Elsevier Editorial System(tm) for BBA - Gene Regulatory Mechanisms
Manuscript Draft

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

Corresponding Author: Dr. Susanna Molinari, Ph.D.

Corresponding Author's Institution: University of Modena and Reggio Emilia

First Author: Massimo Ganassi

Order of Authors: Massimo Ganassi; Sara Badodi; Alessio Polacchini; Fiorenza Baruffaldi; Renata Battini; Simon M Hughes; Yaniv Hinits; 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 ouvert 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 qRTPCR 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 qRTPCR 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.

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

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 3α1 or 3α2 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 3α1 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 3α1 and 3α2 in *mef2cb* transcripts in adult tissues and the results are shown in figure S5B.

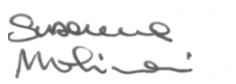
Via G. Campi 287. 41100 Modena. Italy
Tel.: ++39 59 428650-428651-428610 – Fax: ++39 59 428653 – Email: segrdsb@unimo.it

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

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
University of Modena and Reggio Emilia,
Department of Life Sciences, Section of Biochemistry
Via Campi 287, 41100 Modena, Italy
Tel +39 059 2055403, Fax +39 0592055410

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

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
Response to the reviewers.

Dear Dr Reese,

please find below our answers to the specific points raised by the 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.

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

Via G. Campi 287. 41100 Modena. Italy
Tel.: ++39 59 428650-428651-428610 – Fax: ++39 59 428653 – Email: segrdsb@unimo.it

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

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.
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 ouvert 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.

Via G. Campi 287. 41100 Modena. Italy
Tel.: ++39 59 428650-428651-428610 – Fax: ++39 59 428653 – Email: segrdsb@unimo.it

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

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.
Transcripts encoding Mef2cbL and Mef2cbS were quantified by qRTPCR 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.
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.

Via G. Campi 287. 41100 Modena. Italy
Tel.: ++39 59 428650-428651-428610 – Fax: ++39 59 428653 – Email: segrdsb@unimo.it

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

We repeated the experiments and quantified *mef2ca* and *mef2cb* transcript levels by performing overall four independent qRTPCR 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:
1. Line 38, "disregulation" should be dysregulation. **Corrected in the text.**
2. Line 107, missing period after parenthesis. **Corrected in the text.**
3. Line 230, the parenthetical concentration is ambiguous because none is provided for mef2ca 4-6.....
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.
6. Figure S2C is never cited in the text. **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.
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.

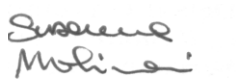
Via G. Campi 287. 41100 Modena. Italy
Tel.: ++39 59 428650-428651-428610 – Fax: ++39 59 428653 – Email: segrdsb@unimo.it

UNIVERSITÀ DEGLI STUDI DI MODENA E REGGIO EMILIA
DIPARTIMENTO DI SCIENZE DELLA VITA
Via G. Campi, 287
41100 MODENA

We would like to thank the reviewers for their useful comments and suggestions that helped us to ameliorate the manuscript.

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
University of Modena and Reggio Emilia,
Department of Life Sciences, Section of Biochemistry
Via Campi 287, 41100 Modena, Italy
Tel +39 059 2055403, Fax +39 0592055410

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.

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

Ganassi M.[1,3], Badodi S. [1], Polacchini A. [1,2], Baruffaldi F. [1], Battini R. [1], Hughes S.M. [3], Hinits Y. [3,§], Molinari S. [1,§]

[1] University of Modena and Reggio Emilia, Department of Life Sciences; [2] Actual address: BRAIN Centre for Neuroscience, Dept. of Biology, University of Trieste, Italy; [3] Randall Division of Cell and Molecular Biophysics, New Hunt's House, Guy's Campus, King's College London, SE1 1UL, UK.

[§] Corresponding Authors. Mailing Adresses: Susanna Molinari, Via Campi 287, 41100, Modena, Italy. Tel.: +39 059 2055403; Fax: +39 059 2055410; Yaniv Hinits: 3rd floor North, New Hunt's House, Guy's Campus, London SE1 1UL, UK. . Tel.: +44 20 7848 6444; Fax: +44 20 7848 6435; *E-mail addresses*: susanna.molinari@unimore.it (S. Molinari), yaniv.hinits@kcl.ac.uk (Y. Hinits)

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.

Abbreviations

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

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.

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

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

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

2. Materials and Methods

2.1. Plasmids

The full-length coding regions of the zebrafish Mef2ca 4-5-6, Mef2ca 4-6, Mef2ca 4'-6' and Mef2cbL variants were amplified from 24 hpf (hours post fertilization) zebrafish embryos cDNAs. The full length cDNA of Mef2cbS was obtained by a PCR reaction starting from a template made of three overlapping PCR products: the exon 5 region amplified from a Mef2cb I.M.A.G.E. clone (clone ID: 6519749, Genbank: CD282884.1), the upstream and downstream regions amplified from the Mef2cbL cDNA. The cDNAs were first inserted in the pCR2.1 vector (Invitrogen) or pGEM-T Easy vector (Promega), then sub-cloned into BamHI/NotI sites of the pcDNA 3.1(+) expression vector (Invitrogen). For RNA injections isoforms were sub-cloned into the XbaI/SalI sites of the βUT-3 vector [8]. Plasmids pGL3(desMEF2)$_3$ and pRSVβ-gal were previously described [28].

2.2. Alternative splicing prediction and multiple alignments

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

2.3. Transcription Reporter Assays

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

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

RNA was isolated using TRIzol® Plus RNA Purification System (Ambion). For each developmental stage, 100 embryos were disrupted using Tissue Raptor (Qiagen). 500 ng of total RNA were reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen). Primers used to detect *myogenin (myog), myod, beta-actin 2 (actb2), mef2ca* and *mef2cb* are listed in Fig. S6A (other primers sequence are available upon request), quantitative Real Time PCR (qRTPCR) was performed on 2,5 ng of Poly A mRNA using SYBR Green method (SYBR® Green PCR Core Reagent, Applied Biosystems). Poly A mRNA has been purified using Ambion's protocol (MicroPoly(A) Purist Kit). To amplify the different *mef2ca* isoforms specific forward primers spanning exon-exon junctions were used with a common reverse primer (Fig. S6B). For each primer combination the optimal $MgCl_2$ concentration was determined to obtain specific and high efficient amplification (slope values between -2.95 and -3.75). Absolute quantification of transcript copy number was achieved by generating calibration curve using plasmid DNA templates (listed above) as previously described [29, 30]. Analysis was performed using PCR ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Student's t-tests were performed for pairwise comparisons to determine significant differences between groups.

2.5. Zebrafish lines, maintenance and embryo manipulation

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

2.6. Whole Mount In Situ Hybridization (WISH)

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

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

2.7. mRNA injection and embryo manipulation

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

2.8. Western blot analysis and antibodies

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

3. Results

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

3.1. Alternative Splicing of zebrafish mef2ca and mef2cb

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

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

One *mef2cb* variant, named Mef2cbL, was obtained by RT-PCR; it includes exon 3α1 and γ but lacks exon β. Mef2cbL arises from the inclusion of an additional sequence from intron 5 (Fig. 1C).

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

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

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

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

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

3.3. Developmentally regulated expression of mef2ca and mef2cb splice variants

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

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

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

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

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

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

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

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

3.6. Mef2cbL has unique myogenic potential in developing zebrafish

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

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

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

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

To gain more insight into the mechanisms underlying the ability of Mef2ca 4-5-6 to disrupt development, the expression levels of genes encoding transcription factors and signaling molecules that are involved in early patterning of the embryo were screened by semi-quantitative RT-PCR. The *chd* gene, encoding a BMP (Bone Morphogenetic Protein) antagonist involved in dorsoventral patterning of early embryos [48] (reviewed in [49], [50]), was up-regulated (2-fold) in embryos injected with the *mef2ca* 4-5-6 mRNA, but not in those injected with the 4-6 spliced isoform (Fig. 7B,C). Mef2ca 4[48]-5-6 also induced the expression of *ndr1* (1.5-fold), *gsc* (2.2-fold) and other dorsally-expressed genes (*no-tail a*, *noggin 1*), and reduced the expression of ventralizing factors such as *bmp7a* (0.4-fold) and *Δnp63* (0.2-fold), but did not alter the transcript level of *myod* or *no-tail b* and *bmp2b* (Fig. 7B,C), suggesting that the protein sequence encoded by exon 5 can modulate the expression level of a specific subset of early embryonic genes.

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

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

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

4. Discussion

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

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

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

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

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

(YTA(A/T)$_4$TAR) (data not shown), raising the possibility that Mef2ca 4-5-6 directly activates their expression during early development. Later in development *chordin* expression may be sustained by Mef2d, which constitutively includes the sequence encoded by exon 5, and which is expressed from mid-gastrulation in adaxial muscle cells that also express *chordin* [32, 60] or by Mef2cb proteins. Interestingly, injection of either *mef2cbS* or *mef2cbL* transcripts, both containing exon5, have resulted in similar developmental defects to that of *mef2ca* 4-5-6 mRNA injection. Future studies will clarify whether these genes are indeed direct targets of a Mef2 protein containing exon 5.

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

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

4.2. Gene duplication and evolutionary partitioning of alternative splicing

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

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

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

5. Conclusions

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

Acknowledgments

We thank Veronica Mantovani for her help in performing qRTPCR and transactivation assays, Isabella Della Noce and Filippo Schepis for their help in RNA and protein extraction from zebrafish embryos, Cristina Valensisi for her help in designing splice variants-specific primers . We are grateful to Sharon Amacher, Tom Gallagher and Tod Gulick for sharing their results before

publication. We are grateful to Andrea Martello, Tommaso Selmi and Tommaso Zanocco-Marani for helpful discussion. We greatly thank Carol Imbriano and Alessandro Magli for reading the manuscript and helpful suggestions.

Research support was provided by: Fondazione Cassa di Risparmio di Modena, progetto internazionale bando 2010, AFM (Association Francaise contre les Myopathies) (grant n°16252) and Optistem (European collaborative project HEALTH-2007-1.4-6). M.G. was a recipient of short term fellowships from EMBO (ref. ASTF 414.00-2009), EufishBioMed (COST action BM0804, ref code 141111-012508), University of Modena and Reggio Emilia (Bando di Mobilità), Myores (Exchange Fund) and CIB (Consorzio Italiano Biotecnologie) that allowed him to visit and perform experiments in the laboratory of S. M. Hughes. A.P. was a recipient of a short-term fellowship from CIB (Consorzio Italiano Biotecnologie) that supported his visit to the laboratory of S. M. Hughes. S. M. Hughes is an MRC Scientist with Programme Grant G1001029 support that funded studies in the Hughes laboratory. Y. Hinits was also supported by a collaborative grant from the UK British Heart Foundation Centres of Excellence at King's College London, Imperial College London and the Universities of Edinburgh and Oxford.

References

[1] Y. Xing, C. Lee, Alternative splicing and RNA selection pressure--evolutionary consequences for eukaryotic genomes, Nature reviews. Genetics, 7 (2006) 499-509.
[2] H. Keren, G. Lev-Maor, G. Ast, Alternative splicing and evolution: diversification, exon definition and function, Nature reviews. Genetics, 11 (2010) 345-355.
[3] S.W. Roy, M. Irimia, Splicing in the eukaryotic ancestor: form, function and dysfunction, Trends in ecology & evolution, 24 (2009) 447-455.
[4] A.R. Kornblihtt, I.E. Schor, M. Allo, G. Dujardin, E. Petrillo, M.J. Munoz, Alternative splicing: a pivotal step between eukaryotic transcription and translation, Nature reviews. Molecular cell biology, 14 (2013) 153-165.
[5] C. Trapnell, B.A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M.J. van Baren, S.L. Salzberg, B.J. Wold, L. Pachter, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, Nature biotechnology, 28 (2010) 511-515.
[6] M. Llorian, C.W. Smith, Decoding muscle alternative splicing, Current opinion in genetics & development, 21 (2011) 380-387.
[7] M. Pistoni, C. Ghigna, D. Gabellini, Alternative splicing and muscular dystrophy, RNA biology, 7 (2010) 441-452.
[8] Y. Hinits, L. Pan, C. Walker, J. Dowd, C.B. Moens, S.M. Hughes, Zebrafish Mef2ca and Mef2cb are essential for both first and second heart field cardiomyocyte differentiation, Developmental biology, 369 (2012) 199-210.
[9] B.L. Black, E.N. Olson, Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins, Annual review of cell and developmental biology, 14 (1998) 167-196.
[10] B.A. Bour, M.A. O'Brien, W.L. Lockwood, E.S. Goldstein, R. Bodmer, P.H. Taghert, S.M. Abmayr, H.T. Nguyen, Drosophila MEF2, a transcription factor that is essential for myogenesis, Genes & development, 9 (1995) 730-741.

[11] B. Lilly, B. Zhao, G. Ranganayakulu, B.M. Paterson, R.A. Schulz, E.N. Olson, Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila, Science, 267 (1995) 688-693.
[12] G. Ranganayakulu, B. Zhao, A. Dokidis, J.D. Molkentin, E.N. Olson, R.A. Schulz, A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in Drosophila, Developmental biology, 171 (1995) 169-181.
[13] Q. Lin, J. Schwarz, C. Bucana, E.N. Olson, Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C, Science, 276 (1997) 1404-1407.
[14] Y. Hinits, S.M. Hughes, Mef2s are required for thick filament formation in nascent muscle fibres, Development, 134 (2007) 2511-2519.
[15] T.K. Ghosh, F.F. Song, E.A. Packham, S. Buxton, T.E. Robinson, J. Ronksley, T. Self, A.J. Bonser, J.D. Brook, Physical interaction between TBX5 and MEF2C is required for early heart development, Molecular and cellular biology, 29 (2009) 2205-2218.
[16] S. Lazic, I.C. Scott, Mef2cb regulates late myocardial cell addition from a second heart field-like population of progenitors in zebrafish, Developmental biology, 354 (2011) 123-133.
[17] M.J. Potthoff, M.A. Arnold, J. McAnally, J.A. Richardson, R. Bassel-Duby, E.N. Olson, Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c, Molecular and cellular biology, 27 (2007) 8143-8151.
[18] E. Dodou, D.B. Sparrow, T. Mohun, R. Treisman, MEF2 proteins, including MEF2A, are expressed in both muscle and non-muscle cells, Nucleic acids research, 23 (1995) 4267-4274.
[19] M.J. Potthoff, E.N. Olson, MEF2: a central regulator of diverse developmental programs, Development, 134 (2007) 4131-4140.
[20] J.C. McDermott, M.C. Cardoso, Y.T. Yu, V. Andres, D. Leifer, D. Krainc, S.A. Lipton, B. Nadal-Ginard, hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors, Molecular and cellular biology, 13 (1993) 2564-2577.
[21] B. Zhu, T. Gulick, Phosphorylation and alternative pre-mRNA splicing converge to regulate myocyte enhancer factor 2C activity, Molecular and cellular biology, 24 (2004) 8264-8275.
[22] B. Zhu, B. Ramachandran, T. Gulick, Alternative pre-mRNA splicing governs expression of a conserved acidic transactivation domain in myocyte enhancer factor 2 factors of striated muscle and brain, The Journal of biological chemistry, 280 (2005) 28749-28760.
[23] S. Sebastian, H. Faralli, Z. Yao, P. Rakopoulos, C. Palii, Y. Cao, K. Singh, Q.C. Liu, A. Chu, A. Aziz, M. Brand, S.J. Tapscott, F.J. Dilworth, Tissue-specific splicing of a ubiquitously expressed transcription factor is essential for muscle differentiation, Genes & development, 27 (2013) 1247-1259.
[24] B. della Gaspera, A.S. Armand, I. Sequeira, S. Lecolle, C.L. Gallien, F. Charbonnier, C. Chanoine, The Xenopus MEF2 gene family: evidence of a role for XMEF2C in larval tendon development, Developmental biology, 328 (2009) 392-402.
[25] S. Ferrari, S. Molinari, R. Melchionna, M.G. Cusella-De Angelis, R. Battini, L. De Angelis, R. Kelly, G. Cossu, Absence of MEF2 binding to the A/T-rich element in the muscle creatine kinase (MCK) enhancer correlates with lack of early expression of the MCK gene in embryonic mammalian muscle, Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research, 8 (1997) 23-34.
[26] Y. Guo, S.J. Kuhl, A.S. Pfister, W. Cizelsky, S. Denk, L. Beer-Molz, M. Kuhl, Comparative analysis reveals distinct and overlapping functions of Mef2c and Mef2d during cardiogenesis in Xenopus laevis, PloS one, 9 (2014) e87294.
[27] G. Genikhovich, U. Technau, Complex functions of Mef2 splice variants in the differentiation of endoderm and of a neuronal cell type in a sea anemone, Development, 138 (2011) 4911-4919.
[28] C. Angelelli, A. Magli, D. Ferrari, M. Ganassi, V. Matafora, F. Parise, G. Razzini, A. Bachi, S. Ferrari, S. Molinari, Differentiation-dependent lysine 4 acetylation enhances MEF2C binding to DNA in skeletal muscle cells, Nucleic acids research, 36 (2008) 915-928.
[29] H.S. Walton, F.M. Gebhardt, D.J. Innes, P.R. Dodd, Analysis of multiple exon-skipping mRNA splice variants using SYBR Green real-time RT-PCR, Journal of neuroscience methods, 160 (2007) 294-301.
[30] Vandenbroucke, II, J. Vandesompele, A.D. Paepe, L. Messiaen, Quantification of splice variants using real-time PCR, Nucleic acids research, 29 (2001) E68-68.

