of the hindbrain (Faucheux et al., 2010). We examine here the function of vgll3 during early development, and show that both gain and loss of vgll3 expression impairs trigeminal placode and nerve development and cranial neural crest (CNC) cell migration. We show that vgll3 can activate pax3 and zic1 expression not only in whole embryo but also in animal cap explants. In addition, vgll3 is able to activate Wnt and FGF signals, providing a model in which vgll3 acts via signaling molecules expressed in the hindbrain. Vgll3 can interact with tead1 and tead2 in the embryo, but this interaction is not sufficient to explain its properties suggesting other potential interacting proteins. We identified ets1 as a new partner of vgll3 that can account for pax3 sustained expression in the embryo. Our results define vgll3 as an essential regulator of trigeminal nerve formation and CNC cell migration.

RESULTS

Restricted spatial expression of vgll3 depends on multiple factors

To determine accurately the onset of vgll3 expression after midblastula transition we performed reverse transcription polymerase chain reaction (RT-PCR) analysis on two-cell stage to stage 20 embryos with narrowing towards close stages between stages 10.5 and 15. Vgll3 mRNA is detected in stage 12 embryos (Fig. 1A). Using whole-mount in situ hybridization (ISH), we detected vgll3 in a single stripe across the neural plate in stage 12.5 (Fig. 1B). Between stage 13 and 17, the vgll3 expression domain follows the neural tube closure as the space between the stripes on each side of the dorsal midline narrows. Vgll3 staining decreases laterally but increases along the anterior-posterior axis. Therefore, vgll3 is one of


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the earliest markers of the hindbrain and, to our knowledge, the only one for which expression is restricted to r2. Such a peculiarity makes it a good model for studying its regulation and function in relation to hindbrain patterning.

Hindbrain patterning depends on an intricate complex regulation network involving signaling pathways, such as those of fibroblast growth factor (FGF) and retinoic acid (RA), which establish a Hox code along the anterior-posterior axis (Alexander et al., 2009). Levels of RA vary along the anterior-posterior axis of the hindbrain, and Xenopus embryos treated with increasing concentrations of retinoic acid (RA). FGF8 mRNA-injected embryos show an anterior-lateral enlargement of vgl13 expression domain. Hoxa2 or hoxb2 mRNA-injected embryos show a strong reduction of vgl13 expression. All views are dorsal-anterior. Asterisks indicate the injected side. Quantification of vgl13 regulation results is shown in the right panel. Three independent experiments were performed. The number of embryos analyzed is indicated on the top of each bar. (D) Vgl13 is induced in animal caps treated with noggin+FGF2 (N+F). (E) Vgl13 expression is induced in early, but not late, animal cap cells overexpressing wnt8a. (F) Overexpression of vgl13 in combination with tead1 in animal cap cells stimulates the expression of wnt5a, wnt8b and fgf8. E, noninjected embryo (number indicates the stage); ni, animal cap from un.injected embryo; N-tub, N-tubulin; –RT, no cDNA. Ornithine decarboxylase (odc) gene expression was used as a control.
with 1996; Schilling et al., 2001). When embryos were injected either
(Baltzinger et al., 2005; Moens and Prince, 2002; Nonchev et al.,
the vertebrate hindbrain are r1/r2 and r2/r3 borders, respectively

from r1 and r3 (Lumsden et al., 1991; Sechrist et al., 1993). In the
will populate pharyngeal arch 1 in coordination with CNC cells
originating from r2
causes defects in their derivatives

All rhombomeres produce CNC cells and those originating from r2
will populate pharyngeal arch 1 in coordination with CNC cells
from r1 and r3 (Lumsden et al., 1991; Sechrist et al., 1993). In the
genetic regulatory network, pax3 and zic1 have been shown to be
esential for specification, differentiation and migration of CNC
cells in Xenopus (Bae et al., 2014; Betancur et al., 2010; Milet et al.,
Stage 19 embryos depleted for vgll3 showed a decrease in
pax3 and zic1 (Fig. 3A). In those embryos, the lateral streams of
CNC cells have either disappeared or have fused (black arrows,
Fig. 3A). This is in agreement with the partial colocalization of vgll3
with pax3 and zic1 expression (Fig. 3B,E). Vgll3 depletion affected
pax3-profundal placode formation, as previously shown

Together, these data suggest that vgll3 expression in hindbrain is
positively regulated by FGF and Wnt signals and negatively by hox
genesis and RA signal. Vgll3 can stimulate secreted molecule
members of the canonical and noncanonical Wnt and FGF
pathways.

