Development of an AP-FRET Based Analysis for Characterizing RNA-Protein Interactions in Myotonic Dystrophy (DM1)

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Abstract

Förster Resonance Energy Transfer (FRET) microscopy is a powerful tool used to identify molecular interactions in live or fixed cells using a non-radiative transfer of energy from a donor fluorophore in the excited state to an acceptor fluorophore in close proximity [1–3]. Since the efficiency of energy transfer (E%) varies inversely with the sixth power of the intermolecular distance, the distance over which FRET can occur is limited to 1–10 nm [1–3], making FRET a powerful technique in identifying molecular interactions [4].

Myotonic Dystrophy type 1 (DM1), a dominantly inherited multisystemic neuromuscular disorder is the first example of RNA-mediated disease amongst genetic disorders [5,6]. DM1 is caused by a CTG repeat expansion in the 3’ untranslated region (3’ UTR) of the DMPK gene [7,8]. As a result, mutant DMPK mRNA is retained in the nucleus as discrete foci, or RNA foci [9]. These RNA foci differ in their shape, size and cellular abundance [10]. Little is known about the composition of RNA foci as there is no method available to purify the foci intact, and nothing is known about RNA-protein and protein-protein interactions at RNA foci in DM1. In DM1, the functions of RNA binding proteins like muscleblind-like protein 1 (MBNL1) and CUG-binding protein-1 (CUGBP1), which are developmental regulators of alternative splicing, are affected resulting in numerous splicing abnormalities [11–17], CUGBP1 levels are elevated in DM1 whereas functional levels of MBNL1 are thought to be depleted due to its sequestration by mutant RNA foci. Though co-localization of MBNL1 with the mutant RNA foci in different DM1 tissues and models of RNA toxicity has been previously demonstrated there is no direct evidence of intracellular interaction [18–23].

In this study, we have developed and used an acceptor photobleaching FRET assay to identify RNA-protein interactions. Using this technique we provide the first direct evidence of intracellular interaction between endogenous MBNL1 and mutant DMPK mRNA foci in cells derived from DM1 patients. We have corroborated our findings with EGFP-fused MBNL1 and have used RNA-IP with anti-MBNL1 antibodies to biochemically corroborate our findings with EGFP-fused MBNL1 and have used RNA-IP with anti-MBNL1 antibodies to biochemically validate the FRET analysis. Further, we have used deletion mutagenesis to provide mechanistic insights by identifying functional domains in MBNL1 involved in this interaction and in regulation of alternative splicing. Taken together these results demonstrate the power of AP-FRET in not only identifying interactions between RNA and proteins but also in determining the functional domains involved in that interaction.
Material and Methods

RNA FISH and immunofluorescence workflow

DM1 cells were grown on a glass coverslip. When the desired cell density was reached the cells were washed in PBS three times then fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. Following fixation they were permeabilized in cold 2% acetone/PBS for 5 min at room temperature. The cells were then washed with PBS three times and incubated with 30% formamide/2x SSC buffer at 37 °C for 10 min. Hybridization was then carried out with either a CY3 or FITC labeled (CAG)90 probe at 0.1 ng/µl for 2 h at 37 °C in the hybridization buffer (30% formamide, 2x SCC, 0.02% BSA, 66 µg/ml yeast tRNA, 2 mM vanadyl complex). After the hybridization, the cells were washed in 30% formamide/2xSSC at 45 °C for 30 min. Next the cells were blocked in 1% BSA/PBS for 1 h at 37 °C. Primary antibody, either MBNL1 rabbit polyclonal antibody (1:2000) [22], RNA Pol II rabbit polyclonal antibody (1:1000) (SCBT, USA) or hnRNPH goat polyclonal antibody (1:200) (SCBT, USA) was used for E% distribution graphs. Primer set 1: FP-Del-C: 5’ GCCACCG-GCATGGCCATGATGAGCTG 3’; RP-Del- C: 5’ GGGTG- CATACCTGCTGCAAGATGCTGCAGTAG 3’. Primer set 2: FP-Del-ZF4-C: 5’ GCCACATTGACCAATGGGGCTGCTGCGG 3’; RP-Del- ZF4-C: 5’ GTTCGATCTGTCGAACTTGGATTTGTTGGCGTGGC 3’. Primer set 3: FP-Del-Linker: 5’ GCGCAATTC- CACCTCGCTGACGAGACTCTGAGTGC 3’; RP-Del-Linker: 5’ CATACCTGCTGTCGAGCTTGGGTTGATGGGCC 3’.

