

Splicing Regulatory Elements and mRNA-abundance of *dlg1* and *capt*, Genetically Interacting with dFMRP in *Drosophila* Brain

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Abstract: Fragile X syndrome (*FraX*) is an inherited disease, caused by the transcriptional inactivation of the gene Fragile X mental retardation 1 (*FMR1*) and the loss of its protein product Fragile X Mental Retardation Protein (*FMRP*). It is an RNA-binding protein involved in the transport, stability and posttranscriptional expression of key neuronal mRNAs.

To further understand the molecular and cellular mechanisms underlying the disease, we used the *Drosophila FraX* model and investigated a not well studied role of *Drosophila* Fragile X Mental Retardation Protein (*dFMRP*) in alternative splicing of neuronal mRNAs to which it binds via a G-quartet sequence.

By means of qRT-PCR we established the relative abundance of some isoforms of the gene *dlg1*, resulting from alternative exon skipping nearby a G-quartet and an exonic ESE-sequence, both acting as exonic splicing enhancers. We also investigated the relative mRNA-abundance of all *capt*-isoforms and the pre-mRNAs of both genes. We proposed a possible involvement of *dFMRP* in alternative splicing of genes, interacting with *dfmr1*. In the absence of *dFMRP* in larval and pupal brains, we found a change in the mRNA-level of one of the studied isoforms of *dlg1* and of its pre-mRNA.

We also established previously reported splicing regulatory elements and predicted computationally novel hexamere sequences in the exonic/intronic ends of both genes with pupative regulatory roles in alternative splicing.

Keywords: *dfmr1*, *dlg1*, *capt*, *dFMRP*, qRT-PCR, Splicing regulatory elements.

Introduction

Drosophila Fragile X Mental Retardation Protein (*dFMRP*) is the *Drosophila* homologue of the protein *FMRP* in humans. The latter is encoded by the gene Fragile X mental retardation 1 (*FMR1*). Abnormal CGG amplification in the promoter region of this gene, followed by its

transcriptional inactivation, is involved in the occurrence of a group of symptoms, described as Fragile X syndrome (FraX) [16, 26]. FraX is the most common cause of mental retardation. It is also characterized by alterations in the circadian rhythms, autism and other abnormal behaviors.

Models of FraX have been created in mice and *Drosophila* [59, 66] with features, similar to those, found in humans.

FMRP is an RNA-binding protein, containing two KH-domains and one RGG-box [3, 54]. It recognizes and binds specific mRNA-targets with G-quartet motives by means of its RGG-domain [8, 13, 45, 52, 53].

It has been suggested that FMRP is associated with a set of brain mRNAs and forms together with other proteins ribonucleoprotein complexes, where it acts as a regulator of their transport [2, 14, 18, 27, 28, 36], stability [15, 63, 65] and translation [21, 22, 25, 32, 34, 46, 47, 49, 55, 64]. These mRNAs are important for synaptic plasticity and neuronal development.

The functions of FMRP in post-transcriptional expression of such RNAs turned out to be far reaching. Recently, Didiot et al. [19] found that FMRP associated with its own mRNA via a G-quartet sequence, whereby this sequence acted as an exonic enhancer of FMRP – alternative splicing in a negative autoregulatory loop. A similar role of the G-quartet was also shown for the FMRP2 protein which is closely related to FMRP [42].

In addition to this, data was obtained, demonstrating an implication of FMRP in the nuclear posttranscriptional metabolism of some neuronal RNAs. It was found that FMRP associated with a novel human protein Simiate which localized to nuclear speckles. These are nuclear domains, enriched in pre-mRNA splicing factors. Simiate and nuclear speckles experienced alterations in FMR1 knock-out mice and this suggested a link between FMRP and transcription and splicing control [17].

To further study the role of FMRP in alternative splicing of its own mRNA and to check its function in others mRNAs as well [19], we used the *Drosophila* model of FraX.

In our work we investigated the possible role of dFMRP in the alternative splicing of brain neuronal mRNAs with G-quartet sequences. We undertook a study of the mRNA abundance of alternatively spliced isoforms, encoded by the genes *discs large 1* (*dlg1*) and *capulet* (*cap1*), at different stages of *Drosophila* development. These genes were chosen, as they had previously shown strong interactions with *dfmr1* (*Drosophila* Fragile X Mental Retardation 1) in the wing imaginal discs (data not shown). Our aim was to see if post-transcriptional expression of specific brain mRNAs, possessing both: G-quartets – as putative dFMRP – binding sites and auxillary exonic/intronic regulatory splicing sequences – enhancers (ESEs/ISEs) or suppressors (ESSs/ISSs) was affected by dFMRP. Such sequences are critical for correct splice-site recognition in pre-mRNA splicing [60]. The chosen approach would give us a hint about the possible regulation of alternative splicing by this protein. Some of the splicing regulatory sequences were identified in previous studies [7, 31, 40, 60]. Another part was computationally predicted as a set of hexamers with significantly increased densities towards the exonic/intronic boundaries of the genes *discs large1* and *capulet*.

discs large 1 encodes *dlg* – a multi-domain containing protein that belongs to the family of molecular scaffolding proteins known as membrane guanylate kinases or MAGUKs.

dlg regulates cell polarity, junctional structure and asymmetric cell divisions [4, 48, 50, 62]. It plays important synaptic role in brain neurons and neuromuscular junctions [9, 23, 33]. *dlg* interacts with multiple proteins, such as ion channels, cell adhesion molecules and other scaffolding proteins, which participate in the organization and function of neuronal synapses [6, 11, 39, 56, 57, 58, 68].

In mice FMRP is shown to stabilize the mRNA of the mammalian homologue of *dlg* – PSD-95 [63].

capulet is an actin-binding protein that regulates actin polymerization and is important for the maintenance of proper neuronal cytoskeleton and for axon guidance during development [41, 61].

Materials and methods

Stocks

In our work we used the following *Drosophila* stocks:

w[1118]

w[1118]; $P\{w[+mc] = UAS - Fmr.Z\}3$

w[*]; $P\{w[+mC] = GAL4-elav.L\}3$

w[1118]; *Fmr1*^{Δ113M}/*TM6B*, *Tb*¹

They all were maintained on corn meal/ yeast extract/raisins at the standard temperature of 25°C.

