Functional characterization of the AFF (AF4/FMR2) family of RNA-binding proteins: insights into the molecular pathology of FRAXE intellectual disability

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INTRODUCTION

AFF (AF4/FMR2) is a family of four genes that comprises: AFF1 [also named ALL1-fused gene from chromosome 4 (AF4)], AFF2 [also named fragile X mental retardation 2 (FMR2)], AFF3 [also named lymphoid nuclear protein related to AF4 (LAF4)] and AFF4 [also named ALL1-fused gene from 5q31 (AF5q31) or major CDK9 elongation factor-associated protein (MCEF)]. They have a common ancestor in Drosophila melanogaster, the Lilliputian gene (1,2).

AFF1/AF4 was first described as a fusion partner with myeloid/lymphoid or mixed-lineage leukemia (MLL) in a patient affected by acute lymphoblastic leukemia (ALL) (3) and MLL-AF4 is the most common fusion gene observed in ALL, with a frequency of 85% (4). AFF1/AF4 is ubiquitously expressed (5) with especially high levels in the lymphoid system (6,7). Indeed, a role for AFF1/AF4 in lymphopoiesis was suggested by an altered lymphoid development in Aff1/Af4-null mice (8). Moreover, a point mutation in Aff1/Af4 causes a dominant form of cerebellar ataxia in mouse (9).

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AFF2/FMR2 is an X-linked gene expressed primarily in placenta and adult and fetal brain. It is silenced in a mild to borderline form of non-syndromic intellectual disability (ID), the FRAXE mental retardation (10–12). The silencing of AFF2/FMR2 in FRAXE patients is due to a CCG expansion (> 200 repeats) located in the 5′-untranslated region of this gene, resulting in the FRAXE fragile site and the lack of AFF2/FMR2 protein (10,11,13). While patients affected by FRAXE ID display a mild form of ID of variable penetrance, Aff2/Fmr2-null mice display a delay-dependent conditioned fear impairment and hippocampal increased long-term potentiation (LTP). This phenotype is supportive of a role for AFF2/FMR2 protein in synaptic plasticity (14).

AFF3/LAF4, which was also found to be fused with MLL in some ALL patients (15), was initially reported to be highly expressed in lymphocytes (16). Furthermore, AFF3 expression is upregulated in cortical neurons during the initial steps of cortical differentiation and is downregulated in postnatal cortex, indicating its involvement in brain development (17). More recently, it was reported to be a novel rheumatoid arthritis susceptibility gene (18) and to be deleted in patients displaying a complex phenotype including brain malformations (19).

AFF4/AF5q31 was identified due to its insertion in the MLL gene [ins(5;11)(q31;q13q23)] in an ALL patient (20). Even though this gene is widely expressed in all human tissues (20,21), Aff4/Af5q31-null mice display azoospermia as their only phenotype, demonstrating the essential role of this protein in Sertoli cells (22).

Previous studies already described that AFF proteins share some conserved functional domains. The N-terminal homology domain is a region of homology shared by the four AFF members whose function is still unknown (16,23,24). The C-terminal homology domain, another region of homology among the AFF proteins, is involved in the intranuclear localization of FMR2 as well as in its ability to bind the G-quadruplex RNA-forming structure (25). The ALF (AF4/LAF4/FMR2) domain mediates the interaction with SIAH ubiquitin ligases that promotes protein degradation through the proteasome pathway (26). The transactivation domain is a serine-rich region that was described as involved in transcriptional activation (16).

AFF1/AF4, AFF3/LAF4 and AFF4/AF5q31 were found to co-immunoprecipitate with ENL, together with enzymes involved in transcription and chromatin remodeling (27). Furthermore, AFF1/AF4 and AFF4/AF5q31 have been reported to interact with the positive transcription elongation factor b (P-TEFb). This association facilitates elongation by RNA polymerase II (28). We have recently shown that AFF2/FMR2 is localized in nuclear speckle domains, and modulates alternative splicing via the interaction with the G-quadruplex-forming RNA structure. We have also shown that one of the targets of AFF2/FMR2 is the pre-mRNA of the Fragile X Mental Retardation 1 (FMR1) gene, the silencing of which causes Fragile X syndrome, the most common form of inherited ID (25).

Here, we explored the functional properties of the AFF family triggered by the observation that AFF3/LAF4 and AFF4/AF5q31, like the AFF2/FMR2 protein, are localized in nuclear speckles and that their overexpression affects the dynamics and/or biogenesis of these structures. We also show that these proteins specifically bind the G-quadruplex-forming RNA structure and modulate the splicing efficiency of a minigene reporter with a G-quadruplex structure in its alternatively spliced exon. Furthermore, we propose that functional redundancy of AFF proteins can explain the mild nature of the cognitive impairment observed in FRAXE patients.