Generation of MBNL1 deletion mutants

The pEGFP-MBNL1 backbone was used for generating the MBNL1 deletion mutants. The Quick exchange mutagenesis kit (Agilent Technologies, USA) was used for generating mutants: Del-C–, Del-Linker and Del-ZF4–C; whereas, a PstI restriction site within the MBNL1 cDNA was used for generating Del-N followed by conventional self ligation and transformation. All deletion mutants were sequence confirmed and were in frame with the N’ terminus EGFP tag. Primes for quick exchange mutagenesis are as follows. Primer set 1: FP-Del-C: 5’ GCCACCG-GCATGGCCATGATGAGCTG 3’; RP-Del- C: 5’ GGGTG- CATACCTGCTGCAAGATGCTGCAGTAG 3’. Primer set 2: FP-Del-ZF4-C: 5’ GCCACATTGACCAATGGGGCTGCTGCGG 3’; RP-Del- ZF4-C: 5’ GTTCGATCTGTCGAACTTGGATTTGTTGGCGTGGC 3’. Primer set 3: FP-Del-Linker: 5’ GCGCAATTC- CACCTCGCTGACGAGACTCTGAGTGC 3’; RP-Del-Linker: 5’ CATACCTGCTGTCGAGCTTGGGTTGATGGGCC 3’.

Transient transfections

Transient transfections in DM1 fibroblasts/myoblasts were done using NucleofectorTM (Amaxa, Germany), and in
HEK293T using Lipofectamine 2000 (Invitrogen, USA). Cells were either fixed for RNA FISH or processed for RNA extraction 48 h post transfection or 24 h for HEK293T. For AP-FRET experiments (1 μg) and for splicing assays (4 μg) of plasmids were used. For the EGFP-tagged RNA binding proteins and for MBNL1-full length (MBNL1-FI) and its deletions, the coverslips were mounted after RNA-FISH and subsequently analyzed by AP-FRET. Plasmids pEGFP-MBNL1, pEGFP-hnRNP-C and pEGFP-CUGBP1 were gifts from Dr. David Brook.

Western blot
Protein expression of FL-MBNL1 and its deletion mutants and their molecular sizes were analyzed and confirmed by western blotting. The blot was probed with anti-EGFP and anti-Dynein antibodies (Invitrogen, USA).

Statistical methods
Standard statistical methods were employed using the software Minitab 16.1.0, produced by Minitab, Inc. Two-sample one-sided T-tests were used to determine differences in E% between donor-acceptor and donor only FRET. A one-sided Fisher’s exact test was used to determine differences in positive E% between groups. A one-way ANOVA with Tukey’s multiple comparison was used to analyze the alternative splicing results. Statistical significance was set at a p-value of <0.05.

Results
AP-FRET provides evidence of interaction between endogenous protein and RNA

