Mol. Cells 30, 65-70, July 31, 2010
DOI/10.1007/s10059-010-0089-9

Molecules
and
Cells

©2010 KSMCB

A Conserved Motif Controls Nuclear Localization of *Drosophila* Muscleblind

Juan M. Fernandez-Costa, and Ruben Artero*

Human Muscleblind-like proteins are alternative splicing regulators that are functionally altered in the RNA-mediated disease myotonic dystrophy. There are different Muscleblind protein isoforms in *Drosophila* and we previously determined that these have different subcellular localizations in the COS-M6 cell line. Here, we describe the conservation of the sequence motif KRAEK in isoforms C and E and propose a specific function for this motif. Different Muscleblind isoforms localize to the peri-plasma membrane (MblA), cytoplasm (MblB), or show no preference for the nuclear or cytoplasmic compartment (MblC and MblD) in *Drosophila* S2 cells transiently transfected with Musclebind expression plasmids. Mutation of the KRAEK motif reduces MblC nuclear localization, whereas fusion of a single KRAEK motif to the heterologous protein β-galactosidase is sufficient to target the reporter protein to the nucleus of S2 cells. This motif is not exclusive to Muscleblind proteins and is detected in several other protein types. Taken together, these results suggest that the KRAEK motif regulates nuclear translocation of Muscleblind and may constitute a new class of nuclear localization signal.

INTRODUCTION

Muscleblind proteins are tissue-specific splicing factors that participate in generating differential developmentally-regulated splice patterns in skeletal muscle, nervous system and cardiac muscle transcripts (Kalsotra et al., 2008; Kanadia et al., 2003; Lin et al., 2006). Muscleblind proteins recognize and bind directly to target transcripts using conserved CCCH motifs. The three-dimensional structure of these motifs has been recently determined (Teplova and Patel, 2008; Warf and Berglund, 2007; Yuan et al., 2007). *Drosophila* Muscleblind zinc fingers bind human Muscleblind-like 1 (MBNL1) targets (Goers et al., 2008), and also regulate the splicing of mouse *cardiac troponin T* and *fast skeletal muscle troponin T* in alternative splicing minigene assays (Kanadia et al., 2003; Vicente-Crespo et al., 2008). This indicates that the target recognition mechanisms are highly conserved. Moreover, human MBNL1 partially rescues the lethality of *Drosophila muscleblind* mutant embryos (Monferrer and Artero, 2006).

Invertebrate genomes contain a single *muscleblind* gene, whereas three paralogs are commonly found in vertebrate species except for *Takifugus rubripes* which contains five paralogs (Fernandes et al., 2007; Pascual et al., 2006). Although human MBNL1, 2 and 3 proteins are all able to regulate exon usage in alternative splicing minigene assays (Ho et al., 2004), additional experimental data suggests that these proteins show functional specializations. In particular, MBNL2 exhibits microtubule-dependent localization near phospho-focal adhesion kinase (pFAK) that is necessary for *integrin $\alpha 3$* mRNA localization in focal adhesion plaques (Adereth et al., 2005).

Human MBNL proteins are associated with the pathogenesis of certain RNA-mediated diseases including Myotonic Dystrophy type 1 (DM1). DM1 originates from an expansion of a non-coding CTG trinucleotide in the *DMPK* gene and is characterized by misregulated alternative splicing of specific pre-RNAs. MBNL proteins aberrantly bind to these mutant CUG expansions leading to the current model of DM1 etiology involving an induced loss of MBNL1 function through sequestration of the protein at the CUG expansion sites. Indeed, a significant fraction of the splice events that are altered in DM1 are also misregulated in *Mbnl1 knockout* mice (Kanadia et al., 2003; Lin et al., 2006). Furthermore, overexpression of MBNL1 in a mouse model of DM1 restores splicing events that are altered in DM1 muscle (Kanadia et al., 2006). MBNL proteins have also been involved in Huntington's disease-like 2 (HDL2) and spinocerebellar ataxias type 3 and type 8 (SCA3 and SCA8) (Li et al., 2008; Mutsuddi et al., 2004; Rudnicki et al., 2007).

Transcripts of *muscleblind* undergo extensive alternative splicing both in vertebrate and invertebrate species giving rise to protein isoforms with a variable number of CCCH motifs as well as isoform-specific C-terminal domains. It has been assumed that protostomal proteins contain only two CCCH motifs, versus the four CCCH motifs found in deuterostome homologs (Pascual et al., 2006). Release 5.1 of the *Drosophila melanogaster* genome annotation identifies Muscleblind protein isoforms containing four zinc fingers (Fig. 2A). Different *Drosophila* Muscleblind isoforms have been hypothesized to have different molecular roles. MblC shows splicing activity in *alpha-actinin* minigene splice assays while MblA, MblB and MblD isoforms show little or no activity. In spite of this, MblA, B and C are all able to efficiently splice out the fetal exon from murine *TnnT3* minigene transcripts in HEK293T cells (Vicente-Crespo et al., 2008).

