**Drosophila Muscleblind Is Involved in troponin T Alternative Splicing and Apoptosis**

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**Abstract**

**Background:** Muscleblind-like proteins (MBNL) have been involved in a developmental switch in the use of defined cassette exons. Such transition fails in the CTG repeat expansion disease myotonic dystrophy due, in part, to sequestration of MBNL proteins by CUG repeat RNA. Four protein isoforms (MblA-D) are coded by the unique *Drosophila muscleblind* gene.

**Methodology/Principal Findings:** We used evolutionary, genetic and cell culture approaches to study muscleblind (mbl) function in flies. The evolutionary study showed that the MblC protein isoform was readily conserved from nematods to Drosophila, which suggests that it performs the most ancestral muscleblind functions. Overexpression of MblC in the fly eye precursors led to an externally rough eye morphology. This phenotype was used in a genetic screen to identify five dominant suppressors and 13 dominant enhancers including *Drosophila* CUG-BP1 homolog aret, exon junction complex components tsunagi and Aly, and pro-apoptotic genes Trafl and reaper. We further investigated Muscleblind implication in apoptosis and splicing regulation. We found missplicing of troponin T in muscleblind mutant pupae and confirmed Muscleblind ability to regulate mouse fast skeletal muscle Troponin T (TnnT3) minigene splicing in human HEK cells. MblC overexpression in the wing imaginal disc activated apoptosis in a spatially restricted manner. Bioinformatics analysis identified a conserved FKRP motif, weakly resembling a sumoylation target site, in the MblC-specific sequence. Site-directed mutagenesis of the motif revealed no change in activity of mutant MblC on TnnT3 minigene splicing or aberrant binding to CUG repeat RNA, but altered the ability of the protein to form perinuclear aggregates and enhanced cell death-inducing activity of MblC overexpression.

**Conclusions/Significance:** Taken together our genetic approach identify cellular processes influenced by Muscleblind function, whereas in vivo and cell culture experiments define *Drosophila troponin T* as a new Muscleblind target, reveal a potential involvement of MblC in programmed cell death and recognize the FKRP motif as a putative regulator of MblC function and/or subcellular location in the cell.

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**Introduction**

Human Muscleblind-like 1, 2 and 3 (MBNL1-3) are RNA binding proteins that have been involved in numerous diseases. Expression levels of human MBNL2 are altered in a number of cancer types [1] and MBNL1 is upregulated in schizophrenia [2] and sporadic idiopathic pulmonary arterial hypertension [3]. MBNL1 function is also impaired in myotonic dystrophy (DM), a multisystemic disease characterized by myotonia, cataracts and muscle weakness (reviewed in [4,5]), and a fly model of spinocerebelar ataxia 8 genetically interacted with *Drosophila muscleblind* mutants [6]. In spite of their biomedical relevance, the processes in which Muscleblind proteins are required and the molecular mechanisms they use to carry out such functions are only beginning to be understood.

MBNL proteins redundantly regulate alternative splicing of cardiac Troponin T (*cTNT* or *TnnT2*) and Insulin Receptor (*IR*) minigenes in cell culture [7]. Only mouse Mbnl1, however, has been found to regulate a developmental switch from a foetal to adult-type alternative splicing pattern in a defined subset of pre-mRNAs [8]. Because Mbnl1 target RNAs are found missspliced in the human disease DM, this and additional evidences strongly support the view that reduction in MBNL1 function contributes to the DM phenotype [9–11]. Maintenance of a foetal splicing pattern in adult DM patients leads to misexpression of protein isoforms, or lack of them, ultimately causing the clinical symptoms [12,13]. The origin of DM is an expansion of the trinucleotide sequence CTG in the 3’-untranslated region (UTR) of the *DMPK* gene. Mutant *DMPK* transcripts accumulate in ribonuclear foci in skeletal and cardiac muscle, fibroblast, and neuron cells of DM.
patients [14–16]. Expansion bearing mRNAs alter expression of specific genes at the level of transcription and alternative splicing [11,17], and might also silence gene expression by RNA interference [19]. CUG repeat RNA becomes retained in nuclear foci, where it sequesters nuclear proteins including transcription factor Sp1 and alternative splicing regulators MBNL1-3 [17,19,20]. MBNL proteins have been shown to extensively co-localize with these ribonuclear foci in vitro and to bind CUG repeat-containing RNA in a repeat length dependent manner in vitro [14,15,21].

Among MBNL1 targets, regulated inclusion of exon 5 during cTNT pre-mRNA alternative splicing has been extensively studied in heart development. A current model proposes that a balance between the antagonistic activities of MBNL1 and polyypyrimidine tract binding protein (PTB), which promote exclusion of exon 5, and CUG binding protein 1 (CUG-BP1) and embryonic lethal abnormal vision type RNA binding protein 3 (ETR-3), which promote exon 5 inclusion, instructs the splicesome to retain or not exon 5 in mature transcripts [10]. A similar antagonism has been demonstrated in normal and DM1 myoblasts between the complex made of hnRNPH and CUG-BP1, and MBNL1, during exon 11 selection in IR splicing [22]. Despite their redundant activity on minigene splicing assays in vitro, MBNL proteins have been related with functions other than splice regulation. MBNL2 participates in the RNA-dependent localization of integrin 23 protein by binding to a specific ACACCC motif in the 3’UTR of the transcript [1]. Furthermore, MBNL3 inhibits muscle differentiation whereas Mbnl1 knockdown mice show muscle development impairment pointing to the implication of Mbnl1 in the myogenic process [11,23,24].

The Drosophila genome contains a single muscleblind gene (mbl) that gives rise to four mature transcripts (mblA-D) through alternative splicing. These encode relatively small proteins that share their amino terminal region (MblA, B, C share 179 amino acids; MblD only the first 63) and differ carboxy-terminally [25]. msculeblind mutants die as late embryos showing disruption of sarcomeric Z bands and lack of extracellular matrix at indirect muscle attachment sites [26]. Mosaic analysis also showed that muscleblind was required for the terminal photoreceptor differentiation. Photoreceptors in mutant retina tissue failed to properly arrange their light harvesting structures, the rhabdomeres [25]. Muscle defects are likely caused by alternative splicing alterations in muscle transcripts of which two have been recently described in muscleblind mutants [27,28]. The expression of human MBNL1 in Drosophila muscleblind embryos rescues embryonic lethality thus demonstrating the functional conservation between human and fly proteins [29]. Activity as alternative splicing factor in a α-actinin minigene splicing assay has also been shown for Drosophila Muscleblind, although protein isoforms were not functionally redundant and MblC showed the highest activity in this assay [27]. muscleblind transcript isoforms are expressed in a developmentally regulated fashion and show preferential subcellular localizations in cell culture being MblA preferentially cytoplasmatic [27].

