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Distinct functions of alternatively spliced isoforms encoded by zebrafish mef2ca and mef2cb.
Ganassi M, Badodi S, Polacchini A, Baruffaldi F, Battini R, Hughes SM, Hinitis Y, Molinari S.
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Manuscript Number: BBAGRM-13-217R1

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

Article Type: Regular Paper

Keywords: *Mef2ca*, *Mef2cb*, zebrafish, skeletal muscle, alternative splicing, development

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First Author: Massimo Ganassi

Order of Authors: Massimo Ganassi; Sara Badodi; Alessio Polacchini; Fiorenza Baruffaldi; Renata Battini; Simon M Hughes; Yaniv Hinitz; Susanna Molinari, Ph.D.

Abstract: In mammals, an array of MEF2C proteins are 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.

Response to Reviewers: Reviewer #1, major issues:

1. The only spatial expression analysis the authors presents in the manuscript is an in situ targeting *mef2ca* exon 5. It is puzzling why the expression of the exon5-specific transcript does not overlap with that of the generic *mef2ca* probe (since the generic probe should bind all variants including the exon 5 transcript). The expression pattern detected by the exon 5 probe (that above the noisy background) detects myosepta, but also suspiciously seem to bind to other domains in the brain, that divides brain structures. The authors need to show that this probe indeed detects the exon 5 transcript and not only bind sticks to grooves and cavities in the embryo, which a non-specific probe can appear to do when over-stained. The idea to analyze the spatial expression patterns of the different splice forms is nevertheless good and the inclusion of more of such experiments would strengthen the paper.

Response to reviewer #1 point 1

We have repeated the Wholemount In situ Hybridization assay on zebrafish embryos. In addition to the *mef2ca* generic probe (3' UTR mainly) and an exon 5-specific probe that recognizes the exon 4/5 boundary (LNA 2), we used another LNA probe (LNA 1) that anneals to a 21 bp sequence located in exon 5. We have changed the technical procedure for the LNA hybridization following recent work (Lagendijk et al., Whole mount microRNA in situ hybridization protocol for zebrafish embryos and adult tissues, *Biology Open* 1 (2012), 566–569). These new assays allowed us to get good and matching results from 2 different non-overlapping LNA probes, exhibiting striking similarities to each other and to the generic probe in the muscle staining in somitic and fin muscles. We strongly believe that these results reflect the real expression of the transcripts that include exon 5. As seen before, heart and branchial arches are either not expressing this form (4-5-6 isoform) or that the signal is too low to be detected.

2. Injection full-length *mef2ca* (4-5-6) gives rise to various malformations including some that often appear after over-dosing. The authors have done a dose response experiment, but only for (4-5-6) and not for (4-6). Inclusion of a dose response experiment where the amount of (4-6) is increased would reveal if the (4-6) transcript also can produce the same phenotype. The fact that none of the lineage markers tested supported the idea of alterations in tissue patterning, rather indicates that the phenotype can be due to toxicity. In fact, small eyes, brain defects and somite malformation are all signs of non-specific effect. The authors must present more rigorous controls. In order to state that *mef2ca* (4-5-6) has important functions during gastrulation, the authors must show that this splice form is expressed at this stage. By the look of the figures (that analyzes expression no earlier than 12 hpf), this is not very clear. To induce the *mef2ca* expression post-gastrulation by an inducible vector would focus the experiment on myogenesis.

Response to reviewer #1 point 2

In figure S6B, we present the effects of increasing amounts (25 and 50 pg/embryo) of both *Mef2ca* 4-5-6 and *Mef2ca* 4-6 mRNAs. It is evident that the effects on embryo development of the longer isoform are dose-dependent, whereas injected *Mef2ca* 4-6 mRNA did not inflict similar developmental defects even at the higher dose. These results taken together with the fact that the two mRNAs are almost identical and that the injected embryos ectopically express similar levels of the encoded proteins (Fig. S6C), support our view of a specific phenotypic effect of this splice variant.

We think that the lack of overt defects of tissue patterning shown in Fig. S6D is due to a specific effect of exogenously expressed *Mef2ca* 4-5-6 earlier in development, during the dorso-ventral patterning of the embryo, as supported by the double-axis phenotype presented by a large proportion of *mef2ca* 4-5-6 mRNA injected embryos and by the array of genes whose expression is specifically altered by the ectopic expression of *Mef2ca* 4-5-6.

In an attempt to gain more insight into a role of *mef2ca* in controlling the expression of early patterning genes, we report here for the first time, a temporal co-expression of *gsc* and *chd* with *mef2ca* transcripts including exon 5 very early during development at the 1K-cell stage of zebrafish development, as shown in figure 8 added to the revised manuscript.

As suggested by the reviewer and discussed in the text, it will be of great interest to define the specific role of the *Mef2ca* 4-5-6 full length protein during myogenesis by inducing its over-expression or knocking down specifically its endogenous expression at 9-10 hpf of development. Nevertheless, these experiments are beyond the scope of this manuscript.

3. The link to BMP signalling is interesting, but very preliminary. For starters, co-expression of *mef2* and *chd* would give the experiment higher impact. It is also not clear if the data in fig 6E is quantitative or semi quantitative. Is it based on a single PCR-experiment? In order to draw any conclusions this needs to be clarified.

Response to reviewer #1 point 3

As stated above we found a temporal co-expression of Mef2ca 4-5-6 transcript and gsc and chd very early in development, supporting the hypothesis of a role of mef2ca in controlling their expression (figure 8).

Moreover, we have repeated the injection of in vitro transcribed mRNAs encoding the two relevant mef2ca isoforms. As shown in figure 7 (figure 6 E in the previous version), we confirmed the up-regulation of chd, gsc, ndr1 and other dorsally expressed genes specifically upon ectopic expression of mef2ca 4-5-6 transcript by semi-quantitative RT-PCR. These results were obtained from three independent sets of experiments, and are statistically significant.

Reviewer #2:

1. Too many data not shown have been described. This should be avoided. qRT-PCR on spliced mef2cb and the phenotype observed in injected embryos following ectopic expression of Mef2c isoform have to be shown.

Response to reviewer #2 point 1

Transcripts encoding Mef2cbL and Mef2cbS were quantified by qRT-PCR and are now shown as percentage of the total number of copies in figure 3F. Similarly, we present the levels of alternatively spliced mef2ca transcripts (shown as absolute copy number in the previous version of the manuscript, figure 3C) as percentage of the total.

As discussed in the text, upon injection of Mef2cb mRNAs, both containing exon 5, we observed developmental defects similar to those observed with Mef2ca 4-5-6 mRNA. In figure 6C are shown the injected embryos.

2. Following the experiment of mef2cbL ectopic expression, the authors stated that injection of the Mef2cbL isoform induced ectopic skeletal muscle in the anterior mesoderm as shown by the myoD mRNA. Nevertheless, the presence of myoD mRNA does not necessarily mean that MyoD is expressed and transcriptionally active in driving the skeletal muscle development. Myogenin and/or MyHC expression should be checked otherwise the third conclusion of the author that the evolutionarily conserved alternate splice of exon 5 in mef2cb transcripts creates a long form that has unique pro-myogenic capacity is a mere speculation. This issue is still to be demonstrated.

Response to reviewer #2 point 2

In figure 6B we now show that forced expression of Mef2cbL mRNA induces not only myoD transcripts but also the expression of muscle markers such as MyHC or smyhc1 (slow myosin heavy chain 1), further supporting the pro-myogenic activity of this specific mef2cb splice variant.

Reviewer #3, Major Concerns:

1. Authors claim statistical significance in the differential expression of the two mef2c genes in Figure 2, but have only performed two independent replicates precluding any meaningful statistical analysis of these results.

Response to reviewer #3 point 1

We repeated the experiments and quantified mef2ca and mef2cb transcript levels by performing overall four independent qRT-PCR experiments using three separate series of RNA extracted from independent groups of staged embryos. The new obtained data (see figure 2A) further confirm that mef2ca is the most abundantly expressed mef2c paralogue during embryogenesis, in line with the results shown in the previous version of the manuscript. Furthermore, statistical tests show that the new obtained data are statistically significant.

2. Authors claim that chd expression is highly upregulated upon injection of high amounts of mef2ca 4-5-6 but appear to base this conclusion on a single set of experiments. Additionally, they suggest that

ndr1 and gsc are mildly upregulated, but the 1.5 fold change observed is unconvincing without replication.

Response to reviewer #3 point 2

As discussed above, we confirmed the up-regulation of chd, gsc, ndr1 and other dorsally expressed genes by semi-quantitative RT-PCR analysis of the RNA extracted from embryos injected in three independent sets of experiments (figure 7C).

Responses to reviewer #3 minor points

1. Line 38, "disregulation" should be dysregulation.

Response: Corrected in the text.

2. Line 107, missing period after parenthesis.

Response: Corrected in the text.

3. Line 230, the parenthetical concentration is ambiguous because none is provided for mef2ca 4-6.....

Response: The absolute copy number of the transcripts variants was substituted for their percentage from the total.

4. Line 236, the sentence beginning with "Transcripts..." is paradoxical because it suggests that both the transcripts with and without the γ -like region disappear after 24 hpf. This is contradicted by the following sentence which asserts the prominence of transcripts with this region.

Response: Corrected in the text.

5. Authors do not clarify the discrepancy between relative expression levels of the mef2ca and mef2cb transcripts in the culture reporter assays.

Response: As discussed in the revised manuscript (lines 345-350), the amount of Mef2cb protein detected in our experiments was low using several antibodies directed against distinct fragments of the protein sequence (our unpublished results), therefore we do not think this is due to a low immunoreactivity to our antibody but instead to a genuine reduced translational efficiency and/or stability of the protein. This point is intriguing but nevertheless is beyond the scope of this paper.

6. Figure S2C is never cited in the text.

Response: It has been eliminated.

7. Figure 3 B + E, better clarification of the control plasmids to the right of each RT-PCR experiment would be beneficial when analyzing the figure. In its current state it is not possible to discern what each control band signifies.

Response: As suggested by reviewer #3, we described in more detail the plasmid vectors used as controls in the figure legends of figures 3B and 3E.



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Dr. Joseph Reese
Executive Editor
BBA - Gene Regulatory Mechanisms

Modena, April, 29th 2014

Object: Submission of the revised version of Manuscript No.: BBAGRM-13-217

Dear Dr Reese,

Please find enclosed the revised version of Manuscript No.: BBAGRM-13-217, Title: Distinct functions of alternatively spliced isoforms encoded by zebrafish *mef2ca* and *mef2cb*, submitted on Dec 27, 2013 to BBA - Gene Regulatory Mechanisms as a regular paper.

We would like to thank the reviewers for their useful comments and suggestions that helped us to ameliorate the manuscript. In the last three months we have performed additional experiments in order to address the issues raised by the reviewers and have incorporated them into the manuscript.

In addition, we have shortened the highlights to match with the length restriction imposed by your journal and corrected some mistakes in the text.

We include a new version of figure 3E where we assess the alternative incorporation of exons 3 α 1 or 3 α 2 into *mef2cb* transcripts. The previous semi-quantitative RT-PCR analysis using isoform-specific primers was moved into the supplementary figures (figure S5A), and in the new version, we present semi-quantitative RT-PCR analysis using common primers that give two amplicons with different sizes. Both approaches revealed the prevalence of the transcript containing exon 3 α 1 during development. The use of common primers has the advantage of allowing a more precise comparison of the relative quantities of the two isoforms, given that the PCR reaction is performed under identical experimental reactions. We also estimated the relative incorporation of alternative exons 3 α 1 and 3 α 2 in *mef2cb* transcripts in adult tissues and the results are shown in figure S5B.



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You will find our answers to specific points raised by the reviewers in the attached file "Response to reviewers".

We hope that our answers are satisfactory and that you can reconsider the revised, new version of our work for publication in your journal.

Yours sincerely,

Dr. Susanna Molinari
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Reviewer #1, major issues:

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We think that the lack of overt defects of tissue patterning shown in Fig. S6D is due to a specific effect of exogenously expressed Mef2ca 4-5-6 earlier in development, during the dorso-ventral patterning of the embryo, as supported by the double-axis phenotype presented by a large proportion of *mef2ca* 4-5-6 mRNA injected embryos and by the array of genes whose expression is specifically altered by the ectopic expression of Mef2ca 4-5-6.

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We repeated the experiments and quantified *mef2ca* and *mef2cb* transcript levels by performing overall four independent qRT-PCR experiments using three separate series of RNA extracted from independent groups of staged embryos. The new obtained data (see figure 2A) further confirm that *mef2ca* is the most abundantly expressed *mef2c* paralogue during embryogenesis, in line with the results shown in the previous version of the manuscript. Furthermore, statistical tests show that the new obtained data are statistically significant.

2. Authors claim that *chd* expression is highly upregulated upon injection of high amounts of *mef2ca* 4-5-6 but appear to base this conclusion on a single set of experiments. Additionally, they suggest that *ndr1* and *gsc* are mildly upregulated, but the 1.5 fold change observed is unconvincing without replication.

As discussed above, we confirmed the up-regulation of *chd*, *gsc*, *ndr1* and other dorsally expressed genes by semi-quantitative RT-PCR analysis of the RNA extracted from embryos injected in three independent sets of experiments (figure 7C).

Minor Concerns:

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2. Line 107, missing period after parenthesis. **Corrected in the text.**
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The absolute copy number of the transcripts variants was substituted for their percentage from the total.

4. Line 236, the sentence beginning with "Transcripts..." is paradoxical because it suggests that both the transcripts with and without the <gamma>-like region disappear after 24 hpf. This is contradicted by the following sentence which asserts the prominence of transcripts with this region. **Corrected in the text.**
5. Authors do not clarify the discrepancy between relative expression levels of the *mef2ca* and *mef2cb* transcripts in the culture reporter assays.
As discussed in the revised manuscript (lines 345-350), the amount of Mef2cb protein detected in our experiments was low using several antibodies directed against distinct fragments of the protein sequence (our unpublished results), therefore we do not think this is due to a low immunoreactivity to our antibody but instead to a genuine reduced translational efficiency and/or stability of the protein. This point is intriguing but nevertheless is beyond the scope of this paper.
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We hope that our answers are satisfactory and that you can reconsider the revised, new version of our work for publication in your journal.

Yours sincerely,

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Highlights

- *mef2ca* and *mef2cb* gene products are alternatively spliced in zebrafish.
- Inclusion of exon 5 in *mef2ca* transcripts is regulated during zebrafish development.
- Exon 5 confers on Mef2ca the ability to activate early patterning genes.
- Mef2cb includes an extra octapeptide encoded by a region of intron 5.
- Inclusion of the extra-octapeptide confers on Mef2cb pro-myogenic activity.

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

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3 Molinari S.^{1,§}

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12

13 **Abstract**

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

32

33 **Abbreviations**

34 AS, Alternative Splicing; MEF2, Myocyte Enhancer Factor 2; BMP, Bone Morphogenetic Protein;
35 MADS, Minichromosome maintenance, Agamous, Deficiens, Serum response factor; TAD,
36 transcription activating domains; PKA, Protein Kinase A; qRTPCR, quantitative Real Time PCR;
37 hpf, hours post fertilization; ss, somitic stage; WISH, Whole Mount In Situ Hybridization; CMV,
38 Cytomegalovirus; LNA, Locked Nucleic Acid; myog, myogenin; actb2, beta-actin 2; chd, chordin;
39 ndr1, nodal related 1; gsc, goosecoid ; nog1, noggin1; ntl, no tail; smyhc1, slow myosin heavy chain
40 1; MyHC, Myosin Heavy Chain; I.M.A.G.E., Integrated Molecular Analysis of Genomes and their
41 Expression; ascl1a, achaete-scute complex-like 1a; kdr1, kinase insert domain receptor; neurog 1,
42 neurogenin 1; myl7, myosin, light polypeptide 7.

43

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46

- 47 • *mef2ca* and *mef2cb* gene products are alternatively spliced in zebrafish.
- 48 • Inclusion of exon 5 in *mef2ca* transcripts is regulated during zebrafish development.
- 49 • Exon 5 confers on Mef2ca the ability to activate early patterning genes.
- 50 • Mef2cb includes an extra octapeptide encoded by a region of intron 5.
- 51 • Inclusion of the extra-octapeptide confers on Mef2cb pro-myogenic activity.

52

53 **Keywords**

54 Mef2ca, Mef2cb, zebrafish, skeletal muscle, alternative splicing, development

55

56 **1. Introduction**

57

58 Alternative splicing (AS) creates diversity within proteins without the need for gene duplication. In
59 addition, AS is also an important mechanism for modulating gene expression and has contributed
60 substantially to the evolution of modern genomes (reviewed in [1-3]). Many transcription factors
61 undergo AS that creates important functional differences in the encoded proteins: altered

62 transcriptional regulation capacity, nuclear trafficking, sensitivity to signals or requirement for co-
63 activators [4]. Splicing-sensitive microarrays and deep sequencing analysis of mRNA from various
64 human tissues have revealed the prevalence of AS in skeletal muscle; dysregulation of AS is
65 associated with human muscle diseases [5] (reviewed in [6, 7]).

66 Genes encoding the Myocyte Enhancer Factor 2 (MEF2) family of transcription factors undergo
67 extensive AS, the function of which is generally unclear. All MEF2 proteins have an N-terminal
68 DNA binding region composed of MADS (Minichromosome maintenance, Agamous, Deficiens,
69 Serum response factor) and MEF2 domains, two central transcription activating domains (TAD1
70 and TAD2) and a C-terminal nuclear localization sequence (Fig. 1B). Invertebrates generally have
71 a single *MEF2* gene, whereas amniotes have four genes (*MEF2A-D*). The teleost-specific genome
72 duplication has led to six *mef2* genes in zebrafish, with two copies of *mef2a* and *mef2c*, designated
73 *mef2aa*, *mef2ab*, *mef2ca* and *mef2cb* [8]. Most MEF2 proteins are highly expressed in muscle
74 tissue, where they regulate heart, skeletal and smooth muscle differentiation [9]. Like *Drosophila*
75 *D-Mef2*, *Mef2c* is particularly important in early heart and skeletal muscle development in both
76 mice and zebrafish [8, 10-17]. *MEF2s* are also more broadly expressed and function to control
77 development and adaptation of brain, immune system, blood vessel and many other tissues [18]
78 (reviewed in [19]). In mammals, *MEF2C* is subjected to three different alternative splices. A
79 mutually exclusive alternative splice occurs between exons $\alpha 1$ and $\alpha 2$, located in the region
80 immediately adjacent to the MEF2 domain [20]. In the central TAD2 region, a skipping-type
81 alternative splice can include exon β and a splice involving alternative 3' splice site selection occurs
82 in the γ region near the C-terminus [21, 22]. In the case of mouse *Mef2d*, AS of the α exon
83 switches the protein from a transcriptional repressor regulated by protein kinase A (PKA), to an
84 activator insensitive to PKA signaling [23]. This switch is thought to drive skeletal muscle terminal
85 differentiation, but how AS in the *Mef2d* α exon relates functionally to AS at other alternate exons
86 is unclear. Developmentally regulated switching of AS of *MEF2* genes has been described during
87 frog and mouse development [24-26]. Involvement of alternative splice variants of *Mef2* in
88 endomesoderm and neuron differentiation in the sea anemone *Nematostella vectensis* has been
89 described recently [27]. However, it has not yet been determined whether functional differences
90 among the splicing variants of the *MEF2C* genes are important in vertebrate development.

91 Here we describe the alternative splicing of the two zebrafish *Mef2c* genes, *mef2ca* and *mef2cb*. In
92 addition to splicing events akin to the α , β , γ splices that were described in mice, we find novel
93 splice forms varying in the region between the two TADs around the fifth coding exon. We provide
94 the first evidence that the developmentally-regulated AS of *mef2ca* in this region affects *Mef2c*

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

103

104 **2. Materials and Methods**

105

106 *2.1. Plasmids*

107 The full-length coding regions of the zebrafish Mef2ca 4-5-6, Mef2ca 4-6, Mef2ca 4'-6' and
108 Mef2cbL variants were amplified from 24 hpf (hours post fertilization) zebrafish embryos cDNAs.
109 The full length cDNA of Mef2cbS was obtained by a PCR reaction starting from a template made
110 of three overlapping PCR products: the exon 5 region amplified from a Mef2cb I.M.A.G.E. clone
111 (clone ID: 6519749, Genbank: CD282884.1), the upstream and downstream regions amplified from
112 the Mef2cbL cDNA. The cDNAs were first inserted in the pCR2.1 vector (Invitrogen) or pGEM-T
113 Easy vector (Promega), then sub-cloned into BamHI/NotI sites of the pcDNA 3.1(+) expression
114 vector (Invitrogen). For RNA injections isoforms were sub-cloned into the XbaI/SalI sites of the
115 β UT-3 vector [8]. Plasmids pGL3(desMEF2)₃ and pRSV β -gal were previously described [28].

116

117 *2.2. Alternative splicing prediction and multiple alignments*

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

123

124 *2.3. Transcription Reporter Assays*

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

127

128 2.4. RNA isolation, RT-PCR and Real Time PCR

129 RNA was isolated using TRIzol® Plus RNA Purification System (Ambion). For each
130 developmental stage, 100 embryos were disrupted using Tissue Raptor (Qiagen). 500 ng of total
131 RNA were reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen).
132 Primers used to detect *myogenin (myog)*, *myod*, *beta-actin 2 (actb2)*, *mef2ca* and *mef2cb* are listed
133 in Fig. S6A (other primers sequence are available upon request), quantitative Real Time PCR
134 (qRT-PCR) was performed on 2,5 ng of Poly A mRNA using SYBR Green method (SYBR® Green
135 PCR Core Reagent, Applied Biosystems). Poly A mRNA has been purified using Ambion's
136 protocol (MicroPoly(A) Purist Kit). To amplify the different *mef2ca* isoforms specific forward
137 primers spanning exon-exon junctions were used with a common reverse primer (Fig. S6B). For
138 each primer combination the optimal MgCl₂ concentration was determined to obtain specific and
139 high efficient amplification (slope values between -2.95 and -3.75). Absolute quantification of
140 transcript copy number was achieved by generating calibration curve using plasmid DNA templates
141 (listed above) as previously described [29, 30]. Analysis was performed using PCR ABI PRISM
142 7900 HT Sequence Detection System (Applied Biosystems). Student's t-tests were performed for
143 pairwise comparisons to determine significant differences between groups.

144

145

146 2.5. Zebrafish lines, maintenance and embryo manipulation

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

149

150 2.6. Whole Mount In Situ Hybridization (WISH)

151 In situ mRNA hybridization was performed as described [14]. Fluorescein- or digoxigenin-tagged
152 probes used were *mef2ca* [32], *mef2cb* [8], *myod* [33], *slow myosin heavy chain 1 (smyhc 1)* [8],
153 *myosin, light polypeptide 7 (myl7)* [34], *kinase insert domain receptor (kdr1)* [35], *neurogenin 1*
154 (*neurog1*) [36] or *achaete-scute complex-like 1a (ascl1a)* [37]. We have also used two non-
155 overlapping dual digoxigenin-labelled custom *mef2ca* exon 5-specific locked nucleic acid (LNA)

156 probes, LNA1 and LNA2 (Exiqon, sequence available upon request) to perform WISH as described
157 [38, 39]. Embryos were photographed as wholemounts on Olympus DP70 or dissected and
158 flatmounted in glycerol and photographed on a Zeiss Axiophot with Axiocam using Improvision
159 Openlab.

160

161 2.7. mRNA injection and embryo manipulation

162 mRNA injection was performed as described previously [40]. β UT-3 vectors encoding Mef2ca and
163 Mef2cb isoforms, were linearized using Sfi/PstI sites. mRNAs were made with mMMESSAGE
164 mMACHINE kit (Ambion). All RNAs were injected at 1-2 cell stage embryos at 10 pg, 25 pg or 50
165 pg/embryo. Tetramethyl-rhodamine Dextran (5% solution in 0.2 M KCl) was co-injected in order
166 to sort phenotypes of injected embryos. At 20-28 hpf injected embryos were analyzed and sorted
167 using a Zeiss Axiophot with Axiocam.