Department of Genetics, University of Valencia, Doctor Moliner 50, Valencia, Spain
*Correspondence: ruben.artero@uv.es

Received February 16, 2010; revised April 6, 2010; accepted April 13, 2010; published online July 14, 2010

Keywords: alternative splicing factor, KRAEK motif, muscleblind, myotonic dystrophy, nuclear localization signal

Springer

Bioinformatic analyses of isoform-specific sequences identified a number of motifs including the KRAEK motif, present in MblC and MblE protein isoforms (Figs. 2A and 2B). The KRAEK motif is referred to as the FKRP motif by Vicente-Crespo et al. (2008) based on bioinformatics predictions that identified the motif as a potential sumoylation site. We have previously shown that site-directed mutagenesis of KRAEK results in a reduction in nuclear localization of GFP-tagged MblC compared to wild-type MblC in the mammalian COSM6 cell line (Vicente-Crespo et al., 2008). Here, we have examined the subcellular localization of Muscleblind proteins in their natural cellular context using the *Drosophila* S2 cell line. We report the preferential localization of MblA-D protein isoforms in S2 cells and confirm that KRAEK is also necessary for the nuclear localization of MblC in *Drosophila*. The fusion of KRAEK to a heterologous cytoplasmic protein is sufficient to target this protein to the nucleus. Therefore, we have demonstrated that this highly conserved motif controls subcellular localization of the *Drosophila* MblC protein and identifies the first functional motif other than CCCH zinc fingers in Muscleblind proteins.

MATERIALS AND METHODS

Constructs

Muscleblind isoforms MblA, B, C and D as well as the mutant protein MblC^{K202I}, were cloned into the pIE vector as described previously (Vicente-Crespo et al., 2008). The coding region of LacZ was amplified from pIE-LacZ (Zhou et al., 2005) using *Pwo* polymerase (Roche Diagnostics, Germany) and primers containing cohesive ends for the restriction sites *Not*I (5′-GCGGCCGCATGAGCGAAAAATACATCG-3′) and *Bam*HI (5′-GGATCCTTATTACGTCGACCCTTTTTGACACCAGACCAAC-3′ and including a *Sal*I restriction site). The resulting PCR product was cloned into the *Not*I and *Bam*HI sites in pIE. The SV40 t-antigen NLS (PKKKRKV) was generated *in vitro* by hybridization of the complementary oligos 5′-ATGCCTAAGAAGAAACGTAAGGTAG-3′ and 5′-GGCCCTACCTTACGTTTCTTCTTAGGCATGC-3′. This synthetic NLS was cloned upstream of *LacZ* using the *Ksp*I and *Not*I sites. A synthetic KRAEK motif was generated by annealing complementary oligonucleo-tides coding for GMVPFKRPAAEKSG: 5′-TCGACGGGCATGGTACCGTTCAAACGTCCAGCTGCCGAAAAGTCTGGCTAATAAG-3′ and 5′-GATCCTTATTAGCCAGACTTTTCGGCAGCTGGACGTTTGAACGGTACCATGCCCG-3′. This synthetic KRAEK-coding motif was inserted downstream of *LacZ* into *Sal*I and *Bam*HI sites to generate a β-galactosidase-KRAEK fusion construct. All constructs in the pIE vector were confirmed by DNA sequencing.

Cell culture and transfection

Drosophila melanogaster Schneider 2 cells (S2) were grown at 27°C in Schneider´s medium (Gibco, UK) supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. For subcellular localization assays, 1×10^6 cells/ml were seeded onto coverslips in 24-well plates. After 24 h cells were transfected with 0.5 μg of DNA for 5 h using 6 μl of Cellfectin reagent (Invitrogen, USA).

Immunochemistry

Cells were fixed 24 h after transfection in 4% paraformaldehyde (0.1 M phosphate buffer pH 7.3) and blocked with 1% donkey serum in PBS. Primary antibodies used were: sheep anti-Mbl (1:5000) (Houseley et al., 2005), rabbit anti-β-Gal (anti-β-galactosidase) (1:5000) (Cappel, France) and mouse monoclonal anti-Lamin (1:200) (Developmental Studies Hybridoma Bank, University of Iowa). Secondary antibodies used were: anti-sheep-FITC (1:200; Sigma, USA), anti-rabbit-FITC (1:200; Calbiochem, Merck KGaA, Germany) and a biotinylated anti-mouse (1:200; Pierce Biotechnologies, USA), which was used in combination with streptavidin-Texas Red (1:200) (Vector Laboratories, USA). Inmunostained cells on coverslips, were mounted in VECTASHIELD with DAPI (Vector Laboratories, USA). Western blots were performed according to Vicente-Crespo et al. (2008). Primary and secondary antibodies were used at a 1:5000 dilution.

