Published in final edited form as:

J Mol Biol. 2011 August 12; 411(2): 397–416. doi:10.1016/j.jmb.2011.06.014.

Facioscapulohumeral muscular dystrophy region gene 1 (FRG1) is a dynamic RNA-associated and actin bundling protein

Chia-Yun Jessica Sun^{a,b}, Silvana van Koningsbruggen^{c,1}, Steven W. Long^a, Kirsten Straasheijm^c, Rinse Klooster^c, Takako I. Jones^{a,b}, Michel Bellini^a, Lyne Levesque^a, William M. Brieher^a, Silvère M. van der Maarel^c, and Peter L. Jones^{a,b,*}

^aDepartment of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, 601 S. Goodwin Ave, B107 Chemical and Life Sciences Laboratory, Urbana, IL 61801 USA ^bBoston Biomedical Research Institute, 64 Grove St, Watertown MA 02472 USA ^cDepartment of Human Genetics, Center for Human and Clinical Genetics, Leiden University Medical Center, Postal zone S-3-P, PO box 9600, 2300RC Leiden, The Netherlands

Abstract

Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) is a dynamic nuclear and cytoplasmic protein that, in skeletal muscle, shows an additional localization to the sarcomere. Maintaining appropriate levels of FRG1 protein is critical for the muscle and vascular development in the vertebrate, however its precise molecular function is unknown. This study investigates the molecular functions of human FRG1 along with mouse and *Xenopus* frg1 using molecular, biochemical and cell-biological approach to provide further insight into its roles in vertebrate development. The nuclear fraction of the endogenous FRG1 is localized in nucleoli, Cajal bodies, and actively transcribed chromatin; however, contrary to overexpressed FRG1, the endogenous FRG1 is not associated with nuclear speckles. We characterize the nuclear and nucleolar import of FRG1, the potential role for phosphorylation, and its interaction with the importin karyopherin α 2 (KPNA2). Consistent with a role in RNA biogenesis, human FRG1 is associated with mRNA *in vivo* and *in vitro* and interacts directly with TAP, the major mRNA export receptor, and is a dynamic nuclear-cytoplasmic shuttling protein supporting a function for FRG1 in mRNA transport. Biochemically, we characterize FRG1 actin binding activity and show that the cytoplasmic pool of FRG1 is dependent on an intact actin cytoskeleton for its localization. These data provide the first biochemical activities - actin binding and RNA binding - for human FRG1 and the characterizations of the endogenous human FRG1, together indicating FRG1 is involved in multiple aspects of RNA biogenesis including mRNA transport and potentially cytoplasmic mRNA localization.

Keywords

TAP; karyopherin alpha 2; actin binding; nucleolus; mRNA transport

© 2011 Elsevier Ltd. All rights reserved.

*Correspondence: pjones@bbri.org, Phone (617) 658-7745, Fax (617) 972-1760.

¹Present address: Division of Gene Regulation & Expression, School of Life Sciences, Wellcome Trust Biocentre, University of Dundee, Dow Street, Dundee DD1 5EH UK

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) in its entirety is very highly conserved from vertebrates to invertebrate (human FRG1 shares 97%, 81%, and 46% homology with mouse, *Xenopus*, and *C. elegans*, respectively) suggesting important biological functions that were conserved throughout evolution¹. A putative domain analysis of the predicted FRG1 protein sequence revealed two nuclear localization signals (NLS) residing in the N-terminus, one bipartite NLS in the C-terminus, and a central fascin-like domain, which is found in the fascin family of actin-bundling and cross-linking proteins². Our previous studies revealed that FRG1 is a nuclear and cytoplasmic protein that shows an additional localization to the sarcomere in skeletal muscle^{3,4}. Consistent with a role at the sarcomere, FRG1 is required for the normal development of the vertebrate musculature and vasculature, and overexpressing FRG1 in vertebrate and invertebrate animal models leads to muscular dystrophy-like phenotypes⁴⁻⁷. However, the precise function of the endogenous human FRG1 is unknown.

Several lines of evidence suggest FRG1 is involved in RNA biogenesis. Although FRG1 has never been reported to be associated with any mRNA *in vitro* or *in vivo*, FRG1 has been found biochemically associated with RNA processing components and large-scale proteomic studies revealed that FRG1 is associated with the human spliceosome complex⁸⁻¹⁰. In addition, epitope-tagged FRG1 overexpressed in certain cultured vertebrate cells appeared almost completely nuclear, and localized to the nucleoli, Cajal bodies, and nuclear speckles, sites where RNA-biogenesis is taking place¹¹. Mis-spliced mRNA transcripts were found in U2OS cells overexpressing FRG1 as well as FRG1 transgenic mice implicating FRG1 may be involved in alternative splicing^{5,9}. Interestingly, we recently characterized the *C. elegans* FRG1 homolog, FRG-1, and in addition to the conserved strong nucleolar localization, we found a cytoplasmic pool of FRG-1 localized to body-wall muscle dense bodies, structures functionally analogous to vertebrate Z-discs and costameres⁴. Functionally, we found FRG-1 is an actin-binding and bundling protein *in vitro* suggesting FRG-1 may be a part of or involved in stabilizing the actin cytoskeleton⁴. This striking Z-disc localization for FRG1 is conserved in mouse and human skeletal muscle³. Thus, these data suggest multiple roles for vertebrate FRG1 in muscle development and function, including aspects of RNA biogenesis and regulation of the actin cytoskeleton, and make FRG1 an intriguing candidate gene for participating in FSHD pathophysiology.

FSHD, the most prevalent form of muscular dystrophy afflicting both children and adults, is a late onset autosomal dominant disease marked by progressive muscle atrophy in specific muscle groups¹²⁻¹⁵. The genetic lesion of FSHD1A is a contraction in a polymorphic array of macrosatellite repeats, termed D4Z4, located in the subtelomeric region of chromosome 4q^{16,17}. The contraction results in a decrease of D4Z4 repeat number to between 1-10, whereas normal individuals carry 11-100 repeats; the contraction leads to an epigenetic mis-regulation of a gene(s) within the 4q35 region whose altered expression ultimately leads to FSHD pathophysiology^{14,18}. *FRG1*, due to its location 125kb centromeric to the D4Z4 repeats, is one such FSHD candidate gene¹⁹, however, expression profile studies fail to show consistent results that FRG1 is mis-regulated in FSHD samples calling into question the viability of FRG1 as a FSHD candidate²⁰⁻²⁶. Recently, the *DUX4* gene located within the affected repeat array has been shown to produce a stable polyadenylated mRNA transcript specifically in FSHD patient muscle suggesting that aberrant expression of the cytotoxic DUX4 protein is the primary mediator of FSHD pathogenesis²⁷, although this result has yet to be independently verified. Therefore, any role for FRG1 in FSHD pathogenesis would likely be secondary, potentially accounting for the high degree of variability in severity, asymmetry, affected muscles, and extra-muscular pathology. FRG1's potential role is further complicated by our lack of understanding of the normal function of

FRG1. Here, we further investigated both the nuclear and cytoplasmic aspects of human FRG1 biology and function.

Results

Endogenous human FRG1 is both nuclear and cytoplasmic, and is prominently localized to the granular component of nucleoli

Previous studies investigating FRG1 by immunocytochemistry (ICC) used transiently expressed and epitope tagged FRG1^{9, 11}. Here, an affinity purified antibody previously shown to be specific for FRG1 in ICC³ was used in HeLa cells to characterize the endogenous human FRG1 (Figure 1). In contrast to the seemingly exclusively nuclear localization often visualized for overexpressed and epitope-tagged FRG1^{11, 9} (Figures 2B and S1), the endogenous FRG1 in HeLa cells is present in both the cytoplasm and nucleus by ICC (Figure 1) and western blotting with multiple FRG1 antibodies (Figure S2). To further confirm the existence of a cytoplasmic pool of FRG1 in HeLa cells, a HA-tagged human FRG1 was expressed in HeLa cells and western blots were performed on purified cytoplasmic and nuclear extracts revealing a mostly nuclear HA-FRG1 with a less abundant cytoplasmic pool of HA-FRG1 (Figure S1), further supporting that FRG1 is both nuclear and cytoplasmic in HeLa cells.

First, an analysis of the nuclear pool of FRG1 was carried out. Consistent with previous data for the overexpressed FRG1, the endogenous nuclear FRG1 is prominently localized in the DAPI-poor foci (Figure 1A–C). Co-immunostaining for FRG1 and fibrillarin, a nucleolar marker²⁸, showed the endogenous FRG1 concentrates in the nucleoli (Figure 1D–G). However, the nucleolar FRG1 associated, but did not completely co-localize with, fibrillarin (Figure 1D–G) suggesting that nucleolar FRG1 was not part of the dense fibrillar component (DFC). The DFC and granular center (GC) of the nucleolus segregate and can be readily distinguished from each other by inhibition of RNA polymerase I transcription using Actinomycin D (AMD)²⁹. HeLa cells treated with AMD showed FRG1 localized to the GC, distinctly separate from the DFC marked by fibrillarin (Figure 1H–K). Beyond the nucleolar localization, FRG1 showed punctate nuclear immunostaining suggestive of nuclear speckles (Figure 1E, I, M), therefore, HeLa cells were co-immunostained for FRG1 and the nuclear speckle marker SC35³⁰. Visualizing 0.2µm focal sections through the nucleus showed that the endogenous FRG1 did not co-localize with SC35 indicating it is not a component of SC35-containing nuclear speckles (Figure 1L–O), contrary to a previous report for the overexpressed FRG1¹¹. To reconcile the data, HeLa cells were transfected to overexpress FRG1 and then co-immunostained for FRG1 and SC35 (Figure S3). Consistent with previous work¹¹, overexpressed FRG1 is predominantly nuclear (Figures S1–S3) and localizes to SC35-containing nuclear speckles in some, but not all, FRG1 overexpressing cells (Figure S3C, F, I). Therefore, we conclude that endogenous nuclear FRG1 is not a component of SC35-containing nuclear speckles.

The FRG1 nuclear and nucleolar localization domains

The endogenous FRG1 protein is both nuclear and cytoplasmic³ (Figures 1 and S2) while most of the detectable overexpressed FRG1 is nuclear and strongly nucleolar (Figures 2B, S1–S3)^{9, 11}. Therefore, transient transfections of mutant FRG1 expression constructs can be used to determine the nuclear and nucleolar localization requirements for FRG1. The *in silico* predicted NLSs of FRG1 (NLS1, amino acids 22–25 and NLS2, amino acids 29–32; bipartite NLS, amino acids 253–261) were proven to be functional for its nuclear and nucleolar localization¹¹. To better characterize these signals, more specific deletion and substitution mutants were generated and fused to a VSV (Vesicular Stomatitis Virus) epitope tag for visualization, including those specifically lacking the first NLS (ΔNLS1), the

second NLS (Δ NLS2), or both (Figure 2A). All constructs were transiently transfected into COS-1 cells and the percent of cells showing cytoplasmic VSV-FRG1 localization (as represented in Figure 2D) was determined for each transfected FRG1 mutant by ICC visualizing the VSV tag (summarized in Figure 2E). The full-length VSV-FRG1 showed a similar localization as described previously¹¹, in the nucleoplasm and more prominently in nucleoli, Cajal bodies and speckles, and devoid of detectable cytoplasmic staining (Figure 2B). FRG1 Δ NLS1 still localized to the nucleus while 43% of the FRG1 Δ NLS2 expressing cells showed the cytoplasmic localization (as per example shown in Figure 2D). However, when both NLSs were deleted, 90% of the cells showed cytoplasmic FRG1 indicating their synergistic effect on FRG1 localization and showing a major role for NLS2 in the nuclear localization of FRG1, which can partly be taken over by NLS1.

