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FSHD region gene 1 (FRG1) is crucial for angiogenesis linking FRG1 to facioscapulohumeral muscular dystrophy-associated vasculopathy

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SUMMARY

The genetic lesion that is diagnostic for facioscapulohumeral muscular dystrophy (FSHD) results in an epigenetic misregulation of gene expression, which ultimately leads to the disease pathology. FRG1 (FSHD region gene 1) is a leading candidate for a gene whose misexpression might lead to FSHD. Because FSHD pathology is most prominent in the musculature, most research and therapy efforts focus on muscle cells. Previously, using Xenopus development as a model, we showed that altering frg1 expression levels systemically leads to aberrant muscle development, illustrating the potential for aberrant FRG1 levels to disrupt the musculature. However, 50-75% of FSHD patients also exhibit retinal vasculopathy and FSHD muscles have increased levels of vascular- and endothelial-related FRG1 transcripts, illustrating an underlying vascular component to the disease. To date, no FSHD candidate gene has been proposed to affect the vasculature. Here, we focus on a role for FRG1 expression in the vasculature. We found that endogenous frg1 is expressed in both the developing and adult vasculature in Xenopus. Furthermore, expression of FRG1 was found to be essential for the development of the vasculature, as a knockdown of FRG1 resulted in decreased angiogenesis and reduced expression of the angiogenic regulator DAB2. Conversely, tadpoles subjected to frg1 overexpression displayed the pro-angiogenic phenotypes of increased blood vessel branching and dilation of blood vessels, and developed edemas, suggesting that their circulation was disrupted. Thus, the systemic upregulation of the FRG1 protein shows the potential for acquiring a disrupted vascular phenotype, providing the first link between a FSHD candidate gene and the vascular component of FSHD pathology. Overall, in conjunction with our previous analysis, we show that FRG1 overexpression is capable of disrupting both the musculature and vasculature, recapitulating the two most prominent features of FSHD.

INTRODUCTION

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant myopathy characterized by progressive atrophy of the facial, shoulder and upper arm muscles. In addition to the muscular dystrophy, 50-75% of FSHD patients have retinal vasculature abnormalities (Fitzsimons et al., 1987; Padberg et al., 1995b), and the upregulation of vasculature system genes is an early event in FSHD muscle pathology (Osborne et al., 2007). Recently, muscle biopsies from FSHD patients revealed that the mesoangioblasts, an adult myogenic mesodermal stem cell occupying the perivascular niche in muscle tissue, were impaired in their ability to differentiate into skeletal muscle (Morosetti et al., 2007). Thus, FSHD progression and pathology, although predominantly evident in skeletal muscle, probably have underlying contributing or causal factors that are associated with the vasculature.

The FSHD genetic lesion is a contraction of the tandem array of 3.3 kb D4Z4 repeats at chromosome 4q35 to below a threshold of 11 copies (Lunt et al., 1995; Wijmenga et al., 1992). This repeat contraction is hypothesized to affect the expression of neighboring gene(s). Based on analysis of gene expression and proximity to the D4Z4 repeats, FRG1 (FSHD region gene 1), which is located 100 kb centromeric to the contracted D4Z4 region, is a leading candidate disease gene for FSHD (van Deutekom et al., 1996). Since FSHD prominently affects voluntary muscles, studies on FRG1 have mainly focused on its role in muscles. Previous work found that FRG1 expression is crucial to the formation of normal muscle structure and that overexpression of the FRG1 protein adversely affects muscles during development (Hanel et al., 2009). Similarly, transgenic mice with a 25-40-fold increase in FRG1 expression, specifically in the skeletal muscle, develop a dystrophic muscle phenotype (Gabellini et al., 2006). However, measurements of FRG1 mRNA levels from the muscles of FSHD patients have varied, including a 25-fold increase in expression, unchanged expression and a 5-fold decrease in expression, when compared with control muscles (van Deutekom et al., 1996; Gabellini et al., 2002; Jiang et al., 2003; Winokur et al., 2003; Osborne et al., 2007). Thus, a correlation between FRG1 mRNA levels in skeletal muscle and FSHD pathology remains inconclusive and controversial.