Though conventional or laser scanning confocal microscopy (LSCM) can be used to detect the co-localization of molecules, its optical resolution (typically 200 nm) limits the ability to make any conclusive statements about molecular interactions. However, FRET microscopy typically detects interactions in the 1–10 nm range. FRET can be analyzed by a number of different methods [1,3]. We have used FRET analysis by acceptor photobleaching (AP-FRET) as it is the most direct method of detecting and/or verifying FRET between molecules labeled with donor and acceptor fluorophores [27] without the need for extensive bleed-through corrections required in spectral and confocal FRET methods. AP-FRET is an intensity based method to study interaction and therefore, the concentrations of the donor and acceptor fluorophores, labeling method, the choice of the right FRET pair, all play a very important role. Also, of importance is the expression level of the targets and their topological arrangement in a RNA-protein complex as this will determine the labeling, the donor and acceptor stoichiometry and therefore detection of their interaction. Another key aspect to successful AP-FRET is to select an appropriate wavelength and laser intensity that selectively excites and photobleaches the acceptor, without affecting the excitation potential of the donor. In steady state when the donor is excited, FRET involves transfer of energy from a donor to an acceptor molecule resulting in decreased fluorescence (quenching) of the donor molecule. In AP-FRET, when the acceptor is specifically excited and photobleached prior to donor excitation, it is no longer capable of receiving transferred energy when the donor is excited, with the end result being increased signal from the donor molecule (dequenching) upon signal acquisition. The relative change in donor signal before and after photobleaching (termed E%) is a measure of FRET efficiency which reflects the relative proximity of the donor and acceptor molecules. To measure and rule out the background noise arising due to instrumental fluctuations and/or due to fluorophore instability and to verify that the observed E% represents a positive interaction, we also measured the distribution of E% from a donor only (no acceptor) labeled control. Positive interactions were scored as any E% over the value that excludes ~95% of the E% measured in experimental negative control. Finally the number of positive interactions was statistically compared between the donor-acceptor labeled samples and the donor only samples. A statistical difference between the groups represent actual FRET while no statistical difference between the donor-acceptor and donor only sections represented a failure to detect an interaction either because no interaction existed or because our assay was unable to accurately identify the interaction.

To demonstrate the utility of AP-FRET in identifying RNA-protein interactions we first turned to the interaction of MBNL1 and DMMPK RNA, which have been extensively studied in myotonic dystrophy. Using fibroblasts from DM1 patients we performed a combined RNA-FISH protocol to simultaneously detect RNA foci with a FITC labeled (CAG)_10 antisense probe (the donor), and MBNL1 (the RNA-binding protein) with an Alexa555 labeled secondary antibody (the acceptor). To optimize of photobleaching for the AP-FRET experiment we avoided any saturation in our Regions of Interest (ROIs) by using low power of the exciting laser below saturation for signal acquisition. These conditions result in images of MBNL1 foci without excessive nucleoplasmic/cytoplasmic MBNL1 staining. After optimizing photobleaching conditions, AP-FRET was performed using a LSCM (Leica SP5X white light laser (WLL)). Dequenched signal from the donor was seen after photobleaching the acceptor, demonstrating FRET and an interaction between MBNL1 and RNA foci (Fig. 1A–B). The specificity of the AP-FRET assay was established using RNA polymerase II (RNA Pol II) as a negative control (Fig. 1C–D). Prebleach and Postbleach images were used to calculate an average E% of 18.11% for MBNL1-RNA foci interactions as compared to a value of only 5.20% for RNA Pol II (Fig. 1E). The variability in E% distribution between various regions of interest (ROIs) is likely the result of observed variability in intensity of RNA foci and MBNL1 aggregates or differences in donor/acceptor stoichiometry at a particular RNA focus, however it was clear that MBNL1 interactions were significantly higher than the donor alone controls. In contrast the RNA Pol II was indistinguishable from its donor only samples verifying it as an appropriate negative control. These results provide the first compelling evidence for an intracellular interaction between MBNL1 and the mutant RNA foci in cells from DM1 patients.

