Fibulin-1 is required for morphogenesis of neural crest-derived structures

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A B S T R A C T

Here we report that mouse embryos homozygous for a gene trap insertion in the fibulin-1 (Fbln1) gene are deficient in Fbln1 and exhibit cardiac ventricular wall thinning and ventricular septal defects with double outlet right ventricle or overriding aorta. Fbln1 nulls also display anomalies of aortic arch arteries, hypoplasia of the thymus and thyroid, underdeveloped skull bones, malformations of cranial nerves and hemorrhagic blood vessels in the head and neck. The spectrum of malformations is consistent with Fbln1 influencing neural crest cell (NCC)-dependent development of these tissues. This is supported by evidence that Fbln1 expression is associated with streams of cranial NCCs migrating adjacent to rhombomeres 2–7 and that Fbln1-deficient embryos display patterning anomalies of NCCs forming cranial nerves IX and X, which derive from rhombomeres 6 and 7. Additionally, Fbln1-deficient embryos show increased apoptosis in areas populated by NCCs derived from rhombomeres 4, 6 and 7. Based on these findings, it is concluded that Fbln1 is required for the directed migration and survival of cranial NCCs contributing to the development of pharyngeal glands, craniofacial skeleton, cranial nerves, aortic arch arteries, cardiac outflow tract and cephalic blood vessels.

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Introduction

Fibulin-1 (Fbln1) is the first described member of a seven-gene family of extracellular matrix (ECM) proteins that have a common structural signature consisting of a series of repeated EGF-like domains followed by a C-terminal element referred to as a ‘fibulin module’ (Argraves et al., 2003; Chu and Tsuda, 2004). Alternative splicing produces two Fbln1 variants in nematodes, chickens and mice and four variant polypeptides in humans. These variants differ at their C-termini and appear to have distinct biological activities based on their ability to differentially bind ECM proteins (Sasaki et al., 1995) and growth factors (Brooke et al., 2002; Perbal et al., 1999) and associate with distinct networks in the ECM (Muriel et al., 2006).

A major function ascribed to Fbln1 is regulation of cell motility and guidance. During Caenorhabditis elegans development, Fbln1 is required for proper guidance of migrating distal tip cells engaged in gonad morphogenesis (Kubota et al., 2004; Kubota and Nishiwaki, 2003). In Fbln1-deficient nematode embryos, an abnormal widening of sheets of gonadal cells occurs combined with a failure of distal tip cells to complete their normal migration to the midline of the animal (Hesselson et al., 2004). Based on findings from in vitro studies, Fbln1 can suppress the motility (i.e., migration velocity and persistence time) of many types of cancer cells (Hayashido et al., 1998; Lee et al., 2005; Qing et al., 1997; Twal et al., 2001). However, Fbln1 alone is apparently neither adhesive nor motility suppressive, but acts to suppress the motility promoting activity of other ECM proteins including fibropectin (FN) (Twal et al., 2001), one of its principal binding proteins (Balbona et al., 1992). Evidence that Fbln1 can inhibit motility promoting activity of other matrix proteins stems from its ability to inhibit the migration of cells through Matrigel (Qing et al., 1997), a basement membrane protein rich extract that lacks FN. This finding is consistent with the requirement for Fbln1 in regulation of distal tip cell guidance in C. elegans, a species whose genome does not encode FN and whose ECM is basement membrane-like (Kramer, 1997).

During vertebrate development, Fbln1 has been indirectly implicated as a regulator of cell motility based on the fact that it is expressed in association with migrating mesenchymal cells including...
endocardial cushion cells and neural crest cells (NCCs) (Bouchey et al., 1996; Kern et al., 2006; Spence et al., 1992; Zhang et al., 1995; Zhang et al., 1993). A number of Fbln1-binding ECM components including FN, versican, perlecan and laminin α5 have been implicated in the regulation of embryonic cell motility, particularly in the migration and guidance of cranial NCCs (Bronner-Fraser, 1993; Perris and Perissinotto, 2000). For example, in mice deficient in laminin α5 there are abnormalities in the migration of NCCs from rhombomeres 6 and 7. This results in improper condensation of the NCC primordia that give rise to cranial nerves IX and X (Coles et al., 2006). Similarly, deficiency of integrin β1, a subunit of integrins which bind both laminin α5 and FN (Kikkawa et al., 2000), leads to defective NCC migration from rhombomeres 6 and 7 and abnormal formation of cranial nerves IX and X (Pietri et al., 2004). Loss of ECM constituents that regulate NCCs can also result in cardiac defects. For example, deficiency in perlecan, a hyaluronan (HA) binding proteoglycan implicated in NCC migration, leads to increased numbers of mesenchymal cells in the cardiac outflow tract (OFT) and transposition of the great arteries (Costell et al., 2002). By contrast, increased expression of the proteoglycan versican, a known inhibitor of NCC migration (Dutt et al., 2006), correlates with a decrease in NCCs lateral to the neural tube and in the pharyngeal arches 4 and 6 of Pax 3 mutants (Henderson et al., 1997). These mutants exhibit a failure of OFT septation owing to insufficient numbers of NCCs in the OFT (Conway et al., 1997).