Quantitative real-time PCR analysis

Total RNA was extracted from third instar larval brains and pupal heads from three different stocks: wild type (*w*¹¹¹⁸), *dfmr1* null mutants (*w*¹¹¹⁸; *Fmr1*^{Δ113M}/*Fmr1*^{Δ113M}) and mutants, over-expressing *dfmr1* in the central nervous system (*w*[1118]; $P\{w[+mc] = UAS - Fmr.Z\}3/P\{w[+mC] = GAL4-elav.L\}3$), using Trizol (Invitrogene) according to the manufacturer's instruction.

cDNA was prepared with RevertAid H Minus First-Strand cDNA Synthesis Kit (Thermo Scientific). The first strand cDNA was used as a template for quantitative real-time PCR (qRT-PCR). qRT-PCR reactions were performed with 2x Sybr Green Master Mix (Thermo Scientific). A standard curve was run in each PCR reactions. Glyceraldehyde 3 phosphate dehydrogenase (*gapdh*) was used for endogenous control. All reactions were done two times in duplicate, and the relative expression of RNA was calculated by Delta-Delta Ct method [37]. Real-time PCR was accomplished with the following PCR program: 95°C for 10 min, then 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The qRT-PCR analysis of mRNA, splice variants and pre-mRNA of the genes *dlg1* and *capt* were carried out with the following primers: *gapdh*F - 5'- TTT GAC CGT GCG CTT GGG CA- 3'; *gapdh*R - 5'- ACC GAC GAG TGG GTG TCG CT- 3'; *dlg* 10-13 F - 5'- AAA GAC AGC GGA GAC CTT GC- 3'; *dlg* 10-13 R - 5'- TTG GAT GAG GCG TCG TTG TT-3'; *dlg* 11-12 F - 5'- AGT AAC AGT CGT GCC GTT GA-3'; *dlg* 11-12 R - 5'- CGG TGG GAT TTG GTT GGT TT-3'; *dlg* 13-14 F - 5'- GAT CCA AAT CGA GGA GCG GG-3'; *dlg* 13-14 R - 5'- GCC GTT GTT ATG GCG AAG TT-3'; *capt* 7-6 F - 5'- TGG AAG CGC ATA ATC CCG AG-3'; *capt* 7-6 R - 5'- TCT TTG GGT GCT AGT GCT GG-3'; *capt* 7-4 F - 5'- TTG GAA GCG CAT AAT CCC GA-3'; *capt* 7-4 R - 5'- GTC TCC AGT CGC TCG CAA AT-3'; *capt* 8-4 F - 5'- CGC AGC TTC TCG TCA GTT CT-3'; *capt* 8-4 R - 5'- GTC TCC AGT CGC TCG CAA AT-3'; *gapdh* 1-3 F - 5'- CCG CGG AAA ACT TTC CAA GT-3'; *gapdh* 1-3 R - 5'- ACG TTG

GCG CCC TTA TCA AT-3'; *dlg* 10-11 F - 5' - AGG CCC TCA GCA GAA CAT TT- 3'; *dlg* 10-11 R - 5' - TTG TGA CAG AAT GGC AGA TG-3'; *capt* 4-3 F - 5' - GCC ATC GTT CAA TTC CGC AA -3'; *capt* 4-3 R - 5' - AAG TCC TGG ATG GCG CTG AT -3'.

Statistics

All statistical calculations were carried out in GraphPad InStat 3.0 (GraphPad Software Inc.) and Excel (Microsoft Corp.). The significance levels of each test are reported as significant for $p < 0.05$ (*), as highly significant for $p < 0.01$ (**) and as extremely significant for $p < 0.001$ (***). A potential difference in the means of the two groups was analysed by a two-tailed Student's t-test (t-test) or when comparing more than two groups ANOVA was applied. Error bars represented standard error of the mean (SEM).

Bioinformatics

Bioinformatic analysis for the G-quartets presence

For the G-quadruplex forming sequences analysis, the *gregexpr* function of seqinR package was used (<http://cran.r-project.org/web/packages/seqinr/>) to access the PERL library and to work with regular expressions. According to previous data, the consensus sequence of the G-quartet is: DWGG – N₀₋₂ – DWGG – N₀₋₁ – DWGG – N₀₋₁ – DWGG, where D means any nucleotide, except C; W means T or A and N means any nucleotide [13].

Bioinformatic analysis for the presence of previously reported auxillary splicing regulatory elements

A list of previously reported auxillary splicing regulatory elements [7, 31, 40, 60] was used. Analysis was made by the function *gregexpr* to find the positions of 24 exonic splicing enhancers (ESEs), 5 exonic splicing suppressors (ESSs), 7 intronic splicing enhancers (ISEs) and 1 intronic splicing suppressor (ISSs) in exonic and intronic sequences of the genes *dlg1* and *capt*.

Computational prediction of auxillary hexanucleotide splicing regulatory elements

Two sets of exonic and intronic sequences were prepared, originating from the *dlg1* and *capt* genes. Each set encompassed segments, located, correspondingly: 50 nt upstream from the 3'-exonic splice signal; 50 nt downstream from the 5'-exonic splice signal; 50 nt upstream from the intronic branch point and 50 nt downstream from the 5'-intronic splice signal. If an exon or intron was shorter than 100 nt, its whole sequence was analyzed.

For the motif search within these four segments, determining the 5'- and 3'-exon/intron boundaries, the MEME algorithm (<http://meme.nbcr.net/meme/>) was used. MEME analyzes a database sequence for similarities among items and produces a description (motif) for each pattern it discovers. Only hexanucleotide motifs (hexamers) were searched.

To determine the statistical significance of the discovered motifs, the *E*-value (or score) for the best motif/alignment from our original sequences was compared with the new *E*-value by using the "Shuffle sequence letters" option and keeping all other parameter settings the same. In the study the shuffling increased several-fold the *E*-value for all motifs of interest in the original run. According MEME documentation if the *E*-values are similar, then the motif is probably not significant.

Results and discussion

Bioinformatic analysis for potential G-quadruplexes and cis-acting splicing regulatory sequences

Screening for G-quadruplexes

The *dlg1* locus is complex and encodes 21 different transcripts, reported in the Flybase (www.flybase.org). They arise due to multiple transcriptional start sites and alternative splicing. Annotated transcripts in the Flybase do not represent all possible combinations of alternative exons and alternative promoters. The longest transcript *dlg1*-RL (7249 bp) contains five different domains: L27, PDZ (Post synaptic density protein – PSD95, *Drosophila* disc large tumor suppressor – *dlg1*, and Zonula occludens-1 protein – zo-1, which share the domain), GMPK (guanosine monophosphate kinase), GUK (guanilate kinase), and SH3-DLG-like (Src homology 3 domain of *dlg* homolog proteins) (<http://www.ncbi.nlm.nih.gov/gene/32083>). These domains are shown in Fig. 1. Exons are represented with boxes and introns – by lines (www.flybase.org); protein domains are represented with grey boxes. The GMPK domain is not presented, as it is located within the domain GUK.