While the interaction of MBNL1 and the mutant DMMPK RNA has been extensively studied we choose to carry out RNA-IP to detect DMMPK transcripts binding to MBNL1 as a way to confirm our assay. We used skeletal muscle tissue from a mouse model expressing a EGFP-DMMPK 3’ UTR (CTG)_200 transgene (termed DM200) [16]. In addition we used DM1 fibroblasts which are polymorphic for a Bpm1 restriction site in exon 10 of the DMMPK gene [24]. This polymorphism enabled us to distinguish between mutant and wildtype transcripts by Bpm1 restriction fragment length polymorphism (RFLP). Since the RNA foci are nuclear, we utilized fractionated nuclear RNA extracts from the DM1 fibroblasts, and total RNA extracts from the DM200 skeletal muscles. RNA-IP with anti-MBNL1 monoclonal antibodies was performed. The immunoprecipitated RNA was extracted and analyzed by RT-PCR for the presence of the EGFP transcript in the mouse extracts (Fig. 2A), or by RT-PCR/BpmI RLFP for the detection of DMMPK transcripts in DM1 fibroblast extracts (Fig. 2B). Both of these assays clearly show immunoprecipitation of either the EGFP mRNA or the mutant DMMPK mRNA by anti-MBNL1 but not by the isotype control antibodies. This provided
Figure 1. Evidence of intracellular interaction between endogenous MBNL1, and hnRNPH with RNA foci using AP-FRET. RNA foci in
confirmatory biochemical evidence for the interaction between MBNL1 and the mutant DMPK transcript in both human cells and mouse tissues.

We next looked at another RNA binding factor with much less evidence for RNA foci binding, hnRNPH, using our AP-FRET assay. There is evidence that hnRNPH is both increased in DM1 but also is pulled down with a modified UV crosslinking assay using DMPK derived RNA [28,29]. Again using fibroblasts from DM1 patients we performed a combined RNA-FISH/immunofluorescence protocol to simultaneously detect RNA foci with a FITC labeled (CAG)10 antisense probe (the donor), and hnRNPH (the RNA-binding protein) with an Alexa555 labeled secondary antibody (the acceptor). After optimizing photobleaching conditions, AP-FRET was performed again using the protocols designed to avoid any saturation in our Regions of Interest (ROIs). The E% was calculated and the number of positive interactions from the donor-acceptor labeled samples was compared to the donor only samples for hnRNPH. We again used FRET results from RNA polymerase as our negative control and MBNL1 as our positive control (Fig. 1F). There was a measurable difference between the E% from the donor-acceptor FRET for hnRNPH compared to the donor only control (Fig. 1F). We observed dequenched signal from the donor after photobleaching of the acceptor at many but not all of the RNA foci (14/34), demonstrating FRET and intracellular interaction between hnRNPH and RNA foci at these RNA foci. To normalize the data and compare MBNL1, RNA Pol II, and hnRNPH we took the proportion of positive interactions measured in the donor-acceptor group and divided it by the proportion of positive interactions measured in the donor only group. When completed we saw a similar ratio in the MBNL1 group (our positive control) and hnRNPH. However, unlike the MBNL1-RNA interactions taking place at all the foci examined (21/21) this more varied pattern of binding may represent a less frequent or transient interaction at the foci. Though unlikely it could also be possible that hnRNPH indirectly interacted with the RNA foci through another protein, such as MBNL1. To address this possibility we measured the E% distribution between hnRNPH and MBNL1 and found no evidence of interaction (Fig. 1F). Using the AP-FRET assay we were able to provide evidence of interaction between endogenous proteins (MBNL1 and hnRNPH) and RNA (DMPK expanded repeat transcripts).

AP-FRET for studying intracellular interaction between RNA foci and fluorescent fusion RNA-binding proteins

Identifying and characterizing RNA-protein interaction can be a difficult undertaking, especially as many techniques to study the process do not provide evidence of direct interaction. FRET is a distance dependent phenomenon [1–3], which allows direct testing of an RNA and protein interaction. However with the use of primary and secondary antibodies, as was done in the previous experiments, steric hindrance could result in no or lower FRET E%. The reporter EGFP is a small protein (~27 kDa) and the chances of steric hindrance may be less with its use. Therefore, we used EGFP fused MBNL1 (EGFP-MBNL1) to independently detect intracellular interactions and study the possibility of RNA-protein interactions by other RNA binding proteins in DM1.