Here we report that mouse embryos deficient in Fbln1 display abnormalities of the OFT, cardiac septa, aortic arch arteries, pharyngeal glands, skull bones, cranial nerves and blood vessels of the head and neck. These and other findings presented support the conclusion that Fbln1 plays an important role in the process of NCC guidance and survival required for proper morphogenesis of cranial neural crest-derived structures.

Materials and methods

Generation of Fbln1 gene trap mutant mice

To develop a Fbln1-deficient mouse strain we used an ES cell line, XST011 (parental cell line E14Tg2a4 derived from 129/OlaHsd strain), which has a gene trap insertion in the Fbln1 gene (Skarnes et al., 1995; Strye et al., 2003) (Bay Genomics, San Francisco, CA). The inserted gene trap element is comprised of a strong splice acceptor, a CD4 transmembrane domain, a β1-galactosidase (β1-gal) cassette and a neuromin resistance cassette (Neo). By inclusion of a CD4 transmembrane domain upstream from the β1-gal element, the truncated Fbln1-CD4-β1-gal fusion protein is targeted for insertion into the endoplasmic reticulum (ER) in a type I configuration such that it is retained tethered to the ER membrane with the β1-gal domain facing the cytosol (Skarnes et al., 1995). The XST011 ES cells were injected into C57BL/6 blastocysts that were then transferred to foster mothers to obtain chimeric mice. Two germ line competent male chimeras were XST011 ES cells were injected into C57BL/6 blastocysts that were then transferred to foster mothers to obtain chimeric mice. Two germ line competent male chimeras were

RT-PCR

To confirm that embryos homozygous for the gene trap insertion were deficient in each of the two mouse Fbln1 splice variants, Fbln1C and D, PCR was performed on cDNA from E9.5 embryos using a sense strand primer, 5′-CCACCAAGGCGCAGAGCGACGAGG-3′ (residues 1398048; Fbln1 and Fbln1; Gi 396820) and two antisense strand primers, 5′-CCCTCATCCCTCCACGCTGCTGAGG-3′ (residues 1960–1934 in Gi 1398048) and 5′-GGAGTCTCGAAGGTTCCCTTCTGTGATG-3′ (residues 7304974). The resulting cDNA were subcloned into pCRII (Invitrogen) and used as templates to generate digoxigenin-labeled antisense riboprobes. Whole-mount in situ hybridizations were carried out essentially according to methods described in Hogan et al. (1994). DIG-labeled antisense RNA probes were detected by RCI/MB-NBT substrate (Roche, Indianapolis, IN).

Histology and immunohistochemistry

Embryos were harvested from pregnant females following matings of mice heterozygous for the Fbln1 gene trap insertion. A piece of the yolk sac was isolated for genotyping. Embryos were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde and then embedded in paraffin and sectioned at 5 μm thickness. Immunohistochemical staining was performed on deparaffinized sections using a high temperature citric acid protocol (H-3300, Vector Laboratories Burlingame, CA). Sections were incubated with rabbit anti-Fbln1 (15 μg/ml) (Argraves et al., 1990), anti-Grb1 (1:100, Abcam, Cambridge, MA), anti-α-sarcosomicin actin (1:500 asces fluid, Sigma Chemical Corp, St. Louis, MO), anti-pax3 (5 μg/ml, Developmental Studies Hybridoma Bank (DSHB) The University of Iowa, Department of Biological Sciences, Iowa City, IA) or anti-α-smooth muscle actin (5 μg/ml, Sigma). For mouse monoclonal anti-Fbln1 (SA11) (Tran et al., 2007) an immunolabeling, a pertussin K treatment (20 μg/ml in PBS) was used as neomycin resistance cassette (Neo). By inclusion of a CD4 transmembrane domain upstream from the β1-gal element, the truncated Fbln1-CD4-β1-gal fusion protein is targeted for insertion into the endoplasmic reticulum (ER) in a type I configuration such that it is retained tethered to the ER membrane with the β1-gal domain facing the cytosol (Skarnes et al., 1995). The XST011 ES cells were injected into C57BL/6 blastocysts that were then transferred to foster mothers to obtain chimeric mice. Two germ line competent male chimeras were generated and bred with C57BL/6 mice.