168

169 2.8. Western blot analysis and antibodies

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

178

179

180 3. Results

181

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

186

187 3.1. Alternative Splicing of zebrafish *mef2ca* and *mef2cb*

188 To predict splice variants of the zebrafish Mef2c proteins, we conducted *in silico* analysis of the
 189 zebrafish *mef2ca* and *mef2cb* genes on public databases (see Methods). In addition to the known
 190 *mef2ca* transcript [32, 42], we detected two alternative 5'UTR sequences and several alternative
 191 splices (Fig. 1A and B). *mef2ca* lacks an alternative exon 3, the α exon, having a single exon most
 192 similar to the 3 α 1 form of amniote *MEF2C*, which has serine residues at positions 98 and 109 [43].
 193 *Mef2ca* contains a putative β exon in intron 6 and, in addition, a γ -region flanked by a non-
 194 canonical 3' splice site (GC) was found at the start of exon 9 (Fig. S2A). Of particular note in the *in*
 195 *silico* analysis, were three alternative splices in the region of exons 4, 5 and 6 (Fig. 1C), two of
 196 which correspond to the alternatively spliced δ exon (exon 5) of the *mef2c* gene product in *Xenopus*
 197 [24] (Fig. S1B,C). By sequence alignment we found that splicing of exon 5 is conserved among
 198 teleosts (Fig. S3). Zebrafish *mef2cb* splicing appeared more similar to amniote *MEF2C* genes than
 199 that of *mef2ca*. We predicted two 5' UTR sequences, alternate exons 3, 3 α 1 and 3 α 2, a putative β
 200 exon, encoding the conserved octapeptide SEDVDLLL in intron 6 of *mef2cb*, and a putative γ
 201 region at the start of exon 9 (Fig. 1B). The sequences of alternative exons 3 α 1 and 3 α 2 are mostly
 202 similar to the corresponding alternate exons of amniote *MEF2C*, although neither of the two
 203 α exons contains a PKA target residue corresponding to serine 120 of MEF2D, which was found to
 204 direct binding of repressive or activating cofactors. [23]. Additionally, we found a *mef2cb* variant
 205 with a long exon 5 resulting from a retained intron 5 sequence. We designate this Mef2cbL to
 206 distinguish it from the conventional exon 5 in Mef2cbS (Fig. 1B, C).

207 To characterize the major *mef2ca* and *mef2cb* spliced isoforms expressed in developing zebrafish
 208 skeletal muscle, we performed RT-PCR on RNA extracted from the dissected tail region of 24 hpf
 209 embryos using primer pairs that target conserved sequences (Fig. 1B). A series of *mef2ca* mRNA
 210 RT-PCR products were amplified, sub-cloned and their sequences compared to the nucleotide
 211 sequence of *mef2ca* genomic DNA, revealing the existence of at least four species of *mef2ca*
 212 mRNAs produced by AS in developing embryos (Fig. 1B). In addition to the transcript encoding the
 213 full length protein, here referred to as Mef2ca 4-5-6 (465 aa), two *mef2ca* isoforms, Mef2ca 4-6
 214 (451 aa) and Mef2ca 4'-6' (413 aa) derive, respectively, from skipping exon 5 or a larger region that
 215 also encompasses part of exons 4 and 6. Another variant, Mef2ca $\Delta\gamma$ -like (411 aa) contains exon 5
 216 but lacks the γ region and further sequences located in exons 8 and 9. The Mef2ca 4'-6' and Mef2ca
 217 $\Delta\gamma$ -like mRNAs are the results of splicing at the non-canonical 5' donor splice sites GG and CA,
 218 respectively (Fig. 1C) (GenBank accession numbers: KF932282 and KF932281 respectively).

219 One *mef2cb* variant, named Mef2cbL, was obtained by RT-PCR; it includes exon 3 α 1 and γ but
 220 lacks exon β . Mef2cbL arises from the inclusion of an additional sequence from intron 5 (Fig. 1C).

221 Indeed, two competing 5' splice sites are present at the end of exon 5, these splice sites direct
222 inclusion or exclusion of 24 nucleotides (nt) encoding the octapeptide KDGIIPTYY (Fig. 1C).
223 When aligned (Fig. S2B), the predicted amino acid sequences of the identified zebrafish *mef2ca* and
224 *mef2cb* isoforms show that the major variation occurs in the exon 4-5-6 region of both genes,
225 located between the two TADs described previously [44, 45].

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

231

232 3.2. *mef2ca* is the main *Mef2c* orthologue expressed during skeletal muscle development

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

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

251

252 3.3. Developmentally regulated expression of *mef2ca* and *mef2cb* splice variants

253 Levels of expression of alternatively spliced *mef2ca* and *mef2cb* during zebrafish development were
254 determined by semi-quantitative RT-PCR and qRT-PCR RNA quantification using SYBR and exon
255 boundary spanning primers, that allow for selective PCR amplification of individual alternative
256 transcripts [30]. At 12 hpf, the amount of *mef2ca* 4-6 transcript (lacking exon 5) represents about
257 30% of the total, whereas the amount of the full length 4-5-6 transcript the remaining 70%. At 24
258 hpf and beyond, *mef2ca* 4-6 expression increased slightly but remained less abundant than the
259 *mef2ca* 4-5-6, whose predominance increases further (80% of the total *mef2ca* transcripts) (Fig. 3B-
260 C). The shortest isoform, *mef2ca* 4'-6' is present at low level (less than 1% of the total *mef2ca*
261 transcripts) at every developmental stage and was therefore not considered further. *mef2ca*
262 transcripts containing the β exon were barely detectable and were found exclusively at 72 hpf after
263 five additional cycles of PCR amplification (data not shown). Transcripts without the γ -like region
264 were expressed at early stages of development. However, they were less abundant, and were not
265 detected beyond 24 hpf (Fig. 3A,B). Thus, almost all *mef2ca* transcripts contain the γ -like region
266 and lack β exon, irrespective of their splicing at the 4-5-6 region.

267 Expression of the *mef2cb* alternatively spliced exons was also determined by semi-quantitative RT-
268 PCR and qRT-PCR amplification. Transcripts containing exons $3\alpha 1$ and $3\alpha 2$ were detected
269 throughout development using common primers that give two amplicons of different size and
270 therefore electrophoretically distinguishable. The transcript that includes the $3\alpha 1$ exon is the most
271 abundant at all the developmental stages beyond 12 hpf (Fig. 3E). This result was also confirmed by
272 using isoform-specific primers, given that, in identical experimental conditions, four additional
273 PCR cycles are required to amplify an amount of exon $3\alpha 2$ -containing DNA similar to that
274 containing exon $3\alpha 1$ (Fig. S5A). Whereas the inclusion of exon $3\alpha 1$ predominates in the
275 developing embryo, RT-PCR analysis revealed that in adult skeletal and cardiac muscle the levels
276 of the two isoforms are comparable (Fig. S5B). We did not detect the $3\alpha 2$ -containing transcript in
277 liver and brain, indicating a muscle-restricted pattern of expression of this splice variant,
278 analogously to what has been reported for the mammalian counterpart (Fig. S5B) [20]. Inclusion of
279 exon β was barely detected. In contrast, the γ region and the extra sequence from intron 5
280 (Mef2cbL) were readily detected at all developmental stages (Fig. 3D,E). qRT-PCR quantification
281 confirmed that more than 90% of *mef2cb* transcripts retain the extra intron 5 sequence, but less than
282 10% encode the Mef2cbS form (Fig. 3F). Thus, both *mef2ca* and *mef2cb* show striking variations
283 in the exon 4-5-6 region.

284
285 *3.4. Exon 5-containing mef2ca transcripts accumulate in skeletal muscle*

286 To examine where *mef2ca* mRNA(s) that include exon 5 are expressed in the developing zebrafish
287 embryo, we performed in situ mRNA hybridization using a probe that recognizes all transcripts
288 (*mef2ca* probe, [32]) and two non-overlapping dual digoxigenin-labelled LNA probes designed to
289 recognize 21 base pair sequences located within exon 5 (LNA1) or within the exon 4/5 boundary
290 (LNA2) (Fig. 4C). In 24 hpf embryos, *mef2ca* transcripts were detected throughout the somitic
291 muscle and preferentially observed at somite borders (Fig. 4A upper panel), as well as in the heart
292 and branchial arches (Fig. 4A upper panel, see also Fig. 2B). Similarly, both the exon 5-specific
293 LNA probes gave signals above background only in skeletal muscle, preferentially observed at
294 somite borders (Fig. 4A, middle and lower panels). By 48 hpf, the signals obtained with the generic
295 and both exon 5-specific probes, are restricted almost entirely to the somite boundaries area (Fig.
296 4B, left and [14]). In addition, signals with all three probes show the typical separate dorsal and
297 ventral muscle signal in the pectoral fin (Fig. 4B, right). Thus, even though we cannot exclude some
298 levels of expression in other tissues, we conclude that the *mef2ca* 4-5-6 transcript is expressed
299 primarily in skeletal muscle and is mainly localized to somite boundaries, suggesting it may have a
300 distinct and specific function.

301

302 3.5. *Mef2ca* 4-5-6 is a potent transactivator

303 The transcriptional activities of *mef2ca* splice variants were tested in vitro by co-transfection into
304 COS-1 cells of each *Mef2c* splice variant with a MEF2-responsive luciferase reporter containing
305 three copies of the MEF2 binding site from the *Desmin* gene regulatory region (pGL3desMEF2)
306 [46]. COS-1 cells have low endogenous MEF2 expression. Immunofluorescence analysis revealed
307 that all *Mef2ca* and *Mef2cb* splice variants efficiently localized to the nucleus (data not shown),
308 congruent with the observation that they all include the sequence corresponding to the nuclear
309 localization signal described in the mouse [47].

310 Compared to other *Mef2c* isoforms tested, the *Mef2ca* 4-5-6 full length protein had the strongest
311 transcriptional activity (Fig. 5A). Deletion of amino acids encoded by exon 5 and neighboring
312 sequences result in a twofold reduction in transcriptional activity, even though the respective
313 protein expression levels were comparable (Fig. 5B). Furthermore, we observed that a *Mef2ca* 4-5-6
314 isoform lacking the γ -like domain had 2-fold higher transcriptional activity than *Mef2ca* containing
315 the γ -like domain, consistent with the finding that this region represses transcription (data not
316 shown; [21]). Upon transfection, the *Mef2cbL* and *Mef2cbS* isoforms, each containing both exon 5
317 and γ , exhibited similar activity (about 70% of that of *Mef2ca* 4-5-6) (Fig. 5A and data not shown).
318 However, *Mef2cbL* immunoreactivity was much lower than the *Mef2ca* isoforms (Fig. 5B). Given

319 that we obtained similar results with other antibodies directed against different regions of MEF2
320 proteins (data not shown), it is unlikely that the low amount of Mef2cb protein detected is due to the
321 low reactivity of our anti-Mef2 antiserum. Additional studies are required to characterize the
322 stability and translational efficiency of Mef2c proteins, but our results suggest that Mef2cbL has
323 higher activity per molecule than Mef2ca 4-5-6. Taken together, these data suggest that inclusion of
324 exon 5 between TAD1 and TAD2 confers increased activity to Mef2ca.

325

326 *3.6. Mef2cbL has unique myogenic potential in developing zebrafish*

327 To investigate the biological significance of Mef2ca and Mef2cb splice variants in zebrafish
328 embryonic development, we determined the effects of their ectopic expression by injecting embryos
329 at the one-cell stage with synthetic Mef2c mRNAs and analyzing them at 24 hpf. We have shown
330 previously that injection of mRNA of *mef2cb* induces ectopic skeletal muscle in embryos [8]. Here
331 we report that injection of 10 pg/embryo of mRNAs of the Mef2cbL isoform induced ectopic
332 skeletal muscle in the anterior mesoderm of 40% of the injected embryos, as revealed by
333 wholemount in situ hybridization for *myod* mRNA in 28 hpf zebrafish embryo, a developmental
334 stage where no endogenous muscle is normally observed in the head (Fig. 6A,B). In addition to
335 *myod* transcripts we detected ectopic expression of *smyhc1* transcripts and MyHC protein, further
336 supporting the pro-myogenic activity of Mef2cbL (Fig. 6B). No induction of ectopic muscle was
337 observed after ectopically expressing any Mef2ca isoform, even when higher quantities of mRNA
338 were injected (Fig. 7). Interestingly, this effect depends on the inclusion of the KDGIPYY
339 octapeptide, because forced expression of the Mef2cbS isoform did not cause ectopic myogenesis
340 (Fig. 6A, B). Thus, the form of Mef2cb that is normally present in developing zebrafish embryos
341 during somitogenesis has unique myogenic potential that is not shared by Mef2ca 4-5-6, the
342 predominant Mef2c isoform in skeletal muscle. Injection of higher amounts (25 pg/embryo) of both
343 Mef2cbS and Mef2cbL mRNAs resulted in head and trunk developmental alterations (Fig. 6C).

344

345 *3.7. Mef2ca 4-5-6 over-expression causes defects in gastrulation*

346 To investigate the functionality of the two main Mef2ca isoforms expressed during development
347 (Mef2ca 4-5-6 and 4-6), high doses of Mef2ca mRNAs were employed. Injection of 25 pg of full
348 length Mef2ca 4-5-6 RNA had dramatic effects on embryonic development, inducing lethality in
349 approximately 30% of the embryos and marked developmental defects in 49% of the surviving
350 embryos, classified as 'severely defective' (Fig. S6A,B). Such embryos already had defects evident
351 at gastrulation stages (6-8 hpf, data not shown). Among the surviving embryos, a further 34%

352 exhibited a milder phenotype classified as 'defective', with trunk convergent extension defects,
353 occasional double axes, and some brain defects such as undeveloped eyes and absence of mid- and
354 forebrain structures (Fig. 7A). Only 16% of embryos appeared unaffected by the Mef2ca 4-5-6
355 RNA. The percentage of severely defective embryos increased in a dose-dependent manner upon
356 increasing the amount of injected RNA (Fig. S6B). In contrast to Mef2ca 4-5-6, forced expression
357 of the Mef2ca 4-6 isoform was less active, having no detectable effect on the development of most
358 (85%) of the injected embryos, even when expressed at comparable levels to Mef2ca 4-5-6 (Figs 7A
359 and S6B,C). These results indicate that ectopic Mef2ca activity in early stages disrupts normal
360 development. The gross defects in gastrulation induced by over-expressed Mef2ca 4-5-6 suggested
361 severe tissue patterning disruption, yet a survey of cell lineage markers revealed no indication of
362 altered cell fates at lower doses of RNA (Fig. S6D).

363 To gain more insight into the mechanisms underlying the ability of Mef2ca 4-5-6 to disrupt
364 development, the expression levels of genes encoding transcription factors and signaling molecules
365 that are involved in early patterning of the embryo were screened by semi-quantitative RT-PCR.
366 The *chd* gene, encoding a BMP (Bone Morphogenetic Protein) antagonist involved in dorsoventral
367 patterning of early embryos [48] (reviewed in [49], [50]), was up-regulated (2-fold) in embryos
368 injected with the *mef2ca* 4-5-6 mRNA, but not in those injected with the 4-6 spliced isoform (Fig.
369 7B,C). Mef2ca 4[48]-5-6 also induced the expression of *ndr1* (1.5-fold), *gsc* (2.2-fold) and other
370 dorsally-expressed genes (*no-tail a*, *noggin 1*), and reduced the expression of ventralizing factors
371 such as *bmp7a* (0.4-fold) and *Anp63* (0.2-fold), but did not alter the transcript level of *myod* or *no-*
372 *tail b* and *bmp2b* (Fig. 7B,C), suggesting that the protein sequence encoded by exon 5 can modulate
373 the expression level of a specific subset of early embryonic genes.

374

375 3.8. Mef2ca 4-5-6 mRNA is the prevalent Mef2c transcript present in the embryo before 376 gastrulation

377 Our data indicate that forced expression of Mef2ca 4-5-6 protein induces the ectopic expression of
378 genes involved in early dorso-ventral patterning of the embryo. In an attempt to get more insight
379 into a putative role of Mef2ca in controlling endogenous patterning genes, we next determined the
380 expression and alternative splicing patterns of *mef2c* genes during early stages of development and
381 compared them to those of two of their putative target genes, i.e. *chd* and *gsc*. To this aim we
382 performed RT-PCR analysis of the RNA from zebrafish embryos harvested at the 1K-cell (3 hpf),
383 50% epiboly (5.25 hpf) and bud (9-10 hpf) stages. Our analysis revealed that *mef2ca* transcripts are
384 already detectable as early as at the 1K-cell stage, with predominant expression of the transcript

385 including exon 5 (Fig. 8). We noticed a rapid loss of the 4-5-6 transcript that became undetectable
386 by 10 hpf when the 4-6 mRNA is the only *mef2ca* transcript detected, inclusion of exon 5 is again
387 detected later, by 12 hpf (Fig. 3) and the 4-5-6 full length transcript predominates upon muscle
388 differentiation. The kinetics of expression of the *mef2ca* 4-5-6 transcript suggests that it might be
389 of maternal origin. The presence of Mef2ca 4-5-6 transcripts early in development, which is
390 temporally coincident with *gsc* expression and overlaps partially with that of *chd* (our data and [51])
391 is consistent with a role of this *mef2ca* splice variant in dorso-ventral patterning. No *mef2cb*
392 expression is detected prior to 50% epiboly, in mid-gastrulation, where only the *mef2cbS* transcript
393 is present. Nonetheless, at the onset of somitogenesis (9-10hpf), we noticed that only the transcript
394 encoding for Mef2cbL, the pro-myogenic variant, is expressed.

395

396

397 **4. Discussion**

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

412

413 *4.1. Regulation of Mef2ca activity by alternative splicing in zebrafish development*

414 In addition to the well documented expression of *mef2ca* starting from 12 hpf [32], we found
415 *mef2ca* transcripts in zebrafish embryo prior to gastrulation at the 1K-cell stage, likely from
416 maternal contribution, with their level declining to a minimum at 9-10 hpf. Starting from 12 hpf we

417 observe an overall increase in expression levels of *mef2ca* mRNA. Besides changes in the
418 abundance of *mef2ca* transcripts, we found a dynamic regulation of the splicing in the exon 5 region
419 : the *mef2ca* variant including exon 5 (*mef2ca* 4-5-6) is the major isoform detected very early in
420 development (1K-cell stage), suggesting that it might play a role prior to gastrulation, by 10 hpf, the
421 *mef2ca* transcripts lacking exon 5 (*mef2ca* 4-6) are predominant. Subsequently, *mef2ca* 4-5-6 again
422 climbs as muscle precursors undergo terminal differentiation, becoming the predominant isoform at
423 24 hpf. Such splicing of exon 5 is evolutionary conserved between *Xenopus* and teleosts,
424 suggesting it has biological significance [24] (Fig. S3). Moreover, muscle differentiation in
425 zebrafish is associated with several other muscle-specific alternative splicing events involving
426 changes in splicing efficiency [54]. Although no specific function was assigned to the exon 5
427 domain by mutational and deletion analysis of the mouse and human protein counterparts [44, 45],
428 our cell culture data indicate that the peptide sequence encoded by exon 5 contributes to the
429 transcriptional activity of Mef2ca. The early expression of *mef2ca* transcripts including exon 5
430 (*mef2ca* 4-5-6) may indicate their early function in embryo patterning. Later in development, the
431 preferential accumulation of full length *mef2ca* 4-5-6 mRNA at skeletal muscle fiber ends, suggests
432 that its normal function is in muscle, a view confirmed by the requirement for Mef2ca function for
433 skeletal muscle fiber growth and heart myogenesis [8, 55]. In the current work the function of
434 Mef2ca isoforms was probed by ectopic over-expression; *mef2ca* 4-5-6 RNA, but not *mef2ca* 4-6
435 RNA, causes gross defects during gastrulation. We suggest that these effects of Mef2ca 4-5-6 are
436 attributable to its ability to activate, directly or indirectly, a specific subset of pivotal genes in
437 gastrulation. We observed the induction in *chd* (2-fold) mRNA and a milder (1.4 fold) increase in
438 *noggin 1* (*nog1*) mRNAs that encode two inhibitors of the BMP signaling. *chd* is required to
439 repress *bmp2b* function in formation of the organizer and dorsoventral patterning of mesoderm and
440 neural tissue [56-58]. Over-expression of *chd* dorsalizes embryos [59, 60], a phenotype present in a
441 fraction of embryos following Mef2ca 4-5-6 over-expression. Thus, up-regulation of these
442 dorsalizing proteins may explain the effects of Mef2ca 4-5-6.

443 In *Xenopus*, MEF2D helps induce mesoderm by driving the expression of the *Nodal-related 1*
444 (*ndr1*) gene [61]. In zebrafish, Mef2ca 4-5-6 over-expression also increases in *gsc* and *ndr1*
445 mRNAs (2.2- and 1.5- folds, respectively), which regulate dorsoventral patterning in organisms
446 ranging from *Drosophila* to mammals [49, 50, 62-64]. In line with our results it has been previously
447 reported that expression of *gsc*, is reduced in *mef2ca*^{-/-} (*hoover*) mutants [42]. Although we cannot
448 exclude off-target effects, this specific ability of Mef2ca 4-5-6, but not of similar amounts of
449 Mef2ca 4-6, suggests distinct transcriptional activity of the former. *In silico* analysis of promoter
450 regions of *chd* and *ndr1* genes revealed the presence of several putative MEF2 binding sites

451 (YTA(A/T)₄TAR) (data not shown), raising the possibility that Mef2ca 4-5-6 directly activates their
 452 expression during early development. Later in development *chordin* expression may be sustained by
 453 Mef2d, which constitutively includes the sequence encoded by exon 5, and which is expressed from
 454 mid-gastrulation in adaxial muscle cells that also express *chordin* [32, 60] or by Mef2cb proteins.
 455 Interestingly, injection of either *mef2cbS* or *mef2cbL* transcripts, both containing exon5, have
 456 resulted in similar developmental defects to that of *mef2ca* 4-5-6 mRNA injection. Future studies
 457 will clarify whether these genes are indeed direct targets of a Mef2 protein containing exon 5.

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

463 The protein sequence encoded by exon 5 might represent a binding motif that mediates protein–
 464 protein interactions with specific co-factors, as one recognized function for alternatively spliced
 465 isoforms is to remodel the protein–protein interaction network [69]. Supporting this hypothesis is
 466 the recent demonstration that the domains encoded by the mutually exclusive $\alpha 1/\alpha 2$ exons of
 467 mouse MEF2D can mediate interactions with different sets of co-repressors or co-activators [23].

468 469 4.2. Gene duplication and evolutionary partitioning of alternative splicing

470 The importance of other splices in Mef2ca remains to be determined. The γ -like and 4'-6' splices
 471 have low abundance and we were unable to display unique activities for these isoforms. On the
 472 other hand, unlike in mammals, exon 3 does not appear to show alternative splicing in Mef2ca, the
 473 gene only having an $\alpha 1$ version. As the $\alpha 1$ exon of mouse Mef2D mediates interactions with
 474 specific transcriptional co-regulators [23], Mef2ca may have a more restricted range of functions
 475 compared to Mef2cb, which retains alternative α exons in its genomic sequence. However, at the
 476 stages examined, transcripts of *mef2cb* containing the $\alpha 2$ exon had low abundance, suggesting that
 477 this splice may be significant in specific cell types or developmental stages. In the adult we found a
 478 high proportion of the *mef2cb* transcripts containing the $3\alpha 2$ exon in striated muscle tissue where it
 479 might play a specific role in mediating muscle gene expression as shown for the analogous splice
 480 variant of *Mef2d* in mammals [23]. Conversely, *mef2cb* transcripts omitting exon 5 were not
 481 observed. Instead, teleost *mef2cb* has evolved a unique splice, possibly derived by exonisation [1]
 482 of a part of intron 5. The addition of this octapeptide and its conservation across teleosts appears to
 483 have conferred myogenic properties to Mef2cbL.

