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Distinct functions of alternatively spliced isoforms encoded by zebrafish *mef2ca* and *mef2cb*

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ABSTRACT

In mammals, an array of MEF2C proteins is generated by alternative splicing (AS), yet specific functions have not been ascribed to each isoform. Teleost fish possess two *MEF2C* paralogues, *mef2ca* and *mef2cb*. In zebrafish, the Mef2cs function to promote cardiomyogenic differentiation and myofibrillogenesis in nascent skeletal myofibers. We found that zebrafish *mef2ca* and *mef2cb* are alternatively spliced in the coding exons 4–6 region and these splice variants differ in their biological activity. Of the two, *mef2ca* is more abundantly expressed in developing skeletal muscle, its activity is tuned through zebrafish development by AS. By 24 hpf, we found the prevalent expression of the highly active full length protein in differentiated muscle in the somites. The splicing isoform of *mef2ca* that lacks exon 5 (*mef2ca 4–6*), encodes a protein that has 50% lower transcriptional activity, and is found mainly earlier in development, before muscle differentiation. *mef2ca* transcripts including exon 5 (*mef2ca 4–5–6*) are present early in the embryo. Over-expression of this isoform alters the expression of genes involved in early dorso-ventral patterning of the embryo such as *chordin*, *nodal related 1* and *goosecoid*, and induces severe developmental defects. AS of *mef2cb* generates a long splicing isoform in the exon 5 region (Mef2cbL) that predominates during somitogenesis. Mef2cbL contains an evolutionarily conserved domain derived from exonization of a fragment of intron 5, which confers the ability to induce ectopic muscle in mesoderm upon over-expression of the protein. Taken together, the data show that AS is a significant regulator of Mef2c activity.

1. Introduction

Alternative splicing (AS) creates diversity within proteins without the need for gene duplication. In addition, AS is also an important mechanism for modulating gene expression and has contributed substantially to the evolution of modern genomes (reviewed in [1–3]). Many transcription factors undergo AS that creates important functional differences in the encoded proteins: altered transcriptional regulation capacity, nuclear trafficking, sensitivity to signals or requirement for co-activators [4]. Splicing-sensitive microarrays and deep sequencing analysis of mRNA from various human tissues have revealed the prevalence of AS in skeletal muscle; dysregulation of AS is associated with human muscle diseases [5] (reviewed in [6,7]).

Genes encoding the Myocyte Enhancer Factor 2 (MEF2) family of transcription factors undergo extensive AS, the function of which is generally unclear. All MEF2 proteins have an N-terminal DNA binding region composed of MADS (Minichromosome maintenance, Agamous, Deficiens, Serum response factor) and MEF2 domains, two central transcription activating domains (TAD1 and TAD2) and a C-terminal nuclear localization sequence (Fig. 1B). Invertebrates generally have a single *MEF2* gene, whereas amniotes have four genes (*MEF2A-D*). The teleost-specific genome duplication has led to six *mef2* genes in zebrafish, with two copies of *mef2a* and *mef2c*, designated *mef2aa*, *mef2ab*, *mef2ca* and *mef2cb* [8]. Most MEF2 proteins are highly expressed in muscle tissue, where they regulate the heart, skeletal and smooth muscle differentiation [9]. Like *Drosophila D-Mef2*, *Mef2c* is particularly important in early heart and skeletal muscle development in both mice and zebrafish [8,10–17]. *MEF2*s are also more broadly expressed and function to

Abbreviations: AS, Alternative Splicing; MEF2, Myocyte Enhancer Factor 2; BMP, Bone Morphogenetic Protein; MADS, Minichromosome maintenance, Agamous, Deficiens, Serum response factor; TAD, transcription activating domains; PKA, Protein Kinase A; qRTPCR, quantitative Real Time PCR; hpf, hours post fertilization; ss, somitic stage; WISH, Whole Mount In Situ Hybridization; CMV, Cytomegalovirus; LNA, Locked Nucleic Acid; myog, myogenin; actb2, beta-actin 2; chd, chordin; ndr1, nodal related 1; gsc, goosecoid; nog1, noggin1; ntl, no tail; smyhc1, slow myosin heavy chain 1; MyHC, Myosin Heavy Chain; I.M.A.G.E, Integrated Molecular Analysis of Genomes and their Expression; ascl1a, achaete-scute complex-like 1a; kdr1, kinase insert domain receptor; neurog 1, neurogenin 1; myl7, myosin, light polypeptide 7

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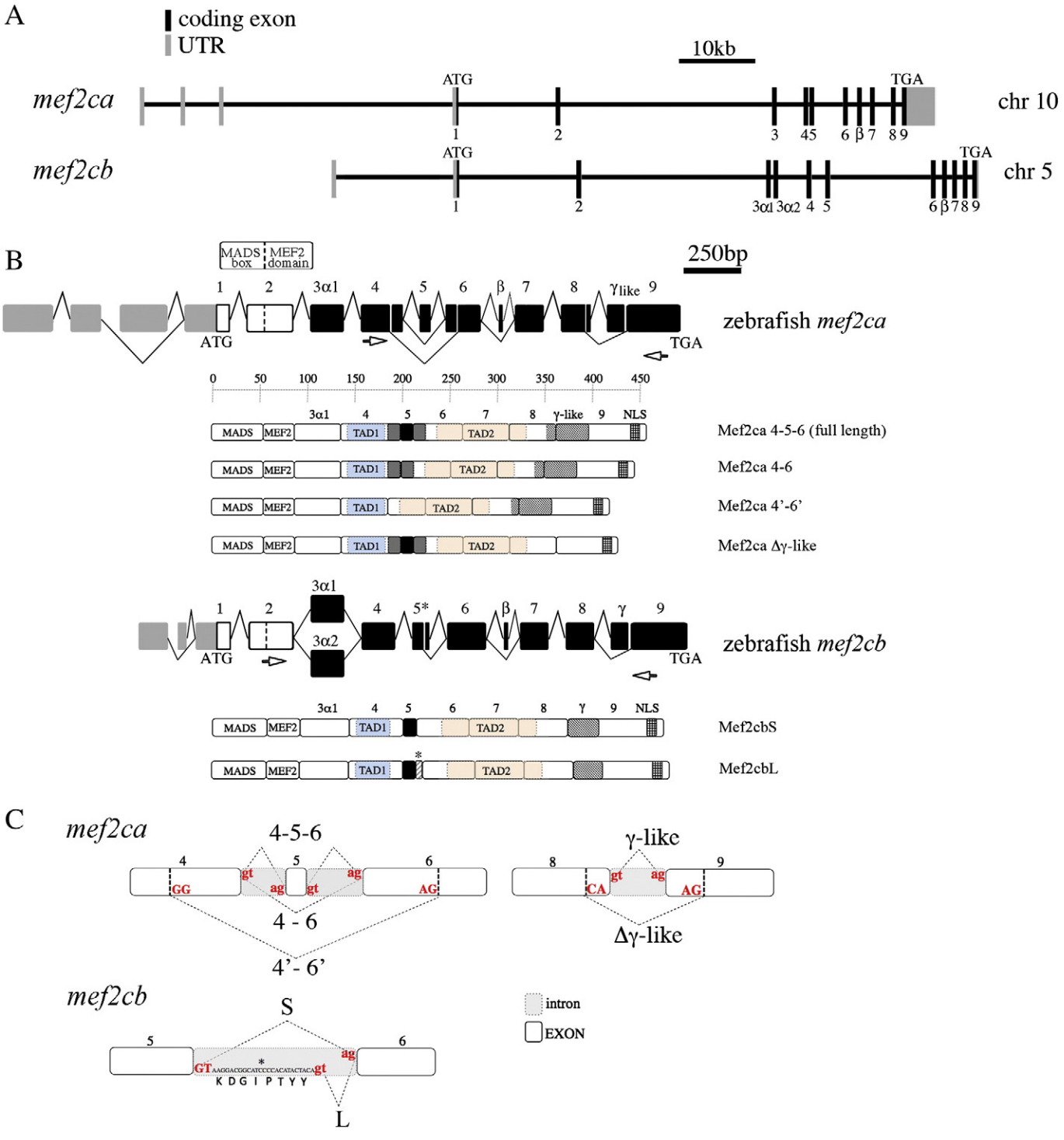
control the development and adaptation of the brain, immune system, blood vessel and many other tissues [18] (reviewed in [19]). In mammals, *MEF2C* is subjected to three different alternative splices. A mutually exclusive alternative splice occurs between exons α1 and α2, located in the region immediately adjacent to the MEF2 domain [20]. In the central TAD2 region, a skipping-type alternative splice can include exon β and a splice involving alternative 3′ splice site selection occurs in the γ region near the C-terminus [21,22]. In the case of mouse *Mef2d*, AS of the α exon switches the protein from a transcriptional repressor regulated by protein kinase A (PKA), to an activator insensitive to PKA signaling [23]. This switch is thought to drive skeletal muscle terminal differentiation, but how AS in the *Mef2d* α exon relates functionally to AS at other alternate exons is unclear. Developmentally regulated switching of AS of *MEF2* genes has been described during frog and mouse development [24–26]. Involvement of alternative splice variants of *Mef2* in endomesoderm and neuron differentiation in the sea anemone *Nematostella vectensis* has been described recently [27]. However, it has not yet been determined whether functional differences among the splicing variants of the *MEF2C* genes are important in vertebrate development.

