The zebrafish van gogh mutation disrupts tbx1, which is involved in the DiGeorge deletion syndrome in humans

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Summary

The van gogh (vgo) mutant in zebrafish is characterized by defects in the ear, pharyngeal arches and associated structures such as the thymus. We show that vgo is caused by a mutation in tbx1, a member of the large family of T-box genes. tbx1 has been recently suggested to be a major contributor to the cardiovascular defects in DiGeorge deletion syndrome (DGS) in humans, a syndrome in which several neural crest derivatives are affected in the pharyngeal arches. Using cell transplantation studies, we demonstrate that vgo/tbx1 acts cell autonomously in the pharyngeal mesendoderm and influences the development of neural crest-derived cartilages secondarily. Furthermore, we provide evidence for regulatory interactions between vgo/tbx1 and edn1 and hand2, genes that are implicated in the control of pharyngeal arch development and in the etiology of DGS.

Key words: van gogh (vgo), tbx1, endothelin1, Pharyngeal arch development, DiGeorge syndrome, Endodermal pouches, Aortic arches, Zebrafish

Introduction

The development of the pharyngeal arches in vertebrates requires an intricate interplay between the pharyngeal endoderm, the mesodermal core of the arches, and neural crest cells. As yet, little is known about the molecular nature of these tissue interactions. The zebrafish mutant van gogh (vgo) is characterized by defects in the pharyngeal arches and associated structures, including fusion and loss of neural crest-derived pharyngeal cartilages, reductions in endodermal pouches and aortic arches, and the absence of the thymus (Piotrowski and Nüsslein-Volhard, 2000; Piotrowski et al., 1996). In addition, the otic vesicles in vgo mutants are reduced in size and some sensory patches within the mature ear are absent (Piotrowski and Nüsslein-Volhard, 2000; Whitfield et al., 1996).

The phenotype of homozygous vgo mutants bears striking resemblance to humans afflicted with DiGeorge syndrome (DGS). DGS is one of the most common developmental diseases in humans, affecting approximately 1 in 4000 live births (Scambler, 2000). Individuals with DGS are characterized by craniofacial defects, aortic arch malformations, thymus hypoplasia, conotruncal heart defects and hearing loss (Ryan et al., 1997). The majority of individuals carry a heterozygous deletion located on chromosomal region 22q11.2. This region comprises ~30 genes and is called the DiGeorge critical region (DGCR). Combined studies of heterozygous deletions within the DGCR and knockout experiments in mice have identified Tbx1 as a major genetic determinant of aortic arch malformations in DGS (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). Tbx1 encodes a transcription factor belonging to a gene family characterized by a highly conserved DNA-binding domain called the T-box. T-box genes are important regulators of embryonic development, examples include the founding family member T or Brachyury, which, when mutated in mice or zebrafish, leads to the loss of notochord and tail structures (Kispert et al., 1995; Schulte-Merker et al., 1994). Other examples are mutations in TBX5 and TBX3, which are responsible for human Holt-Oram and Ulnar-mammary syndromes, respectively, in which formation of limbs and cardiovascular system is disrupted (Bamshad et al., 1997; Basson et al., 1997), and the spadetail mutation in zebrafish, which affects the formation of trunk somites (Griffin et al., 1998).

We show that the zebrafish vgo mutant is defective in tbx1, which is required for interactions between the cranial mesendoderm and the neural crest cells that form the pharyngeal cartilages. Transplantation of wild-type endoderm into vgo/tbx1 mutants induces cartilage formation, which...
indicates that tbx1 acts autonomously in the endoderm. Consequently, the neural crest defects are secondary to endodermal defects in vgo/tbx1 mutants, as has been previously suggested (Piotrowski and Nüsslein-Volhard, 2000). The zebrafish vgo/tbx1 mutant offers the opportunity to study pharyngeal arch development in cellular detail and may contribute further to the understanding of DGS pathogenesis.

Materials and methods
Cloning of tbx1
A zebrafish 24.1 cDNA library (gift from Kai Zinn) constructed from 30- to 33-hour old embryos was screened under low stringency conditions (50°C) using a mixture of 32P-labeled oligonucleotide probes prepared from various mouse T-box sequences. Positive plaques were isolated and the cDNAs subcloned into phBluescript for sequencing. One clone with an open reading frame of 460 amino acids contained a T-box sequence highly similar to the mouse Tbx1 gene, which co-segregated with the mutation. Genomic DNA was amplified by PCR using the N1 restriction site in the tbx1 gene, which co-segregated of an AlwN1 and analyzed independently by repeat PCR reactions.

Sequence analysis of tbx1 in vgo
Primers were designed using the sequences flanking the exon-intron boundaries of tbx1. Genomic DNA was extracted from a pool of 4-day-old homozygous mutant embryos. PCRs were performed separately for each exon and the resulting products were cloned into the pGEM-T-Easy (Promega) vector for sequencing. Mutations were confirmed independently by repeat PCR reactions.