The protein encoded by the FRG1 gene at chromosome 4q35 in humans is highly conserved in vertebrates and invertebrates [97% amino acid (a.a.) identity with the mouse, 81% a.a. identity with X. laevis and 46% a.a. identity with C. elegans] (Grewal et al., 1998). FRG1 expression has been detected in all human tissues that have been tested, including human embryonic brain and muscle, as well as the placenta (van Deutekom et al., 1996), suggesting that it has functions outside of the muscle. Here, we show prominent expression of FRG1 in vascular tissues during Xenopus development and in adult frog muscle capillaries. Knockdown of FRG1 results in decreased angiogenesis and loss of the Xenopus vascular marker DAB2. Overexpression of the FRG1 protein resulted in an opposite phenotype of increased angiogenesis and dilated blood vessels. Identifying FRG1 as a mediator of...
angiogenesis provides insights into the mechanisms of FSHD disease pathology and further supports the FRG1 overexpression disease model for FSHD.

RESULTS
FRG1 is expressed in vascular tissues
Two of the prominent pathological effects in FSHD patients are the progressive appearance of dystrophic skeletal muscle and retinal vasculopathy. Previously, we analyzed FRG1 expression in *X. laevis* during early development of the musculature (Hanel et al., 2009). Here, we analyze the X. laevis vasculature to address any potential vascular role for FRG1. Embryos that were sectioned, either sagitally or transversely, and immunostained for FRG1 (Fig. 1A) show significant FRG1 expression within the pronephros (Fig. 1B,C), posterior cardinal vein and arteries (Fig. 1C), indicating that FRG1 is in fact expressed in the vasculature. However, ubiquitous levels of frg1 transcripts from maternal stores, which progressively decrease during development, potentially introduce an elevated non-tissue-specific background that is not indicative of de novo expression (Hanel et al., 2009).

A transgenic approach was used in order to determine the tissues in which active frg1 expression was taking place. The proximal frg1 promoter sequence from *X. tropicalis*, consisting of a 645 base pair (bp) upstream regulatory region and 636 bps of the transcribed 5’ untranslated region (UTR), was inserted upstream of EGFP (enhanced green fluorescent protein) into a transgene cassette that was flanked by chicken β-globin insulator [hypersensitive site 4 (HS4)] doublets (Fig. 1D) and used for transgenesis. This proximal promoter construct produced a consistent expression pattern in transgenic animals (61% of transgenic tadpoles, n=44), which was visualized by fluorescence (Fig. 1E), with 16% displaying an underlying identical pattern with additional non-uniform spotted expression and 23% showing variable small patches of expression. Overall, the predominant pattern of fluorescence that we observed suggested that promoter expression was heaviest within the eye, dorsal-anterior portion of the head, branchial arches, and at the dorsal and ventral portions of the somites. As EGFP is known for its stability, de novo transcript levels were more clearly examined by in situ hybridization using an egfp probe. When examined in this manner, egfp expression in transgenic embryos coincided largely with fluorescence at stage 32 (Fig. 1E,F). However, as the tadpoles developed, egfp expression became more confined to vascular and muscle tissues (Fig. 1G,H). By stage 42, when much of larval organogenesis is complete, transgenic egfp expression was predominantly vascular, with weak staining throughout the epaxial tail muscle and around the gut (Fig. 1H). Although potentially missing some regulatory elements, the ability of a 645 bp regulatory region of frg1 to direct gene expression specifically within vascular tissues strongly supports a role for frg1 in vascular development.