Genotyping

The genotypes of offspring were determined from tail clip genomic DNA by PCR using three primers. To detect the wild-type Fbln1 allele, PCR was performed using Fbln1 primers, 5′-AGCCCAAGGCTTCTTCATGAGC-3′ (residues 71058–71085 in Gi: 15591330) and 5′-GCAAGCAGCTGTGCTGGAGGAAG-3′ (residues 71366–71339 in Gi: 15591330). To detect homozygotes, the latter primer was used with a CD4 primer, 5′-CTCTCAAGATGTCGACGTTGGC-3′ (residues 743–768 from plasmid pGZT2MPS, Bay Genomics). Cycling parameters for PCR were: 20 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min. The expected size for the amplicon produced from the wild-type allele is 308 bp. The expected size for the amplicon produced from the targeted Fbln1 allele is 414 bp.

In situ hybridization

Whole-mount in situ hybridization of E8.3–10.5 embryos was performed using antisense riboprobes for Fbln1, Sox-10 and Crabp1. cDNA inserts corresponding to each of these genes were generated using the following primer pairs: 5′-GGAATTAATCCATGATGCGTTTCCGAAACG-3′ and 3′-GCACTCTGATATAGCTGCTTGCTTCCG-3′ (Sox10, 1613 bp); 5′-CAGTTTCTCCCGAAGAGGACGAGGACG-3′ and 3′-GCACTCTGATATAGCTGCTTGCTTCCG-3′ (Crabp1, 1993 bp).


To map the position of the gene trap insertion element within the mouse Fbln1 gene

To determine the consequence of the gene trap insertion on Fbln1 mRNA splicing, RT-PCR analysis was performed using RNA isolated from E9.5 heterozygous embryos. A Fbln1 sense strand primer 5′-CCTCATCTGGTACAGCTGAGGCT-3′ (residues 1685–1683 in Gi: 396820) and a CD4 antisense strand primer 5′-GCTCCTAGATTTGAGCAGTG-3′ (residues 1167–1143 in Gi: 7304952) were used to generate a fragment that contains the junctional region between Fbln1 and the CD4 transmembrane region. The deduced amino acid sequence of the resulting 500 bp fragment is presented as Supplementary information (Supplementary Fig. 2).
Whole-mount β-galactosidase analysis

Embryos were fixed in 4% paraformaldehyde/PBS for 1 h followed by a 12 h incubation in PBS containing 0.02% sodium deoxycholate and 0.01% NP-40 at 4°C. To detect β-gal activity, embryos were incubated in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl₂ and 1 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Pierce), for 1–4 h at 37°C. The embryos were then transferred to buffered formalin for 1 h and then stored in PBS with 0.02% sodium azide.

Fbln1 exon 1 deletion mouse

The mouse strain described by Kostka et al. (2001) carrying a targeted deletion of Fbln1 exon 1 (on a mixed C57BL/6 and 129/Sv background) was provided by Dr. Mon-Li Chu (Thomas Jefferson University). Mice were genotyped using the following primer pairs: 5′-TCACCTGACTCTTTGCGTCT-3′ and 5′-CTGGGTGGGGACCTAGTG-3′ for detecting the wild-type Fbln1 allele and 5′-GTCTCTGACCTTGCCCTC-3′ and 5′-TAAAGCGCATGCTCCAGACTGC-3′ for detecting the allele containing the Fbln1 exon 1 deletion.

Results

Fbln1 gene disruption by gene trap insertion

Mapping of the gene trap insertion element within the mouse Fbln1 gene (Fig. 1A) revealed that the start of the element was at position 71146 (GI: 15591330) within intron 14 (Fig. 1B and Supplementary Fig. 1 for the sequence of the 5′ junction region of the insertion within intron 14).

To determine the effect of the gene trap insertion on splicing of the Fbln1 transcript, RT-PCR was performed using a primer from exon 13 and a primer from the CD4 component of the gene trap element. DNA sequencing of the resulting amplicon revealed that the gene trap insertion caused an aberrant splicing of exon 14 to the splice acceptor site of the gene trap cassette. The mis-spliced mRNA encoded the insertion within intron 14.