NLS function can be regulated through the phosphorylation of adjacent amino acids^{31, 32} and FRG1 NLS1 is flanked by serine (S) residues [S(21)KKKKS(26)] that might be subject to phosphorylation. Therefore we generated mutants in which the S21 and/or S26 was substituted with an alanine (A) to mimic the unphosphorylated form or aspartic acid (D) to mimic the phosphorylated form, in all different combinations with the deletion variants. Compared to FRG1 Δ NLS2 alone, an alanine substitution for either one of the serines flanking NLS1 (S21A or S26A) does not affect the cytoplasmic localization of FRG1 Δ NLS2. Interestingly, the function of NLS1 is dramatically enhanced when both adjacent serine residues were mimicking the “unphosphorylated” form (S21A,S26A, Δ NLS2) and perturbed when mimicking the “phosphorylated” form (S21D,S26D, Δ NLS2). These experiments demonstrate an increase in cytoplasmic localization of FRG1 upon the deletion of NLS2 and suggest a phosphorylation-dependent functionality of NLS1.

In addition to the cytoplasmic localization of some FRG1 mutants, a decrease or depletion of FRG1 from nucleoli (Figure 2C) was observed in the majority of mutants (summarized in Figure 2F). Deletion of either NLS1 or NLS2 resulted in a decreased nucleolar FRG1 signal, which was most pronounced with the Δ NLS1 mutant. The NLS sequences act synergistically in the nucleolar localization of FRG1 as deleting both of them resulted in even higher nucleolar depletion frequencies. This nucleolar depletion may be NLS1 phosphorylation dependent as the S21 and S26 mutations affect the nucleolar localization of FRG1 in constructs lacking NLS2. These constructs mimicking the phosphorylated status of NLS1 (S21D, Δ NLS2 and S26D, Δ NLS2) resulted in higher nucleolar depletion frequencies compared to FRG1 Δ NLS2 alone, with the double “phosphorylated” mutant (S21D,S26D, Δ NLS2) showing an extremely high nucleolar depletion rate almost equal to that observed in the double NLS mutant. The FRG1 S21 and S26 mutation constructs containing an intact NLS2 showed no influence on the nucleolar FRG1 localization except for the S21D,S26D mutant, which results in a decreased nucleolar FRG1 staining comparable to full-length FRG1. Both the single and double ‘unphosphorylated’ NLS1 (S21A,S26A, Δ NLS2) do not show differences in nucleolar depletion frequencies compared to NLS1 alone (Δ NLS2). Our results demonstrate a synergistic role of both NLS1 and NLS2 in the nucleolar localization of FRG1, in which NLS1 is the major nucleolar localization signal and its role in nucleolar localization is likely dependent on the phosphorylation status of the flanking serine residues.

Supporting the described nuclear import of FRG1 (Figures 1 and 2), a yeast-two-hybrid screen with FRG1 as bait identified the importin family protein karyopherin alpha 2 (KPNA2), also known as importin alpha 1³³, as a FRG1 binding partner with 40% of the clones sequenced representing KPNA2⁹. We identified full-length KPNA2 clones as well as clones containing only the C-terminal domain of the protein, which included the armadillo repeats. Co-immunoprecipitations (co-IPs) performed on U2OS cell extracts using the affinity purified α FRG1-719 antibody and assaying by western blotting for KPNA2 (Figure

3A) showed the α FRG1-specific co-IP of KPNA2, confirming the interaction *in vivo*. To determine if the interaction between FRG1 and KPNA2 was direct, GST pull-down experiments were performed (Figure 3C–D). Large GST-FRG1 deletion mutants incubated with radiolabeled KPNA2 showed a direct interaction mapped to the N-terminal 80 amino acids (Figure 3D, lanes 4 and 6) and further refined to NLS2 (Figure 3D, lane 9) while Δ NLS1 seems to have no effect on the interaction between the two proteins (Figure 3D, lane 10). This data identifies FRG1-NLS2 as the KPNA2 interaction site and is consistent with previous reports of KPNA2 interacting with NLSs^{32, 34}.

FRG1 nuclear import is impaired when the NLS1 flanking serines are mutated to phosphomimic aspartic acids (Figure 2) and it has been shown that the affinity for KPNA2 binding to a cargo protein NLS is similarly decreased by phosphorylation adjacent to the NLS³⁵. To determine if FRG1 phosphorylation could affect KPNA2 binding, co-IPs were performed on HeLa cells transfected with HA epitope tagged FRG1 or the FRG1 phosphorylation mutants S21A,S26A and S21D,S26D. The *in vivo* FRG1-KPNA2 interaction was confirmed in HeLa cells (Figure 3B, lanes 1 and 2) and mutating the serines flanking NLS1 to alanines, resulting in an unphosphorylatable form of FRG1, consistently enhanced the association with KPNA2 (Figure 3B, lane 4 compared with lane 2). Mutating the NLS1 flanking serines to aspartic acids, mimicking a phosphorylated form of FRG1, abolished the interaction with KPNA2 (Figure 3B, lane 6 compared with lane 2). This *in vivo* interaction data is consistent with the sub-cellular localization data (Figure 2) and together suggests that FRG1 is imported into the nucleus by KPNA2 and this localization could be regulated by NLS1 phosphorylation.

Endogenous FRG1 associates with mRNA transcripts *in vivo*

FRG1 was previously implicated in aspects of RNA biogenesis, in particular pre-mRNA splicing. It is currently well accepted that most RNA processing factors are recruited to nascent transcripts co-transcriptionally, resulting in the formation of export-competent ribonucleoprotein (RNP) complexes at the sites of transcription. Many of these factors, such as the heterogeneous nuclear ribonucleoprotein G (hnRNP G) associate with most transcription units of RNA polymerase II (RNAPII) in amphibian oocytes^{36, 37}. Chromatin in *Xenopus laevis* oocytes is structured within lampbrush chromosomes (LBC) and several thousand non-chromosomal nucleoli. LBCs are often described as extended diplotene bivalent chromosomes, and their characteristic shape is the result of the intense transcriptional activity that is associated with them. Each homologue consists of a condensed chromatin axis, from which are escaping numerous pairs of lateral loops that are active transcription units of RNAPII. Although the chromatin of these loops almost certainly adopts the conformation of a 10nm (or less) fiber³⁷, they are readily distinguishable by light microscopy because the chromatin axis is surrounded by a dense matrix of nascent RNP fibrils. We took advantage of this unique spatial resolution to test whether FRG1 associates with nascent transcripts on nuclear spreads. Both the endogenous frg1, detected using an antibody specific for *X. laevis* frg1⁶ (Figure 4A–L), and an overexpressed HA epitope tagged frg1 (Figure 4M–P) were similarly assayed. The endogenous frg1 (Figure 4E–L) and the HA-frg1 (Figure 4M–P) were detected within the RNP matrix of the LBC loops, which provides strong evidence that frg1 interacts with nascent transcripts within the cell nucleus. In addition, frg1 was also found associated with nucleoli, Cajal bodies, B snurposomes, and interchromatin granule clusters (Figure 4A–D), which is consistent with the subnuclear distribution of FRG1 defined previously in human cells and further supports a role of FRG1 in RNA biogenesis¹¹.

To determine if the endogenous human FRG1 associates with human mRNAs *in vivo*, RNA immunoprecipitations (RIP) were performed on HeLa whole cell extracts using both the HS1 and HS2 FRG1 affinity-purified antibodies previously characterized³. After the RIP

procedure, FRG1 associated RNAs were subjected to reverse transcriptase PCR (RT-PCR), initially assaying for the 3' end of the FXR1, a transcript known to be affected in FSHD³⁸ (Figure 5A and 5B upper panel). All RIP RT-PCR products were sensitive to RNase A treatment of the starting material confirming the RNA (and not DNA) association with FRG1 (Figure 5A, lanes 7 and 9; 5B lower panel). Furthermore, two independent nonspecific antibody controls repeatedly failed to IP any detectable mRNAs illustrating the specificity of the RIP procedure for FRG1 (Figure 5A, lane 3; 5B lanes 3 and 4). Subsequently, new RIP reactions were assayed by RT-PCR for a 5' region of the FXR transcript using PCR primers that spanned an intron and only spliced mRNAs were IP'ed (Figure 5B, upper panel). Analysis for the FRG1 mRNA (Figure 5B, middle panel) produced results similar to that shown for FXR1. These results show that endogenous human FRG1 associates with mature spliced mRNA transcripts *in vivo*.

Recombinant human FRG1 interacts with RNA directly *in vitro*

Although FRG1 does not contain any conventional RNA-binding motif, many RNA associated ribonucleoproteins (RNPs) interact with RNA directly *in vitro*^{39,40}. To determine if FRG1 is capable of binding with RNA directly and specifically, RNA electrophoretic mobility shift assays (REMSA) were performed using recombinant human FRG1. Full-length recombinant FRG1 (Figure S4) formed a protein-RNA complex with *in vitro* transcribed RNA encoding the same FXR1 coding sequence previously shown to be associated with FRG1 *in vivo* by RIP, as indicated by the FRG1-dependent slower migrating RNA species (Figure 6B, arrowheads). Similarly, *in vitro* transcribed RNA encoding the β -globin 5' and 3' UTR sequences (Figure 6C and D, arrowheads), were also bound by recombinant FRG1 *in vitro*. This data indicated that *in vitro* FRG1 binds mRNA with low sequence specificity.

There is no predicted RNA binding domain (RBD) described for FRG1, therefore, recombinant FRG1 proteins from four deletion constructs (Figures 6A and S4) were used for REMSA to identify the FRG1 RBD. The results showed that FRG1 interacts with RNA directly through its N-terminal NLS as deleting this region alone abolishes the RNA-binding activity of FRG1 (Figure 6C, arrowhead) while the other three deletions retained RNA-binding activity similar to full-length FRG1 (lanes 14–16 compared with all other lanes). To determine the specificity of this interaction, binding competition experiments were performed (Figure 6D). To test RNA sequence specificity, competition assays were performed using *in vitro* transcribed but unlabeled UTR RNA probe as a specific competitor (Figure 6D, lanes 6–9) and tRNA as a non-specific competitor (Figure 6D, lanes 10–13). Both specific and non-specific RNAs competed equally well suggesting FRG1 does not bind RNA with sequence specificity *in vitro*. To determine if the FRG1-RNA interaction was specific to RNA, double stranded (ds) T7TS DNA was used as a binding competitor (Figure 6D, lanes 14–17). Interestingly, dsDNA did not compete for FRG1 binding indicating that the FRG1-nucleic acid interaction is specific to RNA *in vitro*.