The expression of the FRG1 protein was characterized further after metamorphosis in cross sections of adult frog gastrocnemius muscle. FRG1 immunostaining showed strong expression in cells at the periphery of muscle fibers (Fig. 1I). This staining pattern shows that FRG1 co-localizes with the capillary marker lectin.
FRG1 links vascular defects to FSHD-like muscle

(Christie and Thomson, 1989), demonstrating that the FRG1 protein is associated with capillaries within frog muscle (Fig. 1J,K). Beyond the capillaries, FRG1 was also strongly expressed in froglet arteries and veins (Fig. 1N). To ensure the specificity of the antibody, peptide competition with the antigenic peptide was performed and resulted in an absence of FRG1 staining (Fig. 1L,M). Control slides, in which the FRG1 primary antibody was omitted, were consistently negative (data not shown). Thus, similar to stage 42 transgenic tadpoles, the strongest expression of FRG1 within frog muscle is associated with the vasculature.

**FRG1 is required for complete angiogenesis and expression of the vascular marker DAB2**

To determine if FRG1 is required for vasculature development, FRG1 levels were reduced by specific morpholino (MO) injection. One cell from each 2-cell stage embryo was injected with an frg1 morpholino (FMO1), which has previously been reported to adversely affect muscle development (Hanel et al., 2009), or with a non-specific control morpholino (CMO). Embryos were then examined at stage 34-36 by whole-mount in situ hybridization for dab2, which encodes a vascular marker that acts as an angiogenic regulator, required for sprouting of intersomitic veins (Cheong et al., 2006). FMO1-injected embryos showed a marked, dose-dependent reduction in dab2 transcript levels (40 ng of FMO1: 100% reduction, n=42; 20 ng of FMO1: 76% reduction, n=38); the embryos were scored based on overall dab2 expression in the areas with the clearest dab2 signal, namely the pronephric sinus, posterior cardinal vein and vascular vitelline network (Fig. 2A-F,M). In the embryos with milder phenotypes, fewer branches of the vascular vitelline network were visible. In many embryos, this reduction was severe enough to result in a complete loss of dab2 in the vascular vitelline network and, strikingly, from the intensely stained pronephric sinus (Fig. 2B,E). Although a small number of CMO-injected embryos displayed a mild reduction in dab2 staining on the injected side (26% reduction, n=42), these embryos did not display the acute loss of dab2 that was seen in FRG1 morphants (Fig. 2C,F). In addition, dab2 loss was specific to FMO1 knockdown of FRG1 because dab2 expression levels and patterns were successfully rescued by co-injection of FMO1 with a non-complementary mRNA encoding the X. tropicalis frg1 protein (Fig. 2H,I,K,L,N).

To determine whether FRG1 knockdown led to a general loss of vasculature and, thus, of all vascular transcripts, or whether this effect was more specific to the transcript encoding FRG1, in situ hybridization was performed on FMO1 morphants for msr, which encodes an early vascular marker and which can be used to identify vascular endothelial cells (Devic et al., 1996). Interestingly, morphant embryo halves did not display an acute loss of msr transcripts, as levels within the posterior cardinal vein were comparable to those in the non-injected control half of the embryo. However, FMO1 injection was observed to cause either partial or complete (20 ng of FMO1: 76% reduction, n=21; 40 ng of FMO1: 75% reduction, n=12) inhibition of intersomitic vein sprouting (Fig. 2G,J) compared with background levels (40 ng of CMO: 18% reduction, n=27). The maintenance of the posterior cardinal vein and lack of intersomitic vein sprouting indicated that, unlike vasculogenesis, angiogenesis is disrupted by FRG1 knockdown. Together, these data suggest that FRG1 is required for the establishment of the intact vasculature, specifically in angiogenic branching from existing vessels.