Vgll3 regulates trigeminal placode and nerve formation
Trigeminal ganglion that will give rise to trigeminal nerve has a dual
embryonic origin being derived from both NC and epidermal
placode (Hamburger, 1961; Steventon et al., 2014). Therefore, we
investigated whether neurogenesis was altered in vgll3-depleted
embryos using markers of early trigeminal placode and the
postmitotic neuronal marker N-tubulin. In stage 14 embryos, vgll3
does not colocalize with expression of the trigeminal placode genes islet1, neuroD, pax3 and foxi1c (Jeong et al., 2014),
while in stage 20 embryos, their expression domains become closer
(Fig. S1). We next used a morpholino (MO) antisense (v3MO) that
blocks vgll3 mRNA translation (Fig. S2). An additional morpholino
was designed to inhibit vgll3-L and vgll3-S splicing
(v3MOsplicing), the efficiency of which was controlled by RT
PCR (Fig. S3). In morphant embryos injected with v3MO or
v3MOsplicing, islet1, neuroD and N-tubulin expression was
partially or totally inhibited in prospective trigeminal and
profundal placentes (arrowhead, Fig. 2A). This effect is dose
dependent (data not shown) and, in stage 28 embryos, the
ophthalmic branch of the trigeminal nerve is shortened (50%,
 n=20, arrowhead, Fig. 2A). This effect is specific since the vgll3-L
splicing morphants can be rescued with the injection of vgll3
mRNA (Fig. 2C). Of note, a stronger effect was observed when both
v3MO splicing were co-injected (Fig. S4). The function of vgll3 on
the trigeminal formation was confirmed at later stages (Fig. S5) and
by using a second translational MO (Fig. S6).

Stage 19 embryos overexpressing vgll3 mRNA showed a dose
dependent decrease of islet1 and N-tubulin expression at the level
of trigeminal placentes (arrowhead, Fig. 2B). The effects observed
did not result from apoptosis as controlled by TUNEL labeling
(Fig. S5). Together, these results indicate that trigeminal placode
and nerve formation requires a strictly controlled vgll3 expression
level.

Knockdown of vgll3 does not affect CNC formation but
causes defects in their derivatives
All rhombomeres produce CNC cells and those originating from r2
will populate pharyngeal arch 1 in coordination with CNC cells
from r1 and r3 (Lumsden et al., 1991; Sechrist et al., 1993). In the
Vgll3 regulates CNC migration
To investigate the implication of vgll3 in CNC migration, we
performed transplantation experiments with green fluorescent
protein (GFP) as a lineage tracer (Borchers et al., 2000). CNC
from v3MO- or vgll3-mRNA injected embryos showed an
inhibition of cell migration (Fig. 5A). To further analyze the role of
vgll3 in cell migratory behavior, CNC explants were cultured on
fibronectin-coated plates (Alfandari et al., 2003). At 3 h after
plating, cells started to spread on their substrate (Fig. 5Ba,d,g,j).
After 18 h, CNC explants from vgll3-depleted embryos displayed a
reduced spreading compared to cMO CNC (Fig. 5B, e versus b). In

contrast, explants from vgl13 mRNA-injected embryos showed an enhanced spreading (Fig. 5B, k versus h). At higher magnification (Fig. 5Bc,f,i,l), only CNC cells from vgl13-depleted embryos seemed to show a spreading failure; instead, cells have tendency to dissociate from each other and remain round (Fig. 5B, f versus c, arrowheads). No apoptotic process was detected at this stage in morphant embryos (Fig. S5). Quantification analysis indicates that explants from vgl13-depleted embryos spread 1.8 less than cMO explants, while vgl13 mRNA injected explants spread 2.6 more than control gfp explants (Fig. 5C). Embryos depleted for vgl13 showed a reduction of myosinX expression, known to be critical for cell-cell adhesion (Nie et al., 2009) at premigratory (stage 16) and migratory stages (stage 28), respectively (Fig. 5D). These findings suggest that vgl13 is required for proper CNC cell migration through alteration in their spreading and adhesion properties.