FRG1 interacts with the nuclear RNA export protein TAP

Considering FRG1 is a nuclear shuttling protein³ and associates with the actively transcribing RNP matrix (Figure 4) as well as mRNAs *in vivo* (Figure 5) and *in vitro* (Figure 6), it seemed reasonable that FRG1 might be involved in mRNA export as part of a hnRNP complex. The majority of mature mRNAs in the nucleus are exported through the TAP:NXT1 pathway^{41,42} involving additional co-adaptor proteins that interact with TAP⁴³. Therefore, FRG1 was tested for a direct interaction with TAP *in vitro* (Figure 7). GST pull-down assays were performed using recombinant GST-TAP (Figure S5) and radiolabeled *in vitro* transcribed and translated (TnT) full-length human FRG1 and FRG1 deletion constructs. The results showed that FRG1 interacts directly with TAP mediated through the

FRG1 N-terminal NLSs (Figure 7A). Since both TAP and FRG1 interact with RNA, and RNA could be present from the recombinant protein purification or TnT reaction, the GST pull-down assay was repeated with benzonase nuclease to digest any RNA or DNA. Benzonase nuclease treatment did not affect the FRG1-TAP interaction indicating that FRG1 interacts with TAP directly and the interaction is independent of RNA (Figure 7B).

To characterize FRG1 nuclear export *in vivo*, a heterokaryon assay was performed using the drug Leptomycin B (LMB), an inhibitor of the CRM1/exportin1 nuclear export pathway but not of the TAP:NXT1 export pathway^{44, 45}. HeLa cells, readily identified by Hoechst staining as containing large DNA-poor nucleoli (Figure 8, blue arrows), were transfected to transiently express HA-FRG1. The overexpressed HA-FRG1 in the HeLa cells showed nuclear localization with enrichment at nucleoli as previously shown (Figure S1). Twenty-four hours post-transfection, cycloheximide (CHX) was added to block *de novo* translation. Subsequently, these transfected HeLa cells were overlaid with non-transfected C2C12 cells, easily identified by the multiple DNA-bright foci visualized in the Hoechst staining (Figure 8, white arrows), and cell fusion was initiated in the continued presence of CHX with or without LMB. Three hours post-fusion, HA-FRG1 staining was detected in C2C12 nuclei (Figure 8A–D), indicating FRG1 nuclear cytoplasmic shuttling and the shuttling was not affected by LMB (Figure 8E–H compared with A–D, CHX alone). Thus FRG1 nuclear export occurs through a CRM1 independent pathway and is consistent with FRG1 exported through the TAP:NXT1 pathway.

Human FRG1 associates with F-actin *in vivo*

Recently, we have shown that the *C. elegans* and human FRG1 bundle F-actin *in vitro*⁴. In order to further analyze the actin binding activity of human FRG1, a high-speed cosedimentation assay using recombinant human FRG1 was performed (Figure 9). Increasing concentrations of FRG1 were incubated with a constant amount of F-actin and subjected to high-speed centrifugation to pellet the actin-associated FRG1. The ratios of FRG1 in the pellets (bound to actin) and supernatants (not bound to actin) were determined by SDS-PAGE and CBBR staining followed by analysis with QuantityOne software. Based on the quantity of G-actin added to the reactions, the results showed that human FRG1 binds actin with a $K_d = 0.16 \pm 0.03 \mu\text{M}$ and $B_{\text{max}} = 1.54 \pm 0.02$ (corresponding to two FRG1 per actin monomer) (Figure 9A). To determine if FRG1 could form the multimers required for F-actin cross-linking, a glutaraldehyde cross-linking assay using recombinant FRG1 was performed. A cross-linking time-course assay showed that FRG1 forms dimers and tetramers *in vitro* (Figure 9B), providing the multiple actin binding sites required for F-actin cross-linking.

To determine if the cytoplasmic FRG1 pool associates with the actin cytoskeleton, as visualized by phalloidin staining, murine C2C12 cells were treated with varying concentrations of Latrunculin B (LatB) for 5 or 15 minutes and immunostained for endogenous FRG1 (Figure 10). The cytoplasmic F-actin was visualized with rhodamine-phalloidin. LatB treatment inhibits actin polymerization resulting in a disrupted actin cytoskeleton as indicated by loss of phalloidin staining (compare Figure 10A to E, I, M). The intensity of the cytoplasmic pool of FRG1 decreased gradually as the intensity of LatB treatment increased, and FRG1 staining was abolished in phalloidin weak areas (Figure 10E–P) indicating that FRG1's localization in the cytoplasm is dependent on the intact actin cytoskeleton *in vivo*.

Discussion

FRG1 is critical for development of the vertebrate musculature and vasculature and has been implicated in mediating FSHD pathophysiology^{5–7}. Still, very little is known about FRG1,

hindering our understanding of how changes in its expression levels might lead to disease. In this study we further investigated human FRG1 by characterizing its subcellular localizations and biological activities, and identifying new interacting proteins.

Focusing on the nuclear aspects of FRG1, we have identified and characterized functional NLS and NoLS domains in FRG1. *In silico* analysis of FRG1 revealed two amino terminal NLSs and a carboxyl bipartite NLS sequence. Our data demonstrate a major role for NLS2 in the nuclear localization of FRG1, which can partly be taken over by NLS1 in a phosphorylation-dependent manner, demonstrating that both are able to function independently in nuclear transport. Similar to FRG1, other proteins including transcription factors, enzymes, and structural proteins consist of multiple NLS sequences^{46–49}. This redundancy ensures their efficient trafficking to the nucleus and likely indicates an essential nuclear function. Although proteins such as FRG1 (29 kb) are small enough to pass through the nuclear pore by diffusion, active nuclear transport via the nuclear import receptor KPNA2 results in rapid and efficient nuclear accumulation. This is the case for FRG1. We previously identified KPNA2 as a prominent and direct binding partner of FRG1 by yeast-two-hybrid screens⁹, and here confirmed the direct interaction by GST pull-down and co-IP experiments. KPNA2 is known to be involved in nuclear import of cargo-proteins through binding to the NLS sequence by means of its armadillo repeats located in the central part of the protein (reviewed in³³). In agreement, all our yeast-two-hybrid clones of KPNA2 contained armadillo repeats, supporting the assumption that KPNA2 binds FRG1 by its armadillo repeats.

Phosphorylation can influence the nuclear transport of proteins by several mechanisms; it can regulate the binding or release of proteins masking a NLS, cause conformational changes in the NLS-containing protein, or alter the affinity of the NLS for nuclear import proteins³⁵. The loss of functionality of NLS1 in nuclear import by mimicking the negatively charged phosphorylation status of this domain is in agreement with previous reports in which the affinity of the basic NLS sequence for the import receptor KPNA2 is decreased by addition of negative charges^{35, 46}. These charges might disturb electrostatic interactions between the NLS-containing protein and KPNA2, thereby preventing the interaction between the two proteins and resulting in a cytoplasmic localization of the hyper-phosphorylated protein³⁵. The balance between phosphorylation and dephosphorylation close to NLS sequences can thereby regulate the subcellular localization of a protein in a spatio-temporal manner^{35, 46, 50}. This strictly regulated system is used by several important regulatory proteins such as the acryl hydrocarbon receptor, adenomatous polyposis coli protein, and Swi6, all showing classic NLS sequences flanked by phosphorylation sites^{46, 51, 52}. Complementary to the direct interaction between FRG1 and KPNA2, we therefore suggest a phosphorylation-dependent localization of FRG1 through a KPNA2-mediated nuclear import mechanism.

In addition to nuclear localization, NLS1 and NLS2 showed synergistic roles in the nucleolar localization of FRG1, however, NLS1 is the major NoLS and its function in nucleolar import is dependent on the phosphorylation status of its flanking serine residues. Although the NoLS motifs are generally not well defined, they are composed mostly of Arg or Lys residues, like FRG1⁵³. Although further experiments are needed to identify the *in vivo* phosphorylation of FRG1, the kinase(s) and phosphatase(s) involved in (de)phosphorylation of FRG1 and their roles in its function, this study provides more insight in the mechanism of nuclear and nucleolar localization of FRG1.

Experiments were also performed to determine the biological function of nuclear FRG1. The nuclear fraction of endogenous FRG1 is concentrated in Cajal bodies (CBs) and the granular component (GC) of nucleoli, as well as associated with nascent mRNA transcripts and the

actively transcribed regions of the euchromatin. The CBs are nuclear bodies rich in factors involved in transcription and RNA processing and are sites of snRNP biogenesis^{54, 55}. Thus, FRG1's presence in CBs is consistent with its proposed role in RNA biogenesis and provides additional similarity with RNPs^{8, 9, 11}. Similarly, FRG1's nucleolar localization supports these functional roles. Nucleoli, the dynamic structures containing tandem chromosomal repeats encoding rRNAs, are sites where pre-ribosomal particles are transcribed and assembled; however, nucleoli have additional roles including maturation of some RNP particles, assembly of the RNA splicing machinery, and sequestration of certain nuclear regulatory factors^{55, 56}. FRG1's nucleolar localization to the GC and not with the fibrillar components suggests FRG1 is involved in later stages of rRNA processing and not directly with rDNA transcription⁵⁷.

LBC spreads and RNA-IPs indicate the endogenous FRG1 associates with numerous nascent RNAPII generated mRNAs *in vivo*. FRG1 has recently been biochemically isolated as a part of the activated human spliceosome, specifically as a component of the B^{act} complex and is lost during the transition to the catalytic C complex¹⁰. Thus, FRG1 is one of the few B^{act} complex proteins only transiently associated with the spliceosome during activation, indicating it is not part of the first catalytic splicing step and suggesting that FRG1 plays a specific role in spliceosome activation¹⁰. This spliceosome activation role is supported by the FRG1 RIP experiments (Figure 5) that did not find FRG1 associated with unspliced transcripts. Interestingly in FSHD-derived muscle cells, the muscle-specific isoforms of *FXR1*, a target mRNA of FRG1 by RIP and REMSA, show an aberrant expression pattern due to decreased mRNA stability³⁸ while the mRNAs from fast skeletal muscle troponin T (*TNNT3*) and myotubularin related protein 1 (*MTMR1*) show aberrant alternative mRNA splicing⁵. However, due to the lack of detectable FRG1 overexpression in FSHD muscle it is not clear that FRG1 would be mediating this effect. Whether FRG1 overexpressing cells or FSHD-derived cells exhibit global aberrant alternative splicing has yet to be addressed.