**Overexpression of the FRG1 protein increases vasculature levels and angiogenesis**

FSHD has been associated previously with elevated levels of FRG1 (Gabellini et al., 2002); therefore, the effects of overexpression of the FRG1 protein were examined. Systemic levels of FRG1 were increased through mRNA injections using the X. tropicalis frg1 mRNA, co-injected with a tracer mRNA. These FRG1-overexpressing embryos were compared with embryos that had...
Expression of the frg1 transgene leads to edemas and vascular abnormalities

To analyze the vascular defects associated with consistent and tissue-specific elevations in FRG1 levels, transgenic animals that overexpress frg1 from the frg1 proximal promoter (FRE-FRG1) were generated and were analyzed for vascular abnormalities. The FRE-FRG1 expression cassette was flanked by HS4 and the Drosophila Fab8 insulator sequences in order to maintain frg1 expression upon genome integration. At stage 34, tadpoles were separated into either transgenic or non-transgenic groups based on expression of gamma crystalline-GFP within the eye. Upon examination of the embryos at stage 46, an increased number of tadpoles had developed ventral edema (Fig. 4B), a phenotype associated with lymphatic system defects or vascular defects (Inui et al., 2006; Rodrigues et al., 2008). Although background levels of this phenotype were 18% (n=37) for non-transgenic sperm recipients, more than a two-fold increase in this phenotype was observed in the transgenic tadpoles (44%, n=32). Animals from both transgenic and non-transgenic pools that appeared normal at stage 46-48 were examined for vascular defects in the tail by using fluorescein-labeled dextran injections into the heart. Animals were examined exclusively for gross vascular abnormalities, which we found to be double branching of the dorsal lateral vein (compare Fig. 4D with Fig. 4E). A two-fold increase in these defects was observed in transgenic animals (45%, n=20) compared with the background levels in non-transgenic animals (22%, n=22). These results confirm that elevated expression of FRG1 within the vascular tissues, where FRG1 is endogenously expressed, is capable of producing significant developmental vascular defects.

DISCUSSION

Epigenetic disregulation leading to the overexpression of FRG1 has been a leading candidate for the mechanism that mediates FSHD pathology; however, all of the previous studies have focused on muscle expression, muscle biopsies and muscle cell culture. Here, a role for FRG1 in the development of vascular structures has been uncovered. We find that an elevated level of FRG1 not only disrupts skeletal muscle (Hanel et al., 2009), consistent with previous work in a mouse model for FSHD (Gabellini et al., 2006), but also leads to an increase in the size and branching of the vasculature structure; both of these results are consistent with data from the tissues of FSHD patients. The vascular component of FSHD has been described in the retina of the eye where the vasculature can be readily visualized; however, the upregulation of gene transcripts in the muscle biopsies from the vascular smooth muscle and endothelial cells (Osborne et al., 2007) imply a more systemic disruption of vasculature in FSHD. One benefit of using Xenopus development for these studies is the ability to visualize intact vascular structures throughout the body and determine the systemic effects of altered FRG1 levels. Our analysis of the eye vasculature was somewhat hindered in the tadpoles that overexpress the FRG1 protein, owing to the more complex nature of the vasculature in the ocular region, which made it difficult to visualize disruption of the architecture. However, consistent with the results from elsewhere in the body, dab2 expression was clearly reduced in the eyes of embryos injected with FMO1 (supplementary material Fig. S1), suggesting that the eye vasculature is similarly regulated. Thus, the frg1 overexpression phenotype in Xenopus correlates strongly with both the afflicted tissues and the clinical...
findings from FSHD patients, consisting of dystrophic muscle with a variable fiber size, retinal vasculopathy, and misregulation of gene transcription in the vascular smooth muscle and endothelial tissues (Fitzsimons et al., 1987; Padberg et al., 1995b; Padberg et al., 1995a; Osborne et al., 2007).