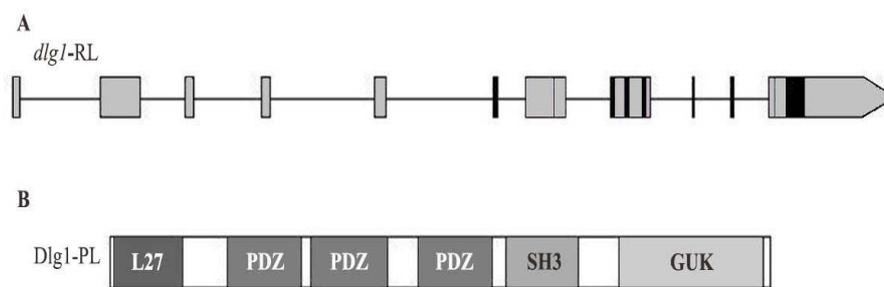


Fig. 1 Schematic representation

- (A) *dlg1* genomic structure for the longest *dlg1*-RL transcript,
(B) protein product, encoded by the transcript *dlg1*-RL

dlg1-mRNA isoforms can be divided arbitrary in several common groups, depending on the domains which they contain. Group I consists of three isoforms which have the domains L27, PDZ, GMPK, GUK and SH3-DLG-like. Group II includes eleven isoforms, all possessing the PDZ, GMPK, GUK and SH3-DLG-like domains. Group III represents one isoform with the L27, PDZ and SH3-DLG-like domains. Group IV represents one isoform with the L27, PDZ domains and group V represents five isoforms only with the L27 domain.

The *capt*-gene encodes 3 so far known mRNA isoforms. The longest mRNA isoform *capt*-RB (3049 bp) contains 3 different domains: CAP-N (adenylate cyclase associated – CAP-N terminal), CARP (domain in CAP – cyclase associated proteins and X-linked retinitis pigmentosa 2 gene product) and CAP-C (adenylate cyclase associated – CAP-C terminal) (<http://www.ncbi.nlm.nih.gov/gene/45233>). These domains are shown in Fig. 2. Exons are represented with boxes and introns by lines (www.flybase.org); protein domains are represented with grey boxes.

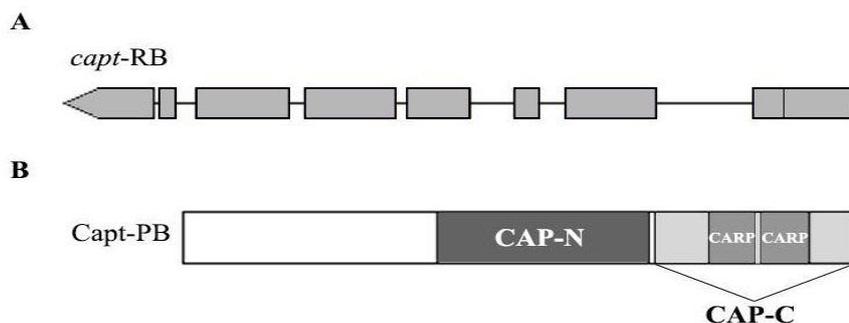


Fig. 2 Schematic representation
(A) *capt* genomic structure for the longest *capt-RB* transcript,
(B) protein product, encoded by the transcript *capt-RB*

We analyzed the transcripts of the genes *dlg1* and *capt* for potential G-quadruplex structures that may mediate an association with the RNA-binding protein dFMRP [5, 13, 19, 51]. Our analysis revealed such structures in the exons in 14 mRNA-isoforms of *dlg1* and in one exon of all 3 mRNA-isoforms of *capt*.

5 exons of the *dlg1* gene out of all 41 displayed G-quartet sequences: exons 12, 38, 39, 40 and 41. Only 1 *capt* – exon – exon 3, out of 8 was found to contain a G-quartet sequence. No G-quartet structures were found within the introns of both genes.

Screening for known auxillary splicing regulatory sequences

Splicing requires conservative splice signals (SS) at the exonic 5'- and 3'-ends. Additional auxillary elements are also involved in the accurate discrimination of exons and introns [10, 24]. These elements have been termed splicing enhancers – exonic (ESEs) and intronic (ISEs) or suppressors (ESSs and ISSs).

We analyzed the transcripts of *dlg1* and *capt* for the presence of known auxillary splicing sequences in *Drosophila*, which were reported by other authors to modulate alternative splicing [7, 31, 40, 60].

The most common type of alternative splicing (AS) in genes of higher eukaryotes is multiple exon skipping, in which one exon, termed exon cassette, or more exons are spliced out of the transcript, together with the flanking introns [29]. Such exons are described as alternative.

Neighbouring exons from a pre-mRNA sequence, which are retained in two or more mature mRNA-isoforms, are described as constitutive exons and the splicing of their flanking introns is classified as constitutive splicing (CS).

We were interested in previously published *Drosophila* regulatory splicing elements [7, 31, 40, 60], which were located in the 3'- and 5'-exon – and – intron boundaries (50 nt upstream/downstream from the 3'- and 5'-exonic splice site and 50 nt upstream from the intronic branch point or 50 nt downstream from the 5'-intronic splice site). Our focus was on regions involved in the alternative exon skipping and located close to the available exonic G-quartets as putative binding sites of dFMRP in *dlg1*- and *capt*-mRNAs.

We also looked for similar regulatory splicing elements in close vicinity to the constitutive splicing regions. These regions of choice for two genes are presented in Fig. 3 and Fig. 4. In Fig. 3 the blue triangles represent alternative RNA splicing of the designated exon

cassettes. The grey box in exon 12 represents the G-quartet sequence. Alternatively skipped exons in the region are: 11 and 12 (retained exons: 10, 13); 1-10 (retained exons: 11, 12 and 13); 10, 11 and 12 (retained exons: 13, 14). In Fig. 4 the blue triangles represent alternative RNA splicing of the designated exon cassettes. The grey box in exon 3 represents the G-quartet sequence. Alternatively skipped exons in the region are: 7, 6 and 5 (retained exon: 8); 6, 5 and 8 (retained exon 7); 8 (retained exons: 7, 6 and 5). The red triangles represent constitutive RNA splicing. Constitutive exons are: 4, 3, 2 and 1.

