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Zebrafish Mef2ca and Mef2cb are essential for both first and second heart field cardiomyocyte differentiation

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Summary

Mef2 transcription factors have been strongly linked with early heart development. *D-mef2* is required for heart formation in Drosophila, but whether Mef2 is essential for vertebrate cardiomyocyte (CM) differentiation is unclear. In mice, although Mef2c is expressed in all CMs, targeted deletion of *Mef2c* causes lethal loss of second heart field (SHF) derivatives and failure of cardiac looping, but first heart field CMs can differentiate. Here we examine Mef2 function in early heart development in zebrafish. Two Mef2c genes exist in zebrafish, *mef2ca* and *mef2cb*. Both are expressed similarly in the bilateral heart fields but *mef2cb* is strongly expressed in the heart poles at the primitive heart tube stage. By using fish mutants for *mef2ca* and *mef2cb* and antisense morpholinos to knock down either or both Mef2cs, we show that Mef2ca and Mef2cb have essential but redundant roles in myocardial differentiation. Loss of both Mef2ca and Mef2cb function does not interfere with early cardiogenic markers such as *nkx2.5, gata4* and *hand2* but results in a dramatic loss of expression of sarcomeric genes and myocardial markers such as *bmp4, nppa, smyd1b* and late *nkx2.5* mRNA. Rare residual CMs observed in *mef2ca;mef2cb* double mutants are ablated by a morpholino capable of knocking down other Mef2s. Mef2cb over-expression activates *bmp4* within the cardiogenic region, but no ectopic CMs are formed. Surprisingly, anterior mesoderm and other tissues become skeletal muscle. *Mef2ca* single mutants have delayed heart development, but form an apparently normal heart. *Mef2cb* single mutants have a functional heart and are viable adults. Our results show that the key role of Mef2c in myocardial differentiation is conserved throughout the vertebrate heart.

Keywords

Second heart field; mef2c; mef2ca; mef2cb; mef2a; Heart; Hand2; Myl7; bulbus arteriosus; outflow tract; cardiomyocyte; differentiation

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Competing interests

Neither author has any competing financial interest.

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Introduction

Congenital heart defects occur in almost 1% of human births, which highlights the complexity of building the heart (Bruneau, 2008). In recent years, it has become clear that mammalian and avian hearts are built from two pools of progenitor cells: first heart field (FHF) cells generate the early cardiac tube and contribute to the left ventricle, atrioventricular canal (AVC) and atria, whereas the second heart field (SHF), also referred to as the anterior heart field, contributes to the outflow tract (OFT), right ventricle, and inflow region (Buckingham et al., 2005; Rochais et al., 2009). The SHF is the main source of myocardial progenitors added at a later stage to the arterial and venous poles of the heart (Cai et al., 2003; Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Recent studies have suggested that, like the amniote heart, zebrafish heart is built from FHF and SHF progenitor cell pools. Whereas the FHF is the source of cells contributing to the primitive heart tube, the putative SHF provides cells that are added to the two poles of the heart tube, and build the structures at the inflow and outflow tract (de Pater et al., 2009; Grimes et al., 2010; Grimes et al., 2006; Hami et al., 2011; Lazic and Scott, 2011; Zhou et al., 2011). This provides a new opportunity to increase understanding of the molecular regulation of cardiomyogenesis.