RESULTS

AFF family protein sequence analysis

We started the characterization of the AFF family of proteins by studying the similarity among the mouse and human paralogs. The level of similarity among the eight proteins is particularly evident at the level of different domains (Supplementary Material, Fig. S1B). A diagram illustrating the functional architecture of AFF/FMR2 domains is presented in Supplementary Material, Figure S1A. Among the human proteins, AFF2/FMR2 shows the highest divergence with AFF1/AF4 (26.8% identity), while AFF3/LAF4 and AFF4/AF5q31 are 35.9 and 33.8% identical to AFF2/FMR2, respectively. This divergence between AFF1/AF4 and AFF2/FMR2 is even higher across the C-terminal domain (Fig. 1A). The identity between AFF1/AF4 and AFF2/FMR2 in this region is 24.7%, while the AFF2/FMR2 C-terminus has 34.6 and 33.8% identity with AFF3/LAF4 and AFF4/AF5q31, respectively (Fig. 1A).

A nuclear localization signal (NLS) is a short peptide driving a protein into the nucleus by interaction with the import machinery (29). By PROSITE (30), two NLS sequences have been identified in these proteins: NLSI is present in AFF2/FMR2, AFF3/LAF4 and AFF4/AF5q31 (Fig. 1B and Supplementary Material, Fig. S1B), while NLSII is present only in AFF1/AF4 and AFF3/LAF4 (Fig. 1C and Supplementary Material, Fig. S1B). Protein localization in the nucleus is not a fully understood mechanism (31). By analyzing a large number of experimentally validated nucleolar localization signals (NoLSs), a predictor of NoLS was created (http://www.compbio.dundee.ac.uk/www-nod/) (32). Using this tool, we identified an NoLS in seven AFF family paralogs (Fig. 1D and Supplementary Material, Fig. S1A).

AFF proteins show different subnuclear localization

We have recently studied the subnuclear distribution of AFF2/FMR2 and shown that it is co-localized with SC35 in nuclear speckle domains (25), the nuclear loci where splicing factors are stored, assembled and modified/recycled (33). The intracellular distribution of the other AFF proteins has not yet been studied. The high level of similarity among the AFF proteins suggests similar functional properties. To address this issue experimentally, we tested recombinant, Flag-tagged AFF1/AF4, AFF3/LAF4 and AFF4/AF5q31 expression in HeLa cells co-stained with anti-Flag and anti-SC35 antibodies (25). The AFF3/LAF4 and AFF4/AF5q31 proteins co-localized with SC35 in nuclear speckles (Fig. 2), similar to AFF2/FMR2 (25) (Supplementary Material, Fig. S2).
Interestingly, the distribution of AFF1/AF4 in the nucleus is diffused, with the appearance of ‘small dots’ (Fig. 3A). Since a similar nuclear distribution was described for AF9/ENL (Fig. 3A), a known AFF1/AF4 protein interactor (34), we also studied its nuclear localization. We confirm similar nuclear distribution of both proteins (Fig. 3B). It is worth noting that the signal for AFF1/AF4 was generally weak (Fig. 3A), improving with the length of the post-transfection time (Fig. 5A, lowest panel).

The domain of AFF2/FMR2 through which the protein is targeted into nuclear speckles is located in its C-terminal region (residues 633–1272) (25). To test whether the paralogous C-terminal domains of AFF3/LAF4 and AFF4/AF5q31 are involved in their subnuclear localization, we subcloned the corresponding regions (residues 647–1224 of AFF3/LAF4 and residues 552–1163 of AFF4/AF5q31) into the Flag-tagged vector. After transient transfection of HeLa cells, we observed that both C-terminal regions co-localized with SC35 (Fig. 4, upper and middle panels), while the C-terminal domain of AFF1/AF4 (residues 628–1210) localized to nucleoli marked by the nucleolar marker fibrillarin (35) (Fig. 4, lower panel).

Previously, we have shown that the N-terminal region of AFF2/FMR2 (residues 1–640) is localized to cytoplasm (25). We subcloned the corresponding fragments of AFF1/AF4 (residues 1–775), AFF3/LAF4 (residues 1–635) and AFF4/AF5q31 (residues 1–715) and, after transfection of HeLa cells (6 or 12 h), we observed similar localization for all three recombinant proteins (Supplementary Material, Fig. S3).