Meiotic and radiation hybrid mapping
The vgo\textsuperscript{m208} allele was generated in the Tübingen background in a large scale mutagenesis screen (Haffter et al., 1996; Piotrowski et al., 1996). To produce a polymorphic strain for genetic mapping, the fish carrying this allele was crossed to wik fish (Knapik et al., 1996; Rauch et al., 1997). Homozygous and sibling embryos were phenotypically scored at 4 dpf and fixed separately in 4% paraformaldehyde overnight. For extraction of genomic DNA individual embryos were placed in 96-well PCR plates and processed as described (Rauch et al., 1997). Bulk segregant analysis with SSLP markers was used to identify the linkage group. Analysis of SSCP markers in individual embryos was used to fine map the mutation. Radiation hybrid mapping of tbx1 was performed as described (Hukriede et al., 1999), using forward primer 5'-AACATGTACTCTGCGAGCTGAC-3' and reverse primer 5’-CACCGGATCTGTAAGGTGGTCTAG-3’ on the LN54 panel. The PCR conditions were 95°C for 4 minutes, 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds each, followed by 72°C for 7 minutes. The results were analyzed by interactive mapping software available at http://www.mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi

Linkage analysis between tbx1 and vgo\textsuperscript{m208}
The A to T transition mutation in the vgo\textsuperscript{m208} allele leads to the loss of an AlwN1 restriction site in the tbx1 gene, which co-segregated with the mutation. Genomic DNA was amplified by PCR using the forward 5’-GCTCTGGAGTGAACTTTA TTACCTG-3' and reverse 5’-AACGTGCTACTCCTGACTAC-3' primers that flank the mutation. The PCR product was digested with AlwN1 and analyzed on a 3% MetaPhor agarose gel (BMA).

In situ hybridization
In situ hybridization experiments were performed as described (Piotrowski and Nüsslein-Volhard, 2000). Probes were used for: hand2 (Angelo et al., 2000), edn1 (Miller et al., 2000), myod (Weinberg et al., 1996), crestin (Luo et al., 2001) and fgf8 (Furthauer et al., 1997).

Confocal microangiography
Labeling of the blood vessels was performed essentially as described (Weinstein et al., 1995) with the following modifications: live, anesthetized 2.5 dpf larvae were mounted on 5% methylcellulose for fluorescent microsphere injections (Molecular Probes). The microspheres were injected into the yolk blood stream that flows into the heart. Images represent projections of z-series stacks that were taken within 15 minutes of the injection.

Transplantation experiments
Wild-type donor embryos were injected with 5% rhodamine-dextran and 3% lysine-fixable biotinylated-dextran (Molecular Probes) at the one- to two-cell stage (diluted in 0.2 mM KCl). Cells were transplanted into the animal pole of unlabeled embryos derived from heterozygous vgo parents at the late blastula stage. Larvae were scored for neural crest and ear clones on day 3 using a BioRad confocal microscope.

edn1 rescue experiments and cartilage rescue experiments
Donor embryos were injected with 2 pg activated Taram A/Alk4 (Tar\textsuperscript* at the one-cell stage to convert most of the donor cells to an endodermal fate (Peyriéres et al., 1998). As lineage tracers, we used 2.5% Alexa rhodamine-dextran and 2% biotinylated-dextran dissolved in 0.2 mM KCl. Donor cells were transplanted at the late blastula stage into the margin of unlabeled embryos derived from heterozygous vgo/tbx1 parents. For transplantation, we used an air-filled standard 10 ml plastic syringe. At 24 hpf, the embryos were scored for endodermal clones under a fluorescent microscope. Embryos with endodermal clones were fixed with 4% paraformaldehyde overnight and processed for in situ hybridization with edn1. The transplanted cells were visualized using an avidin-biotinylated enzyme complex (ABC kit, Vector Laboratories) (Moens and Fritz, 1999). The embryos were mounted ventral side upwards under bridged cover slips after the yolk and the tail were removed. Subsequently the specimens were dehydrated in 100% methanol and cleared in a 1:2 solution of benzyl-benzoate: benzyl-alcohol. They were then photographed on a Zeiss Axioskop using a digital camera (Prog-Res/Kontron).