Myocyte enhancer factor 2 (Mef2) transcription factors are key cardiomyogenic regulators, but their role in vertebrate heart development remains uncertain. In *Drosophila*, the single Mef2, *D-mef2* is essential for the formation of all muscle types, including the heart. In *D-mef2* mutants, cardiomyocyte (CM) precursors are properly specified and positioned, but fail to differentiate (Lilly et al., 1995; Ranganayakulu et al., 1995). In vertebrates, the Mef2 family of transcription factors has four members Mef2A, B, C and D (Breitbart et al., 1993; McDermott et al., 1993; Pollock and Treisman, 1991). Cell culture analyses implicate Mef2 activity, and particularly Mef2c, in CM differentiation (Ieda et al., 2010; Karamboulas et al., 2006). Although *Mef2c* is expressed during development of all murine CMs, FHF CMs differentiate in *Mef2c* mutants, which die at E9.5 from gross heart defects. These mutants have only a single ventricular chamber, abnormalities in the inflow and outflow tracts and defective cardiac looping reminiscent of SHF defects (Bi et al., 1999; Edmondson et al., 1994; Lin et al., 1998; Lin et al., 1997b; Verzi et al., 2005; Vong et al., 2006). Thus, in mice, there is a clear discrepancy between the strong SHF phenotype of Mef2c loss of function and the fact that *Mef2c* is expressed throughout the myocardium. It has been suggested that other Mef2 proteins may help drive FHF CM differentiation (Lin et al., 1997b; Vong et al., 2006). Other *Mef2* genes are expressed in murine CMs, but are not, individually, essential for their formation. Mice mutant in *Mef2d* appear normal, whereas mutants lacking *Mef2a* form a myocardium but exhibit perinatal lethality from late cardiovascular defects (Arnold et al., 2007; Naya et al., 2002). Mef2b is expressed in early heart development (Molkentin et al., 1996b), but mice null for *Mef2b* have not been described. Thus, studies in mice have so far failed to determine whether Mef2 activity is essential for all CM differentiation.

Zebrafish have the advantages of surviving for several days without a heart and having two Mef2c genes, *mef2ca* and *mef2cb* (Hinits and Hughes, 2007; Miller et al., 2007; Ticho et al., 1996). *Mef2ca* is expressed in zebrafish heart (Ticho et al., 1996). *Mef2cb* is expressed in both FHF and SHF myocardium and, like loss of Mef2c function in mouse, *mef2cb* knockdown with morpholinos (MOs) has been shown to eliminate a subset of SHF CMs at the arterial pole (Lazic and Scott, 2011). A combinatorial loss of function analysis is therefore needed to establish the role Mef2 in the early steps of CM differentiation. Here we show that Mef2c activity controls an essential step in CM differentiation throughout the heart, in both FHF and SHF. By using loss- and gain-of-function models, we show that the two zebrafish *Mef2c* paralogues, Mef2ca and Mef2cb, control the expression of myocardial

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sarcomeric genes and other markers of CM maturation, such as *nppa, smyd1b* and *bmp4*, reminiscent of the function of the single Drosophila Mef2. Without Mef2ca and Mef2cb, the heart fails to form, and CMs are specified but developmentally arrested. Embryos lacking either Mef2ca or Mef2cb alone develop a normal heart. Together, our data reveal the essential role of Mef2 factors in the differentiation of FHF and SHF cardiomyocytes.

Materials and methods

Zebrafish lines and maintenance

Mutant and transgenic lines: *mef2ca*^{tn213} (Piotrowski et al., 1996), *mef2ca*^{b1086}(Miller et al., 2007), *Tg(fli1a:EGFP)y1*(Lawson and Weinstein, 2002), *Tg(myl7:EGFP)twu26* (Huang et al., 2003), *Tg(−5.1myl7:nDsRed2)f2* (Mably et al., 2003) and *mef2ca*^{b1086}*;Tg(myl7:EGFP)twu26* were maintained on King's wild type background. *Mef2cb*^{fh288} mutant allele was identified by TILLING (Draper et al., 2004) in the *AB background (http://labs.fhcrc.org/moens/Tilling_Mutants/index.html), and was further cleaned of linked contaminating mutations. Further crossing created the double mutant lines *mef2ca*^{b1086}*;mef2cb*^{fh288} and *mef2ca*^{b1086}*;mef2cb*^{fh288}*;Tg(myl7:EGFP)twu26*. Staging and husbandry were as described (Westerfield, 1995). Genotyping was performed by sequencing of PCR products amplified from fin clip or embryo genomic DNA using primers 5'-AAAGCAGGCAAATAGAAAAACACT-3' and 5'-AAAAGGCCAAACTCAACAGGAACT-3' for *b1086 allele*, 5'-GGAAGAAGCGCTGTATTTAGGAC-3' and 5'-ATATCTGTGCTGGCGTACTGG-3' for *fh288 allele* and other methods in http://labs.fhcrc.org/moens/Tilling_Mutants/index.html).