484

485 *4.3. Alternative splicing of mef2cb gene generates a pro-myogenic transcription factor*

486

487 We detected *mef2cb* transcripts in zebrafish embryo as early as 50% epiboly stage. Mef2cbL is the
488 prevalent Mef2cb isoform starting from 9-10 hpf, concomitantly with the onset of somitogenesis
489 and has a unique pro-myogenic capacity. *mef2cb* mRNA over-expression can convert cells to
490 skeletal muscle (Fig. 6A; [8]). This result suggests a role for Mef2 as a skeletal muscle
491 determination factor in zebrafish head, challenging the classical epistatic relationship between
492 MyoD and MEF2 in which MyoD acts upstream of MEF2 to direct embryonic multipotent
493 progenitors into the myogenic lineage. The myogenic activity of Mef2cbL relies on an octapeptide
494 encoded by a short sequence of intron 5 retained in the transcript. This insert, being too short to
495 form a domain, may act by changing the structural fold and leading to a new function of the protein
496 [70]. Muscle conversion was not observed upon ectopic expression of Mef2cbL in mouse
497 fibroblasts, congruent with previous observations made with the mouse MEF2 proteins [9, 71, 72].
498 Thus, we propose the existence of a specific co-factor expressed in zebrafish head mesoderm that
499 confers myogenic capacity to Mef2cbL. Identifying Mef2cb's molecular partners recruited
500 specifically in the presence of the octapeptide to activate the expression of *myod* and other muscle
501 genes may help in deciphering the molecular mechanisms underlying the pro-myogenic activity of
502 Mef2cbL.

503

504 **5. Conclusions**

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

510

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708

709

710

711 **Figure Legends**

712 **Fig. 1.** Genomic organization, transcripts and protein variants of zebrafish *mef2ca* and *mef2cb*
713 genes. **A)** Schematics of zebrafish *mef2ca* and *mef2cb* genes. Exons are numbered and indicated by
714 boxes. Black boxes indicate the *mef2ca* and *mef2cb* coding exons, whereas grey boxes represent the
715 5'- and 3'-untranslated regions. Introns are indicated by solid lines. The ATG translational start
716 codons and the TGA stop codons of the two genes are also indicated. **B)** Schematic representation
717 of zebrafish *mef2ca* and *mef2cb* transcript variants. Grey boxes represent UTR, white boxes
718 represent the coding regions of the MADS and MEF2 domains in exons 1 and 2, black boxes
719 represent the remaining translated sequence. Structures of zebrafish *mef2ca* and *mef2cb* genes
720 transcripts are similar with the following exceptions: *mef2ca* lacks the 3 α 2, exon 5 alone or together
721 with neighboring sequences from exons 4 and 6 may be excluded from the mature transcripts, the
722 alternatively spliced γ region overlaps with the homologous γ regions of zebrafish *mef2cb* and of the
723 other vertebrates *mef2c* genes, however it extends to neighboring sequences located in exons 8 and
724 9; *mef2cb* transcripts may include a short (24 nt) sequence of intron 5 (*). White arrows indicate the
725 position of the primers used to amplify the cDNAs. The structures of the Mef2ca and Mef2cb
726 protein isoforms deduced from the cloned cDNA sequences are schematized. The N-terminal region
727 of the Mef2c proteins comprises the MADS-box and the MEF2 domain, involved in DNA binding
728 and dimerization. By analogy with the mouse and human proteins, in the C-ter there are two
729 putative transcriptional activation domains, TAD1 (blue) and TAD2 (orange), encoded respectively
730 by exon 4 and by exons 6,7,8, downstream is localized the nuclear localization signal (NLS)
731 (squared box). The position of exon 5 (black) and neighboring sequences that are excluded in the 4'-
732 6' isoform (grey) are indicated as well as the position of the γ -like and γ region of Mef2ca and
733 Mef2cb respectively. Exon numbering is reported and the number of amino acids is indicated on the
734 bar above. Mef2ca forms are named according to whether or not the exon 5 and neighboring regions
735 or the γ -like region are present (Mef2ca 4-5-6, 4-6, 4'-6', Δ γ -like). Mef2cb forms are named
736 according to whether or not the octapeptide (*) in the exon 5 region is present or not (Mef2cbL and
737 Mef2cbS). **C)** Details of the alternative splicing events that take place respectively: i. In the exon 5
738 region of *mef2ca*, showing the consensus and the non-canonical splice sites and the three species of
739 mRNA generated; ii. In the γ region of *mef2ca*, splicing through a non canonical CA alternative 5'
740 splice site in exon 8 and a canonical alternative 3' splice site in exon 9 gives rise to the deletion of
741 the γ -like region; iii. Exon 5 region of *mef2cb* transcript, the cartoon shows the sequence of the
742 intron 5 that can be alternatively included in *mef2cb* transcripts, the competing donor splice sites
743 (GT) and the two species of mRNA generated.

744

745 **Fig. 2.** Expression of *mef2ca* and *mef2cb* genes in zebrafish embryos. **A)** Estimation of absolute
 746 *mef2ca* and *mef2cb* transcripts by qRT-PCR during *D. rerio* development. The graph shows
 747 transcript-specific absolute quantification, reported as \log_2 copy number in equal amount of total
 748 RNA (2,5 ng) extracted from zebrafish embryos at 12, 24, 48 and 72 hpf . Graph showing mean
 749 \pm SE from four independent experiments, ** and *** indicate P-values of ≤ 0.01 and ≤ 0.001
 750 respectively. **B)** Wholemount in situ hybridization for *mef2ca* and *mef2cb* mRNA for embryos at 11
 751 ss (dorsal view, anterior to top) and at 24 hpf (lateral view, anterior to left). At 11 ss, both genes are
 752 expressed in the adaxial cells (black arrowheads). By 24 hpf *mef2ca* is strongly expressed in the
 753 myotome (black arrows) and also in heart (green arrowhead) and branchial arches (blue arrowhead).
 754 *mef2cb* transcripts are detected in the heart (green arrowhead), telencephalon (red arrowhead) and
 755 are weakly detected in the somites (black arrow). Scale bars = 100 μ m.

756

757 **Fig. 3.** Developmental expression profile of *mef2ca* and *mef2cb* alternative splicing products.

758 **A)** Schematic representation of Mef2ca alternative exons. Arrows show primers annealing sites
 759 used in the RT-PCR analysis. **B)** Expression analysis of alternative splicing variants of *mef2ca*
 760 transcripts by RT-PCR. Total RNA was purified from staged embryos at 12, 24, 48 and 72 hpf.
 761 PCR was performed using primers that give amplification products of different sizes depending on
 762 the splice variant. The level of expression of total *mef2ca* transcripts was evaluated by using two
 763 primers (ca1 and ca3) that amplify a region not alternatively spliced between exons 1 and 3.
 764 Amplification of plasmid vectors containing the cDNAs of the various *mef2ca* splice variants
 765 cloned into the pcDNA 3.1 vector were used as controls of the correct size of expected amplicons:
 766 *mef2ca* 4-5-6 γ^+ (lane 1), *mef2ca* 4-6 γ^+ (lane 2), *mef2ca* 4'-6' γ^+ (lane 3), *mef2ca* 4-5-6 γ^- (lane
 767 4). PCR products were separated in 8% polyacrylamide gels. Length of PCR products in base pairs
 768 (bp) is indicated. **C)** Quantitative analysis of the mRNA levels of *mef2ca* exon 5 splice variants
 769 during *D. rerio* development. The amount of the transcripts of each splice variant was estimated by
 770 absolute qRT-PCR. Original data (mRNA levels of each isoform) are reported as % of the total
 771 number of *mef2ca* transcripts (4-5-6 + 4-6 + 4'-6' = 100%). Statistical analysis was performed on
 772 data obtained from three independent experiments , the means \pm SE are represented. *** indicate a
 773 P-value ≤ 0.001 . **D)** Schematic representations of Mef2cb alternative exons. Arrows show primers
 774 annealing sites. **E)** Developmental RT-PCR analysis of *mef2cb* mRNAs. To evaluate the amount of
 775 3 α 1- and 3 α 2-containing *mef2cb* transcripts, we designed common PCR primers (cb2 and cb4Rv)
 776 annealing to flanking regions in exons 2 and 4 to generate two amplicons of different size: a 196-bp

777 (3 α 1) and a 187-bp (3 α 2) RT-PCR products respectively. Flanking primers were also designed to
 778 investigate the expression of exon β , the extra sequence of intron 5 (*) and of the γ region. As
 779 control templates we used the pcDNA 3.1 expression vector containing the cDNAs of Mef2cbL3 α 1
 780 β - γ + (lane 5), Mef2cbL3 α 2 β - γ + (lane 6) and Mef2cbS 3 α 1 β - γ + (lane 7). *actb2* was used as a
 781 control, *myog* was used as a marker for skeletal muscle differentiation.

782

783 **Fig. 4.** WISH analysis of zebrafish *mef2ca* transcripts in developing zebrafish embryos.

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

795

796 **Fig. 5.** Transcriptional activity of zebrafish Mef2ca and Mef2cb splice variants.

797 **A)** COS-1 cells were co-transfected with pGL3(desMEF2)₃ luciferase, the pRSV β -gal reporter
 798 control and CMV (Cytomegalovirus)-driven expression plasmids encoding for the indicated Mef2c
 799 splicing isoforms. Firefly luciferase activities were normalized for transfection efficiency against
 800 the β galactosidase activity and expressed as relative luciferase units of the activity in cells
 801 transfected with the Empty Vector (EV) (= 1.0). Statistical analysis was performed on data obtained
 802 from three independent experiments, the means \pm SE (error bars) are represented. *** indicate a P-
 803 value \leq 0.001. **B)** Extracts from cells transfected in panel A were resolved by SDS PAGE, Mef2ca
 804 and Mef2cb expression was assessed by immunoblotting with anti-MEF2 antibody that recognizes
 805 all Mef2ca and Mef2cb splicing isoforms (upper panel). Sample loading was normalized using
 806 Vinculin immunoreactivity (lower panel).

807

808 **Fig. 6.** Effects of Mef2cbL forced expression in zebrafish embryos.

809 Wholemount in situ mRNA hybridization of zebrafish embryos injected with in-vitro transcribed
 810 mRNA encoding Mef2c isoforms together with Rhodamine dextran at the 1-cell stage. Injected
 811 embryos or uninjected control embryos were analyzed during development.

812 **A)** *Myod* mRNA in 22 hpf embryos injected with 25 pg of *mef2cb* mRNAs. *mef2cbL* but not
 813 *mef2cbS* injected embryos have ectopic *myod* expression in head region (arrowheads). Both groups
 814 show an array of developmental defects in head and trunk regions. **B)** *Myod* mRNA in head region
 815 at 28 hpf (dorsal view, anterior to top). Injection of 10 pg of *Mef2cbL* mRNA induces ectopic *myod*
 816 expression in head mesoderm (arrowheads). **C)** *smyhcl* mRNA and immunofluorescence of MyHC
 817 protein in 28 hpf non injected control embryos or embryos injected with 10 pg of *Mef2cbL* mRNA,
 818 Ectopic muscle is clearly seen in the head region of injected embryos (white arrowheads). While
 819 arrow and green arrowhead indicate somitic muscle and heart respectively. Scale bars = 100 μ m.

820

821 **Fig. 7.** Effects of forced expression of *mef2ca* splice variants on development of zebrafish embryo..

822 **A)** *Myod* mRNA in 22 hpf embryos injected with the mRNAs of *Mef2ca* splice variants or not
 823 injected (control). Forced expression of *Mef2ca* 4-5-6 mRNA resulted in severe developmental
 824 defects: double axis (black arrowheads), trunk and brain defects (white arrowheads). Control
 825 embryos or embryos injected with 25 pg of *Mef2ca* 4-6 mRNA showed normal morphology. **B)**
 826 RT-PCR analysis of the total RNA extracted from 25 pg *mef2ca* mRNA injected or control
 827 uninjected embryos at 22 hpf. *Mef2ca* 4-5-6 injected embryos showed augmented expression of
 828 *chordin*, *no-tail a*, *nodal related 1*, *noggin 1* and *goosecoid*, reduced expression of *Δ np63* and *bmp*
 829 *7a*, whereas *bmp 2b*, *no- tail b* and *myod* expression levels are unaffected. **C)** Densitometric
 830 analysis of the bands shown in B, normalized to *actb2* signal. Expression levels of each gene were
 831 arbitrarily set to a value of 1 in the uninjected control embryos. Statistical analysis was performed
 832 on data obtained from three independent experiments, the means \pm SE (error bars) are represented. *
 833 and ** indicate P-values of ≤ 0.1 and ≤ 0.01 respectively.

834

835 **Fig. 8.** Expression of *mef2ca* and *mef2cb* splice variants during early zebrafish development.

836 Expression levels of the *mef2ca* and *mef2cb* splice variants in exon 5 region were evaluated by RT-
 837 PCR analysis of RNA harvested from zebrafish embryos at the indicated developmental stages.
 838 PCR was performed using primers that give amplification products of different sizes depending on
 839 the splice variant, as schematized in figure 3A. PCR products were separated in 8% polyacrylamide

840 gels. Length of PCR products is indicated. Expression levels of *gsc* and *chd* were also determined.
 841 Expression levels of *actb2* RNA are shown as loading control.

842

843

SUPPLEMENTARY FIGURES

844 Fig. S1. Vertebrate *MEF2* transcripts are alternatively spliced.

845 **A)** Schematic of the highly similar structures of three vertebrate *MEF2C* genes among coding exons
 846 (black boxes). To simplify the comparison, we assigned the number 1 to the exon containing the
 847 first translated ATG. Introns are indicated by solid lines. *MEF2C* genes from the three species share
 848 three alternative exons: the $\alpha 1$ and $\alpha 2$ mutually exclusive exons, the β skipping exon, and 3' splice
 849 site selection at exon 9. **B)** Schematic of the vertebrate *Mef2c* gene exon numbering adopted in this
 850 paper. In the table are reported the exon numbering of the mouse and frog *MEF2C* genes adopted in
 851 the indicated references. **C)** Splicing patterns of frog, mouse and human *MEF2C*. The MADS box
 852 and MEF2 domain are encoded by exons 1 and 2.

853

854 Fig. S2. Amino acid conservation of alternative spliced domains of vertebrate *Mef2c* proteins.

855 **A)** $\alpha 2$ alternative exon, β skipping exon and γ region in *mef2cb* and *mef2ca* genes predicted with the
 856 TBLASTN algorithm. The sequences of bona fide spliced out exons, the percentage of homology
 857 with the mouse sequence and the putative splice sites are indicated. **B)** Comparison of amino acid
 858 sequences for zebrafish *Mef2ca* and *Mef2cb* splice variants. Protein sequence encoded by different
 859 exons is indicated, and alternatively spliced out regions are marked in yellow and green. TADs are
 860 colored in blue and orange.

861 Fig. S3. Amino acid conservation in the exon 5 encoded domain of teleosts *Mef2* proteins.

862 **A)** Comparison of amino acid sequences encoded by exon 5 and surrounding regions for zebrafish
 863 *Mef2ca* and *Mef2cb* proteins and the predicted *Mef2* proteins from cavefish (*S. anophtalmus* and *S*
 864 *angustiporus*), medaka (*O. latipes*), pufferfish (*T. rubripes*) and stickleback (*G. aculeatus*). **B)**
 865 GenBank and NCBI reference accession numbers of the sequences used for the sequence alignment
 866 in A.

867

868 Fig. S4. Developmental expression profile of zebrafish *mef2ca* and *mef2cb*.

869 **A)** Developmental expression profile of *mef2ca* and *mef2cb* transcripts by semi-quantitative RT-
 870 PCR analysis of the RNA extracted from staged zebrafish embryos. To determine the concentration

871 of the transcripts we constructed a standard curve by amplifying serial dilutions of plasmid DNA
 872 templates. As a control for the quantity of substrate RNA, we amplified the same samples for *actb2*.
 873 **B)** Double in situ hybridization for 22 hpf zebrafish embryos for *myod*, *mef2ca* and *mef2cb*
 874 transcripts. Wholemounds shown in lateral view, anterior to left.

875 Fig. S5. Quantitative analysis of the mRNA levels of *mef2ca* and of *mef2cb* exon 3 α splice variants
 876 during *D. rerio* development and in adult tissues.

877 A) Left panel. Schematic representation of *mef2cb* 3 α 1 or 3 α 2 alternative exons. Arrows show
 878 annealing sites of isoform-specific primers used in the RT-PCR analysis they were designed to give
 879 amplification products of the same size (190 bp). Right panel. Expression analysis of *mef2cb*
 880 transcripts including the mutually exclusive 3 α 1 or 3 α 2 exon by RT-PCR. Total RNA was purified
 881 from staged embryos. To amplify an amount of exon 3 α 2 containing DNA similar to that
 882 containing exon 3 α 1, four additional PCR cycles were required. (B) Left panel. Schematic
 883 representation of *mef2ca* 3 α 1 and of *mef2cb* 3 α 1 or 3 α 2 alternative exons. Arrows show annealing
 884 sites of the primers used in the RT-PCR analysis. They give amplification products of distinct sizes.
 885 Right panel. Expression analysis of *mef2ca* and *mef2cb* transcripts including the mutually exclusive
 886 3 α 1 or 3 α 2 exon by RT-PCR in adult tissues. Total RNA was purified from brain, liver, skeletal
 887 and cardiac muscle of adult zebrafish. The level of expression of the transcripts was evaluated by
 888 using primers that anneal to exons 2 and 4 for both *mef2c* genes, in the case of *mef2cb*, they give
 889 two amplification products of distinct sizes: 196 and 187 bp, depending on the incorporation of
 890 3 α 1 or 3 α 2 alternative exons in the transcripts. PCR products were separated in 8% polyacrylamide
 891 gels. Length of PCR products (bp) is indicated.

892

893 Fig. S6. Effects of Mef2ca splice variants overexpression in zebrafish embryos.

894 A) Zebrafish embryos were injected with 25 pg of in vitro-transcribed *mef2ca* 4-5-6 RNA together
 895 with rhodamine dextran at the 1-2 cells stage and analyzed at 20 hpf. Successfully injected
 896 embryos were distinguished on the basis of the red fluorescence (insets) and classified on the basis
 897 of morphology into ‘severely defective’ (blocked development), ‘defective’ (altered development)
 898 or ‘normal’. B) Dose-dependent effects of in vitro-transcribed *mef2ca* mRNAs on embryos
 899 development. The graph reports the quantification of defective embryos upon injection of
 900 increasing doses (25 pg and 50 pg) of RNA encoding Mef2ca 4-5-6 and 4-6 splice variants.
 901 Controls were uninjected embryos (Ctrl). The number of embryos tested in each experiments is
 902 indicated by (n) on top of each column. C) Western blot analysis showing over-expression of

903 Mef2ca 4-5-6 and Mef2ca 4-6 following RNA injection (25 pg) into embryos. COS-1 cell extracts
904 over-expressing Mef2ca 4-5-6 or 4-6 were used as electrophoretic mobility controls (a and b,
905 respectively). α -Tubulin was used as loading control. D) To assess whether injection of 10 pg
906 *mef2ca* 4-5-6 RNA leads to aberrant maturation of vascular, neuronal or cardiac tissues, injected
907 embryos (right panels) or controls (left panels) were subjected to in situ hybridization for *myl7*,
908 *kdrl*, *neurog1* and *ascl1a* mRNAs, respectively.

909

910 Fig. S7 Primers used in semi-quantitative RT-PCR and qRTPCR.

911 A) In the table is reported a restricted list of PCR primer pairs used in the semi-quantitative PCR
912 reaction, missing primers are available on request. B) Schematic drawing of *mef2ca* isoform
913 specific and isoform common primers used in qRTPCR. Sequences are available on request.

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

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14 **Abstract**

15 In mammals, an array of MEF2C proteins are generated by alternative splicing (AS), yet specific
16 functions have not been ascribed to each isoform. Teleost fish possess two *MEF2C* paralogues,
17 *mef2ca* and *mef2cb*. In zebrafish, the Mef2cs function to promote cardiomyogenic differentiation
18 and myofibrillogenesis in nascent skeletal ~~myosele~~ fibers. We found that zebrafish *mef2ca* and
19 *mef2cb* are alternatively spliced in the coding exons 4-6 region and ~~that~~ these splice variants differ
20 in their biological activity. Of the two, *mef2ca* is more abundantly expressed in developing skeletal
21 muscle. ~~We show that~~ its activity is tuned through zebrafish development by AS. ~~By 24 hpf, we~~
22 ~~found the prevalent expression a stage when major muscle differentiation is observed, we found the~~
23 ~~prevalent expression of the highly active full length protein in differentiated muscle specifically in~~
24 ~~the somites. At 12 hours post fertilization (hpf), a high proportion (40%) of *mef2ca* transcripts~~ ~~The~~
25 ~~splicing isoform of *mef2ca* that lacks coding-exon 5 (*mef2ca* 4-6), thereby encodes ing~~ a protein that
26 ~~has exhibits a 50% lower reduction of~~ transcriptional activity, ~~and is found mainly earlier in~~
27 ~~development, before muscle differentiation. in comparison to the full length protein. By 24 hpf, a~~
28 ~~stage when major muscle differentiation is observed, we found the prevalent expression of the~~
29 ~~highly active full length protein specifically in somites. *mef2ca* transcripts including exon 5 (*mef2ca*~~
30 ~~4-5-6) are present early in the embryo. Over-expression of this isoform alters~~ ~~Inclusion of exon 5 in~~
31 ~~Mef2ca protein confers the ability to drive, directly or indirectly,~~ the expression of genes ~~involved~~
32 ~~in early dorso-ventral patterning of the embryo~~ such as *chordin* (~~*chd*~~), *nodal related 1* (~~*ndr1*~~) and

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33 ~~gooseoid (*gsc*), which function in early dorso-ventral patterning of the embryo and induces severe~~
 34 ~~developmental defects. AS of *mef2cb* was not developmentally regulated. However, generates a~~
 35 long splicing isoform in the exon 5 region (Mef2cbL) ~~that predominates during somitogenesis over~~
 36 ~~other isoforms.~~ Mef2cbL contains an evolutionarily conserved domain derived from exonization of
 37 a fragment of intron 5, ~~which that~~ confers the ability to induce ectopic muscle in mesoderm upon
 38 over-expression of the protein. ~~Taken together, the data show that AS is a significant regulator of~~
 39 ~~Mef2c activity.~~

40

41 Abbreviations

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

52

53

54 Highlights

55

- 56 • *mef2ca* and *mef2cb* gene products are alternatively spliced in zebrafish.
- 57 • Inclusion of exon 5 in *mef2ca* transcripts is regulated during zebrafish development.
- 58 • ~~Inclusion of the Exon 5 encoded protein sequence~~ confers ~~on Mef2c to the protein~~ the
 59 ability to activate ~~a subset of early patterning- genes~~ genes involved in dorso-ventral
 60 patterning of the embryo.
- 61 • ~~zebrafish~~ Mef2cb includes an extra octapeptide encoded by a region of intron 5 ~~that is~~
 62 "exonized".

- Inclusion of the "extra-octapeptide" ~~sequence~~ confers on Mef2cb ~~the pro-myogenic activityability to induce myogenic fate in head mesoderm.~~

Keywords

Mef2ca, Mef2cb, zebrafish, skeletal muscle, alternative splicing, development

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 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]. *MEF2s* are also more broadly expressed and function to control development and adaptation of 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 $\alpha 1$ and $\alpha 2$, 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

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95 in the γ region near the C-terminus [21, 22]. In the case of mouse *Mef2d*, AS of the α exon
 96 switches the protein from a transcriptional repressor regulated by protein kinase A (PKA), to an
 97 activator insensitive to PKA signaling [23]. This switch is thought to drive skeletal muscle terminal
 98 differentiation, but how AS in the *Mef2d* α exon relates functionally to AS at other alternate exons
 99 is unclear. Developmentally regulated switching of AS of *MEF2* genes has been described during
 100 frog and mouse development [24-26]. Involvement of alternative splice variants of *Mef2* in
 101 endomesoderm and neuron differentiation in the sea anemone *Nematostella vectensis* has been
 102 described recently [27]. However, it has not yet been determined whether functional differences
 103 among the splicing variants of the *MEF2C* genes are important in vertebrate development.

104 Here we describe the alternative splicing of the two zebrafish *Mef2c* genes, *mef2ca* and *mef2cb*. In
 105 addition to splicing events akin to the α , β , γ splices that were described in mice, we find novel
 106 splice forms varying in the region between the two TADs around the fifth coding exon. We provide
 107 the first evidence that the developmentally-regulated AS of *mef2ca* in this region affects *Mef2c*
 108 protein function. ~~We report that~~ *Over-expression of* *mef2ca* transcripts including exon 5 (*mef2ca* 4-
 109 5-6) **are expressed** early in development, **and their over-expression** causes severe defects in the
 110 embryos related to impaired gastrulation that are not created by variants lacking exon 5. Moreover,
 111 ectopic expression of *Mef2ca* 4-5-6 results in an increase of the transcript levels of genes such as
 112 *chordin* (*chd*), *nodal related 1* (*ndr1*), *no-tail a* (*ntla*) and *goosecoid* (*gsc*), necessary during
 113 gastrulation for correct dorso-ventral patterning. Lastly, we describe a new evolutionarily conserved
 114 alternatively spliced isoform of *mef2cb*, here named *Mef2cbL*, containing an additional octapeptide
 115 in exon 5, that confers on *Mef2cb* the ability to induce ectopic skeletal myogenesis.

