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Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) is a dynamic nuclear and sarcomeric protein

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Abstract

Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) is a candidate gene for mediating FSHD pathophysiology, however, very little is known about the endogenous FRG1 protein. This study uses immunocytochemistry (ICC) and histology to provide insight into FRG1's role in vertebrate muscle development and address its potential involvement in FSHD pathophysiology. In cell culture, primary myoblast/myotube cultures, and mouse and human muscle sections, FRG1 showed distinct nuclear and cytoplasmic localizations and nuclear shuttling assays indicated the subcellular pools of FRG1 are linked. During myoblast differentiation, FRG1's subcellular distribution changed dramatically with FRG1 eventually associating with the matured Z-discs. This Z-disc localization was confirmed using isolated mouse myofibers and found to be maintained in adult human skeletal muscle biopsies. Thus, FRG1 is not likely involved in the initial assembly and alignment of the Z-disc but may be involved in sarcomere maintenance or signaling. Further analysis of human tissue showed FRG1 is strongly expressed in arteries, veins, and capillaries, the other prominently affected tissue in FSHD. Overall, we show that in mammalian cells, FRG1 is a dynamic nuclear and cytoplasmic protein, however in muscle, FRG1 is also a developmentally regulated sarcomeric protein suggesting FRG1 may perform a muscle-specific function. Thus, FRG1 is the only FSHD candidate protein linked to the muscle contractile machinery and may address why the musculature and vasculature are specifically susceptible in FSHD.

Keywords

Facioscapulohumeral muscular dystrophy; FRG1; muscle; Z-disc; sarcomere

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Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is the most prevalent of the adult muscular dystrophies (incidence of 1:7,500 – 1:14,000) and third most common overall [1,2], although its etiology is still not clear. In addition to the muscular dystrophy, 50 to 75% of FSHD patients develop retinal vasculopathy [3,4], highlighting the complex nature of FSHD pathophysiology. The genetic lesion for FSHD1A (OMIM 158900), the most common form of FSHD (~98% of all cases), is a dominant contraction of the large D4Z4 tandem repeat array at chromosome 4q35 [5,6]. Removing this large heterochromatic region alters the chromosome architecture as well as the epigenetic landscape of chromosome 4q35, and in doing so presumably changes localized gene regulation that ultimately leads to the pathology [7]. Multiple candidate genes have been proposed to lead to FSHD pathology based in part on their proximity to the deletion [8–11], their differential expression patterns in FSHD patient versus unaffected controls [12–17], and overexpression phenotypes in animal models [15,18–21]. This study focuses on the FSHD candidate gene *FRG1* (FSHD region gene 1) [22], encoding a highly evolutionarily conserved protein of unknown cellular function (Fig. S1).

FRG1, located 125kb centromeric to the FSHD1A deletion, was one of the early candidate genes for FSHD [9]. However, recent expression studies have failed to find significant *FRG1* misexpression in numerous FSHD patient-derived muscle cells and biopsies casting doubt on its involvement in mediating FSHD pathology [23–26]. Complicating the issue is the lack of understanding towards *FRG1*'s normal spatiotemporal expression, distribution, and cellular function during normal human muscle development. Initial studies using *Xenopus* as a model for vertebrate development found *frg1* was widely expressed early and throughout development, showing elevated levels in vascular tissues and developing muscles with preferential expression in the capillaries, veins, and arteries located between muscle fibers [20,21]. Knockdown and overexpression experiments confirmed a necessary role for *frg1* in development of the musculature and vasculature. Interestingly, systemic increases in *frg1* levels had specific effects on the developing musculature and vasculature, impairing myogenesis and muscle precursor cell migration and causing spurious angiogenesis leading to a tortuous vasculature [20,21]. These phenotypes are consistent with the two major pathologies seen in FSHD patients [3,27]. A similar analysis of the *C. elegans* *FRG1* homolog (*FRG-1*) showed the development, organization, and integrity of the adult body wall musculature is unique in its susceptibility to increased *FRG-1* levels. [18]. Interestingly, *FRG-1* had to be overexpressed in the spatiotemporal pattern dictated by the *FRG-1* promoter and there was no affect on the musculature when *FRG-1* was overexpressed specifically in adult muscle from the *myo-3* promoter. Although *FRG1* may function in many tissues, the developing musculature and vasculature are uniquely susceptible to systemic changes in *FRG1* levels suggesting *FRG1* has tissue specific functions. Thus, in FSHD, small pathogenic changes in *FRG1* expression may be occurring early in muscle development or also involve non-myogenic cell lineages [18,20,21].

FRG1 is proposed to be involved in aspects of RNA biogenesis and it has been identified as a component of the spliceosome [28]. Overexpression studies in cell culture have characterized *FRG1* as a nuclear and predominantly nucleolar protein [29,30]. However, work in *C. elegans* showed that the endogenous *FRG-1* is both a nuclear and cytoplasmic protein, localizing to the nucleoli and body wall muscle dense bodies, respectively [18]. *C. elegans* dense bodies form the muscle attachments and function analogous to the vertebrate Z-discs and costameres combined (reviewed in [31]), structures linked to multiple myopathies (reviewed in [32,33]). Consistent with its localization to muscle attachment sites, *FRG-1* was shown to exhibit F-actin binding and bundling activity and this activity was conserved with its human homolog, *FRG1* [18]. While providing potential insight into

FRG1's function in human muscle development, it is not known how these results translate to the human condition and potentially FSHD. Here, we present an analysis of endogenous FRG1 in muscle cells, during myotube formation, in myofibrils and myofibers, and in adult human muscle tissue biopsies.