[31] M. Westerfield, The zebrafish book a guide for the laboratory use of zebrafish Danio (Brachydanio) rerio, in, Institute of Neuroscience, University of Oregon,, Eugene, OR, 1993.
[32] B.S. Ticho, D.Y. Stainier, M.C. Fishman, R.E. Breitbart, Three zebrafish MEF2 genes delineate somitic and cardiac muscle development in wild-type and mutant embryos, Mechanisms of development, 59 (1996) 205-218.
[33] E.S. Weinberg, M.L. Allende, C.S. Kelly, A. Abdelhamid, T. Murakami, P. Andermann, O.G. Doerre, D.J. Grunwald, B. Riggleman, Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail embryos, Development, 122 (1996) 271-280.
[34] D. Yelon, S.A. Horne, D.Y. Stainier, Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish, Developmental biology, 214 (1999) 23-37.
[35] M.A. Thompson, D.G. Ransom, S.J. Pratt, H. MacLennan, M.W. Kieran, H.W. Detrich, 3rd, B. Vail, T.L. Huber, B. Paw, A.J. Brownlie, A.C. Oates, A. Fritz, M.A. Gates, A. Amores, N. Bahary, W.S. Talbot, H. Her, D.R. Beier, J.H. Postlethwait, L.I. Zon, The cloche and spadetail genes differentially affect hematopoiesis and vasculogenesis, Developmental biology, 197 (1998) 248-269.
[36] P. Blader, N. Fischer, G. Gradwohl, F. Guillemot, U. Strahle, The activity of neurogenin1 is controlled by local cues in the zebrafish embryo, Development, 124 (1997) 4557-4569.
[37] M.L. Allende, E.S. Weinberg, The expression pattern of two zebrafish achaete-scute homolog (ash) genes is altered in the embryonic brain of the cyclops mutant, Developmental biology, 166 (1994) 509-530.
[38] Y. Hinits, V.C. Williams, D. Sweetman, T.M. Donn, T.P. Ma, C.B. Moens, S.M. Hughes, Defective cranial skeletal development, larval lethality and haploinsufficiency in Myod mutant zebrafish, Developmental biology, 358 (2011) 102-112.
[39] A.K. Lagendijk, J.D. Moulton, J. Bakkers, Revealing details: whole mount microRNA in situ hybridization protocol for zebrafish embryos and adult tissues, Biology open, 1 (2012) 566-569.
[40] D.P. Osborn, K. Li, Y. Hinits, S.M. Hughes, Cdkn1c drives muscle differentiation through a positive feedback loop with Myod, Developmental biology, 350 (2011) 464-475.
[41] C.S. Blagden, P.D. Currie, P.W. Ingham, S.M. Hughes, Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog, Genes & development, 11 (1997) 2163-2175.
[42] C.T. Miller, M.E. Swartz, P.A. Khuu, M.B. Walker, J.K. Eberhart, C.B. Kimmel, mef2ca is required in cranial neural crest to effect Endothelin1 signaling in zebrafish, Developmental biology, 308 (2007) 144-157.
[43] A. Magli, C. Angelelli, M. Ganassi, F. Baruffaldi, V. Matafora, R. Battini, A. Bachi, G. Messina, A. Rustighi, G. Del Sal, S. Ferrari, S. Molinari, Proline isomerase Pin1 represses terminal differentiation and myocyte enhancer factor 2C function in skeletal muscle cells, The Journal of biological chemistry, 285 (2010) 34518-34527.
[44] J.D. Molkentin, B.L. Black, J.F. Martin, E.N. Olson, Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C, Molecular and cellular biology, 16 (1996) 2627-2636.
[45] C.G. Janson, Y. Chen, Y. Li, D. Leifer, Functional regulatory regions of human transcription factor MEF2C, Brain research. Molecular brain research, 97 (2001) 70-82.
[46] F.J. Naya, E. Olson, MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation, Current opinion in cell biology, 11 (1999) 683-688.
[47] S. Borghi, S. Molinari, G. Razzini, F. Parise, R. Battini, S. Ferrari, The nuclear localization domain of the MEF2 family of transcription factors shows member-specific features and mediates the nuclear import of histone deacetylase 4, Journal of cell science, 114 (2001) 4477-4483.
[48] P.F. Xu, N. Houssin, K.F. Ferri-Lagneau, B. Thisse, C. Thisse, Construction of a vertebrate embryo from two opposing morphogen gradients, Science, 344 (2014) 87-89.
[49] E.M. De Robertis, Spemann's organizer and self-regulation in amphibian embryos, Nature reviews. Molecular cell biology, 7 (2006) 296-302.
[50] Y.G. Langdon, M.C. Mullins, Maternal and zygotic control of zebrafish dorsoventral axial patterning, Annual review of genetics, 45 (2011) 357-377.
[51] A.M. Branam, G.G. Hoffman, F. Pelegri, D.S. Greenspan, Zebrafish chordin-like and chordin are functionally redundant in regulating patterning of the dorsoventral axis, Developmental biology, 341 (2010) 444-458.

661 [52] V. Infantino, P. Convertini, A. Menga, V. Iacobazzi, MEF2C exon alpha: Role in gene activation and
662 differentiation, Gene, 531 (2013) 355-362.
663 [53] L.L. Bachinski, M. Sirito, M. Bohme, K.A. Baggerly, B. Udd, R. Krahe, Altered MEF2 isoforms in myotonic
664 dystrophy and other neuromuscular disorders, Muscle & nerve, 42 (2010) 856-863.
665 [54] T.L. Gallagher, J.A. Arribere, P.A. Geurts, C.R. Exner, K.L. McDonald, K.K. Dill, H.L. Marr, S.S. Adkar, A.T.
666 Garnett, S.L. Amacher, J.G. Conboy, Rbfox-regulated alternative splicing is critical for zebrafish cardiac and
667 skeletal muscle functions, Developmental biology, 359 (2011) 251-261.
668 [55] O. Yogev, V.C. Williams, Y. Hinits, S.M. Hughes, eIF4EBP3L Acts as a Gatekeeper of TORC1 In Activity-
669 Dependent Muscle Growth by Specifically Regulating Mef2ca Translational Initiation, PLoS biology, 11
670 (2013) e1001679.
671 [56] M. Hammerschmidt, G.N. Serbedzija, A.P. McMahon, Genetic analysis of dorsoventral pattern
672 formation in the zebrafish: requirement of a BMP-like ventralizing activity and its dorsal repressor, Genes &
673 development, 10 (1996) 2452-2461.
674 [57] M. Oelgeschlager, H. Kuroda, B. Reversade, E.M. De Robertis, Chordin is required for the Spemann
675 organizer transplantation phenomenon in Xenopus embryos, Developmental cell, 4 (2003) 219-230.
676 [58] S. Schulte-Merker, K.J. Lee, A.P. McMahon, M. Hammerschmidt, The zebrafish organizer requires
677 chordino, Nature, 387 (1997) 862-863.
678 [59] Y. Sasai, B. Lu, H. Steinbeisser, D. Geissert, L.K. Gont, E.M. De Robertis, Xenopus chordin: a novel
679 dorsalizing factor activated by organizer-specific homeobox genes, Cell, 79 (1994) 779-790.
680 [60] V.E. Miller-Bertoglio, S. Fisher, A. Sanchez, M.C. Mullins, M.E. Halpern, Differential regulation of
681 chordin expression domains in mutant zebrafish, Developmental biology, 192 (1997) 537-550.
682 [61] A. Kolpakova, S. Katz, A. Keren, A. Rojtblat, E. Bengal, Transcriptional regulation of mesoderm genes by
683 MEF2D during early Xenopus development, PloS one, 8 (2013) e69693.
684 [62] S. Dal-Pra, M. Furthauer, J. Van-Celst, B. Thisse, C. Thisse, Noggin1 and Follistatin-like2 function
685 redundantly to Chordin to antagonize BMP activity, Developmental biology, 298 (2006) 514-526.
686 [63] Y. Imai, M.A. Gates, A.E. Melby, D. Kimelman, A.F. Schier, W.S. Talbot, The homeobox genes vox and
687 vent are redundant repressors of dorsal fates in zebrafish, Development, 128 (2001) 2407-2420.
688 [64] T. Tian, A.M. Meng, Nodal signals pattern vertebrate embryos, Cellular and molecular life sciences :
689 CMLS, 63 (2006) 672-685.
690 [65] S.J. Du, S.H. Devoto, M. Westerfield, R.T. Moon, Positive and negative regulation of muscle cell identity
691 by members of the hedgehog and TGF-beta gene families, The Journal of cell biology, 139 (1997) 145-156.
692 [66] A. Meng, B. Moore, H. Tang, B. Yuan, S. Lin, A Drosophila doublesex-related gene, terra, is involved in
693 somitogenesis in vertebrates, Development, 126 (1999) 1259-1268.
694 [67] C. Yin, K. Kikuchi, T. Hochgreb, K.D. Poss, D.Y. Stainier, Hand2 regulates extracellular matrix remodeling
695 essential for gut-looping morphogenesis in zebrafish, Developmental cell, 18 (2010) 973-984.
696 [68] S.E. Patterson, N.C. Bird, S.H. Devoto, BMP regulation of myogenesis in zebrafish, Developmental
697 dynamics : an official publication of the American Association of Anatomists, 239 (2010) 806-817.
698 [69] J.D. Ellis, M. Barrios-Rodiles, R. Colak, M. Irimia, T. Kim, J.A. Calarco, X. Wang, Q. Pan, D. O'Hanlon, P.M.
699 Kim, J.L. Wrana, B.J. Blencowe, Tissue-specific alternative splicing remodels protein-protein interaction
700 networks, Molecular cell, 46 (2012) 884-892.
701 [70] J. Garcia, S.H. Gerber, S. Sugita, T.C. Sudhof, J. Rizo, A conformational switch in the Piccolo C2A domain
702 regulated by alternative splicing, Nature structural & molecular biology, 11 (2004) 45-53.
703 [71] B.L. Black, J.D. Molkentin, E.N. Olson, Multiple roles for the MyoD basic region in transmission of
704 transcriptional activation signals and interaction with MEF2, Molecular and cellular biology, 18 (1998) 69-
705 77.
706 [72] J.D. Molkentin, B.L. Black, J.F. Martin, E.N. Olson, Cooperative activation of muscle gene expression by
707 MEF2 and myogenic bHLH proteins, Cell, 83 (1995) 1125-1136.

708

709

710

Figure Legends

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

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

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

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

(3α1) and a 187-bp (3α2) RT-PCR products respectively. Flanking primers were also designed to investigate the expression of exon β, the extra sequence of intron 5 (*) and of the γ region. As control templates we used the pcDNA 3.1 expression vector containing the cDNAs of Mef2cbL3α1 β- γ+ (lane 5),Mef2cbL3α2 β- γ+ (lane 6) and Mef2cbS 3α1 β- γ+ (lane 7). *actb2* was used as a control, *myog* was used as a marker for skeletal muscle differentiation.

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

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

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

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

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

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

A) *Myod* mRNA in 22 hpf embryos injected with 25 pg of *mef2cb* mRNAs. mef2cbL but not mef2cbS injected embryos have ectopic myod expression in head region (arrowheads). Both groups show an array of developmental defects in head and trunk regions. **B**) *Myod* mRNA in head region at 28 hpf (dorsal view, anterior to top). Injection of 10 pg of Mef2cbL mRNA induces ectopic *myod* expression in head mesoderm (arrowheads). **C)** *smyhc1* mRNA and immunofluorescence of MyHC protein in 28 hpf non injected control embryos or embryos injected with 10 pg of Mef2cbL mRNA, Ectopic muscle is clearly seen in the head region of injected embryos (white arrowheads). While arrow and green arrowhead indicate somitic muscle and heart respectively. Scale bars = 100 μm.

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

A) *Myod* mRNA in 22 hpf embryos injected with the mRNAs of Mef2ca splice variants or not injected (control). Forced expression of Mef2ca 4-5-6 mRNA resulted in severe developmental defects: double axis (black arrowheads), trunk and brain defects (white arrowheads). Control embryos or embryos injected with 25 pg of Mef2ca 4-6 mRNA showed normal morphology. **B)** RT-PCR analysis of the total RNA extracted from 25 pg *mef2ca* mRNA injected or control uninjected embryos at 22 hpf. Mef2ca 4-5-6 injected embryos showed augmented expression of *chordin*, *no-tail a*, *nodal related 1*, *noggin 1* and *goosecoid*, reduced expression of *Δnp63* and *bmp 7a*, whereas *bmp 2b, no- tail b* and *myod* expression levels are unaffected. **C)** Densitometric analysis of the bands shown in B, normalized to *actb2* signal. Expression levels of each gene were arbitrarily set to a value of 1 in the uninjected control embryos. Statistical analysis was performed on data obtained from three independent experiments, the means ±SE (error bars) are represented. * and ** indicate P-values of ≤0.1 and ≤0.01 respectively.

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

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

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

SUPPLEMENTARY FIGURES

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

A) Schematic of the highly similar structures of three vertebrate *MEF2C* genes among coding exons (black boxes). To simplify the comparison, we assigned the number 1 to the exon containing the first translated ATG. Introns are indicated by solid lines. MEF2C genes from the three species share three alternative exons: the α1 and α2 mutually exclusive exons, the β skipping exon, and 3' splice site selection at exon 9. **B)** Schematic of the vertebrate Mef2c gene exon numbering adopted in this paper. In the table are reported the exon numbering of the mouse and frog *MEF2C* genes adopted in the indicated references**. C)** Splicing patterns of frog, mouse and human MEF2C. The MADS box and MEF2 domain are encoded by exons 1 and 2.

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

A) α2 alternative exon, β skipping exon and γ region in *mef2cb* and *mef2ca* genes predicted with the TBLASTN algorithm. The sequences of bona fide spliced out exons, the percentage of homology with the mouse sequence and the putative splice sites are indicated. **B)** Comparison of amino acid sequences for zebrafish Mef2ca and Mef2cb splice variants. Protein sequence encoded by different exons is indicated, and alternatively spliced out regions are marked in yellow and green. TADs are colored in blue and orange.

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

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

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

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

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

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

A) Left panel. Schematic representation of *mef2cb* 3α1 or 3α2 alternative exons. Arrows show annealing sites of isoform-specific primers used in the RT-PCR analysis they were designed to give amplification products of the same size (190 bp). Right panel. Expression analysis of *mef2cb* transcripts including the mutually exclusive 3α1 or 3α2 exon by RT-PCR. Total RNA was purified from staged embryos. To amplify an amount of exon 3α2 containing DNA similar to that containing exon 3α2, four additional PCR cycles were required. (B) Left panel. Schematic representation of *mef2ca* 3α1 and of mef2cb 3α1 or 3α2 alternative exons. Arrows show annealing sites of the primers used in the RT-PCR analysis. They give amplification products of distinct sizes. Right panel. Expression analysis of *mef2ca* and *mef2cb* transcripts including the mutually exclusive 3α1 or 3α2 exon by RT-PCR in adult tissues. Total RNA was purified from brain, liver, skeletal and cardiac muscle of adult zebrafish. The level of expression of the transcripts was evaluated by using primers that anneal to exons 2 and 4 for both *mef2c* genes, in the case of *mef2cb*, they give two amplification products of distinct sizes: 196 and 187 bp, depending on the incorporation of 3α1 or 3α2 alternative exons in the transcripts. PCR products were separated in 8% polyacrylamide gels. Length of PCR products (bp) is indicated.

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

A) Zebrafish embryos were injected with 25 pg of in vitro-transcribed *mef2ca* 4-5-6 RNA together with rhodamine dextran at the 1-2 cells stage and analyzed at 20 hpf. Successfully injected embryos were distinguished on the basis of the red fluorescence (insets) and classified on the basis of morphology into ‘severely defective’ (blocked development), ‘defective’ (altered development) or ‘normal’. B) Dose-dependent effects of in vitro-transcribed *mef2ca* mRNAs on embryos development. The graph reports the quantification of defective embryos upon injection of increasing doses (25 pg and 50 pg) of RNA encoding Mef2ca 4-5-6 and 4-6 splice variants. Controls were uninjected embryos (Ctrl). The number of embryos tested in each experiments is 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.