Cloning of over-expression and other plasmids

pHS-mef2cb-IRES-GFP was made by cloning the full-length coding sequence of *mef2cb* (with four introduced mismatches) generated by PCR using the primers 5'-CGCTCTAGAATGGGACGAAAGAAAATTCAGATCACACGG-3' and 5'-GTCGACTCATGTGGCCCACCCTTCCGAGA-3' into the XbaI and SalI sites of hsp70-4-MCS-IRES-mGFP6 plasmid (Hinits and Hughes, 2007). DNA sequence was verified. *Mef2cb* mRNA was made with mMESSAGE mMACHINE kit from a linear DNA fragment containing the full CDS of *mef2cb* flanked by β-globin UTRs, which was made by a two-step PCR. Firstly, two PCR products were amplified by using the following primers: T3 and 5'-AATTTTCTTTCGTCCCATGAGCTCGATATCTCTCT-3'; T7U and 5'-TCTCGGAAGGGTGGGCCACATGAGTCGACGGATCCAGATCTGG-3' on pβUT3 template (Hinits et al., 2009). Secondly, by using T3 and T7U primers on a template mix of pHS-mef2cb-IRES- GFP plus the two PCR products generated at the first step, a linear template with T7 and T3U ends was generated and confirmed by sequencing. pCMV:mef2cb-GFP was constructed by amplifying a fragment of the *mef2cb* gene 5'UTR including 166 bp immediately upstream and 25 bp downstream from the start codon with primers: 5'-AGATCTAAGCTTGTACAGCTACTGGAATCTTTGAAC-3' and 5'-AGATCTGAATTCTGATCTGAATCTTTTTTCTCCCCAT-3' and cloning into EcoRI/HindIII digested pEGFP-N1 (Clontech) in frame with EGFP and was sequence verified.

mRNA in situ hybridisation

In situ mRNA hybridization was performed as described previously (Hinits et al., 2009). To avoid cross-reactivity between *mef2ca* and *mef2cb* probes, for each gene we used two different probes, one containing only 3'UTR sequences and the other having part of the coding sequence. The two *mef2ca* probes gave identical patterns of expression (data not shown). We therefore used the longer, original probe used by Ticho and colleagues (Ticho et al., 1996). The *mef2cb* probe containing CDS as well as 3'UTR (IMAGE: 6519749) hybridized strongly to all of the domains detected by the 3'UTR only probe and additionally

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marked expression in the cephalic vascular system and was therefore preferred. For *mef2aa*, a plasmid containing the full CDS, MGC:55208, was used for PCR with the following primers (reverse primer contains T3 site) 5’-TGTACGGAAGTGTTACTTCTGCTC-3’ and 5’-GGATCCATTAACCCTCACTAAAGGGAAGGCCGCGACCTGCAGCTC-3’ (Thisse and Thisse, 2004). Other probes used were: *myl7, vmhc* (Yelon et al., 1999), *bmp4* (Walsh and Stainier, 2001), *hand2 (eu880*, Thisse et al., 2005), *nkx2.5* (Lee et al., 1996), *nppa* (Berdougo et al., 2003), *tbx20* (Ahn et al., 2000), *gata4, gata5, gata6* (Reiter et al., 1999) *smbpc, tnnc*, (Xu et al., 2000), *egr2b* (Oxtoby and Jowett, 1993), *ntl* (Schulte-Merker et al., 1992), *kdrl* (Thompson et al., 1998) and *cdh5* (Larson et al., 2004). Embryos were photographed as wholemounts on Olympus DP70 or dissected and flatmounted in glycerol and photographed on a Zeiss Axiophot with Axiocam using Openlab software.