Results
Phenotype of vgo mutants
Zebrafish larvae homozygous for either of two existing vgo mutant alleles (\textit{tm208} and \textit{tu285}) die 6 to 7 days postfertilization (dpf) and appear to have the same phenotypic strength; the mutation is entirely recessive. At 4 dpf, vgo mutants lack segmentation of the posterior pharyngeal arches and have small otic vesicles (Piotrowski and Nüsslein-Volhard, 2000). Alcian Blue staining reveals that neural crest-derived cartilage are reduced (Fig. 1A-C) and often fused (Piotrowski and Nüsslein-Volhard, 2000). The severity of the phenotype depends on the genetic background. In the Tübingen background (in which vgo was first isolated), the first or mandibular arch is rarely affected, whereas in the AB* background it is always highly reduced (Fig. 1C). In the neurocranium surrounding the notochord (Fig. 1B, red elements), the parachordals (pc) are malformed and often not joined to the more anteriorly located neural crest-derived trabeculae, as in wild-type larvae (compare Fig. 1B,D). In addition, pharyngeal muscles associated with posterior arches are undetectable with molecular muscle markers such as myod (Piotrowski and Nüsslein-Volhard, 2000).

In this study, we investigated the aortic arch defects in vgo
vgo represents a null allele of tbx1

Zebrafish tbx1 cDNA encodes an open reading frame of 460 amino acids with extensive sequence similarity to mouse Tbx1 (AF326960) (68.5% overall identity, 98.3% identity within the T-box; Fig. 2A). In addition, the exon-intron structure was identical to that of the mouse gene (data not shown).

Based on phenotypic similarities between vgo and Tbx1 mutant mice, we tested tbx1 as a candidate gene for vgo. Both vgo<sup>tm208</sup> and tbr1 mapped on linkage group 5 (LG5) in meiotic and radiation hybrid panels between markers z7351/fb38h03 and z10456 (Fig. 2B). Sequence analysis of tbx1 in vgo<sup>tm208</sup> revealed an A to T transition at nucleotide position 877 (arrow), which replaces an arginine (AGA) with a stop codon (TGA) near the end of the T-box, leading to the deletion of the entire C-terminus of the protein (Fig. 2C). In many T-box genes, such as Brachyury/T and Tbx2 of the mouse, the effector domains are found in the C-terminal region of the protein (Carreira et al., 1998; Kispert et al., 1995), and thus vgo<sup>tm208</sup> probably represents a null allele of tbx1. This mutation also leads to the loss of an AlwN1 restriction site, and this loss co-segregated with the vgo phenotype in 620 homozygous embryos. We also tested 100 recombinants for the flanking markers z7351 and z6371 that were among 1457 vgo<sup>tm208</sup> embryos used in meiotic mapping and found that the loss of restriction site co-segregated with the mutation in all of these embryos as well. Based on these results, the calculated distance between this mutation and vgo is less than 0.03 cm, consistent with vgo<sup>tm208</sup> being a mutation in tbx1. The tbx1 sequence from the other allele (vgo<sup>ns285</sup>) also harbors a mutation that introduces a premature stop codon. The mutation is a C to T transversion at the nucleotide position 364 (arrow), which deletes 98% of the T-box as well as the whole C terminus of the protein (Fig. 2C). Thus, both alleles of vgo appear to be null mutations in tbx1. However, the dose sensitivity of T-box genes (Hatcher and Basson, 2001; Nikaido et al., 2002) might explain our failure to convincingly rescue vgo by microinjection of tbx1 cDNA or mRNA.

Zebrafish tbx1 is expressed in the ear and in mesendodermal components of pharyngeal arches

Expression of zebrafish tbx1 starts at the beginning of gastrulation (6 hpf) in the involuting cells within the hypoblast (Fig. 3A). These tbx1-expressing cells are likely to be the progenitors of the cranial paraxial mesendoderm. Between 6- and 10-somite stages, tbx1 expression is found in paraxial mesoderm as well as in a pair of bilateral stripes on either side of the neural tube that correspond to the cranial paraxial mesoderm, which extends from just posterior to the eyes (midbrain level) to approximately the level of rhombomere 6 (Fig. 3B,C). At the 20-somite stage, expression is detected in the primordia of the pharyngeal arches (Fig. 3D, p1-p7). By 27 hpf, expression is localized to the mesodermal core of the pharyngeal arches as well as to the arch epithelium, but not to neural crest cells lying in between (Fig. 3E). At 30 hpf, pharyngeal expression marks elongated cell clusters in each arch, which prefigure the forming arch muscles and possibly also mesenchyme cells surrounding the aortic arches (Fig. 3F).