EGFP-MBNL1 corresponds to an isoform of MBNL1 (expressing exons 1–4, 6 and 10) that has previously been shown to co-localize with the RNA foci [19]. Plasmid expressing EGFP-MBNL1 were transfected into DM1 cells and upon RNA-FISH...
with a CY3-(CAG)_{10} antisense probe, AP-FRET was performed using EGFP (donor) and CY3 (acceptor) as the FRET pair. As a negative control, plasmid expressing EGFP alone was used to measure background noise in AP-FRET experiments. Similar experiments were done using EGFP fused versions of CUGBP1 and hnRNP-C, two other RNA-binding proteins thought to have in vivo binding with the DM1 mRNA [12,30]. Only EGFP-MBNL showed interactions with the RNA foci with an average E% of 34.88% and a positive FRET ratio of 49/49 compared to 2.11% for EGFP-alone control and a positive FRET ratio of 4/57 (Fig. 3), confirming the results obtained using antibodies against MBNL1. Notably, we did not detect interaction between RNA foci and EGFP-CUGBP1 or EGFP-hnRNP-C by AP-FRET (Fig. 3). This assay thus allows the detection of RNA binding proteins that bind to RNA, in this case MBNL1.

AP-FRET can be used to identify domains responsible for RNA-protein interactions and function

The domains in a protein that are responsible for binding and the domains in a protein that have other biological functions can be difficult to study independent of each other. Utilizing AP-FRET in the context of the DM1 MBNL1-RNA interaction we examined the different domains present in MBNL1 and how they relate to its RNA binding and mRNA splicing activity.

MBNL1 contains two pairs of zinc-finger motifs, a linker region between zinc-finger pairs and a C’ terminal domain (Fig. 4A) [19]. A previous study using a yeast three-hybrid system showed that all four zinc-finger domains are necessary for interaction with (CUG)_{21} and (CCUG)_{22} RNAs [31]. However, no in vivo data exists defining the domains of MBNL1 necessary for interactions with the mutant DM1 mRNA. To address this we generated a series of MBNL1 deletion mutants using the EGFP-MBNL1 backbone (Fig. 4A). All of the mutants: Del-C’ (lacking the carboxy terminus), Del-Linker (lacking the linker region), Del-214-326 (lacking the fourth zinc finger and C’ terminus) and Del-N’ (lacking all four zinc finger domains and the linker) were expressed in HEK293T cells, and western blotting was used to verify their expression and appropriate size (Fig. 4B). These constructs were also transfected into DM1 fibroblasts and the cells were then analyzed by RNA-FISH and AP-FRET analysis.

The Del-C’ and Del-Linker both of which had all four intact zinc-finger motifs (ZF1-ZF4), showed strong dequenched signal from the donor after photobleaching the acceptor (Fig. 4C and 4D). Their E% distributions were similar to full length-MBNL1 (FL-MBNL1) (Fig. 4C and 4D). On the other hand, deletion of all four zinc-finger motifs (Del-N’) resulted in complete loss of interaction. Deletion of ZF4-C’ (Del-214-326) resulted in a dramatic drop in FRET efficiency values (Fig. 4C and 4D) with an average E% of 8.1% and positive FRET ratio of 6/53 as compared to 28.7% and 29/31 for FL-MBNL1 showing that loss of ZF4 had a significant negative effect on MBNL1-RNA foci interaction. These results are analogous to results using the yeast three-hybrid system where loss of binding with (CUG)_{21} RNAs was observed when ZF4, or the linker region were deleted [31]. However, we did not observe a loss of interaction with RNA foci after deletion of the linker region. Our AP-FRET analysis using MBNL1 deletion mutants underscores that all four zinc-fingers are crucial for interaction with RNA foci.

MBNL1 is also a known regulator of alternative splicing of a number of transcripts whose splicing is mis-regulated in DM1, including insulin receptor (IR), muscle-specific chloride channel (CLCN1) and sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (ATP2A1, also known as SERCA1) [32-36]. MBNL1 in conjunction with other splicing regulators controls the transition from embryonic to adult splicing pattern [29]. Using DM1 myoblasts and HEK293T cells we assessed the role of MBNL1 deletion mutants in the regulation of alternative splicing; specifically of endogenous SERCA1 exon 22, IR exon 11 and NFIX exon 7 [35].