116

117 2. Materials and Methods

118

119 2.1. Plasmids

120 The full-length coding regions of the zebrafish *Mef2ca* 4-5-6, *Mef2ca* 4-6, *Mef2ca* 4'-6' and
 121 *Mef2cbL* variants were amplified from 24 hpf (**hours post fertilization**) zebrafish embryos cDNAs.
 122 The full length cDNA of *Mef2cbS* was obtained by a PCR reaction starting from a template made
 123 of three overlapping PCR products: the exon 5 region amplified from a *Mef2cb* I.M.A.G.E.
 124 (~~Integrated Molecular Analysis of Genomes and their Expression~~) clone (clone ID: 6519749,
 125 Genbank: CD282884.1), the upstream and downstream regions amplified from the *Mef2cbL* cDNA.
 126 The cDNAs were first inserted in the pCR2.1 vector (Invitrogen) or pGEM-T Easy vector

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127 (Promega), then sub-cloned into BamHI/NotI sites of the pcDNA 3.1(+) expression vector
128 (Invitrogen). For RNA injections isoforms were sub-cloned into the XbaI/Sall sites of the β UT-3
129 vector [8]. Plasmids pGL3(desMEF2)₃ and pRSV β -gal were previously described [28].

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131 2.2. Alternative splicing prediction and multiple alignments

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

137

138 2.3. Transcription Reporter Assays

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

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142 2.4. RNA isolation, RT-PCR and Real Time PCR

143 RNA was isolated using TRIzol® Plus RNA Purification System (Ambion). For each
144 developmental stage, 100 embryos were disrupted using Tissue Raptor (Qiagen). 500 ng of total
145 RNA were reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen).
146 Primers used to detect *myogenin* ([myog](#)), *myod*, *beta-actin 2* ([actb2](#)), *mef2ca* and *mef2cb* are listed
147 in Fig. S6A (other primers sequence are available upon request). quantitative Real Time PCR
148 (qRT-PCR) was performed on [2.54](#) ng of Poly A mRNA using SYBR Green method (SYBR® Green
149 PCR Core Reagent, Applied Biosystems). Poly A mRNA has been purified using Ambion's
150 protocol (MicroPoly(A) Purist Kit). To amplify the different *mef2ca* isoforms specific forward
151 primers spanning exon-exon junctions were used with a common reverse primer (Fig. S6B). For
152 each primer combination the optimal MgCl₂ concentration was determined to obtain specific and
153 high efficient amplification (slope values between -2.95 and -3.75). Absolute quantification of
154 transcript copy number was achieved by generating calibration curve using plasmid DNA templates
155 (listed above) as previously described [29, 30]. Analysis was performed using PCR ABI PRISM
156 7900 HT Sequence Detection System (Applied Biosystems). [Student's t-tests were performed for](#)
157 [pairwise comparisons to determine significant differences between groups.](#)

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160 *2.5. Zebrafish lines, maintenance and embryo manipulation*

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

163

164 *2.6. Whole Mount In Situ Hybridization (WISH)*

165 In situ mRNA hybridization was performed as described [14]. Fluorescein- or digoxigenin-tagged
166 probes used were *mef2ca* [32], *mef2cb* [8], *myod*, ~~*myogenin*~~ [33], *slow myosin heavy chain 1*
167 (*smyhc 1*) [8], *myosin, light polypeptide 7 (myl7)* [34], *kinase insert domain receptor (kdr1)* [35],
168 *neurogenin 1 (neurog1)* [36] or *achaete-scute complex-like 1a (ascl1a)* [37]. We have also used
169 ~~two~~ *non-overlapping* dual digoxigenin-labelled custom *mef2ca* exon 5-specific *locked nucleic acid*
170 (LNA) probes, *LNA1* and *LNA2* (Exiqon, sequence available upon request) *to perform WISH* as
171 described [38, 39]. Embryos were photographed as wholemounts on Olympus DP70 or dissected
172 and flatmounted in glycerol and photographed on a Zeiss Axiophot with Axiocam using
173 Improvisation Openlab.

174

175 *2.7. mRNA injection and embryo manipulation*

176 mRNA injection was performed as described previously [40]. β UT-3 vectors encoding Mef2ca and
177 Mef2cb isoforms, were linearized using Sfi/PstI sites. mRNAs were made with mMESSAGE
178 mMACHINE kit (Ambion). All RNAs were injected at 1-2 cell stage embryos at 10 ~~ng~~, *250 ng* or
179 50 ~~ng~~ *ng*/embryo. Tetramethyl-rhodamine Dextran (5% solution in 0.2 M KCl) was co-injected in
180 order to sort phenotypes of injected embryos. At 20-~~28~~ *22* hpf injected embryos were analyzed and
181 sorted using a Zeiss Axiophot with Axiocam.

182

183 *2.8. Western blot analysis and antibodies*

184 Zebrafish embryos were dechorionated and lysed in RIPA buffer (50mM Tris HCl pH 7.5, 150 mM
185 NaCl, 1 mM EDTA, 1% Na Deoxycholate, 1% Igepal, 0.1% SDS, 1 mM DTT) containing 1 mM
186 PMSF and Proteases Inhibitor Complete cocktail (ROCHE). Equal amount of protein extracts were
187 separated by SDS-PAGE and subsequently analyzed by Western blot as previously described [28].
188 The following antibodies were used: rabbit polyclonal anti-MEF2 (sc-313X; Santa Cruz
189 Biotechnology, Inc.), mouse anti- α Tubulin (T6074, Sigma Aldrich), mouse anti-Vinculin (V4505,

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190 Sigma Aldrich). ~~Embryo staining was performed with a primary antibody against sarcomeric~~
 191 ~~myosin heavy chain (MyHC; A4.1025 [41]) as previously described [8].~~

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193 ~~2.9. Statistical Analysis~~

194 ~~Data from qRT-PCR were analyzed using the SigmaPlot 11.0 statistical package (Systat Software,~~
 195 ~~Inc.). Student's t tests were performed for pairwise comparisons, while ANOVAs applying the~~
 196 ~~Holm-Sidak post hoc test were used to determine significant differences between groups.~~

198 3. Results

199
 200 To compare alternative splicing in *MEF2C* genes across species and paralogues, we use a standard
 201 nomenclature numbering exons 5' to 3' from the first coding exon, yet retaining the conventional α ,
 202 β , γ designation for alternatively spliced exons. Each exon number thus corresponds to homologous
 203 sequences (Figs 1A ad S1).

205 3.1. Alternative Splicing of zebrafish *mef2ca* and *mef2cb*

206 To predict splice variants of the zebrafish Mef2c proteins, we conducted *in silico* analysis of the
 207 zebrafish *mef2ca* and *mef2cb* genes on public databases (see Methods). In addition to the known
 208 *mef2ca* transcript [32, 42], we detected two alternative 5'UTR sequences and several alternative
 209 splices (Fig. 1A and B). *mef2ca* lacks an alternative exon 3, the α exon, having a single exon most
 210 similar to the 3 α 1 form of amniote *MEF2C*, which has serine residues at positions 98 and 109 [43].

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211 ~~However~~ *Mef2ca* contains a putative β exon in intron 6 ~~and~~, in addition, a γ -region flanked by a
 212 non-canonical 3' splice site (GC) was found at the start of exon 9 (Fig. S2A). Of particular note in
 213 the *in silico* analysis, were three alternative splices in the region of exons 4, 5 and 6 (Fig. 1C), two
 214 of which correspond to the alternatively spliced δ exon (exon 5) of the *mef2c* gene product in
 215 *Xenopus* [24] (Fig. S1B,C). By sequence alignment we found that splicing of exon 5 is conserved
 216 among teleosts (Fig. S3). Zebrafish *mef2cb* splicing appeared more similar to amniote *MEF2C*
 217 genes than that of *mef2ca*. We predicted two 5' UTR sequences, alternate exons 3, 3 α 1 and 3 α 2, a
 218 putative β exon, encoding the conserved octapeptide SEDVDLLL in intron 6 of *mef2cb*, and a
 219 putative γ region at the start of exon 9 (Fig. 1B). The sequences of alternative exons 3 α 1 and
 220 3 α 2 are mostly similar to the corresponding alternate exons of amniote *MEF2C*, ~~although~~ neither of

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221 the two α exons contains a PKA target residue corresponding to serine 120 of MEF2D, which was
 222 found to direct binding of repressive or activating cofactors. [23]. Additionally, we found a *mef2cb*
 223 variant with a long exon 5 resulting from a retained intron 5 sequence. We designate this Mef2cbL
 224 to distinguish it from the conventional exon 5 in Mef2cbS (Fig. 1B, C).

225 To characterize the major *mef2ca* and *mef2cb* spliced isoforms expressed in developing zebrafish
 226 skeletal muscle, we performed RT-PCR on RNA extracted from the dissected tail region of 24 hpf
 227 embryos using primer pairs that target conserved sequences (Fig. 1B). A series of *mef2ca* mRNA
 228 RT-PCR products were amplified, sub-cloned and their sequences compared to the nucleotide
 229 sequence of *mef2ca* genomic DNA, revealing the existence of at least four species of *mef2ca*
 230 mRNAs produced by AS in developing embryos (Fig. 1B). In addition to the transcript encoding the
 231 full length protein, here referred to as Mef2ca 4-5-6 (465 aa), two *mef2ca* isoforms, Mef2ca 4-6
 232 (451 aa, ~~predicted in silico~~) and Mef2ca 4'-6' (413 aa) derive, respectively, from skipping exon 5 or
 233 a larger region that also encompasses part of exons 4 and 6. Another variant, Mef2ca $\Delta\gamma$ -like (411
 234 aa) contains exon 5 but lacks the γ region and further sequences located in exons 8 and 9. The
 235 Mef2ca 4'-6' and Mef2ca $\Delta\gamma$ -like mRNAs are the results of splicing at the non-canonical 5' donor
 236 splice sites GG and CA, respectively (Fig. 1C) (GenBank accession numbers: KF932282 and
 237 KF932281 respectively).

238 One *mef2cb* variant, named Mef2cbL, was obtained by RT-PCR; it includes exon 3 α 1 and γ but
 239 lacks exon β . ~~Mef2cbL~~, ~~it~~ arises from the inclusion of an additional sequence from intron 5 (Fig.
 240 1C). Indeed, two competing 5' splice sites are present at the end of exon 5, these splice sites direct
 241 inclusion or exclusion of 24 nucleotides (nt) encoding the octapeptide KDGIPTY (Fig. 1C).
 242 When aligned (Fig. S2B), the predicted amino acid sequences of the identified zebrafish *mef2ca* and
 243 *mef2cb* isoforms show that the major variation occurs in the exon 4-5-6 region of both genes,
 244 located between the two TADs described previously [44, 45].

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

250

251 *3.2. mef2ca is the main Mef2c orthologue expressed during skeletal muscle development*

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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 qRT-PCR 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^{86} copies/2.5 ng RNA and *mef2cb* 2^{75} 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^{120} /2.5 ng RNA, *mef2cb* 2^{97} /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.

270

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 qRT-PCR 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 430% of the total, comparable to that of whereas the amount of the full length 4-5-6 transcript the remaining 70% ($\sim 2^7$ copies/ng RNA). At 24 hpf and beyond, *mef2ca* 4-6 expression increased slightly (2^8 copies/ng) but remained less abundant than the *mef2ca* 4-5-6, whose predominance increases further which becomes the predominant isoform (2^{10} copies/ng, 805% of the total *mef2ca* transcripts) (Fig. 3B-A-C). The shortest isoform, *mef2ca* 4'-6' is present at low level (less than 1% of the total *mef2ca* transcripts 2^2 - 2^3 copies/ng) 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 with and without the γ -like region were expressed at early stages of development. H, however, they

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285 | were less abundant, and ~~were not detected disappeared~~ beyond 24 hpf (Fig. 3A,B). Thus, almost all
 286 | *mef2ca* transcripts contain the γ -like region and lack β exon, irrespective of their splicing at the 4-5-
 287 | 6 region.

288 | Expression of the *mef2cb* alternatively spliced exons was also determined by ~~semi-quantitative~~ RT-
 289 | PCR ~~and qRT-PCR~~ amplification. Transcripts containing exons 3 α 1 and 3 α 2 were detected
 290 | throughout development using ~~common primers that give two amplicons of different size and~~
 291 | ~~therefore electrophoretically distinguishable. The transcript that includes the 3 α 1 exon is the most~~
 292 | ~~abundant at all the developmental stages beyond 12 hpf (Fig. 3E). This result was also confirmed by~~
 293 | ~~using~~ isoform-specific primers ~~(Fig. 3D,E).~~ Given that, in identical experimental conditions, four
 294 | additional PCR cycles are required to amplify an amount of exon 3 α 2-containing DNA similar to
 295 | that containing exon 3 α 1 (Fig. S5A). ~~Whereas, it is likely that the~~ inclusion of
 296 | exon 3 α 1 predominates in ~~the developing embryo muscle, RT-PCR analysis revealed that in adult~~
 297 | ~~skeletal and cardiac muscle the levels of the two isoforms are comparable (Fig. S5B). We did not~~
 298 | ~~detect the 3 α 2-containing transcript in liver and brain, indicating a muscle-restricted pattern of~~
 299 | ~~expression of this splice variant, analogously to what has been reported for the mammalian~~
 300 | ~~counterpart (Fig. S5B) [20].~~ Inclusion of exon β was barely detected. In contrast, the γ region and
 301 | the extra sequence from intron 5 (Mef2cbL) were readily detected at all developmental stages (Fig.
 302 | 3D,E). qRT-PCR quantification confirmed that ~~more than~~ 90% of *mef2cb* transcripts retain the extra
 303 | intron 5 sequence, but ~~less than~~ ~10% encode the Mef2cbS form (Fig. 3F ~~data not shown~~). Thus,
 304 | both *mef2ca* and *mef2cb* show striking variations in the ~~exon~~ 4-5-6 region.

305 |
 306 |
 307 |

308 | 3.4. Exon 5-containing *mef2ca* transcripts accumulate in skeletal muscle

309 | To examine where *mef2ca* mRNA(s) that include exon 5 are expressed in the developing zebrafish
 310 | embryo, we performed in situ mRNA hybridization using a probe that recognizes all transcripts
 311 | (*mef2ca* probe, [32]) and ~~two non-overlapping~~ dual digoxigenin-labelled ~~locked nucleic acid~~
 312 | ~~(LNA) probes~~ designed to recognize a 21 base pair sequences ~~located~~ within ~~exon 5 (LNA1) or~~
 313 | ~~within~~ the exon 4/5 boundary (~~exon 5 probe LNA2~~); (Fig. 4C). ~~At 22 ss and In~~ 24 hpf embryos,
 314 | *mef2ca* transcripts were detected throughout the ~~somitic muscle and preferentially observed at~~
 315 | ~~somite border~~ (Fig. 4A upper panel), ~~(Fig. 4A-C)~~ as well as ~~As with the RT-PCR and qRT-PCR~~
 316 | ~~results, both probes suggested an increase in *mef2ca* mRNA levels at 24 hpf, compared to earlier~~

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317 ~~stages. Notably, whereas the *mef2ca* generic probe was also clearly detected~~ in the heart and
 318 branchial arches (Fig. 4A upper panel, see also Fig. 2B) ~~at 24 hpf.~~ Similarly, both the exon 5-
 319 specific LNA probes gave signals above background only in skeletal muscle, ~~and was there~~
 320 preferentially observed at somite borders (Fig. 4A,C,D, middle and lower panels). ~~By 48 hpf, the~~
 321 ~~signals obtained with the generic and both exon 5-specific probes, to which *mef2ca* mRNA, are is~~
 322 restricted ~~almost entirely to the somite boundaries area (Fig. 4B, left and at later stages [14]).~~ In
 323 addition, signals with all three probes show the typical separate dorsal and ventral muscle signal in
 324 the pectoral fin (Fig. 4B, right). Thus, even though we cannot exclude some levels of expression in
 325 other tissues, we conclude that the *mef2ca* 4-5-6 transcript is expressed primarily in skeletal muscle
 326 and is mainly localized to somite boundaries, suggesting it may have a distinct and specific
 327 function.

328

329 3.5. *Mef2ca* 4-5-6 is a potent transactivator

330 The transcriptional activities of *mef2ca* splice variants were tested in vitro by co-transfection into
 331 COS-1 cells of each Mef2c splice variant with a MEF2-responsive luciferase reporter containing
 332 three copies of the MEF2 binding site from the *Desmin* gene regulatory region (pGL3desMEF2)
 333 [46]. COS-1 cells have low endogenous MEF2 expression. Immunofluorescence analysis revealed
 334 that all Mef2ca and Mef2cb splice variants efficiently localized to the nucleus (data not shown),
 335 congruent with the observation that they all include the sequence corresponding to the nuclear
 336 localization signal described in the mouse [47].

337 Compared to other Mef2c isoforms tested, the Mef2ca 4-5-6 full length protein had the strongest
 338 transcriptional activity (Fig. 5A). Deletion of amino acids encoded by exon 5 and neighboring
 339 sequences result in a twofold reduction in transcriptional activity, even though the respective
 340 protein expression levels were comparable (Fig. 5B). Furthermore, we observed that a Mef2ca 4-5-6
 341 isoform lacking the γ -like domain had ~~2-fold~~ ~~twofold~~ higher transcriptional activity than Mef2ca
 342 containing the γ -like domain, consistent with the finding that this region represses transcription
 343 (data not shown; [21]). Upon transfection, the Mef2cbL and Mef2cbS isoforms, each containing
 344 both exon 5 and γ , exhibited similar activity (about 70% of that of Mef2ca 4-5-6) (Fig. 5A and data
 345 not shown). However, Mef2cbL immunoreactivity was ~~much lower than the Mef2ca isoforms (Fig.~~
 346 ~~5B), indicating.~~ ~~Given that we obtained similar results with other antibodies directed against~~
 347 ~~different regions of MEF2 proteins (data not shown), it is unlikely that the low amount of Mef2cb~~
 348 ~~protein detected is due to the low reactivity of either that~~ our anti-Mef2 antiserum. ~~Additional~~
 349 ~~studies are required to characterize the stability and translational efficiency of Mef2c proteins, but~~

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350 ~~our results suggest that Mef2cbL has higher activity per molecule than Mef2ca 4-5-6. reacts poorly~~
 351 ~~to Mef2cb or that the protein has reduced stability and therefore higher activity per molecule than~~
 352 ~~Mef2ca 4-5-6.~~ Taken together, these data suggest that inclusion of exon 5 between TAD1 and
 353 TAD2 confers increased activity to Mef2ca.

354

355 3.6. *Mef2cbL* has unique myogenic potential in developing zebrafish

356 To investigate the biological significance of Mef2ca and Mef2cb splice variants in zebrafish
 357 embryonic development, we determined the effects of their ectopic expression by injecting embryos
 358 at the one-cell stage with synthetic Mef2c mRNAs and analyzing them at 24 hpf. We have shown
 359 previously that injection of mRNA of ~~*mef2cb*~~~~*Mef2cb*~~ induces ectopic skeletal muscle in embryos
 360 [8]. Here we report that injection of ~~10~~ pg/embryo of mRNAs of the Mef2cbL isoform induced
 361 ectopic skeletal muscle in the anterior mesoderm of 40-% of the injected embryos, as revealed by
 362 wholemount in situ hybridization for *myod* mRNA in 28 hpf zebrafish embryo, a developmental
 363 stage where no endogenous muscle is normally observed in the head (Fig. 6A, ~~B~~, ~~black arrows~~). In
 364 addition to *myod* transcripts we detected ectopic expression of *smyhcl* transcripts and MyHC
 365 protein, further supporting the pro-myogenic activity of Mef2cbL (Fig. 6B). No induction of ectopic
 366 muscle was observed after ectopically expressing any Mef2ca isoform, even when higher quantities
 367 of mRNA were injected (~~data not shown~~Fig. 7). Interestingly, this effect depends on the inclusion of
 368 the KDGIPYY octapeptide, because forced expression of the Mef2cbS isoform did not cause
 369 ectopic myogenesis (Fig. 6A, B). Thus, the form of Mef2cb that is normally present in developing
 370 zebrafish embryos during somitogenesis has unique myogenic potential that is not shared by
 371 Mef2ca 4-5-6, the predominant Mef2c isoform in skeletal muscle. Injection of higher amounts (25
 372 pg/embryo) of both Mef2cbS and Mef2cbL mRNAs resulted in head and trunk developmental
 373 alterations (Fig. 6C).

374

375 3.7. *Mef2ca* 4-5-6 over-expression causes defects in gastrulation

376 To investigate the functionality of the two main Mef2ca isoforms expressed during development
 377 (Mef2ca 4-5-6 and 4-6), ~~high~~er doses of Mef2ca ~~m~~mRNAs were employed. Injection of 25 pg of full
 378 length Mef2ca 4-5-6 RNA had dramatic effects on embryonic development, inducing lethality in
 379 approximately 30% of the embryos and marked developmental defects in 49% of the surviving
 380 embryos, classified as ‘severely defective’ (Fig. ~~S6~~5A,B). Such embryos already had defects
 381 evident at gastrulation stages (6-8 hpf, data not shown). Among the surviving embryos, a further
 382 34% exhibited a milder phenotype classified as □-defective’, with trunk convergent extension

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383 defects, occasional double axes, and some brain defects such as undeveloped eyes and absence of
 384 mid- and forebrain structures (Fig. 7A6B,C). Only 16% of embryos appeared unaffected by the
 385 Mef2ca 4-5-6 RNA. The percentage of severely defective embryos increased in a dose-dependent
 386 manner upon increasing the amount of injected RNA (Fig. S6B5C). In contrast to Mef2ca 4-5-6,
 387 forced expression of the Mef2ca 4-6 isoform was less active, having no detectable effect on the
 388 development of most (85%) of the injected embryos, even when expressed at comparable levels to
 389 Mef2ca 4-5-6 (Figs 7A6B,C and S6B,C5D). These results indicate that ectopic Mef2ca activity in
 390 early stages disrupts normal development. The gross defects in gastrulation induced by over-
 391 expressed Mef2ca 4-5-6 suggested severe tissue patterning disruption, yet a survey of cell lineage
 392 markers revealed no indication of altered cell fates at lower doses of RNA (Fig. S65DE).

393 To gain more insight into the mechanisms underlying the ability of Mef2ca 4-5-6 to disrupt
 394 development, the expression levels of genes encoding transcription factors and signaling molecules
 395 that are involved in early patterning of the embryo were screened by semi-quantitative RT-PCR.
 396 The *chd* gene, encoding a BMP (Bone Morphogenetic Protein) antagonist involved in dorsoventral
 397 patterning of early embryos [48] (reviewed in [49], [50]), was highly up-regulated (2-fold) in
 398 embryos injected with the *mef2ca* 4-5-6 mRNA, but not in those injected with the 4-6 spliced
 399 isoform (Fig. 7B,C6D,E). Mef2ca 4[48]-5-6 also mildly induced the expression of *ndr1* (1.5-fold),
 400 *gsc* (2.2-fold) and other dorsally-expressed genes ~~-(no-tail a, noggin 1)involved in embryo~~
 401 ~~patterning, and reduced the expression of ventralizing factors such as *bmp7a* (0.4-fold) and *Anp63*~~
 402 ~~(0.2-fold)~~, but did not alter the transcript level of *myod* or *no-tail b* and *bmp2b* (Fig. 7B,C6D,E),
 403 suggesting that the protein sequence encoded by exon 5 can modulate the expression level of a
 404 specific subset of early embryonic genes.