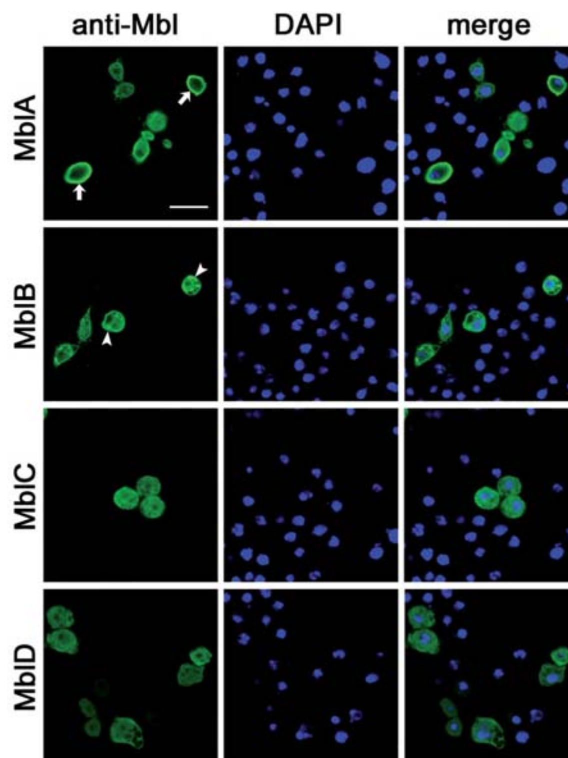

Fig. 1. Subcellular localization of Muscleblind protein isoforms A to D. Confocal micrographs of transiently transfected *Drosophila* S2 cells. Muscleblind proteins were immunostained with an anti-Muscleblind antibody and were detected with a fluorescent-tagged secondary antibody using the green channel in the confocal microscope. Cell nuclei were counterstained with DAPI (blue channel) and the resulting overlap is shown on the right. MblA is preferentially detected in the cytoplasm close to the plasma membrane (arrows). MblB is found in both cellular compartments although the cytoplasmic signal is stronger than the nuclear signal. MblB perinuclear aggregates were frequently observed (arrowheads). Protein isoforms MblC and MblD are detected in both cellular compartments at similar intensities. Scale bar, 20 μm.

Quantitative analysis of nuclear localization

Images of cells were recorded using a Leica SP2 laser confocal microscope using the same acquisition parameters when comparing two conditions. At least 40 cells were examined per construct from quadruplicate experiments. Image analysis was performed with ImageJ (Image processing and analysis in Java, http://rsb.info.nih.gov/ij/) as described in Carmona et al. (2007). The levels of grey obtained with this method represent an evaluation of nuclear localization. Significance analysis was performed using a two-tailed Mann-Whitney test.