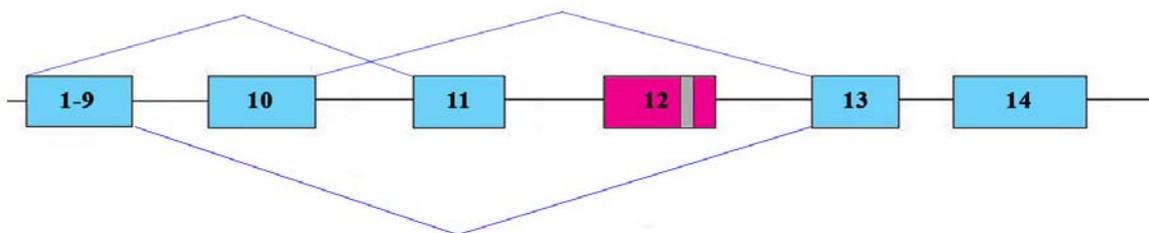


Fig. 3 Regions of genomic *dlgl*-sequence with multiple alternative exon skipping and a G-quartet sequence

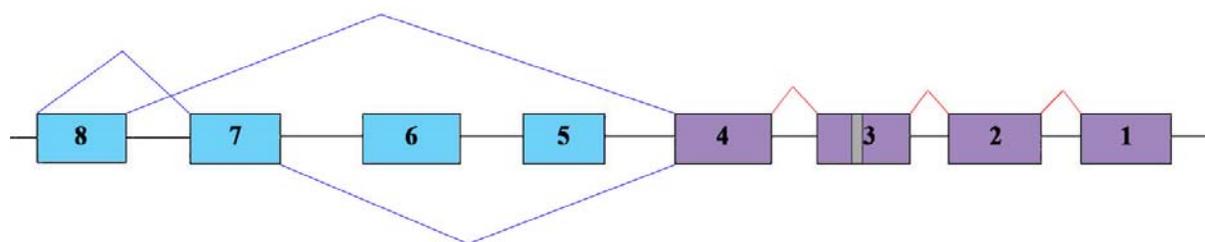


Fig. 4 Regions of genomic *capt*-sequence with alternative exon skipping and a G-quartet sequence

We searched for 37 known regulatory splicing elements. 12 such elements were established: 7 ESE-sequences, 3 ESS-sequences and 2 ISE-sequences. Their distribution in the regions of alternative and constitutive splicing for both genes is presented in Table 1. No ISS-elements were found in the 5'- or 3'-splice site boundaries of the *dlgl* and *capt* genes.

As Table 1 demonstrates, ESE-sequences are the most frequent regulatory elements, found in the splice site boundaries of *dlgl* and *capt*. They are registered 23 times in the sequences analysed.

Most of them are characteristic for both genes but three ESEs – CTGGAG, ATGCGG and TGTGGA are *capt*-specific. One ESE-sequence – AATGGA, though met with low frequency, is *dlgl*-specific and is found in a single exon, involved in AS. All other ESEs are found in both genes.

The ESE-sequence A[AGC]CA[AGC]C displays a high specificity for exons, participating in AS in both genes. The *capt*-specific ESE – TGTGGA is also present only in regions with AS. We assume that these regulatory elements are specific for alternative splicing.

Other ESEs (GGAA, CGCA) do not show any preference in regions with a different type of splicing and are found in exons, involved either in AS or in CS.

Table 1. Distribution of known *Drosophila* splicing regulatory elements in regions with AS and CS of *dlg1* and *capt*-pre-mRNA with available G-quartet sequences

Splicing regulatory sequence	Frequency in <i>dlg1</i> exons in regions with AS	Frequency in <i>dlg1</i> introns alternatively spliced out (AS)	Frequency in <i>capt</i> exons constitutively retained (CS)	Frequency in <i>capt</i> introns constitutively spliced out (CS)	Frequency in <i>capt</i> exons in regions with AS	Frequency in <i>capt</i> introns in regions with AS
ESE (A[AGC]CA [AGC]C)	3	0	0	0	4	0
ESE (GGAA)	3	0	1	0	2	0
ESE (CGCA)	6	0	2	0	1	0
ESE (AATGGA)	1	0	0	0	0	0
ESE (CTGGAG)	0	0	0	0	1	0
ESE (ATGCGG)	0	0	1	0	1	0
ESE (TGTGGA)	0	0	0	0	1	0
<u>ESS (TAGT)</u>	<u>1</u>	0	0	0	0	0
<u>ESS (TGGG)</u>	<u>1</u>	0	0	0	<u>1</u>	0
<u>ESS (GGTT)</u>	<u>1</u>	0	0	0	<u>3</u>	0
ISE (TAAT)	0	3	0	0	0	1
ISE (T[TC]TC)	0	1	0	0	0	4

The ESS-elements, which we found in our search are less frequent and are present 7 times within the splice site boundaries of *dlg1* and *capt*. One of them is characteristic for the *dlg1*-alternative splicing and the other two ESS-elements are also found in exons, involved in AS in both genes. Such regulatory elements were not found in the exons which are constitutively retained and common for the three *capt*-mRNA-isoforms (exons 4, 3, 2 and 1). This finding clearly demonstrates that these exonic sequences, previously reported to suppress splicing [60], are really characteristic for exons, which are always retained by the splicing machinery.

From all 7 ISEs examined we found two sequences – TAAT and T[TC]TC. They were presented within introns of both genes which were alternatively spliced out in the mRNA-isoforms. Besides, these ISEs were not presented in *capt*-introns, which were constitutively removed from mRNAs by splicing.

This finding demonstrates that the ISE-sequences, identified in our bioinformatic analysis, are characteristic only for participating in AS introns.

Computationally predicted auxillary splicing regulatory elements

In order to enrich the 5'- and 3'-exonic and intronic boundaries of *dlg1* and *capt* with additional auxillary control splicing elements, we performed a computational search for hexanucleotide sequences (hexamers) with statistically significant density increase towards these boundaries (see Materials and methods).

We predicted such sequences, clustered in 33 putative core motives, located in the exonic and intronic ends of both genes. These motives are conservative though degenerative.

The comparison of these putative regulatory sequences with known auxillary splicing regulatory elements, discussed above, showed that they all are novel. As their possible function in splicing was experimentally not examined, we did not determine them further as splicing enhancers or suppressors.

We determined the positions of all available hexanucleotide sequences within the regions of *dlg1*- and *capt*-pre-mRNAs with AS or CS and “mapped” them in the exon or intron boundaries. The frequency and distribution of all available hexanucleotide sequences within the regions of *dlg1*- and *capt*-pre-mRNAs with AS or CS are summarized in Table 2.

As the table shows, exonic elements are presented more often (altogether 26 times), while intronic ones are presented with a 15 times lower frequency. We can divide all elements in 6 different and very specific groups (these groups correspond to the Table 2 columns). The exonic sequences are not only gene-specific but also splicing type specific and are different for AS or for CS (*capt*-exonic sequences). The same pattern is also seen in the intronic sequences.