Many RNA-binding proteins and mRNPs are involved in pre-mRNA splicing and processing as well as the transportation, localization, translation and the stability of mRNAs^{40, 58, 59}. We have shown that FRG1 can bind RNA directly *in vitro* and FRG1 is in a complex with mRNA *in vivo*, however, it is still not clear that the *in vivo* RNA association is mediated directly or indirectly through the identified noncanonical FRG1 RBD⁶⁰⁻⁶². Regardless of the nature of the *in vivo* mRNA association, FRG1 interacts directly with TAP supporting a role in mRNA nuclear export⁴¹. TAP:NXT1 heterodimers associate with RNA-binding adaptor proteins, similar to FRG1, in the nucleus to help mediate the nuclear export of the majority of mature mRNAs through the nuclear pore complex. In addition, many adaptor proteins for mRNA export play multiple roles during mRNA biogenesis^{59, 63}. This is likely the case for FRG1. Despite being involved in spliceosome activation, the RIP for FRG1-associated RNAs revealed an association with the spliced *FXR* mRNA supporting FRG1 being involved downstream of mRNA splicing as well. In this light, the abundance of frg1 on the numerous actively transcribing chromatin loops in the LBCs, and in the nucleoli and Cajal bodies (Figure 4) is not surprising. Together, these data support a role for FRG1 in multiple aspects of RNA biogenesis, including RNA splicing and mRNA transport.

The nuclear localization of FRG1 seems to be essential considering its double NLS and bipartite sequences; yet despite these signals, a consistent cytoplasmic pool of FRG1 remains (Figures 1 and 10)^{3, 4}. This suggests that FRG1 is actively being retained in the cytoplasm and that the nuclear function of FRG1 may only be part of the story. We have previously proposed that FRG1 has a role beyond the nucleus as well. The *C. elegans* FRG-1 bundles F-actin *in vitro* and localizes to the body wall muscle dense body, a structure analogous to the vertebrate muscle Z-disk and costameres⁴. Here we show that human FRG1 retains the conserved actin binding activity, binding to actin in a ratio of 2:1, similar

to its *C. elegans* homolog, and forms dimers capable of bundling F-actin⁴. Thus, FRG1 may have a structural role in stabilizing the actin cytoskeleton or may be mediating other cellular functions by associating with the actin cytoskeleton. In support of the latter situation, we showed that FRG1 requires the intact actin cytoskeleton for maintaining its particular punctate cytoplasmic localization suggesting that actin filaments actively anchor FRG1 in the cytoplasm. This may also suggest a mechanism by which overexpressed FRG1 preferentially accumulates in the nucleus; the cytoplasmic binding sites for retaining FRG1 may be occupied with endogenous FRG1.

Considering the associations of FRG1 with mRNA biogenesis and trafficking, we propose a model whereby FRG1's nuclear and cytoplasmic functions are linked through RNA. If FRG1 is functioning as a TAP-mRNA adaptor protein, it would be the first such protein with actin binding activity. FRG1 may associate with nascent mRNA transcripts in the nucleus, which are then exported together to the cytoplasm through the TAP:NXT1 pathway, and the actin cytoskeleton anchors the FRG1-mRNA complex at its designated location. One remaining question, however, revolves around specificity of the FRG1-RNA interaction. How would a protein such as FRG1, which seemingly interacts with most mRNAs in the nucleus based on lampbrush, RIP and spliceosome studies, selectively transport specific mRNAs to locations in the cytoplasm as we propose? Just such a mechanism for regulating cytoplasmic mRNA localization was recently identified in yeast⁶⁴. In yeast, the nuclear mRNA transport proteins, She2p and Puf6p, bind their cargo mRNAs in the nucleus with low affinity; however, specific mRNA recognition takes place after nuclear export when the cytoplasmic protein She3p associates with the complex and provides sequence binding specificity such that only specific mRNAs form a transport complex resulting in those cargo mRNAs being targeted and transported to a specific subcellular localization⁶⁴. Thus, FRG1's mRNA specificity in the nucleus may be similarly non-sequence specific but a cytoplasmic FRG1 complex could provide the high affinity binding for specific mRNAs required for a role in cytoplasmic mRNA transport and localized translation.

FRG1 is clearly an important multifunctional protein involved in muscle development, however a role in FSHD pathophysiology is controversial due to the failure to consistently find any significant changes in FRG1 levels between patients and unaffected individuals. Still, recent data towards understanding FRG1 is compelling in respect to FSHD. Overexpression of FRG1 specifically disrupts development of the vertebrate musculature and vasculature, the two tissues most affected in FSHD^{6,7}. Cytoplasmic FRG1 localizes to the skeletal muscle Z-disc in mouse and humans, the subcellular localization of numerous proteins related to myriad myopathies^{3,65,66}. Here we show FRG1 is a dynamic RNA-associated actin binding protein. In addition, FRG1's interactions with both a nuclear importer (KPNA2) and a nuclear exporter (TAP) indicate its subcellular localization is highly regulated. Interestingly, in all systems tested including mammalian cell culture, *C. elegans*, *Xenopus*, and *Drosophila*, overexpressed FRG1 preferentially accumulates in the nucleus^{4,9,11} (Figure S2). Alterations of FRG1 protein levels could change the subcellular distribution of FRG1, subsequently dysregulating its function. Increasing nuclear levels of FRG1 may ultimately result in mis-spliced mRNA transcripts, altered mRNA stability, or affect mRNA transport and thus translation, any of which could adversely affect the maintenance of muscle integrity.

Material and Methods

DNA constructs

All PCR primers are listed in Table S1. To generate the FRG1 His-tagged bacterial expression constructs, the cDNA for the full-length human FRG1 coding sequence was PCR

amplified (primers #1 and #2) and subcloned between the NdeI and XhoI restriction sites of pET-23b vector (Novagen, Gibbstown, NJ). The pET-FRG1 deletion constructs were made by the same procedure with the following primer sets: #2, #3 for ($\Delta 1-32$), #2, #4 for ($\Delta 1-20$), #1, #5 for ($\Delta 235-258$), and #1, #6 for ($\Delta 183-258$). The pSG8VSV-FRG1, pEGFPc1-FRG1, and pGEX-FRG1 expression constructs were generated by cloning the ORF of FRG1 from fibroblast-derived cDNA, as previously described¹¹. The FRG1 deletion constructs in pSG8VSV incorporating large FRG1 deletions, $\Delta 5-80$, $\Delta 232-257$, $\Delta 5-80, \Delta 232-257$ and $\Delta 48-230$ were described before¹¹. The Δ NLS1 mutant was generated by cloning the NotI/XhoI digested PCR product T7F $\times$ Rfrg1 45/63-XhoI (primer #7) and the XhoI/SacI digested Ffrg1 75/98-XhoI (primer #8) $\times$ 378R (primer #9) PCR product into the NotI/SacI digested pSG8VSV-FRG1 vector. The Δ NLS2 mutant was generated by cloning the NotI/XhoI digested PCR product T7F $\times$ Rfrg1 62/84-XhoI (primer #10) and the XhoI/SacI digested frg1 100/120-XhoI (primer #11) $\times$ 378R (primer #9) PCR product into the NotI/SacI digested pSG8VSV-FRG1 vector. The single amino acid mutants S21A, S21D, S26A and S26D were generated using the site-directed mutagenesis kit (Quick Change XL, Stratagene, La Jolla, CA), according to the manufacturer's instructions. First, the NotI/SacI and the SacI/HindIII FRG1 fragments were cloned from pSG8VSV into the pBluescript (pBS) for generating respectively the serine 21/serine 26 mutants. Briefly, serine 21 was converted into an alanine (A) using the primers FRG1 S21A (primers #12, #13) or into an aspartic acid (D) using primers FRG1 S21D (primers #14, #15). FRG1 S26A and S26D substitutions were generated using primers FRG1 S26A (primers #16, #17) or FRG1 S26D (primers #18, #19). After the conversion of the specific amino acids, the NotI/SacI and the SacI/HindIII FRG1 fragments were recloned into the pSG8VSV vector. For the HA-FRG1 point mutant expression constructs, the pSG8VSV-S21AS26A and S21DS26D were used as templates for PCR (primers #20, #21). The products were TA-cloned into pGEM-T easy (Promega), sequenced, and subcloned into pcDNA3.1HA³ by BamHI/XhoI digestion. The pET28-KPNA2 was generated by PCR (primers #22, #23) on image clone 3867599 (RZPD). The pGEM-FXR was generated by RT-PCR from HSMM (Lonza) RNA (primers #24, #25). Constructs were sequence verified using the BigDye terminator sequencing kit (Perkin Elmer, Foster City, CA, USA) and analyzed on an ABI377 (Perkin Elmer). Extensive cloning strategies are available on request.

Cell Culture

HeLa, C2C12, U2OS, and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (pen/strep). Cells were incubated with 5% CO₂ at 37°C. For Latrunculin B (LatB) treatments, C2C12 cells were treated with indicated concentrations of LatB (Invitrogen Corp., Carlsbad, CA) for 5 or 15 min as indicated. After Lat B treatment, cells were fixed with 2% FA for 15 min at RT and subjected to immunostaining described below. For the nuclear and nucleolar localization studies, COS-1 cells were incubated with 5% CO₂ at 37°C. Twenty hours prior to transfection, 2.4×10^4 cells (24-well plate with coverslips) were seeded. Cells were transfected using FuGENE (Roche, Basel, Switzerland), according to the manufacturer's instructions. Subcellular localization studies were performed under blind conditions in which 200 individual COS-1 cells were analyzed in two independent experiments (2X n=100).

FRG1 Antibodies

Three rabbit polyclonal antibodies were used, all raised against synthetic peptides corresponding to human FRG1. The HS1 (NH₂-CKKDDIPEEDKGNVK) and HS2 (NH₂-CGRSDAIGPREQWEP) FRG1 antibodies were generated by GenScript USA Inc. (Piscataway, NJ), and antisera were affinity purified against the peptide cross-linked to NHS-Sepharose (GE Healthcare, Piscataway, NJ), eluted in 10 mM glycine, pH 2.5,

dialyzed against PBS pH7.4, and characterized for ICC by siRNA knockdown³ and by western blotting (Figure S2). FRG1-718 (NH₂-VGRSDAIGPREQWEPV-CONH₂) and FRG1-719 (NH₂-CETLLDRRAKLKADRY-CONH₂) were generated by Eurogentech Double X program (Seraing, Belgium), and the antisera were pooled and affinity purified against one or the other peptide. As affinity purified antiserum 719 showed the highest and most specific immunodetection of FRG1, this antibody was used in further experiments⁹. FRG1-719 was used at 1:50 for immunostaining.

Protein extracts

HeLa cells were washed 3X in PBS, suspended in 5 packed cell volumes of Buffer A (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1mM DTT, 250 mM sucrose) plus protease inhibitors (1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin) and incubated on ice for 5 min. Cells were lysed by loose pestle Dounce homogenization. Nuclei were pelleted at 1000g for 10 min at 4°C. The supernatant containing the cytosolic fraction was removed to a new tube. Nuclear extraction buffer (20 mM HEPES pH 7.5, 250 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 0.1 % SDS, protease inhibitors) was added to the nuclear pellet (5 packed nuclear volumes), the DNA was fragmented by sonication and the soluble fraction was used as the nuclear fraction (N). The cytosolic fraction was centrifuged at 10,000g for 10 min to pellet the mitochondria. The supernatant was removed to a new tube and used as the cytoplasmic fraction (C). Cell equivalent volumes of fractions were separated by 10% SDS-PAGE, transferred to PVDF membranes and probed for HS1, HS2, PCNA, or β-Tubulin.