Overexpression of AFF2/FMR2, AFF3/LAF4 and AFF4/AF5q31 affects the recycling and organization of nuclear speckles

We have generally analyzed the subcellular localization of the AFF proteins in HeLa cells 6–12 h post-transfection. To test...
the possible impact of AFF proteins on the formation of nuclear speckles, we transfected HeLa cells with AFF2/FMR2, AFF3/LAF4, AFF4/AF5q31 and AFF1/AF4 expression vectors and analyzed them 24 and 48 h post-transfection. For the three proteins localized in nuclear speckles, we observed that under these strong expression conditions, the nuclear distribution of SC35 is altered (Fig. 5A). These data suggest that the overexpression of the AFF2/FMR2, AFF3/LAF4 and AFF4/AF5q31, but not AFF1/AF4 proteins, interferes with the physiological dynamics of these nuclear structures. AFF1/AF4 overexpression has no impact on the distribution of SC35 (Fig. 5A). These data suggest that the overexpression of the AFF2/FMR2, AFF3/LAF4 and AFF4/AF5q31, but not AFF1/AF4 proteins, interferes with the physiological dynamics of these nuclear structures. AFF1/AF4 overexpression has no impact on the distribution of SC35 (Fig. 5A). To assess whether the absence of FMR2 in FRAXE fibroblast cell lines affects the morphology of nuclear speckles, as a consequence of the altered dynamics, we studied these structures by performing an immunofluorescence with SC35 (Supplementary Material, Fig. S4). We analyzed the average size of nuclear speckles in 583 cells of four FRAXE patients’ cell lines and a total of 373 cells in four control fibroblast cell lines. A very highly significant difference between the two groups of cells was observed (Fig. 5B).

**AFF family members bind the RNA G-quadruplex**

The C-terminal domains of AFF3/LAF4 and AFF4/AF5q31 contain the amino acid sequences necessary to direct both proteins into nuclear speckles, similar to the C-terminal domain of AFF2/FMR2. We asked whether these domains also retain the property to bind a G-quadruplex-forming RNA structure, which is preferentially stabilized by K⁺ over Li⁺ or Na⁺ (36).

We produced the C-terminal region of AFF2/FMR2, AFF3/LAF4 and AFF4/AF5q31 as recombinant proteins and we tested their ability to bind the N19 probe (folding in a G-quadruplex structure in the presence of K⁺) (37,38) in the presence of different ions. Interestingly, when K⁺ allows the correct 3D folding of the G-quadruplex in the RNA probe, all three proteins were able to recognize and bind the RNA with a similar apparent affinity (Fig. 6A). The N-terminal region of AFF2/FMR2 is used as a negative control. Conversely, in non-permissive conditions (presence of Li⁺ or Na⁺), G-quadruplex folding was avoided and the proteins did not bind (Fig. 6B, the experiment with Na⁺ is not shown but it produced results similar to those shown in Fig. 6B). As a negative control, we tested the ability of these proteins to bind the N19△35 probe, which we previously described as an RNA fragment originating from N19 and where we deleted the G-quadruplex motif (25). The AFF proteins did not bind this probe in permissive conditions for G-quadruplex formation (Fig. 6C). The same result was obtained repeating this control in the presence of Li⁺ or Na⁺ (data not shown).
Overexpression of AFF3/LAF4 and AFF4/AF5q31 proteins affects splicing of a reporter minigene

We have shown that G-quadruplex RNA has properties of an exonic splicing enhancer (ESE) (25). We have previously shown that the ESE activity of a G-quadruplex structure situated in the mRNA of an alternatively spliced exon of a minigene or of a putative target of AFF2/FMR2 (namely FMR1) appears reduced in the presence of AFF2/FMR2. Since AFF3/LAF4 and AFF4/AF5q31 co-localize with splicing factors and bind the G-quadruplex-forming structure with the same apparent affinity as AFF2/FMR2, we tested their effect on alternative splicing depending upon their binding to a G-quadruplex structure located in an alternatively spliced exon. For this reason, we tested whether AFF3/LAF4 and/or AFF4/AF5q31 could modulate the splicing of the SXN/FMR2-binding site (FBS) minigene (Fig. 6D) (25). We co-transfected HeLa cells with the SXN/FBS minigene and the AFF2/FMR2-, AFF3/LAF4- or AFF4/AF5q31-expressing vector or with an empty vector. We evaluated the relative expression of transcripts with alternative exon 2 inclusion by real-time quantitative PCR (Materials and Methods). As shown in Fig. 6E, inclusion of the exon containing the G-quadruplex, also named AFF2/FBS, was decreased by ~2-fold compared with cells transfected with the empty vector. The level of each AFF protein was tested by western blot and found to be approximately the same (data not shown).