Here we describe the alternative splicing of the two zebrafish Mef2c genes, *mef2ca* and *mef2cb*. In addition to splicing events akin to the α, β, γ splices that were described in mice, we find novel splice forms varying in the region between the two TADs around the fifth coding exon. We



provide the first evidence that the developmentally-regulated AS of *mef2ca* in this region affects Mef2c protein function. We report that *mef2ca* transcripts including exon 5 (*mef2ca* 4–5–6) are expressed early in development, and their over-expression causes severe defects in the embryos related to impaired gastrulation that are not created by variants lacking exon 5. Moreover, ectopic expression of Mef2ca 4–5–6 results in an increase of the transcript levels of genes such as *chordin* (*chd*), *nodal related 1* (*ndr1*), *no-tail a* (*ntla*) and *goosecoid* (*gsc*), necessary during gastrulation for correct dorso-ventral patterning. Lastly, we describe a new evolutionarily conserved alternatively spliced isoform of *mef2cb*, here named Mef2cbL, containing an additional octapeptide in exon 5, that confers on Mef2cb the ability to induce ectopic skeletal myogenesis.

2. Materials and methods

2.1. Plasmids

The full-length coding regions of the zebrafish Mef2ca 4–5–6, Mef2ca 4–6, Mef2ca 4′–6′ and Mef2cbL variants were amplified from 24 hpf (hours post fertilization) zebrafish embryos cDNAs. The full length cDNA of Mef2cbS was obtained by a PCR reaction starting from a template made of three overlapping PCR products: the exon 5 region amplified from a Mef2cb I.M.A.G.E. clone (clone ID: 6519749, GenBank: CD282884.1), the upstream and downstream regions amplified from the Mef2cbL cDNA. The cDNAs were first inserted in the pCR2.1 vector (Invitrogen) or pGEM-T Easy vector (Promega), then sub-cloned into BamHI/NotI sites of the pcDNA 3.1(+) expression vector (Invitrogen). For RNA injections isoforms were sub-cloned into the XbaI/SalI sites of the βUT-3 vector [8]. Plasmids pGL3(desMEF2)$_3$ and pRSVβ-gal were previously described [28].

2.2. Alternative splicing prediction and multiple alignments

TBLASTN (http://blast.ncbi.nlm.nih.gov) was used to predict alternative splicing isoforms of *mef2ca* or *mef2cb* and for multiple alignment to compare *mef2cbL* sequence to available sequences in database (GenBank and NCBI Reference sequence are listed in Fig. S3B). Sequence data were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and then edited using GeneDoc software (http://www.psc.edu/biomed/genedoc).

2.3. Transcription reporter assays

Transactivation assays were performed by co-transfecting COS-1 cells with indicated expression vectors and cell lysates were analyzed as described previously [28].

2.4. RNA isolation, RT-PCR and Real Time PCR

RNA was isolated using TRIzol® Plus RNA Purification System (Ambion). For each developmental stage, 100 embryos were disrupted using Tissue Raptor (Qiagen). 500 ng of total RNA were reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen). Primers used to detect *myogenin* (*myog*), *myod*, *beta-actin 2* (*actb2*), *mef2ca* and *mef2cb* are listed in Fig. S6A (other primer sequences are available upon request), quantitative Real Time PCR (qRTPCR) was performed on 2.5 ng of Poly A mRNA using SYBR Green method (SYBR® Green PCR Core Reagent, Applied Biosystems). Poly A mRNA has been purified using Ambion's protocol (MicroPoly(A) Purist Kit). To amplify the different *mef2ca* isoforms specific forward primers spanning exon-exon junctions were used with a common reverse primer (Fig. S6B). For each primer combination the optimal $MgCl_2$ concentration was determined to obtain specific and high efficient amplification (slope values between −2.95 and −3.75). Absolute quantification of transcript copy number was achieved by generating calibration curve using plasmid DNA templates (listed above) as previously described [29,30]. Analysis was performed using PCR ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Student's t-tests were performed for pairwise comparisons to determine significant differences between groups.

2.5. Zebrafish lines, maintenance and embryo manipulation

Wild-type zebrafish (*Danio rerio*) lines were maintained on King's College wild-type background, and staging and husbandry were as described [31].

2.6. Whole mount in situ hybridization (WISH)

In situ mRNA hybridization was performed as described [14]. Fluorescein- or digoxigenin-tagged probes used were *mef2ca* [32], *mef2cb* [8], *myod* [33], *slow myosin heavy chain 1* (*smyhc 1*) [8], *myosin, light polypeptide 7* (*myl7*) [34], *kinase insert domain receptor* (*kdrl*) [35], *neurogenin* 1 (*neurog1*) [36] or *achaete-scute complex-like 1a* (*ascl1a*) [37]. We have also used two non-overlapping dual digoxigenin-labelled custom *mef2ca* exon 5-specific locked nucleic acid (LNA) probes, LNA1 and LNA2 (Exiqon, sequence available upon request) to perform WISH as described [38,39]. Embryos were photographed as wholemounts on Olympus DP70 or dissected and flatmounted in glycerol and photographed on a Zeiss Axiophot with AxioCam using Improvision Openlab.

2.7. mRNA injection and embryo manipulation

mRNA injection was performed as described previously [40]. βUT-3 vectors encoding Mef2ca and Mef2cb isoforms, were linearized using SfiI/PstI sites. mRNAs were made with mMESSAGE mMACHINE kit

Fig. 1. Genomic organization, transcripts and protein variants of zebrafish *mef2ca* and *mef2cb* genes. A) Schematics of zebrafish *mef2ca* and *mef2cb* genes. Exons are numbered and indicated by boxes. Black boxes indicate the *mef2ca* and *mef2cb* coding exons, whereas gray boxes represent the 5′- and 3′-untranslated regions. Introns are indicated by solid lines. The ATG translational start codons and the TGA stop codons of the two genes are also indicated. B) Schematic representation of zebrafish *mef2ca* and *mef2cb* transcript variants. Gray boxes represent UTR, white boxes represent the coding regions of the MADS and MEF2 domains in exons 1 and 2, black boxes represent the remaining translated sequence. Structures of zebrafish *mef2ca* and *mef2cb* genes transcripts are similar with the following exceptions: *mef2ca* lacks the 3α2, exon 5 alone or together with neighboring sequences from exons 4 and 6 may be excluded from the mature transcripts, the alternatively spliced γ region overlaps with the homologous γ regions of zebrafish *mef2cb* and of the other vertebrates *mef2c* genes, however it extends to neighboring sequences located in exons 8 and 9; *mef2cb* transcripts may include a short (24 nt) sequence of intron 5 (*). White arrows indicate the position of the primers used to amplify the cDNAs. The structures of the Mef2ca and Mef2cb protein isoforms deduced from the cloned cDNA sequences are schematized. The N-terminal region of the Mef2c proteins comprises the MADS-box and the MEF2 domain, involved in DNA binding and dimerization. By analogy with the mouse and human proteins, in the C-ter there are two putative transcriptional activation domains, TAD1 (blue) and TAD2 (orange), encoded respectively by exon 4 and by exons 6,7,8, downstream is localized the nuclear localization signal (NLS) (squared box). The position of exon 5 (black) and neighboring sequences that are excluded in the 4′–6′ isoform (gray) is indicated as well as the position of the γ-like and γ region of Mef2ca and Mef2cb respectively. Exon numbering is reported and the number of amino acids is indicated on the bar above. Mef2ca forms are named according to whether or not the exon 5 and neighboring regions or the γ-like region are present (Mef2ca 4–5–6, 4–6, 4′–6′, Δ γ-like). Mef2cb forms are named according to whether or not the octapeptide (*) in the exon 5 region is present or not (Mef2cbL and Mef2cbS). C) Details of the alternative splicing events that take place respectively: i. In the exon 5 region of *mef2ca*, showing the consensus and the non-canonical splice sites and the three species of mRNA generated; ii. In the γ region of *mef2ca*, splicing through a non-canonical CA alternative 5′ splice site in exon 8 and a canonical alternative 3′ splice site in exon 9 gives rise to the deletion of the γ-like region; iii. Exon 5 region of *mef2cb* transcript, the cartoon shows the sequence of the intron 5 that can be alternatively included in *mef2cb* transcripts, the competing donor splice sites (GT) and the two species of mRNA generated.