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

Ganassi M.[1,3], Badodi S. [1], Polacchini A. [1,2], Baruffaldi F. [1], Battini R. [1], Hughes S.M. [3], Hinits Y. [3,§], Molinari S. [1,§]

[1] University of Modena and Reggio Emilia, Department of Life Sciences; [2] Actual address: BRAIN Centre for Neuroscience, Dept. of Biology, University of Trieste, Italy; [3] Randall Division of Cell and Molecular Biophysics, New Hunt's House, Guy's Campus, King's College London, SE1 1UL, UK.

[§] Corresponding Authors. Mailing Adresses: Susanna Molinari, Via Campi 287, 41100, Modena, Italy. Tel.: +39 059 2055403; Fax: +39 059 2055410; Yaniv Hinits: 3rd floor North, New Hunt's House, Guy's Campus, London SE1 1UL, UK. . Tel.: +44 20 7848 6444; Fax: +44 20 7848 6435; *E-mail addresse*~~e-mail~~: susanna.molinari@unimore.it (S. Molinari), yaniv.hinits@kcl.ac.uk (Y. Hinits)

Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: Italic
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt, English (U.S.)

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 myo~~uscle~~ fibers. We found that zebrafish *mef2ca* and *mef2cb* are alternatively spliced in the coding exons 4-6 region and ~~that~~ these splice variants differ in their biological activity. Of the two, *mef2ca* is more abundantly expressed in developing skeletal muscle. ~~We show that~~ its activity is tuned through zebrafish development by AS. By 24 hpf, we found the prevalent expression ~~a stage when major muscle differentiation is observed, we found the prevalent expression~~ of the highly active full length protein in differentiated muscle ~~specifically~~ in the somites. ~~At 12 hours post fertilization (hpf), a high proportion (40%) of *mef2ca* transcripts~~The splicing isoform of *mef2ca* that lacks ~~coding~~ exon 5 (*mef2ca 4-6*), ~~thereby~~ encodes ~~ing~~ a protein that has~~exhibits a~~ 50% lower ~~reduction of~~ transcriptional activity, and is found mainly earlier in development, before muscle differentiation. ~~in comparison to the full length protein. By 24 hpf, a stage when major muscle differentiation is observed, we found the prevalent expression of the highly active full length protein specifically in somites.~~ *mef2ca* transcripts including exon 5 (*mef2ca 4-5-6*) are present early in the embryo. Over-expression of this isoform alters ~~Inclusion of exon 5 in Mef2ca protein confers the ability to drive, directly or indirectly,~~ the expression of genes involved in early dorso-ventral patterning of the embryo such as *chordin* ~~*(chd)*~~, *nodal related 1* ~~*(ndr1)*~~ and

Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic

goosecoid ~~*(gsc)*~~, ~~which function in early dorso-ventral patterning of the embryo~~and induces severe developmental defects. AS of *mef2cb* ~~was not developmentally regulated. However,~~generates a long splicing isoform in the exon 5 region (Mef2cbL) that predominates during somitogenesis~~d over other isoforms~~. Mef2cbL contains an evolutionarily conserved domain derived from exonization of a fragment of intron 5, which~~that~~ 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.

Abbreviations

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

Highlights

- *mef2ca* and *mef2cb* gene products are alternatively spliced in zebrafish.
- Inclusion of exon 5 in *mef2ca* transcripts is regulated during zebrafish development.
- ~~Inclusion of the~~ Eexon 5 ~~encoded protein sequence~~ confers on Mef2ca~~to the protein~~ the ability to activate ~~a subset of~~early patterning- genes~~genes involved in dorso-ventral patterning of the embryo~~.
- ~~zebrafish~~ Mef2cb includes an extra octapeptide encoded by a region of intron 5~~ that is "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]. *MEF2*s 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 α1 and α2, located in the region immediately adjacent to the MEF2 domain [20]. In the central TAD2 region, a skipping-type alternative splice can include exon β and a splice involving alternative 3' splice site selection occurs

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

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

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

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

Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic

2. Materials and Methods

2.1. Plasmids

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

(Promega), then sub-cloned into BamHI/NotI sites of the pcDNA 3.1(+) expression vector (Invitrogen). For RNA injections isoforms were sub-cloned into the XbaI/SalI sites of the βUT-3 vector [8]. Plasmids pGL3(desMEF2)$_3$ and pRSVβ-gal were previously described [28].

2.2. Alternative splicing prediction and multiple alignments

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

2.3. Transcription Reporter Assays

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

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

RNA was isolated using TRIzol® Plus RNA Purification System (Ambion). For each developmental stage, 100 embryos were disrupted using Tissue Raptor (Qiagen). 500 ng of total RNA were reverse transcribed to cDNA using Superscript III reverse transcriptase (Invitrogen). Primers used to detect *myogenin (myog)*, *myod*, *beta-actin 2 (actb2)*, *mef2ca* and *mef2cb* are listed in Fig. S6A (other primers sequence are available upon request). quantitative Real Time PCR (qRTPCR) was performed on 2,5~~1~~ ng of Poly A mRNA using SYBR Green method (SYBR® Green PCR Core Reagent, Applied Biosystems). Poly A mRNA has been purified using Ambion's protocol (MicroPoly(A) Purist Kit). To amplify the different *mef2ca* isoforms specific forward primers spanning exon-exon junctions were used with a common reverse primer (Fig. S6B). For each primer combination the optimal $MgCl_2$ concentration was determined to obtain specific and high efficient amplification (slope values between -2.95 and -3.75). Absolute quantification of transcript copy number was achieved by generating calibration curve using plasmid DNA templates (listed above) as previously described [29, 30]. Analysis was performed using PCR ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Student's t-tests were performed for pairwise comparisons to determine significant differences between groups.

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

158

159

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 Hybridi~~z~~zation (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* (*kdrl*) [35],
168 *neurogenin* 1 (*neurog1*) [36] or *achaete-scute complex-like 1a* (*ascl1a*) [37]. We have also used
169 two~~a~~ 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 Improvision Openlab.

174

175 *2.7. mRNA injection and embryo manipulation*

176 mRNA injection was performed as described previously [40]. βUT-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 p~~n~~g, 25~~0~~ ~~n~~pg or
179 50 ~~n~~pg/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~~2~~ 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-αTubulin (T6074, Sigma Aldrich), mouse anti-Vinculin (V4505,

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Formatted: Font: Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Italic
Field Code Changed
Formatted: Font: Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Field Code Changed
Formatted: Font: Italic
Field Code Changed
Formatted: Font: Italic
Formatted: Font: Not Italic
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

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

~~*2.9.Statistical Analysis*~~

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

3. Results

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

3.1. Alternative Splicing of zebrafish mef2ca and mef2cb

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

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

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

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

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

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

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

Field Code Changed

Field Code Changed

Field Code Changed

We sought to characterize the temporal and spatial expression patterns of *mef2ca* and *mef2cb* in developing zebrafish skeletal muscle. First, we quantified the expression levels of *mef2ca* and *mef2cb* transcripts by quantitative qRTPCR amplification using paralogue-specific primers, starting from equal amounts of RNA collected from zebrafish embryos at sequential developmental stages (from 12 to 72 hpf). *mef2ca* and *mef2cb* presented a similar profile of expression, with a higher abundance of the transcripts of *mef2ca* at all stages analyzed. Both genes were expressed at low levels at 12 hpf (*mef2ca* 2^{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.

Field Code Changed

Field Code Changed

Field Code Changed

Field Code Changed

3.3. Developmentally regulated expression of mef2ca and mef2cb splice variants

Levels of expression of alternatively spliced *mef2ca* and *mef2cb* during zebrafish development were determined by semi-quantitative RT-PCR and qRTPCR RNA quantification using SYBR and exon boundary spanning primers, that allow for selective PCR amplification of individual alternative transcripts [30]. At 12 hpf, the amount of *mef2ca* 4-6 transcript (lacking exon 5) represents about 430% of the total, ~~comparable to that of~~whereas ~~a~~the amount of the full length 4-5-6 transcript the remaining 70%~~(~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~~, h~~owever, they

Field Code Changed

Formatted: Font: Italic

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

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

Field Code Changed

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

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

Field Code Changed

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

Field Code Changed

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

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

Field Code Changed

Field Code Changed

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

Field Code Changed

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

3.6. Mef2cbL has unique myogenic potential in developing zebrafish

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

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

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

Formatted: Font: Italic

Field Code Changed

Formatted: Font: Italic

Formatted: Font: Italic

Formatted: Font: Not Italic

383 defects, occasional double axeis, and some brain defects such as undeveloped eyes and absence of
384 mid- and forebrain structures (Fig. 76AB,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 *Δnp63*
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.

405

406 *3.8. Mef2ca 4-5-6 mRNA is the prevalent Mef2c transcript present in the embryo before*
407 *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 1K-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 1K-cell stage, with predominant expression of the transcript

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Line spacing: 1.5 lines
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt

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

4. Discussion

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

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

Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt, Italic
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: Italic
Formatted: Font: (Default) Times New Roman, 12 pt, Italic
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt, Italic
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted: Font: (Default) Times New Roman, 12 pt
Formatted
Formatted
Formatted
Formatted
Formatted
Formatted
Formatted
Formatted
Formatted
Formatted: Font: Italic
Formatted
Formatted
Formatted
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

In addition to the well documented expression of *mef2ca* starting from 12 hpf [32], we found *mef2ca* transcripts in zebrafish embryo prior to gastrulation at the 1K-cell stage, likely from maternal contribution, with their level declining to a minimum at 9-10 hpf. Starting from 12 hpf~~During development~~ we observe an overall increase in expression levels of *mef2ca* mRNA. Besides changes in the abundance of *mef2ca* transcripts, we found a dynamic regulation of the splicing in the exon 5 region ~~and, in addition, *mef2ca* transcripts display a switch in isoform dominance. T~~: the *mef2ca* variant including exon 5 (*mef2ca* 4-5-6) is the major isoform detected very early in development (1K-cell stage), suggesting that it might play a role prior to gastrulation, by 10 hpf, the *mef2ca* transcripts lacking exon 5 (*mef2ca* 4-6) are predominant. Subsequently, *mef2ca* 4-5-6 again climbs as muscle precursors undergo terminal differentiation, becominges the predominant~~valent~~ isoform at 24 hpf~~, as muscle precursors undergo terminal differentiation~~. Such splicing of exon 5 is evolutionary conserved between Xenopus and teleosts, suggesting it has biological significance [24] (Fig. S3). Moreover, muscle differentiation in zebrafish is associated with several other muscle-specific alternative splicing events involving changes in splicing efficiency [54]. Although no specific function was assigned to the exon 5 domain by mutational and deletion analysis of the mouse and human protein counterparts [44, 45], our cell culture data indicate that the peptide~~protein~~ sequence encoded by exon 5 contributes to the transcriptional activity of Mef2ca. The early expression of *mef2ca* transcripts including exon 5 (mef2ca 4-5-6) may indicate their early function in embryo patterning. Later in development,

~~T~~the preferential accumulation of full length *mef2ca 4-5-6* mRNA at skeletal muscle fiber ends, suggests that its normal function is in muscle, a view confirmed by the requirement for Mef2ca function for skeletal muscle fiber growth and heart myogenesis [8, 55]. In the current work the function of Mef2ca isoforms was probed by ectopic over-expression; *mef2ca* 4-5-6 RNA, but not *mef2ca 4-6* RNA, causes gross defects during gastrulation. We suggest that these effects of Mef2ca 4-5-6 are attributable to its ability to activate, directly or indirectly, a specific subset of pivotal genes in gastrulation. We observed the~~a robust ten-fold~~ induction in ~~*chordin (chd)*~~*chd* (2-fold) mRNA and a milder (1.4 fold) increase in *noggin 1 (nog1)* mRNAs that encode two inhibitors of the BMP signaling. *ch~~ordin~~dordin* is required to repress *bmp2b* function in formation of the organizer and dorsoventral patterning of mesoderm and neural tissue [56-58]. Over-expression of *ch~~ordin~~dordin* dorsalizes embryos [59, 60], a phenotype present in a fraction of embryos following Mef2ca 4-5-6 over-expression. Thus, up-regulation of these dorsalizing proteins may explain the effects of Mef2ca 4-5-6.

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

Formatted: Font: Italic
Field Code Changed
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Field Code Changed
Field Code Changed
Field Code Changed
Formatted: Font: Italic
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Formatted: Font: Italic

gene [61]. In zebrafish, Mef2ca 4-5-6 over-expression also increases in *gsc-* and *ndr1* mRNAs (2.2-~~1.7~~ and 1.5~~8-~~ folds, respectively), which regulate dorsoventral patterning in organisms ranging from *Drosophila* to mammals [49, 50, 62-64]. In line with our results it has been previously reported that expression of *gsc*, is reduced in *mef2ca* $^{-/-}$ (*hoover*) mutants [42]. Although we cannot exclude off-target effects, this specific ability of Mef2ca 4-5-6, but not of similar amounts of Mef2ca 4-6, suggests distinct transcriptional activity of the former. *In silico* analysis of promoter regions of *chd* and *ndr1-* genes revealed the presence of several putative MEF2 binding sites (YTA(A/T)$_4$TAR) (data not shown), raising the possibility that Mef2ca 4-5-6 directly activates their expression during ~~.~~ early development. Later in development~~Some regions of early~~ *chordin* expression may be sustained by Mef2d, which constitutively includes the sequence encoded by exon 5, and which is expressed from mid-gastrulation in adaxial muscle cells that also express *chordin* [32, 60] or by Mef2cb proteins. Interestingly, injection of either *mef2cbS* or *mef2cbL* transcripts, both containing exon5, have resulted in similar developmental defects to that of *mef2ca* 4-5-6 mRNA injection. Future studies will clarify whether these genes are indeed direct targets of a Mef2 protein containing exon 5.

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

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

4.2. Gene duplication and evolutionary partitioning of alternative splicing

The importance of other splices in Mef2ca remains to be determined. The γ-like and 4'-6' splices have low abundance and we were unable to display unique activities for these isoforms. On the other hand, unlike in mammals, exon 3 does not appear to show alternative splicing in Mef2ca, the gene only having an α1 version. As the α1 exon of mouse Mef2D mediates interactions with specific transcriptional co-regulators [23], Mef2ca may have a more restricted range of functions

Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Formatted: Font: Italic
Field Code Changed
Field Code Changed
Formatted: Font: Italic
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

compared to Mef2cb, which retains alternative α exons in its genomic sequence. However, at the stages examined, transcripts of *mef2cb* containing the α2 exon had low abundance, suggesting that this splice may be significant in specific cell types or developmental stages. In the adult we found a high proportion of the *mef2cb* transcripts containing the 3α2 exon in striated muscle tissue where it might play a specific role in mediating muscle gene expression as shown for the analogous splice variant of *Mef2d* in mammals [23]. Conversely, *mef2cb* transcripts omitting exon 5 were not observed. Instead, teleost *mef2cb* has evolved a unique splice, possibly derived by exonisation [1] of a part of intron 5. The addition of this octapeptide and its conservation across teleosts appears to have conferred myogenic properties to Mef2cbL.

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

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

5. Conclusions

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

Formatted: Font: Italic
Field Code Changed
Field Code Changed
Formatted: Font: Italic
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed
Field Code Changed

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

Acknowledgments

We thank Veronica Mantovani for her help in performing qRTPCR and transactivation assays, Isabella Della Noce and Filippo Schepis for their help in RNA and protein extraction from zebrafish embryos, Cristina Valensisi for her help in designing splice variants-specific primers . We are grateful to Sharon Amacher, Tom Gallagher and Tod Gulick for sharing their results before publication. We are grateful~~,~~ to Andrea Martello, Tommaso Selmi and~~,~~ Tommaso Zanocco-Marani for helpful discussion. We greatly thank Carol Imbriano and Alessandro Magli for reading the manuscript and ~~for their very~~ helpful suggestions.

Research support was provided by: Fondazione Cassa di Risparmio di Modena, progetto internazionale bando 2010, AFM (Association Francaise contre les Myopathies) (grant n°16252) and Optistem (European collaborative project HEALTH-2007-1.4-6). M.G.~~Dr Ganassi~~ was a recipient of short term fellowships from EMBO (ref. ASTF 414.00-2009), EufishBioMed (COST action BM0804, ref code 141111-012508), University of Modena and Reggio Emilia (Bando di Mobilità), Myores (Exchange Fund) and CIB (Consorzio Italiano Biotecnologie) that allowed him to visit and perform experiments in the laboratory of S. M. Hughes. A.P. ~~Dr. Polacchini~~ was a recipient of a short-term fellowship from CIB (Consorzio Italiano Biotecnologie) that supported his visit to the laboratory of S. M. Hughes. S. M. Hughes~~.~~ is an MRC Scientist with Programme Grant G1001029 support that funded studies in the Hughes laboratory. Y. Hinits was also supported by a collaborative grant from the UK British Heart Foundation Centres of Excellence at King's College London, Imperial College London and the Universities of Edinburgh and Oxford.