The finding that FRG1 mediates angiogenesis and not vasculogenesis was deduced from the maintenance of the posterior cardinal vein and loss of intersomitic vein sprouting in FRG1 morphants. In agreement, the opposite effect, overbranching of vascular structures, was observed in animals with elevated levels of FRG1. Similar to our FMO1 injection experiments, the reduction of DAB2 in Xenopus led to an inhibition of intersomitic vein sprouting through the loss of vascular endothelial growth factor (VEGF) induction (Cheong et al., 2006). It is possible that the loss of intersomitic sprouting in the FRG1 depletion experiments occurs as a direct consequence of the reduction in DAB2. However, FRG1 is probably not a direct regulator of dab2, as elevated levels of FRG1 did not appear to lead to increased levels of dab2 staining. Furthermore, whereas overexpression of DAB2 led to a lack of intersomitic vein sprouting (Cheong et al., 2006), elevated levels of FRG1 led to improper branching and dilation of the vasculature. In fact, overexpression of the FRG1 protein in the intersomitic veins more closely resembles the effects of misexpression of ephB4, which encodes an ephrin receptor that is part of a second pro-angiogenic pathway involving ephrins, which are also involved in somitogenesis (Helbling et al., 2000). Our study indicates that, beyond maintaining dab2 transcript levels, FRG1 is functioning as a pro-angiogenic factor.

The maintenance of dab2 transcript levels by FRG1 may affect many tissues beyond the vasculature. DAB2 is highly expressed in many adult mammalian tissues and is most commonly known as a mediator of clathrin-associated endocytosis (Oleinikov et al., 2000; Kowanel et al., 2003) and a key component of the transforming growth factor (TGF)-β signaling pathway. As a component of this signaling pathway, DAB2 has many functions including roles as a tumor suppressor (Fazili et al., 1999), a mediator of epithelial-to-mesenchymal transition (EMT) (Prunier and Howe, 2005), and in cellular migration (Hocevar et al., 2005). Furthermore, DAB2 is also involved in the inhibition of Wnt-stimulated cellular proliferation through its association with Axin (Hocevar et al., 2003; Jiang et al., 2007). In the vasculature, DAB2 functions in endodermal organization, differentiation (Morris et al., 2002; Yang et al., 2002) and in early angiogenesis through induction of VEGF by activin-like signaling (Cheong et al., 2006). Therefore, FRG1 expression is crucial for a wide variety of functions simply by its maintenance of DAB2.

A role for overexpression of the FRG1 protein in FSHD is still controversial, partly because of inconsistencies between gene expression studies in cell lines and FSHD muscle, the lack of information on the precise function of FRG1, and recent studies supporting potential roles for alternative candidate genes. Our studies strongly suggest that misexpression of the FRG1 protein is capable of producing both the musculature and vasculature pathology seen in FSHD (Hanel et al., 2009). In addition, our data are consistent with the disrupted cell signaling pathway model for FSHD that is based primarily on extending the clinical observations of FSHD patient pathology to diseases of known cause that exhibit similar pathology. Coats’ disease is caused by a mutation in Norrin, a ligand to the Wnt receptor Frizzled-4 (Zerlin et al., 2008) and exhibits retinal vascular abnormalities that are highly similar to those found in FSHD. The symptoms of FSHD and Coats’ disease both include the appearance of thick tortuous vessels within the retina, similar to the vessels found in this study with overexpression of FRG1. Frizzled-4 mutations, which are linked to familial exudative vitreoretinopathy, lead to similar retinal vasculopathy along with progressive auditory defects (Zerlin et al., 2008), a symptom that has also been associated with FSHD. Thus, owing to these similarities between FSHD and Coats’ disease, FSHD is speculated to involve a defect in the Wnt signaling pathway, a mechanism that is supported by our data.

Conclusions

In summary, our study has shown that FRG1 is expressed in vascular structures and is essential for angiogenesis. Examination of the tissue-specific effects of frg1 overexpression in our Xenopus system has shown that increased levels of FRG1 can account for the two prominent clinical aspects of FSHD, namely dystrophic muscle and increased angiogenesis.
METHODS

Frog husbandry

Adult *X. laevis* frogs were purchased from Xenopus Express. All procedures were carried out in accordance with established UIUC Institutional Animal Care and Use Committee (IACUC)-approved protocols for animal welfare.