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

408 Our data indicate that forced expression of Mef2ca 4-5-6 protein induces the ectopic expression of
 409 genes involved in early dorso-ventral patterning of the embryo. In an attempt to get more insight
 410 into a putative role of Mef2ca in controlling endogenous patterning genes, we next determined the
 411 expression and alternative splicing patterns of *mef2c* genes during early stages of development and
 412 compared them to those of two of their putative target genes, i.e. *chd* and *gsc*. To this aim we
 413 performed RT-PCR analysis of the RNA from zebrafish embryos harvested at the 1K₂-cell (3 hpf),
 414 50% epiboly (5.25 hpf), and bud (9-10 hpf) stages. Our analysis revealed that *mef2ca* transcripts are
 415 already detectable as early as at the 1K₂-cell stage, with predominant expression of the transcript

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416 including exon 5 (Fig. 8). We noticed a rapid loss of the 4-5-6 transcript that became undetectable
 417 by 10 hpf when the 4-6 mRNA is the only *mef2ca* transcript detected, inclusion of exon 5 is again
 418 detected later, by 12 hpf (Fig. 3) and the 4-5-6 full length transcript predominates upon muscle
 419 differentiation. The kinetics of expression of the *mef2ca* 4-5-6 transcript suggests that it might be
 420 of maternal origin. The presence of Mef2ca 4-5-6 transcripts early in development, which is
 421 temporally coincident with *gsc* expression and overlaps partially with that of *chd* (our data and [51])
 422 is consistent with a role of this *mef2ca* splice variant in dorso-ventral patterning. No *mef2cb*
 423 expression is detected prior to 50% epiboly, in mid-gastrulation, where only the *mef2cbS* transcript
 424 is present. Nonetheless, at the onset of somitogenesis (9-10hpf), we noticed that only the transcript
 425 encoding for Mef2cbL, the pro-myogenic variant, is expressed.

426

427

428 4. Discussion

429 Alternative splicing of transcription factors can have a wide impact on the regulation of
 430 transcriptional networks. However, the relevance of alternative splicing is often unclear as distinct
 431 roles of alternatively spliced isoforms ~~are often have~~ not ~~yet been~~ determined. In this study, we
 432 addressed the functions of alternatively spliced isoforms of zebrafish Mef2ca and Mef2cb, two
 433 transcription factors involved in the development of striated muscle and head skeletal patterning [8,
 434 14, 16, 42]. *Mef2c* mRNA is alternatively spliced in several organisms [20-22, 24, 52], and a recent
 435 report suggests that aberrant splice variants of MEF2C are involved in myogenic disorders [53].
 436 Nevertheless, the functional differences between alternatively spliced Mef2C variants remain
 437 elusive. Our findings make three major points regarding the function of alternative splicing in
 438 Mef2c proteins of teleost fish. Firstly, both *mef2ca* and *mef2cb* gene transcripts undergo specific
 439 alternative splicing and, ~~at least in the case of *mef2ca*, the major Mef2c expressed in differentiated~~
 440 ~~muscle skeletal muscle fibers, their~~ splicing patterns changes during development. Secondly,
 441 splicing of *mef2ca* transcripts to include the exon 5 enhances its positive transcriptional activity and
 442 ability to interfere with gastrulation when over-expressed. Thirdly, an evolutionarily conserved
 443 alternate splice of exon 5 in *mef2cb* transcripts creates a long form that has unique pro-myogenic
 444 capacity.

445

446 4.1. Regulation of Mef2ca activity by alternative splicing in zebrafish development

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447 ~~In addition to the well documented expression of *mef2ca* starting from 12 hpf [32], we found~~
 448 ~~*mef2ca* transcripts in zebrafish embryo prior to gastrulation at the 1K-cell stage, likely from~~
 449 ~~maternal contribution, with their level declining to a minimum at 9-10 hpf. Starting from 12~~
 450 ~~hpf~~ During development we observe an overall increase in expression levels of *mef2ca* mRNA.
 451 ~~Besides changes in the abundance of *mef2ca* transcripts, we found a dynamic regulation of the~~
 452 ~~splicing in the exon 5 region and, in addition, *mef2ca* transcripts display a switch in isoform~~
 453 ~~dominance. The *mef2ca* variant including exon 5 (*mef2ca* 4-5-6) is the major isoform detected~~
 454 ~~very early in development (1K-cell stage), suggesting that it might play a role prior to gastrulation,~~
 455 ~~by 10 hpf, the *mef2ca* transcripts lacking exon 5 (*mef2ca* 4-6) are predominant. Subsequently,~~
 456 ~~*mef2ca* 4-5-6 again climbs as muscle precursors undergo terminal differentiation, becoming the~~
 457 ~~pre~~dominant ~~valent~~ isoform at 24 hpf, ~~as muscle precursors undergo terminal differentiation.~~ Such
 458 splicing of exon 5 is evolutionary conserved between *Xenopus* and teleosts, suggesting it has
 459 biological significance [24] (Fig. S3). Moreover, muscle differentiation in zebrafish is associated
 460 with several other muscle-specific alternative splicing events involving changes in splicing
 461 efficiency [54]. Although no specific function was assigned to the exon 5 domain by mutational and
 462 deletion analysis of the mouse and human protein counterparts [44, 45], our cell culture data
 463 indicate that the ~~peptide~~protein sequence encoded by exon 5 contributes to the transcriptional
 464 activity of Mef2ca. ~~The early expression of *mef2ca* transcripts including exon 5 (*mef2ca* 4-5-6) may~~
 465 ~~indicate their early function in embryo patterning. Later in development,~~
 466 ~~The~~ preferential accumulation of full length *mef2ca* 4-5-6 mRNA at skeletal muscle fiber ends,
 467 suggests that its normal function is in muscle, a view confirmed by the requirement for Mef2ca
 468 function for skeletal muscle fiber growth and heart myogenesis [8, 55]. In the current work the
 469 function of Mef2ca isoforms was probed by ectopic over-expression; *mef2ca* 4-5-6 RNA, but not
 470 *mef2ca* 4-6 RNA, causes gross defects during gastrulation. We suggest that these effects of Mef2ca
 471 4-5-6 are attributable to its ability to activate, directly or indirectly, a specific subset of pivotal
 472 genes in gastrulation. We observed ~~the a robust ten fold~~ induction in *chordin* (*chd*)*chd* (2-fold)
 473 mRNA and a milder (1.4 fold) increase in *noggin 1* (*nog1*) mRNAs that encode two inhibitors of
 474 the BMP signaling. *chordin* is required to repress *bmp2b* function in formation of the organizer
 475 and dorsoventral patterning of mesoderm and neural tissue [56-58]. Over-expression of *chordin*
 476 dorsalizes embryos [59, 60], a phenotype present in a fraction of embryos following Mef2ca 4-5-6
 477 over-expression. Thus, up-regulation of these dorsalizing proteins may explain the effects of
 478 Mef2ca 4-5-6.

479 ~~Ectopic Mef2ca 4-5-6 may mimic the action of other Mef2s during zebrafish gastrulation.~~ In
 480 *Xenopus*, MEF2D helps induce mesoderm by driving the expression of the *Nodal-related 1* (*ndr1*)

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481 gene [61]. In zebrafish, Mef2ca 4-5-6 over-expression also increases in *gsc*- and *ndr1* mRNAs (2.2-
 482 4.7 and 1.58- folds, respectively), which regulate dorsoventral patterning in organisms ranging from
 483 *Drosophila* to mammals [49, 50, 62-64]. In line with our results it has been previously reported that
 484 expression of *gsc*, is reduced in *mef2ca*^{-/-} (*hoover*) mutants [42]. Although we cannot exclude off-
 485 target effects, this specific ability of Mef2ca 4-5-6, but not of similar amounts of Mef2ca 4-6,
 486 suggests distinct transcriptional activity of the former. *In silico* analysis of promoter regions of *chd*
 487 and *ndr1*-genes revealed the presence of several putative MEF2 binding sites (YTA(A/T)₄TAR)
 488 (data not shown), raising the possibility that Mef2ca 4-5-6 directly activates their expression during
 489 -early development. Later in development Some regions of early *chordin* expression may be
 490 sustained by Mef2d, which constitutively includes the sequence encoded by exon 5, and which is
 491 expressed from mid-gastrulation in adaxial muscle cells that also express *chordin* [32, 60] or by
 492 Mef2cb proteins. Interestingly, injection of either *mef2cbS* or *mef2cbL* transcripts, both containing
 493 exon5, have resulted in similar developmental defects to that of *mef2ca* 4-5-6 mRNA injection.
 494 Future studies will clarify whether these genes are indeed direct targets of a Mef2 protein
 495 containing exon 5.

496 After gastrulation, As zebrafish *mef2ca* transcripts accumulate starting from s to be expressed at 12
 497 hpf [14, 32], and *mef2ca* 4-5-6 transcripts are particularly abundant by 24 hpf, after the peak of
 498 *chordin* expression during gastrulation, Wwe suggest that Mef2ca 4-5-6 function might modulates
 499 *chordin* and other target gene expression in the somites at later stages during myotome patterning,
 500 where later muscle differentiation is regulated by BMP~~mp~~ signaling and where *chordin* expression
 501 has been observed [60, 65-68].

502 The protein sequence encoded by exon 5 might represent a binding motif that mediates protein-
 503 protein interactions with specific co-factors, as one recognized function for alternatively spliced
 504 isoforms is to remodel the protein-protein interaction network [69]. Supporting this hypothesis is
 505 the recent demonstration that the domains encoded by the mutually exclusive $\alpha 1/\alpha 2$ exons of
 506 mouse MEF2D can mediate interactions with different sets of co-repressors or co-activators [23].

507 508 4.2. Gene duplication and evolutionary partitioning of alternative splicing

509 The importance of other splices in Mef2ca remains to be determined. The γ -like and 4'-6' splices
 510 have low abundance and we were unable to display unique activities for these isoforms. On the
 511 other hand, unlike in mammals, exon 3 does not appear to show alternative splicing in Mef2ca, the
 512 gene only having an $\alpha 1$ version. As the $\alpha 1$ exon of mouse Mef2D mediates interactions with
 513 specific transcriptional co-regulators [23], Mef2ca may have a more restricted range of functions

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514 compared to Mef2cb, which retains alternative α exons in its genomic sequence. However, at the
 515 stages examined, transcripts of *mef2cb* containing the $\alpha 2$ exon had low abundance, suggesting that
 516 this splice may be significant in specific cell types or developmental stages. In the adult we found a
 517 high proportion of the *mef2cb* transcripts containing the $3\alpha 2$ exon in striated muscle tissue where it
 518 might play a specific role in mediating muscle gene expression as shown for the analogous splice
 519 variant of *Mef2d* in mammals [23]. Conversely, *mef2cb* transcripts omitting exon 5 were not
 520 observed. Instead, teleost *mef2cb* has evolved a unique splice, possibly derived by exonisation [1]
 521 of a part of intron 5. The addition of this octapeptide and its conservation across teleosts appears to
 522 have conferred myogenic properties to Mef2cbL.

523

524 4.3. Alternative splicing of *mef2cb* gene generates a pro-myogenic transcription factor

525

526 We detected *mef2cb* transcripts in zebrafish embryo as early as 50% epiboly stage. Mef2cbL is the
 527 prevalent Mef2cb isoform ~~throughout development~~ starting from 9-10 hpf, concomitantly with the
 528 onset of somitogenesis and has a unique pro-myogenic capacity. *mef2cb* mRNA over-expression
 529 can convert cells to skeletal muscle (Fig. 6A; [8]). This result suggests a role for Mef2 as a skeletal
 530 muscle determination factor in zebrafish head, challenging the classical epistatic relationship
 531 between MyoD and MEF2 in which MyoD acts upstream of MEF2 to direct embryonic multipotent
 532 progenitors into the myogenic lineage. The myogenic activity of Mef2cbL relies on an octapeptide
 533 encoded by a short sequence of intron 5 retained in the transcript. This insert, being too short to
 534 form a domain, may act by changing the structural fold and leading to a new function of the protein
 535 [70]. Muscle conversion was not observed upon ectopic expression of Mef2cbL in mouse
 536 fibroblasts, congruent with previous observations made with the mouse MEF2 proteins [9, 71, 72].
 537 Thus, we propose the existence of a specific co-factor expressed in zebrafish head mesoderm that
 538 confers myogenic capacity to Mef2cbL. Identifying Mef2cb's molecular partners recruited
 539 specifically in the presence of the octapeptide to activate the expression of *myod* and other muscle
 540 genes may help in deciphering the molecular mechanisms underlying the pro-myogenic activity of
 541 Mef2cbL.

542

543 5. Conclusions

544 Our data reveal novel alternative splicing events around exon 5 of zebrafish *mef2ca* and
 545 *mef2cb* transcripts. These various evolutionarily conserved transcripts expand the transcriptional

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546 range of activity of Mef2c proteins. We propose that by excluding or including sequences of the
 547 exon 5 region, Mef2cs can acquire distinct properties, which allow them to regulate different sets of
 548 target genes and execute unique developmental programs *in vivo*.

549

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570

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750 **Figure Legends**

751 **Fig. 1.** Genomic organization, transcripts and protein variants of zebrafish *mef2ca* and *mef2cb*
 752 genes. **A)** Schematics of zebrafish *mef2ca* and *mef2cb* genes. Exons are numbered and indicated by
 753 boxes. Black boxes indicate the *mef2ca* and *mef2cb* coding exons, whereas grey boxes represent the
 754 5'- and 3'-untranslated regions. Introns are indicated by solid lines. The ATG translational start
 755 codons and the TGA stop codons of the two genes are also indicated. **B)** Schematic representation
 756 of zebrafish *mef2ca* and *mef2cb* transcript variants. Grey boxes represent UTR, white boxes
 757 represent the coding regions of the MADS and MEF2 domains in exons 1 and 2, black boxes
 758 represent the remaining translated sequence. Structures of zebrafish *mef2ca* and *mef2cb* genes
 759 transcripts are similar with the following exceptions: *mef2ca* lacks the 3 α 2, exon 5 alone or together
 760 with neighboring sequences from exons 4 and 6 may be excluded from the mature transcripts, the
 761 alternatively spliced γ region overlaps with the homologous γ regions of zebrafish *mef2cb* and of the
 762 other vertebrates *mef2c* genes, however it extends to neighboring sequences located in exons 8 and
 763 9; *mef2cb* transcripts may include a short (24 nt) sequence of intron 5 (*). White arrows indicate the
 764 position of the primers used to amplify the cDNAs. The structures of the Mef2ca and Mef2cb
 765 protein isoforms deduced from the cloned cDNA sequences are schematized. The N-terminal region
 766 of the Mef2c proteins comprises the MADS-box and the MEF2 domain, involved in DNA binding
 767 and dimerization. By analogy with the mouse and human proteins, in the C-ter there are two
 768 putative transcriptional activation domains, TAD1 (blue) and TAD2 (orange), encoded respectively
 769 by exon 4 and by exons 6,7,8, downstream is localized the nuclear localization signal (NLS)
 770 (squared box). The position of exon 5 (black) and neighboring sequences that are excluded in the 4'-
 771 6' isoform (grey) are indicated as well as the position of the γ -like and γ region of Mef2ca and
 772 Mef2cb respectively. Exon numbering is reported and the number of amino acids is indicated on the

773 bar above. Mef2ca forms are named according to whether or not the exon 5 and neighboring regions
 774 or the γ -like region are present (Mef2ca 4-5-6, 4-6, 4'-6', Δ γ -like). Mef2cb forms are named
 775 according to whether or not the octapeptide (*) in the exon 5 region is present or not (Mef2cbL and
 776 Mef2cbS). C) Details of the alternative splicing events that take place respectively: i. In the exon 5
 777 region of *mef2ca*, showing the consensus and the non-canonical splice sites and the three species of
 778 mRNA generated; ii. In the γ region of *mef2ca*, splicing through a non canonical CA alternative 5'
 779 splice site in exon 8 and a canonical alternative 3' splice site in exon 9 gives rise to the deletion of
 780 the γ -like region; iii. Exon 5 region of *mef2cb* transcript, the cartoon shows the sequence of the
 781 intron 5 that can be alternatively included in *mef2cb* transcripts, the competing donor splice sites
 782 (GT) and the two species of mRNA generated.

783

784 **Fig. 2.** Expression of *mef2ca* and *mef2cb* genes in zebrafish embryos. **A)** Estimation of absolute
 785 *mef2ca* and *mef2cb* transcripts by qRT-PCR during *D. rerio* development. The graph shows
 786 transcript-specific absolute quantification, reported as \log_2 Copy number in equal amount of total
 787 RNA (2.54 ng) extracted from zebrafish embryos at 12, 24, 48 and 72 hpf whole zebrafish embryos
 788 Graph showing mean \pm SE from two-four independent experiments, ** and *** indicates a Pp-
 789 values of ≤ 0.015 and ≤ 0.001 respectively. **B)** Wholemount in situ hybridization for *mef2ca* and
 790 *mef2cb* mRNA for embryos at 11 ss (dorsal view, anterior to top) and at 24 hpf (lateral view,
 791 anterior to left). At 11 ss, both genes are expressed in the adaxial cells (black arrowheads). By 24
 792 hpf *mef2ca* is strongly expressed in the myotome (black arrows) and also in heart (green arrowhead)
 793 and branchial arches (blue arrowhead). *mef2cb* transcripts are detected in the heart (green
 794 arrowhead), telencephalon (red arrowhead) and are weakly detected in the somites (black arrow).
 795 Scale bars = 100 μ m.

796

797 **Fig. 3.** Developmental expression profile of *mef2ca* and *mef2cb* alternative splicing products.

798 **A)** Schematic representation of Mef2ca alternative exons. Arrows show primers annealing sites
 799 used in the RT-PCR analysis. **B)** Expression analysis of alternative splicing variants of *mef2ca*
 800 transcripts by RT-PCR. Total RNA was purified from staged embryos at 12, 24, 48 and 72 hpf.
 801 PCR was performed using primers that give amplification products of different sizes depending on
 802 the splice variant. The level of expression of total *mef2ca* transcripts was evaluated by using two
 803 primers (ca1 and ca3) that amplify a region not alternatively spliced between exons 1 and 3.
 804 Amplification of plasmid vectors containing the cDNAs of the various *mef2ca* splice variants
 805 cloned into the pcDNA 3.1 vector were used as controls of the correct size of expected amplicons;

806 *mef2ca* 4-5-6 γ + (lane 1), *mef2ca* 4-6 γ + (lane 2), *mef2ca* 4'-6' γ + (lane 3), *mef2ca* 4-5-6 γ - (lanes
807 4-4). PCR products were separated in 8% polyacrylamide gels. Length of PCR products in base
808 pairs (bp) is indicated. C) Quantitative analysis of the mRNA levels of *mef2ca* exon 5 splice
809 variants during *D. rerio* development. The amount of the transcripts of each splice variant was
810 estimated by absolute qRT-PCR. Original data (mRNA levels of each isoform) are reported as % of
811 the total number of *mef2ca* transcripts (4-5-6 + 4-6 + 4'-6' = 100%). Statistical analysis was
812 performed on data obtained from three independent experiments, the means \pm SE are represented.
813 *** indicate a P-value \leq 0.001. Absolute qRT-PCR estimation of *mef2ca* splicing isoforms during
814 *D. rerio* development. The quantification, reported as \log_2 number of copies, was performed on
815 equal amount (1 ng) of total RNA extracted from staged embryos. The graph reports the mean \pm SE
816 bars of three independent experiments, *** indicates a p value \leq 0.001, ** \leq 0.01, ## \leq 0.01.-D)
817 Schematic representations of Mef2cb alternative exons. Arrows show primers annealing sites. E)
818 Developmental RT-PCR analysis of *mef2cb* mRNAs. To evaluate the amount of α 1- and α 2-
819 containing *mef2cb* transcripts, we designed common PCR primers (cb2 and cb4Rv) annealing to
820 flanking regions in exons 2 and 4 to generate two amplicons of different size: to generate isoform-
821 specific, a 1960-bp (α 1) and a 187-bp (α 2) RT-PCR products respectively. Flanking primers
822 were also designed to investigate the expression of exon β , the extra sequence of intron 5 (*) and of
823 the γ region. As control templates we used the pcDNA 3.1 expression vector containing the cDNAs
824 of Plasmid vectors containing the cDNAs of Mef2cbL α 1 β - γ + (lane 5),-Mef2cbL α 2 β - γ + (lane
825 6) and Mef2cbS α 1 β - γ + (lane 7) were used as control templates (lane 5 and 6, respectively).
826 *β actin 2 (actb2)* was used as a control, *myog* was used as a marker for skeletal muscle
827 differentiation.

828
829 **Fig. 4.** WISH analysis of zebrafish *mef2ca* transcripts in developing zebrafish embryos.

830 In situ hybridization using *mef2ca* (left panels) and *mef2ca*-exon 5 specific probes (right panels)
831 probes as indicated. A, B) Lateral view of 22-ss24 hpf embryos. *mef2ca* mRNA localizes to at both
832 central and peripheral regions of the muscles in the somite, and also to the structure, developing
833 heart and branchial arches (red arrowhead and black arrow respectively). whereas Exon 5 specific
834 transcripts are detected by both LNA probes in a similar way in the muscle, with a slightly stronger
835 expression at localize mainly to somite borders (see insets for magnified somatic muscle area).
836 rectangular areas in A are magnified in B. B, left panels. C) Lateral view of 4824 hpf embryos,
837 anterior to left. *mef2ca* general and both LNA1 and LNA2 exon 5-specific probes shows
838 overlapping strong signals throughout somites, whereas exon5-specific probe is weaker but is

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839 ~~enriched-enriched~~ at fiber ends. ~~Right panels.~~**D**) Dorsal view of ~~the same embryos, -24 hpf~~
 840 ~~embryos,~~ anterior to left. ~~Detection of mef2ca and both LNA probes detect~~ expression in ~~the~~
 841 ~~pectoral fin dorsal and ventral muscle masses heart (red arrowhead) and CNC (cranial neural crest)~~
 842 ~~in pharyngeal arches (black arrowheads, left panel).~~ No expression is detected in these areas with
 843 ~~exon5 specific probe (right panel).~~ (Scale bars = 100µm). ~~C~~**E**) Drawing of the LNA1 and LNA2
 844 probes annealing positions within the exon 4/5 region.

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846 **Fig. 5.** Transcriptional activity of zebrafish Mef2ca and Mef2cb splice variants.

847 **A)** COSes-1 cells were co-transfected with pGL3(desMEF2)₃ luciferase, the pRSVβ-gal reporter
 848 control and CMV (Cytomegalovirus)-driven expression plasmids encoding for the indicated Mef2c
 849 splicing isoforms. Firefly luciferase activities were normalized for transfection efficiency against
 850 the β galactosidase activity and expressed as relative luciferase units of the activity in cells
 851 transfected with the Empty Vector (EV) (= 1.0). Statistical analysis was performed on data obtained
 852 from three independent experiments, the means ±SE (error bars) are represented. *** indicates a
 853 P-value ≤0.001. **B)** Extracts from cells transfected in panel A were resolved by SDS PAGE,
 854 Mef2ca and Mef2cb expression was assessed by immunoblotting with anti-MEF2 antibody that
 855 recognizes all Mef2ca and Mef2cb splicing isoforms (upper panel). Sample loading was normalized
 856 using Vinculin immunoreactivity (lower panel).

857

858 **Fig. 6.** Effects of Mef2cbL forced expression of *mef2cb* and *mef2ca* splice variants on
 859 development in zebrafish embryos.

860 Wholemount in situ mRNA hybridization of zebrafish embryos injected with in-vitro transcribed
 861 mRNA encoding Mef2c isoforms together with Rhodamine dextran at the 1-cell stage. Injected
 862 embryos or uninjected control embryos were analyzed during development. ~~Scale bars = 100 µm.~~

863 **A)** *Myod* mRNA in ~~head at 28/22 hpf embryos injected with 25 pg of mef2cb mRNAs (dorsal view,~~
 864 ~~anterior to top).~~ *mef2cbL* but not *mef2cbS* injected embryos have ectopic *myod* expression in head
 865 region (arrowheads). Both groups show an array of developmental defects in head and trunk
 866 regions. **B)** *Myod* mRNA in head region at 28 hpf (dorsal view, anterior to top). Injection of 10 pg
 867 of Mef2cbL mRNA induces ectopic *myod* expression in head mesoderm (black arrowheads). **B, C)**
 868 *smyhc1* mRNA and immunofluorescence of MyHC protein *myod* and *myogenin* mRNA in 28 hpf
 869 non injected and 30 hpf control embryos (non injected) or embryos injected with 10/25 pg of the
 870 indicated Mef2cbL mRNAs. Ectopic muscle is clearly seen in the head region of injected embryos

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871 (white arrowheads). White arrow and green arrowhead indicate somitic muscle and heart
 872 respectively. Scale bars = 100 μ m.