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Exon 14-Gene Trap Splicing

Gene Trap Element

PCR Genotyping

A

B

C

D

Fig. 1. Disruption of the Fbln1 gene by gene trap insertion. Panel A shows the arrangement of the exons and introns in the mouse Fbln1 gene. The exons that encode the alternatively spliced portions of Fbln1C and D transcripts are connected by lines. Panel B shows the location of the gene trap element within intron 14 and the consequential aberrant splicing of exon 14 to the splice acceptor site (SA) of the gene trap element. Panel C, PCR genotyping of DNA isolated from a wild-type (+/+), embryos heterozygous (+/−) and homozygous (−/−) for the Fbln1 gene trap insertion (E13.5) were subjected to immunoblot analysis using polyclonal antibodies to Fbln1 (A) and FN (B). Panels C and D, anti-Fbln1 (green) and anti-α-sarcomeric actin (blue) immunolabeling of tissue sections containing embryonic hearts from wild-type (+/+) embryos (C) and embryos homozygous for the Fbln1 gene trap insertion (−/−) (D) (E10.5). Nuclei were stained using propidium iodide (red). Bar in panel C=150 μm and applies to panel D. Bar in panel E=10 μm and applies to panel F.

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To assess the effects of the gene trap insertion on in vivo Fbln1 protein expression, immunoblot and immunohistological analyses were performed. By immunoblot analysis using a Fbln1 polyclonal antibody there was no immunoreactive Fbln1 polypeptide in 8 M urea extracts from embryos homozygous for the Fbln1 gene trap insertion (Fig. 2A). Heterozygous embryos showed approximately half the level of Fbln1 present in wild-type embryos extracts.

Embryonic heart tissue sections from wild-type embryos and embryos homozygous for the Fbln1 gene trap insertion were next evaluated by immunohistochemistry. In wild-type embryos, Fbln1 staining is prominent in the ECM surrounding mesenchymal cells of the AV and OFT cushions (Fig. 2C). By contrast, no Fbln1 staining is prominent in the ECM surrounding mesenchymal cells of the AV and OFT cushions (Fig. 2C). Using a recombinant amino terminal fragment of mouse Fbln1 (amino acid residues 1–568), we confirmed that the polyclonal antibody used in immunohistochemistry and immunoblotting was reactive with the truncated portion of Fbln1 in the Fbln1-CD4-β-gal fusion protein

Fig. 2. Embryos homozygous for the Fbln1 gene trap insertion have no immunologically detectable Fbln1. Panels A and B, 8 M urea extracts from wild-type embryos (+/+), embryos heterozygous (+/−) and homozygous (−/−) for the Fbln1 gene trap insertion (E13.5) and (−/−) (F). The epitope for monoclonal antibody 3A11, which has been mapped to amino acid residues 30–153 of Fbln1, is contained within the Fbln1-CD4-β-gal fusion protein expressed in Fbln1 gene trap mutants since the fusion protein contains the first 568 amino acid residues of the Fbln1 polypeptide. Nuclei were stained using propidium iodide (red). Bar in panel C=150 μm and applies to panel D. Bar in panel E=10 μm and applies to panel F.

exons located downstream from the insertion element (i.e., exons 15–18) (Fig. 1D). These findings indicate that homozygous embryos lack Fbln1C and D transcripts.

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Lethality occurs in embryos homozygous for the Fbln1 gene trap insertion

Genotypic analysis was performed on 117 offspring from heterozygous matings. As a result, 37 wild-type, 80 heterozygous and 0 homozygous offspring were detected (Supplementary Table 1). This non-Mendelian ratio indicated that lethality was occurring in offspring homozygous for the gene trap insertion. To determine the stage of lethality, retrograde genotypic analysis was performed on embryos from timed heterozygous matings. From E9.5–18.5, homozygous embryos were detected at a frequency in accordance with Mendelian expectations (Supplementary Table 1). These findings indicated that homozygous embryos were dying at birth. When litters were examined immediately after birth, null embryos were observed that were hemorrhagic and either dead or experiencing labored breathing.

Fbln1−/− embryos have blood vessel and lung anomalies

Morphological analysis revealed that homozygous embryos exhibit bleeding within the eyes and petechial bleeding in the head and neck (Fig. 3). In addition, fewer superficial blood vessels are apparent in the heads of mutant embryos (Fig. 3A). These abnormalities, which were 100% penetrant, could be seen as early as E14.5 and the severity increased with gestational age. In later stage null embryos (E18.5), blood cells could be found in extravascular spaces around the spinal cord. Several homozygous embryos also displayed significant edema on the back of the head overlying the 4th ventricle. Upon histological

![Image](image_url)
examination of the eyes of E15.5 Fbln1-deficient embryos, the hyaloid blood vessels, which extend through the hyaloid cavity to the caudal surface of the lens capsule, appear distended and the hyaloid cavity appeared filled with blood cells (Figs. 3D and E).

The lungs of the Fbln1 null embryos (E17.5) appeared smaller than normal. Histological analysis revealed that the lungs of homozygous embryos have a greater cell density as compared to lungs of wild-type or heterozygote embryos of the same gestational age (Supplementary Fig. 3). Most notable is that lungs of Fbln1 nulls have few saccules.