Material and Methods

Cell Culture

HeLa cells and C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) 2 mM L-glutamine, and 1% penicillin–streptomycin. Proliferating primary human skeletal muscle myoblasts (HSMs) were obtained from Lonza (Walkersville, MD) and were seeded on 0.02% collagen-coated surfaces and maintained in SkBM-2 medium supplemented with SkGM-2 SingleQuots (Lonza) according to the manufacturer's instructions. For myotube formation, HSMs were seeded on 0.02% collagen-coated coverslips at $1.5 \times 10^4/\text{cm}^2$ density for ICC analysis, and the following day were induced to form myotubes by adding fusion medium (DMEM/F-12 50:50 supplemented with 2% horse serum). Murine muscle derived stem cells (MDSC) were isolated and cultured as described [34]. For ICC analysis, MDSCs were seeded on 0.02% collagen-coated coverslips.

Myofiber and myofibril isolation

Mice (C57/B6) were humanely euthanized in accordance with approved UIUC IACUC protocols. Adult mouse muscle fibers were isolated from the flexor digitorum brevis muscle of 1–3 month old female mice. Isolated muscles were washed briefly in DMEM, then incubated in DMEM with 0.2% collagenase type I (Worthington Biochemical, Lakewood, NJ) for 4 hrs at 37°C, with gentle agitation every 15 min and changes into fresh collagenase solution every hour. At the completion of digestion, excess tendon material was carefully dissected away, and fiber bundles were transferred to a dish of myofiber medium (DMEM supplemented with 10mM HEPES, 5% heat-inactivated horse serum, 1% penicillin–streptomycin, and 0.1% amphotericin B). Individual fibers were liberated from the muscle mass by gentle titration and agitation, and cultured overnight in myofiber medium at 37°C, 5% CO₂. The following day, healthy, undamaged myofibers were plated on glass coverslips coated with Geltrex (Invitrogen, Carlsbad, CA) and allowed to adhere for 1–2 hrs before fixation. Myofibrils were isolated as described [35] using skeletal muscle dissected from rectus femoris muscle. Purified myofibrils were plated on coverslips coated with poly-L-lysine and immediately fixed for immunofluorescence staining.

Protein extracts

To generate whole cell extracts (WCE), cells were collected in 1× PBS, pelleted, resuspended in 10 cell volumes of RIPA+ buffer (150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 50 mM Tris pH 8.0, + 1% SDS), and incubated on ice for 10 min. Lysed cells were sonicated briefly, centrifuged at 100,000×g for 10 min and the soluble fraction was used for western blotting analysis. Nuclear and cytoplasmic fractions were generated as described [36]. The PCNA rabbit monoclonal antibody (Epitomics, Inc, 2755-1) was used as marker for the nuclear fraction. Muscle protein extracts (MXT) were generated from humanely euthanized mice. Muscle was freshly dissected from the hindlimbs, snap frozen on dry ice, pulverized into a powder, and incubated in 3 volumes RIPA+ buffer.

FRG1 Antibodies

The HS1, HS2, and DM1 rabbit polyclonal antibodies were made by GenScript USA Inc. (Piscataway, NJ) and generated against synthesized peptide antigens conjugated to KLH.

The HS1 peptide (KKDDIPEEDKGNVK) and HS2 peptide (GRSDAIGPREQWEP) were from the predicted human FRG1 amino acid sequence while the DM1 peptide (TLLDRRSKMKADRYC) was from the predicted *Drosophila melanogaster* FRG1 (CG6480) amino acid sequence (Fig. S1). Antisera were affinity purified against the peptide cross-linked to NHS-Sepharose (GE Healthcare), eluted in 10 mM glycine, pH 2.5, and dialyzed against PBS pH7.4.

FRG1 protein knockdown

For ICC analysis, HeLa cells were plated at less than 50% confluence and transfected three times in 24 hr intervals using oligofectamine (Invitrogen) with the On-Target plus SMARTpool siRNA reagent (100nM final concentration) for human FRG1 (Dharmacon, Inc). This reagent contains four duplex siRNAs (siRNA1: GUUUACGGCUGUCAAAUUA; siRNA2: CGACAGAUACUGCAAGUGA; siRNA3: GGAACCAAGACGAAGAGUA; siRNA4: AAACCCAGCUUGAUUUGU). For western blotting analysis of FRG1 knockdown in HeLa cells, MISSION® short hairpin RNA (shRNA) Lentiviral Transduction Particles (Sigma-Aldrich) for Human FRG1 were purchased. HeLa cells were plated at less than 50% confluent and transfected two times with virus in 24 hr intervals (multiplicity of infection of 3), and transfected cells were selected with puromycin (1.5 µg/ml) for 5 days. Cells were collected and WCE was prepared in RIPA+ buffer as described above. Hairpin sequences in these shRNA constructs are as follows: HsFRG1-1 (Clone ID: NM_004477.1-768s1c1), 5'-CCGGGACATTCCAGAAGAAGACAACTCGAGTTTGTCTTCTTCTGGAATGTCTTTT TG -3'; HsFRG1-2 (Clone ID: NM_004477.1-737s1c1), 5'-CCGGCTGTGCTGAAAGAGAAACCAACTCGAGTTGGTTTCTCTTTCAGCACAGTT TTTG -3'.