Muscleblind proteins contain one (Drosophila) or two (vertebrate) pairs of CCCH zinc fingers [30]. Human MBNL zinc fingers have been shown to be necessary for the binding to both long CUG repeat expansions and physiological targets, whereas a C-terminal region in MBNL1 mediates homotypic interactions [31,32]. Although several targets of MBNL proteins have been recently described [8], very little is known about the cellular processes in which MBNL proteins are implicated, their regulation or the domains required for their functions. As for Drosophila Muscleblind, only α-actinin and CG30084 alternative splicing has been described altered in Drosophila muscleblind mutant embryos [27,28] thus remaining most of mbl targets, and the genetic pathways in which it is involved, largely undefined. A major advantage of Drosophila as a model system is the ability to conduct unbiased genetic screens for enhancers and suppressors of a given phenotype in vivo. Here we present a genetic approach to identify new genes related to muscleblind function. We also used in vitro and cell culture techniques to understand the role of Muscleblind proteins in splicing and programmed cell death, and we define a conserved motif in MblC that impacts on the subcellular distribution of the protein.

Results

MblC is the most ancient isoform

To assess whether Drosophila Muscleblind protein isoforms were evolutionary conserved or were species-specific, we searched the genomes of organisms from representative taxonomic groups for homologous sequences. We were able to detect MblC-like protein isoforms in all species analyzed (Table 1; sequence alignments shown in Figure S1). MblC specific sequences were highly conserved in the Arthropoda group showing percentages of amino acid identity around 70% or higher. The protein was still well conserved in the Hemiptera group (pea aphid) with pairwise alignments up to 60% identical whereas this percentage went down to 40% in the Nematoda group (Caenorhabditis and Ascidia). These observations suggest that an MblC-like protein appeared at least 530 million years ago when the Nematoda group and the Arthropoda lineage split.

In contrast to MblC other Drosophila Muscleblind protein isoforms were far less well conserved. D. simulans is the species most closely related to D. melanogaster and all four Muscleblind isoforms were conserved with an identity of over 90%. MblD, however, was not detected in another closely related species belonging to the melanogaster subgroup, D. yakuba. This suggests that MblD appeared very recently in evolutionary terms within the

Table 1. MblC-specific sequences are found in distinctly related invertebrates.

| Species                | MblA | MblB | MblC | MblD |
|------------------------|------|------|------|------|
| D. melanogaster        |      |      |      |      |
| D. simulans            | 91   | 97   | 100  | 100  |
| D. yakuba              | 68   | 99   | 100  |      |
| D. ananassae           | -    | 81   | 97   |      |
| D. pseudoobscura       | -    | -    | 94   |      |
| D. virilis             | -    | 70   | 97   |      |
| D. majavensis          | -    | 73   | 97   |      |
| Anopheles gambiae      | -    | -    | 84a |      |
| Anis mettillera        | -    | -    | 81   |      |
| Bombyx mori            | -    | -    | 68   |      |
| Acrhythosphan pisuma   | -    | 56b  |      |      |
| C. elegans             | -    | 40c  |      |      |
| Acris suum            | 49d  |      |      |      |

Numbers indicate percentage of identity (best local alignment) between dynamically translated genomic DNA and isoform-specific Muscleblind protein sequences, except for species marked with an asterisk (*) for which only Expressed Sequence Tag (EST) sequences were available in databases. Accession numbers of ESTs containing isoform-specific sequences are: *BM605179, *BM619051.1, *CV835929.1, *yk381a7.3, *BQ094923.1. Non-significant similarity is indicated by a minus sign (-) and data not available, at the time of the analysis, by an empty cell. Species are listed by phylogenetic distance to Drosophila melanogaster [65]. Multiple alignments are shown in Figure S1.

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The isoform-specific sequences of MblA and Drosophila genus. The isoform-specific sequences of MblA and MblB were detected in the D. yakuba genome with identity percentages of 68 and 99% respectively, although MblB-specific sequence in D. yakuba was notably shorter (30 amino acid long) compared to D melanogaster (93 amino acid). D. ananassae, viridis, and mojavensis also showed an MblB-like isoform, but the conservation was almost restricted to a run of 16 consecutive alanine residues (Figure S1). These observations suggest that MblC is under strong evolutionary pressure since it has not changed significantly over at least 13 million years (almost 100% protein identity in melanogaster group species) and can be found in Nematoda. Contrarily, MblA, B and D are Muscleblind protein isoforms restricted to different melanogaster and viridis group species, probably carrying out specialized functions within these species. In summary, our data indicate that MblC isoform is the most ancient in evolutionary terms.

MblC generates the strongest developmental defects when overexpressed

To test the functional inferences we made from the evolutionary results, we targeted Muscleblind protein isoform expression to imaginal disc tissue, muscle, photoreceptor precursors and posterior compartment within segments using the Gal4/UAS system [33] (Table S1). The strongest developmental defects, including lethality, were observed by overexpressing mblC. Overexpression of mblA and mblB gave rise to far more limited defects including vein patterning defects (T80-Gal4>UAS-mblA) and abnormal wing position (T80-Gal4>UAS-mblB), thus suggesting a more restricted developmental role. mblD overexpression gave no phenotype in all tissue types tested. As there was no antibody available to detect Muscleblind, we confirmed expression from the UAS transgenes by in situ hybridization. All transgenes were expressed to comparable levels including UAS-mblD [27] and data not shown). However, neither small differences in transcript transcription (because of the qualitative nature of the experimental approach) nor differences in translation efficiency or protein turnover can be excluded. Therefore, these data suggest that MblC isoform performs important roles during Drosophila development, which is consistent with the broad developmental expression pattern and the high ability to rescue lethality of muscleblind mutant embryos that we previously described for mblC [27].