Figure 3. Demonstration of intracellular interaction between RNA foci and EGFP-MBNL1 using AP-FRET. DM1 fibroblasts were transfected with plasmids encoding EGFP-MBNL1, EGFP-hnRNPC, EGFP-CUGBP1 and EGFP alone. RNA-FISH was carried out 48 h post-transfection with CY3-(CAG)_{10} antisense probe. EGFP-CY3 was used as FRET pair. Representative donor and acceptor (A) pre-bleach and (B) post-bleach images for each experiment are shown. Strong dequenched signal from the donor could be seen only for EGFP-MBNL1 after photobleaching the acceptor. (C) E% distribution for different ROIs shows E% for EGFP-MBNL1 with an average of 34.8% (n = 49) and lower or background level E% distribution for EGFP-hnRNPC (n = 49) and EGFP-CUGBP1 (n = 49) similar to EGFP alone (n = 57). Line on graphs represents the positive FRET threshold level.

doi:10.1371/journal.pone.0095957.g003
HEK293T cells have a high level of CUGBP1 and relatively low level of MBNL1, analogous to the DM1 condition, and express an embryonic pattern of splicing with a predominance of SERCA1 without exon 22, IR without exon 11 and NFIX with exon 7. Western blotting and qPCR were used to verify that the transfected cells were over-expressing the relevant MBNL1 constructs (Fig. 5).

RT-PCR showed that over-expression of FL-MBNL1 resulted in 17.82 fold increase in SERCA1 exon 22 inclusion (Fig. 6A) and 10.79 fold increase in NFIX exon 7 exclusion (Fig. 6E). The deletion mutants of MBNL1 showed progressive loss of positive splicing regulation for SERCA1 exon 22 and NFIX exon 7: with the Del-C' showing a 40% reduction, Del-Linker a 51% reduction in splicing regulation for SERCA1 exon 22; and Del-C' showing a 25% reduction and Del-Linker a 46% reduction in splicing regulation for NFIX exon 7 as compared to FL-MBNL1; Del-N' showed almost complete loss of splicing regulation (Fig. 6A and 6E). A similar incremental loss in positive regulation was observed in DM1 myoblasts for endogenous SERCA1 with the toxic RNA foci that occur in DM1 and then continued to study other RNA binding proteins such as hnRNPH, CUGBP1, and hnRNPC.

The main pathogenic process in DM1 is described as the nuclear retention of mutant DMPK transcripts into discrete RNA foci which are thought to be deleterious due to their interactions with RNA binding proteins. The role of MBNL1 in RNA foci formation in DM1 has been implicated ever-since its identification as the “EXP” protein in 2000 [11]. Since then, MBNL1 has been a subject of intense study using various models for DM1: from human tissues [20,21], to mice [14,23] to flies [38,39]. However, no studies have shown direct intracellular interaction between MBNL1 and RNA foci. The AP-FRET analysis of deletion mutants versus FL-MBNL1 was performed with line averaging (Line Av = 2). Representative donor and acceptor pre-bleach and post-bleach images for each experiment are shown.

**Discussion**

Regulation of RNA processing, stability, and localization are essential for the proper functioning of the cell. Many of these functions such as mRNA splicing, microRNA processing, RNA shuttling, and RNA sequestration are regulated by RNA-protein interactions. Biochemical studies aimed at understanding RNA-protein interaction are limited by *in vitro* conditions while techniques like immunofluorescence can only suggest co-localization not actual interaction. When an aspect of normal RNA regulation is defective numerous diseases can arise. This is especially true in many of the expanded RNA repeats disease, of which DM1 is a member. We developed a technique combining RNA-FISH and AP-FRET to study the function and interactions of RNA and proteins. We have demonstrated the utility of this assay by studying the well characterized interaction of MBNL1 with the toxic RNA foci that occur in DM1 and then continued to study other RNA binding proteins such as hnRNPH, CUGBP1, and hnRNPC.