References

[1] Y. Xing, C. Lee, Alternative splicing and RNA selection pressure--evolutionary consequences for eukaryotic genomes, Nature reviews. Genetics, 7 (2006) 499-509.
[2] H. Keren, G. Lev-Maor, G. Ast, Alternative splicing and evolution: diversification, exon definition and function, Nature reviews. Genetics, 11 (2010) 345-355.
[3] S.W. Roy, M. Irimia, Splicing in the eukaryotic ancestor: form, function and dysfunction, Trends in ecology & evolution, 24 (2009) 447-455.
[4] A.R. Kornblihtt, I.E. Schor, M. Allo, G. Dujardin, E. Petrillo, M.J. Munoz, Alternative splicing: a pivotal step between eukaryotic transcription and translation, Nature reviews. Molecular cell biology, 14 (2013) 153-165.

[5] C. Trapnell, B.A. Williams, G. Pertea, A. Mortazavi, G. Kwan, M.J. van Baren, S.L. Salzberg, B.J. Wold, L. Pachter, Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation, Nature biotechnology, 28 (2010) 511-515.
[6] M. Llorian, C.W. Smith, Decoding muscle alternative splicing, Current opinion in genetics & development, 21 (2011) 380-387.
[7] M. Pistoni, C. Ghigna, D. Gabellini, Alternative splicing and muscular dystrophy, RNA biology, 7 (2010) 441-452.
[8] Y. Hinits, L. Pan, C. Walker, J. Dowd, C.B. Moens, S.M. Hughes, Zebrafish Mef2ca and Mef2cb are essential for both first and second heart field cardiomyocyte differentiation, Developmental biology, 369 (2012) 199-210.
[9] B.L. Black, E.N. Olson, Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins, Annual review of cell and developmental biology, 14 (1998) 167-196.
[10] B.A. Bour, M.A. O'Brien, W.L. Lockwood, E.S. Goldstein, R. Bodmer, P.H. Taghert, S.M. Abmayr, H.T. Nguyen, Drosophila MEF2, a transcription factor that is essential for myogenesis, Genes & development, 9 (1995) 730-741.
[11] B. Lilly, B. Zhao, G. Ranganayakulu, B.M. Paterson, R.A. Schulz, E.N. Olson, Requirement of MADS domain transcription factor D-MEF2 for muscle formation in Drosophila, Science, 267 (1995) 688-693.
[12] G. Ranganayakulu, B. Zhao, A. Dokidis, J.D. Molkentin, E.N. Olson, R.A. Schulz, A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in Drosophila, Developmental biology, 171 (1995) 169-181.
[13] Q. Lin, J. Schwarz, C. Bucana, E.N. Olson, Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C, Science, 276 (1997) 1404-1407.
[14] Y. Hinits, S.M. Hughes, Mef2s are required for thick filament formation in nascent muscle fibres, Development, 134 (2007) 2511-2519.
[15] T.K. Ghosh, F.F. Song, E.A. Packham, S. Buxton, T.E. Robinson, J. Ronksley, T. Self, A.J. Bonser, J.D. Brook, Physical interaction between TBX5 and MEF2C is required for early heart development, Molecular and cellular biology, 29 (2009) 2205-2218.
[16] S. Lazic, I.C. Scott, Mef2cb regulates late myocardial cell addition from a second heart field-like population of progenitors in zebrafish, Developmental biology, 354 (2011) 123-133.
[17] M.J. Potthoff, M.A. Arnold, J. McAnally, J.A. Richardson, R. Bassel-Duby, E.N. Olson, Regulation of skeletal muscle sarcomere integrity and postnatal muscle function by Mef2c, Molecular and cellular biology, 27 (2007) 8143-8151.
[18] E. Dodou, D.B. Sparrow, T. Mohun, R. Treisman, MEF2 proteins, including MEF2A, are expressed in both muscle and non-muscle cells, Nucleic acids research, 23 (1995) 4267-4274.
[19] M.J. Potthoff, E.N. Olson, MEF2: a central regulator of diverse developmental programs, Development, 134 (2007) 4131-4140.
[20] J.C. McDermott, M.C. Cardoso, Y.T. Yu, V. Andres, D. Leifer, D. Krainc, S.A. Lipton, B. Nadal-Ginard, hMEF2C gene encodes skeletal muscle- and brain-specific transcription factors, Molecular and cellular biology, 13 (1993) 2564-2577.
[21] B. Zhu, T. Gulick, Phosphorylation and alternative pre-mRNA splicing converge to regulate myocyte enhancer factor 2C activity, Molecular and cellular biology, 24 (2004) 8264-8275.
[22] B. Zhu, B. Ramachandran, T. Gulick, Alternative pre-mRNA splicing governs expression of a conserved acidic transactivation domain in myocyte enhancer factor 2 factors of striated muscle and brain, The Journal of biological chemistry, 280 (2005) 28749-28760.
[23] S. Sebastian, H. Faralli, Z. Yao, P. Rakopoulos, C. Palii, Y. Cao, K. Singh, Q.C. Liu, A. Chu, A. Aziz, M. Brand, S.J. Tapscott, F.J. Dilworth, Tissue-specific splicing of a ubiquitously expressed transcription factor is essential for muscle differentiation, Genes & development, 27 (2013) 1247-1259.
[24] B. della Gaspera, A.S. Armand, I. Sequeira, S. Lecolle, C.L. Gallien, F. Charbonnier, C. Chanoine, The Xenopus MEF2 gene family: evidence of a role for XMEF2C in larval tendon development, Developmental biology, 328 (2009) 392-402.
[25] S. Ferrari, S. Molinari, R. Melchionna, M.G. Cusella-De Angelis, R. Battini, L. De Angelis, R. Kelly, G. Cossu, Absence of MEF2 binding to the A/T-rich element in the muscle creatine kinase (MCK) enhancer correlates with lack of early expression of the MCK gene in embryonic mammalian muscle, Cell growth &

634 differentiation : the molecular biology journal of the American Association for Cancer Research, 8 (1997)
635 23-34.
636 [26] Y. Guo, S.J. Kuhl, A.S. Pfister, W. Cizelsky, S. Denk, L. Beer-Molz, M. Kuhl, Comparative analysis reveals
637 distinct and overlapping functions of Mef2c and Mef2d during cardiogenesis in Xenopus laevis, PloS one, 9
638 (2014) e87294.
639 [27] G. Genikhovich, U. Technau, Complex functions of Mef2 splice variants in the differentiation of
640 endoderm and of a neuronal cell type in a sea anemone, Development, 138 (2011) 4911-4919.
641 [28] C. Angelelli, A. Magli, D. Ferrari, M. Ganassi, V. Matafora, F. Parise, G. Razzini, A. Bachi, S. Ferrari, S.
642 Molinari, Differentiation-dependent lysine 4 acetylation enhances MEF2C binding to DNA in skeletal muscle
643 cells, Nucleic acids research, 36 (2008) 915-928.
644 [29] H.S. Walton, F.M. Gebhardt, D.J. Innes, P.R. Dodd, Analysis of multiple exon-skipping mRNA splice
645 variants using SYBR Green real-time RT-PCR, Journal of neuroscience methods, 160 (2007) 294-301.
646 [30] Vandenbroucke, II, J. Vandesompele, A.D. Paepe, L. Messiaen, Quantification of splice variants using
647 real-time PCR, Nucleic acids research, 29 (2001) E68-68.
648 [31] M. Westerfield, The zebrafish book a guide for the laboratory use of zebrafish Danio (Brachydanio)
649 rerio, in, Institute of Neuroscience, University of Oregon,, Eugene, OR, 1993.
650 [32] B.S. Ticho, D.Y. Stainier, M.C. Fishman, R.E. Breitbart, Three zebrafish MEF2 genes delineate somitic
651 and cardiac muscle development in wild-type and mutant embryos, Mechanisms of development, 59 (1996)
652 205-218.
653 [33] E.S. Weinberg, M.L. Allende, C.S. Kelly, A. Abdelhamid, T. Murakami, P. Andermann, O.G. Doerre, D.J.
654 Grunwald, B. Riggleman, Developmental regulation of zebrafish MyoD in wild-type, no tail and spadetail
655 embryos, Development, 122 (1996) 271-280.
656 [34] D. Yelon, S.A. Horne, D.Y. Stainier, Restricted expression of cardiac myosin genes reveals regulated
657 aspects of heart tube assembly in zebrafish, Developmental biology, 214 (1999) 23-37.
658 [35] M.A. Thompson, D.G. Ransom, S.J. Pratt, H. MacLennan, M.W. Kieran, H.W. Detrich, 3rd, B. Vail, T.L.
659 Huber, B. Paw, A.J. Brownlie, A.C. Oates, A. Fritz, M.A. Gates, A. Amores, N. Bahary, W.S. Talbot, H. Her,
660 D.R. Beier, J.H. Postlethwait, L.I. Zon, The cloche and spadetail genes differentially affect hematopoiesis and
661 vasculogenesis, Developmental biology, 197 (1998) 248-269.
662 [36] P. Blader, N. Fischer, G. Gradwohl, F. Guillemot, U. Strahle, The activity of neurogenin1 is controlled by
663 local cues in the zebrafish embryo, Development, 124 (1997) 4557-4569.
664 [37] M.L. Allende, E.S. Weinberg, The expression pattern of two zebrafish achaete-scute homolog (ash)
665 genes is altered in the embryonic brain of the cyclops mutant, Developmental biology, 166 (1994) 509-530.
666 [38] Y. Hinits, V.C. Williams, D. Sweetman, T.M. Donn, T.P. Ma, C.B. Moens, S.M. Hughes, Defective cranial
667 skeletal development, larval lethality and haploinsufficiency in Myod mutant zebrafish, Developmental
668 biology, 358 (2011) 102-112.
669 [39] A.K. Lagendijk, J.D. Moulton, J. Bakkers, Revealing details: whole mount microRNA in situ hybridization
670 protocol for zebrafish embryos and adult tissues, Biology open, 1 (2012) 566-569.
671 [40] D.P. Osborn, K. Li, Y. Hinits, S.M. Hughes, Cdkn1c drives muscle differentiation through a positive
672 feedback loop with Myod, Developmental biology, 350 (2011) 464-475.
673 [41] C.S. Blagden, P.D. Currie, P.W. Ingham, S.M. Hughes, Notochord induction of zebrafish slow muscle
674 mediated by Sonic hedgehog, Genes & development, 11 (1997) 2163-2175.
675 [42] C.T. Miller, M.E. Swartz, P.A. Khuu, M.B. Walker, J.K. Eberhart, C.B. Kimmel, mef2ca is required in
676 cranial neural crest to effect Endothelin1 signaling in zebrafish, Developmental biology, 308 (2007) 144-
677 157.
678 [43] A. Magli, C. Angelelli, M. Ganassi, F. Baruffaldi, V. Matafora, R. Battini, A. Bachi, G. Messina, A. Rustighi,
679 G. Del Sal, S. Ferrari, S. Molinari, Proline isomerase Pin1 represses terminal differentiation and myocyte
680 enhancer factor 2C function in skeletal muscle cells, The Journal of biological chemistry, 285 (2010) 34518-
681 34527.
682 [44] J.D. Molkentin, B.L. Black, J.F. Martin, E.N. Olson, Mutational analysis of the DNA binding, dimerization,
683 and transcriptional activation domains of MEF2C, Molecular and cellular biology, 16 (1996) 2627-2636.
684 [45] C.G. Janson, Y. Chen, Y. Li, D. Leifer, Functional regulatory regions of human transcription factor
685 MEF2C, Brain research. Molecular brain research, 97 (2001) 70-82.

Formatted: Italian (Italy)

[46] F.J. Naya, E. Olson, MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation, Current opinion in cell biology, 11 (1999) 683-688.
[47] S. Borghi, S. Molinari, G. Razzini, F. Parise, R. Battini, S. Ferrari, The nuclear localization domain of the MEF2 family of transcription factors shows member-specific features and mediates the nuclear import of histone deacetylase 4, Journal of cell science, 114 (2001) 4477-4483.
[48] P.F. Xu, N. Houssin, K.F. Ferri-Lagneau, B. Thisse, C. Thisse, Construction of a vertebrate embryo from two opposing morphogen gradients, Science, 344 (2014) 87-89.
[49] E.M. De Robertis, Spemann's organizer and self-regulation in amphibian embryos, Nature reviews. Molecular cell biology, 7 (2006) 296-302.
[50] Y.G. Langdon, M.C. Mullins, Maternal and zygotic control of zebrafish dorsoventral axial patterning, Annual review of genetics, 45 (2011) 357-377.
[51] A.M. Branam, G.G. Hoffman, F. Pelegri, D.S. Greenspan, Zebrafish chordin-like and chordin are functionally redundant in regulating patterning of the dorsoventral axis, Developmental biology, 341 (2010) 444-458.
[52] V. Infantino, P. Convertini, A. Menga, V. Iacobazzi, MEF2C exon alpha: Role in gene activation and differentiation, Gene, 531 (2013) 355-362.
[53] L.L. Bachinski, M. Sirito, M. Bohme, K.A. Baggerly, B. Udd, R. Krahe, Altered MEF2 isoforms in myotonic dystrophy and other neuromuscular disorders, Muscle & nerve, 42 (2010) 856-863.
[54] T.L. Gallagher, J.A. Arribere, P.A. Geurts, C.R. Exner, K.L. McDonald, K.K. Dill, H.L. Marr, S.S. Adkar, A.T. Garnett, S.L. Amacher, J.G. Conboy, Rbfox-regulated alternative splicing is critical for zebrafish cardiac and skeletal muscle functions, Developmental biology, 359 (2011) 251-261.
[55] O. Yogev, V.C. Williams, Y. Hinits, S.M. Hughes, eIF4EBP3L Acts as a Gatekeeper of TORC1 In Activity-Dependent Muscle Growth by Specifically Regulating Mef2ca Translational Initiation, PLoS biology, 11 (2013) e1001679.
[56] M. Hammerschmidt, G.N. Serbedzija, A.P. McMahon, Genetic analysis of dorsoventral pattern formation in the zebrafish: requirement of a BMP-like ventralizing activity and its dorsal repressor, Genes & development, 10 (1996) 2452-2461.
[57] M. Oelgeschlager, H. Kuroda, B. Reversade, E.M. De Robertis, Chordin is required for the Spemann organizer transplantation phenomenon in Xenopus embryos, Developmental cell, 4 (2003) 219-230.
[58] S. Schulte-Merker, K.J. Lee, A.P. McMahon, M. Hammerschmidt, The zebrafish organizer requires chordino, Nature, 387 (1997) 862-863.
[59] Y. Sasai, B. Lu, H. Steinbeisser, D. Geissert, L.K. Gont, E.M. De Robertis, Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes, Cell, 79 (1994) 779-790.
[60] V.E. Miller-Bertoglio, S. Fisher, A. Sanchez, M.C. Mullins, M.E. Halpern, Differential regulation of chordin expression domains in mutant zebrafish, Developmental biology, 192 (1997) 537-550.
[61] A. Kolpakova, S. Katz, A. Keren, A. Rojtblat, E. Bengal, Transcriptional regulation of mesoderm genes by MEF2D during early Xenopus development, PloS one, 8 (2013) e69693.
[62] S. Dal-Pra, M. Furthauer, J. Van-Celst, B. Thisse, C. Thisse, Noggin1 and Follistatin-like2 function redundantly to Chordin to antagonize BMP activity, Developmental biology, 298 (2006) 514-526.
[63] Y. Imai, M.A. Gates, A.E. Melby, D. Kimelman, A.F. Schier, W.S. Talbot, The homeobox genes vox and vent are redundant repressors of dorsal fates in zebrafish, Development, 128 (2001) 2407-2420.
[64] T. Tian, A.M. Meng, Nodal signals pattern vertebrate embryos, Cellular and molecular life sciences : CMLS, 63 (2006) 672-685.
[65] S.J. Du, S.H. Devoto, M. Westerfield, R.T. Moon, Positive and negative regulation of muscle cell identity by members of the hedgehog and TGF-beta gene families, The Journal of cell biology, 139 (1997) 145-156.
[66] A. Meng, B. Moore, H. Tang, B. Yuan, S. Lin, A Drosophila doublesex-related gene, terra, is involved in somitogenesis in vertebrates, Development, 126 (1999) 1259-1268.
[67] C. Yin, K. Kikuchi, T. Hochgreb, K.D. Poss, D.Y. Stainier, Hand2 regulates extracellular matrix remodeling essential for gut-looping morphogenesis in zebrafish, Developmental cell, 18 (2010) 973-984.
[68] S.E. Patterson, N.C. Bird, S.H. Devoto, BMP regulation of myogenesis in zebrafish, Developmental dynamics : an official publication of the American Association of Anatomists, 239 (2010) 806-817.