A genetic screen for modifiers of a mblC overexpression phenotype

The mechanism by which Muscleblind proteins regulate alternative splicing is largely unknown both in Drosophila and vertebrates. Because mblC overexpression generated phenotypes amenable to genetic analysis, in particular eye morphology defects [34], and the evolutionary analysis suggested that MblC carried out critical muscleblind functions, we decided to perform a genetic screen for dominant modifiers of such phenotype. These modifiers provide an unbiased approach to genes potentially involved in muscleblind function. sevenless (sev) Gal4 UAS-mblC recombinant chromosomes showed a moderately rough eye phenotype (Figure 1A,B), which facilitated identification of both enhancers and suppressors. In a deficiency screen for dominant modifiers 13 out of 109 assayed deficiencies (covering around a 55% of the genome), visibly modified the phenotype being most of them dominant suppressors (Table S2). Deficiencies Df(2L)sc19-8 and Df(2L)TW137 strongly suppressed external eye morphology and were selected for further analyses. Both of them cover a wide chromosomal region including dozens of genes. By using overlapping deletions we could delineate a candidate interacting region to 24E2; 25A3 for Df(2L)sc19-8 and to 36F1; 37B9-10 for Df(2L)TW137. Described genetic entities within these regions were individually tested for their ability to modify the sev-Gal4>UAS-mblC eye phenotype. In addition, several genes mapping within other interacting deletions, or genes known or putatively involved in RNA or DNA binding, in the DM pathogenesis or apoptosis [35], or potentially related with muscleblind function [6], were also tested (Table 2 and Table S3).

Four out of the 14 P-element insertions mapping to 2HD5; 25A2 and four of the 16 insertions mapping to 36F10; 37B-C showed interaction (Table 2). Because most P insertions tested were predicted to generate overexpression phenotypes (GS and NP lines), loss-of-function alleles of candidate genes were also checked for interaction whenever possible. All five alleles tested confirmed the interactions previously detected. Loss of function alleles of CG15435 and CG15433 interacted in the same direction than their corresponding NP and GS lines. CG17323 and turtle loss-of-function interaction opposite to the strains initially tested. Finally, both Traf11502178 and Traf11502174 suppressed the mblC overexpression phenotype.

The candidate approach identified five suppressors and 13 enhancers of the mblC overexpression eye phenotype (Table 2;
Table 2. Suppressors and enhancers of a mblC overexpression phenotype.

| Line       | M Gene     | Description                                      |
|------------|------------|--------------------------------------------------|
| His70Ab    | His70Ab    | chaperone                                        |
| jumua      | jumua      | jumua chromatin remodelling and t.f.            |
| Dp[5970ES]| Dp[5970ES] | apoptosis/cell cycle regulator                   |
| amos[am1];UAS-amos | amos[am1];UAS-amos | amos transcript factor                           |
| A[22875]  | A[22875]   | A splicing factor                                |
| tsu[2007]  | tsu[2007]  | tsu exon junction complex                        |
| are[aret]  | are[aret]  | are splicing factor                              |
| non[aret]  | non[aret]  | non splicing factor                              |
| rpr[P{GS}]| rpr[P{GS}] | rpr pro-apoptotic                               |
| tin[aret]  | tin[aret]  | tin adhesion and motor control                   |
| CG15432    | CG15432    | CG15432 C2H2 zinc finger protein                 |
| CG17424    | CG17424    | CG17424 stomatin-like protein (cytoskeleton)    |
| CG15432    | CG15432    | CG15432 acetyltransferase activity              |
| CG5790     | CG5790     | CG5790 Ser/Thr kinase                            |
| CG17322    | CG17322    | CG17322 UDP-glucosyltransferase                  |
| CG15625    | CG15625    | CG15625 methyltransferase activity               |

Modification (M) of phenotype is denoted as E (enhancer), S (suppressor) or L (lethal). Iof designates other loss-of-function alleles of the modifier gene.

Muscleblind Activate Apoptosis

Figure 1A-H, whereas 33 mutations did not interact including bruno-2, bruno-3, and Diap2 alleles (Table S3). CG5790NP12535 was synthetic lethal with the sv-Gal4 UAS-mblC chromosome, but this could be due to the additional overexpression of mblC driven by the NP line. For 13 of the interacting genes we tested additional alleles, which in all cases confirmed the genetic interaction. Overexpression of Heat shock protein 70Ab (His70Ab), a gene encoding a protein with chaperone activity enhanced the rough eye phenotype. Four genes related to RNA metabolism, Aly and nonA (both components of the exon junction complex), CUG-BP1 Drosophila homolog aret (splicing and translation regulation factor) and aret (splicing factor) modified mblC overexpression phenotype thus reinforcing previous data that implicate MblC in pre-mRNA metabolism [27]. Interestingly, several genes involved in the apoptotic process such as Diap1, Tsg1, Dp and reaper strongly interacted. Reduction in the dose of proapoptotic genes hid, spr and grim (Df[3L]4W) dominantly suppressed a mblC overexpression phenotype. spr[P{GS}] allele, which overexpresses sprer from a glass mutliemer reporter (GMR) fusion construct [36], strongly enhanced the phenotype (not shown). Crosses had actually to be made at a lower temperature (19°C) because simultaneous overexpression of reaper and mblC was larval lethal, possibly due to leaky expression in tissues other than eye imaginal discs. Consistently, halving the dose of antiapoptotic gene thread, the Drosophila inhibitor of apoptosis 1 (Diap1) ortholog, enhanced the rough eye phenotype further suggesting that MB1C overexpression sensitized cells to apoptosis. Specificity of the interaction of mblC overexpression with antiapoptotic genes is supported by the inability of Diap2 dose reduction to modify the rough eye phenotype. Also amos and jumua, transcription and chromatin remodelling factors respectively [37-40], interacted with mblC overexpression in the eye. jumua overexpression was further confirmed in wing imaginal disc tissue. Targeted mblC expression to posterior wing compartment by engrailed-Gal4 originated vein fusions and lack of intervein material, affecting specially L4/L5 veins (Figure 11-I). Simultaneous reduction of jumua dose reduced L4/L5 fusion events from 91.6 to 70.6% and also ameliorated several other aspects of the phenotype such as loss of laminar wing tissue or anterior intervein.