For western blotting analysis of FRG1 knockdown in C2C12 cells, three pLKO.1-puro plasmid constructs containing shRNAs for mouse FRG1 (MmFRG1) were purchased (Sigma-Aldrich MISSION® shRNA). Each construct was packaged for viral production and infection for knockdown. As negative control, pLKO.1-puro empty vector and pLKO.1-puro containing scrambled shRNA were obtained from Addgene [37]. For viral packaging, pLKO-shRNA, pCMV-dR8.91 and pCMV-VSV-G were co-transfected into 293T cells using TransIT-LT1 (Mirus Bio LLC) at 1 µg, 0.95 µg and 0.05 µg, respectively (in 4 ml for a 6-cm plate). Viruses produced between 48 and 60 hrs after transfection were used for infection. C2C12 cells were infected with the viruses in the presence of 8µg/ml polybrene (Sigma-Aldrich) for 24 hrs and selected with 2 µg/ml puromycin for 5 days. Cells were collected and WCE was prepared in RIPA+ buffer as described above. Hairpin sequences in these shRNA constructs are as follows: MmFRG1-1 (Clone ID: NM_013522.1-233s1c1); 5'-CCGGCCAACTTGATATTGTGGGAATCTCGAGATTCCCACAATATCAAGTTGGTTT TTG -3'; MmFRG1-2 (Clone ID: NM_013522.1-862s1c1), 5'-CCGGCCAAATTGAAAGCTGACCGATCTCGAGATCGGTCAGCTTTCAATTGGTT TTTG -3'; MmFRG1-3 (Clone ID: NM_013522.1-320s1c1), 5'-CCGGCTATATCCATGCACTGGACAACCTCGAGTTGTCCAGTGCATGGATATAGTT TTTG -3'; Scrambled (Scr), 5'-CCTAAGGTAAAGTCGCCCTCGCTCTAGCGAGGGCGACTTAACCTTAGG-3'.

Nuclear shuttling assay

The assay was carried out essentially as described [38]. The HA-FRG1 expression plasmid was generated by subcloning the human FRG1 coding sequence into pcDNA3.1 HA [39]. Murine C2C12 cells (~60% confluent) were transfected with pcDNA3.1HA-FRG1 using TransIT-LT1 reagent and allowed to grow for 24 hrs. The cells were removed by trypsinization, washed with PBS, plated on poly-L-lysine coated coverslips ($1 \times 10^5/\text{cm}^2$)

and allowed to adhere for 2 hrs before non-transfected HeLa cells were overlaid ($5 \times 10^4/\text{cm}^2$) onto the transfected C2C12 cells for 3 hrs. The co-cultures were incubated with 100 $\mu\text{g}/\text{ml}$ Cycloheximide (CHX) for 15 min to stop translation, and the cells were fused by adding 50% (wt/vol) polyethylene glycol 4000 in DMEM for 2 min. The fusions were immediately washed with DMEM and then incubated with 100 $\mu\text{g}/\text{ml}$ CHX for 2, 3, or 4 hrs followed by ICC analysis. The cells were fixed with 4% formaldehyde (FA) in PBS for 15 min, immunostained with HA monoclonal antibody clone 3F10 (1:100) (Roche) as described below, and co-stained with 5 $\mu\text{g}/\text{ml}$ Hoechst 33342.

Immunocytochemistry (ICC) staining

HeLa, C2C12, and MDSC, were fixed in 4% FA in PBS and HSMM were fixed in 2% FA in PBS, for 15 min at room temperature (RT). After fixation, cells were permeabilized with 0.25% Triton-X 100 in PBS for 10 min on ice, and subsequently incubated with 2% BSA in PBS for 30 min at RT. Primary antibody incubations were carried out at RT for 1 hr up to overnight at 4°C and secondary antibody incubations were for 40 min at RT. Mouse myofibers were fixed in 2% paraformaldehyde for 15 minutes, rinsed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. Fibers were incubated in TBS-T +5% milk powder and 0.02% sodium azide for 1 hr at RT, or alternatively, overnight at 4°C, and then incubated with diluted primary antibodies and secondary antibodies as above. Mouse myofibrils were fixed with 2% FA in PBS for 15 min at RT, rinsed with PBS and permeabilized with 0.1% Triton X-100 for 10 min. Myofibrils were incubated with normal goat serum for 30 min at RT then incubated with FRG1 primary antibody followed by Alexa 488-conjugated goat anti-rabbit IgG secondary antibody as described above. Cryosections of human skeletal muscle were obtained from Telethon network of biobanks and Eurobiobank (Italy) and fixed in 10% neutral buffered formaldehyde (NBF) for 15 min at RT. After fixation, cells were permeabilized with 0.25% Triton-X 100 in PBS for 10 min on ice, then stained using Alexa Fluor SFX Kits as according to manufacturer's instruction. The antibodies and their dilutions were as follows: desmin monoclonal antibody [D9] (Santa Cruz Biotechnology Inc, sc-52326), 1:1000; sarcomeric α -actinin mouse monoclonal antibody [EA-53] (Abcam Inc, ab-9465), 1:200; COX IV mouse monoclonal antibody (Abcam Inc, ab-33985), 1:500; fast twitch myosin [A4.714], developed by Helen M. Blau [40], 1:5; FRG1 HS1 (1:100), HS2 (1:200), and DM1(1:200). Secondary antibodies used were FITC-conjugated goat anti-mouse, FITC-conjugated donkey anti-rabbit (pre-cleared), and rhodamine-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc) used at 1:100. Alexa488 or Alexa594-conjugated goat anti-rabbit IgG, highly absorbed (Invitrogen) used at 1:800. Alexa488-conjugated goat anti-mouse IgG, highly absorbed (Invitrogen) used at 1:800. F-actin was visualized with 5 units/ml rhodamine-phalloidin (Invitrogen) incubated for 30 min at RT for cell culture staining, and with 1 unit/ml rhodamine-phalloidin incubated for 2 min at RT for myofibrils. DAPI was used at 0.5 $\mu\text{g}/\text{ml}$.