These patches are separated by the endodermal pouches that also express tbx1, which are more clearly observed in horizontal sections (Fig. 3G; ‘e’). From 48 hpf onwards, tbx1 expression is seen in individual arch muscles, which co-express...
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**VT**

**PSC**

**FGMDPMADYMLLMDFVL**

**---ENNGFGSK**

**TKAGRRMFPSYK**

**FPD**

**VSFS**

**GHIILNSMHK**

**FIS**

**DVI**

**LLMDIV**

**VYI**

**LLAVTAYQNEE**

**KLTNKMNG-G**

**GRRMFPVLK**

**YI**

**PKTKPA**

**VTAYQNPQ**

**YLF**

**AVTS**

**VKVTGAH**

**VSVT**

**GKPE**

**ITQLKIAA**

**VFP**

**EFSSV**

**ETA**

**FR**

**QP**

**VKA**

**FI**

**RWKYVNGE**

**YE**

**KTFVFEETRFTAVTAYQHRITQLKIAASNP**

**FAKGFR**

**QL**

**WMRSMHKPR**

**HDKDN---H**

**SQHWGG----MAS**

**PAMYTV**

**RYRWQGRRNPFAKGF**

**HL**

**GHL**

**DD--H**

**GL**

**VSFHRVETQDPE**

**NTAFCYQ**

**PVDDKRYRYAFHSSS**

**HI**

**HI**

**TKAGRRMFPSYK**

**GKAEHPDSPATV**

**VSMS**

**GL**

**GAH**

**LL**

**KLKLTNNH**

**FGMDPMADYMLLMDFV**

**YQNHK**

**DPNPFI**

**VRAA-QLCNPFAKGFR**

**WMKDPFL**

**VR**

**FADNKFHEVIM**

**PQA**

**FKEILHRVYI**

**FP**

**LKIKH**

**LNSL**

**EMIVTKNPGRLYV**

**ETT**

**VG---GTQRMI**

**------SHS**

**PVDG--A**

**GTEMII**

**GAHRYKDFVAA**

**LDPF**

**GQ**

**HPDSPNFKV**

**LDPH**

**LTN**

**GTEMII**

**AM**

**VSFQAR**

**WMR**

**ITQLKIENHPDSPAT**

**vgo/tbx1** is required within the surrounding mesendoderm. To

**mutants. Expression in the otic vesicles begins at approximately the eight-somite stage and persists in 72 hpf larvae (Fig. 3C,D,J-L). **tbx1** is initially expressed throughout the ear epithelium (Fig. 3J) as well as in the apical poles of the cells within the sensory patches (christae, Fig. 3K,L; asterisks) and semicircular canals (Fig. 3K,L; arrows). Expression is absent in the anteroventral pole of the otocyst (Fig. 3J, arrow), which may correspond to the primordium of the anterior macula, the only sensory patch that forms in the **vgo** mutant.

**vgotbx1** is required non-cell autonomously in neural crest cells and cell autonomously in the ear

As **vgo/tbx1** mutants exhibit defects in the neural crest-derived head skeleton where **tbx1** is not expressed, we tested whether **vgo/tbx1** is required within the surrounding mesendoderm. We attempted to rescue **vgo/tbx1** mutant cartilages (AB* background) by placing wild-type cells into the pharyngeal mesendoderm. Transplantation of unmanipulated wild-type cells usually results in small mesendodermal clones. To achieve larger clone sizes in the pharynx, we injected the wild-type donors prior to transplantation with Tar*, an activated version of the type 1 TGF-β-related receptor Taram-A (Tar) (David and Rosa, 2001; David et al., 2002; Peyrieras et al., 1998). Tar is normally expressed in endodermal precursor cells and injection of its activated form converts blastomeres to an endodermal fate. Tar*-injected cells behave like endogenous endodermal cells in transplantation experiments (David and Rosa, 2001; Peyrieras et al., 1998; Renucci et al., 1996). Relevant to our experiments is the fact that transplanted cells do not induce ectopic expression of downstream nodal targets (David and Rosa, 2001). As expected, donor cells in the mosaic host embryos contribute largely to the pharynx, endodermal pouches of the pharyngeal arches and the digestive tract. In 12 out of 43 **vgo** larvae (28%) in which transplanted wild-type cells contributed to the mesendoderm of the pharyngeal arches, we observed partial rescue of the neural crest-derived arches, and injection of its activated form converts blastomeres to an endodermal fate. Tar*-injected cells behave like endogenous endodermal cells in transplantation experiments (David and Rosa, 2001; Peyrieras et al., 1998; Renucci et al., 1996). Relevant to our experiments is the fact that transplanted cells do not induce ectopic expression of downstream nodal targets (David and Rosa, 2001). As expected, donor cells in the mosaic host embryos contribute largely to the pharynx, endodermal pouches of the pharyngeal arches and the digestive tract. In 12 out of 43 **vgo** larvae (28%) in which transplanted wild-type cells contributed to the mesendoderm of the pharyngeal arches, we observed partial rescue of the neural crest-derived pharyngeal cartilages (Fig. 4A,B). Rescue was generally unilateral, corresponding to the side to which most transplanted cells contributed, and in separate cases included restoration of cartilages in the mandibular (Fig. 4B, n=4) and branchial arches (Fig. 4A; n=3). These results suggest that cartilage defects in **vgo/tbx1** result from defective signaling from the endoderm.
7/21 = 30\%). The semicircular canals on the contralateral side (Fig. 4C), leading to an increase in ear size at 5 dpf (Fig. 4E; to the semicircular canals of just one ear of the mutant embryo injected transplanted wild-type cells occasionally contributed Fig. 3.