In situ hybridizations, immunohistochemistry and *X. laevis* FRG1 antibody

In situ hybridizations were carried out as described (Hanel et al., 2009). Treatment and immunostaining of embryos using the XL FRG1 antibody were performed as described previously (Hanel et al., 2009). The previously characterized XL FRG1 rabbit polyclonal antibody was raised against the peptide EREAKDDDNSIPED and was produced by GenScript Corp (Hanel et al., 2009).

Sectioning and immunohistochemistry

Leg muscles dissected from froglet and adult frogs were snap frozen in isopentane, cooled in liquid nitrogen and cryosectioned into 10 μm sections. FRG1 immunostaining gave identical staining after acetone or formaldehyde fixation. Double staining with FRG1 and rhodamine-labeled *Griffonia simplicifolia* lectin I (GSL I) (Vector laboratories) was performed on sections fixed in acetone for 5 minutes. Slides were blocked in 2% donkey serum and 1% BSA. The XL FRG1 antibody was used at a dilution of 1/100 and detected with secondary donkey anti-rabbit fluorescein (1/100). After washing, GSL I was applied to slides at a concentration of 40 μg/ml and incubated for 5 minutes. For peptide competition experiments, sections were treated with 0.5% Triton X for 5 minutes, followed by fixation in 10% formaldehyde in PBS for 10 minutes. Slides were blocked in 2% donkey serum, 1% BSA, 0.1% Triton X, 0.05% Tween 20. XL FRG1 antibody was incubated with, and without, a 50-fold excess of peptide at 37°C for 2 hours, followed by 16 hours at 4°C with rocking. The antibody-peptide mixture was centrifuged at 20,000 g for 15 minutes and the supernatant was diluted in the blocking solution and used for immunostaining.

Transgenic construct cloning

All PCR products were initially cloned into pGEM-T easy vectors (Promega) and sequenced. All PCR primers are listed in the supplementary material Table S1. The pEGFP-N1 (Clontech) vector was digested with restriction enzyme (FRE) was amplified by genomic PCR (using primers 3 and 4) and reamplified (using primers 5 and 6) for cloning.

pCAIN

The pEFGP-N1 (Clontech) vector was digested with AseI and XhoI, filled in using the Klenow fragment of *Escherichia coli* DNA polymerase I and self-ligated. The plasmid was digested with AflII, filled in using the Klenow fragment and self-ligated. The chicken β-globin insulator (HS4) (Prioleau et al., 1999) was amplified by genomic PCR (using primers 7 and 8) from chicken blood DNA and reamplified using primers 9 and 10, or 11 and 12. The PCR products were digested with EcoRI and SpeI, or BamHI and XbaI, and cloned by triple ligation into the modified pEGFP-N1 vector that had been digested with EcoRI and BamHI; this process was repeated by using primers 13 and 10, or 11 and 14, and cloning into the BamHI–NotI-digested vector, resulting in pCAIN.

pCAIN FRE-EGFP

FRE was isolated from FRE-pGEM by AseI-BamHI digestion and ligated into the similarly digested pEGFP-N1 vector. The resulting vector, FRE-EGFP, was digested with AseI and AflIII, and cloned into similarly cut pCAIN.

pCAIN GC FRE-FRG1

PCR was performed on purified Drosophila genomic DNA using primers 15 and 16 to produce the Fab8 insulator. This was then reamplified using primers 17 and 18, and 19 and 20, and cloned into pCAIN. The gamma crystalline-GFP reporter was cloned from pGCG (a gift from Dr Yun-Bo Shi) (Fu et al., 2002). This vector was then digested with AseI-AflIII and ligation was performed with the similarly digested FRE-FRG1 vector.