The main pathogenic process in DM1 is described as the nuclear retention of mutant DMPK transcripts into discrete RNA foci which are thought to be deleterious due to their interactions with RNA binding proteins. The role of MBNL1 in RNA foci formation in DM1 has been implicated ever-since its identification as the “EXP” protein in 2000 [11]. Since then, MBNL1 has been a subject of intense study using various models for DM1: from human tissues [20,21], to mice [14,23] to flies [38,39]. However, no studies have shown direct intracellular interaction between MBNL1 and RNA foci. The AP-FRET assay used in this study provides the first compelling evidence for this interaction between MBNL1 and RNA foci in DM1 cells (Fig. 1). Using an
independent biochemical approach, RNA-IP with an anti-MBNL1 antibody clearly shows the pull down of mutant DMPK transcripts from extracts made from DM1 cells and from skeletal muscles of transgenic mice expressing the EGFP-DMPK	extsuperscript{319}(CTG)	extsuperscript{200} transgene (Fig. 2). Together, these two assays along with data generated by other groups on the co-localization, biochemical interaction, and preferred binding substrate of MBNL1 provides strong support for the physical interaction between MBNL1 and DMPK mRNAs.

Interestingly, the RNA-IP from DM1 cells also revealed that the normal DMPK transcript interacts with MBNL1. The functional relevance of this is currently unknown, but in the context of a cryptic splice site within the DMPK 3’ UTR [30] and a transgenic mouse model over-expressing a normal DMPK 3’ UTR that develops DM1 pathology [16], this is an intriguing finding. Furthermore, we took this assay and explored the less characterized but previously identified potential interaction between hnRNPH and DMPK RNA foci. Using the AP-FRET assay we found evidence of an interaction between the toxic RNA foci that was independent of an interaction with MBNL1.

Having established the validity of the AP-FRET assay to detect intracellular interactions, we next used the technique to explore fluorescent fusion RNA-binding proteins. We chose proteins shown by in vitro binding assays to interact with the RNA foci in DM1 cells. Both CUGBP1 and hnRNP-C are distributed throughout the nucleoplasm and previous studies did not identify co-localization with RNA foci [19], but this does not preclude interactions that may not be detectable due to the high background signal from the nucleoplasmic signal. AP-FRET has the advantages of focusing on just the pixels where the RNA foci are and the proximity limits of FRET (i.e. 1–10 nm). Using our AP-FRET assay, it was clear that CUGBP1 and hnRNP-C do not interact with or were incapable of being detected in RNA foci with our assay (Fig. 3).
Our AP-FRET data also provides insights into the molecular basis of the RNA foci-MBNL1 interaction. Using deletion mutants of MBNL1, it is evident that all four zinc finger domains are necessary for this interaction (Fig. 4D). This is consistent with previous data from a three-hybrid assay which showed that all four ZF domains were necessary for strong interactions with CUG21 oligonucleotides [31], and more recently a study that showed that MBNL1 isoforms with all four ZF domains co-localized with RNA foci [40]. The MBNL1 gene consists of at least 10 exons, and MBNL1 is expressed as many different alternatively spliced isoforms [34]. The isoform of MBNL1 used in this study encodes exons 1 through 4, 6 and 10. The four zinc finger domains present in all isoforms are encoded by exon 1 and 2 (ZF1, ZF2) and exon 4 (ZF3, ZF4). Exon 3 encodes a "linker" domain connecting the two pairs of zinc fingers. It has been reported that this "linker" region was essential for the interaction of MBNL1 with CUG repeats based on three-hybrid assays [31]. In contrast, our AP-FRET assay shows that deletion of the "linker" region has negligible effects on the physical interaction between MBNL1 and RNA foci (Fig. 4C–D). The Del-C9 mutant shows that the carboxy terminus of MBNL1 including domains encoded by exon 6 and 10 are also dispensable for the interaction of MBNL1 to RNA foci.

