A defect in the RNA-processing protein HNRPPDL causes limb-girdle muscular dystrophy 1G (LGMD1G)
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Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of genetically determined muscle disorders with a primary or predominant involvement of the pelvic or shoulder girdle musculature. More than 20 genes with autosomal recessive (LGMD2A to LGMD2Q) and autosomal dominant inheritance (LGMD1A to LGMD1H) have been mapped/identified to date. Mutations are known for six among the eight mapped autosomal dominant forms: LGMD1A (myotilin), LGMD1B (lamin A/C), LGMD1C (caveolin-3), LGMD1D (desmin), LGMD1E (DNAJB6), and more recently for LGMD1F (transportin-3). Our group previously mapped the LGMD1G gene at 4q21 in a Caucasian-Brazilian family. We now mapped a Uruguayan family with patients displaying a similar LGMD1G phenotype at the same locus. Whole genome sequencing identified, in both families, mutations in the HNRPDL gene. HNRPDL is a heterogeneous ribonucleoprotein family member, which participates in mRNA biogenesis and metabolism. Functional studies performed in S. cerevisiae showed that the loss of HRP1 (yeast orthologue) had pronounced effects on both protein levels and cell localizations, and yeast proteome revealed dramatic reorganization of proteins involved in RNA-processing pathways. In vivo analysis showed that hnrpdl is important for muscle development in zebrafish, causing a myopathic phenotype when knocked down. The present study presents a novel association between a muscular disorder and a RNA-related gene and reinforces the importance of RNA binding/processing proteins in muscle development and muscle disease. Understanding the role of these proteins in muscle might open new therapeutic approaches for muscular dystrophies.

INTRODUCTION

The limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of genetically determined progressive disorders of the muscle with a primary or predominant involvement of the pelvic or shoulder girdle musculature. Mutations in > 20 genes with autosomal recessive (LGMD2A-Q) and autosomal dominant inheritance (LGMD1A-H) have been mapped/identified to...
date (1–6). The autosomal dominant forms are relatively rare and represent <10% of all LGMD (2). Among the eight autosomal dominant forms that have been mapped, only six genes have been identified to date: LGMD1A (myotilin), LGMD1B (lamin A/C), LGMD1C (caveolin-3), LGMD1D (desmin), LGMD1E (DNAJB6) and more recently LGMD1F [transportin-3 (TNPO)] (5,6). The causal genes still remained unknown for LGMD1G and LGMD1H (3).

We have previously mapped LGMD1G at 4q21 in a four-generation Brazilian family of European ancestry, with a maximum logarithm of odds (LOD)-score of 6.6. The disease was characterized by late and variable muscular weakness. The initial symptoms were proximal lower limbs involvement and weakness followed by upper limbs proximal weakness. As detailed in the first report (4), limitation of finger and toe flexion was also a characteristic of this condition. Type II diabetes was seen in 5 out of 10 patients, but also in three unaffected family members. No cataracts, clinical myotonia or electroneuromyography-detected myotonic phenomena were found when the family was first described. Biceps muscle biopsy taken from one more severely affected patient showed a predominantly myopathic histopathological pattern associated with rimmed vacuoles (4).

Subsequently, we have identified a genealogy from Uruguay with 18 affected members by an autosomal dominant form of LGMD1G which was mapped in the same region. Whole genome sequencing allowed us to identify the mutation in both families in the HNRPD1 gene.

RESULTS

Mapping and clinical findings

The genealogies of the Brazilian and Uruguayan families are depicted in Figure 1A and B, respectively.

Linkage analysis in the unrelated LGMD1G Uruguayan family, using flanking microsatellite markers for the 4q21 region showed a maximum LOD-score of 4.8 also at 4q21. Just like the original Brazilian family, the Uruguayan clinically affected members presented an adult-onset disease with proximal limb-girdle weakness that included finger and toe flexion limitation. In 8 out of 11 examined patients, the onset was in proximal lower limbs involving subsequently the upper limbs. In three of them it started with proximal weakness in the upper limbs (difficulty to raise the arms). The age of onset varied from 15 to 53 years old (36.6 ± 13.7). As an additional phenotype, 6 out of 11 clinically affected individuals presented cataracts (three bilateral and three unilateral) with onset before the age of 50. Type II diabetes, with onset at the age of 53, was present in one patient. A careful clinical and neurological examination revealed that none of the patients had myotonic phenomena.

