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Vestigial-like-2b (VITO-1b) and Tead-3a (Tef-5a) expression in zebrafish skeletal muscle, brain and notochord

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Abstract

The *vestigial* gene has been shown to control skeletal muscle formation in *Drosophila* and the related Vestigial-like 2 (Vgl-2) protein plays a similar role in mice. Vgl-family proteins are thought to regulate tissue-specific gene expression by binding to members of the broadly expressed Scalloped/Tef/TEAD transcription factor family. Zebrafish have at least four Vgl genes, including two Vgl-2s, and at least three TEAD genes, including two Tead3s. We describe the cloning and expression of one member from each family in the zebrafish. A novel gene, *vgl-2b*, with closest homology to mouse and human *vgl-2*, is expressed transiently in nascent notochord and in muscle fibres as they undergo terminal differentiation during somitogenesis. Muscle cells also express a TEAD-3 homologue, a possible partner of Vgl-2b, during myoblast differentiation and early fibre assembly. *Tead-3a* is also expressed in rhombomeres, eye and epiphysis regions.

Keywords: Muscle; Adaxial; Zebrafish; Vestigial-like; Transcription enhancer factor; TEAD domain; Vgl; Skeletal muscle; VITO; Vestigial; Notochord; TEAD; Tef; Scalloped; Rhombomere; Eye; Epiphysis; Somite; Gene expression; Floatinghead; Cyclopamine; Smoothened; After eight; Fused somites; Danio rerio

1. Results and discussion

1.1. Background

Drosophila vestigial (*vg*) encodes a 453 amino acid (aa) 46 kDa protein (Williams et al., 1991) required for acquisition of muscle fibre identity in a subset of muscle founder cells during embryonic myogenesis and plays a role in conferring indirect flight muscle identity in the pupa (Bate et al., 1993; Bate and Rushton, 1993; Bernard et al., 2003; Sudarsan et al., 2001; Williams et al., 1991). *Vg* also functions elsewhere, most notably to control wing outgrowth where it appears to control cell survival and proliferation (Agrawal et al., 1995; Zider et al., 1996). Vg heterodimerizes with Scalloped (Sd) protein, and the correct balance of these factors is required for normal wing development. Vg has two identified sequence motifs, a Sd-interacting domain and an N-terminal domain similar to that of *Paired*, a *Drosophila* homologue of mammalian *Pax3*. In the wing disc, Sd promotes Vg nuclear accumulation and the complex is thought to target *serum response factor*, *spalt* and other genes (Halder et al., 1998). The targets of Vg/Sd in muscle are still unclear.

In mammals, several *vestigial* homologues have been identified. Murine *vestigial-like 2 (vgl-2)*, also known as *VITO1*, is expressed exclusively in skeletal muscle (Mielcarek et al., 2002; Maeda et al., 2002a). Vgl-4 controls gene expression in heart (Chen et al., 2004b), whereas expression of *vgl-1* and *vgl-3* appears largely restricted to the placenta (Maeda et al., 2002a). Vgls interact with vertebrate Scalloped homologues, such as Transcriptional enhancer factor-1 (Tef-1, also known as TEAD-1 and belonging to a gene family hereafter called TEADs) (Chen et al., 2004a; Gunther et al., 2004). TEADs bind strongly to MCAT and A/T rich sequences important for muscle-specific expression of certain genes, possibly sometimes alone, but

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often in combination with Vgls, MEF2s or serum response factor (Azakie et al., 1996; Farrance and Ordahl, 1996; Gunther et al., 2004; Gupta et al., 2001; Maeda et al., 2002b; Mahoney et al., 2005). Functional studies have revealed that *vgl-2* is required for normal skeletal myogenesis and loss of function can reduce myoblast terminal differentiation in cell culture. However, over-expression of *vgl-2* alone is not myogenic (Maeda et al., 2002a; Gunther et al., 2004).

1.2. Identification and family analysis of vestigial-like proteins in zebrafish

We searched for zebrafish homologues of vestigial-like and TEAD-like proteins *in silico* using GenBank and the Sanger zebrafish genome assembly (Zv3–Zv7; www.ensembl.org). An EST clone (Accession No. CN504929), named *vgl-2b*, with homology to mouse and human *vgl-2* genes (Fig. 1A) localises to chromosome 17 (Fig. 1B). Additionally, a second locus, designated *vgl-2a*, with a higher degree of conservation to amniote *vgl-2* is found on chromosome 20 of the zebrafish genome (Fig. 1B). Thus, both proteins appear to be homologues/orthologues of *vgl-2* that have arisen by gene duplication (Taylor et al., 2001). To elucidate genomic relationships we compared synteny using neighbouring known genes between fish, human and mouse *vgl-2* loci. *Vgl-2a* shows syntenic conservation with human and mouse by proximity to *Rfxdc1*, *Dcbld1* and *Gopc* genes, whereas *vgl-2b* exhibits a divergence (Fig. 1B). We were unable to find evidence for a genomic duplication of the Vgl-2 locus in either *Fugu rubripes* or *Tetraodon nigroviridis* (data not shown).