DISCUSSION

AFF family proteins: a link between transcription and splicing

With the exception of AFF2/FMR2, all members of the AFF family have been reported to form fusion genes with MLL in ALL (16,39,40). For this reason, the main focus has been so far on the function of the fusion MLL/AFF genes and only a few studies aimed to address the specific function of each member of the AFF family. The function of AFF1/AF4 and AFF2/FMR2 in transcription regulation was promptly recognized soon after their cloning (16,24). Further studies allowed us to dissect the dynamics of AFF member-containing multimolecular complexes, even if the precise function of these proteins is still unknown (41).

AFF1/AF4, AFF3/LAF4 and AFF4/AF5q31 associate with ENL family proteins and p-TEFb (27,28,34,42). By a yeast two-hybrid assay, we have also found a direct interaction between ENL/AF9 and AFF2/FMR2 (B.B. and M.M., unpublished results), suggesting that all AFF family members interact with the ENL proteins (43). AFF1/AF4 functions as a positive regulator of P-TEFb kinase (28), which, in turn,
controls the transactivation activity of ENL family proteins (27, 44). Recently, the participation of different members of the AFF family members within the complex including P-TEFb and ENL/AF9 was proposed (41). ENL family proteins also associate with Dot1, a major histone methyltransferase responsible for the H3K79 methylation mark (28, 45). Additionally, we showed that AFF2/FMR2 is also involved in the regulation of alternative splicing. In the present study, we show that two other members of the AFF family, AFF3/ LAF4 and AFF4/AF5q31, share these same functional properties with AFF2/FMR2. Our study also suggests that there is a link between alternative splicing, chromatin remodeling and transcriptional elongation. Cyclin T1 and CDK9, which make up P-TEFb, localize to nuclear speckles as do three-quarters of the members of the AFF family (46), but not AFF1/AF4. This latter protein appears to occupy the entire nucleus in a diffuse dot-like pattern, similar to AF9/ENL proteins and Dot1 (Fig. 3) (27). The above data would suggest that the AFF family members are components of distinct subcomplexes that under specific conditions can be recruited by RNA PolIII and Dot1. The implication of chromatin modifications in splicing modulation was initially suggested by the use of drugs inhibiting histone acetylases (47), and other links between transcription and splicing have been described recently (48, 49). These findings led to the speculation that the splicing machinery relies on chromatin regulators, which are able to read the “histone code” to locate and access pre-mRNAs ready to be spliced. Thus, it is not surprising that other complexes involved in chromatin remodeling show the same behavior.

**Organization of AFF proteins and nuclear speckles**

In this study, we also observed that the overexpression of AFF2/3/4 causes disaggregation of nuclear speckles. A similar effect on nuclear speckles was reported for other proteins such as the X-linked cyclin-dependent kinase-like 5 (CDKL5), which is mutated in an early-onset variant of Rett syndrome (50). DYRK1A kinase, which is present in three copies in Down syndrome (the most common form of ID), has also been reported to disaggregate nuclear speckles when overexpressed (51). On the same path, the absence of AFF2/FMR2 determines a small but highly significant alteration of nuclear speckles size. These findings suggest a role for these proteins (DYRK1A, CDKL5 and AFF2/3/4) in the structural organization of nuclear speckles. Since nuclear
Figure 5. Dynamics and morphology of AFF proteins and nuclear speckles. (A) Twenty-four hour overexpression of AFF proteins affects the dynamics of nuclear speckles. HeLa cells were transfected with pTL1-Flag-AFF2/FMR2, pTL1-Flag-AFF3/LAF4, pTL1-Flag-AFF4/AF5q31 or pTL1-Flag-AFF1/AF4 and analyzed 24 h post-transfection. The AFF proteins were revealed by a polyclonal anti-Flag antibody and nuclear speckles were detected using a monoclonal anti-SC35 antibody. Overexpression of AFF2/FMR2, AFF3/LAF4 or AFF4/AF5q31 determines an altered distribution of SC35. No impact was observed on SC35 distribution for AFF1/AF4 overexpression. Magnification 63×; scale bars, 10 μm. (B) Increased size of nuclear speckles in FRAXE patient fibroblasts. Control (four cell lines) and FRAXE patient (four cell lines) fibroblasts were fixed and then immunolabeled with the mouse anti-SC35 antibody to detect nuclear speckle domains. After microscopy acquisition, the size of nuclear speckles in control (n = 373) and FRAXE patient (n = 583) cells was analyzed using the ImageJ software (64). Results are presented as the mean ± SEM (t-test, ***,P < 0.0001).
speckles are dynamic structures whose morphology also depends on transcription and splicing activity of the cell (25), these abnormalities could also be due to the nuclear trafficking of splicing factors from nuclear speckles to chromatin or to an abnormal transcriptional activity. AFF proteins have a role in both of these cell functions, thus also interfering with nuclear speckle morphology also in an indirect manner.