In the Brazilian family, there are 18 affected members distributed in three generations (Fig. 1A), and at least two asymptomatic cases: II-2 died in her 90s and never showed any muscular weakness; her son, III-1 who should also be a carrier of the mutation died at the age of 63 without any reported symptoms. Three additional asymptomatic individuals carried the causative variant; IV-23, IV-25 and V-1, but they are still within the range of age of onset (30–47 years old). Recently, two patients presented bilateral cataract with onset at ages 35 and 56 years. In the Uruguayan family (Fig. 1B), there are 18 affected patients. Here, also three clinically unaffected members (III-5, IV-1 and IV-7) were found to carry the causative variant but were still within the age of manifestation when examined. Nonetheless, it is clear from both families that the disease progresses with age in most patients with no evidence of clinical anticipation, does not display full penetrance and that both sexes are equally affected.

Two distinct mutations in the same codon

The combined analysis of both families reduced the candidate region to <3.5 MB (positions 81 985 539 to 85 468 682 in University of California Santa Cruz (UCSC) Human Genome v19). Whole genome sequencing was undertaken for three affected patients (two Brazilian and one Uruguayan) by an Illumina HiSeq2000 platform. In each family, a different variant in chromosome 4, position 83 347 676 was identified (Supplementary Material, Table S1). The base is located within the HNRPD1 gene (heterogeneous nuclear ribonucleoprotein D-like), also known as JKTBP (NM_031372.3, c.1667, p.D378). The mutation in exon 6 was G > A (p.D378N) in the Brazilian family, causing an amino acid change from aspartate to asparagine, while in the Uruguayan family the mutation was G > C (p.D378H), causing a change to histidine (Fig. 1C). These mutations were confirmed using Sanger sequencing in 13 Brazilian (10 affected and 3 asymptomatic) and 12 Uruguayan (9 affected and 3 asymptomatic) members and neither present in 14 non-affected adult members from both families nor in 604 Brazilian controls older than 60. The conservation profile of the residue was performed in PolyPhen2 (7) and its alignment is indicated in Supplementary Material, Figure S1. Sequencing of 70 isolated undiagnosed LGMD cases from Brazil or atypical forms of myotonic-like phenotype failed to identify any further mutations in the HNRPD1 gene.

Loss of hrp1 in yeast affects RNA-processing proteins

The yeast HNRPD1 orthologue is called hrp1. These proteins are 42% identical over a 66% query cover of the human protein (P-value of 1e-45) with overlaps in functions, as shown by a Gene Ontology enrichment analysis (Supplementary Material, Table S2). Using automated mating technologies, we assessed the loss of HRP1 on the levels and localization of all yeast proteins (8,9). A mutation in Δhrp1 was introduced into almost 6000 yeast strains each carrying a fusion of one yeast protein with a c-terminal Green fluorescent protein (GFP) tag (10). The hrp1 mutation had pronounced effects on both protein levels and cell localizations demonstrating that loss of this protein is detrimental to cellular physiology. Interestingly, the loss of HRP1 on the yeast proteome revealed dramatic reorganization of proteins involved in RNA-processing pathways. The RNA helicases Prp43 and Dpb2, which are both involved in rRNA maturation, were completely excluded from their natural place in the nucleolus and now reside in the cytosol rendering them, most probably, inactive. A similar phenotype was observed for Urb2, a nucleolar protein required for normal metabolism of the rRNA primary transcript (Fig. 2). In addition, a large number of proteins were up-/down-regulated indicating the extent of cellular stress (Supplementary Material, Table S3).
Hnrpdl is important for zebrafish muscle development