Development and disease
clearly

The endodermal pouches (e) and mesodermal cores (m) of the arches are (G) Horizontal section through the pharyngeal region of a 36 hpf embryo. In the posterior arches, the endodermal pouches are visible. (F) Lateral view of vgo/tbx1 primordia of the pharyngeal arches (p1-p7). OV, otic vesicle. (E) 27 hpf. vgo/tbx1 expression commences within the otic placode (arrow). (D) Twenty-somite stage (19 hpf). vgo/tbx1-expressing cells are now organized into the primordia of the pharyngeal arches (p1-p7). In the anterior arches, the mesodermal core is in focus. In the posterior arches, the endodermal pouches are visible. (G) Horizontal section through the pharyngeal region of a 36 hpf embryo. The endodermal pouches (e) and mesodermal cores (m) of the arches are clearly vgo/tbx1 positive. (H,I) tbx1 is expressed in pharyngeal arch muscles. (H) At 48 hpf, vgo/tbx1 is expressed in most of the pharyngeal arch muscles that also express myod (I). (J-L) High magnification views of vgo/tbx1 expression within the otic vesicle. (J) 24 hpf. (K) 48 hpf. (L) 72 hpf. Expression is initially found throughout the otic vesicle except for the anteroventral corner (arrow in J). Strong expression is maintained in the developing cristae (asterisks in K,L) and semicircular canals (arrows in K,L). ah, adductor hyoideus; am, adductor mandibulae; ih, interhyoideus; hh, hyohyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; sh, sternohyoideus; tv, transversus ventralis.

Fig. 3. Expression of vgo/tbx1 during zebrafish embryogenesis. (A) Animal pole view. (B-E,G) Dorsal views and (F,H-L) lateral views. (A) In a shield stage embryo (6 hpf), expression is confined to the hypoblast cells of the shield (arrow) and flanking region. (B) Six-somite stage (12 hpf). Expression cells are now organized into the cranial paraxial mesoderm (thin lateral stripe, marked by an arrowhead) and a more medial group of mesenchyme cells consisting of prospective pharyngeal endodermal cells and cells of parachordal mesoderm (arrow). (C) Ten-somite stage (14 hpf). New expression commences within the otic placode (arrow). (D) Twenty-somite stage (19 hpf). vgo/tbx1-expressing cells are now organized into the primordia of the pharyngeal arches (p1-p7). OV, otic vesicle. (E) 27 hpf. vgo/tbx1 expression within the mesodermal core and endodermal epithelia of individual arches. Anterior towards the left. (F) Lateral view of vgo/tbx1 expression in a 30 hpf embryo. In the anterior arches, the mesodermal core is in focus. In the posterior arches, the endodermal pouches are visible. (G) Horizontal section through the pharyngeal region of a 36 hpf embryo. The endodermal pouches (e) and mesodermal cores (m) of the arches are clearly vgo/tbx1 positive. (H,I) tbx1 is expressed in pharyngeal arch muscles. (H) At 48 hpf, vgo/tbx1 is expressed in most of the pharyngeal arch muscles that also express myod (I). (J-L) High magnification views of vgo/tbx1 expression within the otic vesicle. (J) 24 hpf. (K) 48 hpf. (L) 72 hpf. Expression is initially found throughout the otic vesicle except for the anteroventral corner (arrow in J). Strong expression is maintained in the developing cristae (asterisks in K,L) and semicircular canals (arrows in K,L). ah, adductor hyoideus; am, adductor mandibulae; ih, interhyoideus; hh, hyohyoideus; ima, intermandibularis anterior; imp, intermandibularis posterior; sh, sternohyoideus; tv, transversus ventralis.

In another set of experiments we observed that non-Tar*-injected transplanted wild-type cells occasionally contributed to the semicircular canals of just one ear of the mutant embryo (Fig. 4C), leading to an increase in ear size at 5 dpf (Fig. 4E; 7/21 = 30\%). The semicircular canals on the contralateral side that did not receive wild-type cells remained small (Fig. 4D).

This indicates that in contrast to neural crest cells, cells in the semicircular canals require vgo/tbx1 cell-autonomously to develop properly.