Embryo staining—Anti-Mef2 (Santa Cruz) and Anti-Mef2c (McDermott et al., 1993) were used as described (Hinits and Hughes, 2007). Anti-Mef2 can detect Mef2cb protein, as high-level nuclear expression was detected in a mosaic fashion after injecting BAC CH211-202E12 DNA, containing the *mef2cb* locus into embryos (Fig. S1A-E). Anti-Mef2ca/cb (1:200, Anaspec) reacted similar to anti-Mef2c and did not cross-react with other Mef2s (Fig. S2A-C). It also detects Mef2cb, as injecting either hs-mef2cb-IRES-GFP plasmid DNA (followed by a heat shock) or *mef2cb* mRNA at 1-2 cell stage, resulted in many cells co-expressing strong nuclear Mef2c and GFP (Fig. S1F,G). Other primary antibodies used were against sarcomeric myosin heavy chain (MyHC; A4.1025 (Blagden et al., 1997) or MF20 (DSHB, Iowa)), slow MyHC (F59, (Devoto et al., 1996)), DM-Grasp (zn5, ZIRC), Elastin (Miao et al., 2007), GFP (rabbit, Torrey Pines or chicken, Abcam ab13970) or RFP (rabbit PM0005, Medical and Biological Laboratories). Secondary antibodies were either HRP-conjugated (Vector) or Alexa dye-conjugated (Invitrogen). Embryos for immunohistochemistry were fixed in 4% PFA for 30 min to 2 h (except embryos stained with DAF2DA were fixed in 2% PFA for 30 min) and stained as described (Hinits and Hughes, 2007). Embryos were mounted in Citifluor (Agar) or low melting point agarose. Confocal images collected on a Zeiss LSM510 and some processed using Volocity software. DAF-2 DA (Santa Cruz) was used as described (Grimes et al., 2006).

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Embryo manipulation

All morpholinos, plasmid and BAC DNA were injected into 1-2 cell stage embryos. *mef2d/c* MO and *mef2ca* ATG MO were described (Hinits and Hughes, 2007). *Mef2cb* ATG MO (5’TGTCCCCGTCTTTTCGTCTCTCTCT3’, Gene-Tools, 0.25 ng) and *mef2cb* E1I1 MO (5’TTCCGGTCAGCGTGACTCACCTGTC3’, Gene-Tools, 1 ng) were used for Mef2cb knockdown. To evaluate the effectiveness of *mef2cb* ATG MO, w*e* co-injected pCMV:mef2cb-GFP with *mef2cb* ATG MO or other control MOs (Fig. S3A,B). M*ef2cb* E1I1 MO was checked by RT-PCR using primers 5’-CACACGGATTATGGATGAACG-3’ and 5’-TCCTTTGACTCTGGGCTGTGG-3’ matching the first and the third exons of *mef2cb*, which produces the 321 bp normal splicing product and an additional PCR product of 403 bp. Sequencing indicated the use of a hidden splice donor site inside intron 1, and so creating an aberrant transcript with a premature stop codon (Fig. S3C). *Hand2* MO 5’-CCTCCAACTAAACTCATGGCGACAG-3’) was used as described (Maves et al., 2009).

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Results

Mef2ca and *mef2cb* are the only Mef2 orthologues expressed in the early phases of heart development in zebrafish

Developing zebrafish hearts were screened for expression of Mef2 family transcription factors. Four Mef2 orthologues are known in zebrafish: *mef2aa* (previously named *mef2a*), *mef2ca* (previously named *mef2c*), *mef2cb* and *mef2d* (Hinits and Hughes, 2007; Lazic and