Vgl13 regulates a specific subset of genes and interacts with tead in the embryo
We turned to the animal cap assay to gain further insight into the regulatory interplay between vgl13, pax3 and zic1 (Fig. 6A). Animal caps from embryos injected with pax3GR and zic1GR mRNAs in combination or not with v3MO expressed the CNC markers foxD3

Fig. 2. Vgl13 knockdown or overexpression impairs trigeminal placode and nerve formation. (A) Embryos injected with v3MO or v3MOsplicing (v3MOsp and v3MOsp, 20 ng each) exhibit reduced expression of islet1, neuroD and N-tubulin in the trigeminal placodes (arrowheads). (B) Overexpression of increasing amounts of vgl13 mRNA reduces islet1 and N-tubulin expression in stage 19 embryos. Double ISH shows no prominent overlapping staining between vgl13 (red) and N-tubulin (blue) (arrow). The injected side (indicated with asterisks) was traced by lacZ staining. Gene expression was assayed by ISH. Arrowheads indicate the trigeminal placodes. (C) Quantification of results. Images at the top of bars indicate v3MOsp defects rescued with increasing amounts of vgl13 mRNA injections. Three independent experiments were performed. The number of embryos analysed is indicated on the top of each bar. Views are dorsal-anterior excepted for lateral views for stage 28 embryos.
and snail2 (lanes 4-5). This indicates that the activation of foxD3/snail2 downstream of pax3/zic1 is independent of vgll3. However, pcdh18, N-cadherin (N-cad) and myosinX expression is significantly reduced in the presence of v3MO (compare lane 4 to lane 5 in Fig. 6A). In all experiments, no significant effect was observed in cMO injections (lane 6). We may conclude that although vgll3 is not essential for CNC induction, it is required for the full expression of genes involved in adhesion and migration of CNC downstream of pax3/zic1.

We next tested the effect of vgll3 overexpression on gene targets in combination with tead (Naye et al., 2007). None of the genes tested is activated by vgll3, tead1 or tead2 alone, except for myosinX that is induced by tead2 (data not shown and Fig. 6B, lanes 2 and 5). However, pax3, zic1, snail2, myosinX and N-cadherin are robustly expressed when vgll3 is co-expressed with tead1 (lanes 2 and 3). The co-expression of vgll2 with tead1 gave the same results (lane 4). Surprisingly, co-expression of vgll3 and tead2 did not stimulate any of the genes analyzed while vgll2 and tead2 did, albeit at different levels (lanes 6 and 7). Together, these results indicate that vgll3/tead1 can stimulate the expression of members of the gene regulatory network that orchestrate CNC formation and development.

Tead1 has been previously shown to expand pax3-expressing CNC progenitors in Xenopus embryos and Tead2 has been found to...
Fig. 4. Vgll3 knockdown and overexpression do not affect CNC formation but block their migration. Embryos were injected with v3MO (40 ng or cMO) or vgll3 mRNA (1 ng, or lacZ mRNA) and analysed at different stages for snail2, twist, PCNS or pcdh18 expression. Pharyngeal arches are indicated (a, anterior; br, branchial; hy, hyoid; ma, mandibular; p, posterior). Arrowheads indicate the mandibular branch of the trigeminal nerve. Vgll3 knockdown and overexpression block migration of CNC streams. Arrows indicate overlapping expression of vgll3 and snail2. White lines indicate the plane of agarose section. Asterisks indicate the injected side. Dashed lines indicate the midline of embryos. The oval indicates the lateral CNC stream. All views are dorsal-anterior except lateral views for stage 25 and 28 embryos. Quantification of results is shown in the lower panels. Three independent experiments were performed. The number of embryos analysed is indicated at the top of each bar.
be an endogenous activator of Pax3 in mouse NC cells (Gee et al., 2011; Milewski et al., 2004). Therefore, we asked whether vgll3-dependent stimulation of pax3 required tead1 or tead2. Embryos injected with vgll3 mRNA and depleted for tead1, tead2 or both showed an extended pax3 expression domain similar to embryos overexpressing vgll3 alone or injected with cMO (100%, n = 50, Fig. 7A). We next demonstrated by immunoprecipitation that vgll3 could interact efficiently with tead1/tead2 (Fig. 7B). The above finding led us to hypothesize that even in the absence of tead1 and tead2, vgll3 is still able to activate pax3 expression through a tead-independent mechanism.