Regulatory interactions between vgo/tbx1, edn1 and hand2
Edn1 and Hand2 (previously known as dHand) are expressed in the pharyngeal arches and cause phenotypes similar to DGS if knocked-out in mice (Kurihara et al., 1995; Kurihara et al., 1994; Srivastava et al., 1997; Thomas et al., 1998) or mutated in zebrafish (Miller et al., 2000). Edn1 is a small signaling peptide which regulates Hand2 (called hand2 in zebrafish), a bHLH transcription factor expressed in the neural crest cells surrounding the mesodermal core of the arches (Charite et al., 2001; Miller et al., 2000). In both mouse and zebrafish, edn1 is expressed in a similar pattern to tbx1, in pharyngeal arch epithelia (both surface ectoderm and pharyngeal endoderm) and in the mesodermal core (Fig. 3E, Fig. 5A). This prompted us to test whether vgo/tbx1, edn1 and hand2 interact in a regulatory pathway. The existence of zebrafish mutants with mutations in all three genes (vgo/tbx1, suc/edn1, and han/hand2) facilitates the study of their epistatic relationships.

In situ hybridization experiments revealed that edn1 expression is strongly reduced in vgo/tbx1 mutants, particularly in the posterior arches (Fig. 5A,B). This lack of expression is not due to the lack of cells that normally express edn1, as mesenchymal cells and the ectodermal lining of the pharyngeal arches, where edn1 is normally expressed, are present in the posterior arches of vgo mutant embryos (Piotrowski and Nüsslein-Volhard, 2000). In addition, edn1 and tbx1 are co-expressed in the pharyngeal arches (Fig. 3E, Fig. 5A,C,E). Even though disorganized, tbx1 mRNA persists in cells of the pharyngeal arches of vgo mutant embryos up to ~28 hpf (Fig. 5C,D), whereas edn1 expression in vgo mutants is reduced or absent in these cells (Fig. 5E,F). This demonstrates that many cells that normally express edn1 are still present in vgo, yet fail to express edn1 when tbx1 is mutated.

In vgo/tbx1 mutants, the expression of hand2 in neural crest cells is reduced in the two anterior pharyngeal arches and absent in the posterior arches (Fig. 5G,H). However, expression in the cardiac mesoderm and fin buds is unaffected, supporting studies showing that Hand2 expression is controlled by different enhancers in these two regions (Charite et al., 2001; Thomas et al., 1998). Defects in hand2 expression in vgo/tbx1 mutants again are not simply due to the absence of neural crest cells, as in situ hybridization experiments with crestin, a pan-neural crest marker (Luo et al., 2001; Rubinstein et al., 2000) shows the presence of neural crest cells even posterior to the otic vesicle of mutant embryos (Fig. 5J,L, arrows).

Therefore, reductions in edn1 and hand2 expression in vgo/tbx1 mutants are probably due to gene regulation defects. A simple regulatory cascade in which vgo/tbx1 is upstream of edn1 and hand2 predicts that vgo/tbx1 expression is unaffected in edn1/suc and hand2/suc mutant embryos, which is indeed
the case (Fig. 6A,B). In addition, *hand2* is downregulated in *edn1/suc* mutants (Miller et al., 2000), whereas in *hand2/han* mutants, *edn1* expression is normal (Fig. 6C). The results of these in situ hybridization experiments suggest that *vgo/tbx1* acts upstream of *edn1* and *hand2*.

A conditional knockout of *Fgf8* in mouse has demonstrated that in the mandibular arch *Edn1* is regulated by *Fgf8* (Trumpp et al., 1999; Tucker et al., 1999). In zebrafish, we did not detect a regulation of *edn1* by *fgf8*, as *edn1* expression is normal in *fgf8* mutant *acerebellar* embryos (ace) (Reifers et al., 1998) (data not shown). In mice, it has also been shown that *Fgf8* acts downstream of *Tbx1* (Vitelli et al., 2002). We analyzed *fgf8* expression in *vgo/tbx1* mutants (*vgo*<sup>208</sup> and *vgo*<sup>tu285</sup>, AB* background). However, in zebrafish, no alterations of *fgf8* expression were detected (Fig. 6D) suggesting that *fgf8* is not downstream of *vgo/tbx1*. We also tested whether *fgf8* might be upstream of *vgo/tbx1*. However, *tbx1* expression is normal in *ace/fgf8* mutants, suggesting that *fgf8* is not upstream of *vgo/tbx1* (Fig. 6E,F). Thus, it is likely that in zebrafish other *Fgf* genes are functionally redundant with *fgf8* in pharyngeal arch development.