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Scott; Miller et al., 2007; Ticho et al., 1996). Two more, *mef2ab* and *mef2b*, were identified bioinformatically as orthologues of mammalian *Mef2a* and *Mef2b*, respectively. Only *mef2ca, mef2cb* and *mef2aa* genes were detectably expressed in the developing zebrafish heart (Fig. 1 and data not shown). *Mef2ca* mRNA accumulated in cells of the anterior lateral plate mesoderm (ALPM) from around 6ss (6 somite stage, data not shown), and was strong at 10-13ss. Expression persisted as the heart fields fuse to form the primitive heart tube (Fig. 1A,D) (Hinits and Hughes, 2007; Ticho et al., 1996). At 24 hpf, *mef2ca* was still detected weakly throughout the heart but was downregulated thereafter (Fig. 1H and data not shown). *Mef2cb* mRNA accumulated weakly in differentiated somitic adaxial cells in the bilateral heart fields as early as 7-8ss (Fig. 1B,E and data not shown, see also (Lazic and Scott, 2011)). During the heart cone stage *mef2cb* mRNA and Mef2c protein had accumulated in the differentiating CMs (Fig. 1F,G). Around 24 hpf, *mef2cb* mRNA pattern became distinct from that of *mef2ca* mRNA. *Mef2cb* was detected in the arterial and venous poles of the heart, in addition to the overall weak signal in the rest of the heart tube (Fig. 1I). *Mef2cb* mRNA was present without detectable *mef2ca* mRNA in the telencephalon and vascular system (Fig. S4A). Conversely, *mef2cb* mRNA was absent in the branchial arches that express *mef2ca* highly (Miller et al., 2007; Ticho et al., 1996). *Mef2aa* mRNA was not apparent before FHF CMs differentiate, but was detected in the heart at later stages than *mef2ca* and *mef2cb* and remained until at least 48 hpf (Fig. 1C and data not shown). Thus, the main Mef2 genes expressed during early cardiac development are *mef2ca* and *mef2cb*.

Mef2ca and *mef2cb* are required for myocardial differentiation

To test the role of Mef2c during early cardiac development, we analysed a double *mef2ca*^{b1086};*mef2cb*^{fh288} mutant made by crossing double heterozygotes for *mef2ca*$^{+/b1086}$ (*hoover*) (Hinits and Hughes, 2007; Miller et al., 2007) and a novel *mef2cb*^{fh288} allele with an A to T single point mutation changing Arg at residue 24 of the Mef2cb protein to a stop codon early in the MADS domain, thereby generating a predicted null mutation (http://labs.fhcrc.org/moens/Tilling_Mutants/index.html; (Molkentin et al., 1996a; Yu et al., 1992)). The *mef2cb*^{fh288} mutation was cleaned of linked contaminating mutations. In wild type embryos, differentiation of myocardial precursors begins around 14ss in ALPM, and is marked by accumulation of mRNAs encoding sarcomeric protein genes such as *myl7* and *vmhc* (Fig. 2A and (Yelon et al., 1999)). Close to 1/16th of embryos from a *mef2ca*$^{+/b1086}$;*mef2cb*$^{+/fh288}$ in-cross entirely lacked these markers at 15ss (Fig. 2F, quantification of all experiments is presented in Table S1). We confirmed this result in a *mef2ca* single mutant injected with *mef2cb* MO (either of two *mef2ca* null mutant alleles injected with either of two *mef2cb* MOs lacked markers in one quarter of embryos), the *mef2cb* null mutant injected with *mef2ca* MO, or dual *mef2ca* and *mef2cb* MOs (Figs. 2B, S5A,B). Hereafter, we refer to embryos lacking Mef2c activity as ‘dual loss of function’ irrespective of the combination of mutant allele and/or MOs employed; all lack the vast majority of differentiated CMs. We also injected wild type embryos with a single *mef2d/c* ATG MO which we previously showed ablates several Mef2 proteins due to the high degree of conservation between various Mef2 genes at the beginning of the coding region (Hinits and Hughes, 2007)). This MO also ablated all CMs differentiation at 15ss (Fig. S5A,B).

Zebrafish *smyd1b* mRNA normally marks differentiating CMs in the FHF from 13ss, and is an orthologue of mouse *Smyd1/Bop*, a known Mef2c target (Phan et al., 2005). *Mef2ca;mef2cb* dual loss of function embryos lacked *smyd1b* mRNA both before (13ss) and after (17ss) the time of CM differentiation (Fig. 2A,B and data not shown). At 24ss, such embryos had no sarcomeric myosin heavy chain (MyHC) protein in heart, whereas this protein was present in skeletal muscle (Fig. 2G). At 24 hpf, *mef2ca;mef2cb double mutants* and other dual loss of function embryos had no beating heart cells, and lacked almost all *myl7* and *vmhc* mRNA and MyHC protein in heart; however, a few residual CMs were