Immunohistochemical staining

Formalin-fixed and paraffin-embedded normal human skeletal muscle soft tissue slides (ProSci Inc. Poway, CA) were treated with sodium citrate buffer (10mM sodium citrate, 0.05% Tween20, pH 6.0) at 90°C for 30 min for antigen retrieval. Cryosections of non-FSHD human skeletal muscle (Telethon network of biobanks and Eurobiobank, Italy) were fixed in 10% NBF for 15 min at RT. After fixation, both paraffin and cryosections were permeabilized with 0.25% Triton-X 100 in PBS for 10 min on ice. Immunohistochemical staining was performed with the ImmPress kit (Vector Laboratories) as per the manufacturer's instruction using the following antibody dilutions: 1:100 for HS1 and HS2, 1:500 for COX IV, 1:5 for fast twitch myosin [A4.714]. After the signal was developed with 3,3'-Diaminobenzidine (DAB) for 15 min, sections were counterstained with hematoxylin QS (Vector Laboratories) for nuclear staining if necessary.

Microscopy

Fluorescence images were taken by fluorescence microscopy using an Olympus BX60 microscope equipped with a SpotRT monochrome model 2.1.1 camera and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI). Confocal microscopy was carried out using Zeiss LSM510. Immunohistochemical images were acquired with Olympus BX60 microscope equipped with a Leica DFC290 camera using Leica Application Suit software (Leica microsystems). Deconvolution images were taken using a wide-field DeltaVision microscope (Applied Precision, Olympus IX71 microscope, Roper Scientific CoolSNAP HQ camera) with a 60 $\times$ (1.42 NA) oil objective. Images were deconvolved using Sedat/Agard algorithms with Applied Precision SoftWoRx v5.0 software. All images were processed using Adobe Photoshop to adjust brightness, contrast, size, and merged or split channels.

Results

Nuclear and cytoplasmic localization of endogenous FRG1

To assess the potential involvement of FRG1 in FSHD pathophysiology we first need to understand the normal cellular and developmental function of FRG1 in mammalian muscle. We have recently characterized the *C. elegans* FRG1 homolog as being both a nuclear protein and also cytoplasmically associated with body-wall muscle sarcomeres [18]. To characterize the endogenous human FRG1 protein in respect to subcellular localization in skeletal myoblasts and through myogenesis into myotubes, we generated three independent highly specific anti-FRG1 antibodies, HS1, HS2, and DM1 (Figs. 1 and S1). Western blotting of HeLa whole cell extracts showed each affinity-purified antibody reacted to the predicted 29kDa FRG1 polypeptide, however, the DM1 antibody also recognized a smaller ~18kDa polypeptide (Fig. 1M). Assaying HeLa protein fractionated into nuclear and cytoplasmic pools shows that the 29kDa FRG1 exists in the nucleus and the cytoplasm (Figure 1N), a subcellular distribution never previously reported for vertebrate FRG1 but consistent with the *C. elegans* FRG-1 localization, while the ~18kDa DM1 reactive polypeptide was exclusively nuclear (Figure 1N). BLAST searches of the NCBI human non-redundant protein database indicated that the peptides used for immunization are each unique in the human genome for FRG1. In humans, *FRG1* exists at several genomic loci due to partial duplications leaving the 4q35-localized *FRG1* as the only locus containing all nine exons and predicting a 29kDa polypeptide. Thus, the HS1 and HS2 antibodies are only detecting the FRG1 protein originating from chromosome 4q35 while the DM1 antibody may be reactive to an alternative FRG1 as well. Alternatively spliced FRG1 transcripts have been reported [9] that, if stable, could generate the smaller DM1 reactive polypeptide. The mouse genome contains one *Frg1* locus and western blotting of C2C12 cells with the DM1 antibody (Figure S2) similarly reveals the 29kDa polypeptide and a smaller polypeptide reactive only to the DM1 antibody suggesting that these smaller polypeptides originate from the conserved full-length *Frg1/FRG1* loci. The specificity of the antibodies for mammalian FRG1 was confirmed by assaying the effects of FRG1 siRNAs on FRG1 protein levels by western blotting (Figure S2).

Although all three antibodies appear highly specific for FRG1 by western blotting, the primary technique used in this study is ICC. Therefore, to characterize the specificity of the antibodies for ICC, a specific siRNA-mediated knockdown approach was used. HeLa cells were transfected three consecutive times with a pool of 4 siRNAs specific to human FRG1, subjected to immunostaining with each of the FRG1 antibodies, and compared to controls (Fig. 1A–L). Each of the FRG1 antibodies showed similar immunostaining patterns with varying intensities of both cytoplasmic and nuclear staining. In all cases the FRG1 signal was severely depleted or absent in the siRNA treated cells (Fig. 1, compare C, D with A, B; G, H with E, F; and K, L with I, J). Due to the fact that transfection efficiency was less than

100%, some cells were still positive for FRG1 staining and served as positive controls for the immunostaining procedure. We conclude that all three of our FRG1 antibodies are specific for FRG1 in the ICC procedure.

As opposed to overexpressed epitope-tagged FRG1 that appears exclusively nuclear in cell culture [29], the endogenous FRG1 in HeLa cells clearly appears to be both nuclear and cytoplasmic by immunostaining. Western blotting of HeLa cell extract fractionated into nuclear and cytoplasmic pools confirmed the dual subcellular localization of FRG1 (Fig. 1N). Although HeLa cells are a commonly used human cell line for many studies of protein function because they are fast growing and easily transfected, they are HPV17 transformed immortal adenocarcinoma cells, quite distant from muscle cells. Therefore, the unexpected cytoplasmic subcellular localization of human FRG1 was further addressed in multiple cell types including HeLa cells, primary human skeletal myoblasts, murine muscle derived stem cells (MDSC), and murine C2C12 cells (Fig. 2). In HeLa and C2C12 cells, FRG1 was predominantly localized to nuclei, however the cells displayed distinct, non-uniform fiber-like cytoplasmic FRG1 immunostaining strongly suggesting an association with a subcellular architecture. In the human myoblasts (Fig. 2Q) and the murine MDSCs (Fig. 2U) the nuclear FRG1 was much less pronounced compared with the cytoplasmic staining which appeared to surround the nucleus and was very granular. We conclude that endogenous FRG1 exists in both a nuclear and cytoplasmic pool in all cell types tested, however its cytoplasmic to nuclear distribution is cell type dependent.