Specific functions of AFF proteins

The differences in function we observed among the AFF proteins probably reflect their level of similarity across the relevant functional domains. Indeed AFF3/LAF4 and AFF4/AF5q31 display a higher level of identity with AFF2/FMR2 than AFF1/AF4 does (Fig. 1A and Supplementary Material, Fig. S1B) (52). According to PROSITE, AFF2/3/4 share the same NLSI (Fig. 1 and Supplementary Material, Fig. S1B) while AFF1/AF4 harbors NLSII, also shared with the second site of AFF3/LAF4 (Fig. 1C and Supplementary Material, Fig. S1B). Originally, two NLSs have been suggested for AFF2/FMR2, the first one corresponds to NLSI and, indeed, the AFF2/FMR2-NLSI sequence is able to localize GFP in the nucleus in a diffuse distribution (52). Interestingly, the AFF4/AF5q31-NLS is not sufficient to direct this protein in the nucleus (Supplementary Material, Fig. S2), suggesting that additional sequences, or NLSII, are needed in this protein for its correct subcellular localization. We have now found that the second NLS motif, NLSII, corresponds to an NoLS in seven of these proteins (Fig.1D and Supplementary Material, Fig. S1B). Since the ability of AFF2/FMR2 to enter the nucleolus has already been shown (25,52), here we show that the C-terminus of AFF1/AF4 (containing the NLSII/NoLS sequence) co-localizes with fibrillarin in the nucleolus (Fig. 4). The similarity between NLS and NoLS motifs has been recognized by others (32). Together with in silico prediction, we can conclude that the NLS/NoLS of AFFs is probably a complex motif

Figure 6. AFF proteins are RNA-binding proteins affecting alternative splicing. (A–C) Binding specificity of AFF proteins to the G-quadruplex-forming RNA structure. Filter-binding assay using increasing amounts of AFF2/FMR2 C-ter, AFF3/LAF4 C-ter, AFF4/AF5q31 C-ter and AFF2/FMR2 N-ter with 32P-labeled N19 RNA in the presence of K+ (A) or Li+ (B) ions and with 32P-labeled N19△35 RNA in the presence of K+ ions (C). All data shown in (A–C) are listed in Supplementary Material, Table. (D) Schematic representation of the SXN13 minigene including the G-quadruplex (FBS) in exon 2, which is alternatively spliced. (E) AFF proteins affect the splicing efficiency of the SXN13/FBS minigene. HeLa cells were co-transfected with the pTL1-Flag empty vector, pTL1-Flag-AFF2/FMR2, pTL1-Flag-AFF3/LAF4 or pTL1-Flag-AFF4/AF5q31 and the SXN13/FBS minigene and analyzed 16 h post-transfection. Relative expression of the exon 2-FBS transcript was evaluated using RT–qPCR. Arrows indicate primer positions; GAPDH was used for normalization. Error bars represent standard error of the mean (SEM) (t-test, P < 0.05).
endowed with both functions, as also proposed for other proteins (32). Further characterization of the NLS and NoLS of the AFF proteins is required, as well as the characterization of other NLS/NoLS motifs of other proteins. The role of AFF proteins in the nucleus is unknown; however, it is interesting that they are directed to this subcompartment and they are not retained by an interaction with a nucleolar component. Furthermore, using the same in silico tool a nucleolar signal is also predicted in the AFF1/AF4 protein, a common interactor of all AFF members (27,28). These findings suggest that further investigations into the role of AFF proteins in the nucleus are warranted. It is also unlikely that these sequences direct AFF2/3/4 into nuclear speckles. In DYRK1A it is a histidine-rich sequence which directs the protein into nuclear speckles (51); however, such a sequence motif was not observed in the AFF proteins. Thus, most probably, another mechanism may explain the different subcellular localization of AFF2/3/4 and AFF1/AF4, as, for instance, it is shown for P-TEFb, which is sequestered into nuclear speckles by its interactor 7SK RNA (53). Thus, in the future, a detailed analysis of proteins specifically interacting with individual AFF members will help to dissect their specific functions and intranuclear dynamics.