(Ambion). All RNAs were injected at 1–2 cell stage embryos at 10 pg, 25 pg or 50 pg/embryo. Tetramethyl-rhodamine Dextran (5% solution in 0.2 M KCl) was co-injected in order to sort phenotypes of injected embryos. At 20–28 hpf injected embryos were analyzed and sorted using a Zeiss Axiophot with AxioCam.

2.8. Western blot analysis and antibodies

Zebrafish embryos were dechorionated and lysed in RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Na Deoxycholate, 1% Igepal, 0.1% SDS, 1 mM DTT) containing 1 mM PMSF and Proteases Inhibitor Complete cocktail (ROCHE). Equal amounts of protein extracts were separated by SDS-PAGE and subsequently analyzed by Western blot as previously described [28]. The following antibodies were used: rabbit polyclonal anti-MEF2 (sc-313X; Santa Cruz Biotechnology, Inc.), mouse anti-αTubulin (T6074, Sigma Aldrich), mouse anti-Vinculin (V4505, Sigma Aldrich). Embryo staining was performed with a primary antibody against sarcomeric myosin heavy chain (MyHC; A4.1025 [41]) as previously described [8].

3. Results

To compare alternative splicing in *MEF2C* genes across species and paralogues, we use a standard nomenclature numbering exons 5′ to 3′ from the first coding exon, yet retaining the conventional α, β, γ designation for alternatively spliced exons. Each exon number thus corresponds to homologous sequences (Figs. 1A and S1).

3.1. Alternative splicing of zebrafish mef2ca and mef2cb

To predict splice variants of the zebrafish Mef2c proteins, we conducted in silico analysis of the zebrafish *mef2ca* and *mef2cb* genes on public databases (see Materials and methods). In addition to the known *mef2ca* transcript [32,42], we detected two alternative 5′UTR sequences and several alternative splices (Fig. 1A and B). *mef2ca* lacks an alternative exon 3, the α exon, having a single exon most similar to the 3α1 form of amniote *MEF2C*, which has serine residues at positions 98 and 109 [43]. *Mef2ca* contains a putative β exon in intron 6 and, in addition, a γ-region flanked by a non-canonical 3′ splice site (GC) was found at the start of exon 9 (Fig. S2A). Of particular note in the in silico analysis, were three alternative splices in the region of exons 4, 5 and 6 (Fig. 1C), two of which correspond to the alternatively spliced δ exon (exon 5) of the *mef2c* gene product in Xenopus [24] (Fig. S1B,C). By sequence alignment we found that splicing of exon 5 is conserved among teleosts (Fig. S3). Zebrafish *mef2cb* splicing appeared more similar to amniote *MEF2C* genes than that of *mef2ca*. We predicted two 5′ UTR sequences, alternate exons 3, 3α1 and 3α2, a putative β exon, encoding the conserved octapeptide SEDVDLLL in intron 6 of *mef2cb*, and a putative γ region at the start of exon 9 (Fig. 1B). The sequences of alternative exons 3α1 and 3α2 are mostly similar to the corresponding alternate exons of amniote *MEF2C*, although neither of the two α exons contains a PKA target residue corresponding to serine 120 of MEF2D, which was found to direct binding of repressive or activating cofactors [23]. Additionally, we found a *mef2cb* variant with a long exon 5 resulting from a retained intron 5 sequence. We designate this Mef2cbL to distinguish it from the conventional exon 5 in Mef2cbS (Fig. 1B, C).

To characterize the major *mef2ca* and *mef2cb* spliced isoforms expressed in developing zebrafish skeletal muscle, we performed RT-PCR on RNA extracted from the dissected tail region of 24 hpf embryos using primer pairs that target conserved sequences (Fig. 1B). A series of *mef2ca* mRNA RT-PCR products were amplified, sub-cloned and their sequences compared to the nucleotide sequence of *mef2ca* genomic DNA, revealing the existence of at least four species of *mef2ca* mRNAs produced by AS in developing embryos (Fig. 1B). In addition to the transcript encoding the full length protein, here referred to as Mef2ca 4–5–6 (465 aa), two *mef2ca* isoforms, Mef2ca 4–6 (451 aa) and Mef2ca 4′–6′ (413 aa) derive, respectively, from skipping exon 5 or a larger region that also encompasses part of exons 4 and 6. Another variant, Mef2ca Δγ-like (411 aa) contains exon 5 but lacks the γ region and further sequences located in exons 8 and 9. The Mef2ca 4′–6′ and Mef2ca Δγ-like mRNAs are the results of splicing at the non-canonical 5′ donor splice sites GG and CA, respectively (Fig. 1C) (GenBank accession numbers: KF932282 and KF932281 respectively).

One *mef2cb* variant, named Mef2cbL, was obtained by RT-PCR; it includes exon 3α1 and γ but lacks exon β. Mef2cbL arises from the inclusion of an additional sequence from intron 5 (Fig. 1C). Indeed, two competing 5′ splice sites are present at the end of exon 5, these splice sites direct inclusion or exclusion of 24 nucleotides (nt) encoding the octapeptide KDGIPTYY (Fig. 1C). When aligned (Fig. S2B), the predicted amino acid sequences of the identified zebrafish *mef2ca* and *mef2cb* isoforms show that the major variation occurs in the exon 4–5–6 region of both genes, located between the two TADs described previously [44,45].

The splicing pattern and the octapeptide sequence of Mef2cbL, appear to be conserved in other teleosts (Fig. S3). cDNA sequences from medaka (*Oryzias latipes*) and cavefish (*Sinocyclocheilus anophtalmus* and *Sinocyclocheilus angustiporus*) have a similar sequence at the end of intron 5 as in the *mef2cbL* homologue (Fig. S3). Such sequence conservation across the major teleost clades, combined with the location between TAD1 and TAD2, suggest that AS in the exon 5 region is functionally significant.

3.2. mef2ca is the main Mef2c orthologue expressed during skeletal muscle development

We sought to characterize the temporal and spatial expression patterns of *mef2ca* and *mef2cb* in developing zebrafish skeletal muscle. First, we quantified the expression levels of *mef2ca* and *mef2cb* transcripts by quantitative qRTPCR amplification using paralogue-specific primers, starting from equal amounts of RNA collected from zebrafish embryos at sequential developmental stages (from 12 to 72 hpf). *mef2ca* and *mef2cb* presented a similar profile of expression, with a higher abundance of the transcripts of *mef2ca* at all stages analyzed. Both genes were expressed at low levels at 12 hpf (*mef2ca* 2^6 copies/2.5 ng RNA and *mef2cb* 2^5 copies/2.5 ng RNA). The total number of mRNA copies increased by 24 hpf, when the first massive wave of muscle fibers differentiates (*mef2ca* 2^{10}/2.5 ng RNA, *mef2cb* 2^7/2.5 ng RNA) and stayed stable at later stages (Fig. 2A). These results were confirmed by a semi-quantitative PCR experiment (Fig. S4A).

These observations were confirmed by whole mount in situ mRNA hybridization on developing zebrafish embryos using probes specific for either *mef2ca* or *mef2cb* transcripts (Figs. 2B and S4B). At 11 somite stage (ss), *mef2ca* and *mef2cb* transcripts display an overlapping expression pattern in the adaxial cells next to the notochord and in the bilateral heart fields (Fig. 2B) [8]. At 24 hpf, most *mef2ca* mRNA is skeletal muscle-specific where it follows the expression of *myod* [14,32] (Figs. 2B and S4B). In contrast, the transcripts of *mef2cb* are detected in the developing heart, blood vessels and telencephalon, as well as somitic muscle [8] (Figs. 2B and S4B). In summary, *mef2ca* is the more abundantly expressed in skeletal muscle of the two *Mef2c* paralogues.