Our bioinformatic analysis, aimed to identify within the *dlg1*- and *capt*-splice site ends familiar *Drosophila* splicing regulatory elements and to predict novel putative regulatory sequences in AS or CS regions, where we first identified the G-quadruplex sequences. In the next step we looked for the mRNA-abundance of different *dlg1*- and *capt*-isoforms, arising in these regions as splice variants.

Relative mRNA abundance of alternatively spliced isoforms of capulet and discs large1 in mutants with different expression of dFMRP

In order to experimentally study the possible influence of the dFMRP protein on the alternative splicing, occurring in regions, close to the FMRP putative binding site (G-quartet) and to an ESE-sequence, we chose the qRT-PCR approach. By means of this approach we studied the relative amount of different mRNA-isoforms of *capt* and *dlg1*, which were encoded by alternative or constitutive splicing in the selected appropriate gene regions.

As already mentioned, there are 3 *capt*-mRNA-isoforms. They all contain exon 3, which is the only one, found to contain a G-quartet. This sequence starts at position 266 nt of the exon and is not within the 5'- or 3'- 50 bp regions, immediately neighbouring the exonic splice sites. There are also two previously published ESE-sequences: GGAA and TGTGGA [7], located in close proximity to the G-quartet and startig at positions 273 and 297, correspondingly. Upstream from exon 4, there is a region of exon cassette alternative splicing, whereby either two exons – exon 6 and exon 5 are omitted in the mature transcript (designated as isoform 1, presented in Fig. 5), or three exons – 7, 6 and 5, are omitted in the processed transcript (isoform 3 in Fig. 5).

Table 2. Distribution of computationally predicted *Drosophila* splicing regulatory elements in the regions of AS and CS of the *dlg1* and *capt*-pre-mRNA with available exonic G-quartets

Computational predicted splicing regulatory sequence	Frequency in <i>dlg1</i> exons in regions with AS	Frequency in <i>dlg1</i> introns alternatively spliced out (AS)	Frequency in <i>capt</i> exons constitutively retained (CS)	Frequency in <i>capt</i> introns constitutively spliced out (CS)	Frequency in <i>capt</i> exons in regions with AS	Frequency in <i>capt</i> introns in regions with AS
e-ACATAC	2	0	0	0	0	0
e-ACGCAC	2	0	0	0	0	0
e-CTGCTG	1	0	0	0	0	0
e-AAGTGA	1	0	0	0	0	0
e-GAGTGA	1	0	0	0	0	0
e-AATTGA	1	0	0	0	0	0
i-AACGAT	0	2	0	0	0	0
i-ATCGAA	0	1	0	0	0	0
i-CCAAAC	0	1	0	0	0	0
i-CGCACA	0	1	0	0	0	0
i-GCCACC	0	1	0	0	0	0
e-GCGGCG	0	0	2	0	0	0
e-AAGCCA	0	0	1	0	0	0
e-CTTGAA	0	0	2	0	0	0
e-ATAAAA	0	0	2	0	0	0
e-ATAAGA	0	0	1	0	0	0
i-GAGTCA	0	0	0	2	0	0
i-CCGCAG	0	0	0	1	0	0
i-CCGCAA	0	0	0	1	0	0
e-GGATTA	0	0	0	0	2	0
e-AGCAAA	0	0	0	0	2	0
e-AACCAA	0	0	0	0	1	0
e-CAAAGA	0	0	0	0	1	0
e-CTTGAC	0	0	0	0	2	0
e-CAAATT	0	0	0	0	2	0
i-GTGAAG	0	0	0	0	0	2
i-CTAGCG	0	0	0	0	0	1
i-CCCACC	0	0	0	0	0	1
i-CCCATC	0	0	0	0	0	1

“e” means exonic sequence

“i” means intronic sequence

When only the introns between exons 7-6; 6-5 and 5-4 are spliced out, they give rise to the longest isoform 2, in which all the consecutive exons from 7 to 1 are retained. This isoform is also shown in Fig. 5, where 5'- and 3'- means the 5'- and 3'- UTR (untranslated region) of the *capt*-pre-mRNA and of *capt*-isoforms. The blue triangles represent alternative RNA splicing

of the designated exon cassettes. The red triangles represent constitutive RNA splicing of the designated introns between exons 4-3; 3-2. The G-quartet in exon 3 is presented as a grey box and the downstream ESE-sequences are presented as black boxes. The arrows indicate the position of the primer pairs, used for amplification of the corresponding mature transcript or the pre-mature mRNA.

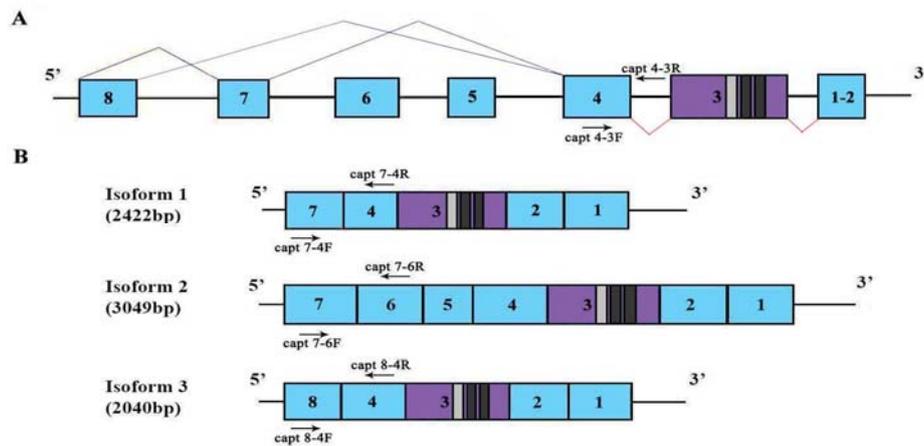


Fig. 5 *capt*-mRNA-isoforms, encoded by alternative splicing of the genomic *capt*-sequence (A) *capt*-pre-mRNA, (B) *capt*-mRNA-isoforms

To investigate the relative amounts of the three *capt*-isoforms we designed appropriate primer pairs and analyzed the mRNA-levels by qRT-PCR. The results from these experiments are shown in Fig. 6.

The three *capt*-isoforms – 1, 2 or 3, designated also as *capt* 7-4, *capt* 7-6 and *capt* 8-4, did not change significantly their abundance in larval brains from *dfmr1*-null mutants with no dFMRP expression (*Fmr1*^{Δ113M}/*Fmr1*^{Δ113M}), as compared to the wild type (Fig. 6A).