ICC

For endogenous FRG1, HeLa and C2C12 cells were fixed in 2% formaldehyde (FA) in PBS for 15 min at room temperature (RT). For overexpressed FRG1, HeLa cells (~60% confluent) were transfected with pcDNA3.1HA-FRG1³ using TransIT-LT1 reagent (Mirus Bio, Madison, WI) and allowed to grow for 24 hrs prior to fixation as above. After fixation, cells were made permeable with 0.25% Triton-X 100 in PBS for 10 min on ice, and subsequently blocked with 2% BSA in PBS for 30 min at RT. Primary antibody (FREG1 HS1) incubation was carried out overnight at 4°C in PBS with 2% BSA, and secondary antibody incubation was for 40 min at RT in PBS. Co-immunostaining experiments were performed using FRG1 HS1, HA rat monoclonal (Roche, clone 3F10), mouse monoclonal fibrillarin (ab18380, Abcam, Cambridge, MA) and SC-35 (S4045, Sigma-Aldrich, St. Louis, MO) antibodies at 1: 500, 1:100, 1:100 and 1:200 dilutions, respectively. Secondary antibodies used were FITC-conjugated donkey anti-rabbit (pre-cleared) and rhodamine-conjugated goat anti-mouse, or rhodamine-conjugated goat anti-rabbit and FITC-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories Inc) used at 1:100 or Alexa594-conjugated goat anti-rat IgG (Invitrogen, Corp) used at 1:1000. F-actin was visualized with 5 U/ml rhodamine-phalloidin (Invitrogen Corp) incubated for 30 min at RT. The 4,6-diamidino-2-phenylindole (DAPI) was used at 1µg/ml.

COS-1 cells were fixed (24 hrs post-transfection) with 4% FA and permeabilized in 0.1% triton-X100 in PBS for 20 min at RT. To detect the transfected FRG1 constructs, a mouse anti-VSV primary antibody (P5D4; gift from Dr. J. Franssen, Nijmegen, The Netherlands) was used at 1:100 for 1 hr at RT. Goat anti-mouse Alexa 488 secondary antibody was used at 1:250 for 1 hr at RT.

Lampbrush Chromosome Spreads

Female adult frogs (*Xenopus laevis*) were anesthetized in 0.15% tricaine methanesulfonate (Sigma-Aldrich), and small fragments of ovary were surgically removed. Oocytes were defolliculated for 1 hr in saline buffer OR2⁶⁷ containing 0.15% collagenase type II (Sigma-

Aldrich). Stage IV-V oocytes were selected and maintained in OR2 at 18°C. Nuclear spreads were prepared as described⁶⁸. Fixed nuclear spreads were rinsed in PBS and blocked in PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) plus 0.5% gelatin (from cold-water fish). Spreads were incubated with primary antibody, XTB-FRG1⁶, for 1 hr at RT, washed for 30 min with two changes of PBS, incubated with secondary antibody Alexa 488 conjugated goat anti-rabbit antibody (Invitrogen Corp.) for 1 hr at RT, and washed again for 30 min with two changes of PBS. Spreads were mounted in 50% glycerol containing 1mg/ml phenylenediamine and 10 pg/ml DAPI.

For the HA epitope tagged FRG1 immunostaining, an HA peptide sequence was added in-frame to the amino terminus of frg1 by inserting the annealed oligonucleotide primers #26 and #27 in between the *HindIII* and *SalI* restriction sites of the pMS2-xt-frg1 vector⁶. The vector was linearized and capped RNA was generated by *in vitro* transcription using the SP6 mMessage mMachine kit (Ambion). RNA (1 mg/ml) was injected (30 nl) into the cytoplasm of oocytes and incubated 16 hrs at 18°C to allow for translation. Oocytes were processed for nuclear spreads and HA immunostaining as described⁶⁹. The rat HA high affinity monoclonal antibody (clone 3F10, Roche) was used (20 ng/μl) as the primary antibody and Alexa 594-labeled goat anti rat IgG (Invitrogen) was used (2.5 μg/ml) as the secondary antibody.

Nuclear shuttling assay

The assay was carried out essentially as described³. HeLa cells (~60% confluent) were transfected with pcDNA3.1HA-FRG1³ using TransIT-LT1 reagent (Mirus Bio, Madison, WI) and allowed to grow for 24 hrs. The cells were removed with trypsin, washed in PBS, plated (1×10^6) on glass coverslips and allowed to adhere for 2 hrs before non-transfected murine C2C12 cells (5×10^5) were overlaid onto the transfected HeLa cells for 3 hrs. Translation was stopped with cycloheximide (CHX) (100 μg/ml) treatment for 15 min prior to fusion. After removing the media, cells fused by adding 50% polyethylene glycol 4000 in DMEM for 2 min. The fusions were immediately washed with DMEM and then incubated for three hrs in the presence of CHX (100 μg/ml) or CHX (100μg/ml) + leptomycin B (2 ng/ml) and processed for ICC using a HA (clone 3F10, Roche) monoclonal antibody and co-staining with Hoechst 33342 (5 μg/ml).

Co-immunoprecipitations (co-IPs)

For co-IPs with endogenous FRG1, nuclear extracts were obtained from untransfected U2OS cells as described⁷⁰. Nuclear pellets were washed in PBS and lysed with 1 ml cold CHAPS buffer [50 mM Tris pH 7.5; 150 mM NaCl; 0.15% CHAPS; and protease inhibitor cocktail (Roche)] for 30 min on ice. Subsequently, cells were snap frozen, thawed, then spun down at maximum speed for 10 min at 4°C. The lysates were incubated with 5 μl rabbit αFRG1-719 antibody with rotation at 4°C overnight. Subsequently, 25 μl of washed Protein-A sepharose 4B beads (GE Healthcare) in CHAPS buffer were added and incubated with rotation for 1 hr at 4°C. The beads were washed 4 times with cold CHAPS buffer, and once with 50 mM Tris pH 7.5, after which the beads and the non-bound (NB) fractions were boiled in sample buffer for 8 min and run in duplicate on a 10% SDS-PAGE protein gel (15 μl per lane), transferred to PVDF membrane and probed with mouse anti-KPNA2 (BD Biosciences Pharmingen, San Diego, CA).

For co-IPs of the HA-FRG1 series, 1×10^7 HeLa cells were transfected with 10 μg of pcDNA3.1HA-FRG1, pcDNA3.1HA-S21AS26A, or pcDNA3.1HA-S21DS26D using TransIT-LT1 transfection reagent (Mirus), respectively. Cells were lysed 48 hours post-transfection in 800 μl IPH buffer (50 mM Tris 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1 mM PMSF and protease inhibitors) as described⁷¹. Lysates were incubated at

4°C for 40 min before centrifugation at 12,000g for 15 min to remove the debris. The soluble protein was incubated with HA rat monoclonal antibody (clone 3F10, Roche) at 4°C overnight with rotation. Immune complexes were collected using protein G Dynabeads (Invitrogen) as per manufacture's instructions and eluted by boiling in Laemmli sample buffer. Input (5%) and bound fractions were assayed using SDS-PAGE and followed by western analysis using HS1 or KPNA2 (#GTX106323, GeneTex) antibodies.

RNA-IP

These experiments were carried out essentially as described⁷². HeLa cells (1×10^8) were suspended in 40 ml 1x PBS with 4 ml cross-linking buffer (100 mM NaCl, 50 mM HEPES, 1 mM EDTA, 0.5 mM EGTA, 11% FA) for 30 min at RT with rocking. The reaction was quenched with 2.2 ml 2.5 M glycine (pH 7.0) for 5 min at RT then washed with 1X PBS and pelleted. Cells were lysed by suspending in FA buffer (50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, protease inhibitors) + RNase inhibitor (50 U/500 μ l) and sonicated on a Branson sonifier at 25% power for 20 pulses of 20 sec with 30 sec rest on ice between each pulse. The solution was made up to 25 mM MgCl₂ and 5 mM CaCl₂ then RNase-free DNase I (100 U/500 μ l) and RNase inhibitor (3 μ l/500 μ l) were added and incubated at 37°C for 30 min. The reactions were centrifuged at 20,000 g for 15 min at 4°C, keeping the supernatant for IPs.

For each RNA-IP reaction, 100 μ l cleared extract was diluted with 900 μ l ChIP buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.5% NP-40) to which 6 μ l antibody (FRG1 antibodies α HS1 or α HS2, or the control IP antibodies α PAT-9, α HSB, which do not IP FRG1) and 4 μ l RNase inhibitor were added then rotated for 12 hrs at 4°C. Protein A Dynabeads (40 μ l/IP) were washed twice in ChIP buffer then added to the IP reactions for 1 hr. Dynabead IPs were washed 3X in 1 ml wash buffer (50 mM Tris pH 7.4, 500 mM NaCl, 1% Triton X-100, 0.1% SDS) and RNA was eluted in 200 μ l Elution Buffer (200 mM NaCl, 50 mM Tris pH 7.4, 20 μ g Proteinase K) for 1 hr at 42°C. Cross-linking was reversed by heating at 65°C for 5 hrs, and the RNA was extracted with acid equilibrated (pH 4.8) phenol:chloroform (5:1), ethanol precipitated, and brought up in DEPC-dH₂O for use in RT-PCR (FXR1 primers #24, #25 or primers #28, #29; FRG1 primers #30, #31).

REMSA

To generate full-length and deletion-containing FRG1 recombinant proteins, the pET23 plasmid constructs described above were transformed into *E. coli* BL21(DE3) cells and induced with 1 mM IPTG. The protein was purified using TALON resin (Clontech, Mountain View, CA) as per the manufacturer's instructions, further purified by ion exchange chromatography (MonoS resin, GE Healthcare), dialyzed to 50 mM NaCl, aliquoted and analyzed for integrity and purity by SDS-PAGE and Coomassie Blue R250 staining (Fig S5). Two templates for radiolabeled RNA probes were used, pGEM FXR containing part of the human FXR coding region and T7TS⁷³, containing the 5' and 3' untranslated regions (UTRs) of the *Xenopus* β -globin mRNA. To generate the FXR probe, pGEM-FXR was linearized with SpeI and to generate the UTR probe, the T7TS plasmid was linearized with BglII. RNA was *in vitro* transcribed using T7 RNA polymerase and [³²P]-UTP according to the manufacturer's instructions, treated with TURBO DNase (Ambion, Inc., Austin, TX) for 15 min at 37°C, purified on a 5% TBE gel and the extracted radiolabeled RNA was quantified by scintillation counter (Beckman LS6500). The RNA-protein binding assay was performed in REMSA buffer (1 mM MgCl₂, 10 mM Tris 7.4, 5% glycerol, 0.1% IGEPAL/NP-40, 50 mM NaCl, and 20 u RNasin (Promega, Madison, WI) with 30,000 CPM labeled FXR probe or 50,000 CPM [³²P] labeled UTR probe. For the deletion series, recombinant full-length FRG1 and deletion constructs were used at 150, 300, and 450 ng. Reactions were incubated on ice for 20 min, analyzed by 5% TBE gel, and

visualized by autoradiography. Unlabeled RNA probe, tRNA (Sigma-Aldrich), and T7TS plasmid, added to the reactions prior to the specific radiolabeled probe, were used as competitors.