FRG1 is a nuclear-cytoplasmic shuttling protein

The endogenous FRG1 is localized in both the nucleus and cytoplasm. Nuclear shuttling assays were performed (Fig. 3) to determine if these two pools of FRG1 were linked. Murine C2C12 cells, easily identifiable by their DNA-dense nuclear foci, were transfected with a plasmid expressing epitope tagged HA-FRG1 and allowed to accumulate HA-FRG1 overnight. Cycloheximide (CHX) was added to the culture media to block translation and the cells were fused with non-transfected HeLa cells, readily identifiable by their DNA poor nucleoli, in continued presence of CHX, and HA-FRG1 localization was monitored over time by ICC probing for HA. Thus, any HA signal in the HeLa cells represents FRG1 protein synthesized in the C2C12 cells. Within two hours of the fusion initiation FRG1 synthesized in a C2C12 cell (Fig. 3A–D, white arrow) had begun to accumulate in the nuclei and concentrate in the nucleoli of a fused HeLa cell (Fig. 3A–D, blue arrow). This nuclear import of FRG1 was more evident at three hours (Fig. 3E–H) and at four hours (Fig. 3I–L). As the amount of cytoplasmic HA-FRG1 is almost undetectable, we deduce that much of the HeLa nuclear HA-FRG1 came from the C2C12 nuclear HA-FRG1 and conclude that FRG1 shuttles between the nucleus and cytoplasm.

FRG1's subcellular localization changes during myogenesis

FRG1 is critical for development of the musculature and the vasculature [20,21]; therefore, the newly described subcellular dynamics for the endogenous FRG1 were examined during myogenesis (Fig. 4). Primary myoblasts from human skeletal muscle were stimulated to undergo differentiation to fuse each other and form myotubes by serum depletion, and analyzed by ICC at various time points to determine FRG1's subcellular distribution. In undifferentiated myoblasts, FRG1 was almost exclusively cytoplasmic (Fig. 4A, B), however within 24 hours after initiation of differentiation FRG1 became predominantly nuclear, and strongly nucleolar (Fig. 4C, D) in early myotubes, and by five days post-differentiation the majority of FRG1 appeared to be predominantly cytoplasmic again (Fig. 4E, F). Interestingly, by eight days post-differentiation the cytoplasmic FRG1 appeared in a striated pattern reminiscent of sarcomeres with the immunostaining being consistent between two FRG1 antibodies (Fig. 4 G, J). Co-staining for sarcomeric α -actinin showed

clear co-localization of the striated FRG1 signal with α -actinin (Fig. 4 G–L, white arrows), indicating that FRG1 was in fact localizing to the Z-disk in the sarcomere of matured myotubes. Although the majority of FRG1 immunostaining aligned with the α -actinin, a fraction of FRG1 immunostaining appeared aligned between the Z-disks, resembling M-lines (Fig. 4 G–L, blue arrows). Considering that from 2–5 days post-differentiation, α -actinin was aggregating at the sarcolemma (Fig. 4D, F), forming Z-disc-like structures in the absence of any detectable localized FRG1, we conclude that during myogenesis FRG1 associates with more matured Z-disks, after adjacent myofibrils are aligned, and is not likely involved in their establishment or initial assembly. In addition, a fraction of the sarcomeric FRG1 is not aligned with the Z-disk.

Sarcomeric FRG1 is associated primarily with the Z-disk

The previous work was carried out in cell culture system. To determine if FRG1 was a new sarcomere-associated protein in mature muscle tissue, the FRG1 antibodies were first screened by western blotting against mouse muscle extract (Fig. S3). All three antibodies react to low abundance 29kDa polypeptide as expected as well as a prominent ~40kDa polypeptide not seen in the cell culture extracts. The HS1 and HS2 antibodies showed overall similar patterns while the DM1 antibody appeared to interact with several additional smaller muscle-specific polypeptides as well. Therefore, only HS1 and HS2 were used for the muscle ICC and histology studies. Intact mouse myofibers were immunostained for FRG1, sarcomeric α -actinin, and desmin (Fig. 5). Two FRG1-specific antibodies (HS1 and HS2) showed intense striated patterns of FRG1 (Fig. 5A, E, I, M) as well as some nuclear staining (Fig. 5I, M, blue arrows) with the striated FRG1 immunostaining overlapping with sarcomeric α -actinin (Fig. 5D, H). Higher resolution confocal images revealed the precise co-localization of FRG1 with α -actinin (Fig. 5I–L) however FRG1 and desmin, while displaying highly similar patterns, did not precisely co-localize by confocal microscopy (Fig. 5M–P). As opposed to the newly formed myotubes from primary myoblasts in cell culture (Fig. 4), there was no evidence of FRG1 M-line staining in isolated myofibers suggesting the FRG1 seen in the developing myotubes may reflect differences in maturity or contraction. Negative controls using secondary antibodies alone showed no signal (data not shown). To further characterize the sarcomeric FRG1, purified myofibrils were immunostained for FRG1 and co-stained with rhodaminephalloidin, which binds both ends of the actin thin filament but with sharper staining at the Z-disk [41]. Here, FRG1 showed a much more diffuse pattern (Fig. 5Q–S) than seen on the intact myofibers suggesting FRG1 is less stably associated with the individual myofibrils than the Z-disks of intact myofibers.