Muscleblind proteins regulate troponinT alternative splicing

Four different loss-of-function alleles of aret (a Bruno protein) dominantly enhanced the mblC overexpression phenotype in the Drosophila eye. Human MBNL1 and Bruno homologs CUGBP1 and ETR3 act antagonistically on human cTNT transcripts to regulate inclusion of foetal exon 5 [10]. This prompted us to hypothesize that the underlying origin of the genetic interaction observed between aret and muscleblind might be conservation of their antagonism over the selection of alternative exons. Mbnl1 knockout mice show splicing defects in both cardiac Troponin T (Tnn2) and fast skeletal muscle Troponin T (TnnT3) [11]. The Drosophila genome presents a unique troponin T (tntT) gene, which was found to undergo tissue-specific alternative splicing during development [41]. Four splicing variants differ in the inclusion or exclusion of exons E3, E4 and E5. We performed RT-PCR amplifying exons E2 to E6 of tntT mRNA to detect all described splicing isoforms in muscleblind mutant embryo, pupae and adults. Embryo and adult RNA extractions showed no detectable differences. Early mutant pupae, however, showed an increment in the tntT isoform specific of tergal depressor of...
trochanter (TDT) and indirect flight muscles (IFM; Figure 2A) when compared to controls. The alteration was also clearly detected in muscleblind null heterozygotes (mbt<sup>277</sup>/CyO, ubi-GFP), but not in hypomorphic heterozygotes (mbt<sup>103</sup>/CyO, ubi-GFP), thus suggesting a muscleblind dose effect.

Regulation of in1T alternative splicing by Drosophila Muscleblind isoforms was confirmed using an already available mouse TnnT3 minigene whose splicing has been previously described to depend on Mbnl1 [9]. Because they were technically more amenable than Drosophila S2 cells, human HEK293T cells were transiently co-transfected with the TnnT3 minigene and plasmids expressing all four Muscleblind protein isoforms fused to GFP. We also tested the possibility that Drosophila Bruno proteins influenced splicing of TnnT3 minigene transcripts by co-transfecting Bruno proteins similarly fused to GFP. The analysis showed that MblA, MblB and MblC shifted TnnT3 splicing pattern from preferential inclusion of a foetal exon to its exclusion. Bruno proteins, however, did not significantly modify foetal exon usage (Figure 2B,C). Western blotting of HEK293T protein extracts with an anti-GFP antibody consistently failed due to protein degradation (not shown) thus precluding drawing any conclusions as for differences in splicing activity between Muscleblind protein isoforms. Titration of transfected DNA, however, showed a clear response to concentration in splicing activity, which further supports activity of Muscleblind protein isoforms in this assay (not shown).

Taken together these results demonstrate that muscleblind function is required for alternative splicing control of Drosophila tropion T mRNA and that Muscleblind protein isoforms promote the exclusion of murine TnnT3 foetal exon from mature mRNAs in human HEK cell cultures.

MblC overexpression activates apoptosis in vivo

Genetic interactions with key regulators of apoptosis prompted the possibility that muscleblind could directly or indirectly participate in the apoptotic process. In order to confirm this possibility in vivo, we first analyzed the phenotype brought about by overexpression of mblC in the posterior compartment of the wing imaginal disc (en-Gal4>UAS-mblC). Lack of laminar tissue could originate from reduced proliferation of disc cells or from an excess of cell death (Figure 1J). Immunostaining with anti mammalian Caspase-3 antibody showed a robust activation of caspase-3 in cells overexpressing MblC under the control of the en-Gal4 driver (Figure 3A–C), decapentaplegic-Gal4 or patched-Gal4 (not shown). However, not all cells overexpressing MblC showed the same susceptibility to caspase cleavage. Within the fate map of the wing imaginal disc, whereas posterior notum or ventral pleura cells did not significantly promote caspase-3 activation, posterior wing margin and pouch cells strongly activated caspase 3. In order to confirm that caspase activation was due to the activation of the apoptotic pathway, and not to other functions described for caspases (see [42] for examples), we used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) to detect DNA fragmentation that results from apoptotic signaling cascades. Using this assay we detected several apoptotic cells in the posterior compartment of the wing disc when en-Gal4 drove MblC overexpression. The assay also confirmed the spatially restricted susceptibility to enter apoptosis, in particular lack of apoptosis in posterior notum and pleura (Figure 3D,E).

Transfection of pro-apoptotic genes hid, reaper and grim into Drosophila S2 cells induce cell death [43]. Individual Muscleblind isoforms were similarly tested for their ability to induce apoptosis in cell culture. We first characterized endogenous expression of each muscleblind transcript isoform in Drosophila S2 cells by semiquantitative RT-PCR using isoform specific primer pairs (Figure S2). We detected predominant expression of mRNA mblC, similar levels of mblA and mblB, and a barely visible band for mblD. Next we over-expressed myc-tagged MblA-D isoforms in S2 cells and monitored cell viability by counting LacZ-expressing cells 48 h after transfection (Figure 3F,G). In these experiments we detected a reduction in the average of viable cells overexpressing MblA, MblB and MblC, whereas MblD slightly increased cell viability when compared to vector alone controls. Differences, however, were not statistically significant.

Altogether our results demonstrate that an increase of cells entering apoptosis contributes to lack of tissue in the adult wing blade in MblC-overexpressing flies and that either expression levels, or factors other than MblC, are critical to activate apoptosis in vivo under our experimental conditions.

A conserved FKRP motif influences protein distribution and cell death-inducing activity of MblC

Drosophila Muscleblind protein isoforms sharing both their zinc fingers (MblA-C) showed different behaviour in various functional
weights are: MblA, 22.65 kDa; MblB, 34.46 kDa; MblC 26.91 kDa.

could not be detected by western blotting. Predicted molecular weight bands in lanes MblA and MblB are degradation products. MblD are standard deviations. (G) Western blot of protein extracts from S2 cells transfected with myc-tagged Muscleblind proteins, however, did not reveal bands of higher than predicted molecular weight (Figure 3G). Therefore, if sumoylation is actually taking place, must affect a very small proportion of the MblC protein isoform.