3.3. Developmentally regulated expression of mef2ca and mef2cb splice variants

Levels of expression of alternatively spliced *mef2ca* and *mef2cb* during zebrafish development were determined by semi-quantitative RT-PCR and qRTPCR RNA quantification using SYBR and exon boundary spanning primers, that allow for selective PCR amplification of individual alternative transcripts [30]. At 12 hpf, the amount of *mef2ca* 4–6 transcript (lacking exon 5) represents about 30% of the total, whereas the amount of the full length 4–5–6 transcript the remaining 70%. At 24 hpf and beyond, *mef2ca* 4–6 expression increased slightly but

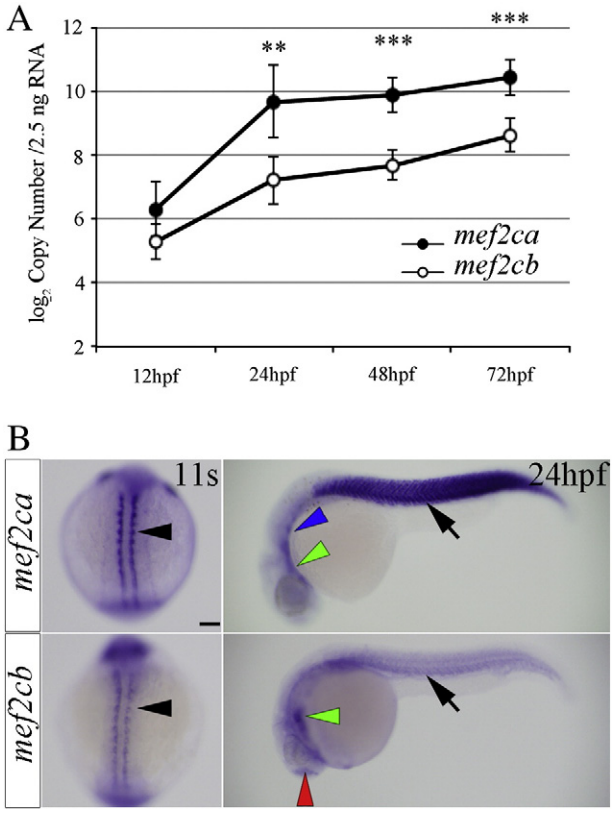


Fig. 2. Expression of *mef2ca* and *mef2cb* genes in zebrafish embryos. A) Estimation of absolute *mef2ca* and *mef2cb* transcripts by qRTPCR during *D. rerio* development. The graph shows transcript-specific absolute quantification, reported as $\log_2$ copy number in equal amount of total RNA (2.5 ng) extracted from zebrafish embryos at 12, 24, 48 and 72 hpf. Graph showing mean ± SE from four independent experiments, ** and *** indicate P-values of ≤0.01 and ≤0.001 respectively. B) Wholemount in situ hybridization for *mef2ca* and *mef2cb* mRNA for embryos at 11 ss (dorsal view, anterior to top) and at 24 hpf (lateral view, anterior to left). At 11 ss, both genes are expressed in the adaxial cells (black arrowheads). By 24 hpf *mef2ca* is strongly expressed in the myotome (black arrows) and also in the heart (green arrowhead) and branchial arches (blue arrowhead). *mef2cb* transcripts are detected in the heart (green arrowhead), telencephalon (red arrowhead) and are weakly detected in the somites (black arrow). Scale bars = 100 μm.

remained less abundant than the *mef2ca* 4–5–6, whose predominance increases further (80% of the total *mef2ca* transcripts) (Fig. 3B–C). The shortest isoform, *mef2ca* 4′–6′ is present at low level (less than 1% of the total *mef2ca* transcripts) at every developmental stage and was therefore not considered further. *mef2ca* transcripts containing the β exon were barely detectable and were found exclusively at 72 hpf after five additional cycles of PCR amplification (data not shown). Transcripts without the γ-like region were expressed at early stages of development. However, they were less abundant, and were not detected beyond 24 hpf (Fig. 3A,B). Thus, almost all *mef2ca* transcripts contain the γ-like region and lack β exon, irrespective of their splicing at the 4–5–6 region.

Expression of the *mef2cb* alternatively spliced exons was also determined by semi-quantitative RT-PCR and qRTPCR amplification. Transcripts containing exons 3α1 and 3α2 were detected throughout development using common primers that give two amplicons of different size and therefore electrophoretically distinguishable. The transcript that includes the 3α1 exon is the most abundant at all the developmental stages beyond 12 hpf (Fig. 3E). This result was also confirmed by using isoform-specific primers, given that, in identical experimental conditions, four additional PCR cycles are required to amplify an amount of exon 3α2-containing DNA similar to that containing exon 3α1 (Fig. S5A). Whereas the inclusion of exon 3α1 predominates in the developing embryo, RT-PCR analysis revealed that in adult skeletal and cardiac muscle the levels of the two isoforms are comparable (Fig. S5B). We did not detect the 3α2-containing transcript in the liver and brain, indicating a muscle-restricted pattern of expression of this splice variant, analogously to what has been reported for the mammalian counterpart (Fig. S5B) [20]. Inclusion of exon β was barely detected. In contrast, the γ region and the extra sequence from intron 5 (Mef2cbL) were readily detected at all developmental stages (Fig. 3D, E). qRTPCR quantification confirmed that more than 90% of *mef2cb* transcripts retain the extra intron 5 sequence, but less than 10% encode the Mef2cbS form (Fig. 3F). Thus, both *mef2ca* and *mef2cb* show striking variations in the exon 4–5–6 region.

3.4. Exon 5-containing mef2ca transcripts accumulate in skeletal muscle

To examine where *mef2ca* mRNA(s) that include exon 5 are expressed in the developing zebrafish embryo, we performed in situ mRNA hybridization using a probe that recognizes all transcripts (*mef2ca* probe, [32]) and two non-overlapping dual digoxigenin-labelled LNA probes designed to recognize 21 base pair sequences located within exon 5 (LNA1) or within the exon 4/5 boundary (LNA2) (Fig. 4C). In 24 hpf embryos, *mef2ca* transcripts were detected throughout the somitic muscle and preferentially observed at somite borders (Fig. 4A upper panel), as well as in the heart and branchial arches (Fig. 4A upper panel, see also Fig. 2B). Similarly, both the exon 5-specific LNA probes gave signals above background only in skeletal muscle, preferentially observed at somite borders (Fig. 4A, middle and lower panels). By 48 hpf, the signals obtained with the generic and both exon 5-specific probes, are restricted almost entirely to the somite boundaries area (Fig. 4B, left and [14]). In addition, signals with all three probes show the typical separate dorsal and ventral muscle signal in the pectoral fin (Fig. 4B, right). Thus, even though we cannot exclude some levels of expression in other tissues, we conclude that the *mef2ca* 4–5–6 transcript is expressed primarily in skeletal muscle and is mainly localized to somite boundaries, suggesting it may have a distinct and specific function.

3.5. Mef2ca 4–5–6 is a potent transactivator

The transcriptional activities of *mef2ca* splice variants were tested in vitro by co-transfection into COS-1 cells of each Mef2c splice variant with a MEF2-responsive luciferase reporter containing three copies of the MEF2 binding site from the *Desmin* gene regulatory region (pGL3desMEF2) [46]. COS-1 cells have low endogenous MEF2 expression. Immunofluorescence analysis revealed that all Mef2ca and Mef2cb splice variants efficiently localized to the nucleus (data not shown), congruent with the observation that they all include the sequence corresponding to the nuclear localization signal described in the mouse [47].

Compared to other Mef2c isoforms tested, the Mef2ca 4–5–6 full length protein had the strongest transcriptional activity (Fig. 5A). Deletion of amino acids encoded by exon 5 and neighboring sequences results in a twofold reduction in transcriptional activity, even though the respective protein expression levels were comparable (Fig. 5B). Furthermore, we observed that a Mef2ca 4–5–6 isoform lacking the γ-like domain had 2-fold higher transcriptional activity than Mef2ca containing the γ-like domain, consistent with the finding that this region represses transcription (data not shown; [21]). Upon transfection, the Mef2cbL and Mef2cbS isoforms, each containing both exon 5 and γ, exhibited similar activity (about 70% of that of Mef2ca 4–5–6) (Fig. 5A and data not shown). However, Mef2cbL immunoreactivity was much lower than the Mef2ca isoforms (Fig. 5B). Given that we obtained similar results with other antibodies directed against different regions of MEF2 proteins (data not shown), it is unlikely that the low amount of Mef2cb protein detected is due to the low reactivity of our anti-Mef2 antiserum. Additional studies are required to characterize the stability and translational efficiency of Mef2c proteins, but our results suggest that Mef2cbL has higher activity per molecule than Mef2ca 4–5–6. Taken