**Outflow tract abnormalities in Fbln1−/− embryos**

Abnormalities of the remodeling of the OFT were evidenced by the high incidence of double outlet right ventricle (DORV) (Fig. 4B and Supplementary Figs. 4A–F) and overriding aorta in homozygotes (Table 1). Persistent truncus arteriosus (PTA), resulting from a failure of OFT septation, was not observed in Fbln1−/− embryos. Together, these observations indicate that Fbln1 is not required for the septation of the aorta and pulmonary artery, but that it is required for the proper rotation of the aorta and pulmonary arteries during remodeling of the OFT.

**Cardiac septation defects in Fbln1−/− embryos**

Ventricular septal defects (VSD) (both muscular and membranous) were seen in 86% of the hearts from Fbln1−/− embryos (Fig. 4D, Supplementary Figs. 4E and F and Table 1). The majority of these VSDs (53%) occurred in combination with either DORV or overriding aorta. Atrial septal defects (ASD) were also observed in 40% (6 out of 15) of Fbln1−/− hearts and included septum primum and septum secundum anomalies (Supplementary Fig. 4G). The prevalence of VSD and ASD in Fbln1−/− hearts emphasizes the important role of Fbln1 in proper cardiac septation.

**Aortic arch abnormalities in Fbln1−/− embryos**

Abnormalities of morphology of the aortic arch arteries were apparent in Fbln1 null embryos. The right subclavian artery (RSA), which is derived from the right 4th-pharyngeal arch artery and branches off the brachiocephalic artery was missing from its normal position in 20% of the Fbln1−/− mutants (Fig. 4F). Moreover, in several of these mutants, there was an anomalous blood vessel that emerged from the aorta, caudal to the left subclavian, that passed behind the esophagus (i.e., retroesophageal RSA). In addition, constrictions of the right common carotid artery were also apparent in one Fbln1 null (Fig. 4H). These anomalies are consistent with defective remodeling of the pharyngeal arch arteries, a process that requires proper NCC investment into the arch artery sheath layers (Bockman et al., 1989).

**Myocardial wall defects in Fbln1−/− embryos**

In 40% of Fbln1−/− hearts (6 of 15 at E16.5–18.5), the compact layer of the ventricular wall myocardium of both ventricles was thinner compared to that of wild-type hearts (Supplementary Fig. 5).

**Pharyngeal gland abnormalities in Fbln1−/− embryos**

Examination of the pharyngeal glands of Fbln1-deficient embryos revealed a number of morphological abnormalities. In all Fbln1 null embryos examined (E16.5–18.5, n=15), the thymus, which is derived from the third pharyngeal pouch, was hypoplastic (Fig. 5A). Thymic aplasia or uni-lobe thymus was not observed in null embryos. Fbln1−/− embryos also displayed hypoplasia of the thyroid, which is derived from the 3rd and 4th pharyngeal arches and pouches (Fig. 5A).

**Craniofacial skeletal abnormalities in Fbln1−/− embryos**

Analysis of the ossified and cartilaginous tissues of the craniofacial skeleton of Fbln1 mutant embryos (E17.5) revealed decreased ossification of most cranial bones, particularly the frontal, parietal, tympanic ring, nasal and premaxillary bones (Figs. 5B–D). There was also reduction in the size of many skull bones in the nulls including the mandible (micrognathia) (Fig. 5C) and tympanic rings (Fig. 5D) as well as an overall reduced cranial size as compared to littermate controls. No cleft palate was observed in the Fbln1 nulls. The neural crest-derived elements of the cartilages of the throat (i.e., hyoid and thyroid) also appear reduced in the nulls as compared to wild-type embryos (data not shown).

**Fbln1 expression in the neural crest, somites, pharyngeal arches and distal OFT/secondary heart field**

As a result of the gene trapping, β-gal is placed under the control of the Fbln1 promoter. X-gal staining was performed to establish the pattern of Fbln1 expression in heterozygous embryos. X-gal staining was apparent in presomitic mesoderm from E8.75 to E9.0 (Figs. 6A–D).
X-gal staining was also prominent in somites from E8.75 to E10.5 (Figs. 6A–E). X-gal staining was also apparent along the margins of neural folds in caudal regions of the embryo and dorsal regions of the neural tube (Figs. 6A and C, arrowheads). At E10.5, the dorsal neural tube region was relatively free of X-gal stained cells, however, pronounced X-gal staining was apparent in populations of cells lateral to the hindbrain, around the otic vesicle, in pharyngeal arches 1–3, in anterior and posterior regions of the somites, in the limb buds and in the region of the cardiac inflow tract (Fig. 6E).