As an alternative approach, and in order to test the relevance of the FKRP site in MblC function, we mutated lysine 201 into isoleucine by site-directed mutagenesis (Figure 4A) and tested the mutant protein (MblC<sup>K201I</sup>) in the functional assays we performed before in vertebrate and <i>Drosophila</i> cells with wild type MblC.

Transfection of COSM6 cells with GFP-tagged MblC<sup>K201I</sup> protein showed a higher frequency of perinuclear protein foci and reduction in nuclear signal when compared to transfection of wild type MblC (Figure 4B). To check whether MblC aggregation and subcellular localization depended on the amount of transfected plasmid or were cell-type specific, we transfected HEK293T cells with a third of the constructs used initially (Figure 4B). No prominent perinuclear foci were observed when transfecting wild type MblC-GFP and the signal concentrated in the nucleus, but MblC<sup>K201I</sup>-GFP continued forming perinuclear aggregates and was also detected in the cytoplasm. We previously showed that wild type MblC co-localises in vivo with CUG repeat-containing ribonuclear foci [27]. Mutant MblC was similarly tested in COSM6 cells for its ability to co-localize with expanded CUG ribonuclear foci. We detected several examples of co-localization of CUG repeat RNA and MblC<sup>K201I</sup>-GFP in the cell nucleus and number and morphology of foci did not qualitatively differ from aggregates formed in the presence of wild type MblC (Figure 4C). Alternative splicing activity of mutant MblC was assessed using the mouse <i>Tun</i>T3 minigene in HEK293T cells. In this assay MblC<sup>K201I</sup> promoted foetal exon exclusion from mature transcripts to the same extent than wild type MblC (Figure 4D). Finally, we tested the ability of mutant MblC to induce cell death in <i>Drosophila</i> S2 cells. Whereas MblC only marginally reduced the average number of viable cells upon transfection, reduction of cell viability 48 h after MblC<sup>K201I</sup> overexpression was to approximately 52 % of control and statistically significant (Figure 4E).

Taken together these results identify the FKRP motif in MblC as a putative site for post-translational modification. They also show that the FKRP sequence influenced subcellular localization of the protein and that mutation of the motif enhances the cell death inducing activity of MblC overexpression in cell culture.

**Discussion**

Using <i>Drosophila</i> as a model organism, here we report the first screen specifically addressed to identify gene functions related to the biomedically important protein Muscleblind. In support of the relevance of our results, we show the strong functional conservation between fly and vertebrate Muscleblind proteins. Furthermore, we generated data supporting that Muscleblind can induce apoptosis in vivo in imaginal disc tissue and identified a conserved motif in the MblC protein isoform that conferred pro-apoptotic activity in <i>Drosophila</i> cell culture when mutated. Noteworthy, this is the first conserved motif (besides CCCH zinc fingers) that is associated with a particular function in Muscleblind proteins.

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**Figure 3.** <i>mbLC</i> overexpression activates apoptosis in vivo, but not significantly in cell culture. Confocal micrographs of third instar wing imaginal discs from <i>en-Gal4 mbLC/+</i> (A,C,E) and <i>en-Gal4+</i> controls (B,D) stained with an anti-Mbl (A), anti mammalian Caspase-3 antibody (B,C), or TUNEL assay (D,E). Wing imaginal discs of <i>en-Gal4 UAS-mblC</i> (C) flies show activation of executioner caspase-3 in cells over-expressing MblC (A) in the posterior compartment where the <i>en-Gal4</i> driver is active. Despite the fact that MblC overexpression levels are similar in posterior pouch (A, bent arrow) and notum cells (A, arrowhead), caspase-3 is not detected activated in prospective notum cells (C). A TUNEL assay to detect DNA fragmentation that results from apoptosis signalling cascades reproduced the same pattern of apoptotic cells (D,E) detected by caspase-3 activation. (F) Bar graph representing the average number (from quadruplicates) of live cells 48 h after transfection of plasmids expressing the indicated Muscleblind protein isoforms. Overexpression of Muscleblind isoforms did not significantly reduce <i>Drosophila</i> S2 cell viability in cell culture conditions. Error bars are standard deviations. (G) Western blot of protein extracts from S2 cells transfected as in (F) with the indicated Muscleblind proteins and detected with an anti-Muscleblind antibody [47]. Lower molecular weight bands in lanes MblA and MblB are degradation products. MblD could not be detected by western blotting. Predicted molecular weights are: MblA, 22.65 kDa; MblB, 34.46 kDa; MblC 26.91 kDa.
MblC-specific sequences, but not other isoforms, are detected in several protostomes and human MBNL1

Whereas most vertebrates include three muscleblind paralogues in their genomes, a single muscleblind gene carries out all muscleblind-related functions in Drosophila. These functions are probably accomplished through alternative splicing, which generates four Muscleblind protein isoforms with different carboxy-terminal regions. We performed an evolutionary analysis with isoform-specific protein sequences in order to assess conservation of alternative splicing within protostomes. We detected MblC-like isoforms even in the nematodes C. elegans and Ascaris suum but not MblA, B, or D, that were only consistently found within Drosophilidae. Interestingly, also vertebrate Mbl1 genes included MblC-like sequences (Figure 4A).

This finding, together with previous studies where we reported that mblC was the isoform with the strongest activity in a muscleblind mutant rescue experiment and α-actinin minigene splicing assay [27] point to mblC as the isoform performing most of muscleblind functions in the fly. Despite this Muscleblind isoforms are partially redundant. Both mblD and B partially rescued the embryonic lethality of muscleblind mutant embryos [27] and were able to similarly promote foetal exon exclusion in murine Tnnt3 minigene splicing assays. MblD showed no activity in splicing assays or in vivo overexpression experiments. However, we show a marginal increase in cell viability in cell death assays. Using isoform-specific RNAs we construct we plan to re-evaluate the function of Muscleblind isoforms both in vivo and in cell culture.