**Endodermal *vgo/tbx1*-expressing cells induce *edn1***

To confirm the result from the in situ hybridization experiments, we tested if *vgo/tbx1* regulates *edn1* by transplantsing wild-type endodermal cells at the blastula stage into *vgo/tbx1* mutants. In three out of 20 *vgo/tbx1* mutants that had transplanted cells in the pharynx, we observed
upregulation of edn1 (Fig. 7B). However, transplantation of
cells at this early stage leads to small mesendodermal clones,
because many cells also contribute to the ectoderm. The small
number of transplanted endodermal cells in vgo/tbx1
mutants might explain why we only observed rescue of edn1 expression
in a relatively low number of mutant embryos. Therefore, we
also transplanted Tar* injected wild-type cells into vgo
mutants to ensure that the transplanted cells contributed to
the endoderm. In this experiment, 119 embryos received
transplanted cells in the pharyngeal region. Among these were
34 vgo/tbx1 mutants, out of which 32 showed an increase in
the expression level of edn1 close to the transplanted cells
(94.7%, Fig. 7C, arrows indicate transplanted cells). In many
embryos, edn1 expression was not only detected in the
transplanted cells themselves but also in several tiers of cells
adjacent to the transplanted cells. As vgo/tbx1 is a transcription
factor, an intermediate gene product must exist that transduces
the signal over several cell diameters. Consequently, our data
suggests that vgo/tbx1 is regulating edn1 indirectly, although
we cannot rule out that vgo/tbx1 also is regulating edn1 directly
in a subset of cells.

Discussion

vgo encodes tbx1

In this report, we show that the vgo mutation affects the
zebrafish tbx1 gene. In vgo mutant embryos, pharyngeal
pouches 2-6 do not develop, the pharyngeal cartilages of the
different pharyngeal arches fuse with each other, thymus
development is impaired and the ear remains very small. With
the exception of the ear defect, all other defects can be
attributed to abnormal development of the pharyngeal
endoderm, specifically to defective endodermal pouch
development (Piotrowski and Nüsslein-Volhard, 2000). In
agreement with the mutant phenotype, tbx1 is expressed in
cranial paraxial mesoderm, pharyngeal endoderm, the
mesenchyme surrounding the aortic arches and in the otic
vesicle, all of which are disrupted in vgo mutants. The
homozygous phenotype of zebrafish vgo mutants closely
resembles the phenotypes of individuals with DiGeorge
Syndrome (DGS), including craniofacial, thymic, aortic arch
and ear defects (Ryan et al., 1997). Even though a deletion that
includes Tbx1 accounts for most malformations in the DGS,
not all patients carry a mutation in that gene (Lindsay, 2001),
and mutations in several other genes cause phenotypes
associated with DGS. Nevertheless, recent studies in mice have
shown that Tbx1 is one of the main candidates for causing the
aortic arch phenotype in DGS (Jerome and Papaioannou, 2001;
Lindsay et al., 2001; Merscher et al., 2001). An important
difference between the different species is that DGS is caused
by heterozygous deletions in humans, and in mice homozygous
null mutations in Tbx1 lead to an almost complete set of DGS-
like phenotypes whereas only the aortic arch phenotype is
observed in heterozygotes. By contrast, vgo is a recessive gene
in zebrafish and no phenotype is observed in the heterozygous
embryos. T-box genes have been shown to be dose sensitive
(Hatcher and Basson, 2001) and different sensitivities to Tbx1

Fig. 6. Expression analysis of tbx1, edn1 and fgf8 in mutants.
(A,B) Expression of vgo/tbx1 in 29 hpf suc (A) and 28 hpf han (B)
embryos. (C) Expression of edn1 in han embryos. (D) fgf8
expression in the mandibular arch of vgo/tbx1 (30 hpf).
(E,F) vgo/tbx1 expression in 36 hpf wild-type (E) and ace (F)
embryos.

Fig. 7. Rescue of edn1 expression by transplanted endodermal cells in vgo/tbx1 embryos. Transplanted cells in brown (labeled with biotin) and
edn1 expression in blue. (A) Expression of edn1 in a 27 hpf wild-type control embryo. (B) vgo/tbx1 host embryo in which transplanted wild-
type cells induced upregulation of edn1 (arrows). (C) vgo/tbx1 host embryo, in which Tar*-injected endodermal cells contributed to the pharynx
on only one side. On the control side, edn1 expression is weak and disorganized. The side that received endodermal cells (brown cells, arrows)
shows much stronger expression of edn1, which includes cells located several cell diameters away from the transplanted cells (arrowheads point
at pigment cells). Flat mounts in dorsal views with anterior towards the left in all panels.
dose may account for these differences. Nevertheless, given the similarity of the observed defects, both the mouse and the zebrafish models present valuable opportunities for the study of DGS.

**vg/o/tbx1 function in the endoderm is required for normal development of neural crest-derived pharyngeal structures**

In our description of the vgo mutant phenotype, we hypothesized that the neural crest defects are secondary to defects in the pharyngeal endoderm (Piotrowski and Nüsslein-Volhard, 2000). This suggestion is supported by the expression pattern of tbx1, which is not expressed in neural crest cells. Furthermore, we showed that transplantation of wild-type endodermal cells can rescue cartilage formation in vgo/tbx1 mutants. The fact that neural crest induction and migration appear normal in vgo (Piotrowski and Nüsslein-Volhard, 2000) also supports this conclusion. These three results suggest that the mesendoderm is the source of a secreted signal required for the proper differentiation of neural crest cells in the pharyngeal arches.