Consistent with the X-gal staining, immunohistological analysis showed pronounced Fbln1 immunolabeling in the ECM of the mesenchyme lateral to the neural tube at the level of the pharyngeal arches in E9.5–10.5 embryos (Figs. 7A and B). By contrast, relatively low levels of Fbln1 immunolabeling were detected in the arch mesenchyme directly adjacent to the aortic sac/distal OFT (Figs. 7A and B, brackets). At E9.5, this area was rich in Pax3-expressing NCCs (Fig. 7C, brackets). Prominent Fbln1 labeling was evident in the ECM of the distal OFT, in the region of the SHF (Figs. 7C and D, arrowheads). Pharyngeal arch mesenchyme underlying the neural tube had high levels of Fbln1 and few Pax3-positive cells (Fig. 7C, asterisk). Little or no Fbln1 labeling was apparent in pharyngeal arch ectoderm or endoderm at E9.5–10.5.

Whole-mount in situ RNA hybridization analysis of wild-type E8.3–9.5 embryos showed that Fbln1 mRNA is expressed in presomitic mesoderm and somites, similar to what was observed in X-gal stained embryos heterozygous for the gene trap insertion (Fig. 8). Fbln1 RNA was also detected in rhombomeres 2, 4, 6/7 and in pharyngeal arches 1–4 (Figs. 8C and D). The rhombomere expression of Fbln1 is similar to that of the NCC marker Crabp1 (Fig. 8E). Together, the findings from Fbln1 expression analysis indicate that Fbln1 expression is associated with migratory pathways of NCCs derived from rhombomeres 2–7, which contribute to morphogenesis of the OFT and pharyngeal glands. However, it is not known whether Fbln1 is expressed by migrating NCCs or by non-neural crest derived-mesenchymal cells located along the path of migrating NCCs.

**Fbln1 gene trap nulls have cranial nerve patterning abnormalities**

NCCs derived from rhombomeres 2, 4, 6 and 7 also contribute to the formation of the cranial nerves. In situ hybridization analysis using a Sox-10 probe revealed abnormalities in the patterning of cranial nerve NCCs in Fbln1 null embryos (Figs. 9A–C). In particular, interruptions and inappropriately directed streams of Sox-10-positive NCCs that comprise the primordia for cranial nerves IX (glossopharyngeal) and X (vagus) were observed (Figs. 9B and C). The apparent misguidance of NCCs resulted in abnormal mixing of the distal regions of the glossopharyngeal and vagal streams (Figs. 9B and C, brackets), which may be interpreted to have resulted from a caudal misdirection of the glossopharyngeal stream. Abnormal branching of the proximal region of the vagal stream was also observed in some embryos (data not shown).

To evaluate the impact of Fbln1 deficiency on cranial nerves, E10.5 embryos were immunostained to detect neurofilament-M. The results reveal abnormalities in cranial nerves IX and X (Figs. 9D–F). Null embryos displayed an interruption in the proximal portion of cranial nerve IX and a less than normal degree of branching in the distal segment (Figs. 9E and F). Furthermore, there was an abnormal fusion of cranial nerves IX and X in the epibranchial placode region (Fig. 9E). Cranial nerve X in Fbln1 null embryos also appeared more compact and less reticulated than in wild-type embryos (Figs. 9E and F).

**Fbln1 gene trap nulls have increased apoptosis of NCCs in the hindbrain**

TUNEL analysis was performed to assess the consequence of Fbln1 deficiency on NCC survival. Null E10.5 embryos displayed increased
levels of TUNEL-positive cells in the hindbrain region as compared to wild-type embryos (Figs. 9G and H). In particular, relatively high levels of TUNEL-positive cells were apparent in the otic vesicle epithelium and around rhombomeres 4, 6 and 7 as compared to wild-type embryos. To determine if the apoptotic cells observed in Fbln1 nulls might correspond to NCCs, immunolabeling was performed using antibodies to the NCC marker, Crabp1. Crabp1-positive cells were observed in null embryos in streams extending from rhombomeres 4, 6 and 7, which corresponded to areas having high levels of TUNEL-positive cells (Fig. 9I).

**Genetic background influences penetrance of cardiac anomalies in Fbln1 nulls**

While the bleeding, lung abnormalities and perinatal lethality in Fbln1 gene trap homozygotes are in agreement with an earlier analysis of mouse Fbln1 deficiency (Kostka et al., 2001), abnormalities of the OFT, arch arteries, pharyngeal arches and cephalic skeleton were not previously reported. Differences in genetic background could account for the phenotypic disparities given that the Fbln1 gene trap homozygotes were a mixed C57BL/6 and 129P2/OlaHsd background whereas embryos homozygous for Fbln1 exon 1 deletion were a mixed C57BL/6 and 129Sv background (Kostka et al., 2001). When we examined embryos homozygous for the Fbln1 exon 1 deletion we found hypoplastic thymus (100% penetrance) as well as cardiac defects corresponding to those observed in embryos homozygous for the Fbln1 gene trap mutation (e.g., VSD associated with DORV) (Supplementary Fig. 6). However, the penetrance of cardiac defects in the Fbln1 exon 1 mutants was lower than that of the Fbln1 allele carrying the Fbln1 gene trap insertion or homozygous for Fbln1 exon 1 deletion.