Vgll3 interacts with ets1 and requires a highly conserved histidine repeat to activate pax3

Tead transcription factors bind the so-called MCAT sequence [5′-(AGGAATGT)-3′] present in non-muscle and muscle genes (Pasquet et al., 2006; Yoshida, 2008). For instance, tead binding sites have been identified in Xenopus and mouse pax3 gene regulatory regions (Gee et al., 2011; Milewski et al., 2004). Surprisingly, the core sequence of TEAD binding site, 5′-GGAA-3′, is a perfect recognition sequence for members of the ETS domain transcription factor family (Sharrocks, 2001). Ets1, the prototype of the ETS family, is specifically expressed by CNC in the chick embryo and is necessary for their proper delamination (Théveneau et al., 2007). In Xenopus, ets1 is expressed in neural tube and CNC and has been shown to be an immediate-early target gene of pax3 (Meyer et al., 1997; Plouhinec et al., 2014). Indeed, embryos overexpressing ets1 showed an ectopic pax3 expression (100%, n = 30, Fig. 8A). A synergic effect of both ets1 and vgll3 on pax3 expression is barely detectable owing to their strong effect when proteins are expressed alone (Fig. 8A). However, immunoprecipitation revealed that vgll3 could interact with ets1 in the embryo (Fig. 8B). To address the functionality of vgll3/ets1 complex, we turned to a gene reporter analysis. We have previously shown that a 284 bp sequence of the α-tropomyosin gene contained a MCAT binding site that could recapitulate endogenous gene expression pattern in a tead1-dependent way (Fig. 8C) (Pasquet et al., 2006). A luciferase reporter gene driven by this 284 bp fragment (pGL284LUC) was co-transfected in HEK293 cells with plasmids encoding HA-vgll3, myc-ets1 or myc-tead1. In those experiments, Ets1, vgll3 and tead1 are expressed at basal level in nontransfected cells and expressed at similar protein levels in transfected cells (Fig. 8D). Tead1 overexpressing cells showed a basal luciferase activity that is stimulated 1.35-fold upon
co-expression of vgl3 (Fig. 8E), while luciferase activity of ets1-overexpressing cells is stimulated 1.7-fold. This difference might reflect a preferential activation of the reporter gene in favor of vgl3/ets1 rather than vgl3/tead1. Vgll proteins interact physically and functionally with TEAD proteins though their conserved tondu (TDU) domain (Vaudin et al., 1999). Vgll3 protein deleted from its TDU domain (V3ΔTDU) did not stimulate the luciferase activity in the presence of tead1 or ets1 (Fig. 8E). These results demonstrate that vgll3 can interact with ets1 and stimulate a MCAT element-dependent gene promoter. Moreover, the TDU domain of vgll3 is necessary for both ets1, and tead1-dependent gene activation.

All vertebrate Vgll3 proteins have in common a histidine tract, a feature that is shared by a limited number of proteins in mammals, the function of which is still speculative (Fig. S8A) (Salichs et al., 2009). When the protein is deleted from its histidine repeat (vgll3Δhis), it cannot stimulate anymore pax3 expression (Fig. S8B) while its nuclear localization is unchanged (white arrow, Fig. S8C). In conclusion, the histidine repeat of vgl3 is required for its transcriptional activity but does not influence its nuclear localization.

**DISCUSSION**

In the present study, we described vestigial-like 3 (vgll3) as a novel factor that has a dual role in trigeminal placode and nerve formation and NC migration. We identified vgll3 as a new cofactor of ets1 that can regulate, through its association, MCAT-dependent gene activation. Moreover, the TDU domain of vgll3 is necessary for both ets1, and tead1-dependent gene activation.