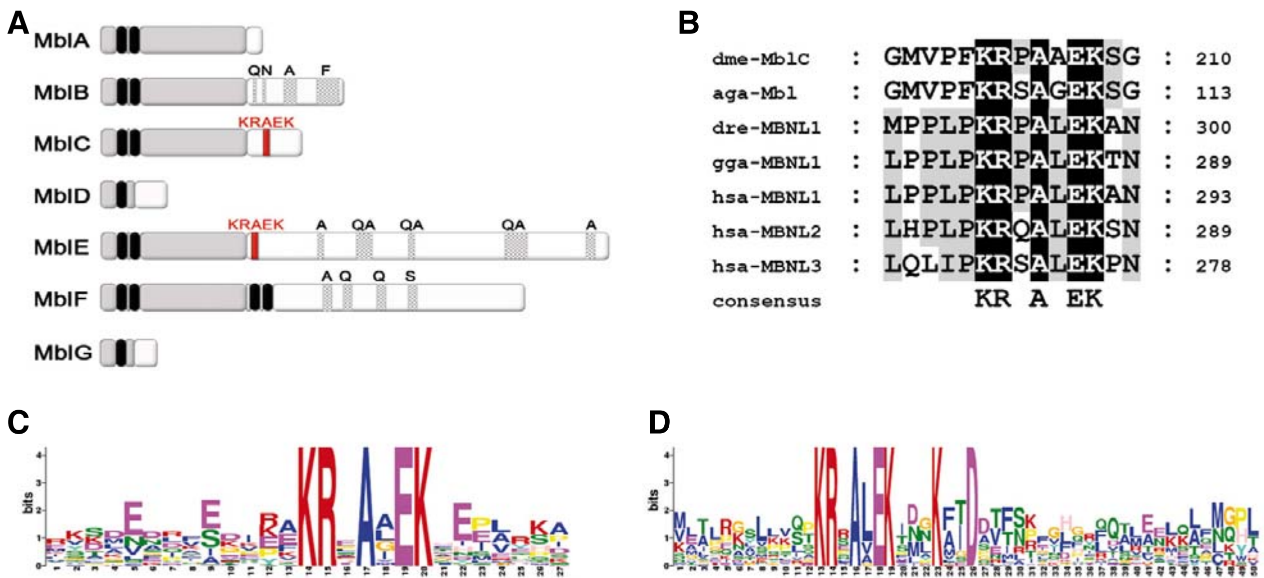

Fig. 2. The KRAEK motif is found in vertebrate and invertebrate proteins including Muscleblind family members. Sequence motifs detected in *Drosophila* Muscleblind protein isoforms A to G (A; representation to scale). Rectangles shaded in grey indicate identical sequences between isoforms whereas empty rectangles are isoform-specific regions. Black, stippled and red bars denote zinc finger (CCCH), repetitive, or KRAEK motifs, respectively. Multiple alignment showing the conservation of KRAEK motif in Muscleblind family members (B; dme: *Drosophila melanogaster*, aga: *Anopheles gambiae*; dre: *Danio rerio*; gga: *Gallus gallus*; hsa: *Homo sapiens*). GLAM2 output for invertebrate (C) and vertebrate (D) proteins containing the KRAEK motif. Note strong conservation of K and D amino acids C-terminal to KRAEK in vertebrate proteins.

Database and motif searches

The GENPEPT database (invertebrate and vertebrate sections; GenBank) was searched with the KRXA$EK string (where X = any amino acid and $ = uncharged amino acid) using the Scansite program (http://scansite.mit.edu; Quick matrix method). The GLAM2 program from the MEME suite (http://meme.nbcr. net) provided a pictogram of conserved amino acids around KRAEK (number of alignments = 100).

RESULTS AND DISCUSSION

Subcellular distribution of MblA-D in *Drosophila* S2 cells

Drosophila Muscleblind isoforms MblA-D were transiently transfected into *Drosophila* S2 cells and were detected immunochemically using an anti-Muscleblind antibody, which is capable of recognizing all four proteins (Houseley et al., 2005). The isoforms were observed to preferentially localize to different subcellular compartments (Fig. 1). MblA is exclusively cytoplasmic and accumulates closely apposed to the plasma membrane. MblB localizes in both cellular compartments, nucleus and cytoplasm, but accumulates in the cytoplasm to form perinuclear aggregates. Although these aggregates might form under normal conditions, the hydrophobic alanine and phenylalanine-rich regions present in MblB could mediate self-interactions when the protein is overexpressed. MblC and MblD are distributed uniformly in both the nuclear and cytoplasmic compartments.

The distribution of Muscleblind isoforms in *Drosophila* S2 cells is consistent with previous studies that expressed Green Fluorescent Protein (GFP)-tagged isoforms MblA-D in a mammalian cell line (COS-M6) (Vicente et al*.,* 2007), although some differences are evident. MblA preferentially localizes to the cytoplasm and forms perinuclear aggregates in COS-M6, but accumulates in the plasma membrane in S2 cells. MblB and MblC preferentially localize to the nucleus in COS-M6 cells but, in S2, MblB occurs predominantly in the cytoplasm while MblC shows no clear preference for a cellular compartment. The preferential sub-cellular distribution of Muscleblind isoforms clearly points to specialized molecular roles, which we propose might correspond to the activities described for human MBNL proteins. The nuclear localization of MblC as well as its ability to regulate both the *alpha-actinin* and murine *TnnT3* minigenes suggests that MblC is specialized in alternative splicing regulation. However, the peri-cytoplasmic localization of MblA suggests that it might be the counterpart for human MBNL2 that targets focal adhesion components including the integrin mRNAs. Despite the detection of MblD in the nucleus when it is overexpressed, it did not work in splicing assays (Vicente et al*.,* 2007; Vicente-Crespo et al., 2008). The presence of a single CCCH motif in MblD (as well as in MblG) suggests that it might have a regulatory role, such as binding pre-mRNA targets in an unproductive manner.

