RESEARCH ARTICLE

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

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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, Wnt8a, 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 Tondo 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 either a role 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 mid-blastula 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
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 vgll3 expression domain. Hoxa2 or hoxb2 mRNA-injected embryos show a strong reduction of vgll3 expression. All views are dorsal-anterior. Asterisks indicate the injected side. Quantification of vgll3 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) Vgll3 is induced in animal caps treated with noggin+FGF2 (N+F). (E) Vgll3 expression is induced in early, but not late, animal cap cells overexpressing wnt8a. (F) Overexpression of vgll3 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 uninjected embryo; N-tub, N-tubulin; −RT, no cDNA. *Omitoeh decarboxylase* (*odc*) gene expression was used as a control.

Fig. 1. Temporal expression and spatial regulation of vgll3 in *Xenopus* embryo. (A) Vgll3 expression detected by RT-PCR starts between stages 11 and 12. (B) Vgll3 is detected by ISH in r2 during neural tube closure. Dashed lines indicate the midline of embryos. (C) Vgll3 expression decreases in stage 18 embryos treated with increasing concentrations of retinoic acid (RA). FGF8 mRNA-injected embryos show an anterior-lateral enlargement of vgll3 expression domain. Hoxa2 or hoxb2 mRNA-injected embryos show a strong reduction of vgll3 expression. All views are dorsal-anterior. Asterisks indicate the injected side. Quantification of vgll3 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) Vgll3 is induced in animal caps treated with noggin+FGF2 (N+F). (E) Vgll3 expression is induced in early, but not late, animal cap cells overexpressing wnt8a. (F) Overexpression of vgll3 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 uninjected embryo; N-tub, N-tubulin; −RT, no cDNA. *Omitoeh decarboxylase* (*odc*) gene expression was used as a control.
marker otx2 or the posterior marker hoxb9, respectively, but not vgll3 nor krox20 (Fig. 1D). Animal caps derived from noggin mRNA-injected embryos and treated with FGF2 expressed both vgll3 and krox20 (Fig. 1D). Neural induction is independent of mesoderm as controlled by the absence of vgll2 muscle-specific expression (Fig. 1D).

We next determined whether vgll3 expression could be regulated by hox genes. The anterior limits of hoxa2 and hoxb2 expression in the vertebrate hindbrain are r1/r2 and r2/r3 borders, respectively (Baltzinger et al., 2005; Moens and Prince, 2002; Nonchev et al., 1996; Schilling et al., 2001). When embryos were injected either with hoxa2 or hoxb2 mRNAs they showed reduced vgll3 expression in r2 (Fig. 1C). We next studied the effects of secreted signaling Wnt proteins involved in many aspects of neural development (Baker et al., 1999). Wnt8 overexpression in animal cap cells stimulates vgll3 expression (Fig. 1E) and, conversely, vgll3 stimulates wnt8, and also wnt5a and fgf8 (Fig. 1F).

Together, these data suggest that vgll3 expression in hindbrain is positively regulated by FGF and Wnt signals and negatively by hox genes 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 expression 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 placodes (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 placodes (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 genetic regulatory network, pax3 and zic1 have been shown to be essential for specification, differentiation and migration of CNC cells in Xenopus (Bae et al., 2014; Betancur et al., 2010; Milet et al., 2013). 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 (arrowhead, Fig. 3A).

Vgll3 mRNA-injected embryos showed strong ectopic expression of pax3 and zic1, while no change was observed in embryos injected with cMO or lacZ mRNA (Fig. 3A,D). Because pax3 and zic1 are expressed earlier than vgll3 in the developing embryo we examined whether they could regulate its expression (Hong and Saint-Jeannet, 2007). Embryos injected with inducible pax3GR/zic1GR mRNAs showed a faint focalized lateral expansion of vgll3 expression after dexamethasone treatment (Fig. 3C). Together, these data suggest that vgll3 regulates pax3 and zic1 expression and can be stimulated, albeit very faintly, by pax3 and zic1. Snail2 is one of the earliest CNC specifiers expressed in the embryo followed by twist (Lander et al., 2013; Mayor et al., 1995). Whole-mount ISH for vgll3/snail2 shows a lateral and partial overlapping expression at the r2 level (arrows, Fig. 4). In stage 16 vgll3-depleted embryos, expression of snail2 is not affected, but the onset of CNC migration is blocked, and this is more conspicuous in stage 21 embryo (Fig. 4). Stage 19 and stage 25 vgll3-depleted embryos display a reduction of twist expression in mandibular, hyoid and branchial segments (Fig. 4).