Recently, the domains encoded by exons 3 and 6 were shown to be involved in the splicing of human cTNT and IR minigenes using co-transfection assays [40]. Though mini-genesis are useful in mapping domains regulating splicing, they may not reflect intracellular behavior of the splicing targets. Instead, in our study we chose to look at endogenous splicing targets of MBNL1 that are implicated in DM1 pathology. Over-expression of MBNL1 in DM1 myoblasts rescued the splicing of SERCA1 exon 22 and also increased exon 22 inclusion in HEK293T cells (Fig. 6A–B). In contrast, deletion of MBNL1-exon 6 (Del-C9) dramatically reduced the efficiency of MBNL1 in rescuing splicing defects in both DM1 myoblasts and the HEK293T cells (Fig. 6A–B). We also observed a similar effect in rescuing splicing defects for NFIX exon 7 (Fig. 6E). However, neither of these deletions resulted in a notable decrease in interactions with the RNA foci (Fig. 4C–D). In combination with the AP-FRET data, the results show that the ZF4 region of MBNL1 is important for both RNA foci interactions and regulation of alternative splicing. Both the Del-C9 and the Del-Linker constructs showed a significant reduction in splicing regulation for SERCA1 and NFIX with no major change in RNA foci interaction which suggests that the domains required for MBNL1-RNA foci interaction can be different from the domains required for the regulation of alternative splicing events. The splicing targets analyzed in this study could be differentially regulated by MBNL1 and its mutants possibly by altering the recruitment or interactions of other splicing factors at the MBNL1-RNA splicing complex. RNA splicing requires the recruitment of multiple proteins to the splicing substrate. Recently, a number of RNA-binding proteins including hnRNPH, hnRNP-F and several others were identified as interacting partners of MBNL1 [41]. It may be that by deleting the sequences in MBNL1 encoded by exons 3 and/or 6, or even ZF4-C9 the recruitment of crucial components of the splicing machinery (i.e. other splicing factors) to the MBNL1-RNA complex is compromised, resulting in decreased splicing efficiency. The fact that SERCA1 and NFIX splicing are more affected than IR exon 11 (Fig. 6C–D) suggests that different splicing targets are variably sensitive to these changes in stoichiometry.

FRET is one of the most sensitive techniques available for studying molecular interactions in cells and AP-FRET is an ideal method to investigate protein interactions in fixed cells. We have...
now developed and validated a novel FRET based assay for studying intracellular RNA-protein interactions. Furthermore, in validating this assay we have provided the first intracellular evidence of interaction between the mutant DMPK mRNA and MBNL1 and the mutant DMPK mRNA and hnRNP A1. RNA foci have been found in a growing number of disorders including DM1, DM2, Fragile X Tremor and Ataxia Syndrome (FXTAS), Huntington Disease Like-2 (HDL-2), Spinocerebellar Ataxias (SCA8, SCA10) and more recently in Amyotrophic Lateral Sclerosis/Frontotemporal Dementia (ALS/FTD). Our study opens up new means of identifying and characterizing RNA-protein interactions in RNA foci complexes in such disease states and has the potential utility as an assay screening for compounds capable of disrupting deleterious RNA-protein interactions in DM1 and other disorders where these interactions play a key role in disease pathogenesis.

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Acknowledgments

We thank Dr. Charles Thornton (University of Rochester, NY) for kindly providing us with anti-MBNL1 rabbit polyclonal antibodies, DM1 human and HSA4th mouse tissues; Dr. David Brook (University of Nottingham, UK) for pEGFP-MBNL1, pEGFP-hnRNP-C, pEGFP-CUGBP1 and DM1 fibroblasts with Bpmil polymorphism; Dr. Scott Vande Pol (University of Virginia, VA) for pEGFP empty vector and the HEK293T cell line and Dr. I. H. Engels (Genomics Institute of the Novarits Research Foundation, CA) for immortalized DM1 fibroblasts/myoblasts.

Author Contributions

Conceived and designed the experiments: SR JTG AP YS MSM. Performed the experiments: SR JTG AP YS. Analyzed the data: SR JTG AP MSM. Contributed reagents/materials/analysis tools: AP MSM. Wrote the paper: SR JTG AP MSM.
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