GST pull-down

To test the FRG1-KPNA2 interaction, recombinant GST protein inductions (50 ml) were performed in *E. coli* DH5 α bacterial cells using 1mM IPTG. Bacterial cells were dissolved in NETN buffer [20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% IGEPAL, protease inhibitor cocktail (Roche)] 3 hrs after induction and lysed by French press (American Instrument Company, Haverhill, MA, USA). Lysates were centrifuged for 30 min at 7000 *g* to obtain the soluble GST or GST-FRG1 fractions. These soluble fractions (100 μ l) were incubated with 40 μ l of a 75% glutathione bead slurry (4B, Amersham Pharmacia Biotech, Buckinghamshire, UK), rotating for 1 hr at 4°C. To test the FRG1-TAP interaction, recombinant GST and GST-Tap proteins were purified and bound to glutathione beads as described ⁷⁴. Meanwhile, pET28-KPNA2 or pET23-FRG1 constructs were used for *in vitro* transcription/translation (TnT) according to the manufacturer's instructions (TnT-kit, Promega). After washing the glutathione beads bound to GST proteins, the ³⁵S methionine labeled proteins were added and subsequently incubated with the GST, GST-FRG1P, or GST-TAP beads with rotation for 3 hrs at 4°C. The FRG1-KPNA2 reactions were carried out in 500 mM NaCl and the FRG1-TAP interactions were carried out in 150 mM NaCl as described ⁷⁴. The beads were washed and proteins eluted by boiling with 20 μ l of 2x Laemmli sample buffer, after which the whole samples were loaded onto a SDS-PAGE gel. The gels were dried and exposed to film overnight.

Actin binding assay

Actin was purified from rabbit skeletal muscle as described ⁷⁵. Recombinant full-length FRG1 generated as described above was further purified by ion exchange chromatography, binding to MonoS resin using an AKTA-FPLC (GE Healthcare), step eluted between 350 and 700 mM NaCl in 10 mM HEPES pH 7.5 and 10% glycerol, and dialyzed to 50 mM NaCl. Actin co-sedimentation assays were carried out as described with minor modifications ⁷⁶. For high-speed sedimentation assays, increasing amounts of recombinant FRG1 (7–30 mM as monomer) were incubated with G-actin (final concentration 6 μ M) in 50 mM NaCl in a total volume of 50 μ l at RT for 2 hrs and then centrifuged at 100,000 *g* for 20 min at RT. The amounts of FRG1 and F-actin in the supernatants and pellets were determined by densitometry using Coomassie-Blue-stained 10% SDS-PAGE. The intensities of the stained polypeptide bands were quantified by volume integration after local background subtraction using Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA). Subtracting the percentage of FRG1 in the pellet without actin was used to normalize the FRG1. The binding data from four independent experiments were analyzed by fitting to the Michaelis-Menton equation, $Y=B_{max} \cdot X/(K_d+X)$, using the nonlinear regression function of Prism 5 (GraphPad Software), where Y is FRG1 per F-actin (mol/mol) in the pellet and X is the FRG1 concentration (micromolar) remaining in the supernatant.

Glutaraldehyde cross-linking

Recombinant full-length FRG1 protein (0.1 mg/ml) was incubated with 0.01% glutaraldehyde in 1X PBS in 1 ml at RT. Samples (50 μ l) were removed at the indicated time intervals and the reactions were stopped by the addition of 2X Laemmli sample buffer, boiled, and separated by SDS-PAGE (12%). Protein was detected by western blotting using the HS2 antibody described above.

Microscopy

Standard fluorescence microscopy was carried out using a HCL FL Fluotar 100X oil objective (NA=1.30) on an upright Leica DMR microscope. Images were captured using a monochrome Retiga EXI Charge-Coupled Device (CCD) camera (Qimaging) driven by the In vivo software (version 3.2.0, Media Cybernetics). All images were processed using Adobe Photoshop to adjust brightness, contrast, size, and merged or split channels. Applied Precision Personal Deltavision was used for deconvolution images.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AMD	actinomycin D
CHX	cycloheximide
co-IP	co-immunoprecipitation
DAPI	4,6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DUX4	double homeobox 4
FBS	fetal bovine serum
FRG1	FSHD region gene 1
FSHD	facioscapulohumeral muscular dystrophy
HA	hemagglutinin
ICC	immunocytochemistry
IP	immunoprecipitation
KPNA2	karyopherin alpha 2
LatB	latrunculin B
LMB	leptomycin B
NLS	nuclear localization signal
NoLS	nucleolar localization signal
RIP	RNA IP
TAP	Tip-associated protein
VSV	Vesicular Stomatitis Virus

Acknowledgments

We would like to thank Dr. Y.W. Lam for helping with the optimization of the α FRG1-719 antibody in immunofluorescence. In addition, we thank Daniel Perez and the FSH Society for their continued support. This work was funded by the National Institutes of Health (NIAMS grants #RO1 AR055877, #R21 AR48327 and #R21 AR055876), the Prinses Beatrix Fonds, the Muscular Dystrophy Association (USA), The Dutch FSHD Foundation, the Stichting Spieren voor Spieren, the European Union (#QLTR-2000-01673), the Shaw family and the FSH Society.

References

1. Grewal PK, Todd LC, van der Maarel S, Frants RR, Hewitt JE. FRG1, a gene in the FSH muscular dystrophy region on human chromosome 4q35, is highly conserved in vertebrates and invertebrates. *Gene*. 1998; 216:13–19. [PubMed: 9714712]
2. Kureishy N, Sapountzi V, Prag S, Anilkumar N, Adams JC. Fascins, and their roles in cell structure and function. *Bioessays*. 2002; 24:350–361. [PubMed: 11948621]
3. Hanel ML, Sun CY, Jones TI, Long SW, Zanutti S, Milner D, Jones PL. Facioscapulohumeral muscular dystrophy (FSHD) region gene 1 (FRG1) is a dynamic nuclear and sarcomeric protein. *Differentiation*. 2011; 81:107–118. [PubMed: 20970242]
4. Liu Q, Jones TI, Tang VW, Briehner WM, Jones PL. Facioscapulohumeral muscular dystrophy region gene-1 (FRG-1) is an actin-bundling protein associated with muscle-attachment sites. *J Cell Sci*. 2010; 123:1116–1123. [PubMed: 20215405]
5. Gabellini D, D'Antona G, Moggio M, Prella A, Zecca C, Adami R, Angeletti B, Ciscato P, Pellegrino MA, Bottinelli R, Green MR, Tupler R. Facioscapulohumeral muscular dystrophy in mice overexpressing FRG1. *Nature*. 2006; 439:973–977. [PubMed: 16341202]
6. Hanel ML, Wuebbles RD, Jones PL. Muscular dystrophy candidate gene FRG1 is critical for muscle development. *Dev Dyn*. 2009; 238:1502–1512. [PubMed: 19097195]
7. Wuebbles RD, Hanel ML, Jones PL. FSHD region gene 1 (FRG1) is crucial for angiogenesis linking FRG1 to facioscapulohumeral muscular dystrophy-associated vasculopathy. *Dis Model Mech*. 2009; 2:267–274. [PubMed: 19383939]
8. Rappsilber J, Ryder U, Lamond AI, Mann M. Large-scale proteomic analysis of the human spliceosome. *Genome Res*. 2002; 12:1231–1245. [PubMed: 12176931]
9. van Koningsbruggen S, Straasheijm KR, Sterrenburg E, de Graaf N, Dauwerse HG, Frants RR, van der Maarel SM. FRG1P-mediated aggregation of proteins involved in pre-mRNA processing. *Chromosoma*. 2007; 116:53–64. [PubMed: 17103222]
10. Bessonov S, Anokhina M, Krasauskas A, Golas MM, Sander B, Will CL, Urlaub H, Stark H, Luhrmann R. Characterization of purified human Bact spliceosomal complexes reveals compositional and morphological changes during spliceosome activation and first step catalysis. *RNA*. 2010; 16:2384–2403. [PubMed: 20980672]
11. van Koningsbruggen S, Dirks RW, Mommaas AM, Onderwater JJ, Deidda G, Padberg GW, Frants RR, van der Maarel SM. FRG1P is localised in the nucleolus, Cajal bodies, and speckles. *J Med Genet*. 2004; 41:e46. [PubMed: 15060122]
12. Tawil R. Facioscapulohumeral muscular dystrophy. *Neurotherapeutics*. 2008; 5:601–606. [PubMed: 19019312]
13. van der Maarel SM, Frants RR, Padberg GW. Facioscapulohumeral muscular dystrophy. *Biochim Biophys Acta*. 2007; 1772:186–194. [PubMed: 16837171]
14. van der Maarel SM, Frants RR. The D4Z4 repeat-mediated pathogenesis of facioscapulohumeral muscular dystrophy. *Am J Hum Genet*. 2005; 76:375–386. [PubMed: 15674778]
15. Orphanet. Prevalence of rare diseases: Bibliographic data. Orphanet Report Series: Rare Diseases collection. 2009. [cited November; Available from: http://www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_alphabetical_list.pdf]
16. Wijmenga C, Hewitt JE, Sandkuijl LA, Clark LN, Wright TJ, Dauwerse HG, Gruter AM, Hofker MH, Moerer P, Williamson R, et al. Chromosome 4q DNA rearrangements associated with facioscapulohumeral muscular dystrophy. *Nat Genet*. 1992; 2:26–30. [PubMed: 1363881]
17. van Deutekom JC, Wijmenga C, van Tienhoven EA, Gruter AM, Hewitt JE, Padberg GW, van Ommen GJ, Hofker MH, Frants RR. FSHD associated DNA rearrangements are due to deletions of integral copies of a 3.2 kb tandemly repeated unit. *Hum Mol Genet*. 1993; 2:2037–2042. [PubMed: 8111371]
18. Zeng W, de Greef JC, Chen YY, Chien R, Kong X, Gregson HC, Winokur ST, Pyle A, Robertson KD, Schmiesing JA, Kimonis VE, Balog J, Frants RR, Ball AR Jr, Lock LF, Donovan PJ, van der Maarel SM, Yokomori K. Specific loss of histone H3 lysine 9 trimethylation and HP1 gamma/

cohesin binding at D4Z4 repeats is associated with facioscapulohumeral dystrophy (FSHD). *PLoS Genet.* 2009; 5:e1000559. [PubMed: 19593370]