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**Vgll3 expression is strictly restricted to rhombomere 2 and regulates trigeminal placode and nerve formation**

We showed that vgll3 expression is spatially restricted in the hindbrain through a combination of multiple signals including retinoic acid (RA), FGF8, Wnt, hoxa2 and hoxb2. This is consistent with previous findings that showed that FGF8 restricts the caudal boundary of anterior neural gene and our observation where engrailed2 overexpression switched off vgll3 (Faucheux et al., 2010; Fletcher et al., 2006). We found that Vgll3 expression is caudally restricted by hoxb2. Surprisingly, hoxa2 overexpression also switches off vgll3 expression, suggesting that vgll3 is not subject to this repression in the normal development or is countered by positive signals. Both gain- and loss-of-function of hoxa2 in *Xenopus* embryos phenocopies our results on vgll3. Indeed, in both cases, embryos displayed skeletal head defects and NC cell migration impairment (Baltzinger et al., 2005; Pasqualetti et al., 2000). This fits with the hypothesis that hoxa2 could be a repressor of vgll3 in r2.

Vgll3 gain- and loss-of-function clearly affected the expression of the specific placode genes islet1 and neuroD. Consequently, N-tubulin expression is affected leading to a reduction in ophthalmic and maxillo-mandibular branches and in axonal outgrowth of trigeminal nerve. We hypothesize that vgll3 regulates trigeminal placode development through pax3 and zic1, two genes that are associated with placode development (Jaurena et al., 2015; Schlosser, 2006). Vgll3-depleted embryos show a downregulation of pax3 at the level of trigeminal placode, while vgll3 overexpression induces pax3 and zic1 ectopic expression. In pluripotent animal cap cells, vgll3 overexpression also stimulates pax3 and zic1 expression. Surprisingly, vgll3 is not expressed in placode domain and therefore we may suggest that it acts in a non-cell autonomous manner. Indeed, it is known that Wnt and FGF signals cooperate in the formation and differentiation of the otic and trigeminal placoDes (Canning et al., 2008; Park and Saint-Jenne, 2008). Since we have showed that vgll3 stimulates both Wnt and FGF expression, we hypothesize that vgll3 regulates trigeminal placode and nerve formation through these signals.

That similar phenotypes in vgll3 gain- or loss-of-function studies are observed may be conceivable if we consider a functional dependence on protein-protein interaction where proper stoichiometry is essential (Lander et al., 2013). In our case, this could be related to the formation of the complex between vgll3 and tead1 (or ets1) and several mechanisms of repression can be proposed such as competition, quenching or squelching of the transcriptional complex.

**Vgll3 is implicated in signaling pathways that control migration of CNC cells**

Although vgll3 is a strong activator of pax3 and zic1, its temporal expression precludes any role in the early NC gene regulatory network. However, from *in vivo* and *in vitro* analysis of morphant embryos, we may conclude that vgll3 is required for normal CNC migration as shown by the analysis of snail2-positive cells that do not migrate. How can we reconcile the broad effect of vgll3...
knockdown that affect all segments of the migrating CNC and their derivatives, while its expression is restricted to r2? We propose that vgll3 can act on target genes through secreted molecules. Indeed, we have showed that vgll3 stimulates wnt5a, wnt8b and fgf8, supporting the hypothesis of a nonautonomous role through those signals. Moreover, this ensures the maintenance of pax3 and zic1 expression levels.

It is interesting to note that in zebrafish, vgll2a, a paralog of vgll3, has been shown to regulate CNC derivatives formation in a nonautonomous manner (Johnson et al., 2011). A recent report demonstrates that both activation and inhibition of canonical Wnt signaling results in severe NC migration in Xenopus embryo (Maj et al., 2016). This may explain our results since we have shown that vgll3 stimulates Wnt expression supporting a role through secreted molecules. We may also hypothesize a paracrine action like the one observed for en2 and pax2/5 that regulates wnt-1 and its target Tcf-4 in a nonautonomous manner during brain patterning (Koenig et al., 2010). Vgll3 can also regulate cell fate in the hindbrain in a non-cell autonomous manner, as has been shown for meis3 (Dibner et al., 2001).