To analyse the in vivo role of HNRPD1 in muscle, we used a loss of function approach in zebrafish. Fish hnrpdl (GenBank Accession: NP_998557.1) was found to have 85% identity with the human sequence. An antisense oligonucleotide morpholino (MO) was designed to specifically target the translation start site of hnrpdl. Wild-type (AB) zebrafish embryos were injected with a translation blocking MO designed to knockdown hnrpdl expression in parallel with a control MO. Microinjection hnrpdl MO at the one-cell stage leads to a dose-dependent downregulation of the expression of the hnrpdl gene (Fig.3A). Injected fish were quantified 4 days post-fertilization (4 dpf) based on abnormal phenotypes (Fig.3B). Depending on MO dosage, injected embryos showed a range of severity such as body shape defects or twisted tails (Fig. 3C). Additionally, movements of hnrpdl morphants were restricted and uncoordinated, which is consistent with a myopathy. Immunohistochemical staining of MO injected fish showed abnormalities in the V-shaped chevron pattern typically associated with a normal fish. The myofibres were also disorganized as shown by birefringence and myosin heavy chain (MCH) immunofluorescence (IF) (Fig. 3D).

LGMD1G patients show diffuse HNRPD1 in their muscle

Muscle histological features revealed variable findings apparently related to the progression of the disease in two patients from the Brazilian family (Fig. 4A). While the most severely affected case (IV-9) showed a clear myopathic pattern, with degeneration, fibre size variation with discrete perimysial fibrosis, several necrotic fibres and rimmed vacuoles, the other less severely affected (III-33) showed an almost normal histological pattern. Furthermore, evidence of neurogenic involvement was observed in both patients, including the presence of small angulated fibres, predominance of type II fibres and a discrete fibre type grouping pattern on the ATPases reaction, compatible with a neurogenic involvement.

Immunohistochemical and western blot (WB) analyses revealed normal pattern for dystrophin, calpain3, dysferlin, telethonin and α-, β-sarcoglycans [previously shown in our original report (4)] and γ-sarcoglycans as shown in Figure4A.

Since HNRPD1 is a candidate to physically interact with TNP01 in RNA-processing events (11,12) analysing the distribution patterns of these proteins in the muscle of patients versus controls is of great interest.

Immunohistochemical analysis identified the presence of HNRPD1 protein in the nuclei of the control as well as in patients, co-localizing with TNP01 (Fig. 4B). However, an apparently higher variability in nuclear labelling was observed in the patients: some nuclei presented a strong condensed HNRPD1 labelling while in others, HNRPD1 showed a diffused pattern around the nuclei more frequently than the observed in normal muscle. Interestingly, a similar observation was previously reported in conditions where there is no transportation of HNRPD1 by TNP01 (11).

DISCUSSION

HNRPD1 is a heterogeneous ribonucleoprotein (hnRNP) family member, along with HNRNPD, HNRNPL, SRSF3 and PTBP1. This family has been shown to function in mRNA biogenesis and mRNA metabolism, including alternative splicing, mRNA
nuclear export, translational regulation and turnover (11). HNRPDs participate in the splicing of specific exons in pre-mRNA of transcripts from important muscle-related genes. HnRNPF, hnRNPG and hnRNPH regulate alternative splicing of alpha- and beta-tropomyosin and hnRNPG is also involved in dystrophin splicing (13). HnRNP A1 and hnRNP A/B interact with PABPN1, a gene that when mutated causes adult-onset oculopharyngeal muscular dystrophy (OPMD). In OPMD patients, hnRNP A1 is sequestered in intranuclear insoluble aggregates interfering with RNA export and causing muscle cell death (14). Abnormal hnRNPH also inhibits nuclear export of mRNA containing expanded CUG repeats (15) and is also associated with aberrant splicing in myotonic dystrophy type 1 (16). Cataracts, a feature of the myotonic dystrophy spectrum, were the present in six members of the Uruguayan LGMD1G family and more recently in two of the Brazilian family.

Interestingly, Drosophila smooth gene mutant, orthologue of the mammalian hnRNPL, shows decreased motor function and a progressive dystrophic muscle phenotype (17). Our in vivo study in zebrafish showed that movements of hnrpdl morphants were restricted and uncoordinated, especially in response to touch, which supports the importance of hnrpdl in muscle development and function, consistent with a myopathy (18). Thus, our findings reinforce the role of the hnRNP family in muscle pathology.