CNC migration is regulated by cell-cell interaction mediated by cadherins such as PCNS (protocadherin in NC and somites) and pcdh18 (Aamar and Dawid, 2008; Rangarajan et al., 2006). In stage 19 vgll3-depleted embryos, PCNS expression is less extended along the different streams that will form pharyngeal arches and the embryos showed a loss of PCNS expression in stage 25 (Fig. 4). Likewise, pcdh18 expression is not detected in the CNC lateral streams in stage 20 morphant embryos (circle, Fig. 4) and absent in the mandibular branch of trigeminal nerve in stage 28 (arrowhead, Fig. 4). Similar results were obtained with a second translational morpholino (Fig. S6) and in v3MOsplicing morphants (data not shown).

Stage 19 and stage 21 embryos overexpressing vgll3 mRNA showed a clear impairment of cell migration expressing snail2 and twist (Fig. 4). CNC cell migration into pharyngeal arches is also inhibited in vgll3-overexpressing embryos as revealed by PCNS staining (Fig. 4). Taken together, these results suggest that the absence of vgll3 does not affect CNC formation but impairs their migration. Because CNC are the source of most of the cranial cartilages and play an important role in determining the head shape, we further observed that vgll3 depletion or overexpression induced abnormal cartilage and impaired head structures (Fig. S7).

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. 5B,a,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.](http://bio.biologists.org/)

(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/snaill2 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. 3. Vgll3 stimulates pax3 and zic1 expression. (A) Embryos were injected with v3MO or vgll3 mRNA and analysed at stage 19 for pax3 and zic1 expression. Defects of CNC lateral streams and trigeminal placode are shown by black arrows and arrowhead, respectively. (B) ISH for vgll3 and pax3 shows overlapping expression at the r2 level (blue arrows indicate vgll3 expression domain). (C) Stage 19 embryos injected with pax3GR/zic1GR mRNAs show a faint vgll3 and a strong snail2 expression after dexamethasone treatment (+dex). (D) cMO/LacZ mRNA control and quantification. Three independent experiments were performed (n=40). Asterisks indicate the injected side. All views are dorsal-anterior. Dashed lines indicate the midline of embryos. (E) ISH for vgll3 and zic1 shows overlapping expression at the r2 level (blue arrows). Zic1 expression in stage 21 embryo.
Vgll3 knockdown and overexpression do not affect CNC formation but block their migration. Embryos were injected with v3MO (40 ng or cMO) or vgl3 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. Vgl3 knockdown and overexpression block migration of CNC streams. Arrows indicate overlapping expression of vgl3 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.

Fig. 4. Vgl3 knockdown and overexpression do not affect CNC formation but block their migration. Embryos were injected with v3MO (40 ng or cMO) or vgl3 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. Vgl3 knockdown and overexpression block migration of CNC streams. Arrows indicate overlapping expression of vgl3 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 vgll3 (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 vgll3/ets1 rather than vgll3/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 vgll3 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 promoter. Moreover, the TDU domain of vgll3 is necessary for both ets1, and tead1-dependent gene activation.

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 engerailed2 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 counteracted 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-Jeannet, 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
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 Scallop can switch the DNA-binding selectivity of Scallop (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; Gambero 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 tead1, tead2 and ets1 were subcloned in pCS2+MT vector. Cloning strategies for HA-vgll3 cDNAs are indicated in Table S1.

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**Fig. 8. Vgll3 interacts with ets1 and activates a tead-dependent luciferase reporter gene.** (A) Pax3 expression in embryos injected with vgll3 or ets1 mRNA. Ets1 overexpression induces ectopic Pax3 expression as well as co-expression of ets1 and vgll3. Asterisks indicate the injected side. (B) Embryos were injected with myc-ets1 or HA-vgll3 mRNAs. Immunoprecipitation with myc (IP myc) or HA antibodies (IP HA) was followed by IB with antibodies. (C) Schematic representation of the pGL284LUC reporter plasmid used in transfection assay. The different cis-sequences are depicted together with the MCAT cis-sequence (black box). (D) Left panel: ets1, tead1 and vgll3 are expressed in HEK293 cells when analysed by RT-PCR. (−), no cDNA. Right panel: HEK293 cells were transfected with HA-vgll3, HA-V3ΔTDU, myc-tead1 or myc-ets1 plasmids and similar amounts of proteins were checked by IB with myc and HA antibodies. Gapdh and erk2 are used as control. (E) HEK293 cells were co-transfected with pGL284LUC and HA-vgll3, HA-V3ΔTDU, myc-tead1 and myc-ets1 plasmids. Relative luciferase activity is expressed as fold change in luciferase activity compared to tead1 or ets1 alone. Data are mean±s.e.m. from three independent experiments carried out in duplicate. *P<0.05; Student’s t-test.
Embryo and explant manipulation