DGS often has been described as being caused by autonomous defects in the development of neural crest cells, an explanation supported by the fact that neural crest ablation in chicks results in very similar phenotypes, as have been reported for DGS. By contrast, similar to our findings in vgo/tbx1 mutant fish, a study in Tbx1/–/– mice suggests that neural crest defects are indirect (Kochilas et al., 2002). However, as TBX1 is not the only gene responsible for causing DGS, downstream targets of TBX1 that are expressed in neural crest cells may also represent candidate genes for causing DGS. In contrast to what we found for neural crest cells, we have demonstrated that vgo/tbx1 is required cell autonomously in the outgrowing semicircular canals of the ear. Therefore, TBX1 may not only be responsible for the pharyngeal arch defects characteristic of DGS but also for the ear defects found in these individuals (Reyes et al., 1999; Funke et al., 2001).

**Genetic components of the tbx1 pathway in the pharyngeal arches**

A large number of genes are expressed in the pharyngeal arches, underscoring the genetic complexity of their development (Garg et al., 2001). vgo/tbx1 appears to be one of the earliest genes involved in this process as disruption of its function affects the segmentation of the endodermal pouches (Piotrowski and Nüsslein-Volhard, 2000), which is one of the earliest events in arch morphogenesis. In mice and chickens, Shh (Garg et al., 2001; Yamagishi et al., 2003), Fgf8 (Frank et al., 2002; Vitelli et al., 2002) and Vegf (Stalmans et al., 2003) have been suggested to be part of the Tbx1 pathway.

Among genes implicated in DGS and in pharyngeal development, zebrafish mutations have been isolated in fgf8, tbx1, edn1 and hand2, and therefore we focused our study on these loci. Our experiments suggest regulatory interactions between the tbx1 and the edn1 pathways. In mice, such a relationship might not exist, as Edn1 expression has been reported to be normal in the aortic arch endothelial cells of Tbx1/–/– animals (Kochilas et al., 2002). However, expression of Edn1 in the pharyngeal arches of Tbx1/–/– mice has not been directly tested. In zebrafish, transplantation of wild-type cells into vgo/tbx1 mutant embryos showed a clear increase in edn1 expression in the pharynx in cells close to the transplanted cells (Fig. 7B,C). The fact that edn1 expression was also induced in cells up to several cell diameters away from the transplanted cells implies that tbx1 regulates one or several intermediate factors that in turn regulate edn1, possibly in addition to direct regulation in certain cell types.

We found that gene regulation within the anterior and posterior arches differs slightly (Fig. 8C). This result is not surprising, given that most jaw mutants mainly affect either the anterior or the posterior arches (Piotrowski et al., 1996; Schilling et al., 1996). A model for genetic interactions between vgo/tbx1, edn1 and hand2 in the pharyngeal arches and a summary of expression data are presented in Fig. 8. Within the first arch of the mouse, Edn1 has been shown to be regulated by Fgf8 (Trump et al., 1999; Tucker et al., 1999), and Tbx1 and Fgf8 have been shown to interact genetically (Funke et al., 2001; Vitelli et al., 2002).
zebrafish, fgf8 does not appear to regulate edn1, as fgf8/ace mutants (Reifers et al., 1998) do not show defects in edn1 gene expression (data not shown). Likewise, vgo/tbx1 does not appear to be upstream of fgf8, as fgf8 expression is normal in vgo/tbx1 mutants (Fig. 6F). Owing to gene duplication events, the zebrafish genome contains more redundant genes than mice, and it is possible that other Fgfs play a role in zebrafish pharyngeal arch development. This is likely because, in contrast to mice and chicks, in which fgf8 is expressed in all endodermal pouches, fgf8 is only expressed in the mandibular arch in zebrafish. Regulation of edn1 by another member of the Fgf family may be responsible for the remaining, although reduced, edn1 and hand2 expression still present in the first arch of vgo/tbx1 (Fig. 5B; see Fig. 8C).

Within the posterior arches, vgo/tbx1 might play the dominant role in regulating edn1, as edn1 expression is strongly reduced in the posterior arches of vgo mutant fish (Fig. 5B). We further suggest that in the posterior arches hand2 is not only regulated by edn1 but also by other genes, because in suc/edn1 mutants, hand2 expression is still detectable in this region (Miller et al., 2000). We hypothesize that hand2 might also be regulated by tbx1 independently of edn1 via an unknown intermediate gene (‘?’ in Fig. 8C). This hypothesis is supported by the finding that in vgo/tbx1 mutants, hand2 expression is strongly reduced in the posterior arches (Fig. 5H), whereas suc/edn1 embryos still show expression (Miller et al., 2000).

In summary, the identification of tbx1 as the gene responsible for the vgo mutation allows for mechanistic studies of pharyngeal development in zebrafish and adds a potentially useful model system to the available tools for understanding the etiology of DGS.

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