Xenopus transgenesis

Xenopus transgenesis was carried out essentially as described previously (Kroll and Amaya, 1996; Wuebbles and Jones, 2007);

TRANSATIONAL IMPACT

Clinical issue

Facioscapulohumeral muscular dystrophy (FSHD) is the third most common myopathy, affecting approximately one out of 20,000 people worldwide. The most characteristic symptom is a progressive atrophy of facial, shoulder and upper arm muscles. Additionally, over half of FSHD patients have abnormal blood vessels in the back of the eye, which causes vision problems in some patients.

Over 95% of FSHD cases carry a genetic abnormality within a series of DNA repeats at chromosome 4q35. Although the unaffected population contains between 11 and 150 copies of this DNA repeat (named D4Z4), FSHD patients have deletions resulting in between one and ten D4Z4 repeats. This mutation is not in a known protein-encoding gene, thus these deletions are expected to disrupt the normal regulation of gene expression. However, there is little agreement regarding which gene or genes are misregulated in FSHD, thus complicating the creation of appropriate FSHD animal models, and hindering advances in understanding and treating this disease. Previous studies demonstrate that altered expression of FRG1 disrupts the musculature in developing *Xenopus laevis*, thus supporting a role for FRG1 in FSHD pathology.

Results

In this study, the spatiotemporal expression of FRG1 is analyzed during Xenopus development, revealing prominent expression in the vasculature. The authors show that FRG1 is required for the growth of new blood vessels from pre-existing vessels, but is not needed for spontaneous blood vessel formation. Conversely, increasing FRG1 levels promotes angiogenesis and disrupts the organization of the developing vasculature. Thus, normal levels and patterns of FRG1 expression are crucial for proper vascularization.

Implications and future directions

This study, combined with previous work analyzing the effect of FRG1 on the developing musculature, demonstrates that FRG1 affects the skeletal musculature and the vasculature when its expression levels are altered in an animal model. Thus, FRG1 expression is a causal factor in FSHD pathology. Prominent expression of FRG1 in the vasculature suggests that future FSHD research should examine vascular cell types and tissue, as well as skeletal muscle.

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however, the sperm nuclei were not digested with restriction enzymes. Plasmids were linearized, purified and incubated (100 ng per experiment) with sperm nuclei for 5 minutes. Xenopus egg extract (10 μl) and 20 μl of sperm dilution buffer (SDB: 250 mM sucrose, 75 mM KCl, 0.5 mM spermidine, 0.2 mM spermine) were mixed and incubated at 65°C for 5 minutes, then centrifuged at 16,000 g for 3 minutes to remove the precipitate. The soluble fraction (6 μl) was diluted to 22 μl with SDB plus 10 mM of MgCl₂, then added to the nuclei-sperm mix and incubated for 15 minutes at room temperature. The swollen nuclei were added to 170 μl of SDB and used for microinjection at a rate of 0.586 μl/minute using a microliter syringe pump (Harvard Apparatus).

**Probe constructs**

The PCRs for cloning used the Triplemaster polymerase enzyme mixture (Eppendorf); the RT-PCRs used the Superscript III HiFi one-step RT-PCR kit (Invitrogen); and restriction enzymes were purchased from New England Biolabs. All oligonucleotide primers are listed in supplementary material Table S1. Total RNA was extracted from the ovaries of *X. laevis* and *X. tropicalis* frogs with Trizol (Invitrogen), as per the manufacturer's instructions, and used into pGEM T-Easy vectors (Promega) and sequenced.

**RNA and morpholino microinjections**

Morpholino and mRNA injections were performed as described previously (Hanel et al., 2009).

**Vascular labeling in tadpoles**

Fluorescein-labeled dextran (9.2 nl) was injected into the heart of stage 46 transgenic and non-transgenic tadpoles, incubated for 30 minutes at 16°C, and visualized using a Leica dissecting microscope.

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**Author contributions**

R.D.W., M.L.H. and P.L.J. performed the experiments and analyzed data; R.D.W., M.L.H. and P.L.J. wrote the paper.

**Supplementary material**

Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.002261/-/DC1

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