Formatted: Italian (Italy)

737 [69] J.D. Ellis, M. Barrios-Rodiles, R. Colak, M. Irimia, T. Kim, J.A. Calarco, X. Wang, Q. Pan, D. O'Hanlon, P.M.
738 Kim, J.L. Wrana, B.J. Blencowe, Tissue-specific alternative splicing remodels protein-protein interaction
739 networks, Molecular cell, 46 (2012) 884-892.
740 [70] J. Garcia, S.H. Gerber, S. Sugita, T.C. Sudhof, J. Rizo, A conformational switch in the Piccolo C2A domain
741 regulated by alternative splicing, Nature structural & molecular biology, 11 (2004) 45-53.
742 [71] B.L. Black, J.D. Molkentin, E.N. Olson, Multiple roles for the MyoD basic region in transmission of
743 transcriptional activation signals and interaction with MEF2, Molecular and cellular biology, 18 (1998) 69-
744 77.
745 [72] J.D. Molkentin, B.L. Black, J.F. Martin, E.N. Olson, Cooperative activation of muscle gene expression by
746 MEF2 and myogenic bHLH proteins, Cell, 83 (1995) 1125-1136.

747

748

749

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 3α2, 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

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

Fig. 2. Expression of *mef2ca* and *mef2cb* genes in zebrafish embryos. **A)** Estimation of absolute *mef2ca* and *mef2cb* transcripts by qRTPCR during *D. rerio* development. The graph shows transcript-specific absolute quantification, reported as $\log_2$ ~~C~~copy number in equal amount of total RNA (2,5~~1~~ ng) extracted from zebrafish embryos at 12, 24, 48 and 72 hpf ~~whole zebrafish embryos~~ . Graph showing mean ±SE from ~~two~~ four independent experiments, ** and *** indicate~~s a~~ P~~p~~-value~~s~~ of -≤0.01~~5~~ and ≤0.001 respectively. **B)** Wholemount in situ hybridization for *mef2ca* and *mef2cb* mRNA for embryos at 11 ss (dorsal view, anterior to top) and at 24 hpf (lateral view, anterior to left). At 11 ss, both genes are expressed in the adaxial cells (black arrowheads). By 24 hpf *mef2ca* is strongly expressed in the myotome (black arrows) and also in heart (green arrowhead) and branchial arches (blue arrowhead). *mef2cb* transcripts are detected in the heart (green arrowhead), telencephalon (red arrowhead) and are weakly detected in the somites (black arrow). Scale bars = 100µm.

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

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

Formatted: Font: Italic

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 1- 4). PCR products were separated in 8% polyacrylamide gels. Length of PCR products in base pairs (bp) is indicated. **C)** Quantitative analysis of the mRNA levels of *mef2ca* exon 5 splice variants during *D. rerio* development. The amount of the transcripts of each splice variant was estimated by absolute qRTPCR. Original data (mRNA levels of each isoform) are reported as % of the total number of *mef2ca* transcripts (4-5-6 + 4-6 + 4'-6' = 100%). Statistical analysis was performed on data obtained from three independent experiments , the means ± SE are represented. *** indicate a P-value ≤0.001. Absolute qRTPCR estimation of *mef2ca* splicing isoforms during *D.rerio* development. The quantification, reported as log_2 number of copies, was performed on equal amount (1 ng) of total RNA extracted from staged embryos. The graph reports the mean ±SE bars of three independent experiments,*** indicates a p-value ≤0.001, ** ≤0.01, ## ≤0.01. **D)** Schematic representations of Mef2cb alternative exons. Arrows show primers annealing sites. **E)** Developmental RT-PCR analysis of *mef2cb* mRNAs. To evaluate the amount of 3α1- and 3α2-containing *mef2cb* transcripts, we designed common PCR primers (cb2 and cb4Rv) annealing to flanking regions in exons 2 and 4 to generate two amplicons of different size: to generate isoform-specific, a 1960-bp (3α1) and a 187-bp (3α2) RT-PCR products respectively. Flanking primers were also designed to investigate the expression of exon β, the extra sequence of intron 5 (*) and of the γ region. As control templates we used the pcDNA 3.1 expression vector containing the cDNAs of Plasmid vectors containing the cDNAs of Mef2cbL3α1 β- γ+ (lane 5),-Mef2cbL3α2 β- γ+ (lane 6) andand Mef2cbS 3α1 β- γ+ (lane 7)were used as control templates (lane 5 and 6, respectively). *β actin 2* (*actb2*) was used as a control, *myog* was used as a marker for skeletal muscle differentiation.

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

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

Formatted: Font: Italic
Formatted: Font: Symbol
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Symbol
Formatted: Font: Symbol
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Bold

839 ~~enriched~~ enriched at fiber ends. Right panels.~~D~~**)** Dorsal view of ~~a~~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~~s, left panel~~). ~~No expression is detected in these areas with~~
843 ~~exon5 specific probe (right panel). (s~~Scale bars = 100µm~~)~~. **C~~E~~)** Drawing of the LNA1 and LNA2
844 probes annealing positions within the exon 4/5 region.
845
846 **Fig. 5.** Transcriptional activity of zebrafish Mef2ca and Mef2cb splice variants.
847 **A)** COS~~os~~-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. *** indicate~~s~~ a
853 P~~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~~C~~ 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~~s~~). ~~**B,**~~ **C)**
868 *smyhc1* mRNA and immunofluorescence of MyHC protein ~~*myod* and *myogenin* mRNA~~ in 28~~2~~ hpf
869 non injected ~~and 30 hpf~~ control embryos ~~(non injected)~~ or embryos injected with 10~~25~~ pg of ~~the~~
870 ~~indicated~~Mef2cbL mRNA~~s~~.~~,~~ Ectopic muscle is clearly seen in the head region of injected embryos

Formatted: Font: Bold

Formatted: Font: Italic

Formatted: Font: Bold

(white arrowheads). While arrow and green arrowhead indicate somitic muscle and heart respectively. Scale bars = 100 µm.

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

A) *Myod* mRNA in 22 hpf embryos injected with the mRNAs of Mef2ca splice variants or not injected (control). Forced expression of Mef2ca 4-5-6 mRNA resulted in severe developmental defects: double axis (black arrowheads), trunk and brain defects (white arrowheads). Control embryos or ~~E~~embryos injected with 25 pg of Mef2ca 4-6 mRNA showed normal morphology. **B~~D~~)** RT-PCR analysis of the total RNA extracted from 25 pg *mef2ca* mRNA injected or control uninjected embryos at 22 hpf. Mef2ca 4-5-6 injected embryos showed augmented expression of *chordin* ~~*(chd)*~~, *no- tail a*~~*(ntla)*~~, *nodal related 1* ~~*(ndr1)*~~, *noggin 1* ~~*(nog1)*~~ and *goosecoid* ~~*(gsc)*~~, reduced expression of *Δnp63* and *bmp 7a* ~~show augmented expression in Mef2ca 4-5-6 injected embryos~~, whereas *bmp 2b*, *no- tail b* and *myod* expression levels are unaffected. **C~~E~~)** Densitometric analysis of the bands shown in B~~D~~, normalized to *actb2* signal. Expression levels of each gene were arbitrarily set to a value of 1 in the uninjected control embryos. Statistical analysis was performed on data obtained from three independent experiments, the means ±SE (error bars) are represented. * and ** indicate P-values of ≤0.1 and ≤0.01 respectively.

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

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

SUPPLEMENTARY FIGURES

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

Formatted: Font: Bold
Formatted: Font: Bold
Formatted: Font: Italic
Formatted: Font: Symbol, Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Bold
Formatted: Font: Not Bold, Italic
Formatted: Font: Not Bold
Formatted: Font: Not Bold, Italic
Formatted: Font: Not Bold
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic
Formatted: Font: Italic

A) Schematic of the highly similar structures of three vertebrate *MEF2C* genes among coding exons (black boxes). To simplify the comparison, we assigned the number 1 to the exon containing the first translated ATG. Introns are indicated by solid lines. MEF2C genes from the three species share three alternative exons: the α1 and α2 mutually exclusive exons, the β skipping exon, and 3' splice site selection at exon 9. **B)** Schematic of the vertebrate Mef2c gene exon numbering adopted in this paper. In the table are reported the exon numbering of the mouse and frog *MEF2C* genes adopted in the indicated references**. C)** Splicing patterns of frog, mouse and human MEF2C. The MADS box and MEF2 domain are encoded by exons 1 and 2.

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

A) α2 alternative exon, β skipping exon and γ region in *mef2cb* and *mef2ca* genes predicted with the TBLASTN algorithm. The sequences of bona fide spliced out exons, the percentage of homology with the mouse sequence and the putative splice sites are indicated. **B)** Comparison of amino acid sequences for zebrafish Mef2ca and Mef2cb splice variants. Protein sequence encoded by different exons is indicated, and alternatively spliced out regions are marked in yellow and green. TADs are colored in blue and orange. ~~**C)** Comparison of amino acid sequences for mouse (Mm), human (Hs), frog (Xl) and zebrafish (Dr) Mef2ca exon 5 and surrounding regions. Asterisks design fully conserved amino acids.~~

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

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

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

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

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

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

A) Left panel. Schematic representation of *mef2cb* 3α1 or 3α2 alternative exons. Arrows show annealing sites of isoform-specific primers used in the RT-PCR analysis they were designed to give amplification products of the same size (190 bp). Right panel. Expression analysis of *mef2cb* transcripts including the mutually exclusive 3α1 or 3α2 exon by RT-PCR. Total RNA was purified from staged embryos. To amplify an amount of exon 3α2 containing DNA similar to that containing exon 3α2, four additional PCR cycles were required. (B) Left panel. Schematic representation of *mef2ca* 3α1 and of mef2cb 3α1 or 3α2 alternative exons. Arrows show annealing sites of the primers used in the RT-PCR analysis. They give amplification products of distinct sizes. Right panel. Expression analysis of *mef2ca* and *mef2cb* transcripts including the mutually exclusive 3α1 or 3α2 exon by RT-PCR in adult tissues. Total RNA was purified from brain, liver, skeletal and cardiac muscle of adult zebrafish. The level of expression of the transcripts was evaluated by using primers that anneal to exons 2 and 4 for both *mef2c* genes, in the case of *mef2cb*, they give two amplification products of distinct sizes: 196 and 187 bp, depending on the incorporation of 3α1 or 3α2 alternative exons in the transcripts. PCR products were separated in 8% polyacrylamide gels. Length of PCR products (bp) is indicated.

Fig. S~~6~~5. Effects of Mef2ca splice variants overexpression in zebrafish embryos.

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

Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Symbol, Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Symbol, Not Italic
Formatted: Font: Not Italic
Formatted: Font: Symbol, Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: Font: Not Italic
Formatted: English (U.S.)