The KRAEK motif is necessary for MblC nuclear localization

Unlike the CCCH motifs, KRAEK motifs are not encoded in all Muscleblind splice variants. In *Drosophila*, MblC and MblE have a KRAEK motif whereas other isoforms do not (Fig. 2A). To assess the functional relevance of the KRAEK motif we used a previously described mutant MblC construct, in which lysine-202 is changed to isoleucine ($MblC^{K202I}$) (Vicente-Crespo et al., 2008) in subcellular localization assays. Confocal microscopy analysis revealed that $MblC^{K202I}$, in contrast to the wild type protein, preferentially localized to the cytoplasm (Fig. 3A). In order to quantify the nuclear distribution of MblC and $MblC^{K202I}$, confocal images were processed with ImageJ software for co-localization with the nucleic acid stain DAPI as described (Carmona et al., 2007). In this analysis, lower mean grey values

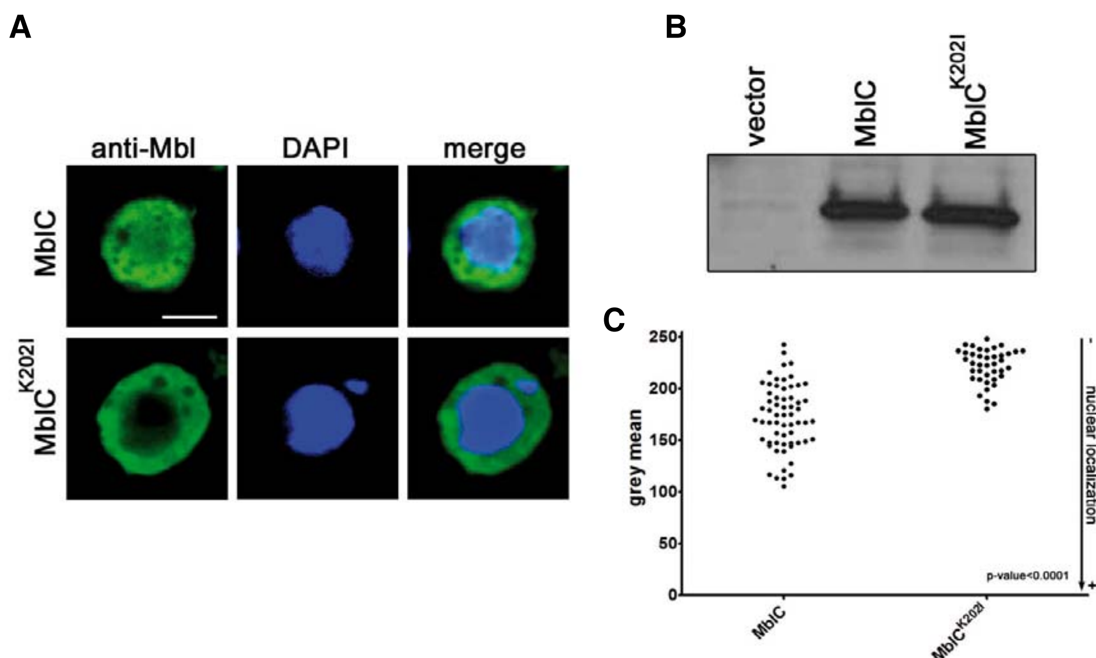

Fig. 3. The KRAEK motif is necessary for MblC nuclear localization. Confocal fluorescence micrographs of *Drosophila* S2 cells transiently transfected with wild type MblC and KRAEK-mutated $MblC^{K202I}$ cDNAs (A). Muscleblind was detected with an anti-Mbl antibody in the green channel and nuclei were stained with DAPI. MblC protein distributes to both compartments whereas mutant $MblC^{K202I}$ protein is preferentially cytoplasmic. Scale bar, 5 μm. (B) Western blot of protein extracts from S2 cells transfected as in (A) and detected with an anti-Muscleblind antibody. MblC migrated at the molecular weight predicted for the wild type protein (27 kDa). The empty vector was used as negative control. Quantification of MblC and $MblC^{K202I}$ nuclear localization (C). The overlap between green (detecting Mbl) and DAPI (detecting the nucleus) fluorescence signals was transformed into values of grey (the lower the grey the higher the overlap). Mean grey values are plotted for at least 40 representative cells per condition. Grey values for MblC transfections are significantly lower than for mutant $MblC^{K202I}$ in a two-tailed Mann-Whitney test.

indicate higher overlap between the MblC and DAPI fluorescence signals (Figs. 3A and 3C, green and blue channels, respectively). Correct expression and lack of degradation of both proteins was confirmed by Western blotting (Fig. 3A). The distribution of mean grey values per nucleus was found to be significantly lower for wild type MblC than for $MblC^{K202I}$. This indicates that the KRAEK motif is necessary for wild type MblC nuclear localization. Interestingly, nuclear localization signal (NLS) prediction programs do not identify KRAEK as a described NLS (not shown) thus suggesting that it constitutes a new class of NLS motif.