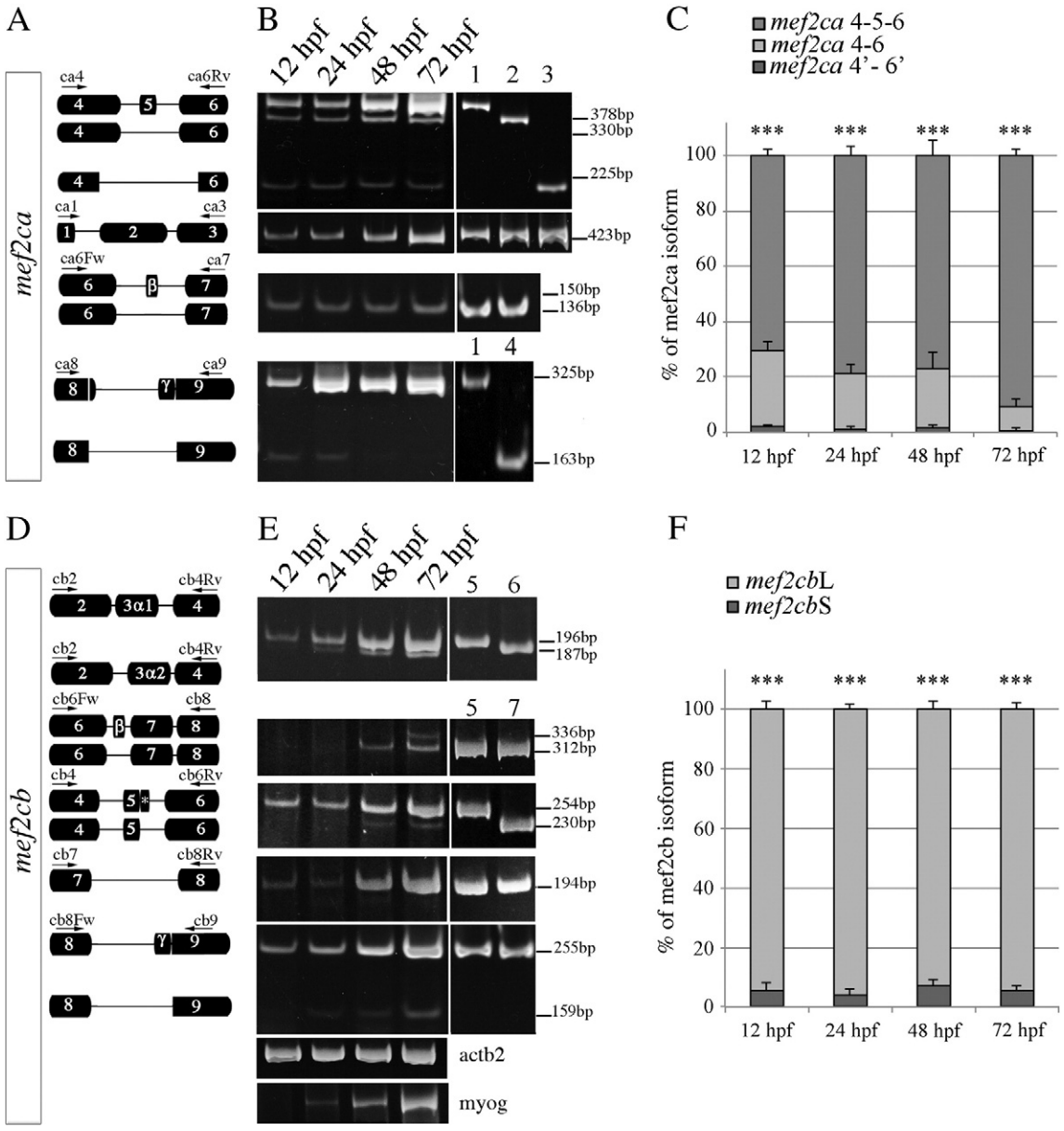


Fig. 3. Developmental expression profile of *mef2ca* and *mef2cb* alternative splicing products. A) Schematic representation of Mef2ca alternative exons. Arrows show primers annealing sites used in the RT-PCR analysis. B) Expression analysis of alternative splicing variants of *mef2ca* transcripts by RT-PCR. Total RNA was purified from staged embryos at 12, 24, 48 and 72 hpf. PCR was performed using primers that give amplification products of different sizes depending on the splice variant. The level of expression of total *mef2ca* transcripts was evaluated by using two primers (ca1 and ca3) that amplify a region not alternatively spliced between exons 1 and 3. Amplification of plasmid vectors containing the cDNAs of the various *mef2ca* splice variants cloned into the pcDNA 3.1 vector was used as controls of the correct size of expected amplicons: *mef2ca* 4–5–6 γ + (lane 1), *mef2ca* 4–6 γ + (lane 2), *mef2ca* 4′–6′ γ + (lane 3), and *mef2ca* 4–5–6 γ- (lane 4). PCR products were separated in 8% polyacrylamide gels. Length of PCR products in base pairs (bp) is indicated. C) Quantitative analysis of the mRNA levels of *mef2ca* exon 5 splice variants during *D. rerio* development. The amount of the transcripts of each splice variant was estimated by absolute qRTPCR. Original data (mRNA levels of each isoform) are reported as % of the total number of *mef2ca* transcripts (4–5–6 + 4–6 + 4′–6′ = 100%). Statistical analysis was performed on data obtained from three independent experiments, the means ± SE are represented. *** indicates a P-value ≤ 0.001. D) Schematic representations of Mef2cb alternative exons. Arrows show primers annealing sites. E) Developmental RT-PCR analysis of *mef2cb* mRNAs. To evaluate the amount of 3α1- and 3α2-containing *mef2cb* transcripts, we designed common PCR primers (cb2 and cb4Rv) annealing to flanking regions in exons 2 and 4 to generate two amplicons of different sizes: a 196-bp (3α1) and a 187-bp (3α2) RT-PCR products respectively. Flanking primers were also designed to investigate the expression of exon β, the extra sequence of intron 5 (*) and of the γ region. As control templates we used the pcDNA 3.1 expression vector containing the cDNAs of Mef2cbL3α1 β- γ + (lane 5), Mef2cbL3α2 β- γ + (lane 6) and Mef2cbS 3α1 β- γ + (lane 7). *actb2* was used as a control, *myog* was used as a marker for skeletal muscle differentiation.

together, these data suggest that inclusion of exon 5 between TAD1 and TAD2 confers increased activity to Mef2ca.

3.6. Mef2cbL has unique myogenic potential in developing zebrafish

To investigate the biological significance of Mef2ca and Mef2cb splice variants in zebrafish embryonic development, we determined the effects of their ectopic expression by injecting embryos at the one-cell stage with synthetic Mef2c mRNAs and analyzing them at 24 hpf. We have shown previously that injection of mRNA of *mef2cb* induces ectopic skeletal muscle in embryos [8]. Here we report that injection of 10 pg/embryo of mRNAs of the Mef2cbL isoform induced ectopic skeletal muscle in the anterior mesoderm of 40% of the injected embryos, as revealed by wholemount in situ hybridization for *myod* mRNA in 28 hpf zebrafish embryo, a developmental stage where no endogenous muscle is normally observed in the head (Fig. 6A,B). In addition to *myod* transcripts we detected ectopic expression of *smyhc1* transcripts and MyHC protein, further supporting the pro-myogenic activity of Mef2cbL (Fig. 6B). No induction of ectopic muscle was observed after ectopically expressing any Mef2ca isoform, even when higher quantities of mRNA

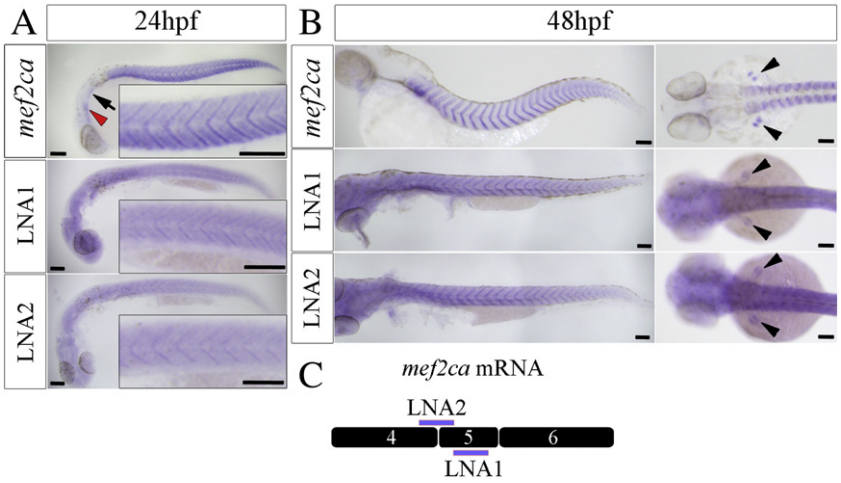


Fig. 4. WISH analysis of zebrafish *mef2ca* transcripts in developing zebrafish embryos. In situ hybridization using *mef2ca* and *mef2ca*-exon 5 specific probes as indicated. A) Lateral view of 24 hpf embryos. *mef2ca* mRNA localizes to both central and peripheral regions of the muscles in the somite, and also to the developing heart and branchial arches (red arrowhead and black arrow respectively). Exon 5 specific transcripts are detected by both LNA probes in a similar way in the muscle, with a slightly stronger expression at somite borders (see insets for magnified somatic muscle area). B, left panels.) Lateral view of 48 hpf embryos, anterior to left. *mef2ca* general and both LNA1 and LNA2 exon 5-specific probes show overlapping signals enriched at fiber ends. Right panels.) Dorsal view of the same embryos, anterior to left. *mef2ca* and both LNA probes detect expression in the pectoral fin dorsal and ventral muscle masses (black arrowheads). Scale bars = 100 μm. C) Drawing of the LNA1 and LNA2 probes annealing positions within the exon 4/5 region.