When dFMRP was overexpressed, there was also no change in the relative amounts of the alternatively spliced isoforms *capt* 7-4 and *capt* 8-4 but that of the constitutive spliced isoform *capt* 7-6 (containing exons 7, 6, 5) increased more than two-fold ($p < 0.01$) (Fig. 6A).

It can be concluded that, the results from our qRT-PCR-experiments on larval brains did not show a simple connection between the amount of the dFMRP protein and the splicing events in its genetic interactor *capulet*.

In contrast, when pupal brains from the same genotypes were used, all three *capt*-isoforms dramatically decreased their relative abundance in the absence of dFMRP (Fig. 6B).

It is tempting to speculate that such findings argue for a positive regulation of *capulet* expression at mRNA level by dFMRP.

dFMRP has long been attributed to function in different aspects of RNA metabolism (see Introduction). Recently, an autosomal paralogue of FMRP – FMR2P have been shown to be involved in transcription and alternative splicing [42]. The other FMRP paralogue – FXR1P was found to interact genetically and to co-immunoprecipitate with the BTF-transcription factor [38]. Some FMRP mRNA-isoforms were found in nuclear structures, termed Cajal bodies, which are involved in histone pre-mRNA transcription and 3'-end

processing [20]. All these data provide a new clue on the relation between FMRP and its paralogues on the transcription events.

In the studied pupal brains, overexpressing dFMRP, one of the alternatively spliced isoforms – *capt* 8-4, displayed a seven-fold increase in the mRNA abundance ($p < 0.01$), another one – *capt* 7-4, showed a twofold decrease ($p < 0.01$), and *capt* 7-6, which arises by splicing out of all introns between exons 7, 6, 5, 4 and 3 – all upstream from G-quartet possessing exon 3, displayed no change. These observations show that the increased amount of dFMRP increased the level of the mRNA-isoform, which was produced by exon cassette splicing between exons 8-4 and decreased the level of the splice-isoform, obtained by exon cassette splicing between exons 7-4.

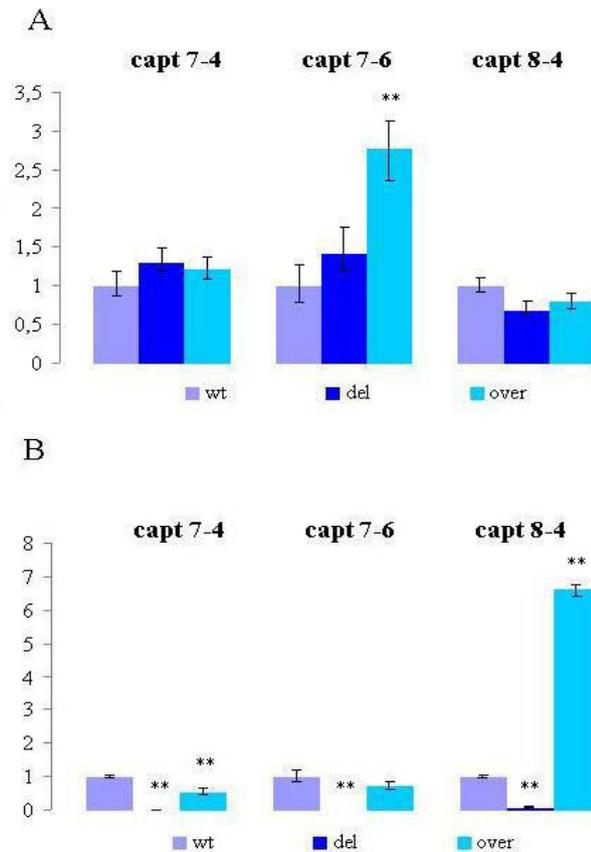


Fig. 6 Relative levels of *capt*-mRNA-isoforms in wild type (wt), in mutants with no dFMRP (del) and with increased amount of dFMRP (over) at different developmental stages:

(A) in larval brains, (B) in pupal brains.

wt-w[1118]; del-Fmr1^{Δ113M}/Fmr1^{Δ113M}, over-GAL4-elav.L/UAS – Fmr.Z

The mRNA values are the means \pm SEM, * $p < 0.05$; ** $p < 0.01$.

Altogether, our data show that the expression levels of alternatively spliced *capt*-mRNA-isoforms in *Drosophila* brains were affected by dFMRP in a developmentally specific pattern. The *dlg1* gene encodes multiple mRNAs, whose functions are poorly studied. We were interested in the abundance of those isoforms which arise due to multiple alternative exon skipping in the vicinity of a G-quadruplex forming sequence and an ESE sequence/sequences. As mentioned in the previous section of our results, exon 12 contains these sequences, close to the 3'-end of the exon. The G-quartet starts at the position 316 nt from the exonic 5'-end and the sequence GGAAAC, located at position 366 nt, is an ESE, which was identified in

previous studies [7]. There are 3 isoforms which are encoded by alternative multiple exon skipping in the region of exons 10-13, presented in Fig. 7. In the figure 5'- and 3'- means the 5'- and 3'- UTR (untranslated region) of the *dlg1*-pre-mRNA and of *dlg1*-isoforms. The blue triangle represents a RNA splicing of the designated exon cassettes. The G-quartet in exon 12 is presented as a grey box and the downstream ESE-sequence is presented as black box. The arrows indicate the position of the primer pairs, used for amplification of the corresponding mature transcript or the pre-mature mRNA.

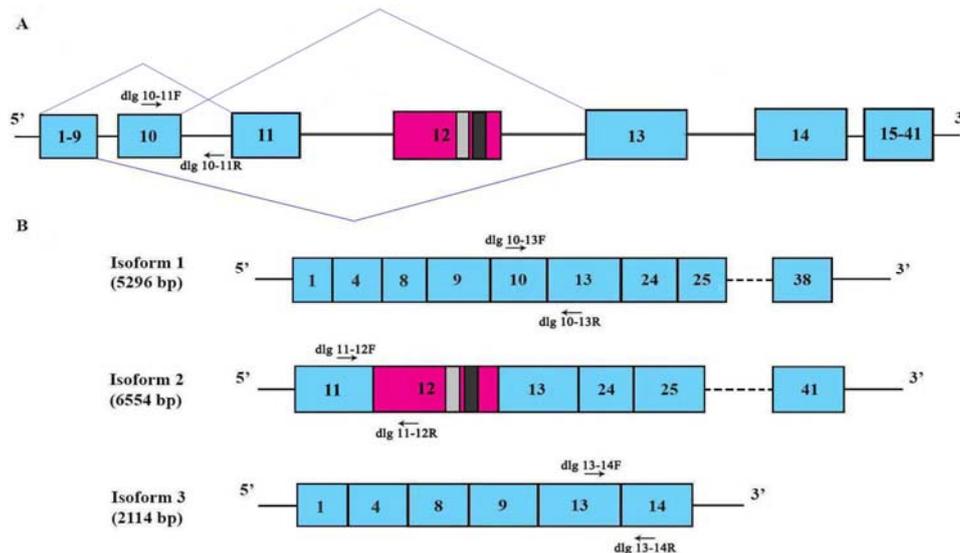


Fig. 7 *dlg1*-mRNA-isoforms, encoded by alternative splicing of the genomic *dlg1*-sequence (A) *dlg1*-pre-mRNA, (B) *dlg1*-mRNA-isoforms