A genetic screen involves Muscleblind in defined cellular processes

Although the regulation of alternative splicing by Muscleblind proteins is an established fact, the cellular processes in which the protein participates are largely unknown. Genetic screens provide a way to approach those processes as they interrogate a biological system as a whole. Overexpression of MblC in the Drosophila eye originated an externally rough eye phenotype that was temperature sensitive, thus indicating that was sensitized to the muscleblind dose. We performed a deficiency screen and tested several candidate mutations for dominant modification of the phenotype. We identified 19 genes of which more that half can be broadly classified as involved in apoptosis regulation (syr, th and Traf1), RNA metabolism (Aly, tsu, aret and nonA) or transcription regulation (jumu, amos, Dp, CG15435 and CG15433), whereas the rest do not easily fall into defined classes. muscleblind has been shown to regulate α-actinin and troponinT alternative splicing both in vivo and in cell culture ([27,28]; this work). The genetic interaction with the Drosophila homolog of human splicing factor CUG-BP1 (aret) and nonA supports a functional relationship in flies. The antagonism between MBNL1 and CUG-BP1 has actually been shown in humans [10], whereas RNA-binding protein NonA might be relevant to Muscleblind sequestration by CUG repeat RNA in flies [47].

Reduction of dose of exon junction complex (EJC) components tsu and Aly also modified MblC overexpression phenotype. EJC provides a binding platform for factors involved in mRNA splicing, export and non-sense mediated decay (NMD). This suggests a previously unforeseen relationship between Muscleblind and EJC, perhaps helping to couple splicing to mRNA export. Consistently, Aly mutations enhanced a CUG repeat RNA phenotype in the Drosophila eye [33]. A similar coupling between transcription and splicing might explain the identification of a number of transcription factors in our screen. Of these, we studied in some detail the effect of jumu alleles in the eye and wing MblC overexpression phenotypes. Loss of function jumu mutations suppressed both wing defects and rough eye, whereas had no effect on unrelated overexpression phenotypes (not shown) thus suggesting that the interaction was specific.
Mutations in the *Drosophila* homolog of vertebrate *Inhibitor of Apoptosis* (*Diap1* or *thread*) dominantly enhanced the rough eye phenotype. Consistently with the specificity of the interaction, a second *Drosophila* paralog, *Diap2*, did not interact. Also, a deficiency that removes the *Drosophila* proapoptotic genes *hid*, *reaper* and *grim* (which inhibit thread) was a dominant suppressor while *tiger* overexpression in eye disc enhanced the phenotype. Interestingly the human homolog of *Drosophila* Hsp70Ah, Hsp70, has been related to apoptosis as it directly interacts with Apaf-1 and Apoptosis Inducing Factor (AIF) resulting in the inhibition of caspase-dependent and caspase-independent apoptosis [48]. All these genetic data are consistent with MblC overexpressing eye discs being sensitized to enter apoptosis, although we did not detect increase in caspase-3 activation in third instar eye imaginal disc overexpressing MblC (not shown).

**Do Bruno proteins antagonize Muscleblind activity in flies?**

Human MBNL1 and CUB-BP1 cooperate to regulate the splicing of cardiac *TroponinT* (*cTNT*, [7]). We detected splicing defects in *Drosophila troponin T* mRNA in muscleblind mutant pupae. Interestingly, we detected an abnormal exclusion of exon 3 in muscleblind mutant pupae, encoding a glutamic acid-rich domain homologous to the foetal exon of *cTNT* regulated by human MBNL1 [41]. *Drosophila* exon 3 is only absent in the *tropomin T* isoform expressed in TDT and IFM muscles and probably confers specific functional properties much like the foetal exon does in humans [49]. This identifies *tropomin T* as a new target of Muscleblind activity in flies.

CUG-BP1 protein has been described to antagonize MBNL1 exon choice activity in IR and *cTNT* pre-mRNAs. Moreover, we detected a genetic interaction between MblC overexpression and arte loss of function mutations. In order to further characterize the functional interaction between Muscleblind and Bruno proteins we checked their ability to regulate murine *TnnT3* in human cell culture. MblA, B and C showed strong activity on *TnnT3* mRNA but no significant activity was detected for any Bruno protein. This shows a strong functional conservation between fly and vertebrate Muscleblind proteins as *Drosophila* isoforms can act over a murine target in a human environment. In contrast, Bruno proteins might not conserve the regulatory activity over *tropomin T* mRNA described for their vertebrate homologues or at least they were not functional in the cellular environment used in this assay. Because GFP-tagged Bruno proteins were only weakly expressed in HEK cells under our experimental conditions, the level of expression might be insufficient to overcome endogenous Muscleblind activity in cell culture. Furthermore, Bruno proteins might antagonize Muscleblind on a different subset of RNA targets. Although Bruno1 has been shown to regulate splicing of some transcripts in S2 cell culture [50] and Bruno3 binds the same EDEN sequence than human CUG-BP [51], no in vivo experiments have addressed the functional conservation between fly and vertebrate Brunos. Bruno1 is expressed in the germ line [52] where it acts as translational repressor of *oskar* and *gurken* mRNAs [53,54].

**Muscleblind overexpression activates apoptosis**

Wing imaginal discs stained with anti-caspase-3 and with TUNEL showed that activation of apoptosis was not general in cells expressing MblC but restricted to defined regions within the disc, in particular the wing blade. The spatial constraints that we observed within the imaginal disc might explain the small effect detected when expressing Muscleblind proteins in S2 cells. MblC might require the presence of other factors to be able to unleash programmed cell death. Alternatively, the level of overexpression may be critical and transfected Muscleblind proteins may not reach a critical threshold in *Drosophila* S2 cells. MblC activation of apoptosis could reveal a direct regulation of apoptotic genes at RNA level or be an indirect effect. Several apoptotic genes produce pro-apoptotic or anti-apoptotic isofoms depending on the regulation of their alternative splicing [55]. MblC could be similarly regulating protein isoforms originating from one or a number of key apoptotic genes at the level of pre-mRNA splicing. Alternatively, MblC could be regulating isoform ratio of a molecule indirectly related to programmed cell death, for example a cell adhesion molecule causing apoptosis by inefficient cell attachment to the substrate. Furthermore, human MBNL1 proteins are implicated not only in splicing but also in RNA localization [1,7], a process that if conserved in flies can potentially impinge in apoptosis regulation.