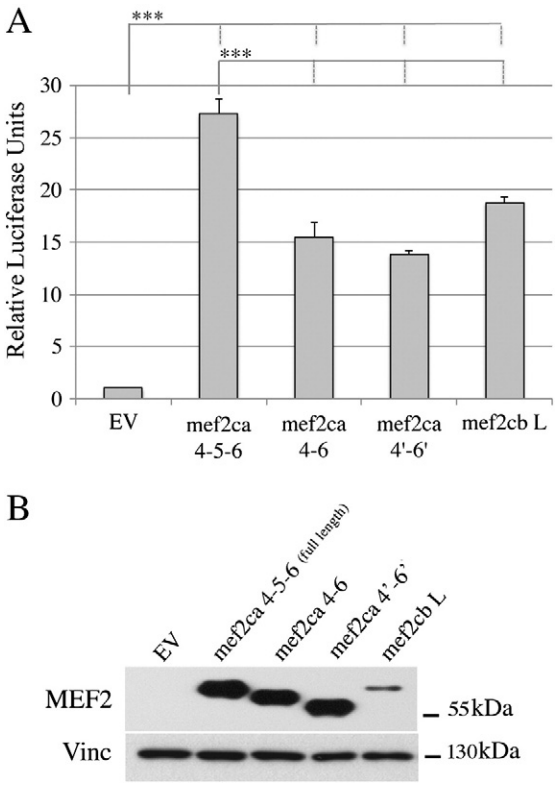


Fig. 5. Transcriptional activity of zebrafish Mef2ca and Mef2cb splice variants. A) COS-1 cells were co-transfected with pGL3(desMEF2)$_3$ luciferase, the pRSVβ-gal reporter control and CMV (Cytomegalovirus)-driven expression plasmids encoding for the indicated Mef2c splicing isoforms. Firefly luciferase activities were normalized for transfection efficiency against the β galactosidase activity and expressed as relative luciferase units of the activity in cells transfected with the Empty Vector (EV) (=1.0). Statistical analysis was performed on data obtained from three independent experiments, the means ± SE (error bars) are represented. *** indicates a P-value ≤0.001. B) Extracts from cells transfected in panel A were resolved by SDS PAGE, Mef2ca and Mef2cb expression was assessed by immunoblotting with anti-MEF2 antibody that recognizes all Mef2ca and Mef2cb splicing isoforms (upper panel). Sample loading was normalized using Vinculin immunoreactivity (lower panel).

were injected (Fig. 7). Interestingly, this effect depends on the inclusion of the KDGIPTYY octapeptide, because forced expression of the Mef2cbS isoform did not cause ectopic myogenesis (Fig. 6A, B). Thus, the form of Mef2cb that is normally present in developing zebrafish embryos during somitogenesis has unique myogenic potential that is not shared by Mef2ca 4–5–6, the predominant Mef2c isoform in skeletal muscle. Injection of higher amounts (25 pg/embryo) of both Mef2cbS and Mef2cbL mRNAs resulted in head and trunk developmental alterations (Fig. 6C).

3.7. *Mef2ca 4–5–6 over-expression causes defects in gastrulation*

To investigate the functionality of the two main Mef2ca isoforms expressed during development (Mef2ca 4–5–6 and 4–6), high doses of Mef2ca mRNAs were employed. Injection of 25 pg of full length Mef2ca 4–5–6 RNA had dramatic effects on embryonic development, inducing lethality in approximately 30% of the embryos and marked developmental defects in 49% of the surviving embryos, classified as 'severely defective' (Fig. S6A,B). Such embryos already had defects evident at gastrulation stages (6–8 hpf, data not shown). Among the surviving embryos, a further 34% exhibited a milder phenotype classified as 'defective', with trunk convergent extension defects, occasional double axes, and some brain defects such as undeveloped eyes and absence of mid- and forebrain structures (Fig. 7A). Only 16% of embryos appeared unaffected by the Mef2ca 4–5–6 RNA. The percentage of severely defective embryos increased in a dose-dependent manner upon increasing the amount of injected RNA (Fig. S6B). In contrast to Mef2ca 4–5–6, forced expression of the Mef2ca 4–6 isoform was less active, having no detectable effect on the development of most (85%) of the injected embryos, even when expressed at comparable levels to Mef2ca 4–5–6 (Figs. 7A and S6B,C). These results indicate that ectopic Mef2ca activity in early stages disrupts normal development. The gross defects in gastrulation induced by over-expressed Mef2ca 4–5–6 suggested severe tissue patterning disruption, yet a survey of cell lineage markers revealed no indication of altered cell fates at lower doses of RNA (Fig. S6D).

To gain more insight into the mechanisms underlying the ability of Mef2ca 4–5–6 to disrupt development, the expression levels of genes encoding transcription factors and signaling molecules that are involved

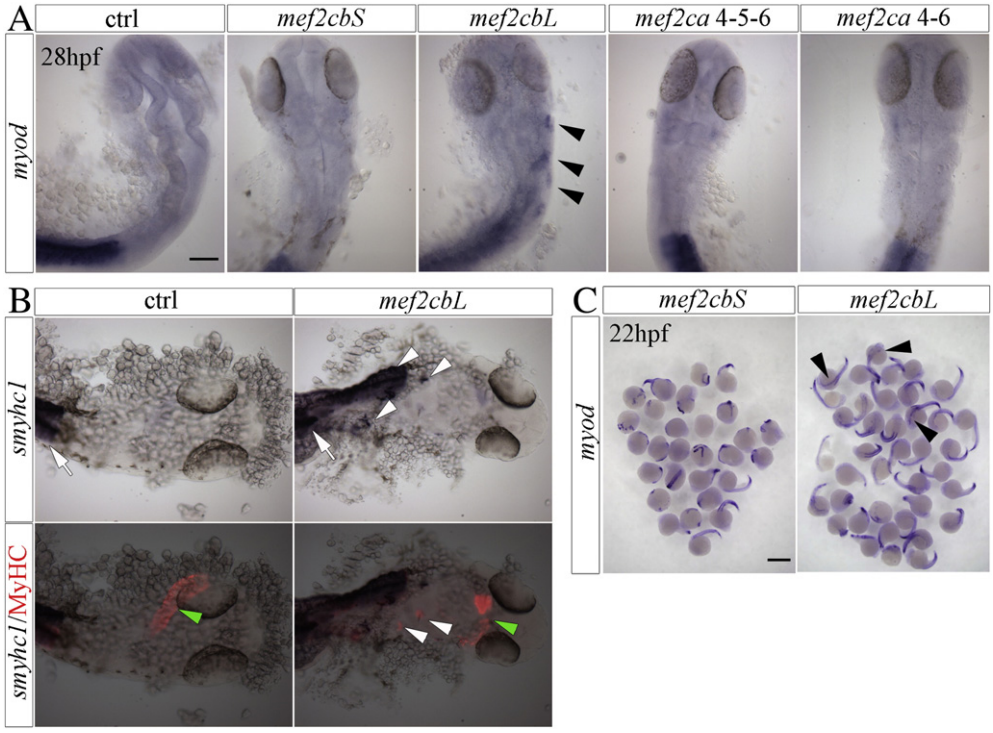


Fig. 6. Effects of Mef2cbL forced expression in zebrafish embryos. Wholemount in situ mRNA hybridization of zebrafish embryos injected with in-vitro transcribed mRNA encoding Mef2c isoforms together with Rhodamine dextran at the 1-cell stage. Injected embryos or uninjected control embryos were analyzed during development. A) *Myod* mRNA in 22 hpf embryos injected with 25 pg of *mef2cb* mRNAs. mef2cbL but not mef2cbS injected embryos have ectopic myod expression in head region (arrowheads). Both groups show an array of developmental defects in the head and trunk regions. B) *Myod* mRNA in head region at 28 hpf (dorsal view, anterior to top). Injection of 10 pg of Mef2cbL mRNA induces ectopic *myod* expression in head mesoderm (arrowheads). C) *smyhc1* mRNA and immunofluorescence of MyHC protein in 28 hpf non-injected control embryos or embryos injected with 10 pg of Mef2cbL mRNA, ectopic muscle is clearly seen in the head region of injected embryos (white arrowheads). While arrow and green arrowhead indicate somitic muscle and heart respectively. Scale bars = 100 μm.

in early patterning of the embryo were screened by semi-quantitative RT-PCR. The *chd* gene, encoding a BMP (Bone Morphogenetic Protein) antagonist involved in dorsoventral patterning of early embryos [48] (reviewed in [49,50]), was up-regulated (2-fold) in embryos injected with the *mef2ca* 4–5–6 mRNA, but not in those injected with the 4–6 spliced isoform (Fig. 7B,C). Mef2ca 4–5–6 also induced the expression of *ndr1* (1.5-fold), *gsc* (2.2-fold) and other dorsally-expressed genes (*no-tail a*, *noggin 1*), and reduced the expression of ventralizing factors such as *bmp7a* (0.4-fold) and Δ*np63* (0.2-fold), but did not alter the transcript level of *myod* or *no-tail b* and *bmp2b* (Fig. 7B,C), suggesting that the protein sequence encoded by exon 5 can modulate the expression level of a specific subset of early embryonic genes.