The divergence between *vgl-2a* and *vgl-2b* is also reflected in the extent of conservation of the protein sequence: Vgl-2a is more similar than Vgl-2b to mammalian Vgl-2s (Fig. 1C). Whereas mouse and human sequences are ~85% identical, zebrafish Vgl-2a is ~57% identical to both human and mouse Vgl-2 proteins at the amino acid level. By contrast, Vgl-2b is only ~35% identical to mouse and human Vgl-2, ~32% identical to mouse and human Vgl-3 and only slightly more (37%) identical with zebrafish Vgl-2a. However, strong Vgl-2 homology was observed in the Tef-interacting domain (also known as TDU motif; Vaudin et al., 1999), which is completely conserved between human, mouse and zebrafish (Fig. 1A). We also observed strong homology in the N-terminal region, which acts as a signature to distinguish Vgl-sub-family members (Fig. 1A and data not shown). Searching the Pfam and SMART databases for additional motifs only identified the Tef-interaction domain. The paired domain is not readily detected in the fish Vgl-2s. Despite the low overall identity of Vgl-2b and other Vgl-2s, conservation in specific regions is sufficient to assign orthology to Vgl-2.

Mouse Vgl-2 is known to shuttle in and out of the nucleus in cultured muscle cells according to differentiation status (Maeda et al., 2002a). In zebrafish, a proposed nuclear export signal (NES) is retained in Vgl-2b, but not in Vgl-2a (Fig. 1A, underlined; Maeda et al., 2002a). However, the putative nuclear localisation sequence (NLS) KRRRE in the mouse sequence (Maeda et al., 2002a) is absent from human and zebrafish Vgl-2 family members (Fig. 1A, bold). We focused on *vgl-2b* because *vgl-2a* is being analysed by others (X. Cousin, personal communication).

1.3. Expression of vgl-2b in somites

In-situ hybridisation of staged embryos revealed that *vgl-2b* mRNA is undetectable during gastrulation (data not shown) and becomes detectable in somitic adaxial cells adjacent to the notochord at the ~7 somite stage (7s) (Fig. 2A and B). This timing and the absence of signal in adaxial cells of presomitic mesoderm indicate that expression commences with the first terminal differentiation of slow muscle fibres (Fig. 2C and I; Devoto et al., 1996). Co-expression of *vgl-2b* with myosin heavy chain (MyHC) at 14s supports this view (Fig. 2D–F). Subsequently, *vgl-2b* is expressed in the region where slow fibres are differentiating as each somite forms (Fig. 2G–J, M and R).

To confirm that *vgl-2b* is expressed in slow fibres, we examined mutant fish lacking slow fibre formation. Hedgehog (Hh) signalling is required for slow myogenesis (Blagden et al., 1997; Barresi et al., 2000). Prevention of Hh signalling either in the signalling pathway mutant *smoothened* or by application of the Smoothened inhibitor cyclopamine ablated somitic *vgl-2b* mRNA at 15s (Fig. 3A–C and J–M). However, *u-boot* (*ubo* = *prdm1*; Baxendale et al., 2004) mutants or embryos injected with *prdm1* morpholino, which undergo adaxial myogenesis, but ultimately fail to produce slow muscle, show *vgl-2b* expression similar to wild type (Fig. 3D–F). Similarly, *fused somites* (*fss* = *tbx24*; van Eeden et al., 1996; Nikaido et al., 2002) and *after eight* (*aei* = *deltaD;* van Eeden et al., 1996) mutants which have slow fibres, despite defective somite border formation, retain adaxial *vgl-2b* expression (Fig. 3G and H). Thus, early somitic *vgl-2b* expression is in differentiating adaxial muscle fibres.

Fast muscle also expresses *vgl-2b*. Fast muscle differentiation begins around 16s, when most slow fibres move from their medial location to the superficial surface of the myotome (Devoto et al., 1996). By 25s, *vgl-2b* mRNA is detected in the lateral somite and transverse sections reveal expression in most, if not all, muscle, including fast fibres (Fig. 2N–P). Somitic *vgl-2b* mRNA is retained until at least 24 h post fertilization (hpf), after which it declines rapidly (Fig. 2Q–S and data no shown). Thus, fast muscle fibres express *vgl-2b* as they differentiate. *Fgf8 (acerebellar*; *ace)* mutants lack a proportion of fast fibres and express reduced levels of *vgl-2b* mRNA (Fig. 3N–P data not shown; Groves et al., 2005). Mouse Vgl-2 can bind to Mef2 proteins (Maeda et al., 2002a,b). In zebrafish, the expression of *vgl-2b* in the myotome coincides with the expression of *mef2c* mRNA and protein (Ticho et al., 1996; Thisse and Thisse, 2004; Hinits and Hughes, 2007).