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variably present (Fig. 3A,B,F and Fig. S6A). The residual cells had both ventricular and atrial character, having *vmhc* mRNA caudally and *myl7* mRNA alone anteriorly. At this stage, *mef2cb*^{fh288} mutant embryos injected with *mef2ca* MO had somewhat more cells expressing *myl7* mRNA than the double *mef2ca;mef2cb* mutants, and their heart had a thin string-like shape (Fig. S6B). Although *mef2ca* morphants phenocopied the jaw defects seen in the mutant at 5 dpf ((Miller et al., 2007) and data not shown), this finding suggests incomplete knockdown of Mef2ca protein by *mef2ca* MO. Conversely, using the *mef2d/c* MO we observed no cardiac cells expressing MyHC or a variety of other myocardial markers (Fig. 3G-I and data not shown). In addition, mRNAs encoding *bmp4, smyd1b* and atrial natriuretic factor (*nppa*), all markers of maturing CMs, were absent (Fig. 3B,F,J). Thus, lack of both Mef2ca and Mef2cb results in failure of CM differentiation and heart formation.

By 48 hpf, double *mef2ca;mef2cb* mutant embryos had pericardial oedema accompanied by lack of most myocardial cells (Fig. 4A, S6C). Around 30 residual CMs were present in small beating tube-shaped structure(s) forming either one or two zones (usually in the location of the venous pole, occasionally in both poles, and, more rarely, in a single string-like structure; Fig. 4B,C and data not shown). Wild type hearts had undergone looping by this stage and two chambers are readily distinguished. Double in situ hybridisation for *myl7* and *vmhc* marks the ventricular cells with both mRNAs, whereas atrial cells express *myl7* only. Each small residual tube in a *mef2ca;mef2cb* double mutant had either the ventricular or the atrial expression pattern appropriate to its position in the cardiac region (Fig. 4B). *nppa* and *bmp4* mRNAs are missing or weakly restricted to residual regions (Fig. 4E,F). At 72 hpf, an incross of *mef2ca*$^{+/b1086}$*;mef2cb*$^{+/fh288}$*;Tg(myl7:EGFP)twu26* had one or two residual GFP^{+} tube-shaped structures in double mutant embryos, of which ~80% (n=17) were beating (Fig. 4G and data not shown). Thus, lack of both Mef2ca and Mef2cb results in failure of most CM differentiation and heart formation, although a few cells showing either atrial or ventricular fates are able to differentiate after 24 hpf.

In contrast, hearts of *mef2d/c* morphant embryos had a more severe phenotype; neither MyHC nor atrial-specific MyHC immunostaining was detected in *mef2d/c* morphants (Fig. 4D). No Mef2 immunoreactivity was observed in the cardiac region of *mef2d/c* morphants (data not shown). Lack of additional Mef2s in *mef2d/c* MO appears to prevent residual CM differentiation.

We also tested whether non-myocardial cells that form the bulbus arteriosus can differentiate when lacking Mef2ca and Mef2cb. Double mutant embryos had a smaller than normal bulbus arteriosus structure marked by Elastin immunostaining (Grimes et al.; Grimes et al., 2006; Hami et al.; Lazic and Scott; Miao et al., 2007), but the bulbus size was reduced in proportion to that of the residual CM tube structure in the arterial pole (Fig. 4G). Thus, Mef2c activity directly or indirectly controls bulbus arteriosus size.