Additionally, our functional studies show the importance of hnrpdl in RNA-processing pathways in yeast. Interestingly, the diffuse hrp1 labelling found in the yeast mutant was also observed in the nucleus of LGMD1G patients’ muscle fibres, which might suggest an impaired traffic of the mutant HNRPD1 into the nuclei. Recently, TNPO3 was identified as the gene responsible for LGMD1F. It belongs to a family of importin β super-family proteins that import numerous proteins into the nucleus, including serine/arginine-rich proteins (SR proteins) that control mRNA splicing (5,6). On the other hand TNPO1 mediates the nuclear import of M9-bearing proteins and has been shown to bind the nuclear localization signal in the C-terminal of HNRPD1 (11,12). LGMD1F and 1G might share a common and novel pathological mechanism, still unrelated to the known LGMD molecular mechanisms.

In conclusion, we have identified mutations in HNRPD1 associated with LGMD1G. We also provide evidence that the impairment of HNRPD1 homologues cause a rearrangement of RNA-related proteins both in localization and level parameters in yeast and a muscular phenotype in zebrafish. Our study may provide a link between this disease and the group of myopathies caused by impaired RNA binding/processing proteins, which had been previously associated with oculopharyngeal muscular dystrophy as well as myotonic dystrophies type 1 and 2, disorders caused by dynamic mutations. This work significantly contributes to our understanding of inherited muscular dystrophies.

MATERIAL AND METHODS

The study was approved by the institutional ethics committee and all patients signed informed consent forms.

Muscle IF analyses

From the Brazilian family, a muscle biopsy was taken from biceps muscle from the index case affected brother at the age of 38 (patient IV-3, Fig. 1), and from one additional affected relative (II-33). The muscles were cryo-protected and snap frozen in liquid nitrogen. Routine histological and histochemical analyses include staining for HE, modified Gomori trichrome, SDH, NADH, acid phosphatases and ATPase at pH 9.4 and 4.3.

Protein analyses were done through IF and WB methodologies, as previously described (4). The following antibodies

![Figure 2. Images of seven proteins involved in RNA processing, whose localization was shifted in the absence of HRP1. A null mutation in HRP1 was introduced into almost 6000 yeast strains each carrying a fusion of one yeast protein with a c-terminal GFP tag. All proteins levels and localizations were followed and mutants (right column) were compared with control cells with a normal copy of HRP1 (left column). Loss of HRP1 caused a dramatic reorganization in the subcellular localization of proteins involved in RNA-processing pathways demonstrating a central role for this protein in determining RNA-processing physiology. Loss of the HRP1, the yeast homologue of HNRPDL, causes dramatic changes in the localization of proteins required for RNA processing.](http://hmg.oxfordjournals.org/)

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were used: dystrophin, sarcoglycans α, β, γ and δ, calpain-3, dysferlin and telethonin. Additionally, the following primary antibodies were used: polyclonal rabbit anti-HNRPDL (1/100, Sigma), monoclonal anti- TNPO1 (1/20, Novus Biologicals). As secondary antibodies, anti-mouse-FITC and anti-rabbit-Cy3 (Sigma) in the concentration of 1/100 were used.

Figure 3. hnrpdl knockdown in zebrafish. (A) Western blot confirming a dose-dependent hnrpdl MO knockdown with either 6, 9 or 12 ng hnrpdl translation blocking MO. Beta-actin was used as a loading control. (B) Injected fish were quantified 4 days post-fertilization (4dpf) based on abnormal phenotypes, which were defined by body shape defects like shortened or twisted tails, and swimming impairments, such as slow motility in response to touch, or rotating in circles. Compared with fish injected with control MO, hnrpdl morphants were more abnormal in the 9 ng concentration, while the number of dead embryos increased at the 12 ng concentration, which is likely due to toxicity. Three separate experiments were performed; graph shows the average number of fish per group. (C) Phase images of injected fish with hnrpdl MO (9 ng) and control MO. Note abnormal body shape seen in majority of injected fish. (D) Phase image, birefringence and IF staining with MCH antibody of hnrpdl knockdown in zebrafish. Note the dose-dependent effect of low birefringence and disorganized muscle fibres in the hnrpdl morphant fish muscle when compared with the control MO and non-injected fish (n = 10). Non-injected, AB zebrafish; CoMo, control morpholino injected fish; TBMO, translational blocking hnrpdl MO; ng, nanograms.
Genome sequencing