The analysis of MblC-specific sequence revealed a region conserved in Muscleblind proteins from nematodes to humans. Post-translational prediction programs found a motif (FKRP) weakly resembling a sumoylation target site. However, our results in S2 cells suggest that sumoylation, if actually taking place, modifies only a small fraction of MblC proteins. FKRP may alternatively participate in an interaction with a Muscleblind partner potentially regulating activity or location in cell compartments, assist in protein dimerization [32], or others functions. We mutated the FKRP site and performed a number of functional assays using the mutant MblC. Whereas MblC<sup>5069T</sup> excluded foetal exon in *TnnT3* minigene splicing assays and bound CUG repeat RNA like its wild type counterpart, the mutant protein showed a different preferential distribution in human cells and significantly increased cell death activation upon overexpression. The mechanism by which the FKRP site influences subcellular distribution and cell death-inducing activities is currently unknown, but nevertheless constitutes the first motif, other than zinc fingers, that is associated with a function within Muscleblind proteins.

**Materials and Methods**

**Evolutionary study of Muscleblind isoforms**

The tBLASTn algorithm [56] was used to search isoform-specific Muscleblind protein sequences in insect and *C. elegans* genome assembly DNA and Genbank EST databases using the BLAST tools posted in Flybase [57] and Wormbase [58], respectively. *Acrithosiphon pisum* ESTs searches (tBLASTn) were performed using the aphidbase (www.aphidbase.org). *Ascaris suum* ESTs searches (tBLASTn) were performed using the corresponding Wellcome Trust Sanger Institute project (www.sanger.ac.uk/) and the University of Edinburgh nematode database (www.nematodes.org). Searches were performed without filtering for low complexity sequences.

**Drosophila genetics and phenotypic analysis**

The strong hypomorph, possibly null, allele *mbf<sup>E27</sup>* was reported in [25], whereas the weak allele *mbf<sup>E701</sup>* was in [59]. *UAS-mblD* and *UAS-mblC* flies are described in [34], and *UAS-mblB* in [27]. To generate *UAS-mblD* transgenic flies, a 2 kb EcoRI fragment from *mblD* cDNA [25], including the putative open reading frame, was released from pBluescript by *Nol*I/EcoRI digestion and subcloned into pUAST opened up with *Nol*I and *EcoRI*. The transgene was microinjected into *yw* flies following standard methods for *Drosophila* germline transformation.

Expression under the control of the *se* enhancer in *R3/R4*, *R1/R6* and ectopically in *R7*, the mystery and cone cells was achieved with the *se-Gal4* driver (gift from M. Mlodzik; Mount Sinai School of Medicine, New York), and in the posterior compartment of every segment with the *en-Gal4*. Transgenic lines A1 and E1 of *UAS-mblC*, and Gal4 drivers *se-Gal4* and *en-Gal4*,
were respectively combined by meiotic recombination using standard procedures. Recombinant chromosomes se-Gal4 UAS-mblC(A1), and en-Gal4 UAS-mblC(E1) were subsequently used in this work. Repositories Drosophila Genomics Resource Center (DGRC), Bloomington Drosophila Stock Center (BDSC), and Kyoto fly were used to obtain the deficiency kit (BDSC), mutant stocks and en-Gal4 driver. All P-element insertion lines screened were GS (two UAS sequences in opposite directions [60]) or NP (Gal4 enhancer trap [61]). See-Gal4 UAS-mblC(A1), Tm3 females were crossed to deficiency or P-element carrying males. All crosses were maintained at 25°C except for the interaction with Gal4-tpr that was at 19°C. Oregon-R (Or-R) was used as reference strain.

For phenotypic analysis flies were incubated in SH buffer (25% glycerol, 75% ethanol) overnight. After a rinse with water, wings were dissected out in water and mounted in Faure’s mounting media [62]. For quantification, wing phenotypes of F1 flies with the genotypes en-Gal4 UAS-mblC(E1).1/+ (controls; n = 12) and en-Gal4 UAS-mblC(E1).1/+; jamu270/+ (n = 17) were scored according to the type and degree of alteration observed in the posterior wing margin (lack of tissue and ectopic vein material). For scanning electron microscopy adult flies were treated as described in [63].

Immunohistochemistry and Terminal transferase dUTP Nich End Labeling (TUNEL) assay

For immunodetection of executioner caspases in Drosophila we used an anti mammalian caspase-3 active polyclonal antibody from R&D systems (AF835). Third instar wing discs were dissected out in cold PBS, fixed for 30 min at room temperature in a 1:1 mixture of 8% formaldehyde: PEM 2x (0.2 M PIPES, 2 mM MgCl2, 2 mM EGTA) and permeabilized in PBS + Triton 0.3% (PBT) at room temperature (3 washes, 20 min each). Following blocking in PBT + 10% goat serum, discs were incubated with a 1:100 dilution of primary antibody for 2 h at room temperature or overnight at 4°C. After 8 washes with PBT (15 min each), discs were incubated for 1–2 h with anti-rabbit secondary antibody conjugated to biotin at a 1:200 dilution. Secondary was washed 8 times with PBT (15 min each) and incubated with streptavidin-FITC (1:200) for 30 min. After three washes (20 min each), discs were postfixed for 30–60 min in fixation mixture as above. Fixative was washed 3 times with PBT and discs were mounted in mounting media (Dako Cytomation). All steps were with gentle agitation (mutarot mixer).

TUNEL labelling was performed using an in situ cell death detection kit (Roche) adapted to Drosophila imaginal disc tissue according to [64]. Briefly, third instar larvae were dissected in PBS and fixed in 0.1 M PIPES, pH6.9, 1 mM EDTA, 1% Triton X-100, 2 mM MgSO4, 1% formaldehyde for 30 min. Samples were then washed three times in PBS+0.1% Triton X-100, twice in PBS+0.5% Triton (10 min each) and transferred to permeabilization solution (sodium citrate 0.1 M in PBT) for 30 min at 65°C. Discs were washed twice in 5X PBT (10 min each), rinsed three times in 1X PBT, and incubated in 50, 50 and 45 μl of labelling solution for 10 and 20 min (at room temperature), and 10 min (at 37°C), respectively. Terminal transferase (enzyme solution) was added to a concentration of 1X and incubated at 37°C for 3 h. Reaction was stopped by transferring discs to 5X PBT and three rinses in 1X PBT. Images were taken on a Leica TCS SP confocal microscope.