Skeletal muscle FRG1 is predominantly cytoplasmic and localizes to sarcomeres

Cryosections and paraffin-embedded sections from human skeletal muscle biopsy (Figs. 6A–H and 6I–O, respectively) were immunostained for FRG1 using the HS1 and HS2 antibodies. Immunostaining of semi-consecutive sections with an antibody recognizing adult fast (type II) fibers showed that FRG1 stained both type I and type II fibers similarly (compare Fig. 6E with A–C), although especially HS2 showed slight difference in intensity between myofibers. To determine if FRG1 immunostaining is correlated with mitochondrial distribution, skeletal muscle cryosections were immunostained for cytochrome c oxidase subunit IV (COX IV), part of the COX enzyme complex localized to the inner mitochondrial membrane [42] (Fig. 6D). Mitochondria are usually enriched in slow (type I) fibers, but it is not a precise feature to distinguish the two fiber types in humans. When compared with serial sections immunostained for FRG1 (Fig. 6A–C), the COX IV antibody intensely recognized type I myofibers (compare Fig. 6A and D) and the pattern did not resemble FRG1 immunostaining (see also Fig. S4). The signals from both antibodies were further determined to be specific by comparing with cryosections incubated without primary antibody (Fig. 6A and F). In paraffin embedded sections, after antigen retrieval, the HS1

signal (Fig. 6J) was similarly absent in the secondary alone staining (Fig. 6I). Taken together, both FRG1 antibodies, regardless of histological technique, showed that skeletal muscle myofibers were generally lightly stained throughout their cytoplasm (Fig. 6B,C, G, H, J, L, and M), with some nuclear and perinuclear staining in myonuclei (Fig. 6J). In some cases FRG1 appeared to be surrounding a single myonucleus (Fig. 6L). There were also regions of very intense FRG1 staining concentrated at the periphery of some muscle fibers where the structure was not homogenous with the rest of the muscle fiber, indicating a different cellular organization or niche (Fig. 6O).

The vast majority of the tissue on these slides was sectioned transverse to the myofibers although occasionally some longitudinal sections of muscle were found. As predicted by the mouse myofiber data, FRG1 appeared in sarcomeric-like striations in longitudinal sections visualized with both the HS1 (Fig. 6G, O) and HS2 antibodies (Fig. H), compared with negative controls (Fig. 6F and N). Although mitochondria also have sarcomeric-like striation pattern, we confirmed that COX IV staining does not overlap with FRG1 signals by confocal and deconvolution microscopy (Fig. S4), confirming the FRG1 antibodies were not cross-reacting with mitochondria. These data indicate that the FRG1 is not a component of or associated with mitochondria, and that the sarcomeric localization of FRG1 is conserved in human skeletal muscle.

FRG1 is expressed in the vascular smooth muscle and epithelial tissues

In addition to myofibers, the paraffin embedded skeletal muscle sections showed strong FRG1 immunostaining in interstitial cells, within the walls of capillaries, and the nuclei of pericytes, cells closely associated with the capillaries (Fig. 6). Considering these results and that the second most prominent pathology seen in FSHD is retinal vasculopathy with an accompanying tortuous vasculature, the FRG1 expression in vascular smooth muscle was examined in more detail (Fig. 7A–E). The HS1 antibody detected intense specific expression of FRG1 in the smooth muscle of arteries (Fig. 7A) and veins (Fig. 7B and D) compared with negative controls (Fig. 7C and E). Compared with the skeletal muscle sections in Figure 6, FRG1 consistently appears to be both cytoplasmic and nuclear but stains more prominently in the nuclei of vascular smooth muscle and stain a much greater majority of the nuclei.

To investigate FRG1's expression and subcellular distribution in additional tissue types, muscle sections containing portions of the dermis were immunostained for FRG1 (Fig. 7F–I). FRG1 showed strong expression in sweat glands (Fig. 7F compared to negative control G) and the staining appeared particularly strong on the inner layer of epithelial cells with the staining for showing FRG1 present in both the nuclei and the cytoplasm. In the epithelial cells of the epidermis, anti-FRG1 HS1 (Fig. 7H) staining was uniform throughout the tissue and again appeared both cytoplasmic and nuclear. Overall, FRG1 is both cytoplasmic and nuclear in all endogenous tissues tested, although its specific distribution between the two can vary.

Discussion

Expression analyses have failed to produce consistent, reproducible results showing any 4q35 FSHD candidate gene, including FRG1, is misexpressed in FSHD muscle biopsies or patient-derived myocytes [15,17,24–26]. An alternative approach using overexpression of FSHD candidate genes in animal models has singled out FRG1 alone as being able to recapitulate both muscular and vascular FSHD-like pathology when overexpressed systemically [15,18,20,21]. However, these models have been criticized for exaggerated levels of expression beyond what would be expected in FSHD and therefore potentially leading to artificial phenotypes resulting in an inconclusive verdict. To gain further insight

on the viability of FRG1 misexpression being involved in FSHD, we have sought here to further understand the endogenous FRG1 protein in mouse and human muscle. Although FRG1 is ubiquitously expressed in all tissues tested by mRNA analysis [9], our ICC analyses showed the FRG1 protein is specifically spacially localized within myotubes and myofibers at the sarcomere (Figs. 4, 5 and 6) an interesting aspect from muscle development and muscular dystrophy perspectives.