*Xenopus laevis* embryos were obtained and staged using current protocols (Nieuwkoop and Faber, 1975; Sive et al., 2000). All mRNAs were synthesized using the Message Machine kit (Ambion, Foster City, USA) and injected at the following doses: *noggin* (500 pg), FGFR9, wnt1b (100 pg), *pax3Gr/zic1Gr* (100 pg each), *hoxa2* (70 pg), *hoxb2*, *tead1*, *tead2* (50 pg), *vgll3* (0.25-1 ng), *vgll3mis* (0.5 ng) and *vgll3hia*, *vgll2*, *ets1* (1 ng). For retinoic acid (RA) (Sigma-Aldrich) treatment, embryos were treated at stage 8 with 10^{-6} M to 10^{-8} M or with DMSO for control. *Pax3Gr* and *zic1Gr*-injected embryos were cultured in 0.1× MMR with or without 10 µM dexamethasone from stage 10.5. Silencing of selected genes was performed using antisense morpholino oligonucleotides (GeneTools) (Table S2).

Synthetic mRNAs or MOs were co-injected with 250 pg β-galactosidase (lacZ staining) or gfp mRNA as a lineage tracer. Animal caps were dissected from early stage 9 embryos and cultured until appropriate stages before 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 three independent experiments.

Whole-mount ISH and immunostaining

Whole-mount ISH was carried out using a standard protocol (Harland, 1991). For double ISH, probes were labelled with DIG and fluorescein or both with DIG. Staining was performed with Fast Red and BM-Purple or both with BM-Purple. Immunostaining was performed following standard procedures (Sive et al., 2000). Antibodies are described in Table S4. Embryos were embedded in agarose before sectioning.

Immunoblotting

Embryos were lysed in RIPA buffer (PBS, 1% triton, 1% NP40, 0.05% SDS, 1 mM PMSF and proteasme inhibitors) (Roche, Boulogne-Billancourt, France). Proteins extracted from the equivalent of one embryo were loaded on 12% SDS-PAGE and transferred on nitrocellulose membranes. Proteins were reacted with the antibodies (Table S4) and staining was visualized using the enhanced chemiluminescence detection kit (GE Healthcare, Velzy-Villacoublay, France).

In vitro translation

*In vitro* transcribed mRNAs (0.5 µg) were translated in lyse reticulocytes (Promega, Charbonneries les bains, France) according to the manufacturer’s instructions and in the presence of 100-200 ng of MOs and [35S]methionine. The reaction products were analyzed by 12% SDS-PAGE followed by autoradiography.

Alcian Blue staining

Stage 47 embryos were fixed in MEMFA and stained in 0.05% Alcian 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 (Hensley 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 proteasme 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-HA) were detected with HRP-conjugated EasyBlot anti-mouse IgG (diluted at 1/1000) (GeneTex, Wembly, UK) and visualized as before.

Cell transfection and reporter gene analysis

HEK293 cells were seeded at 6×10^3 cells/cm² and co-transfected with a TK-driven renilla construct (pRL-TK, Promega) for normalization of transfection efficiency, together with the pGL284LUC construct (Passquet et al., 2006), or the pGL284LUC construct in addition to DNA constructs expressing tead1, *ets1*, *vgll3* or *vgll3ATDU* (500 ng/well). Transfection assay was performed using X-treme gene (Roche) according to the manufacturer’s instructions. Luciferase activity (Dual Luciferase, Promega) was quantified with a Varioskan Flash (Thermo Fisher Scientific) and results were calculated from duplicate samples of three independent repeats.

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

original draft: N.T., P.T., C.F.; Writing - review & editing: N.T., P.T., C.F.; Project administration: P.T., C.F.; Funding acquisition: N.T., P.T., C.F.