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

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

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

Figure 1

Click here to download high resolution image

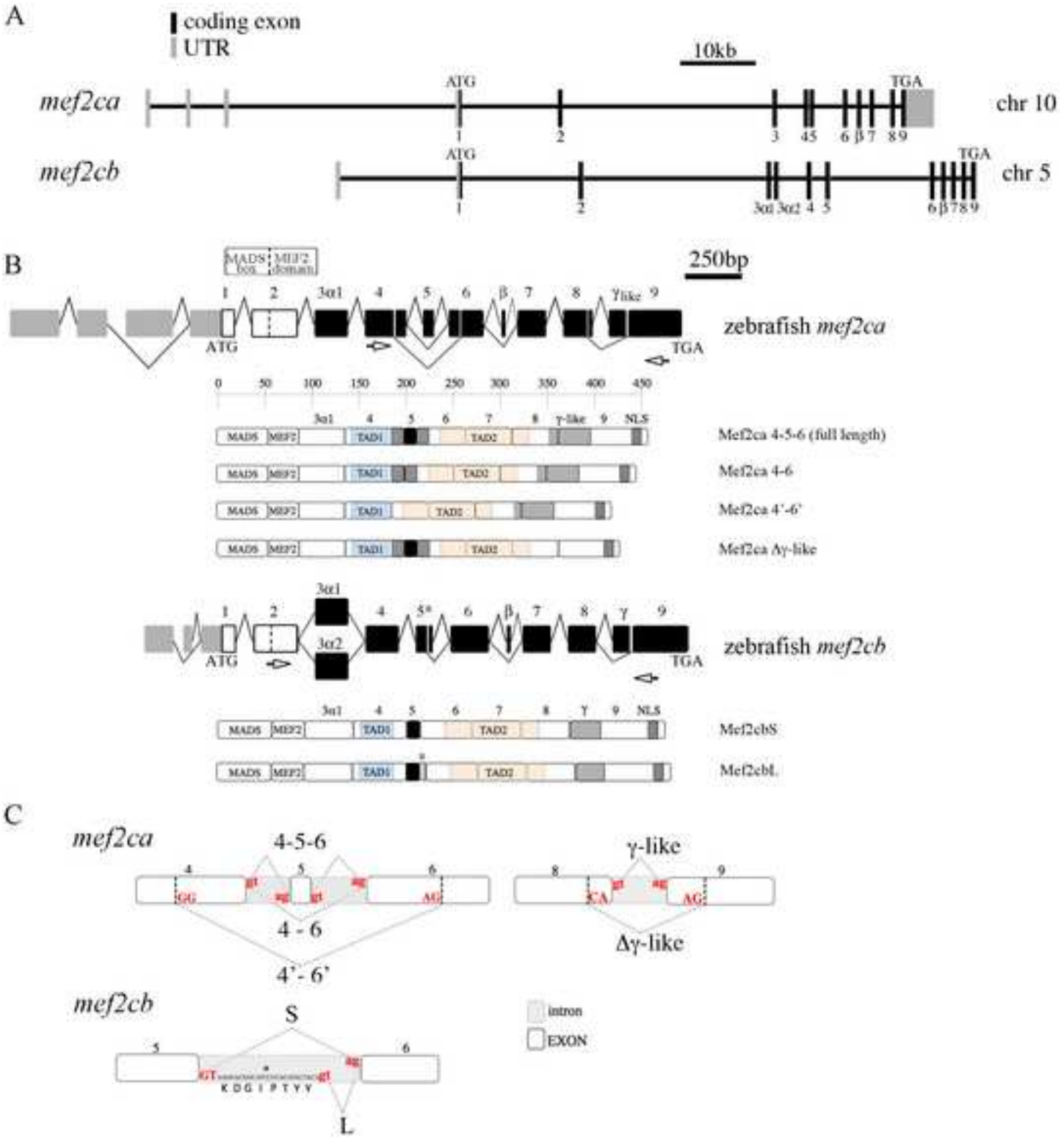


Figure 2

Click here to download high resolution image

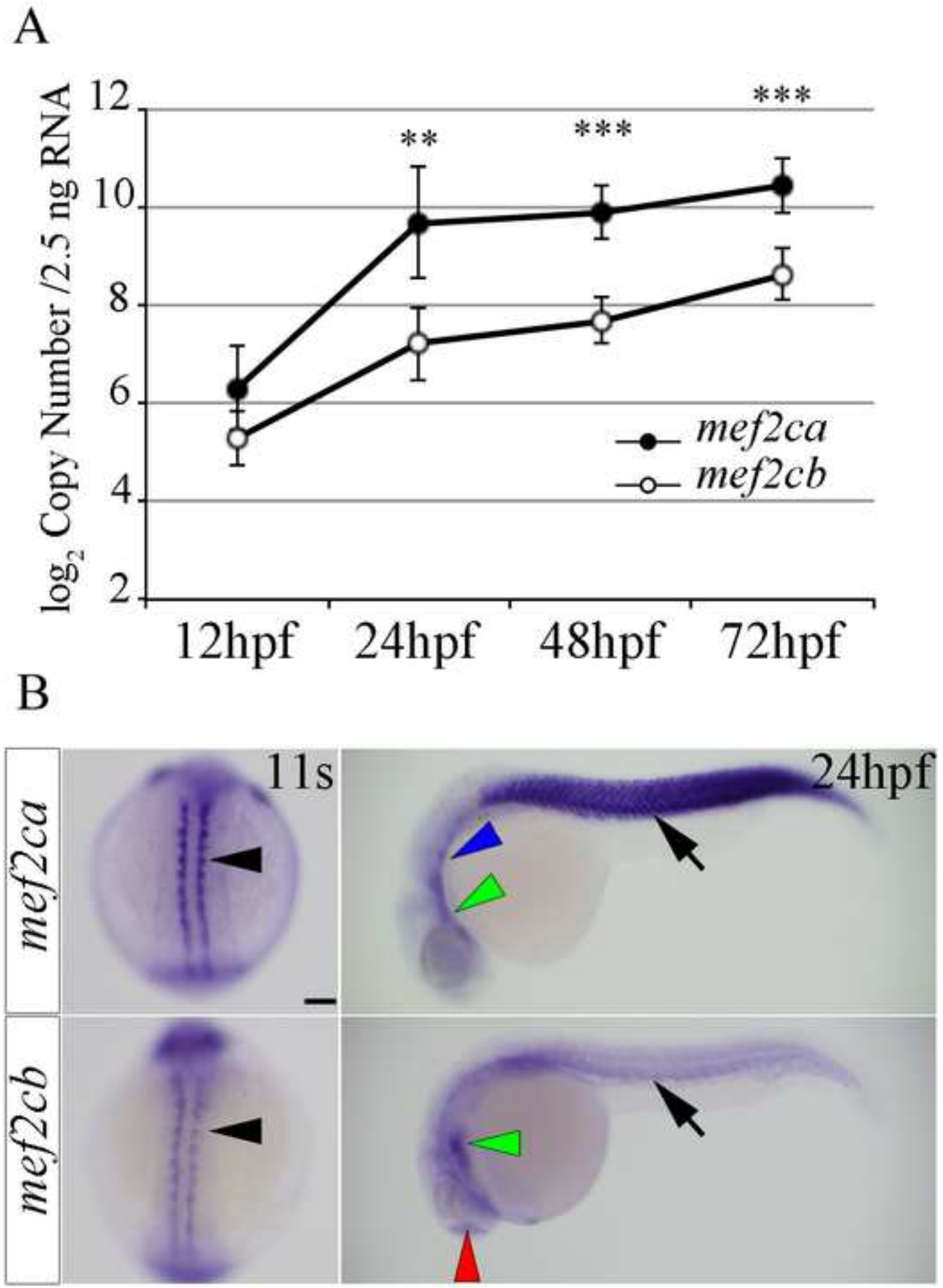


Figure 3

Click here to download high resolution image

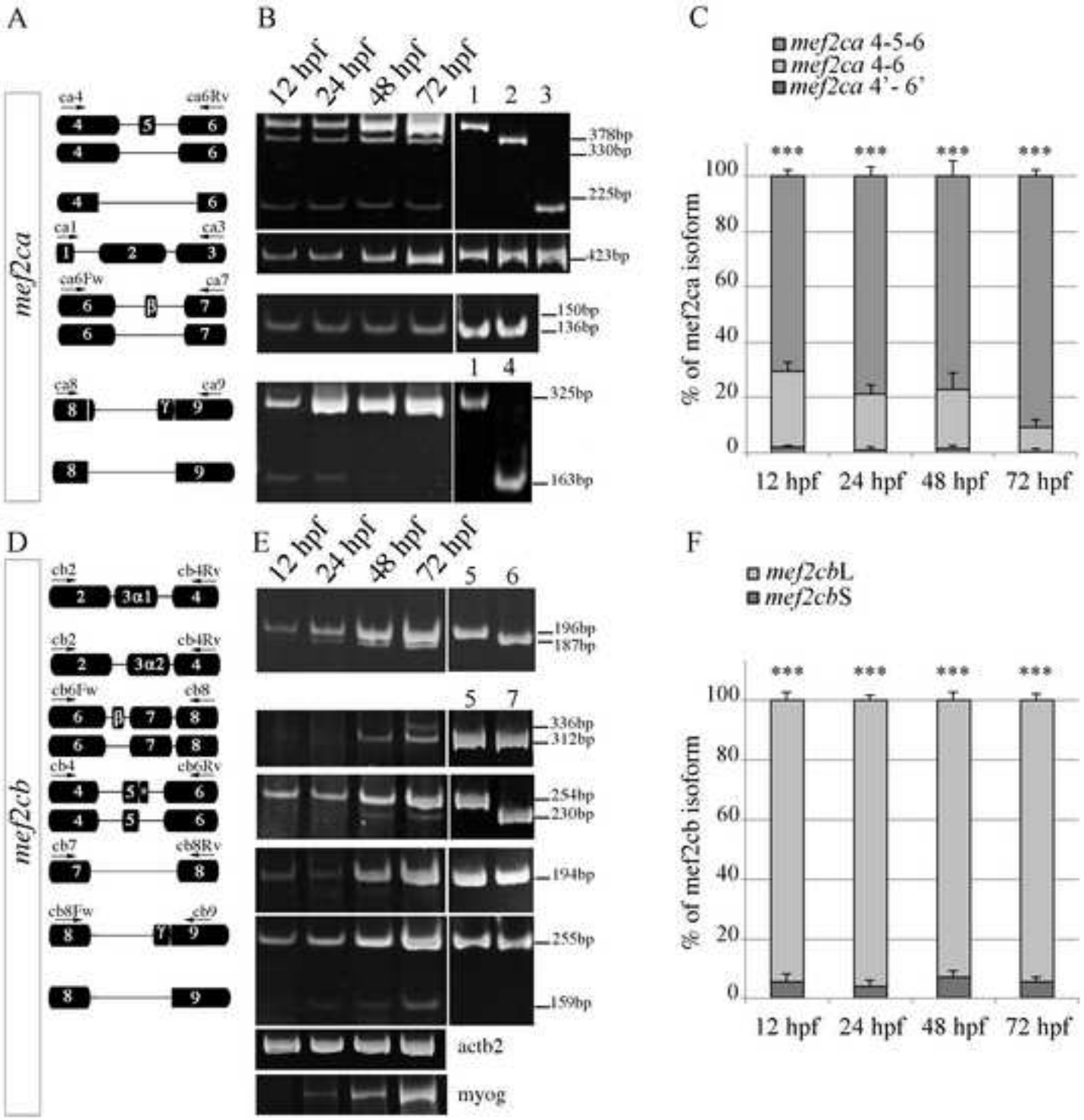


Figure 4

Click here to download high resolution image

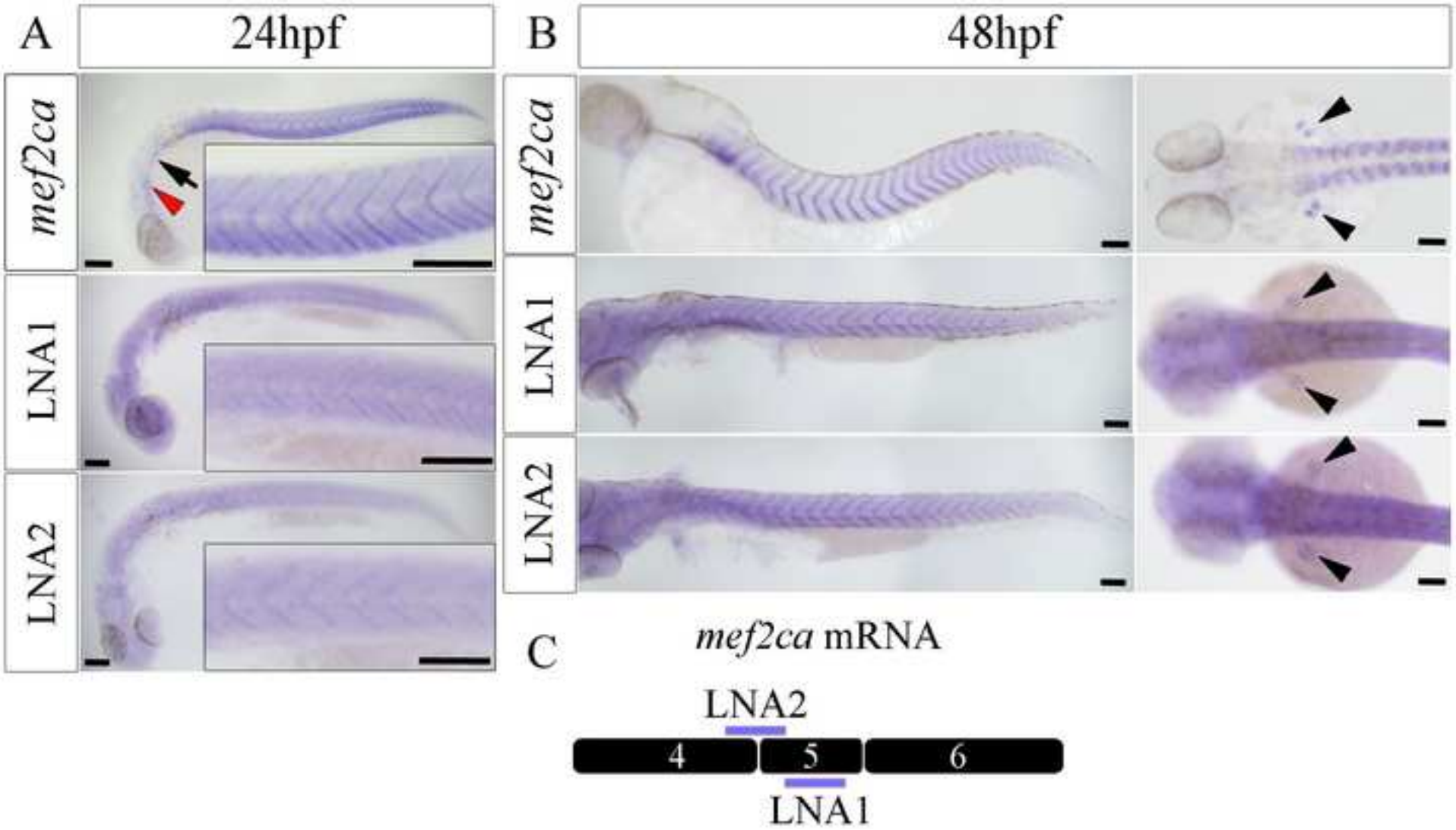
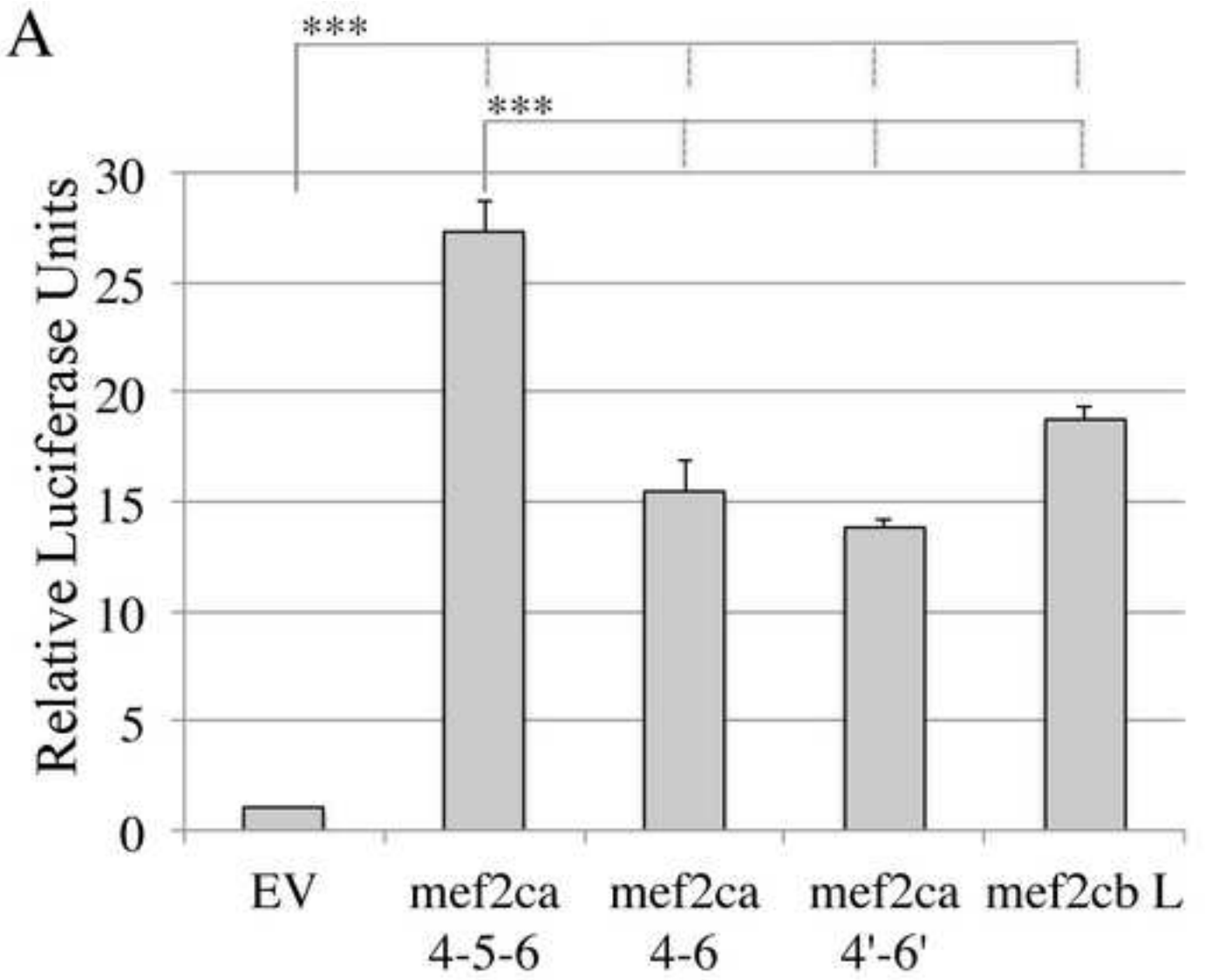