Previous cell culture studies using epitope-tagged FRG1 transgenes characterized FRG1 as near exclusively nuclear with strong nucleolar and nuclear speckle concentrations implicating FRG1 in RNA biogenesis [29,30]. Although our analysis of the endogenous FRG1 in cell culture and myofibers clearly contradict the characterization of an exclusive nuclear localization for FRG1 (Figs. 1, 2, 4, 5, and 6), endogenous FRG1 does accumulate in the nucleoli during myotube formation supporting the claim for a role in RNA biogenesis. It should be noted that in our nuclear shuttling assays, HA-FRG1 recipient cells (HeLa) accumulated the transiently expressed FRG1 almost exclusively in their nuclei and specifically in the nucleoli (Fig. 3), despite the endogenous FRG1 showing both cytoplasmic and nuclear staining (Figs. 1 and 2). This data indicates that the majority of overexpressed FRG1 protein is nuclear and preferentially nucleolar. Thus, this raises the question of how is the exogenous or overexpressed FRG1 different from the endogenously regulated FRG1? It is interesting to note that different cell types showed different ratios of nuclear to cytoplasmic FRG1 with undifferentiated and fully differentiated muscle cells showing the greatest amount in the cytoplasm. Since exogenous or overexpressed FRG1 preferentially accumulates in the nucleus, potentially a certain cell-type specific level of endogenous FRG1 is capable of being actively maintained in the cytoplasm (FRG1 is ~29kDa) at any one time and any increases in FRG1 protein levels result in default FRG1 nuclear localization. We suggest that in our nuclear shuttling assay and published overexpression studies, the overexpressed FRG1 is actively shuttling between the nucleus and cytoplasm but is visualized exclusively in the nuclei because it is not being readily retained in the cytoplasm. Conversely, the endogenous FRG1 is stably maintained in the cytoplasm awaiting a signal to release it to the nucleus. This cytoplasmic retention model is supported by the dramatic change in endogenous FRG1 localization to the nucleus in myoblasts upon stimulation of myogenic differentiation (Fig. 4). This model further predicts that even small changes in FRG1 levels would alter its subcellular distribution, aberrantly increasing its levels in the nucleus.

Active cytoplasmic retention of FRG1 likely involves interaction with other proteins to anchor it. Recently we showed that FRG1 is a bona fide F-actin binding and bundling protein [18], further supporting a cytoplasmic role for FRG1. Here we have characterized FRG1 as a sarcomeric Z-disc associated protein in mouse and human skeletal muscle (Figs. 4,5 and 6). This highlights that FRG1, although ubiquitously expressed in respect to tissues, has cell type specific, and particularly muscle specific, functions. If FRG1 were dysregulated in FSHD, this could explain why the genetic lesion presents skeletal muscle specific pathophysiology. The Z-disc localization is additionally intriguing in respect to FSHD since in FSHD patient muscle some of the structures at the sarcolemma are misaligned and the association of the sarcolemma with contractile structures is altered [43]. Numerous other myopathies can trace their molecular defects to proteins associated with the contractile apparatus and force generating structures of skeletal muscle [32,33,44]. This work places FRG1 as the only current FSHD candidate gene whose product is directly linked to the skeletal muscle contractile apparatus.

Identifying FRG1 as a dynamic nuclear and sarcomere-associated protein may suggest a link between the two known biological activities for FRG1, F-actin binding/bundling and RNA biogenesis [18,29,30]. Potentially FRG1 could be transducing signals from the Z-disc to the

nucleus directly and affecting mRNA biogenesis, as is the case for the dynamic Z-disk protein MLP [45,46]. Conversely, FRG1 may be involved in trafficking molecules such as RNAs to the Z-disc for site specific translation. Interestingly, several Z-disc proteins have been shown to co-localize with their cognate mRNAs in cultured skeletal muscle [47]. In either case, one can imagine how aberrantly altering the levels of FRG1, and thus affecting FRG1-mediated signaling or transport, could disrupt the efficiency of myogenic differentiation, muscle regeneration, and maintenance of muscle integrity over time as seen in the animal models overexpressing or depleting FRG1, and as proposed for FSHD [21].

Overall, we provide the first characterization of endogenous FRG1 protein in mammalian skeletal and smooth muscle. We further identify FRG1 as a dynamic nuclear and cytoplasmic protein exhibiting developmental regulation of subcellular localization during myogenesis. Significantly, FRG1 is associated with mature, aligned sarcomeres in differentiated human and mouse skeletal muscle. Thus, FRG1, an actin bundling protein previously shown to be critical for muscle and vascular development, provides the only link between a FSHD candidate gene and the muscle contractile machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

FSHD	Facioscapulohumeral muscular dystrophy
FRG1	FSHD region gene 1
HSMM	human skeletal muscle myoblast
ICC	immunocytochemistry
MDSC	muscle-derived stem cell

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Figure 1. FRG1 antibodies are highly specific

ICC on HeLa cells (A, B, E, F, I, J) or HeLa cells transfected with a FRG1-specific pool of siRNAs (C, D, G, H, K, L) using the HS1 (A–D), HS2 (E–H), or DM1 (I–L) FRG1 antibodies show a specific reduction in both the cytoplasmic and nuclear FRG1 antibody signal intensities in the siRNA treated cells. The merged images (B, D, F, H, J, L) show FRG1 (A, C, E, G, I, K) in green, DAPI in blue, and phalloidin in red. Images are taken under the same parameters. Bars = 10 µm. M) Western blot analysis of HeLa whole-cell extract (200 µg/lane) probed with HS1, HS2, and DM1 as indicated. PS indicates Ponceau S staining of membranes. N) Western blot analysis of HeLa cell extract fractionated into nuclear and cytoplasmic pools and probed with DM1 and HS2 as indicated. A monoclonal antibody against proliferating cell nuclear antigen (PCNA) was used to control for contamination of the cytoplasmic protein pool with nuclear proteins.