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Supplementary information
Supplementary information available online at http://bio.biologists.orglookup doi:10.1242/bio.026153.supplemental

References


Table S1. PCR primers used for the engineering of vgll3 proteins. The different tagged vgll3 proteins were made by PCR cloning into pCS2-HA vector with primers listed. Restriction sites are in bold and HA tag sequence is underlined. F, Forward; R, Reverse.

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<th>Primer name</th>
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<td>Vgl3-HA-XhoI R</td>
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Table S2. Morpholinos sequences used in the study.

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**Table S3. PCR primers used in the study.** Accession number, sequence and origin of primers used are listed (sp, splicing).
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Table S4. Antibodies used in the study. The origin and use of the different antibodies are listed. The 3A10 antibody was obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. AP, Alkaline phosphatase; DAB, Diaminobenzidine; DIG, Digoxigenin; HRP, Horseradish peroxidase; *, SIGMA; **, Roche. JIRL, Jackson ImmunoResearch Laboratories; IB, Immunoblotting; ICH, Immunocytochemistry; IP, Immunoprecipitation; ISH; In situ hybridization. Reference products are indicated in italic.
Figure S1. Vgl13 does not co-localise with trigeminal placode markers. (A) Whole-mount ISH for *vgl13* and trigeminal placode genes *islet1*, *neuroD*, *pax3* and *foxi1c*. (B) Whole-mount ISH for trigeminal placodal genes *islet1*, *neuroD*, *pax3* and *foxi1c*. Blue arrow indicates the position of *vgl13* expression domain. All staining are visualized in blue. Analysed embryos are from stage 14 to stage 20 depending on the trigeminal markers used. All views are dorsal-anterior.
Figure S2. In vivo specificity of vglL3 morpholinos. Embryos were injected with 500 pg of vglL3, vglL3 mismatch (mis) or vglL2 mRNAs in the presence (+) or not (-) of 40 ng of vglL3 (v3MO) or control (cMO) morpholino oligonucleotide antisenses. Stage 15 embryos were stopped and analysed by immunoblotting with HA antibody to detect vglL3. v3MO efficiently blocks the translation of vglL3 mRNA but has no effect on vglL3 mismatch mRNA neither on vglL2 mRNA. Erk2 is used for loading controls. Right panel, control MO (cMO) has no effect on vglL3 mRNA translation in an in vitro translation assay.
Figure S3. *In vivo* specificity and efficiency of v3MOsplicing. (A) Schematic representation of the *vgll3* locus. There are two *vgll3* homeolog genes, *vgll3*.L and *vgll3*.S, that are expressed in *Xenopus laevis*. Specific primers for *vgll3*.L and *vgll3*.S (v3Lsp F and v3Ssp F respectively) and common primer for both mRNAs (v3sp R) were chosen to detect normal and altered splicing for *vgll3*.L and *vgll3*.S mRNAs. The position of the splicing blocking MO specific to *vgll3*.L pre-mRNA (v3LMOsp for v3LMOe2i2) or to *vgll3*.S pre-mRNA (v3SMOsp for v3SMOi1e2) is indicated by a black box. (B) Embryos were co-injected with 10, 20 or 40 ng of each morpholino splicing (v3MOsplicing) and gfp mRNA as lineage tracer. Stage 15 embryos were analysed by RT-PCR to detect *vgll3* expression. V3MOsplicing decreases normal spliced *vgll3*.L RNA or *vgll3*.S level compared to non-injected embryos in a dose-dependent manner. PCR fragments corresponding to the altered splicing mRNAs appeared at expected size for both *vgll3* mRNAs. *Odc* is used for loading controls.-RT, no DNA for each condition.
Figure S4. Dose dependent effect of v3MO splicing on N-tubulin and snail2 expression domain. (A) Embryos injected with v3LMOsp, specific to vgll3 form L or v3SMOsp, specific to vgll3 form S or both (v3MOsplicing) exhibit a reduced expression of N-tubulin in the trigeminal placodes (arrowhead) and an inhibition of CNC migration as shown snail2 expression. Stronger effect was observed when both splicing MOs were injected at 10ng each compared to single MO. Injected side (*) was traced by lacZ staining. Gene expression was assayed by ISH. Arrowhead indicates the trigeminal placodes. (B) Quantification of results. Three independent experiments were performed. The number of embryos analysed is indicated on the top of each bar. All views are dorsal-anterior. sp, splicing.