19. van Deutekom JC, Lemmers RJ, Grewal PK, van Geel M, Romberg S, Dauwerse HG, Wright TJ, Padberg GW, Hofker MH, Hewitt JE, Frants RR. Identification of the first gene (FRG1) from the FSHD region on human chromosome 4q35. *Hum Mol Genet.* 1996; 5:581–590. [PubMed: 8733123]
20. Gabellini D, Green MR, Tupler R. Inappropriate gene activation in FSHD: a repressor complex binds a chromosomal repeat deleted in dystrophic muscle. *Cell.* 2002; 110:339–348. [PubMed: 12176321]
21. Winokur ST, Chen YW, Masny PS, Martin JH, Ehmsen JT, Tapscott SJ, van der Maarel SM, Hayashi Y, Flanigan KM. Expression profiling of FSHD muscle supports a defect in specific stages of myogenic differentiation. *Hum Mol Genet.* 2003; 12:2895–2907. [PubMed: 14519683]
22. Jiang G, Yang F, van Overveld PG, Vedanarayanan V, van der Maarel S, Ehrlich M. Testing the position-effect variegation hypothesis for facioscapulohumeral muscular dystrophy by analysis of histone modification and gene expression in subtelomeric 4q. *Hum Mol Genet.* 2003; 12:2909–2921. [PubMed: 14506132]
23. Osborne RJ, Welle S, Venance SL, Thornton CA, Tawil R. Expression profile of FSHD supports a link between retinal vasculopathy and muscular dystrophy. *Neurology.* 2007; 68:569–577. [PubMed: 17151338]
24. Arashiro P, Eisenberg I, Kho AT, Cerqueira AM, Canovas M, Silva HC, Pavanello RC, Verjovski-Almeida S, Kunkel LM, Zatz M. Transcriptional regulation differs in affected facioscapulohumeral muscular dystrophy patients compared to asymptomatic related carriers. *Proc Natl Acad Sci U S A.* 2009; 106:6220–6225. [PubMed: 19339494]
25. Klooster R, Straasheijm K, Shah B, Sowden J, Frants R, Thornton C, Tawil R, van der Maarel S. Comprehensive expression analysis of FSHD candidate genes at the mRNA and protein level. *Eur J Hum Genet.* 2009; 17:1615–1624. [PubMed: 19809486]
26. Masny PS, Chan OY, de Greef JC, Bengtsson U, Ehrlich M, Tawil R, Lock LF, Hewitt JE, Stocksdales J, Martin JH, van der Maarel SM, Winokur ST. Analysis of allele-specific RNA transcription in FSHD by RNA-DNA FISH in single myonuclei. *Eur J Hum Genet.* 2010; 18:448–456. [PubMed: 19888305]
27. Lemmers RJ, van der Vliet PJ, Klooster R, Sacconi S, Camano P, Dauwerse JG, Snider L, Straasheijm KR, van Ommen GJ, Padberg GW, Miller DG, Tapscott SJ, Tawil R, Frants RR, van der Maarel SM. A unifying genetic model for facioscapulohumeral muscular dystrophy. *Science.* 2010; 329:1650–1653. [PubMed: 20724583]
28. Aris JP, Blobel G. cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. *Proc Natl Acad Sci U S A.* 1991; 88:931–935. [PubMed: 1846968]
29. Olson MO, Dundr M. The moving parts of the nucleolus. *Histochem Cell Biol.* 2005; 123:203–216. [PubMed: 15742198]
30. Spector DL, Fu XD, Maniatis T. Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* 1991; 10:3467–3481. [PubMed: 1833187]
31. Kaffman A, O'Shea EK. Regulation of nuclear localization: a key to a door. *Annu Rev Cell Dev Biol.* 1999; 15:291–339. [PubMed: 10611964]
32. Jans DA, Xiao CY, Lam MH. Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays.* 2000; 22:532–544. [PubMed: 10842307]
33. Chook YM, Blobel G. Karyopherins and nuclear import. *Curr Opin Struct Biol.* 2001; 11:703–715. [PubMed: 11751052]
34. Fontes MR, Teh T, Kobe B. Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin- α . *J Mol Biol.* 2000; 297:1183–1194. [PubMed: 10764582]
35. Harreman MT, Kline TM, Milford HG, Harben MB, Hodel AE, Corbett AH. Regulation of nuclear import by phosphorylation adjacent to nuclear localization signals. *J Biol Chem.* 2004; 279:20613–20621. [PubMed: 14998990]

36. Soulard M, Della Valle V, Siomi MC, Pinol-Roma S, Codogno P, Bauvy C, Bellini M, Lacroix JC, Monod G, Dreyfuss G, et al. hnRNP G: sequence and characterization of a glycosylated RNA-binding protein. *Nucleic Acids Res.* 1993; 21:4210–4217. [PubMed: 7692398]
37. Morgan GT. Lampbrush chromosomes and associated bodies: new insights into principles of nuclear structure and function. *Chromosome Res.* 2002; 10:177–200. [PubMed: 12067208]
38. Davidovic L, Sacconi S, Bechara EG, Delplace S, Allegra M, Desnuelle C, Bardoni B. Alteration of expression of muscle specific isoforms of the fragile X related protein 1 (FXR1P) in facioscapulohumeral muscular dystrophy patients. *J Med Genet.* 2008; 45:679–685. [PubMed: 18628314]
39. Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG. hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem.* 1993; 62:289–321. [PubMed: 8352591]
40. Dreyfuss G, Kim VN, Kataoka N. Messenger-RNA-binding proteins and the messages they carry. *Nat Rev Mol Cell Biol.* 2002; 3:195–205. [PubMed: 11994740]
41. Gruter P, Tabernero C, von Kobbe C, Schmitt C, Saavedra C, Bachi A, Wilm M, Felber BK, Izaurralde E. TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol Cell.* 1998; 1:649–659. [PubMed: 9660949]
42. Katahira J, Strasser K, Podtelejnikov A, Mann M, Jung JU, Hurt E. The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human. *EMBO J.* 1999; 18:2593–2609. [PubMed: 10228171]
43. Katahira J, Inoue H, Hurt E, Yoneda Y. Adaptor Aly and co-adaptor Thoc5 function in the Tap-p15-mediated nuclear export of HSP70 mRNA. *EMBO J.* 2009; 28:556–567. [PubMed: 19165146]
44. Nishi K, Yoshida M, Fujiwara D, Nishikawa M, Horinouchi S, Beppu T. Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. *J Biol Chem.* 1994; 269:6320–6324. [PubMed: 8119981]
45. Wolff B, Sanglier JJ, Wang Y. Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol.* 1997; 4:139–147. [PubMed: 9190288]
46. Ikuta T, Kobayashi Y, Kawajiri K. Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. *Biochem Biophys Res Commun.* 2004; 317:545–550. [PubMed: 15063792]
47. Mirski SE, Gerlach JH, Cole SP. Sequence determinants of nuclear localization in the alpha and beta isoforms of human topoisomerase II. *Exp Cell Res.* 1999; 251:329–339. [PubMed: 10471318]
48. Mo YY, Wang C, Beck WT. A novel nuclear localization signal in human DNA topoisomerase I. *J Biol Chem.* 2000; 275:41107–41113. [PubMed: 11016921]
49. Schwamborn K, Albig W, Doenecke D. The histone H1(0) contains multiple sequence elements for nuclear targeting. *Exp Cell Res.* 1998; 244:206–217. [PubMed: 9770363]
50. Jans DA, Hubner S. Regulation of protein transport to the nucleus: central role of phosphorylation. *Physiol Rev.* 1996; 76:651–685. [PubMed: 8757785]
51. Sidorova JM, Mikesell GE, Breeden LL. Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. *Mol Biol Cell.* 1995; 6:1641–1658. [PubMed: 8590795]
52. Zhang F, White RL, Neufeld KL. Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein. *Proc Natl Acad Sci U S A.* 2000; 97:12577–12582. [PubMed: 11050185]
53. Emmott E, Hiscox JA. Nucleolar targeting: the hub of the matter. *EMBO Rep.* 2009; 10:231–238. [PubMed: 19229283]
54. Gall JG. [A role for Cajal bodies in assembly of the nuclear transcription machinery]. *Tsitologiia.* 2003; 45:971–975. [PubMed: 14989168]
55. Austin CM, Bellini M. The dynamic landscape of the cell nucleus. *Mol Reprod Dev.* 2010; 77:19–28. [PubMed: 19650114]
56. Boisvert FM, van Koningsbruggen S, Navascues J, Lamond AI. The multifunctional nucleolus. *Nat Rev Mol Cell Biol.* 2007; 8:574–585. [PubMed: 17519961]

57. Sirri V, Urcuqui-Inchima S, Roussel P, Hernandez-Verdun D. Nucleolus: the fascinating nuclear body. *Histochem Cell Biol.* 2008; 129:13–31. [PubMed: 18046571]
58. Aguilera A. Cotranscriptional mRNP assembly: from the DNA to the nuclear pore. *Curr Opin Cell Biol.* 2005; 17:242–250. [PubMed: 15901492]
59. Kelly SM, Corbett AH. Messenger RNA export from the nucleus: a series of molecular wardrobe changes. *Traffic.* 2009; 10:1199–1208. [PubMed: 19552647]
60. Valverde R, Edwards L, Regan L. Structure and function of KH domains. *FEBS J.* 2008; 275:2712–2726. [PubMed: 18422648]
61. Clery A, Blatter M, Allain FH. RNA recognition motifs: boring? Not quite. *Curr Opin Struct Biol.* 2008; 18:290–298. [PubMed: 18515081]
62. Lunde BM, Moore C, Varani G. RNA-binding proteins: modular design for efficient function. *Nat Rev Mol Cell Biol.* 2007; 8:479–490. [PubMed: 17473849]
63. Le Hir H, Izaurralde E, Maquat LE, Moore MJ. The spliceosome deposits multiple proteins 20–24 nucleotides upstream of mRNA exon-exon junctions. *EMBO J.* 2000; 19:6860–6869. [PubMed: 11118221]
64. Muller M, Heym RG, Mayer A, Kramer K, Schmid M, Cramer P, Urlaub H, Jansen RP, Niessing D. A cytoplasmic complex mediates specific mRNA recognition and localization in yeast. *PLoS Biol.* 2011; 9:e1000611. [PubMed: 21526221]
65. McNally EM, Pytel P. Muscle diseases: the muscular dystrophies. *Annu Rev Pathol.* 2007; 2:87–109. [PubMed: 18039094]
66. Selcen D, Carpen O. The Z-disk diseases. *Adv Exp Med Biol.* 2008; 642:116–130. [PubMed: 19181098]
67. Wallace RA, Ho T, Salter DW, Jared DW. Protein incorporation by isolated amphibian oocytes. IV. The role of follicle cells and calcium during protein uptake. *Exp Cell Res.* 1973; 82:287–295. [PubMed: 4203066]
68. Patel SB, Novikova N, Bellini M. Splicing-independent recruitment of spliceosomal small nuclear RNPs to nascent RNA polymerase II transcripts. *J Cell Biol.* 2007; 178:937–949. [PubMed: 17846169]
69. Beenders B, Jones PL, Bellini M. The tripartite motif of nuclear factor 7 is required for its association with transcriptional units. *Mol Cell Biol.* 2007; 27:2615–2624. [PubMed: 17261593]
70. Waisfisz Q, de Winter JP, Kruyt FA, de Groot J, van der Weel L, Dijkmans LM, Zhi Y, Arwert F, Schepers RJ, Youssoufian H, Hoatlin ME, Joenje H. A physical complex of the Fanconi anemia proteins FANCG/XRCC9 and FANCA. *Proc Natl Acad Sci U S A.* 1999; 96:10320–10325. [PubMed: 10468606]
71. Long SW, Ooi JY, Yau PM, Jones PL. A brain-derived mecp2 complex supports a role for MeCP2 in RNA processing. *Biosci Rep.* 2010
72. Lin C, Yang L, Yang JJ, Huang Y, Liu ZR. ATPase/helicase activities of p68 RNA helicase are required for pre-mRNA splicing but not for assembly of the spliceosome. *Mol Cell Biol.* 2005; 25:7484–7493. [PubMed: 16107697]
73. Pickard B, Damjanovski S. Overexpression of the tissue inhibitor of metalloproteinase-3 during *Xenopus* embryogenesis affects head and axial tissue formation. *Cell Res.* 2004; 14:389–399. [PubMed: 15538971]
74. Matzat LH, Berberoglu S, Levesque L. Formation of a Tap/NXF1 homotypic complex is mediated through the amino-terminal domain of Tap and enhances interaction with nucleoporins. *Mol Biol Cell.* 2008; 19:327–338. [PubMed: 17978099]
75. Pardee JD, Spudich JA. Purification of muscle actin. *Methods Enzymol.* 1982; 85(Pt B):164–181. [PubMed: 7121269]
76. Ono S, Yamakita Y, Yamashiro S, Matsudaira PT, Gnarr JR, Obinata T, Matsumura F. Identification of an actin binding region and a protein kinase C phosphorylation site on human fascin. *J Biol Chem.* 1997; 272:2527–2533. [PubMed: 8999969]
77. Patel S, Novikova N, Beenders B, Austin C, Bellini M. Live images of RNA polymerase II transcription units. *Chromosome Res.* 2008; 16:223–232. [PubMed: 18320360]
78. Austin C, Novikova N, Guacci V, Bellini M. Lampbrush chromosomes enable study of cohesin dynamics. *Chromosome Res.* 2009; 17:165–184. [PubMed: 19308699]