873
 874 **Fig. 7. Effects of forced expression of *mef2ca* splice variants on development of zebrafish**
 875 **embryo. Injection with *Mef2ca* 4-5-6 mRNA.**

876 **A) *Myod* mRNA in 22 hpf embryos injected with the mRNAs of *Mef2ca* splice variants or not**
 877 **injected (control). Forced expression of *Mef2ca* 4-5-6 mRNA resulted in severe developmental**
 878 **defects: double axis (black arrowheads), trunk and brain defects (white arrowheads). **Control****
 879 **embryos or **B) Embryos injected with 25 pg of *Mef2ca* 4-6 mRNA showed normal morphology. **BD******
 880 **RT-PCR analysis of the total RNA extracted from 25 pg *mef2ca* mRNA injected or control**
 881 **uninjected embryos at 22 hpf. *Mef2ca* 4-5-6 injected embryos showed augmented expression of**
 882 ***chordin* (*chd*), *no-tail* (*ntl*), *nodal related 1* (*ndr1*), *noggin 1* (*nog1*) and *goosecoid* (*gsc*), reduced**
 883 **expression of *App63* and *bmp 7a* show augmented expression in *Mef2ca* 4-5-6 injected embryos,**
 884 **whereas *bmp 2b*, *no-tail b* and *myod* expression levels are unaffected. **CE) Densitometric analysis****
 885 **of the bands shown in **BD**, normalized to *actb2* signal. Expression levels of each gene were**
 886 **arbitrarily set to a value of 1 in the uninjected control embryos. **Statistical analysis was performed****
 887 **on data obtained from three independent experiments, the means \pm SE (error bars) are represented. ***
 888 **and ** indicate P-values of ≤ 0.1 and ≤ 0.01 respectively.**

889
 890 **Fig. 8. Expression of *mef2ca* and *mef2cb* splice variants during early zebrafish development.**
 891 **Expression levels of the *mef2ca* and *mef2cb* splice variants in exon 5 region were evaluated by RT-**
 892 **PCR analysis of RNA harvested from zebrafish embryos at the indicated developmental stages.**
 893 **PCR was performed using primers that give amplification products of different sizes depending on**
 894 **the splice variant, as schematized in figure 3A. PCR products were separated in 8% polyacrylamide**
 895 **gels. Length of PCR products is indicated. Expression levels of *gsc* and *chd* were also determined.**
 896 **Expression levels of *actb2* RNA are shown as loading control.**

897
 898
 899 SUPPLEMENTARY FIGURES

900 Fig. S1. Vertebrate *MEF2* transcripts are alternatively spliced.

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901 **A)** Schematic of the highly similar structures of three vertebrate *MEF2C* genes among coding exons
 902 (black boxes). To simplify the comparison, we assigned the number 1 to the exon containing the
 903 first translated ATG. Introns are indicated by solid lines. *MEF2C* genes from the three species share
 904 three alternative exons: the $\alpha 1$ and $\alpha 2$ mutually exclusive exons, the β skipping exon, and 3' splice
 905 site selection at exon 9. **B)** Schematic of the vertebrate *Mef2c* gene exon numbering adopted in this
 906 paper. In the table are reported the exon numbering of the mouse and frog *MEF2C* genes adopted in
 907 the indicated references. **C)** Splicing patterns of frog, mouse and human *MEF2C*. The MADS box
 908 and MEF2 domain are encoded by exons 1 and 2.

909

910 Fig. S2. Amino acid conservation of alternative spliced domains of vertebrate *Mef2c* proteins.

911 **A)** $\alpha 2$ alternative exon, β skipping exon and γ region in *mef2cb* and *mef2ca* genes predicted with the
 912 TBLASTN algorithm. The sequences of bona fide spliced out exons, the percentage of homology
 913 with the mouse sequence and the putative splice sites are indicated. **B)** Comparison of amino acid
 914 sequences for zebrafish *Mef2ca* and *Mef2cb* splice variants. Protein sequence encoded by different
 915 exons is indicated, and alternatively spliced out regions are marked in yellow and green. TADs are
 916 colored in blue and orange. ~~**C)** Comparison of amino acid sequences for mouse (Mm), human (Hs),
 917 frog (Xl) and zebrafish (Dr) *Mef2ca* exon 5 and surrounding regions. Asterisks design fully
 918 conserved amino acids.~~

919

920 Fig. S3. Amino acid conservation in the exon 5 encoded domain of teleosts *Mef2* proteins.

921 **A)** Comparison of amino acid sequences encoded by exon 5 and surrounding regions for zebrafish
 922 *Mef2ca* and *Mef2cb* proteins and the predicted *Mef2* proteins from cavefish (*S. anoptalmus* and *S*
 923 *angustiporus*), medaka (*O. latipes*), pufferfish (*T. rubripes*) and stickleback (*G. aculeatus*). **B)**
 924 GenBank and NCBI reference accession numbers of the sequences used for the sequence alignment
 925 in A.

926

927 Fig. S4. Developmental expression profile of zebrafish *mef2ca* and *mef2cb*.

928 **A)** Developmental expression profile of *mef2ca* and *mef2cb* transcripts by semi-quantitative **RT-**
 929 **PCR** analysis of the RNA extracted from staged zebrafish embryos. To determine the concentration
 930 of the transcripts we constructed a standard curve by amplifying serial dilutions of plasmid DNA
 931 templates. As a control for the quantity of substrate RNA, we amplified the same samples for *actb2*

932 (*b-actin-2*). B) Double in situ hybridization for 22 hpf zebrafish embryos for *myod*, *mef2ca* and
 933 *mef2cb* transcripts. Wholemounts shown in lateral view, anterior to left.

934 Fig. S5. Quantitative analysis of the mRNA levels of *mef2ca* and of *mef2cb* exon 3 α splice variants
 935 during *D. rerio* development and in adult tissues.

936 A) Left panel. Schematic representation of *mef2cb* 3 α 1 or 3 α 2 alternative exons. Arrows show
 937 annealing sites of isoform-specific primers used in the RT-PCR analysis they were designed to give
 938 amplification products of the same size (190 bp). Right panel. Expression analysis of *mef2cb*
 939 transcripts including the mutually exclusive 3 α 1 or 3 α 2 exon by RT-PCR. Total RNA was purified
 940 from staged embryos. To amplify an amount of exon 3 α 2 containing DNA similar to that
 941 containing exon 3 α 1, four additional PCR cycles were required. (B) Left panel. Schematic
 942 representation of *mef2ca* 3 α 1 and of *mef2cb* 3 α 1 or 3 α 2 alternative exons. Arrows show annealing
 943 sites of the primers used in the RT-PCR analysis. They give amplification products of distinct sizes.
 944 Right panel. Expression analysis of *mef2ca* and *mef2cb* transcripts including the mutually exclusive
 945 3 α 1 or 3 α 2 exon by RT-PCR in adult tissues. Total RNA was purified from brain, liver, skeletal
 946 and cardiac muscle of adult zebrafish. The level of expression of the transcripts was evaluated by
 947 using primers that anneal to exons 2 and 4 for both *mef2c* genes, in the case of *mef2cb*, they give
 948 two amplification products of distinct sizes: 196 and 187 bp, depending on the incorporation of
 949 3 α 1 or 3 α 2 alternative exons in the transcripts. PCR products were separated in 8% polyacrylamide
 950 gels. Length of PCR products (bp) is indicated.

951
 952 Fig. S65. Effects of Mef2ca splice variants overexpression in zebrafish embryos.

953 A) Zebrafish embryos were injected with 25 pg of in vitro-transcribed *mef2ca* 4-5-6 RNA together
 954 with rhodamine dextran at the 1-2 cells stage and analyzed at 20 hpf. Successfully injected
 955 embryos were distinguished on the basis of the red fluorescence (insets) and classified on the basis
 956 of morphology into 'severely defective' (blocked development), 'defective' (altered development)
 957 or 'normal'. B) Dose-dependent effects of in vitro-transcribed *mef2ca* mRNAs on embryos
 958 development. The graph reports the Θ quantification of defective embryos upon injection of
 959 increasing doses (25 pg and 50 pg) of RNA encoding Mef2ca 4-5-6 and 4-6 splice variants.
 960 Controls were uninjected embryos (Ctrl). The number of embryos tested in each experiments is
 961 indicated by (n) on top of each column. Data were averaged from two independent experiments. C)
 962 Dose dependence effect of in vitro transcribed *mef2ca* 4-5-6 RNA on embryos development. D)
 963 Western blot analysis showing over-expression of Mef2ca 4-5-6 and Mef2ca 4-6 following RNA

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964 | injection (25 pg) into embryos. COS-1 cell extracts over-expressing Mef2ca 4-5-6 or 4-6 were used
965 | as electrophoretic mobility controls (a and b, respectively). α -Tubulin was used as loading control.
966 | DE) To assess whether injection of 10 pg *mef2ca* 4-5-6 RNA leads to aberrant maturation of
967 | vascular, neuronal or cardiac tissues, injected embryos (right panels) or controls (left panels) were
968 | subjected to in situ hybridization for *myl7*, *kdrl*, *neurog1* and *ascl1a* mRNAs, respectively.

969

970 | Fig. S76 Primers used in semi-quantitative RT-PCR and qRTPCR.

971 | A) In the table is reported a restricted list of PCR primer pairs used in the semi-quantitative PCR
972 | reaction, missing primers are available on request. B) Sschematic drawing of *mef2ca* isoform
973 | specific and isoform common primers used in qRTPCR. Sequences are available on request.

Figure 1
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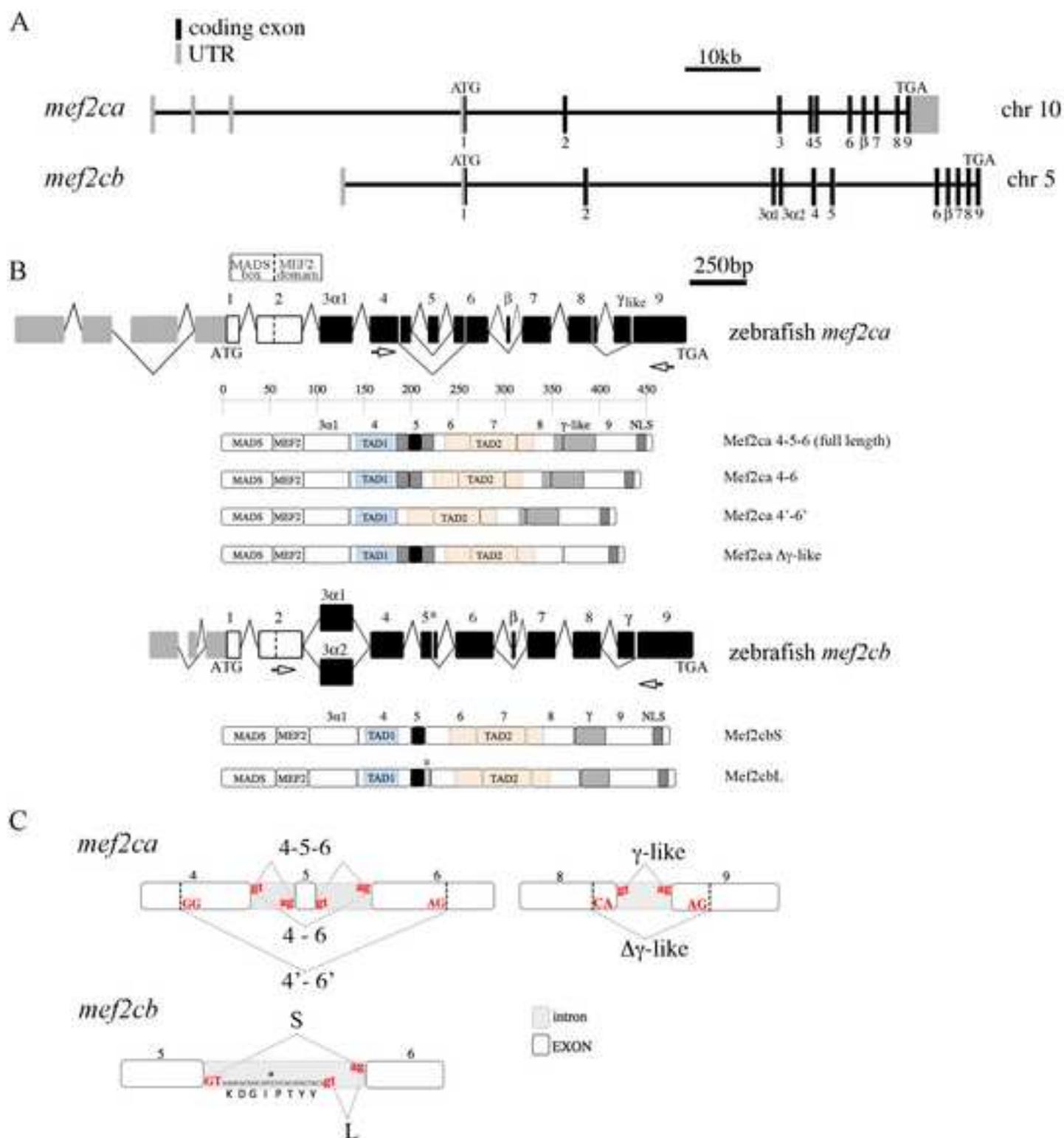
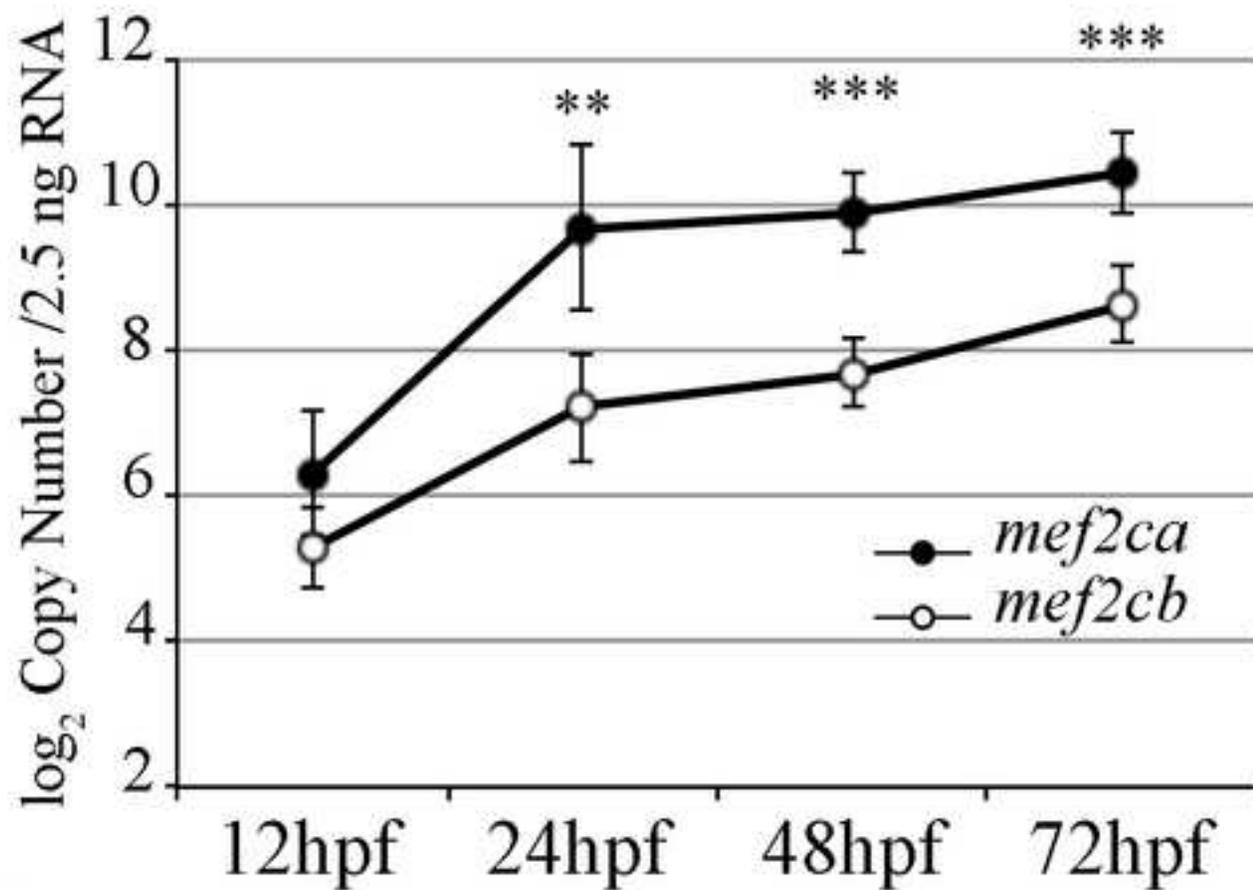


Figure 2
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A



B

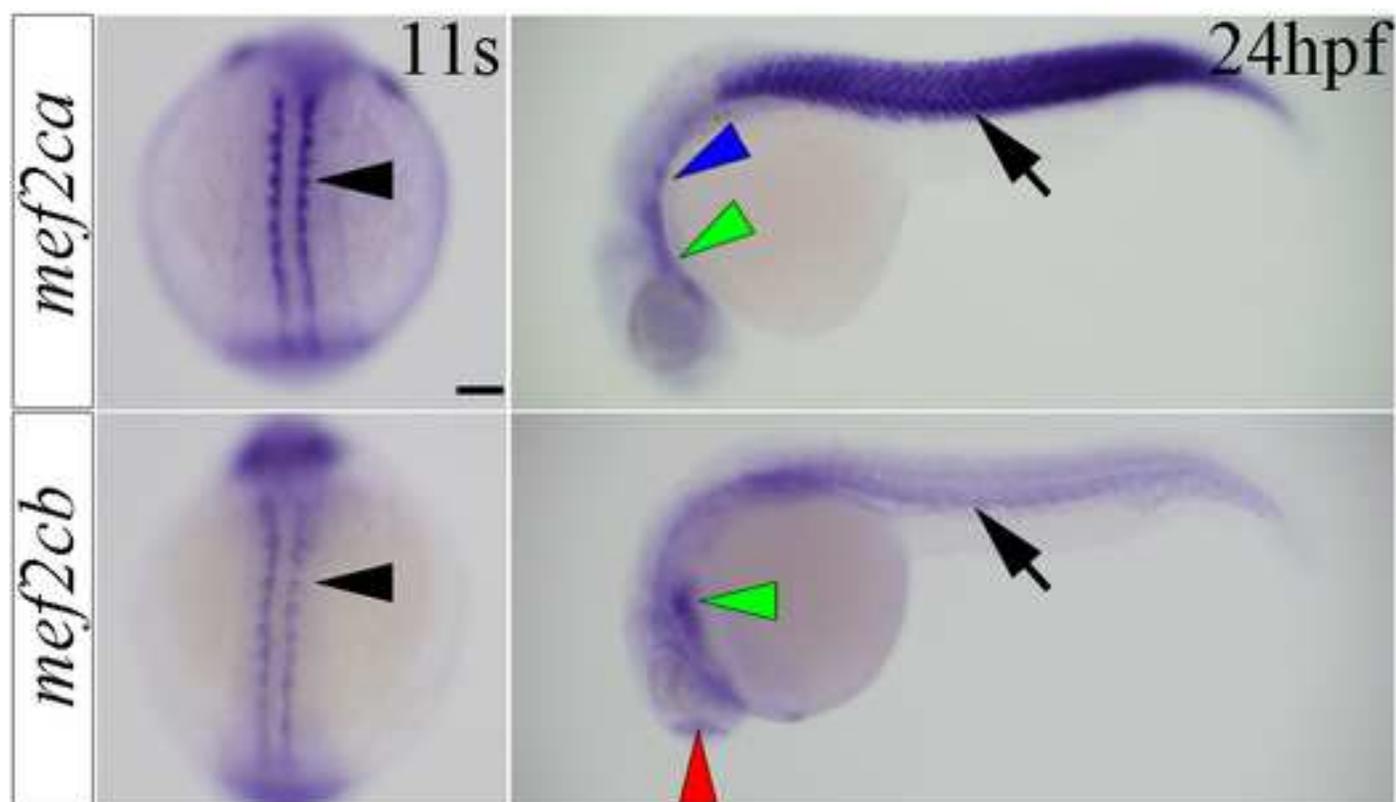


Figure 3
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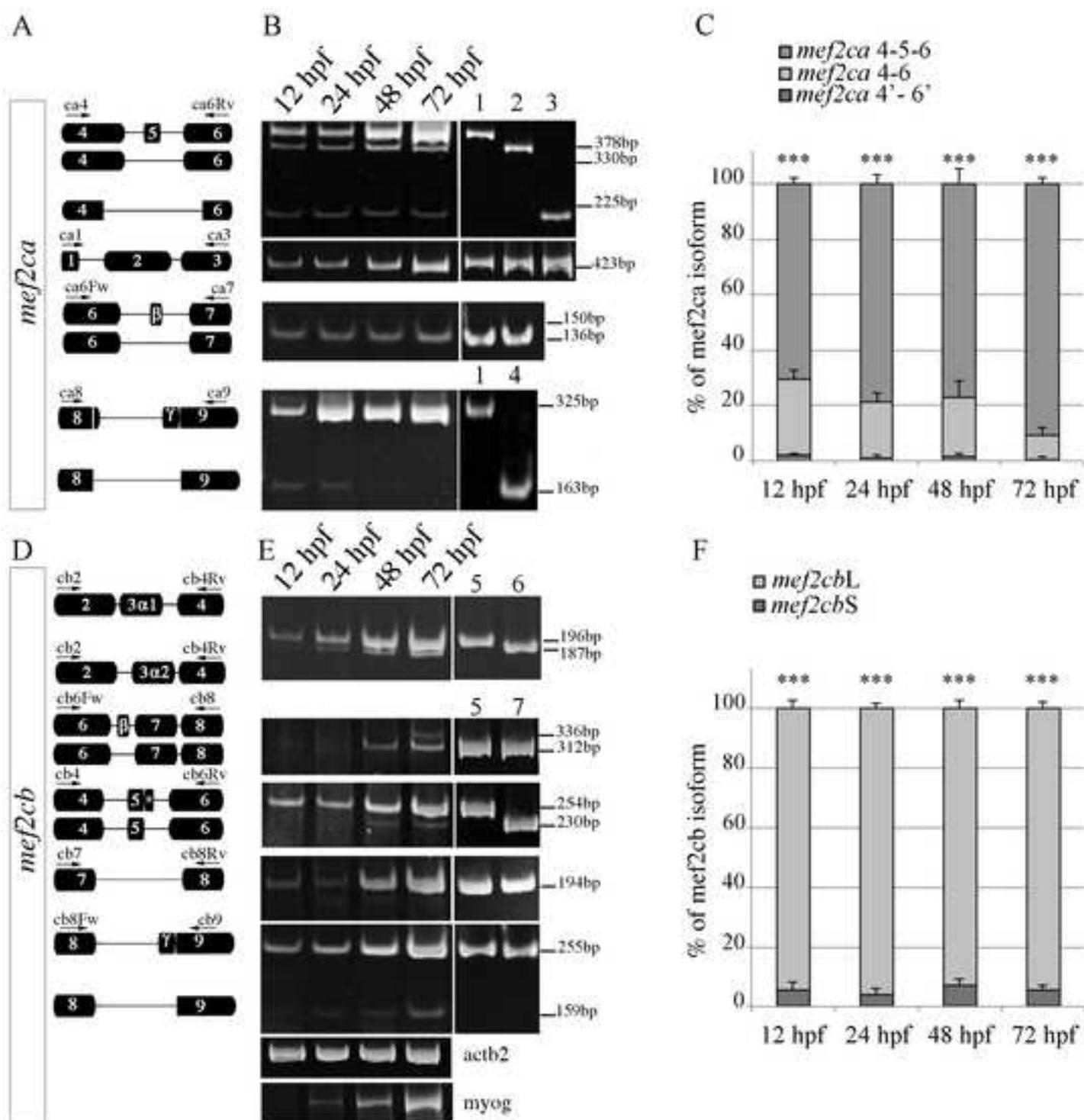