Mef2ca and *mef2cb* are required for a late step in cardiomyocyte differentiation

To define the role of Mef2 proteins in the cascade of events leading to CM differentiation, we examined *mef2ca;mef2cb* dual loss of function embryos at early stages when myocardial precursors reside bilaterally within the ALPM. Specified CMs in the ALPM express a transcriptional program that includes several GATA, Tbx, Nkx2 and Hand2 factors (Begemann and Ingham, 2000; Reiter et al., 1999; Ruvinsky et al., 2000; Serbedzija et al., 1998; Yelon et al., 1999; Yelon et al., 2000). At 12ss, *nkx2.5, gata4,5,6* and *hand2* mRNAs appeared indistinguishable in dual loss of function embryos compared to controls (Fig. 5A-C and S5E). Congruently, all embryos from *mef2cb*$^{+/b1086}$*;mef2cb*$^{+/fh288}$ incross analysed at this stage showed similar expression of these mRNAs (data not shown). By 22ss, expression of various genes that are part of the myocardial program, such as *tbx20* and *gata6*, began to

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show defects in dual loss of function embryos and mef2d/c MO by lacking the characteristic ring of expression around the endocardium and had a disorganised pattern at the midline (Fig. 5D,E). Paralleling this defect there was a failure of *nkx2.5* mRNA maintenance. Whereas early *nkx2.5* expression was unaffected by loss of Mef2ca and Mef2cb function, its mRNA was later lost, presumably due to the lack of differentiated CMs (Fig. 5A,F). Similarly, *smyd1b* and most *bmp4* mRNA was lost (Figs 2B and S5C,D), indicating their role in the maturation of differentiated CMs that are lost after dual loss of function. Thus, Mef2cs function late, during sarcomeric differentiation of CMs.

In order to place the function of Mef2cs in relation to other factors implicated in CM differentiation, we examined the bHLH transcription factor *hand2*, which promotes CM differentiation, especially in ventricle (Yelon et al., 2000). In mice, it has been suggested that Mef2c is upstream of *Hand2*, as expression of *Hand2* was reduced in *Mef2c* null mice (Lin et al., 1997b). However, we found that, at 20-25ss, *hand2* mRNA levels appear up-regulated after *mef2ca;mef2cb* dual loss of function in a wide sheet cells in the prospective cardiac region (Fig. 5G). Although *hand2* mRNA normally persists in the heart tube, its levels are down-regulated in the pre-cardiac region as CMs differentiate (Yelon et al., 2000). These data indicate that CM development was arrested in the absence of Mef2 function. Although *hand2* mRNA expression precedes that of *mef2ca* and *mef2cb in the ALPM*, both are expressed in *hand2* mutants and morphants, ((Yelon et al., 2000), and Fig. S7A,B). Thus, it is not likely that *mef2ca/mef2cb* are targets for Hand2 in zebrafish.

Endothelial expression of Mef2c is not required for endocardium formation

The lack of a clear cardiac cone revealed by *tbx20* and *gata6* mRNA in dual loss of function embryos suggested that embryos lacking Mef2ca and Mef2cb may have defects in the endocardium or, more generally, in endothelium (Fig. 5D,E). Indeed, *mef2cb* mRNA is clearly detected in vasculature (Fig. S4A), consistent with the presence in *mef2ca, mef2cb* and mouse *Mef2c* upstream regulatory regions of a FOX:ETS binding motif capable of driving GFP in zebrafish endothelium (De Val et al., 2008). However, endothelial markers *fli-1, kdr* (*flk-1*) and *cdh5* are relatively unperturbed in vasculature of double *mef2ca;mef2cb* mutants and in *mef2d/c* MO embryos (Fig. S4B-E). Endocardial markers were also present in dual loss of function embryos. Whereas in 22ss control embryos a ring of *myl7*$^{+}$ CMs surrounds a small circle of *cdh5*$^{+}$ endocardial cells, in dual loss of function and *mef2d/c* MO embryos lacking heart *myl7* mRNA, *cdh5* mRNA was not only present but even expanded and up-regulated (Fig. 5H). Intriguingly, by 48 hpf, endothelial cells marked with *cdh5* mRNA were abnormally organised in the cardiac region where the heart had failed to form (Fig. 5I). Thus, lack of myocardial differentiation leads to the disorganisation of other layers of the heart.