Functional redundancy of AFF proteins

Taken together, our data suggest functional redundancy for AFF2/AFF3/AFF4 proteins during brain development. We also observed upregulation of the expression of AFF4/AFF3/AFF5q31 in FRAXE patient fibroblasts, suggesting a feedback loop at least between AFF4/AFF5q31 and AFF2/FMR2 and functional compensation as a consequence. Further evidence suggesting AFF2/AFF3/AFF4 functional redundancy comes from co-expression studies during mouse brain development. At embryonic day E13.5, when the neurogenesis peaks in the developing mouse brain, the expression level of Aff2/Aff3/Aff4 genes is high, while the level of Aff1/Af4 is low (GEO at NCBI). The expression overlap during pre-natal development was independently shown also for Aff3/Laf4 and Aff2/Fmr2 (17). More specifically, these authors proposed a role for Aff3/Laf4 in the early steps of cortical differentiation (17). It was hypothesized that the silencing of AFF2/FMR2 in FRAXE patients might not be complete and that is why the FRAXE phenotype is mild (14). In agreement with previous studies (9), in our FRAXE patient fibroblasts, we could not detect any residual expression of AFF2/FMR2. Based on the similarity and functional properties, we can speculate that AFF3/LAF4 or AFF4/AFF5q31 may have a similar, crucial role in normal neuronal function to AFF2/FMR2. The Aff4/Aff5q31-null mouse has a testis phenotype but, to our knowledge, detailed learning and memory or behavioral studies are yet to be performed (22). Furthermore an abnormal expression of AFF3/LAF4 has been associated with limb, brain, urogenital and tibia malformations (19). It is also interesting to notice that a fragile site was described on chromosome 2 upstream of the AFF3/LAF4 gene in patients affected by FRAXA (54), leading to the suggestion that a mutation similar to that observed in FRAXE patients could generate another ID reducing the expression of AFF3/LAF4.

In conclusion, comparative and subsequent functional analysis of the AFF proteins provided us with a new insight into their role in the regulation of gene expression. Further studies to identify bona fide targets of AFF2/FMR2 and its paralogs will give more accurate indications on the function of each AFF family member and enable us to clarify the potential compensatory activity of AFF3/LAF4 or AFF4/AFF5q31 in FRAXE ID.

MATERIALS AND METHODS

Sequence alignment and secondary structure prediction

Human and mouse sequences were extracted from the Pfam database of the protein family (Pfam accession number PF05110; 116 sequences in May 2010). The multiple alignment has been performed with the program Muscle (55) at Mobyle (56). The program Mview (57) produced the consensus tags: columns are shaded by the amino acid property color code if the sequence identity is ≥70% at the aligned position (which means that six amino acids out of the eight must be conserved to be shaded). The eight aligned sequences are in the order of human FRM2 (18–1309) (AFF2_HUMAN; UniProt P51816), mouse FMR2 (1–1270) (AFF2_MOUSE; UniProt O55112), human MCEF (2–1160) (AFF4_HUMAN; UniProt Q9UHB7), mouse MCEF (2–1157) (AFF4_MOUSE; UniProt Q9ESC8), mouse AF4 (8–1215) (AFF1_MOUSE; UniProt O88573), human AF4 (8–1208) (AFF1_HUMAN; UniProt P51825), mouse LAF4 (44–1252) (AFF3_MOUSE; UniProt P51827) and human LAF4 (19–1224) (AFF3_HUMAN; P51826). The secondary structure prediction for hFMR2 is a consensus from four predictors (DSC, PREDATOR, PSIPRED and SIMPA96) offered by the analysis toolkit PAT (30). Similarly, the PAT toolkit has been used to analyze the sequences to identify NLSs by using the program ScanProsite. The ‘nuclear bipartite nuclear targeting sequence’ motif PS00015 is indicated by blue boxes.
enzymes and cloned into the pTL1-Flag plasmid (58) for PCR fragments were digested with appropriate restriction sites (Table 1). The FL, N-ter and C-ter of AFF2/FMR2 Xho (TaKaRa) and primers containing Xho provided by Professor R.G. Roeder (21) using LA Taq AF5q31 (Takara Bio Inc., Otsu, Shiga, Japan) and the appropriate Blast restriction sites, respectively. The N-ter of AFF4/AF5q31 and C-ter of AFF2/FMR2 cDNA were used to digest and then religate FL AFF4/AF5q31 cDNA clone in the pTL1-N19 (37) and SXN13-FBS minigene (25) constructs. Gateway destination vector pcDNA-DEST26 (Invitrogen). The N-ter and C-ter constructs of the respective gene. HindIII/BamHI and NheI/BamHI restriction sites were used to digest and then religate FL AFF1/AF4 in the pTL10-Flag AFF1/AF4 N-ter construct and FL AFF4/AF5q31 in the pTL1-Flag AFF4/AF5q31 N-ter construct, respectively. The FL AFF3/LAF4 cDNA clone in the pCMV6 Flag-tagged vector was purchased from Origene Technologies. N-ter and C-ter AFF3/LAF4 were amplified from pCMV6 Flag-AFF3/LAF4 FL using Pfx DNA polymerase (Invitrogen) and attB PCR primers for the Gateway system (Table 1). attB-flanked PCR products were cloned into the pDONR221 vector (Invitrogen) by the Gateway recombinant cloning technique and then subcloned into the Gateway destination vector pcDNA-DEST26 (Invitrogen). pTL1-N19 (37) and SXN13-FBS minigene (25) constructs were described previously.