The migration default of CNC induced by vgll3 depletion can be correlated to myosinX which is required for adhesion of CNC cells to the extracellular matrix (Nie et al., 2009). Interestingly, myosinX and vgll3 knockdown affect migration (this study and Grenier et al., 2009). Moreover, vgll3 knockdown in embryos and in animal cap cells induced a specific decrease in myosinX expression, which may explain the inhibition of CNC cell migration in vivo. Together, our data establish a potential link between vgll3 and the myosinX-dependent migration processes (Nie et al., 2009; Zhu et al., 2007). After induction, CNC cells leave their original territory followed by a cadherin-dependent migration process (Théveneau and Mayor, 2012). Vgll3 downregulation decreases N-cadherin and pcdh18 expression in animal cap explants and PCNS and pcdh18 expression in the embryo. Interestingly, vgll3-depleted embryos phenocopied twist1-depleted embryos leading to abnormal cartilage development (Lander et al., 2013). Surprisingly, a potential involvement of vgll3 in NC cells emerged from the report on a human patient that presents a microdeletion of chromosomal region 3p11.2-p12.1, including the VGLL3 gene (Gat-Yablonski et al., 2011). The patient presented a face dysmorphic development suggesting alteration in the NC cell formation/migration. Curiously, VGLL3 gene was also found to be significantly higher in human cartilage presenting endemic osteoarthritis, suggesting its implication in cartilage development (Wang et al., 2009). Our results emphasize the role of vgll3 in the genetic regulatory network that controls cell-cell and cell-matrix interactions that could explain its essential function in CNC migration.

**Ets1 is a new partner of vgll3**

We have shown that vgll3 can interact in the embryo with tead1 or tead2 as expected (Chen et al., 2004; Kitagawa, 2007). However, we found that the complex vgll3/tead2, unlike vgll3/tead1, is unable to induce pax3, zic1, snail2 or N-cadherin expression in animal cap cells. This suggests that the protein complexes vgll3/tead1 and vgll3/tead2 have distinct cis-regulatory targets or that animal cap cells are missing factors, present in the embryo that are required for pax3 induction by vgll3/tead2. Alternatively, this might be reminiscent to what has been observed in Drosophila where the binding of Vestigial to Scalloped can switch the DNA-binding selectivity of Scalloped (Halder and Carroll, 2001).

Our results establish that tead1 is not the only transcription factor that conveys vgll3 activity in vivo. Indeed, vgll3 and ets1 can interact in the embryo and, when co-expressed, can stimulate a MCAT-luciferase reporter gene. Therefore, it is conceivable that vgll3 can bind either to tead or ets1 depending on both cell context and relative affinity of partners. A recent report has shown that ets1 represses NC formation through downregulation of BMP signaling (Wang et al., 2015). Whether this effect is modulated by vgll3 is unknown but it may be noted that gain- or loss-of-function of vgll3 and ets1 give the same phenotype with regard to trigeminal nerve formation, NC migration and defects in its derivatives (this work and Wang et al., 2015). Vgll3 as a new partner of ets1 was unexpected and is very challenging as ets1 is also a proto-oncogene and VGLL3 has been proposed to play a role in tumor progression (Antonescu et al., 2011; Cody et al., 2007, 2009; Gambaro et al., 2013; Hallor et al., 2009; Helias-Rodzewicz et al., 2010). In the
future, it will be interesting to determine the relative affinity of vgll3 for tead and ets1 and the repertoire of target genes for the two complexes. Finally, we have evidence that the conserved histidine repeat in vgll3 protein is required for its transcriptional activity suggesting that this region is part of the transcriptional activation domain.

In summary, our results provide the first evidence of the function of vgll3 during vertebrate development. Clearly, vgll3 is critical for trigeminal placode and nerve formation. Moreover, although vgll3 does not play a direct role in NC formation, it is required for their migration. We propose that vgll3 fulfill all these properties mainly through the activation of both wnt and FGF signals (Fig. 9). One major finding of our work is that ets1 is a novel partner of vgll3. This suggests that vgll3 can regulate distinct gene targets and activate or repress signaling pathways depending on its association with different transcription factors. This should be helpful in our exploration of its function in mammalian cells and for scientific community to provide new target genes for vestigial-like members associated with the new transcription factor, ets1.

MATERIALS AND METHODS

Ethics statement

This study was carried out in accordance with the European Community Guide for Care and Use of Laboratory Animals and approved by the Comité d’éthique en expérimentation de Bordeaux (No. 33011005-A).