3.8. Mef2ca 4–5–6 mRNA is the prevalent Mef2c transcript present in the embryo before gastrulation

Our data indicate that forced expression of Mef2ca 4–5–6 protein induces the ectopic expression of genes involved in early dorso-ventral patterning of the embryo. In an attempt to get more insight into a putative role of Mef2ca in controlling endogenous patterning genes, we next determined the expression and alternative splicing patterns of *mef2c* genes during early stages of development and compared them to those of two of their putative target genes, i.e. *chd* and *gsc*. To this aim we performed RT-PCR analysis of the RNA from zebrafish embryos harvested at the 1K-cell (3 hpf), 50% epiboly (5.25 hpf) and bud (9–10 hpf) stages. Our analysis revealed that *mef2ca* transcripts are already detectable as early as at the 1K-cell stage, with predominant expression of the transcript including exon 5 (Fig. 8). We noticed a rapid loss of the 4–5–6 transcript that became undetectable by 10 hpf when the 4–6 mRNA is the only *mef2ca* transcript detected, inclusion of exon 5 is again detected later, by 12 hpf (Fig. 3) and the 4–5–6 full length transcript predominates upon muscle differentiation. The kinetics of expression of the *mef2ca* 4–5–6 transcript suggests that it might be of maternal origin. The presence of Mef2ca 4–5–6 transcripts early in development, which is temporally coincident with *gsc* expression and overlaps partially with that of *chd* (our data and [51]) is consistent with a role of this *mef2ca* splice variant in dorso-ventral patterning. No *mef2cb* expression is detected prior to 50% epiboly, in mid-gastrulation, where only the *mef2cbS* transcript is present. Nonetheless, at the onset of somitogenesis (9–10 hpf), we noticed that only the transcript encoding for Mef2cbL, the pro-myogenic variant, is expressed.

4. Discussion

Alternative splicing of transcription factors can have a wide impact on the regulation of transcriptional networks. However, the relevance of alternative splicing is often unclear as distinct roles of alternatively spliced isoforms are often not determined. In this study, we addressed the functions of alternatively spliced isoforms of zebrafish Mef2ca and Mef2cb, two transcription factors involved in the development of striated muscle and head skeletal patterning [8,14,16,42]. *Mef2c* mRNA is alternatively spliced in several organisms [20–22,24,52], and a recent report suggests that aberrant splice variants of MEF2C are involved in myogenic disorders [53]. Nevertheless, the functional differences between alternatively spliced Mef2C variants remain elusive. Our findings make three major points regarding the function of alternative splicing in Mef2c proteins of teleost fish. Firstly, both *mef2ca* and *mef2cb* gene transcripts undergo specific alternative splicing and their splicing patterns change during development. Secondly, splicing of *mef2ca* transcripts to include the exon 5 enhances its positive transcriptional activity and ability to interfere with gastrulation when over-expressed. Thirdly, an evolutionarily conserved alternate splice of exon 5 in *mef2cb* transcripts creates a long form that has unique pro-myogenic capacity.

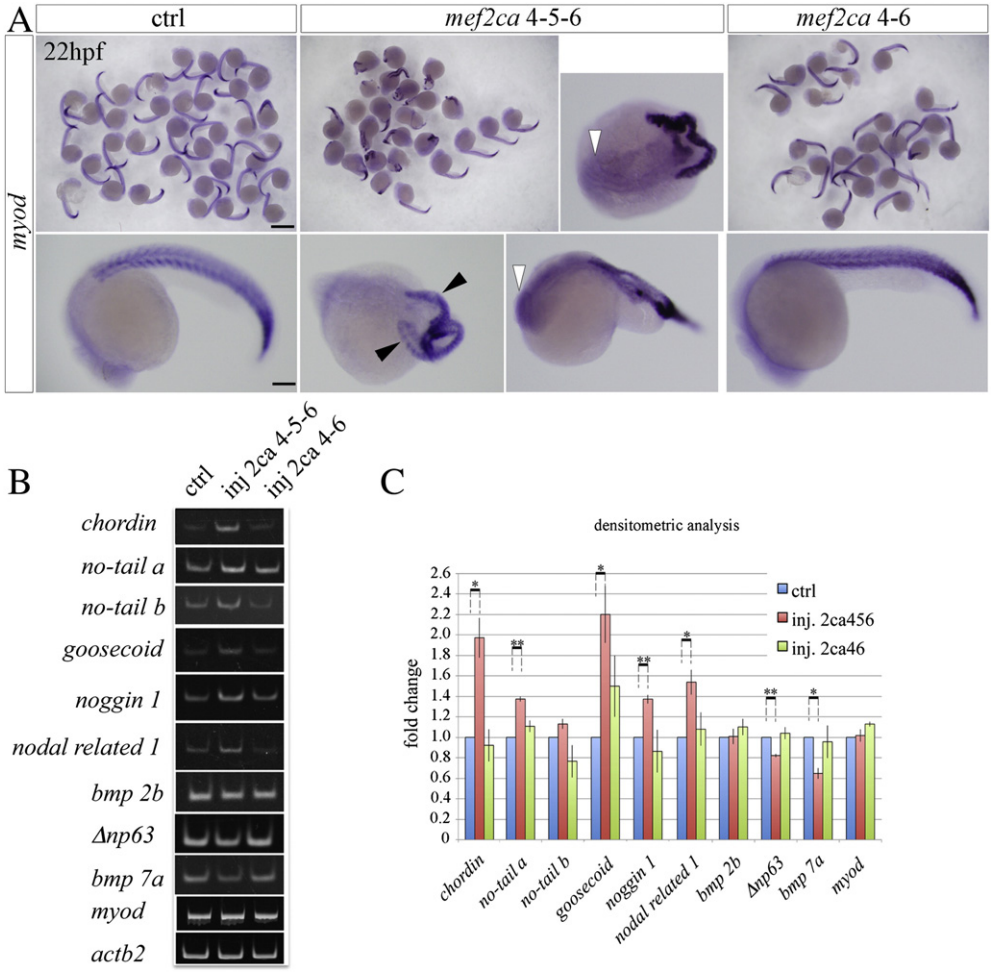


Fig. 7. Effects of forced expression of mef2ca splice variants on development of zebrafish embryo. A) *Myod* mRNA in 22 hpf embryos injected with the mRNAs of Mef2ca splice variants or not injected (control). Forced expression of Mef2ca 4–5–6 mRNA resulted in severe developmental defects: double axis (black arrowheads), trunk and brain defects (white arrowheads). Control embryos or embryos injected with 25 pg of Mef2ca 4–6 mRNA showed normal morphology. B) RT-PCR analysis of the total RNA extracted from 25 pg *mef2ca* mRNA injected or control uninjected embryos at 22 hpf. Mef2ca 4–5–6 injected embryos showed augmented expression of *chordin*, *no-tail a*, *nodal related 1*, *noggin 1* and *goosecoid*, reduced expression of *Δnp63* and *bmp 7a*, whereas *bmp 2b*, *no-tail b* and *myod* expression levels are unaffected. C) Densitometric analysis of the bands shown in B, normalized to *actb2* signal. Expression levels of each gene were arbitrarily set to a value of 1 in the uninjected control embryos. Statistical analysis was performed on data obtained from three independent experiments, the means ± SE (error bars) are represented. * and ** indicate P-values of ≤0.05 and ≤0.01 respectively.