When exons 11 and 12 are skipped in the mature transcript, a mRNA-isoform is produced, designated as isoform 1 or *dlg 10-13* (Fig. 7). Isoform 2 or *dlg 11-12* arises due to splicing out the exon cassette 1-10 and isoform 3 or *dlg 13-14* – due to skipping of exons 10, 11 and 12. All *dlg1*-isoforms and the pre-mature *dlg1*-RNA are presented in Fig. 7.

We designed appropriate primer pairs (see Materials and methods) and examined by qRT-PCR the relative amount of the *dlg1*-alternative spliced mRNA-isoforms in the selected region of exons 9-14. The results from these experiments are shown in Fig. 8.

In preliminary experiments we analyzed the total abundance of the *dlg1*-mRNAs and observed a positive control of their relative amounts by dFMRP (data not shown).

As Fig. 8A shows, in the larval brains with no dFMRP protein, there is a significant (three-fold) increase of the mRNA abundance of the *dlg1*-isoform 1, retaining exons 10 and 13, while the abundance of isoform 2, retaining exons 11 and 12, remains unchanged. In the pupal brains we observed the opposite effect – the mRNA abundance of the *dlg1*-isoforms with exons 10 and 13 was significantly decreased in *dfmr1*-null mutants. The levels of isoform 2 with exons 11 and 12 and of isoform 3 with exons 9, 13 and 14, remained unchanged. When *dfmr1* was overexpressed in pupal brains, the relative amount of this isoform was significantly increased, while that of the other two isoforms did not show any change.

Based on the increased abundance of the isoforms, containing exons 10 and 13 in larval brains when dFMRP is absent, we can assume that the frequency of splicing out exons 11 and 12

occurs more often than in the wild type. On the contrary, the frequency of the same exon cassette splicing in pupal brains is lower than that in the wild type.

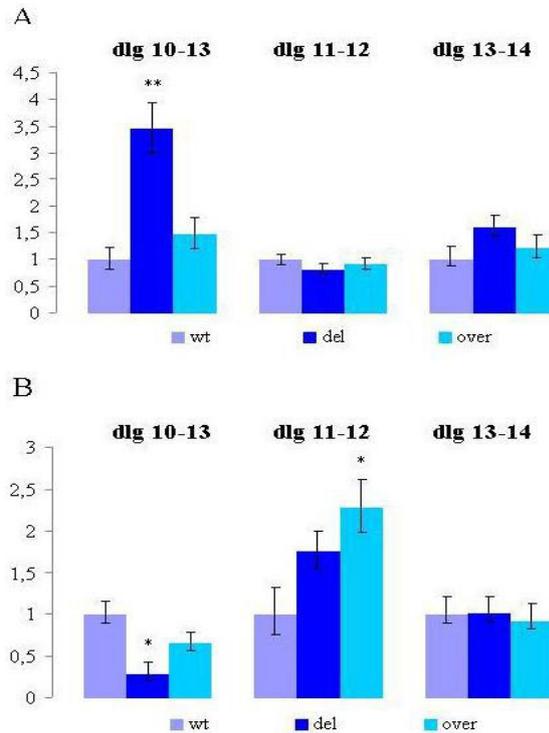


Fig. 8 Relative levels of *dlg1*-mRNA-isoforms in wild type (wt), in mutants with no dFMRP (del) and with increased amount of dFMRP (over) at different developmental stages:

(A) in larval brains, (B) in pupal brains.

wt-*w[1118]*; del-*Fmr1^{Δ113M}/Fmr1^{Δ113M}*; over-*GAL4-elav.L/UAS-Fmr.Z*

The mRNA values are the means ± SEM, * $p < 0.05$; ** $p < 0.01$.

Similarly to *capulet* mRNA-isoforms, the alternatively spliced isoforms of *discs large 1* show a dFMRP – dependent abundance pattern, which differs in the context of neuronal development (larval and pupal brains).

Analyzing the results from our qRT-PCR experiments, we can hypothesize that the presence of an exonic G-quartet and nearby ESE-sequences in a specific mRNA, encoded by a genetic interactor of dFMRP, influences the alternative splicing in neighboring regions, only if these sequences are located in the 5'- or 3'-ends of the exon. In the case of the *capt* gene, these sequences are located in the middle of exon 3. In that case we did not observe a connection between the change of the dFMRP-amount (in null-mutants or in mutants overexpressing the protein) and the mRNA level of the alternatively spliced isoforms in larval brains.

In the *dlg1* exon 12 the G-quartet and the near-by ESE are located within its 3'-end. In this case we observed that the abundance of dFMRP influences the mRNA level of alternatively spliced isoforms.

At the present, the functional significance of the change in abundance of mRNA-isoforms, arisen in the regions with AS, is not clear. For the gene *capt* a link between AS and a functional switch to expression of specific mRNA-isoforms is hard to observe, as all

3 *capt*-isoforms contain the same 3 protein domains, described in the first section of Results and discussion.

For the gene *dlg1* multiple isoforms are observed, which originate through alternative splicing but the functions of these variants are not well known [1, 35, 43, 44]. The most studied among them are DlgA and DlgS97. DlgS97 has distinct neuronal functions, as it is differentially expressed in the CNS (central nervous system) in embryos and larvae, but not in epithelial tissues [43]. This protein isoform contains all 5 functional domains, encoded by *dlg1*. DlgA contains 4 of these domains – PDZ, SH3, GMPK and GUK, and lacks the domain L27, which is thought to act as determinant for synaptic targeting and scaffold formation [12].

Isoform 1 of *dlg1* belongs to the arbitrary group I, containing 5 Dlg1 protein domains (see the first section of Results and discussion). Isoform 2 belongs to the group II isoforms, characterized by the presence of 4 Dlg1 protein domains, lacking L27 domain.