Figure S5. *Vgli3* impairs trigeminal nerve formation. (A) Embryos injected with 40 ng of v3MO exhibit a decrease of HNK-1 staining (arrowhead) and a quivering phenotype for 3A10 staining (bottom panel) in the trigeminal ophthalmic and maxillo-mandibular branches (50%, n=30 for both staining). (B) Embryos injected with *vgll3* mRNA (1ng) exhibit a similar decrease of staining and a quivering phenotype in the trigeminal branches for HNK-1 (50%, n=25) and 3A10 staining (80%, n=25). Major branch connected to trigeminal ganglia disappeared (arrowhead). (C) Upper row: Embryos injected with cMO or *lacZ* mRNA show no difference in HNK-1 staining between non injected side (ni) or injected side (*). Lower row: Stage 14 embryos injected with cMO, v3MO, *gfp* or *vgll3* mRNAs show no apoptotic cells when analysed by TUNEL. The median line of the embryo is indicated with a dotted line. The injected side was traced by *lacZ* staining. HNK1 (specifically labelling axonal outgrowth antibody) and 3A10 (neurofilament specific antibody) expression was assayed by immunohistochemistry on stage 28 and stage 42 respectively. (D) TUNEL apoptosis analysis of stage 17 embryos. V, trigeminal nerve; VII, facial nerve; ey, eye; ov, otic vesicle.
Figure S6. A second vgll3 morpholino (v3MO2) phenocopies v3MO effects. (A) Embryos were injected with 10 ng of a second non overlapping vgll3 (v3MO2) morpholino oligonucleotide antisense, then fixed at stage 16 or 19 and stained for snail2 expression. No significant effect was detected at stage 16 but defects in neural crest migration were observed at stage 19 (brackets). (*) injected side. (B) Specificity of v3MO2 in in vitro translation experiments. (C) Immunohistochemistry analysis of HNK-1 and 3A10 (stage 28 and stage 35 respectively). White and black arrowheads show defects of the ophthalmic and maxillo-mandibular branches of the trigeminal nerve respectively. (*) injected side.
Figure S7. *Vgll3* is required for the formation of neural crest derivatives. Embryos were injected with 40 ng of v3MO (or cMO) or 1 ng of *vgll3* mRNA (or *gfp* mRNA) and fixed at stage 47 before alcian blue staining (A-I). (A, B, C, D) The formation of cranial cartilage is impaired when *vgll3* is knocked-down when compared to control MO injected embryo [A compared to D (embryos injected with cMO show no alteration)]. This is more obvious on dissected embryos that revealed on the injected side a high rate of cranial cartilage hypoplasia including severe loss of Meckel’s (MC), ceratohyal (CH) and branchial cartilages (Br) (100%, n=15, Fig. 6B,C). (E, F) Transversal sections of paraffin embedded tissues showed that the ceratohyal cartilage on the injected side was shorter than on the uninjected control side (Fig. 6E) and displayed numerous smaller chondrocytes (Fig. 6F, enlarged view of E indicated in the square line. Scale bars represent 500 µm (E) and 130 µm (F). (G, H, I, J) Embryos injected with *vgll3* mRNA showed an impaired cartilage development with the ceratohyal cartilage being severely disorganized (100%, n=15, Fig. 6G, H). In control experiments, embryos injected with *gfp* mRNA showed no change in cartilage head morphology (Fig. 6I, J). ey, eye. Ventral views with anterior to the top (A, D, G, I) and dorsal views (J). (*) injected side. Midline embryo is indicated by a dotted line.
Figure S8. The histidine repeat in vgll3 is required for pax3 activation. (A) Sequence alignment of histidine (H) tract in Vgll3 proteins from different vertebrate species. Numbers indicate the relative position of the histidine tract. Xenlae, *Xenopus laevis*, Xentrop, *Xenopus tropicalis*. (B) Embryos were injected with 1 ng of vgll3 (vgll3\textit{wt}) or vgll3\textit{Dhis}, fixed at stage 11 and analysed for pax3 expression. Vgll3\textit{Dhis} cannot activate pax3 (100%, n=30). (*) injected side. (C) HEK293 cells, recently tested for contamination, were transfected with 1µg of plasmid encoding HA-vgll3\textit{wt} or HA-vgll3\textit{Dhis}, fixed and analysed by immunocytochemistry using HA antibody. The deletion of histidine tract from vgll3 does not impair its nuclear localization. White arrow indicates nuclear staining. Scale bars, 10 µm.