Figure 4
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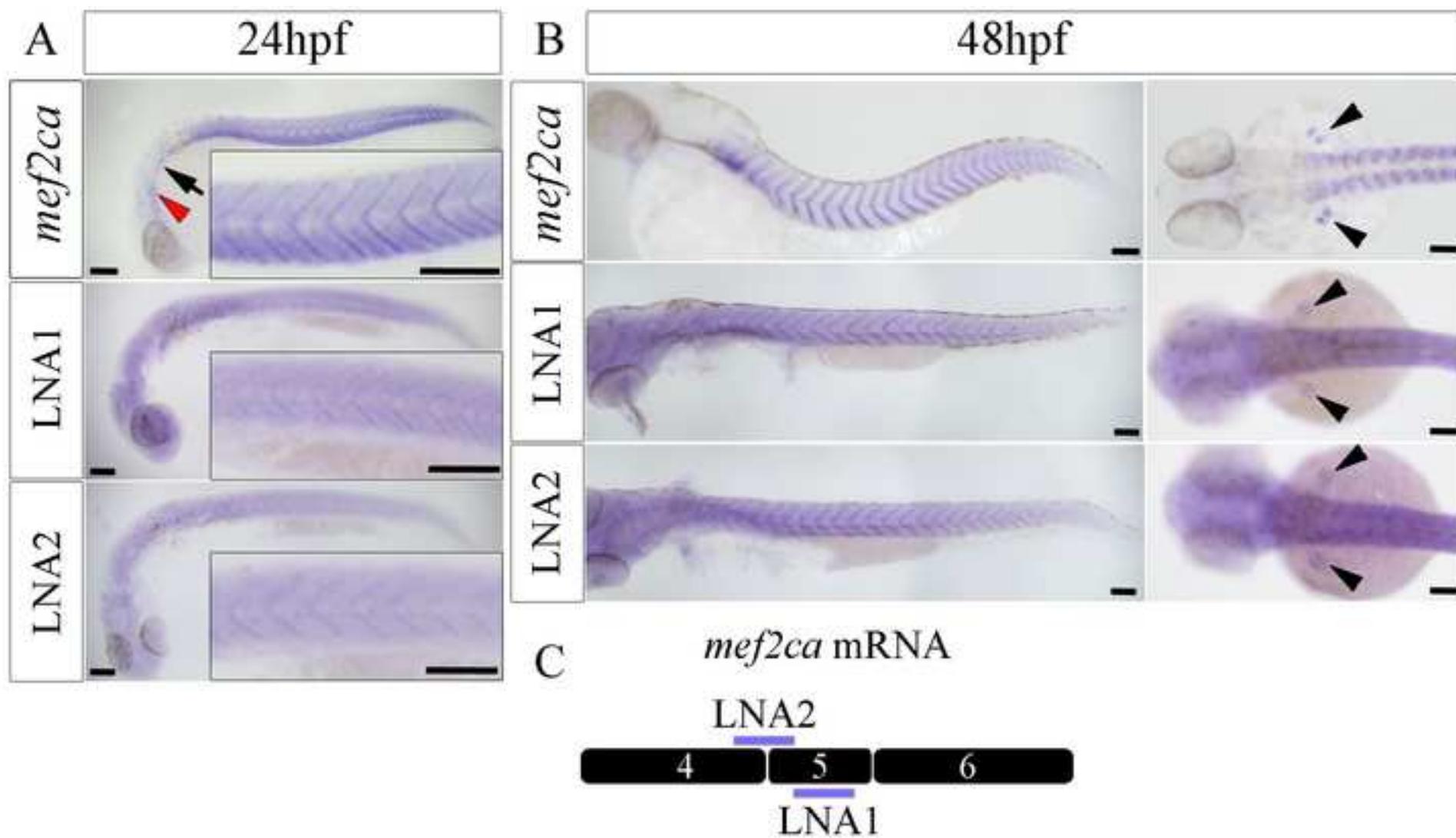


Figure 5
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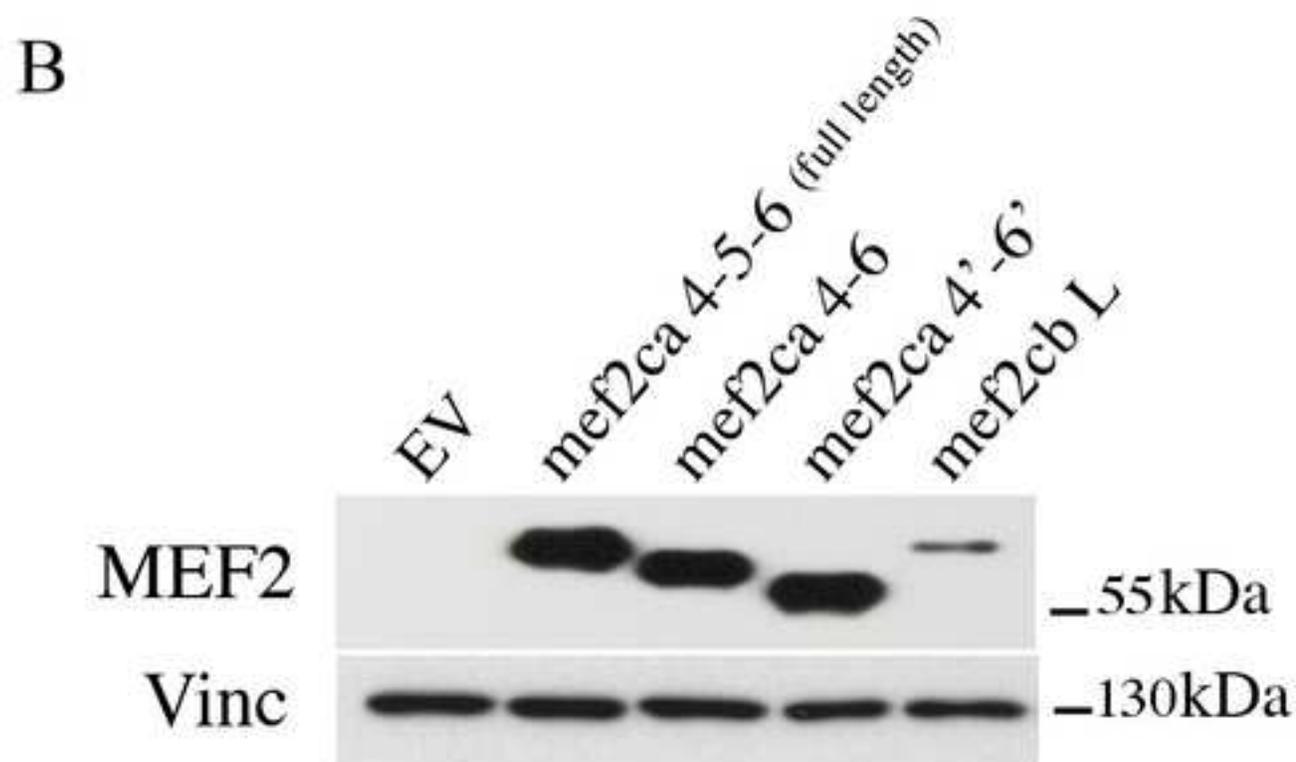
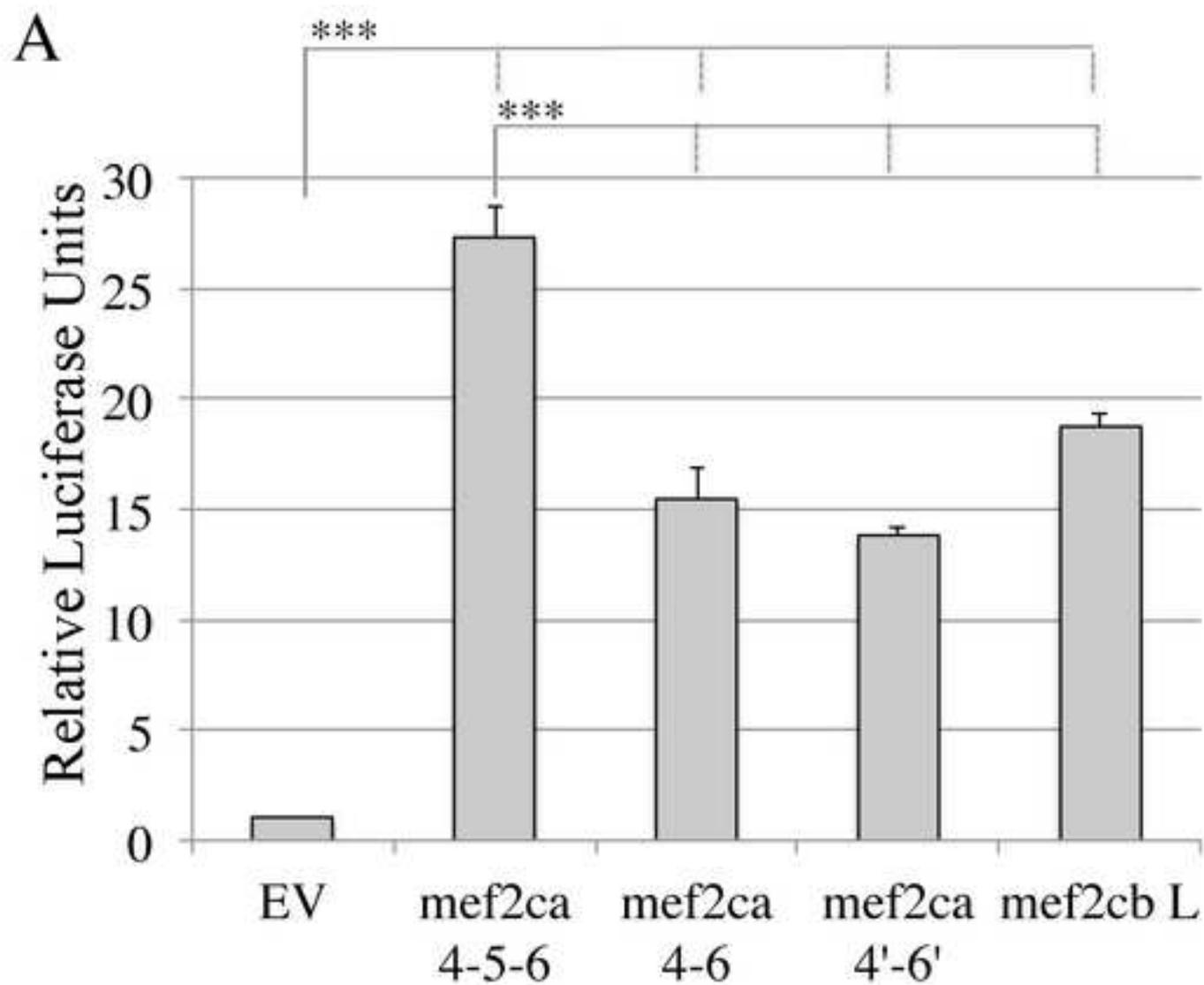


Figure 6
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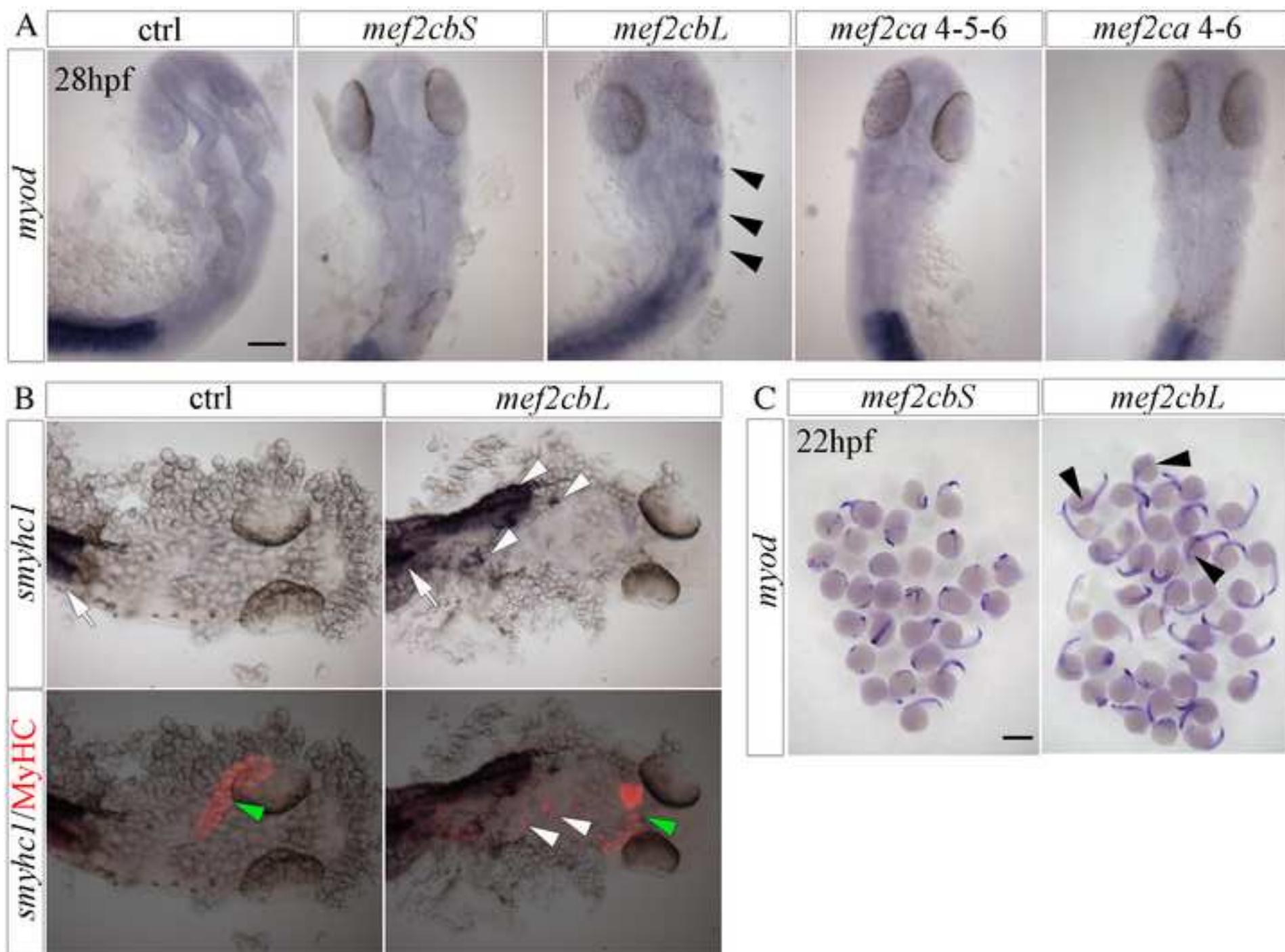


Figure 7

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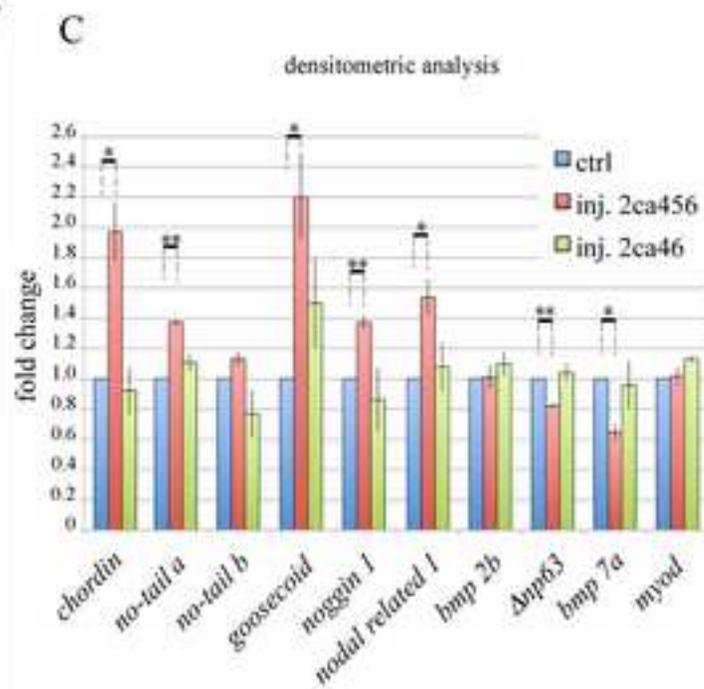
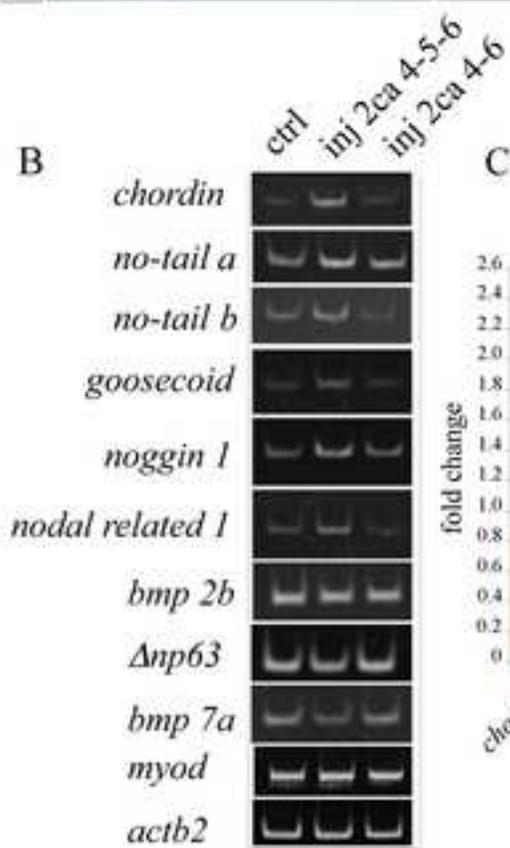
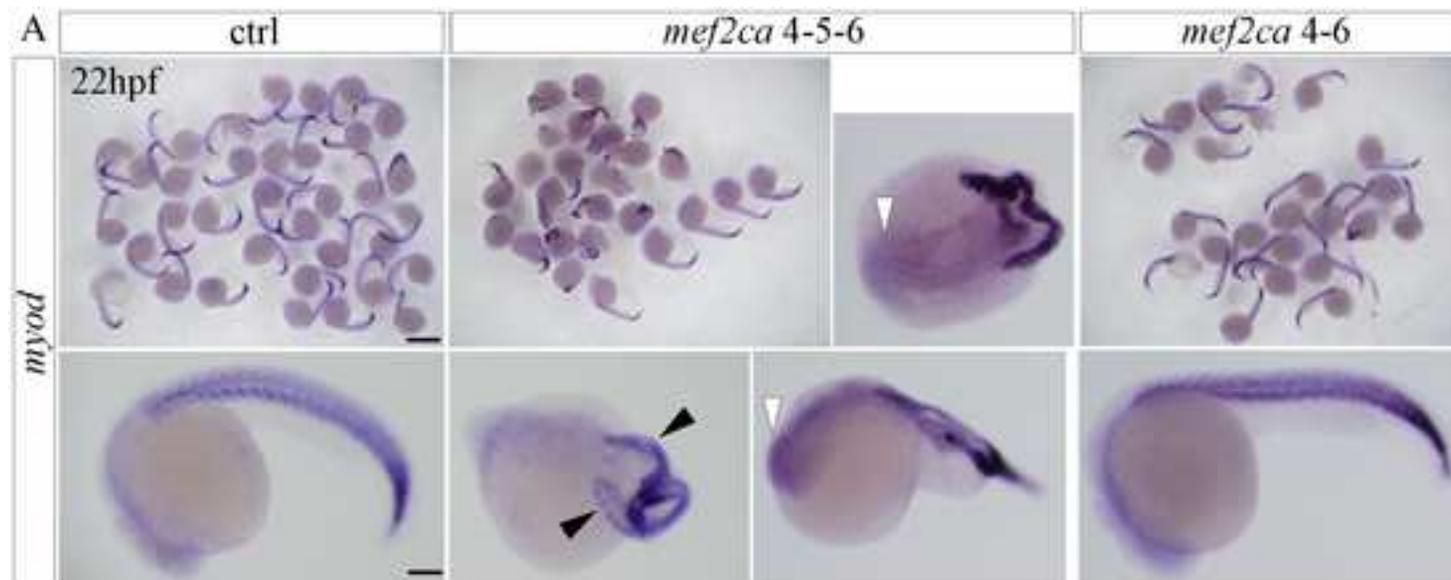