Plasmids and probes

Plasmid containing cDNAs encoding *X. laevis* vgll2 (IMAGE clone 4930090, accession number BC056001) and ets1 (IMAGE clone 8549297, NM_001087613) were obtained from Geneservice and Source BioScience, respectively. cDNA encoding *Xenopus laevis* vgll3 (XL405a05ex, accession number BP689606) was obtained from the National BioResource Project (www.nbrp.jp). The 5'-sequence of vgll3 mRNA was obtained by 5'-RACE (Invitrogen). Coding sequences for HA-vgll3 cDNAs are indicated in Table S1.
three independent experiments. Primers are listed in Table S3. All results shown are representative of RNA extraction and RT-PCR analysis (Naye et al., 2007; Tréguer et al., 2009). Primers are listed in Table S3. All results shown are representative of RNA extraction and RT-PCR analysis (Naye et al., 2007; Tréguer et al., 2009). Using antisense morpholino oligonucleotides (GeneTools) (Table S2). Injected embryos were cultured in 0.1× MMR with or without 10 µM (lacZ staining) or [35S]methionine. The reaction products were analyzed by 12% SDS-PAGE followed by autoradiography.

**Acian Blue staining**

Stage 47 embryos were fixed in MEMFA and stained in 0.05% Acian Blue/30% acetic acid in ethanol. Embryos were washed through a glycerol series before manual cartilage dissection. Cartilages were embedded in paraplast for serial sections.

**Migration assay**

Migration assay was performed from CNC explants as described before (Borchers et al., 2000; Alfandari et al., 2003). CNC explants from GFP-labeled embryos were grafted homotypically into unlabeled host embryos (in vivo) or plated on bovine plasma fibronectin (in vitro) (10 µg/ml, Sigma-Aldrich). The ratio of spreading of the explants was measured by comparing the relative surface area at 18 h of culture to that at 3 h. The surface area of individual CNC explants was performed using the Image J plugin (http://rsb.info.nih.gov/ij/features.html). Student’s t-test was performed to determine significant effects of vgll3 mRNA (n=15) compared to gfp mRNA (n=19) injections, and the effect of v3MO (n=24) compared to cMO (n=12) injections.

**TUNEL**

TUNEL assay was completed using a protocol previously described (Hensey and Gautier, 1997).

**Immunoprecipitation**

Batches of 30 embryos injected with relevant mRNAs were lysed at gastrula stage in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF and proteasine inhibitors (Roche). Pre-cleared proteins were incubated with appropriate antibodies (2 µg) (Table S4) and then incubated with protein A sepharose beads (Sigma-Aldrich). Bead pellets were boiled in SDS sample buffer before loading onto 10% SDS-PAGE gels. Bound antibodies (anti-myc or anti-αHA) were detected with HRP-conjugated EasyBlot anti-mouse IgG (diluted at 1:1000) (GeneTex, Wembley, UK) and visualized as before.

**Cell transfection and reporter gene analysis**

HEK293 cells were seeded at 6×10⁴ cells/cm² and co-transfected with a TK-driven renilla construct (pRL-TK, Promega) for normalization of transfection efficiency, together with the pGL284LUC construct (Pasquest et al., 2006), or the pGL284LUC construct in addition to DNA constructs expressing tead1, etsl, vgll3 or vgll3Δhis, FGF8, wnt8a, tead1, vgll3 or vgll3Δhis, β-galactosidase (β-gal) and human cytomegalovirus (CMV) promotor. Luciferase activity (Dual Luciferase, Promega) was quantified with a Varioskan Flash (Thermo Fisher Scientific). Stably transfected HEK293 cell lines were harvested and seeded into 96-well plates in triplicates. The ratio of spreading of the explants was measured by comparing the relative surface area at 18 h of culture to that at 3 h. The surface area of individual CNC explants was performed using the Image J plugin (http://rsb.info.nih.gov/ij/features.html). Student’s t-test was performed to determine significant effects of vgll3 mRNA (n=15) compared to gfp mRNA (n=19) injections, and the effect of v3MO (n=24) compared to cMO (n=12) injections.

**Statistical analysis**

Quantitative data are presented as mean±s.e.m. and were analyzed using Student’s unpaired two-tailed test. Statistical significance was defined at *P*<0.05.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

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Supplementary information
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References