4.1. Regulation of Mef2ca activity by alternative splicing in zebrafish development

In addition to the well documented expression of *mef2ca* starting from 12 hpf [32], we found *mef2ca* transcripts in zebrafish embryo prior to gastrulation at the 1K-cell stage, likely from maternal contribution, with their level declining to a minimum at 9–10 hpf. Starting from 12 hpf we observe an overall increase in expression levels of *mef2ca* mRNA. Besides changes in the abundance of *mef2ca* transcripts, we found a dynamic regulation of the splicing in the exon 5 region: the *mef2ca* variant including exon 5 (*mef2ca* 4–5–6) is the major isoform detected very early in development (1K-cell stage), suggesting that it might play a role prior to gastrulation, by 10 hpf, the *mef2ca* transcripts lacking exon 5 (*mef2ca* 4–6) are predominant. Subsequently, *mef2ca* 4–5–6 again climbs as muscle precursors undergo terminal differentiation, becoming the predominant isoform at 24 hpf. Such splicing of exon 5 is evolutionary conserved between Xenopus and teleosts, suggesting it has biological significance [24] (Fig. S3). Moreover, muscle differentiation in zebrafish is associated with several other muscle-specific alternative splicing events involving changes in splicing efficiency [54]. Although no specific function was assigned to the exon 5 domain by mutational and deletion analysis of the mouse and human protein counterparts [44,45], our cell culture data indicate that the peptide sequence encoded by exon 5 contributes to the transcriptional activity of Mef2ca. The early expression of *mef2ca* transcripts including exon 5 (mef2ca 4–5–6) may indicate their early function in embryo patterning. Later in development, the preferential accumulation of full length *mef2ca 4–5–6* mRNA at skeletal muscle fiber ends, suggests that its normal function is in muscle, a view confirmed by the requirement for Mef2ca function for skeletal muscle fiber growth and heart myogenesis [8,55]. In the current work the function of Mef2ca isoforms was probed by ectopic over-expression; *mef2ca* 4–5–6 RNA, but not *mef2ca 4–6* RNA, causes gross defects during gastrulation. We suggest that these effects of Mef2ca 4–5–6 are attributable to its ability to activate, directly or indirectly, a specific subset of pivotal genes in gastrulation. We observed the induction in *chd* (2-fold) mRNA and a milder (1.4 fold) increase in *noggin 1* (*nog1*) mRNAs that encode two inhibitors of the BMP signaling. *chd* is required to repress *bmp2b* function in formation of the organizer and dorsoventral patterning of mesoderm and neural tissue [56–58]. Over-expression of *chd* dorsalizes embryos [59,60], a phenotype present in a fraction of embryos following Mef2ca 4–5–6 over-expression. Thus, up-regulation of these dorsalizing proteins may explain the effects of Mef2ca 4–5–6.

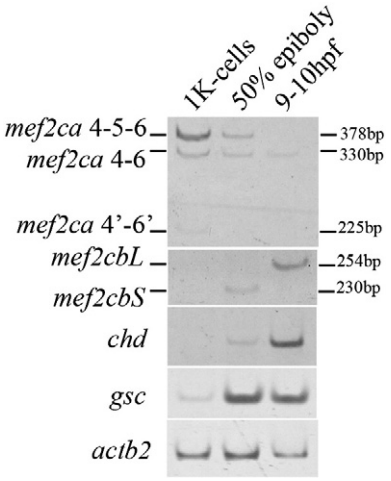


Fig. 8. Expression of *mef2ca* and *mef2cb* splice variants during early zebrafish development. Expression levels of the *mef2ca* and *mef2cb* splice variants in exon 5 region were evaluated by RT-PCR analysis of RNA harvested from zebrafish embryos at the indicated developmental stages. PCR was performed using primers that give amplification products of different sizes depending on the splice variant, as schematized in Fig. 3A. PCR products were separated in 8% polyacrylamide gels. Length of PCR products is indicated. Expression levels of *gsc* and *chd* were also determined. Expression levels of *actb2* RNA are shown as loading control.

In Xenopus, MEF2D helps induce mesoderm by driving the expression of the *Nodal-related 1* (*ndr1*) gene [61]. In zebrafish, Mef2ca 4–5–6 over-expression also increases in *gsc* and *ndr1* mRNAs (2.2- and 1.5-folds, respectively), which regulate dorsoventral patterning in organisms ranging from *Drosophila* to mammals [49,50,62–64]. In line with our results it has been previously reported that expression of *gsc*, is reduced in *mef2ca* $^{-/-}$ (*hoover*) mutants [42]. Although we cannot exclude off-target effects, this specific ability of Mef2ca 4–5–6, but not of similar amounts of Mef2ca 4–6, suggests distinct transcriptional activity of the former. In silico analysis of promoter regions of *chd* and *ndr1* genes revealed the presence of several putative MEF2 binding sites (YTA(A/T)$_4$TAR) (data not shown), raising the possibility that Mef2ca 4–5–6 directly activates their expression during early development. Later in development *chordin* expression may be sustained by Mef2d, which constitutively includes the sequence encoded by exon 5, and which is expressed from mid-gastrulation in adaxial muscle cells that also express *chordin* [32,60] or by Mef2cb proteins. Interestingly, injection of either *mef2cbS* or *mef2cbL* transcripts, both containing exon 5, has resulted in similar developmental defects to that of *mef2ca* 4–5–6 mRNA injection. Future studies will clarify whether these genes are indeed direct targets of a Mef2 protein containing exon 5.

After gastrulation, zebrafish *mef2ca* transcripts accumulate starting from 12 hpf [14,32], and *mef2ca* 4–5–6 transcripts are particularly abundant by 24 hpf, We suggest that Mef2ca 4–5–6 function might modulate *chordin* and other target gene expression in the somites at later stages during myotome patterning, where later muscle differentiation is regulated by BMP signaling and where *chordin* expression has been observed [60,65–68].

The protein sequence encoded by exon 5 might represent a binding motif that mediates protein–protein interactions with specific co-factors, as one recognized function for alternatively spliced isoforms is to remodel the protein–protein interaction network [69]. Supporting this hypothesis is the recent demonstration that the domains encoded by the mutually exclusive α1/α2 exons of mouse MEF2D can mediate interactions with different sets of co-repressors or co-activators [23].

4.2. Gene duplication and evolutionary partitioning of alternative splicing

The importance of other splices in Mef2ca remains to be determined. The γ-like and 4′–6′ splices have low abundance and we were unable to display unique activities for these isoforms. On the other hand, unlike in mammals, exon 3 does not appear to show alternative splicing in Mef2ca, the gene only having an α1 version. As the α1 exon of mouse Mef2D mediates interactions with specific transcriptional co-regulators [23], Mef2ca may have a more restricted range of functions compared to Mef2cb, which retains alternative α exons in its genomic sequence. However, at the stages examined, transcripts of *mef2cb* containing the α2 exon had low abundance, suggesting that this splice may be significant in specific cell types or developmental stages. In the adult we found a high proportion of the *mef2cb* transcripts containing the 3α2 exon in striated muscle tissue where it might play a specific role in mediating muscle gene expression as shown for the analogous splice variant of *Mef2d* in mammals [23]. Conversely, *mef2cb* transcripts omitting exon 5 were not observed. Instead, teleost *mef2cb* has evolved a unique splice, possibly derived by exonisation [1] of a part of intron 5. The addition of this octapeptide and its conservation across teleosts appears to have conferred myogenic properties to Mef2cbL.

4.3. Alternative splicing of mef2cb gene generates a pro-myogenic transcription factor

We detected *mef2cb* transcripts in zebrafish embryo as early as 50% epiboly stage. Mef2cbL is the prevalent Mef2cb isoform starting from 9 to 10 hpf, concomitantly with the onset of somitogenesis and has a unique pro-myogenic capacity. *mef2cb* mRNA over-expression can convert cells to skeletal muscle (Fig. 6A; [8]). This result suggests a role for Mef2 as a skeletal muscle determination factor in zebrafish head, challenging the classical epistatic relationship between MyoD and MEF2 in which MyoD acts upstream of MEF2 to direct embryonic multipotent progenitors into the myogenic lineage. The myogenic activity of Mef2cbL relies on an octapeptide encoded by a short sequence of intron 5 retained in the transcript. This insert, being too short to form a domain, may act by changing the structural fold and leading to a new function of the protein [70]. Muscle conversion was not observed upon ectopic expression of Mef2cbL in mouse fibroblasts, congruent with previous observations made with the mouse MEF2 proteins [9,71,72]. Thus, we propose the existence of a specific co-factor expressed in zebrafish head mesoderm that confers myogenic capacity to Mef2cbL. Identifying Mef2cb's molecular partners recruited specifically in the presence of the octapeptide to activate the expression of *myod* and other muscle genes may help in deciphering the molecular mechanisms underlying the pro-myogenic activity of Mef2cbL.

5. Conclusions

Our data reveal novel alternative splicing events around exon 5 of zebrafish *mef2ca* and *mef2cb* transcripts. These various evolutionarily conserved transcripts expand the transcriptional range of activity of Mef2c proteins. We propose that by excluding or including sequences of the exon 5 region, Mef2cs can acquire distinct properties, which allow them to regulate different sets of target genes and execute unique developmental programs in vivo.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2014.05.003.

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