Figure 1. Endogenous human FRG1 is both nuclear and cytoplasmic in HeLa cells and localizes predominantly to the nucleoli but not nuclear speckles

(A–C) Immunostaining for FRG1 (α HS1) shows the endogenous FRG1 (green) is both nuclear and cytoplasmic. Nuclear FRG1 is prominent in the DAPI-poor nucleoli (white arrows). (D–G) Co-immunostaining for FRG1 (green) and the nucleolar marker fibrillarin (red) confirms the nucleolar localization of FRG1. (H–K) HeLa cells treated with AMD show FRG1 localizes to the granular component (GC) of the nucleoli (white arrow) and not the fibrillarin stained dense fibrillar component (DFC) (white arrowhead). (L–O) A 0.2 μ m thick deconvolution section through HeLa cells co-immunostained for FRG1 (green) and SC35 (red) shows endogenous FRG1 does not co-localize with SC35 positive nuclear speckles. (P–R) HeLa cells overexpressing FRG1 (green) and co-immunostained for SC35 (red) show FRG1 localizes to nuclear speckles in cells overexpressing FRG1 at high levels (blue arrow) but not at low levels (white arrows). * Nontransfected HeLa cell. Bars = 10 μ m.

Figure 2. Characterization of FRG1's nuclear and nucleolar localizations

(A) Schematic representations of FRG1 deletion and point mutation constructs used to analyze nuclear and nucleolar localization requirements. (B–D) COS-1 cells showing the three major localization patterns that were observed in different ratios after transient transfection. (B) A representative image showing the full-length transfected FRG1 signal is nuclear and prominently nucleolar with no detectable cytoplasmic staining. This localization was also observed for all single phosphorylation site mutants and S21AS26A double mutant. (C) A representative image showing depletion of FRG1 from nucleoli, but no cytoplasmic signal, observed with different frequencies in cells transfected with constructs lacking either NLS1 or NLS2, but also in the S21DS26D double mutant. (D) A representative image showing cells expressing FRG1 in the nucleus and cytoplasm as observed with different frequencies for some of the Δ NLS2 mutants. Bars = 10 μ m. (E) Graph representing the percentages of COS-1 cells that show cytoplasmic staining (as illustrated in D) after transient transfection of the FRG1 mutant constructs. (F) Graph showing the percentages of COS-1 cells with a nucleolar FRG1 signal that is higher, equal, or lower than the nucleoplasmic signal after transient transfection of the different FRG1 mutant constructs. Slides were blinded and, on average, 100 cells per construct were analyzed. NOL = nucleolus, NP = nucleoplasm.

Figure 3. Human FRG1 interacts with KPNA2 *in vivo* and *in vitro*

(A) Co-IP experiments were performed on U2OS cell lysates using the αFRG1-719 antibody or protein A beads alone (Control). Western blot analysis shows the specific co-IP of KPNA2 with FRG1. (B) To determine a potential role for NLS phosphorylation in the KPNA2 interaction, co-IP experiments were performed using HeLa cells transfected with HA tagged FRG1 and two FRG1 point mutants, the unphosphorylatable HA-FRG1 S21A,S26A, and the phospho-mimic HA-FRG1 S21D,S26D. Input (In) and the HA IP are shown probed for KPNA2 or FRG1 (using αFRG1-HS1). The FRG1 protein in the IP lane runs slightly slower due to the large amount of antibody light chain present. (C) The schematic diagram showing protein domains of full-length FRG1 and the deletion constructs. (D) GST pull-down experiments show direct interaction between FRG1 and KPNA2 *in vitro* and localize the KPNA2 interaction domain to NLS2 on FRG1.

Figure 4. FRG1 associates with nucleoli, Cajal bodies, and nascent RNA transcripts on lampbrush chromosome spreads in *Xenopus laevis* oocytes

X. laevis oocyte nuclei isolated under oil and immunostained using antibodies specific for *X. laevis* FRG1 show endogenous FRG1 (green) localizes to (A–D) the nucleoli (arrow), Cajal bodies (arrowhead) with associating B snurposomes (indicated by *), and (E–L) nascent mRNAs that are being actively transcribed (blue arrowhead). DAPI staining (red) labels the DNA, marking the chromosome axis (blue arrow in F) and distinguishing nucleoli from Cajal bodies (B). (I–L) Focus on FRG1 localizing to the transcriptionally active chromosome loops, indicated by the blue arrowhead in L. The boxed area in K is shown magnified 3X in L. (M–P) *X. laevis* oocyte nuclei expressing HA-FRG1 and immunostained for HA (O) show HA-FRG1 localized to the actively transcribed ribonuclear matrix (P). HA immunostaining on lampbrush spreads from uninjected oocytes have no detectable signal (data not shown)^{69, 77, 78}. Bars = 10 μm (D, H, K) and 1 μm (P).

Figure 5. Endogenous human FRG1 is associated with mRNA *in vivo*

A) RNA-IPs using the HS1 FRG1 antibody show FRG1 associates with *FXR1* mRNA transcripts *in vivo* in HeLa cells as assayed by RT-PCR. The FRG1 antibody specifically IP'ed *FXR1* mRNA (lanes 2 and 6) while a control non-specific antibody (lane 3) did not. The unbound fraction confirms the presence of *FXR1* mRNA in the control reaction (lane 5). The RT-PCRs are sensitive to RNase treatment (lanes 7 and 9), confirming that RNA is the IP'ed nucleic acid. B) RT-PCRs of RIPs from HeLa cells using the HS1 or HS2 FRG1 antibodies, as indicated, compared with RIPs using two control antibodies that do not IP FRG1 show spliced *FXR1* mRNA and *FRG1* mRNA specifically associate with FRG1 protein *in vivo*. All RIPs were sensitive to RNase A treatment.

Figure 6. FRG1 specifically associates with RNA *in vitro* in a sequence independent manner
 (A) A schematic diagram showing protein domains of full-length human FRG1 and the deletion constructs used for REMSA. (B) REMSA experiment, using an *FXR1* RNA coding fragment as a probe (arrow), shows a direct *in vitro* FRG1-RNA interaction (arrowhead). (C) REMSA experiment to identify the FRG1 RNA binding domain (RBD) using β -globin UTR RNA as a probe (arrow) showed FRG1 forms RNA complexes (arrowhead) through its N-terminal NLS domains as deleting this region abolished the FRG1-RNA interaction. (D) REMSA competition experiment to determine specificity of the FRG1-RNA interaction, using radiolabeled 5' and 3' UTR mRNA as a probe (arrow) and full-length FRG1 protein, shows the FRG1-RNA interaction (arrowhead) is competed away by both unlabeled RNA probe and tRNA while dsDNA does not compete with the interaction. Box in A shows the region responsible for FRG1-RNA interaction (RBD).

Figure 7. FRG1 directly associates with TAP through the N-terminal NLS

(A) Radio-labeled, *in vitro* transcribed and translated FRG1 and deletion constructs of FRG1 were incubated with GST-TAP or GST alone. Bound proteins were resolved by SDS-PAGE followed by autoradiography. (B) The FRG1-TAP interaction is independent of nucleic acids as the interaction is insensitive to benzonase nuclease treatment. Stained protein gel for Gst input in Figure S5.

Figure 8. FRG1 nuclear-cytoplasmic shuttling is CRM1-independent

HeLa cells, distinguished by clear DNA-poor regions (blue arrows), expressing HA-FRG1 were fused to murine C2C12 cells distinguished by DNA-rich foci (white arrows) in the presence of the translation inhibitor CHX for 3 hours with or without LMB. C2C12 cells alone (examples indicated by *) do not express HA-FRG1. (A–D) HA-FRG1 expressed in HeLa cells localizes to C2C12 nuclei 3 hours after inducing fusion, showing that FRG1 shuttles between the nucleus and the cytoplasm. However, the presence of LMB to block CRM1-mediated nuclear export had no effect on FRG1 shuttling (E–H). Bars = 10 μ m.

Figure 9. Human FRG1 binds F-actin and forms multimers *in vitro*

(A) High speed cosedimentation assay using an increasing amount of recombinant human FRG1 with a constant concentration of F-actin shows saturation. (B) Western blot analysis of a glutaraldehyde crosslinking time course assay using recombinant human FRG1 shows FRG1 can readily form dimers and tetramers *in vitro*.

Figure 10. Endogenous FRG1 requires the actin cytoskeleton for cytoplasmic localization in murine C2C12 cells

(A–D, Q–S) Cytoplasmic pool of FRG1 appeared granular and associated with actin filaments labeled by rhodamine-phalloidin staining (arrowheads in S). Boxed area in D is shown magnified 2X in Q–S. (E–P, T–V) Cells were treated with variable concentrations of Latrunculin B (LatB) for 5 or 15 minutes as indicated. FRG1 staining decreased or was abolished (arrows) in areas of LatB disrupted actin filaments as indicated by weak phalloidin staining. Boxed area in P is shown magnifies 2X in T–V.