Figure 2. Endogenous FRG1 is both a nuclear and cytoplasmic protein in multiple cell types ICC on (A–L) HeLa cells, and (M–P) murine C2C12 cells reveal intense nuclear FRG1 immunostaining accompanied by cytoplasmic FRG1 immunostaining (A, E, I, M; green in merge). (Q–T) HSMs, and (U–X) murine MDSCs show much more prominent cytoplasmic FRG1 immunostaining (Q, U, T, X; green in merge). Rhodaminephalloidin staining (B, F, J, N; red in merge) labeled the cytoplasmic actin filaments while DAPI (C, G, K, O, S, W; blue in merge) labeled nuclei. Desmin immunostaining (R, V; red in merge) was used to confirm the myoblast phenotypes. (U–X) White arrow indicates a desmin negative MDSC and blue arrow indicates a desmin positive MDSC beginning differentiation. Overall, the immunostaining patterns within a cell type are consistent between HS1 (A–D, Q–T), HS2 (E–H), and DM1 (I–L, M–P), three independent FRG1 antibodies raised against peptides from different regions of FRG1, as shown in Figure S1. Bars = 10 μ m.

Figure 3. FRG1 shuttles between the nucleus and cytoplasm

Murine C2C12 cells, morphologically distinguished by their DNA-bright foci (white arrow), expressing HA-FRG1 (red) and treated with CHX were fused with HeLa cells, distinguished by their DNA-poor nucleoli (blue arrows) in the presence of CHX. (A–D) Two hours into the fusion process FRG1 translated in the C2C12 cells begins to localize in the HeLa cell nuclei (C', longer exposure of C) and specifically the nucleoli (D, blue arrows). This translocation of FRG1 from C2C12 to HeLa nuclei is more evident at 3 hours (E–H) and at 4 hours (I–L), appearing to have reached equilibrium between the two cell type nuclei (K). Hoechst 33342 staining (green) identified nuclei. Bars = 10 μ m

Figure 4. FRG1 subcellular localization changes dramatically when primary human skeletal myocytes are stimulated to undergo myogenic differentiation

FRG1 subcellular localization was monitored using the HS1 antibody (A–I red) in normal HSMs (A, B), 2 days post-differentiation (C, D), 5 days post-differentiation (E, F), and 8 days post-differentiation (G–I). Developing Z-discs were monitored by α -actinin immunostaining (D, F, I, L; green). FRG1 was similarly monitored with the HS2 (J–L; red) at 8 days post-differentiation. Using all three antibodies FRG1 is detected co-localized with α -actinin at Z-discs 8 days post-differentiation, but not earlier (I, L; white arrows). Desmin immunostaining (B; green) confirmed the myoblast phenotype and DAPI identified the nuclei (B, D, F; blue). Bars = 10 μ m.

Figure 5. FRG1 is a sarcomeric protein localized to myofiber Z-discs

To characterize FRG1's localization at the sarcomere, intact mouse muscle fibers were isolated and subjected to ICC using the HS1 (A, I, M) and HS2 (E) FRG1 antibodies and co-staining for α -actinin (B, F, J) or desmin (N). Images were visualized by standard fluorescent microscopy (A–H) or by fluorescent confocal microscopy (I–P). When merged (D, H, L,) α -actinin (green) appears overlapping and flanked by the FRG1 (red) around the Z-lines. However, confocal images clearly show the precise co-staining of FRG1 and α -actinin (K, L) while FRG1 and desmin only partially overlap (O, P). Overall, FRG1 was detectable in myofiber nuclei (blue arrows) and Z-discs (white arrows). Bars = 10 μ m. (Q–S) Isolated myofibrils were immunostained with HS1 (Q), counter stained with rhodamine-phalloidin (R), and images merged (S; green = FRG1, red = F-actin). Bar = 10 μ m.

Figure 6. Human skeletal muscle biopsies show FRG1 is nuclear, cytoplasmic, and sarcomeric Immunohistochemistry on human gastrocnemius muscle cryosections (A–H) and paraffin embedded sections (I–O) from human skeletal muscle using FRG1 antibodies (brown). Paraffin sections were counterstained with hematoxylin (blue). (A–E) Serial cryosections of human quadriceps immunostained for (A) secondary antibody alone, (B) FRG1 HS1, (C) FRG1 HS2, (D) COX IV, and (E) fast twitch myosin type II. (F–H) Longitudinal human quadriceps muscle sections immunostained for (F) secondary alone or (G and H) FRG1 HS1. (G' and H') are 2× magnifications (I–K) Serial paraffin sections of human skeletal muscle showing (I) secondary antibody alone negative control, (J) FRG1 HS1 antibody staining, and (K) fast twitch myosin. (L–O) Additional immunohistochemistry panels using the FRG1 HS1 antibody shows features of FRG1 subcellular localization. (O) Rare longitudinal muscle sections show striated FRG1 immunostaining (black arrow) compared with (N) secondary antibody alone. * red = slow twitch fibers; p = pericyte; n = perinuclear staining; ni = niche; black arrow = sarcomeric striations. Bars = 100 μm (A–E) and 10 μm (F–O).

Figure 7. FRG1 is prominently expressed in vascular smooth muscle and dermal tissues
 Immunohistochemistry on paraffin embedded sectioned normal human skeletal muscle sections probing with FRG1 HS1 shows strong staining of (A) arteries and (B, D) veins, indicating cytoplasmic and nuclear pools of FRG1. (C, E) Negative controls omitting primary antibodies. (D, E) 2.5× magnifications of B and C, respectively. Immunostaining of human skeletal muscle sections containing dermal tissue with FRG1 HS1 shows FRG1 is expressed in the sweat glands (F) and the epidermis (H). Negative controls omitting the primary antibody (G) or pre-incubating the primary antibody with antigenic peptide (I) did not show reactivity. (A, B, C) Bars = 50 µm. (F–I) Bars = 20 µm. (D, E) Bars = 10 µm.