The KRAEK motif is sufficient for nuclear localization of a heterologous reporter

Other sequence motifs or structural features in addition to the KRAEK motif may be necessary for nuclear localization of MblC. To test this possibility we generated a protein expression construct that fused a single KRAEK motif (14 amino acids, as shown in Fig. 2B) to a heterologous protein. Although the GFP reporter protein is widely used in cell culture experiments, it is not useful for nuclear-import assays because it shows restricted responsiveness to fused NLS in *Drosophila* (Chan et al., 2007). For this reason, we used β-galactosidase as the reporter protein in our experimental design. Firstly, we transiently transfected S2 cells with the pIE-LacZ vector and immunostained them with an anti-β-galactosidase antibody. This confirmed that β-galactosidase is localized to the cytoplasm in S2 cells. As a result, we proceeded to generate a construct that fused the β-galactosidase open reading frame to the KRAEK coding sequence. As a control for nuclear import, we also generated a construct that fused β-galactosidase to the SV40 virus t-antigen NLS, which has been shown to target proteins to the cell nucleus *in vivo* in *Drosophila* (Sisson et al., 2006). Only a small fraction of S2 cells targeted the control β-galactosidase-NLS to the nucleus in transiently transfected S2 cells. In S2 cells similarly transfected with the β-galactosidase-KRAEK construct, the protein distributed to both the cell nucleus and the cytoplasm, showing the same localization as wild type MblC (Figs. 4A and 4B). Consistent with this qualitative analysis, quantification of the nuclear signal from confocal microscope images revealed significantly lower mean grey values for β-galactosidase-KRAEK compared to wild type β-galactosidase (Fig. 4C). Thus, the KRAEK motif controls nuclear localization of Muscleblind protein isoform C in the S2 cell line.

Conservation of the KRAEK motif

We performed GENPEPT database searches with the SCANSITE software to assess whether proteins other than Muscleblind have KRAEK motifs (Obenauer et al., 2003). A total of 162 proteins contain the KRAEK motif, of which 45 were from invertebrates and 123 were from vertebrates. The *Drosophila* proteome included 16 KRAEK-containing proteins arising from seven genes. These included three novel genes (*CG34404*, *CG5626* and *CG12584*) and four known genes (*Topoisomerase 1*, *Minichromosome maintenance 2*, *nervous wreck* and *muscleblind*). The KRAEK motif in these proteins may control nuclear localization of topoisomerase 1 or Minichromosome maintenance 2. The presence of a KRAEK motif is not predic-