DNA was isolated from the blood by the standard methodology. Libraries were subsequently sequenced as 100-base paired-end runs (average insert size of 300 bases). Read alignment and variant calling was performed by the Illumina CASAVA 1.9 pipeline software. An average of 120 GB in aligned sequences was produced per sample, yielding a genome sampling depth of \( \approx 40 \times \) and >95% of the UCSC hg19 reference genome sampled at 10 reads or more. Moreover, samples were genotyped using the HumanOmni2.5-8v1 microarray. NGS alignment and genotyping pipeline was also redone using Broad Institute’s Best Practices (BWA 0.7; GATKv2.4; HaplotypeCaller), but no further variants in the linked region were identified.

As a downstream variant analysis, the resulting variant list for each sample was filtered using the 1000 Genomes variants (phase 1 release v3), the NHLBI Exome Sequencing Project (ESP6500) variants and 604 high-coverage exomes from randomized, population-based Brazilian controls. Variants with minor allele frequency >0.01 were removed from analysis.

Insertion of \( \Delta hrp1 \) into the yeast GFP library expressing cytosolic mCherry

The synthetic genetic array (SGA) technique was performed between a MATa haploid strain harbouring the \( \Delta hrp1::kan^{R} \) deletion from the deletion library collection (19) and the GFP collection (::HIS3) (10) expressing a TEF2-er-mCherry::URA3 integrated into the URA3 locus (20). In this collection of strains, a GFP was integrated carboxy-terminally into the genomic locus of each yeast open reading frame, maintaining the chromosomal context and preserving the natural promoter of each gene. The cytosolic fluorescence allows us to segment the images into hundreds of individual yeast cells for each strain and to extract protein abundance (detected as GFP.

Figure 4. Muscle analyses in two Brazilian affected members. (A) HE staining showing histopathological alterations in patient IV-9 and in less affected patient III-33, as compared with a normal control, and an immunohistochemical reaction for \( \gamma \)-SG to illustrate the pattern of sarcolemmal labelling. Magnification: 200 \( \times \). (B) Double immunohistohchemical analysis for HNRPD1 and TNPO1. Magnification 400 \( \times \). Arrow showing nuclei with peripheral diffused labelling, and detail showing a nucleus with strong condensed labelling.
fluorescence) under the desired condition. Mating was performed on rich-media plates, selection for diploid cells was performed on plates lacking HIS and URA and containing G418. Sporulation was then induced by transferring cells to nitrogen starvation plates for 5 days. Haploid cells containing all desired mutations were selected by transferring cells to plates containing all selection markers alongside the toxic amino acid derivatives Canavanine and Thialysine (Sigma-Aldrich) to select against remaining diploids and lacking leucine to select for only spores with an ‘alpha’ mating type (8,9). The SGA procedure was validated by inspecting representative strains for the deletion and the presence of the GFP-tagged strains and for the cytosolic mCherry expression. To manipulate the collection in high-density format (384), we used a RoToR bench top colony arrayer (Singer Instruments, UK).

High-throughput fluorescence microscopy

The manipulated 5330 strains as described above were grown in 50 l synthetic (SD) medium [0.67% yeast nitrogen base without amino acids (Conda Pronadisa) and 2% dextrose] containing the 50 l synthetic (SD) medium [0.67% yeast nitrogen base without amino acids (Conda Pronadisa) and 2% dextrose] containing the appropriate supplements for selection in 384-well plates (Cat. Number 781 162, Greiner). Microscopic screening was performed using an automated microscopy set-up as previously described (9,20). Cells were moved from agar plates into liquid 384-well polystyrene growth plates using the RoToR arrayer (Singer). Liquid cultures were grown over night in SD medium in a shaking incubator (LiCONIC Instruments) at 30°C. A JANUS liquid handler (PerkinElmer), which is connected to the incubator, was used to back-dilute the strains to ~0.25 OD into plates containing the same medium. Plates were then transferred back to the incubator and were allowed to grow for 3.5 h at 30°C to reach the logarithmic growth phase, as was validated in preliminary calibration. The liquid handler was then used to transfer strains into glass bottom 384-well microscope plates (Matrical Bioscience) coated with Concanavalin A (Sigma-Aldrich) to allow cell adhesion. Wells were washed twice in medium to remove floating cells and reach cell monolayer. Plates were then transferred into an automated inverted fluorescent microscopic ScanR system (Olympus) using a swap robotic arm (Hamilton). Imaging of plates was performed in 384-well format using a 60× air lens (NA = 0.9) in SD medium at 24°C with a cooled CCD camera (Hamamatsu ORCA-ER). Images were acquired at GFP (excitation at 490/20 nm, emission at 535/50 nm) and mCherry (excitation at 572/35 nm, emission at 632/60 nm) channels.