Constructs

bruno open reading frames were amplified by high fidelity PCR (TripleMaster PCR system, Eppendorf) from cDNAs LD29068 (aret/bruno1), LD19052 (bruno2) and LD31834 (bruno3) using primers carrying adaptors for enzymatic restriction (Table S4) and cloned into pGFP-N3 linearized with the same enzymes. Myc-specific FKRIP site was mutated by oligonucleotide-directed mutagenesis. MblCK202I and MblC-PstI primers were used to amplify a fragment of MblC, which substituted A by a T that changed Lys202 into Ile202. For convenience, the MblCK202I primer also introduced a silent PstI restriction site that facilitated recognition of mutated construct. PCR product and vector pEGFP-N3 were digested with BglII and NotI and ligated in a standard reaction. For cell culture experiments, myc-tagged muscleblind constructs [27] were BglII and NotI digested from pMV vector and cloned into the Drosophila expression vector pIE14 opened up by BamHI and NotI digestion. All constructs were confirmed by sequencing.

Cell culture assays and western blotting

Minigene splicing assays were carried out in human HEK293T cells as previously described [27] using 0.5 μg of a mouse TnuTi minigene [9] and 0.25 μg and 0.5 μg of pEGFP-N3 (Clontech) plasmids expressing Muscleblind or Bruno proteins fused to GFP (see [27] and above for a description of these constructs) using 1 ml of Lipofectamine in presence of Optimem Media. COSM6 cells were transfected with 1 μg of plasmids expressing GFP-tagged Muscleblind proteins and 1 μg of candidate DNA (pSP72) for subcellular localization studies, or 1 μg of plasmid expressing CUG197 plus 1 μg of plasmid expressing GFP-tagged Muscleblind proteins for colocalization assays. HEK293T cells were transfected with 300 ng of plasmids carrying the Muscleblind:GFP fusion. Detection was carried out as described previously [27]. For cell death assay S2 cells were transfected and treated as described [43] using 0.9 μg of plasmids expressing Myc-tagged Muscleblind isoforms [27].

Total protein extracts from S2 Drosophila cells were performed 24 h after transfection by resuspending cells in loading buffer. Samples were denatured for 5 min at 100°C, electrophoresed on 12% PAGE-SDS gels, transferred to PVDF membranes and immunodetected following standard procedures. Primary anti-Muscleblind antibody, with the capacity to recognize all four protein isoforms [47], was used at 1:5000 in blocking solution (3% BSA). Chemiluminescent detection was with the ECL substrate (Pierce) following recommendations by the provider.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of alternative splicing

Individuals with the mblR27/mblR7105 genotype were obtained by crossing mblR27/GyO; ubiquitous-GFP and mblR7105/GyO; ubiquitous-GFP flies and identified by the lack of fluorescence using a GFP fluorescence module mounted on a Leica MZ APO stereo microscope. 12 to 24 h after egg laying embryos, early pupae (1–2 days after pupation) and adults (6 h after emergence from puparium or older) were collected to analyze in situ splicing pattern.

Total RNA from flies or minigene splicing assays in cell culture was extracted using Tri-reagent (Sigma) and treated with DNase I or 0.25 μg of carrier DNA (pSP72) for subcellular localization studies, or 1 μg of plasmid expressing CUG197 plus 1 μg of plasmid expressing GFP-tagged Muscleblind proteins for colocalization assays. HEK293T cells were transfected with 300 ng of plasmids carrying the Muscleblind:GFP fusion. Detection was carried out as described previously [27]. For cell death assay S2 cells were transfected and treated as described [43] using 0.9 μg of plasmids expressing Myc-tagged Muscleblind isoforms [27].

Total RNA from flies or minigene splicing assays in cell culture was extracted using TRI-reagent (Sigma) and treated with DNase I (Invitrogen or Roche). Reverse transcription (RT) was performed using 1 μg (flies) or 5 μg (cell cultures) of total RNA, Superscript II RNase H- and random hexamers following instructions from the provider (Invitrogen). 1 μl of cDNA was used in a standard 20 μl PCR reaction with Thermus thermophilus DNA polymerase (Netzyme, NEED) and primers TNTE2 and TNTE6 for fly samples, 1938/1956 for minigene assays, or isoform-specific primer pairs to amplify muscleblind isoforms (Table S4). Cycling conditions were 94°C for 2 min and 25 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. 1 μl of a 1:100 dilution of cDNA was used in a similar 25 cycle PCR with primers Rp49 for/reverse control for reverse transcriptase reaction and RNA input. Rp49 primers were
designed encompassing an intronic region to detect contamination by genomic DNA. PCR products were resolved in 2% agarose gels. ID-Manager software (TDBa) was used to quantify gel images. The presence of the foetal exon of TnnT3 in the PCR products was confirmed by sequencing.

Supporting Information

Figure S1 Clustal W multiple sequence alignment of isoform-specific Muscleblind sequences. Clustal W (1.82) multiple sequence alignment of evolutionarily conserved Muscleblind protein isoforms. Coordinates refer to the isoform-specific sequence. The FKRP site in the MblC specific sequence is highlighted in grey. Forms. Coordinates refer to the isoform-specific sequence. The

Figure S2 Drosophila S2 cells express mblA, B, C and D mature transcripts to different levels. A semiquantitative RT-PCR amplified isoform-specific regions from two independent RNA samples. Rp49 is shown as control.

Table S1 Targeted expression of mbl transcript isoforms originates markedly different morphological phenotypes. Ga4 lines used drive expression ubiquitously to imaginal (T80-Gal4) or muscular tissue (Mhc-Gal4), to specific eye cell types (sev-Gal4) or to the posterior compartment of segments (en-Ga4).

Table S2 Interacting deficiencies. Deficiencies that dominantly modified the mblC eye overexpression phenotype. S denotes suppression, E enhancement and -no interaction. Number of + signs qualitatively indicate the strength of the phenotypic modification. (*) Genetic data (Flybase) indicate that region from approximately 24D2 to 24E2 is actually present in the deletion.

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