Figure 8
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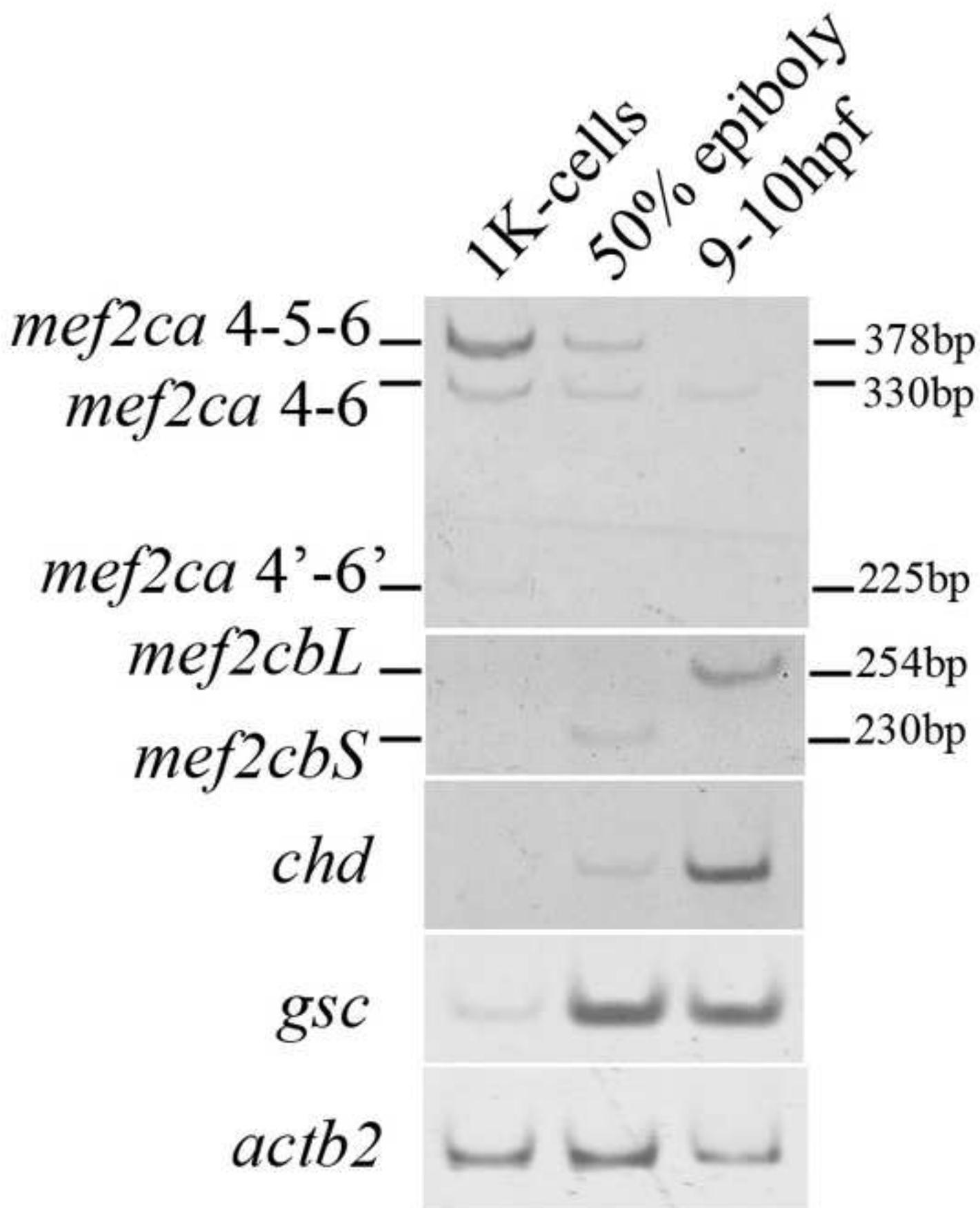
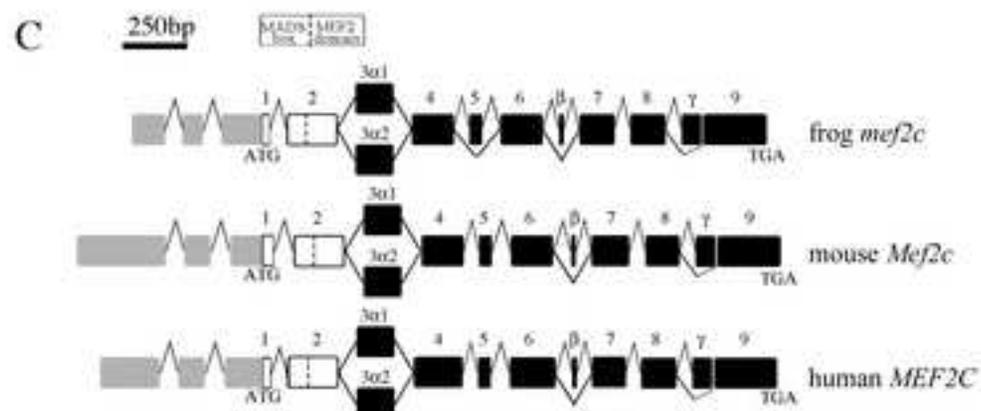
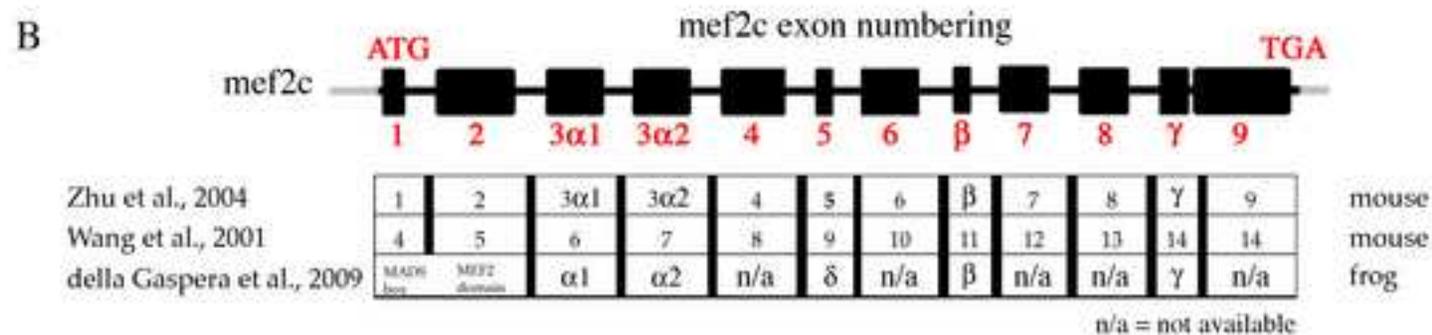
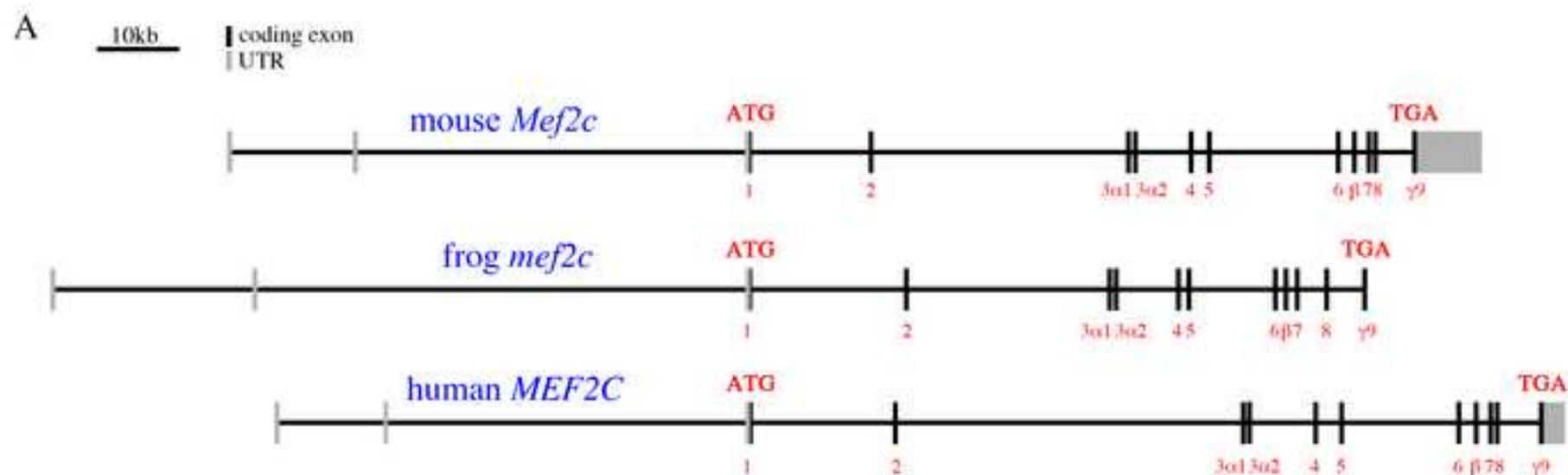


Figure S1

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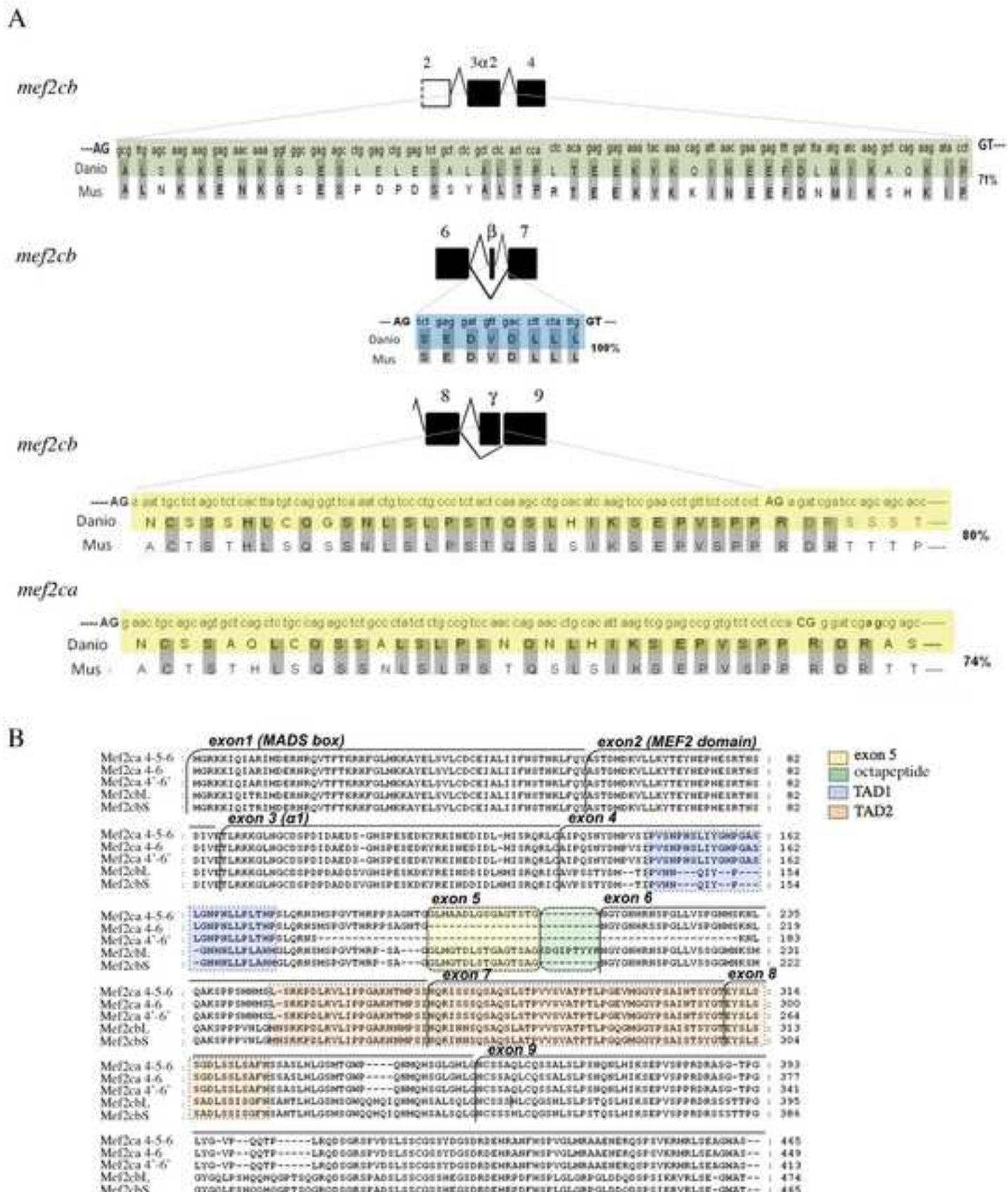
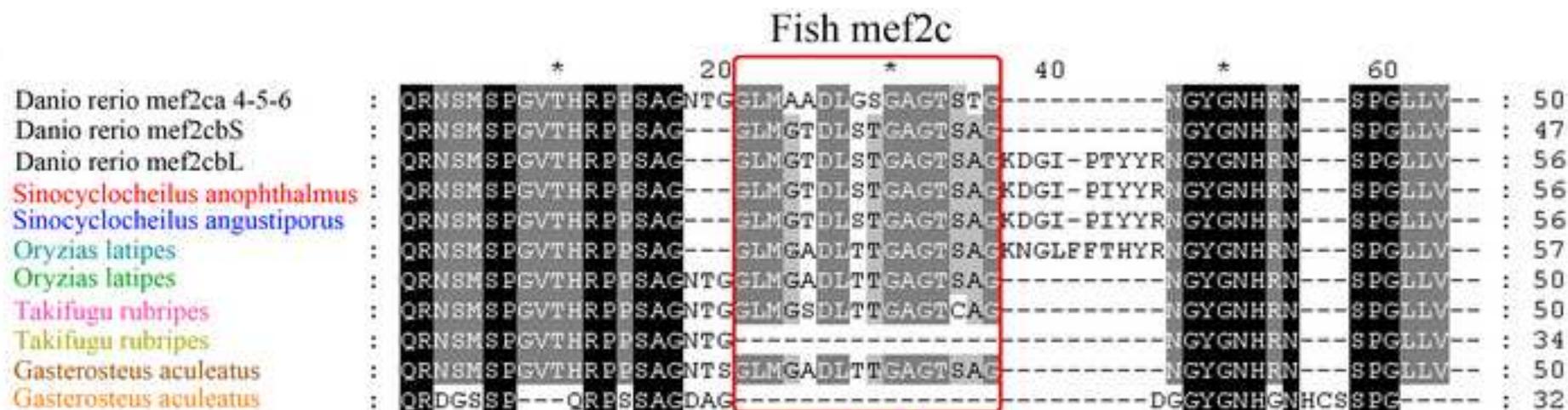


Figure S3

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A

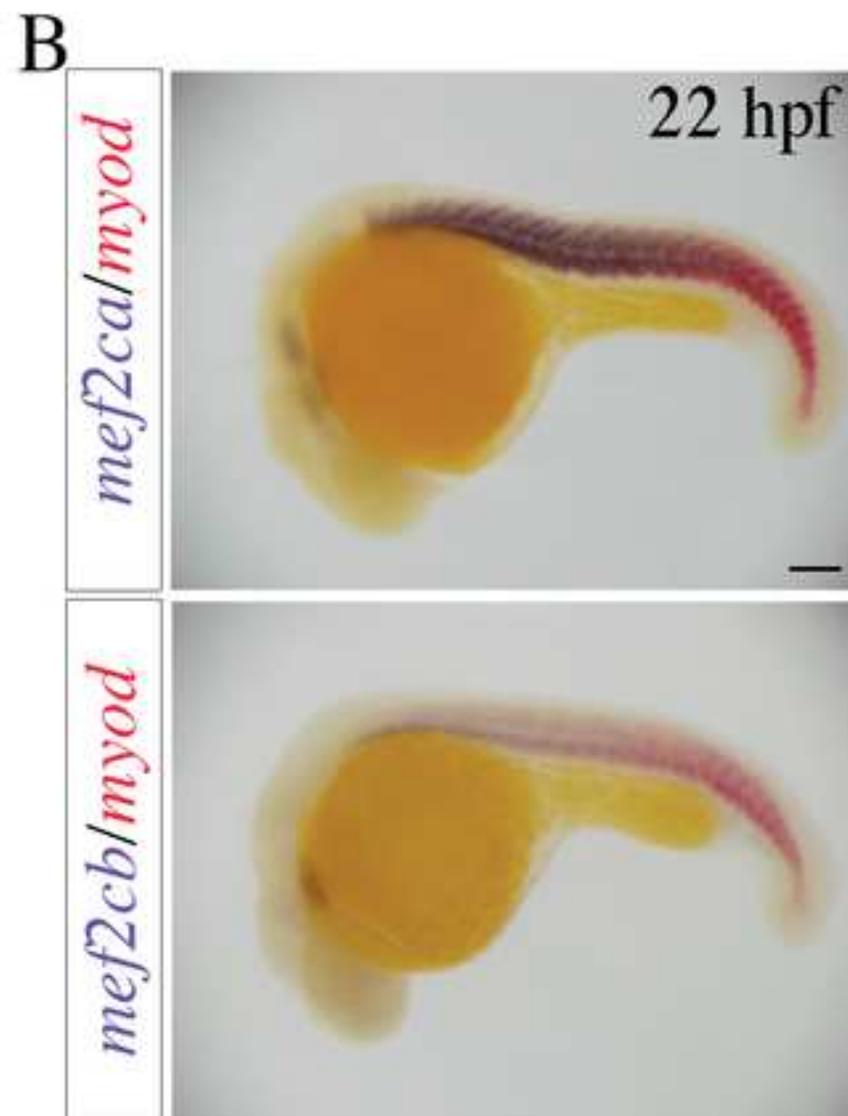
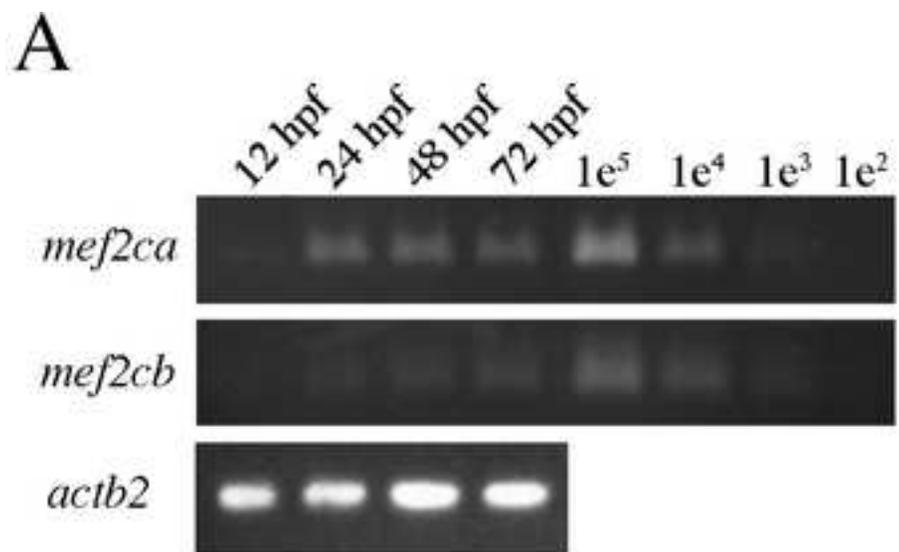


B

GenBank sequence IDs:

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[gb|GAHO01128571.1|](#)
[ref|XP_004072496.1|](#)
[ref|XP_004072494.1|](#)
[ref|XP_003975084.1|](#)
[ref|XP_003975082.1|](#)
[gb|BT026989.1|](#)
[gb|BT026990.1|](#)

exon 5



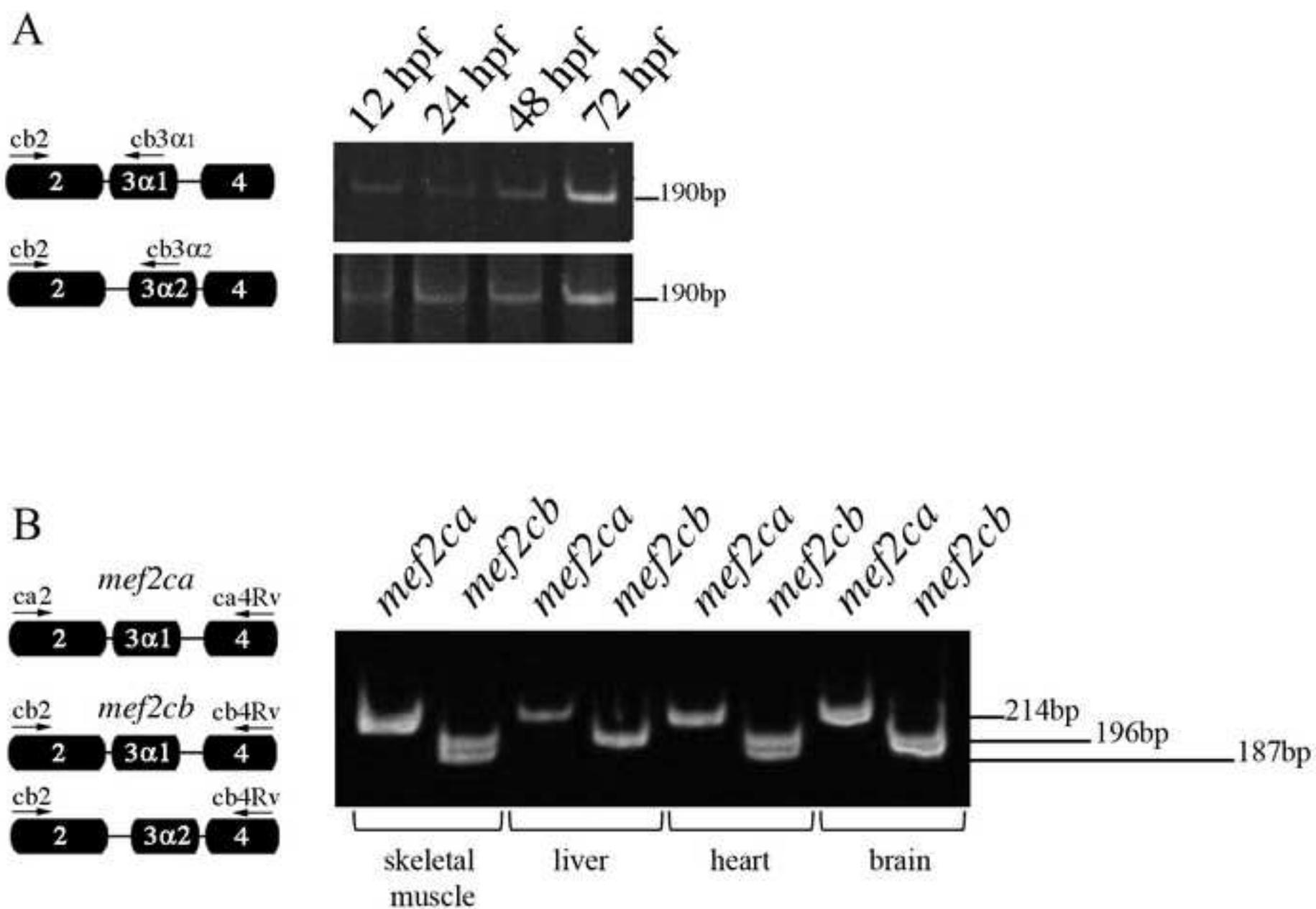
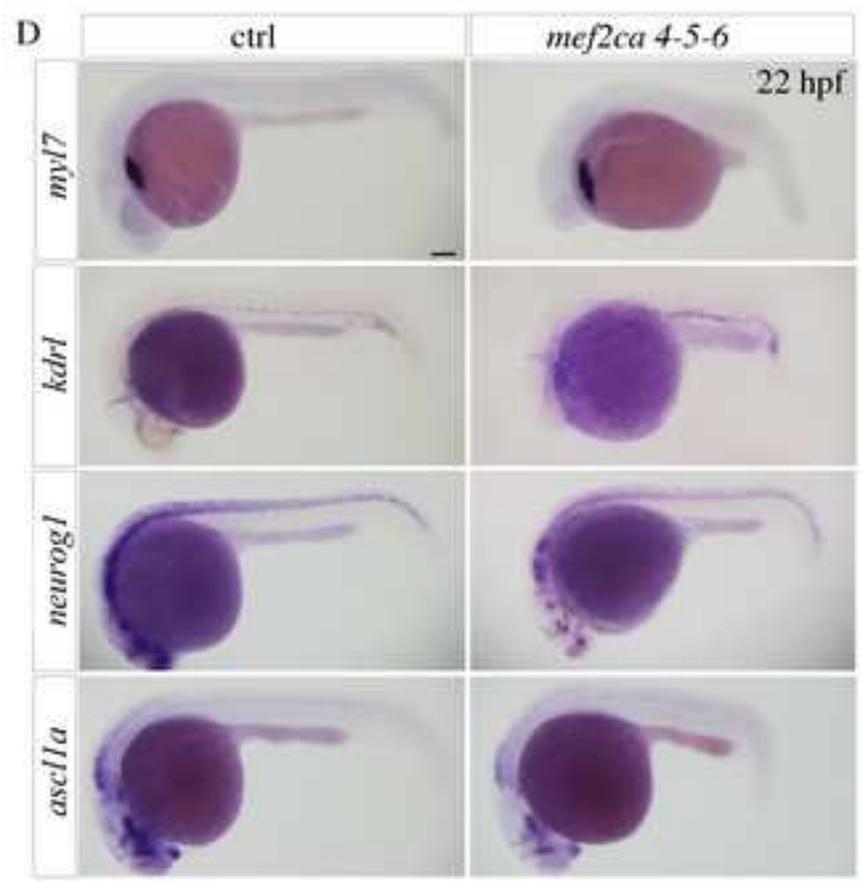
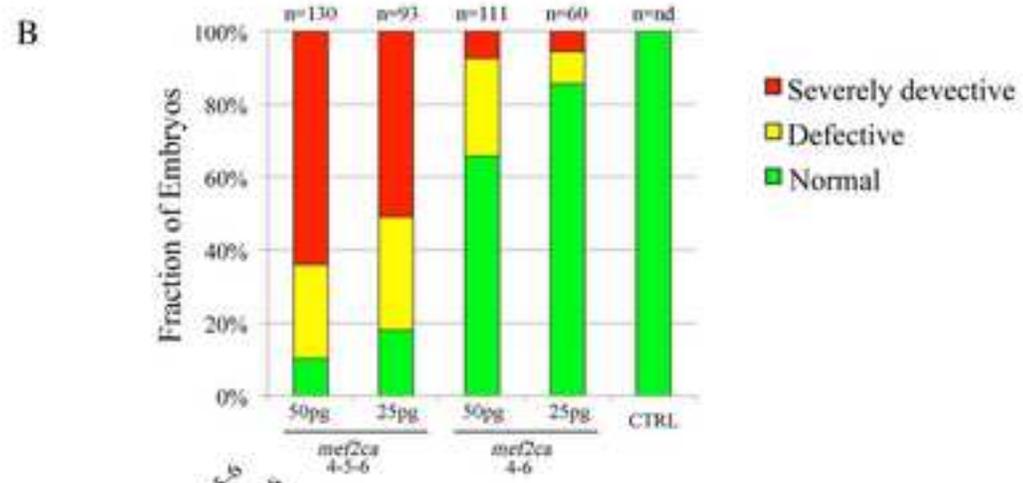


Figure S6

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A

| Oligo Name | Sequence 5' - 3' |
|---------------------|--------------------------|
| mef2ca FW | tgggactaattcagacatcgtg |
| mef2ca REV | gtagatcaggctgttgggggtt |
| mef2cb FW | gaccaactcggacatagtggag |
| mef2cb REV | tgcctgggtagatctgggttatt |
| β actin-2 FW | gcagaaggagatcacatccctggc |
| β actin-2 REV | cattgccgctcaccttcaccgttc |
| myog FW | gcttcgagaccaaccctact |
| myog REV | tcactagaggacgacacccca |
| myod FW | tcgcaaagccgccaccatga |
| myod REV | cacgggctctcttcgtgccc |