Image analysis

Our screening assay was designed to explore yeast cell biology by assessing two cellular key features of interest: subcellular localization and fluorescence intensity. To analyse these images, we have utilized an in-house script to browse manually and assign localization rapidly and efficiently. To extract proteomic abundance from images, we utilized the Olympus ScanR analysis software. This allows for the pre-processing of images by background subtraction, and segmentation of images to identify individual cells as separate objects. Data on expression intensity were compared with a WT library that does not have the Δhrp1 background. In addition, manual inspection of the images allows us to assign localization for each protein relative to a control strain.

Zebrafish knockdown

A translation blocking MO directed at the zebrafish hnrpd gene (TBMO) (5′-TCTAAA(ATG)CAGGCGAGAGCAAG-3′) was designed against the initiation methionine shown in parenthesis. A standard negative control MO (5′-CCTTACCCTCA GTTACAATTATA-3′) was used for control injections. All MOs were ordered from Gene Tools. Zebrafish were injected at the 1–2 cell stage with either 6, 9 or 12 and were assessed for phenotypic changes at 4 days post-fertilization (4 dpf), each injection was performed three times. Approximately 200 embryos were injected at each dosage.

Birefringence assay

The muscle phenotype was detected by using a birefringence assay, a technique used to analyse muscle quality due to the unique ability of highly organized sarcomeres to rotate polarized light. The birefringence assay is performed by placing anaesthetized embryos on a glass polarizing filter and covering them with a second polarizing filter. The filters are placed on an underlit dissecting scope and the top polarizing filter is twisted until the light refracting through the zebrafish’s striated muscle is visible. The fish do not need to be sacrificed for this technique.

Zebrafish immunostaining

Immunostaining was performed in 4 dpf embryos. Embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight and dehydrated in 100% methanol. After rehydration, 4 dpf embryos were incubated in 0.1% collagenase (Sigma) in PBS for 60 min. Blocking solution containing 0.2% saponin was used for 4 dpf embryos. Anti-slow muscle MCH antibody (F59, Developmental Studies Hybridoma Bank; 1:50) was used for 4 dpf embryos. Anti-slow muscle MCH antibody (F59, Developmental Studies Hybridoma Bank; 1:50) was used. The embryos were placed in 3% methyl cellulose or mounted on a glass slide and observed with fluorescent microscopes (Nikon Eclipse E1000 and Zeiss Axioplan2).

AUTHORS’ CONTRIBUTIONS

Study concept and design were performed by: N.V., M.S.N., F.K., L.L., J.K., D.S., M.V., M.S., L.K., M.Z. Acquisition of data was carried out by N.V., M.S.N., F.K., L.L., N.S., L.G., D.S., N.C., P.S., S.C., C.V., A.M., M.V., R.P., M.Z. Statistical analysis and interpretation of the data were performed by N.V., M.S.N., F.K., L.L., J.K., L.G., D.S., N.C., P.S., S.C., C.V., A.M., V.N., M.V., R.P., M.S., L.K., M.Z. The manuscript was drafted by N.V., M.S.N., M.V., M.S., N.C., M.Z., Critical revision of the manuscript was performed by N.V., M.S.N., V.N., M.V., M.S., L.K., M.Z. Funding was obtained by D.S., V.N., M.V., M.S., L.K., M.Z.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.
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Conflict of Interest statement. None declared.

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