**Cell culture**

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 100 mg/ml penicillin/streptomycin. Human fibroblasts were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 mg/ml penicillin/streptomycin.

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**Table 1. Primers used to clone AFF members into mammalian or bacterial expression vectors and primers used in real-time quantitative PCR**

<table>
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<th>Genes</th>
<th>Forward reverse primers</th>
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<tr>
<td>AFF2/FMR2 FL in pTL1</td>
<td>5′-GGCTCGAGGATCTATTTCCGACATTACG-3′ / 5′-ACACCATCTCCAAGCAAGGT-3′</td>
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<tr>
<td>AFF2/FMR2 N-ter in pTL1</td>
<td>5′-GGGGATCCACCATCTTCTGTACAGCAG-3′ / 5′-CCCAGAGTTCGAGTACACG-3′</td>
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<tr>
<td>AFF2/FMR2 C-ter in pTL1</td>
<td>5′-GGCTCGAGGACAGTGTACTGCAG-3′ / 5′-GGGATCCCTCAGAAGAAGGT-3′</td>
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<td>5′-GGCTCGAGCTTGTTCTGAGG-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
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<tr>
<td>AFF3/LAF4 C-ter in pDONR221</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
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<tr>
<td>AFF4/AF5q31 C-ter in pTL1</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF1/AF4 N-ter in pTL10</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF3/LAF4 N-ter in pDONR221</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF4/AF5q31 N-ter in pTL1</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF9 FL in pTL1</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF2/FMR2 N-ter in pET151</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF2/FMR2 C-ter in pET151</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF3/LAF4 C-ter in pDONR221</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF4/AF5q31 C-ter in pET16b</td>
<td>5′-GGGGATCCCTCAGAAGAAGGT-3′ / 5′-GGGGATCCCTCAGAAGAAGGT-3′</td>
</tr>
<tr>
<td>AFF1/AF4 for qPCR</td>
<td>5′-GACGGCACGCTGCTCTG-3′ / 5′-GACGGCACGCTGCTCTG-3′</td>
</tr>
<tr>
<td>AFF3/LAF4 for qPCR</td>
<td>5′-CAACATATAACACACAC-3′ / 5′-CAACATATAACACACAC-3′</td>
</tr>
<tr>
<td>AFF4/AF5q31 for qPCR</td>
<td>5′-GACGGCACGCTGCTCTG-3′ / 5′-GACGGCACGCTGCTCTG-3′</td>
</tr>
</tbody>
</table>

**Plasmids and constructs**

Mammalian expression vectors. cDNAs were synthesized from human lymphoblastoma cell line RNA and human fetal brain RNA (Clontech Laboratories, Inc., Palo Alto, CA, USA) using the SuperScript II first-strand synthesis system for RT–PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. cDNA was used as a template to amplify full-length (FL) AFF2/FMR2 cDNA (from human fetal brain cDNA), FL AF9, C-terminus (C-ter) and N-ter of AFF1/AF4 and C-ter of AFF4/AF5q31 cDNAs (from lymphoblastoma cell line cDNA) by PCR using TaKaRa LA Taq (Takara Bio Inc., Otsu, Shiga, Japan) and the appropriate primers, the sequences of which are reported in Table 1, containing Xhol/BamHI, SmaI/SmaI, Xhol/BamHI, NheI/Xhol and Xhol/BamHI restriction sites, respectively. The N-ter of AFF4/AF5q31 was amplified by PCR from the cDNA clone kindly provided by Professor R.G. Roeder (21) using LA Taq (TaKaRa) and primers containing Xhol/BamHI restriction sites (Table 1). The AFF2/FMR2 N-ter and C-ter were amplified from the FL gene using Xhol/BamHI restriction sites. All PCR fragments were digested with appropriate restriction enzymes and cloned into the pTL1-Flag plasmid (58) for FL, N-ter and C-ter of AFF2/FMR2, FL AF9, C-ter of AFF1/AF4, N-ter and C-ter of AFF4/AF5q31 and into the pTL10-Flag plasmid for N-ter of AFF1/AF4, FL AFF1/AF4 and AFF4/AF5q31 were generated by the digestion subcloning method using the N-ter and C-ter constructs of the respective gene. HindIII/BamHI and NheI/BamHI restriction sites were used to digest and then religate FL AFF1/AF4 in the pTL10-Flag AFF1/AF4 N-ter construct and FL AFF4/AF5q31 in the pTL1-Flag AFF4/AF5q31 N-ter construct, respectively. The FL AFF3/LAF4 cDNA clone in the pCMV6 Flag-tagged vector was purchased from Origene Technologies. N-ter and C-ter AFF3/LAF4 were amplified from pCMV6 Flag-AFF3/LAF4 FL using Pfx DNA polymerase (Invitrogen) and attB PCR primers for the Gateway system (Table 1). attB-flanked PCR products were cloned into the pDONR221 vector (Invitrogen) by the Gateway recombinant cloning technique and then subcloned into the Gateway destination vector pcDNA-DEST26 (Invitrogen). pTL1-N19 (37) and SXN13-FBS minigene (25) constructs were described previously.
Transfection and immunofluorescence

HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were seeded either on coverslips in 24-well cell culture plates or into 6-well cell culture plates, cultured for 6, 16, 24 or 48 h. Immunofluorescence was carried out as previously described (59) using the following antibodies: monoclonal anti-Flag M2 (Sigma) at 1:1000, polyclonal anti-Flag (Sigma) at 1:1000, monoclonal anti-SC35 (Abcam) at 1:2000, monoclonal anti-AF4 (60) at 1:500, monoclonal anti-fibrillarin (1:20), polyclonal anti-AF9 (Bethyl Laboratories) at 1:2000, monoclonal anti-histidine (1:500) and the following secondary antibodies: Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes) or Zenon Alexa Fluor 594 mouse IgG1 labeling kit (Invitrogen).

Production and purification of recombinant proteins

Escherichia coli BL21 star (DE3) cells (Invitrogen) were transformed with the histidine-tagged pET151/D-Topo AFF2/FMR2 N-ter, pET151/D-Topo AFF2/FMR2 C-ter, pDEST17 AFF3/LAF4 C-ter or pET16b AFF4/AF5q31 C-ter construct. The cells were grown in LB medium supplemented with ampicillin (100 mg/ml) or with kanamycin (50 mg/ml) for the pET16b AFF4/AF5q31 C-ter construct. Cells were induced for 4 h by addition of 1 mM isopropyl-β-D-thiogalacto-pyranoside until the cultures reached an OD of 0.4 at 600 nm. The cells were harvested and resuspended in lysis buffer [25 mM Tris–HCl, pH 7.6, 300 mM KCl or LiCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20% glycerol, 5% NP-40, 0.5 mM urea, complete protease inhibitor cocktail (Roche), 1 mM phenylmethylsulfonyl fluoride (PMSF)] sonicated for 5 min and centrifuged at 15 000 rpm for 30 min at 4°C. The supernatant was incubated with Ni-NTA agarose beads (Qiagen, Hilden, Germany) for 2 h at 4°C by agitation. Beads were washed four times at 4°C with washing buffer (25 mM Tris–HCl, pH 7.6, 300 mM KCl or LiCl, 1 mM DTT, 0.5 mM urea, 20% glycerol, 20 mM imidazole). Fusion proteins were eluted from the beads with elution buffer (25 mM Tris–HCl, pH 7.6, 500 mM KCl or LiCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Triton, 0.5 mM urea, 250 mM imidazole) for 30 min on ice.

RNA filter-binding assays

RNA filter-binding assays were performed as previously described using the two probes N19 and N19△35 (25,37,38).

RT–PCR and quantitative real-time PCR

Total RNAs were extracted from HeLa cells, control and FRAXE skin fibroblasts using the RNeasy kit (Qiagen) and retro-transcribed by the SuperScript II first-strand synthesis system (Invitrogen). We evaluated the inclusion of exon 2-FBS of the SXN13 minigene and the relative mRNA expression of AFF members in fibroblasts by quantitative real-time PCR as we have described previously (61). The primers used in this set of experiments are reported in Table 1. Real-time PCR was performed using LightCycler 480 Real-Time PCR System (Roche) using the cDNA, qPCR Core kit for SYBR Green (Eurogentec, Seraing, Belgium) according to the manufacturer’s instructions and 200 nM of each primer. All experiments were performed in triplicate. The relative expressions of transcripts were quantified using the 2–ΔΔCT method (62). The human GAPDH gene was used as a housekeeping gene for normalization. Real-time PCR amplification was performed as we have described recently (25).

Statistical analysis of mRNA levels in patients’ cell lines versus controls was performed by the Relative expression software tool (63).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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