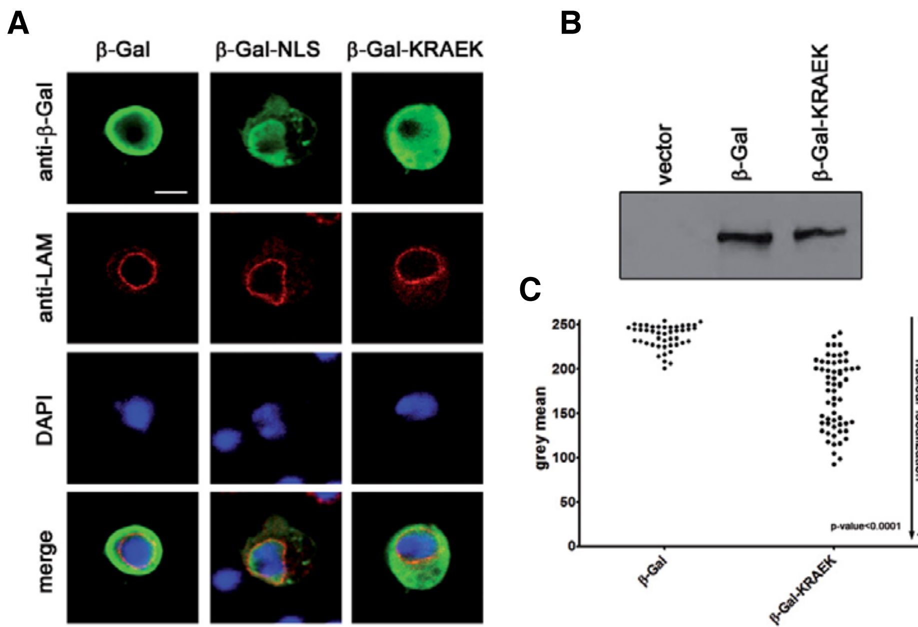

Fig. 4. Fusion of KRAEK to a heterologous protein (β-galactosidase) targets the reporter to the cell nucleus. Confocal fluorescence micrographs of *Drosophila* S2 cells transiently transfected with the β-galactosidase fusion proteins indicated (A). β-galactosidase was detected with an anti-β-galactosidase antibody (green channel), nuclei were stained with DAPI (blue channel) and delineated with the nuclear membrane marker anti-Lamin (red channel), and the overlap is shown in the lower panels. Wild type β-galactosidase is localized in the cytoplasm but fusion to the KRAEK motif or the SV40 virus t-antigen NLS (as a positive control) increased nuclear distribution of the reporter. Scale bar, 4 μm. (B) Western blot of β-galactosidase fusion proteins from S2 cell extracts. Empty vector transfection was used as negative control. The molecular weights of β-galactosidase and β-galactosidase-KRAEK (123 kDa) confirmed that both proteins were full length. (C) Quantification of β-galactosidase and β-galactosidase-KRAEK fusion proteins in the nuclear cell compartment was performed as in Fig. 3. Grey values for β-galactosidase-KRAEK are significantly lower than for wild type β-galactosidase in a two-tailed Mann-Whitney test.

tive of nuclear localization because Nervous wreck is a protein with SH3/SH2 adaptor activity that participates in the negative regulation of synaptic growth at neuromuscular junction and is cytoplasmic in localization. Our results demonstrate that the KRAEK motif is sufficient to target a heterologous protein to the nucleus of *Drosophila* cells but in other organisms additional regions may be necessary. The GLAM2 program from the MEME suite (Frith et al., 2008) was used to identify conserved amino acids surrounding the KRAEK motif in both invertebrate and vertebrate proteins (Figs. 2C and 2D). Apart from KRAEK we found no additional amino acid residues conserved in invertebrate proteins but analysis of vertebrate proteins with GLAM2 detected an additional lysine and aspartate on the C-terminal side of the KRAEK core sequence. The physicochemical properties of these amino acids are similar to residues conserved in KRAEK suggesting that they may indeed be part of the motif in vertebrates.

ACKNOWLEDGMENTS

We thank J. Terol for help with the bioinformatics analysis of the KRAEK motif. The anti-Lamin antibody (ADL67.10) developed by Paul A. Fisher was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA 52242. *Drosophila* S2 cells were a gift from F. Gebauer. This work was supported by research grants SAF2006-09121 and BFU2009-10940 from the Ministerio de Ciencia e Innovación to R.A. and a predoctoral fellowship from the Conselleria d'Educació de la Generalitat Valenciana to J.M.F.

REFERENCES

Adereth, Y., Dammai, V., Kose, N., Li, R., and Hsu, T. (2005). RNA-dependent integrin alpha3 protein localization regulated by the Muscleblind-like protein MLP1. Nat. Cell Biol. *7*, 1240-1247.

Carmona, R., Macias, D., Guadix, J.A., Portillo, V., Perez-Pomares, J.M., and Munoz-Chapuli, R. (2007). A simple technique of image analysis for specific nuclear immunolocalization of proteins. J. Microsc. *225*, 96-99.

Chan, W.M., Shaw, P.C., and Chan, H.Y. (2007). A green fluorescent protein-based reporter for protein nuclear import studies in *Drosophila* cells. Fly *1*, 340-342.

Fernandes, J.M., Kinghorn, J.R., and Johnston, I.A. (2007). Characterization of two paralogous muscleblind-like genes from the tiger pufferfish (Takifugu rubripes). Comp. Biochem. Physiol. B Biochem. Mol. Biol. *146*, 180-186.

Frith, M.C., Saunders, N.F., Kobe, B., and Bailey, T.L. (2008) Discovering sequence motifs with arbitrary insertions and deletions. PLoS Comput. Biol. *4*, e1000071.

Goers, E.S., Voelker, R.B., Gates, D.P., and Berglund, J.A. (2008). RNA binding specificity of *Drosophila* muscleblind. Biochemistry *47*, 7284-7294.

Ho, T.H., Charlet, B.N., Poulos, M.G., Singh, G., Swanson, M.S., and Cooper, T.A. (2004). Muscleblind proteins regulate alternative splicing. EMBO J. *23*, 3103-3112.