The increase of the relative mRNA level of isoform 1 in *dfm1*-null mutant larvae might represent a switch to elevated functions in larval brain neurons, accomplished by the domain L27 – synaptic functions [43]. On the other side, it is well known that dFMRP also has synaptic functions, negatively regulating synaptic growth and development [67]. We could speculate that this dFMRP function might be accomplished via an expression control of isoform 1 in a developmentally dependent pattern.

Relative amount of the pre-mature mRNAs of the genes capulet and discs large1 in mutants with different expression of dFMRP

In order to confirm that the changes in the relative amounts of the alternatively spliced *dlg1*- and *capt*-mRNA-isoforms, reported in the previous section of our results, are due to exon skipping/exon inclusion and not to differential mRNA-stability, additional qRT-PCR – experiments with appropriate primer pairs were carried out.

The primer pair *dlg* 10-11F and *dlg* 10-11R (forward and reversed, see Materials and methods) encompasses the region between exon 10 and its downstream intron to amplify premature mRNAs in the previously selected region of *dlg1* gene, where multiple exon skipping/inclusion take part. This premature mRNAs are designated as *dlg* 10-11 (see Fig. 7).

The primer pair *capt* 4-3F and *capt* 4-3R encompasses the region between exon 4 and the downstream intron, involved in constitutive splicing to amplify premature mRNAs, designated as *capt* 4-3 (see Fig. 5).

The primer pair *gapdh* 1-3F and *gapdh* 1-3R amplifies premature mRNAs of the housekeeping gene, encoding glyceraldehyde 3 phosphate dehydrogenase (see Material and methods), which is chosen as a control gene.

The results from this experiment are presented in Fig. 9.

We observed a decrease of the relative levels of the premature *dlg1*-mRNAs in larval brains with no dFMRP (*Fmr1^{A113M}/Fmr1^{A113M}*) or with an increased amount of this protein (*GAL4-elav.L/UAS-Fmr.Z*) (Fig. 9A). At the same time, the relative amounts of the *gapdh*-premature mRNAs remained unchanged in all larval genotypes analyzed. Unchanged was also the pre-mRNA-abundance of *capt* in all larval genotypes – with no dFMRP or with an increased amount of the protein (Fig. 9A).

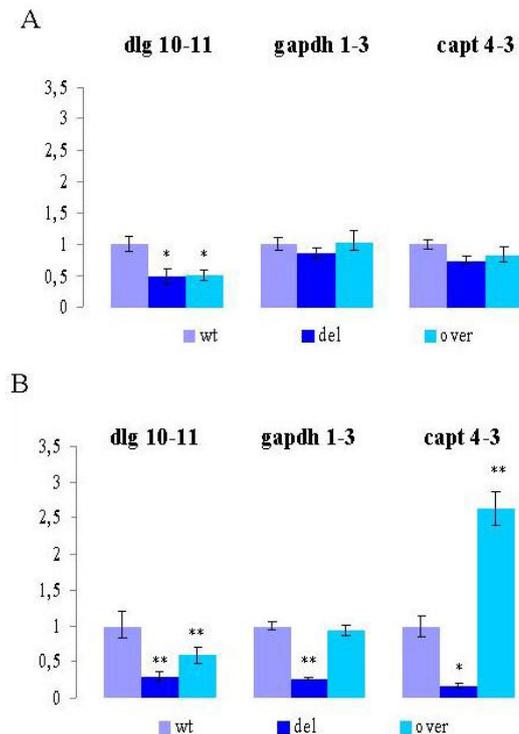


Fig. 9 *dlg1*-, *capt*- and *gapdh*-pre-mature mRNA abundance in genotypes with different amounts of dFMRP (A) in larval brains, (B) in pupal brains. wt-*w[1118]*; del-*Fmr1^{Δ113M}/Fmr1^{Δ113M}*; over-*GAL4-elav.L/UAS – Fmr.Z*. The mRNA values are the means ± SEM, **p* < 0.05; ** *p* < 0.01.

When dFMRP was absent in pupal brains, a significant decrease of the pre-mRNA abundance for both genes *dlg1* and *capt* was found. Surprisingly, the same was noticed for the control gene (Fig. 9B). In comparison to the wild type, the overexpression of dFMRP at this developmental stage was accompanied by a decrease of the pre-mRNA amount for the *dlg1* gene and by an increase of the premature mRNA for the *capt* gene (Fig. 9B).

Summarizing all our qRT-PCR results, we assume that dFMRP, most probably, does not affect the stability of *dlg1* and *capt*-mRNA-isoforms. In favor of this suggestion is the fact that we did not find an increase of the pre-mRNA abundance in *dfmr1*-null mutant larval brains, as was the case with the mature isoform 1 (retaining exons 10 and 13) (Fig. 7A). If dFMRP affected mRNA-stability, a change would be noticed – either an increase or a decrease of both – the premature and the mature forms of RNA.

As the expression level of *dlg1*-pre-mRNAs in *dfmr1*-null mutant larvae decreased (Fig. 9A), we speculate that dFMRP might be involved not only in splicing events (see previous section), but probably in its transcriptional regulation as well. In the light of the recent findings that most often *Drosophila* splicing occurs co-transcriptionally [30] its putative dual role could find a plausible explanation.

capt-pre-mRNAs and its three alternatively spliced isoforms decreased their abundance in pupal brains in the absence of dFMRP (*Fmr1^{Δ113M}/Fmr1^{Δ113M}*) (Fig. 9B, Fig. 6B). As all types of mRNAs studied showed the same type of change without dFMRP, we interpret these results, speculating that in the case of the gene *capt* dFMRP might regulate transcription only.

The significant increase of the *capt*-pre-mRNA-abundance in pupal brains, when dFMRP was overexpressed (Fig. 11B), might also favour this speculation.

As the initial goal of this study, was to estimate whether dFMRP has influence on alternative splicing of some brain neuronal mRNAs with G-quartet sequences as putative binding sites of this protein, our qRT-PCR – results gave a piece of evidence, that dFMRP influences alternative splicing of mRNAs, encoded by genetic interactors of *dfmr1*. This influence spreads to regions with alternative exon skipping, located close to an exonic G-quartet and an ESE-sequence, available within the 3'- or the 5'-ends of the exon.

Conclusions

To summarize, our results demonstrate a possible involvement of dFMRP in the regulation of alternative splicing and/or transcription of some neuronal mRNA-isoforms, encoded by genes, which interact with *dfmr1* – *dlg1* and *capt*. We did not find an effect of this protein on the mRNA stability of the *dlg1* and *capt*-isoforms analysed.

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