### Table 2

<table>
<thead>
<tr>
<th>Cardiac/OFT anomaly</th>
<th>Anomaly penetrance (%)</th>
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<tr>
<td>Fbln1 gene trap homozygotes (129P2/OlaHsd: C57BL/6)</td>
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<td>Fbln1 gene trap homozygotes (C57BL/6)</td>
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<td>Fbln1 Δ exon 1 homozygotes (C57BL/6)</td>
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<tr>
<td>Fbln1 gene trap (129P2/OlaHsd: C57BL/6)/Fbln1 Δ exon 1 (C57BL/6)</td>
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1The background of embryos homozygous for the Fbln1 gene trap insertion was 129P2/OlaHsd:C57BL/6 backcrossed to C57BL/6 for 2 generations. Percentages indicated are based on evaluation of 15 embryos homozygous for the Fbln1 gene trap insertion.

2The background of embryos homozygous for the Fbln1 gene trap insertion was 129P2/OlaHsd:C57BL/6 backcrossed to C57BL/6 for 5 generations. Percentages indicated are based on evaluation of 13 embryos (E15.5–18.5) homozygous for the Fbln1 gene trap insertion.

3The background of embryos homozygous for the Fbln1 gene trap insertion was 129P2/OlaHsd:C57BL/6 backcrossed to C57BL/6 for 5 generations. Percentages indicated are based on evaluation of 14 embryos (E15.5–18.5) homozygous for the Fbln1 gene trap insertion.

4Percentages indicated are based on evaluation of 9 embryos (E15.5–18.5) each having one Fbln1 allele carrying the Fbln1 gene trap insertion (129P2/OlaHsd:C57BL/6 background as described in column 1) and the other Fbln1 allele carrying the Fbln1 exon 1 deletion (C57BL/6 background as described in column 3). Note that all embryos of this genotype displayed thymic hypoplasia.
Cardiac neural crest ablation is also associated with an absence or poor development of cranial nerves IX and X (Kuratani et al., 1991). The IXth cranial nerve, the glossopharyngeal nerve, derives from NCCs of rhombomere 6 and innervates the third pharyngeal arch (D’Amico-Martel and Noden, 1983). The Xth cranial nerve, the vagus nerve, derives from NCCs of rhombomere 7 and innervates the fourth pharyngeal arch. In Fbln1 nulls, hypoplasia and patterning anomalies of the NCC precursors for these cranial nerves is observed in E10.5 embryos as revealed by Sox-10 in situ hybridizations. The patterning anomalies include interruptions and apparent misguidance in the streams of Sox-10-positive NCCs that comprise each nerve primordia. The apparent misguidance is manifested by abnormal mixing of the distal regions of the glossopharyngeal and vagal streams of Sox-10-positive NCCs. These abnormalities in Sox-10-expressing NCCs are mirrored by abnormalities in the cranial nerves of Fbln1 nulls as revealed by neurofilament-M immunolabeling. Specifically, the proximal portion of cranial nerve IX in nulls was missing and the distal epibranchial placode-derived region was often fused with the vagus nerve. Given the evidence that post otic rhombomere-derived NCCs (i.e., rhombomere 6–7 Sox-10-positive cells) contribute to the heart (Montero et al., 2002), the observed abnormalities in rhombomere 6–7 NCC patterning might also be a contributing factor to the OFT defects in Fbln1 nulls.

Abnormalities in Fbln1 nulls, including patterning defects of cranial nerves IX and X, are also seen in Hoxa3 null mice (Chisaka and Capecchi, 1991). Like the Fbln1 nulls, mice deficient in Hoxa3 die at or shortly after birth and display hypoplastic thymus and thyroid (Chisaka and Capecchi, 1991). Hoxa3 nulls also display arch artery and heart abnormalities resembling those seen in Fbln1 nulls (Chisaka and Capecchi, 1991). Hoxa3 is expressed in rhombomeres 5–8, which contribute NCCs to the formation of cranial nerves IX–XII. In Hoxa3 nulls, the formation of cranial nerves IX and X is affected in a manner similar to that seen in Fbln1 nulls, with a fusion of the epibranchial placode regions of the two nerves and a gap in the proximal portion of IX (Watari et al., 2001). Evidence that cranial nerve anomalies in Hoxa3 nulls are a consequence of NCCs defects comes from the finding of reduced Sox-10–positive cells in streams emanating from rhombomeres 6/7 (Watari et al., 2001). Again, these findings are consistent with our results showing an apparent reduction in the level of Sox-10 expression in the stream of NCCs that comprise the primordia for cranial nerve IX as well as a mixing of Sox-10 positive cells in distal regions of the glossopharyngeal and vagal streams.