Houseley, J.M., Wang, Z., Brock, G.J., Soloway, J., Artero, R., Perez-Alonso, M., O'Dell, K.M., and Monckton, D.G. (2005). Myotonic dystrophy associated expanded CUG repeat muscleblind positive ribonuclear foci are not toxic to *Drosophila*. Hum. Mol. Genet. *14*, 873-883.

Kalsotra, A., Xiao, X., Ward, A.J., Castle, J.C., Johnson, J.M., Burge, C.B., and Cooper, T.A. (2008). A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. Proc. Natl. Acad. Sci. USA *105*, 20333-20338.

Kanadia, R.N., Johnstone, K.A., Mankodi, A., Lungu, C., Thornton, C.A., Esson, D., Timmers, A.M., Hauswirth, W.W., and Swanson, M.S. (2003). A muscleblind knockout model for myotonic dystrophy. Science *302*, 1978-1980.

Kanadia, R.N., Shin, J., Yuan, Y., Beattie, S.G., Wheeler, T.M., Thornton, C.A., and Swanson, M.S. (2006). Reversal of RNA missplicing and myotonia after muscleblind overexpression in a mouse poly(CUG) model for myotonic dystrophy. Proc. Natl. Acad. Sci. USA *103*, 11748-11753.

Li, L.B., Yu, Z., Teng, X., and Bonini, N.M. (2008). RNA toxicity is a component of ataxin-3 degeneration in *Drosophila*. Nature *453*, 1107-1111.

Lin, X., Miller, J.W., Mankodi, A., Kanadia, R.N., Yuan, Y., Moxley, R.T., Swanson, M.S., and Thornton, C.A. (2006). Failure of MBNL1-dependent post-natal splicing transitions in myotonic dystrophy. Hum. Mol. Genet. *15*, 2087-2097.

Monferrer, L., and Artero, R. (2006). An interspecific functional complementation test in *Drosophila* for introductory genetics laboratory courses. J. Hered. *97*, 67-73.

Mutsuddi, M., Marshall, C.M., Benzow, K.A., Koob, M.D., and Rebay, I. (2004). The spinocerebellar ataxia 8 noncoding RNA causes neurodegeneration and associates with staufen in *Drosophila*. Curr. Biol. *14*, 302-308.

Obenauer, J.C., Cantley, L.C., and Yaffe, M.B. (2003). Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res. *31*, 3635-3641.

Pascual, M., Vicente, M., Monferrer, L., and Artero, R. (2006). The Muscleblind family of proteins: an emerging class of regulators of developmentally programmed alternative splicing. Differentiation *74*, 65-80.

Rudnicki, D.D., Holmes, S.E., Lin, M.W., Thornton, C.A., Ross, C.A., and Margolis, R.L. (2007). Huntington's disease-like 2 is associated with CUG repeat-containing RNA foci. Ann. Neurol. *61*, 272-282.

Sisson, B.E., Ziegenhorn, S.L., and Holmgren, R.A. (2006) Regulation of Ci and Su(fu) nuclear import in *Drosophila*. Dev. Biol. *294*, 258-270.

Teplova, M., and Patel, D.J. (2008). Structural insights into RNA recognition by the alternative-splicing regulator muscleblind-like MBNL1. Nat. Struct. Mol. Biol. *15*, 1343-1351.

Vicente, M., Monferrer, L., Poulos, M.G., Houseley, J., Monckton, D.G., O'Dell K, M., Swanson, M.S., and Artero, R.D. (2007). Muscleblind isoforms are functionally distinct and regulate alpha-actinin splicing. Differentiation *75*, 427-440.

Vicente-Crespo, M., Pascual, M., Fernandez-Costa, J.M., Garcia-Lopez, A., Monferrer, L., Miranda, M.E., Zhou, L., and Artero, R.D. (2008). *Drosophila* muscleblind is involved in troponin T alternative splicing and apoptosis. PloS one *3*, e1613.

Warf, M.B., and Berglund, J.A. (2007). MBNL binds similar RNA structures in the CUG repeats of myotonic dystrophy and its pre-mRNA substrate cardiac troponin T. RNA *13*, 2238-2251.

Yuan, Y., Compton, S.A., Sobczak, K., Stenberg, M.G., Thornton, C.A., Griffith, J.D., and Swanson, M.S. (2007). Muscleblind-like 1 interacts with RNA hairpins in splicing target and pathogenic RNAs. Nucleic Acids Res. *35*, 5474-5486.

Zhou, L., Jiang, G., Chan, G., Santos, C.P., Severson, D.W., and Xiao, L. (2005). Michelob_x is the missing inhibitor of apoptosis protein antagonist in mosquito genomes. EMBO Rep. *6*, 769-774.