Figure 5

Click here to download high resolution image



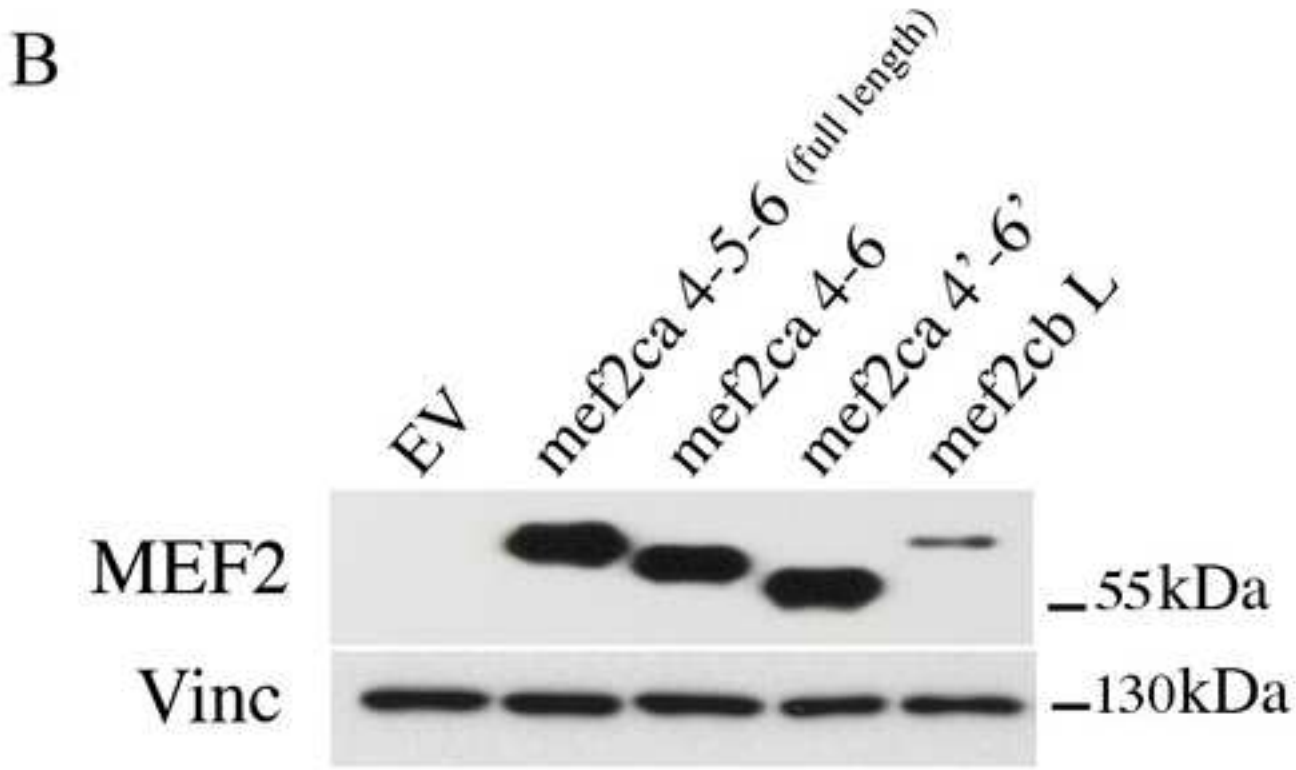


Figure 6

Click here to download high resolution image

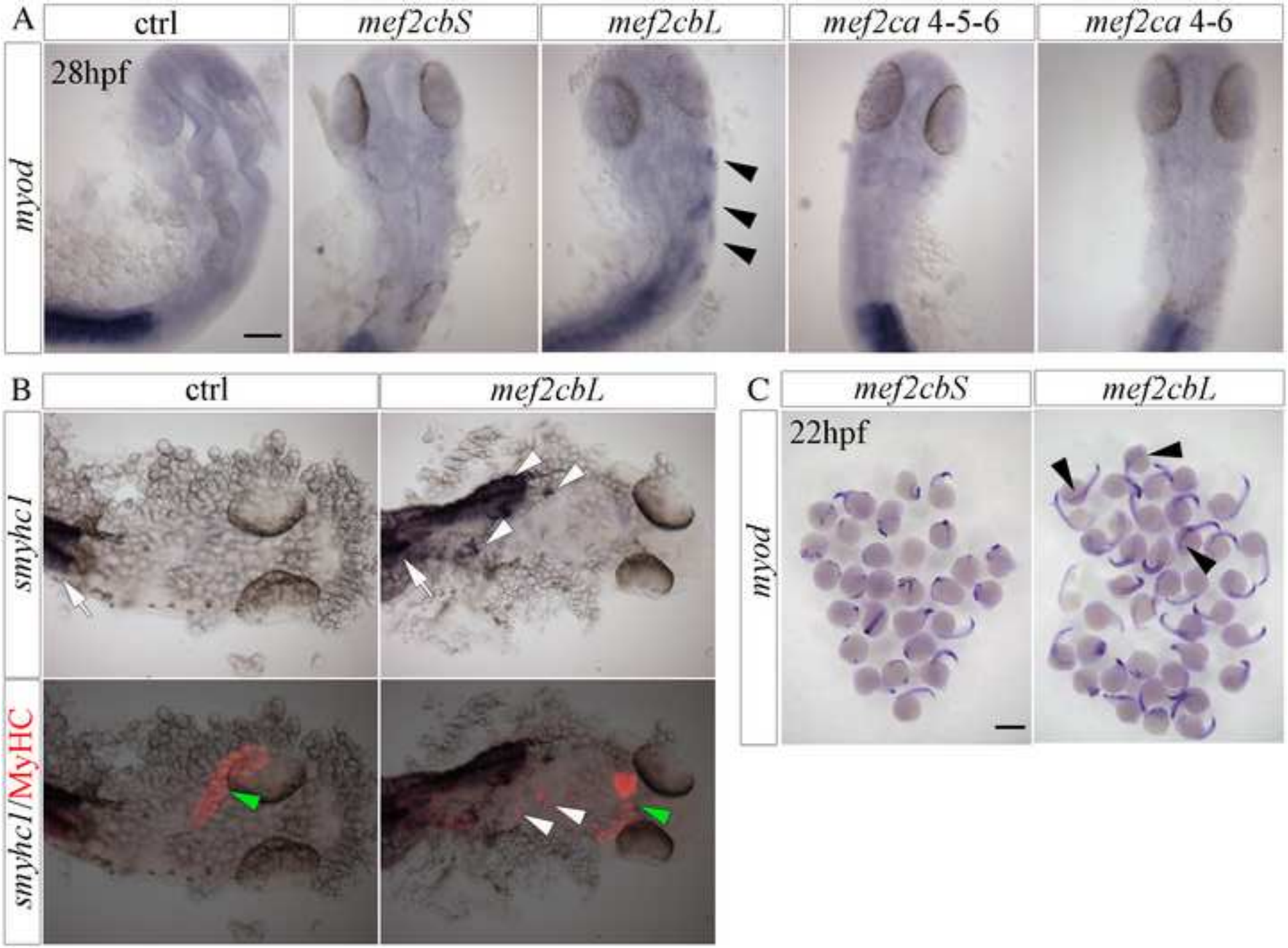


Figure 7

Click here to download high resolution image

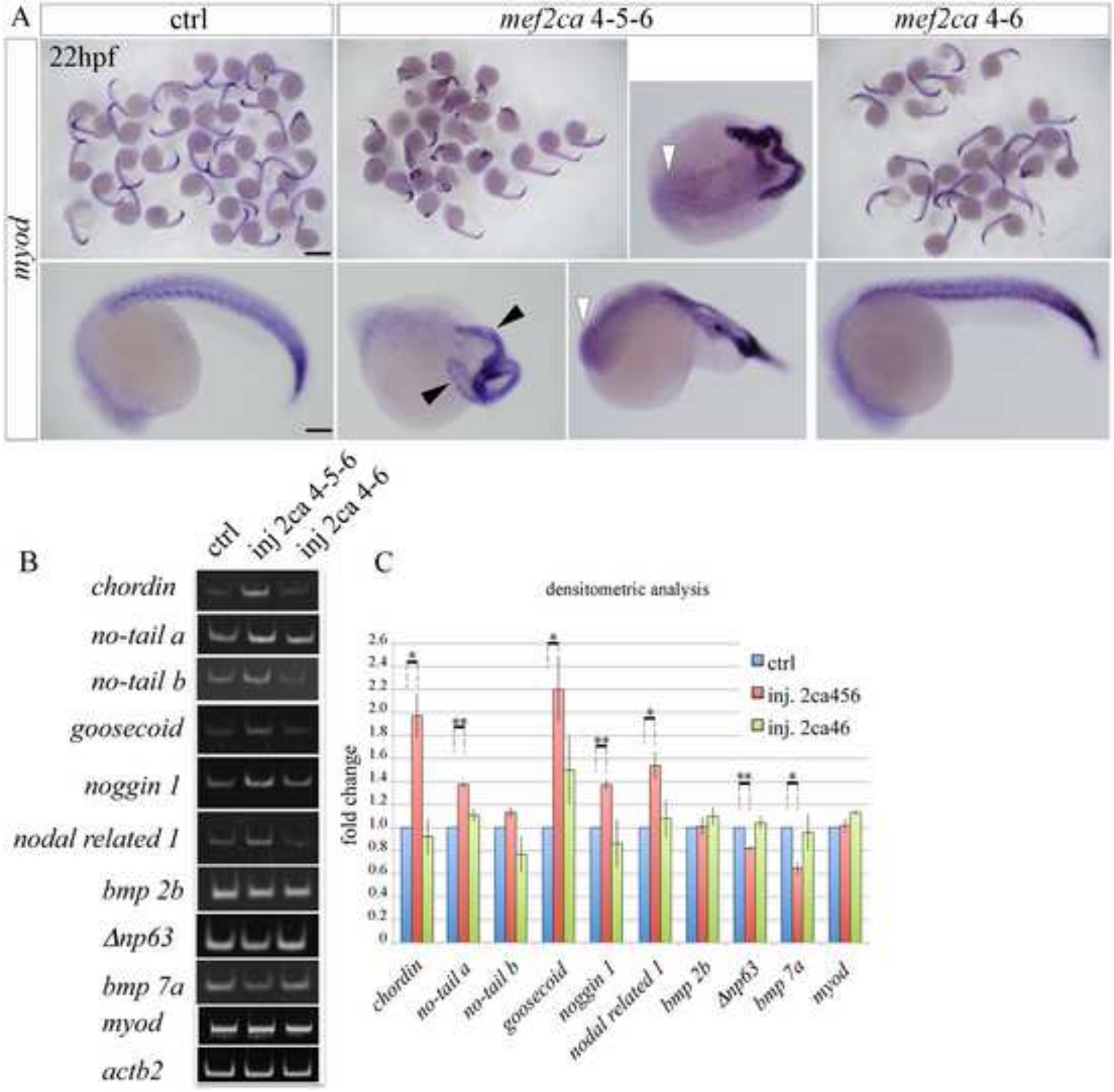


Figure 8

Click here to download high resolution image

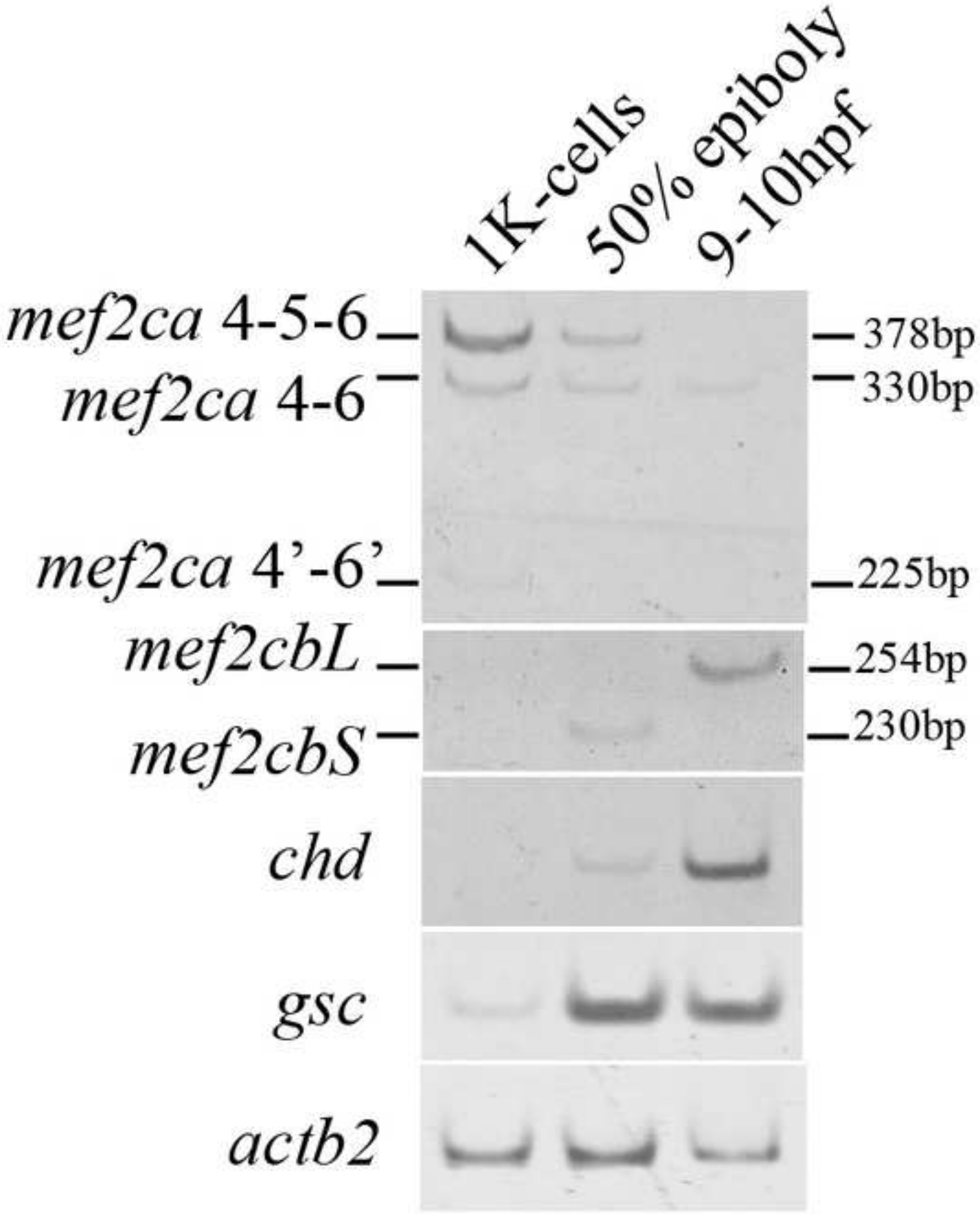


Figure S1

Click here to download high resolution image

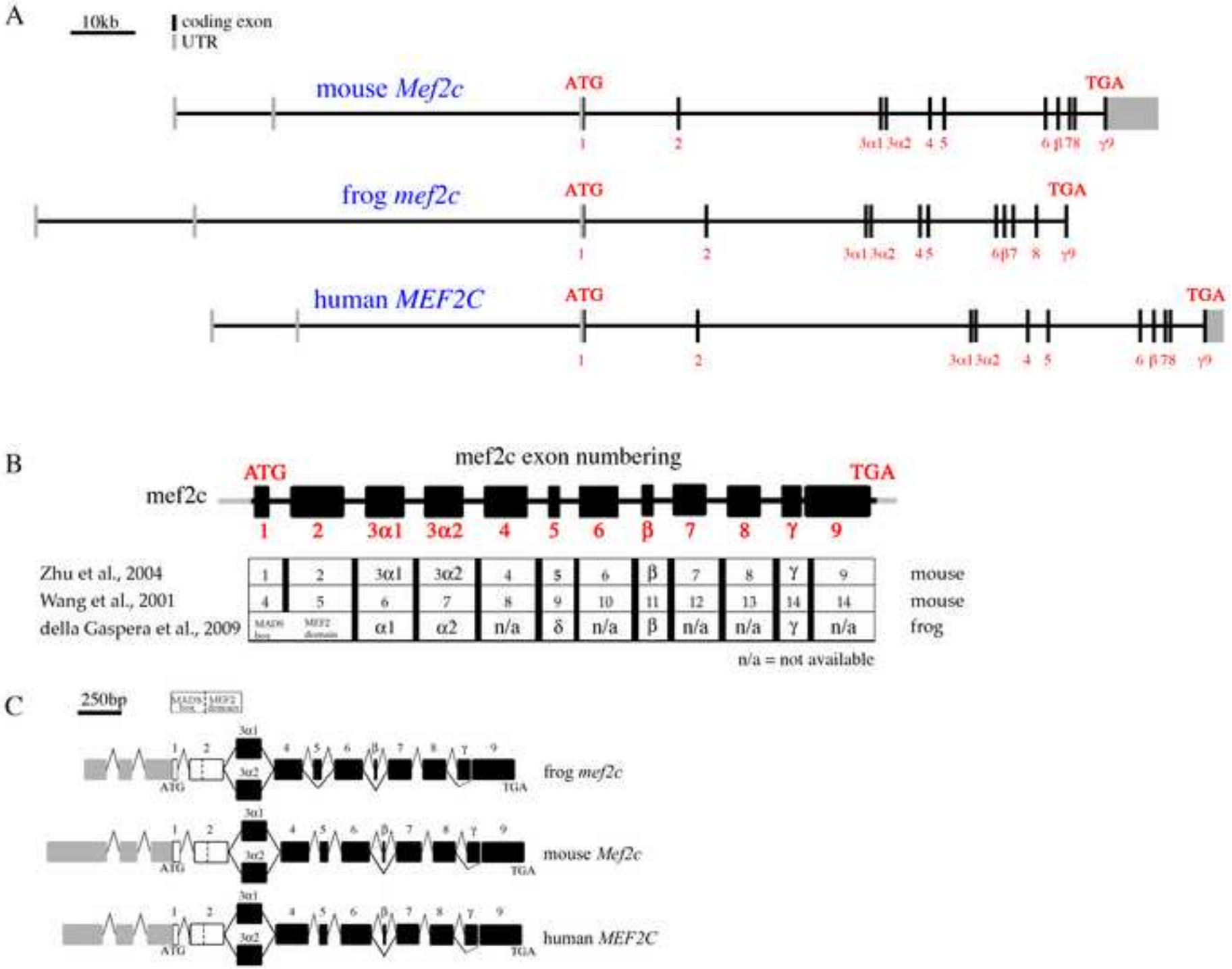


Figure S2

Click here to download high resolution image

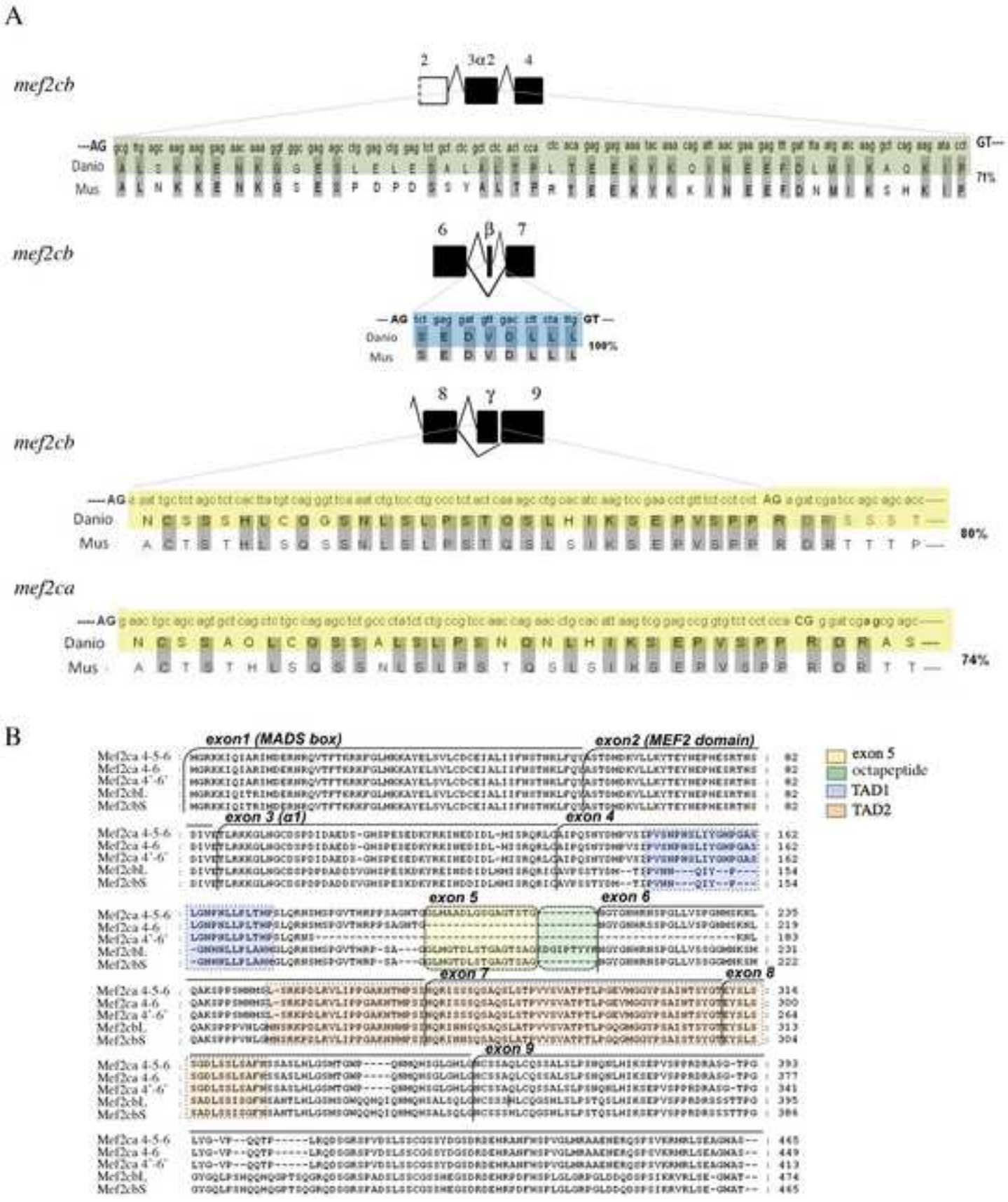


Figure S3

Click here to download high resolution image

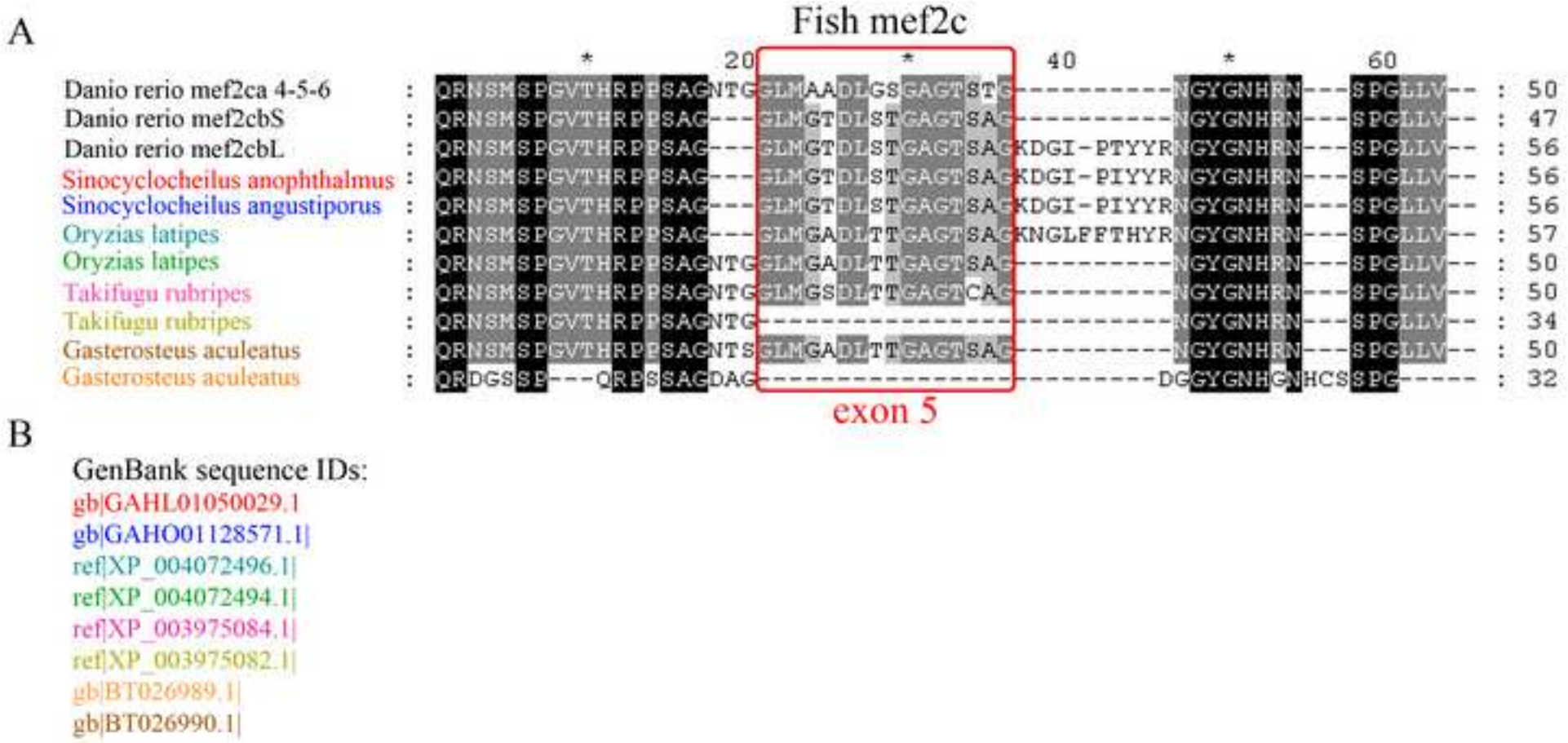


Figure S4