An underlying basis for the mixing and interruptions of NCC streams emanating from rhombomeres 6 and 7 seen in Fbln1 nulls may be defects in NCC motility and guidance. Defective migration of Sox-10-positive NCCs emanating from rhombomeres 6 and 7 has been implicated in cranial nerve IX interruptions observed in mice deficient in Hoxa3 (Watari et al., 2001). Migration defects of rhombomere 6–7–derived, Sox-10-positive NCCs have also been speculated to account for the gap in glossopharyngeal nerves observed in mice doubly deficient for the Nk-related homedomain transcription factors Msx1 and Msx2 (Ishii et al., 2005). Additional evidence for NCCs of Msx1/ Msx2 double mutants having migration problems comes from the finding of Sox-10–positive NCCs in the normally NCC-free zone of rhombomere 3 (Ishii et al., 2005). As a result of this apparent misguidance/unrestricted migration, there is a fusion of the trigeminal and facial cranial nerves. Indeed, in Fbln1 nulls we observed Sox-10–positive cells in the normally NCC-free zone between the glossopharyngeal and vagal NCCs streams and a subsequent fusion of the two nerves. These findings are consistent with the known roles of Fbln1 as a suppressor of motility (Hayashido et al., 1998; Lee et al., 2005; Qing et al., 1997; Twal et al., 2001) and a regulator of directed cell migration (Kubota et al., 2004).

The abnormalities of Fbln1 nulls are similar to the constellation of congenital malformations of the aortic arch, heart, thymus, thyroid and bones of the head that are associated with DiGeorge syndrome.
(DGS) (Baldini, 2004; Liao et al., 2004). The majority of DGS individuals are heterozygous for a microdeletion of human chromosome 22q11.2; however, the remaining individuals do not possess a 22q11.2 deletion indicating that mutation of genes outside of the region may cause the disorder. The Fbn1 gene is located outside of the 22q11.2 deletion region at 22q11.3 (Korenberg et al., 1995). Since heterozygous deletion of Fbn1 in mice did not lead to OFT, aortic arch, cardiac, pharyngeal gland or craniofacial anomalies, Fbn1 haploinsufficiency does not lead to the same outcome as occurs with haploinsufficiency of genes located within the 22q11.2 deletion region. The variable penetrance and severity of DGS also indicate that genes from outside of the microdeletion region may act as modifiers of the DGS pathogenesis pathway. Several candidate DGS modifier genes have been identified through gene deletion studies in mice. For example, Hoxa3 deficiency in mice leads to a DGS-like phenotype (Chisaka and Capeschi, 1991). In addition, FgB8, and Gbx2, an Fgb8-inducible transcription factor (Liu and Joyner, 2001), when deleted individually or in combination in mice leads to cardiac, aortic arch and craniofacial anomalies consistent with features of 22q11 deletion in humans (Abu-Issa et al., 2002; Byrd and Meyers, 2005; Frank et al., 2002).

The similarities that exist between the DGS phenotype and that of Fbn1-deficient embryos raises the possibility that Fbn1 has links to the mechanism by which 22q11 deletion causes DGS pathogenesis. Interaction with the FgB8 signaling pathway represents one plausible mechanism. Fgb8 signaling is mediated by Fgfr1 and 2 interaction with the adaptor protein, Crkl (Moon et al., 2006), whose deficiency in mice causes many of the anomalies seen in DGS (Guris et al., 2001) and in Fbn1 nulls. Mechanistically, Crkl participates in activation of the Raf-1/MEK/Erk signaling pathway (Aral et al., 2006) and mediates FN-integrin-induced cell migration (Li et al., 2003) and cell survival. Indeed, Crkl deficiency in mice leads to decreased survival of NCCs that contribute to the pharyngeal arches and OFT (Moon et al., 2006), an outcome similar to what is observed in the Fbn1 nulls. Although Fbn1 is not known to interact with FgB8, Fbn1 does regulate Erk activation and FN-induced cell migration (Twal et al., 2001). Future studies will need to test this and other possible interactions between Fbn1 and the repertoire of genes associated with DGS.

Acknowledgments

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Appendix A. Supplementary data


References