Click here to download high resolution image

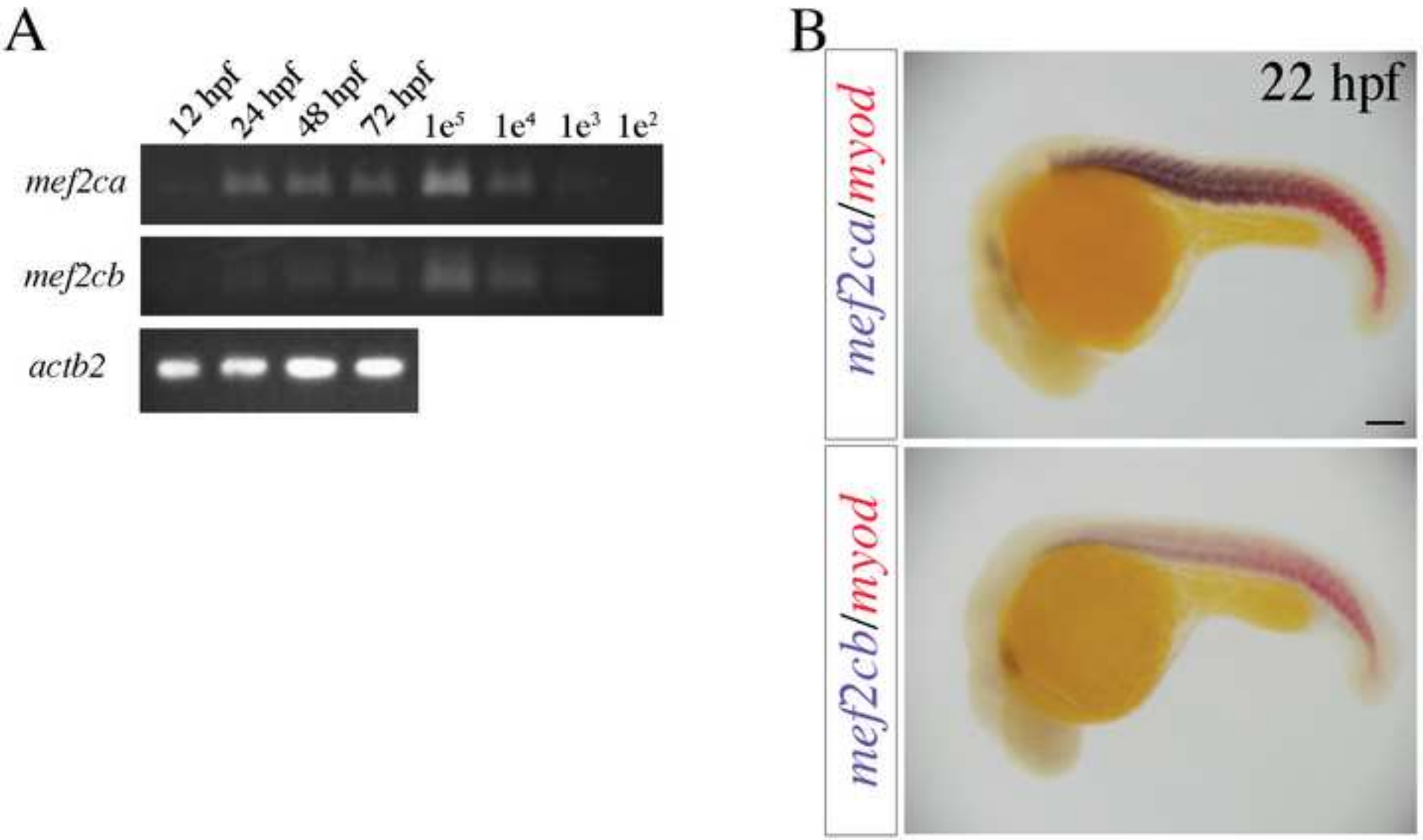


Figure S5

Click here to download high resolution image

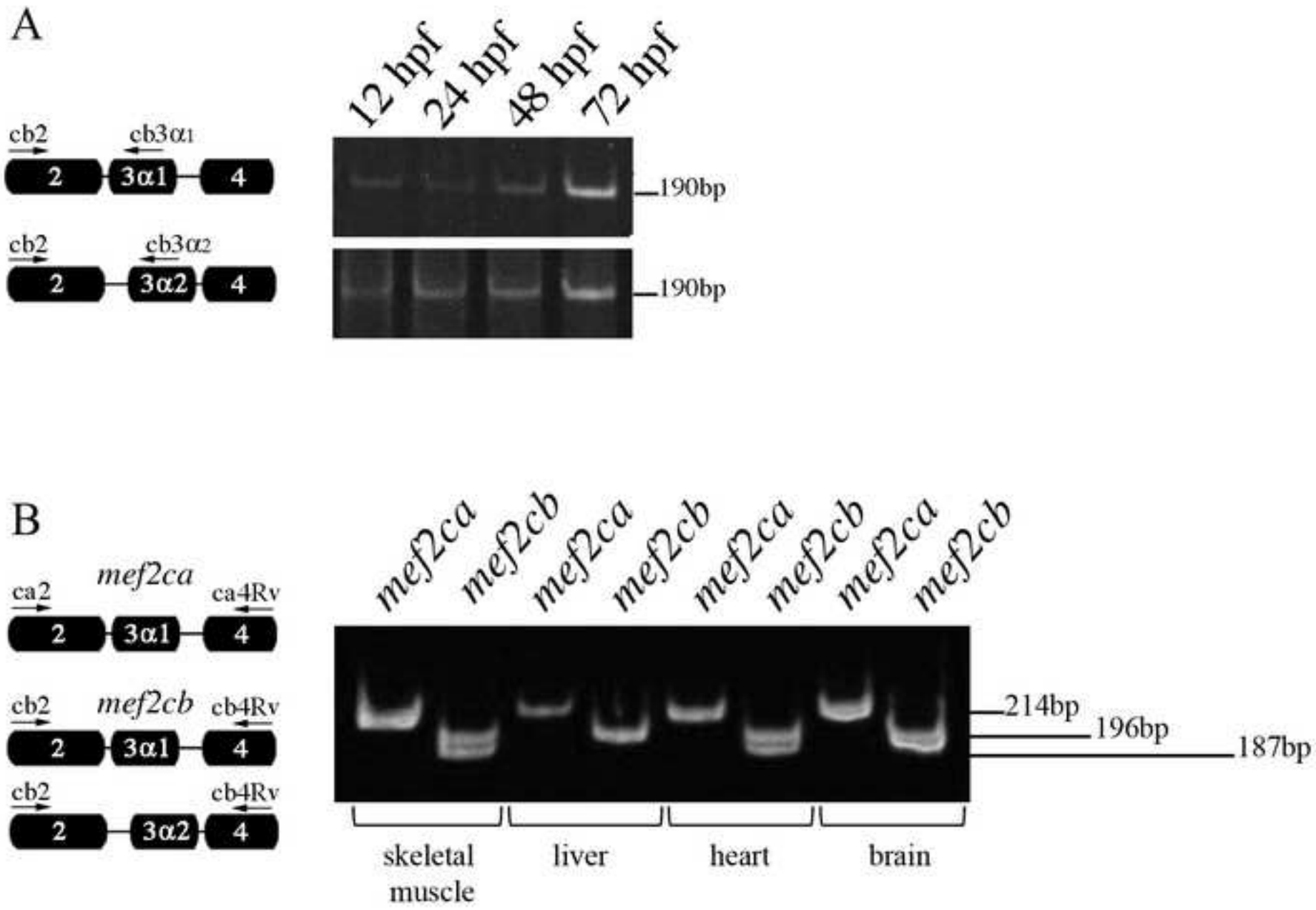


Figure S6

Click here to download high resolution image

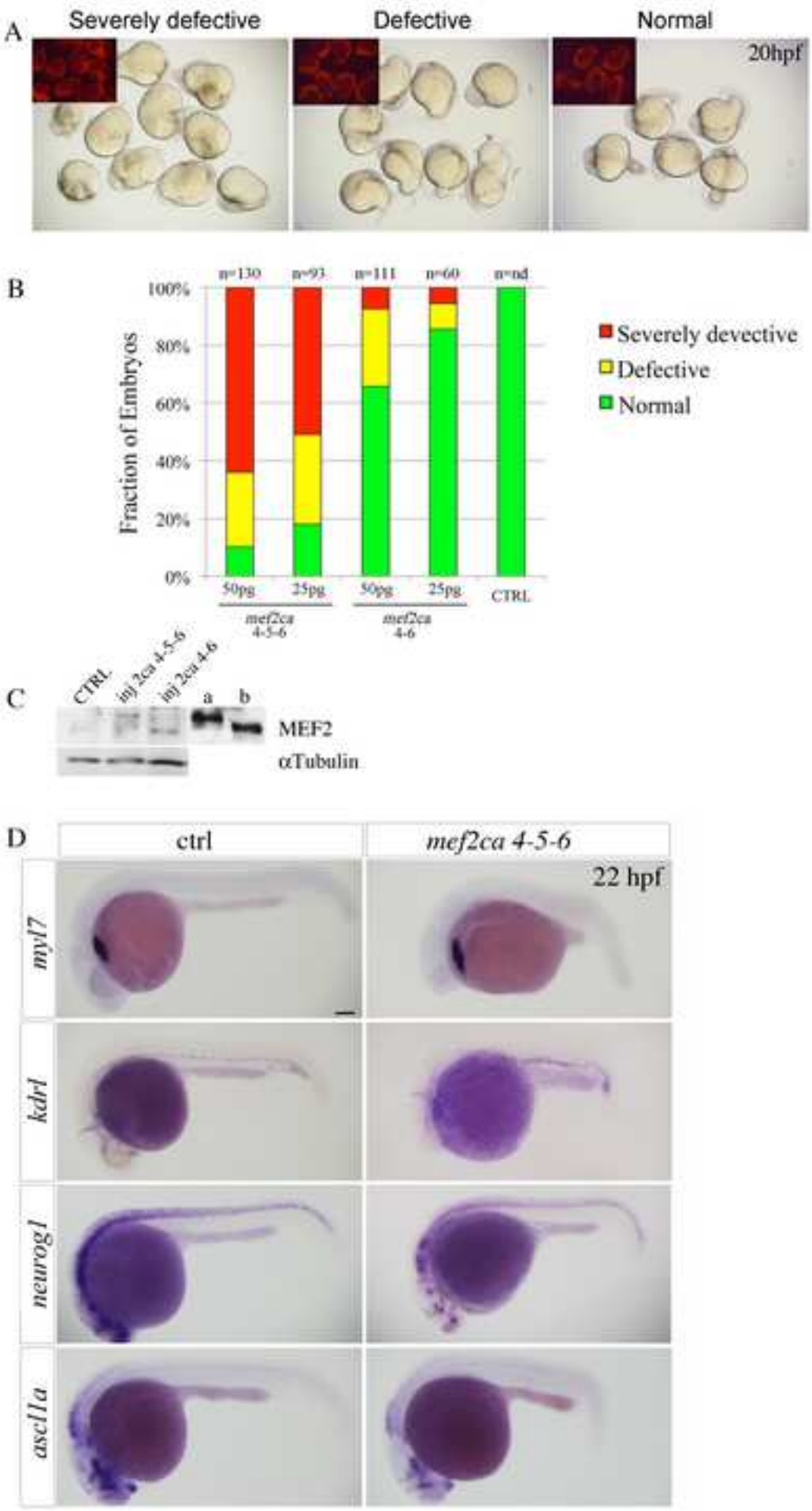


Figure S7

Click here to download high resolution image

A

Oligo Name	Sequence 5 '- 3'
mef2ca FW	tcggactaattcagacatcgtg
mef2ca REV	gtagatcaggctgttggggtt
mef2cb FW	gaccaactcggacatagtggag
mef2cb REV	tgcctgggtagatctggttatt
β actin-2 FW	gcagaaggagatcacatccctggc
β actin-2 REV	cattgccgtcaccttcaccgttc
myog FW	gcttcgagaccaaccccctact
myog REV	tcactagaggacgacacccca
myod FW	tcgcaaagccgccaccatga
myod REV	cacgggctctcttcgtgccc

B