Over-expression of *mef2cb* dominantly changes cell fate

Dual loss of function shows Mef2c factors are necessary for myocardial differentiation. To test whether Mef2 is also sufficient for CM differentiation, we used a gain of function approach. Embryos injected at the 1-2 cell stage with RNA encoding *mef2cb* had widespread ectopic muscle in the head region (Figs 6A and S1G). Most ectopic muscle was composed of elongated cells expressing high levels of MyHC and *smyd1b* mRNA and lacking *bmp4* mRNA, and therefore may differ from CMs (Fig. 6A-C). Interestingly, *mef2cb* mRNA injection did up-regulate *bmp4* mRNA in many cells in a rectangle within the cardiogenic region, but not elsewhere in the embryo (Fig. 6C). Nevertheless, few cells in the rectangle appeared to form CMs, and hearts in such embryos were not bigger than normal (Fig. 6A). Taken together, these data indicate that although Mef2cb can induce *bmp4* in the cardiogenic region, Mef2cb alone is insufficient to drive widespread ectopic CM differentiation.

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The elongated nature of ectopic muscle cells induced outside the cardiogenic region by Mef2cb over-expression was reminiscent of skeletal muscle. To test this possibility, we used double in situ hybridisation for *myod* and *myl7*, which exclusively mark skeletal muscle or myocardial cells, respectively. Injection of 20 pg of *mef2cb* RNA caused high levels of ectopic myogenesis accompanied by a dramatic failure of brain morphogenesis (Fig. 6D,E). At both 14ss and 23ss, no ectopic *myl7* expression was found in embryos injected with *mef2cb* RNA, whereas many cells in the head region expressed ectopic *myod* (Fig. 6D,E). Moreover, at 23ss, fewer CMs expressed *myl7* in Mef2cb-overexpressing embryos (Fig. 6E). In addition, *cdh5*$^{+}$ endothelial cells were also reduced in both endocardium and vasculature in such embryos (Fig. 6G). The ectopic expression of the skeletal muscle marker *myod* in mesodermal and probably other territories, together with the depletion of cardiac and endothelial markers, indicates that high levels of Mef2cb can dramatically alter cell fate in the early embryo.

In order to restrict our overexpression analysis to cells normally expressing *mef2cb*, we drove mosaic over-expression of *mef2cb* by injection of a BAC CH211-202E12 DNA, containing the *mef2cb* locus. This approach leads to greatly increased expression of genes contained within the BAC, but in normal locations (Minchin and Hughes, 2008). The BAC also contains Dre-mir-9-5 microRNA. mir-9 is highly expressed in the brain, and is suggested to regulate neurogenesis, but is not expressed in zebrafish heart (Leucht et al., 2008; Wienholds et al., 2005), and thus is not predicted to effect the heart. Injection of the BAC increased levels of *mef2cb* mRNA and Mef2 protein in scattered somitic muscle, heart, telencephalon and vasculature, regions were *mef2cb* is normally detected (Fig. S1A-D). Within the cardiogenic region, patches of ectopic m*yl7* mRNA appeared around the heart (Fig. 6G), and could reflect either ectopic induction of CMs or their aberrant migration. Ectopic skeletal muscle was not observed elsewhere (data not shown). Thus, the skeletal myogenic effect of Mef2cb over-expression appears to be restricted to certain cells in early embryos.

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Lack of Mef2ca delays cardiomyocyte maturation

Dual loss of function of both *mef2ca* and *mef2cb* blocked CM differentiation, but loss of function of either gene alone did not prevent heart formation (Figs 2,3). Analysis of two mutant alleles of *mef2ca, b1086* and *tn213*, revealed weaker *myl7* mRNA accumulation in FHF CMs at 15ss, suggesting a decreased rate of differentiation in the absence of Mef2ca (Fig. 2C and data not shown). However, *vmhc* expression was indistinguishable from that in sibling and control wild type embryos (Fig. 2C and data not shown). Consistent with the view that FHF differentiation is retarded, *smyd1b* mRNA was not detected in the bilateral heart of a quarter of *mef2ca*$^{+/-}$ incross progeny at 13ss, but appeared normal by 17ss (Fig. 2C). By 24 hpf, *mef2ca* mutant hearts had normal shape and size as revealed by various sarcomeric genes and *bmp4* mRNA accumulation (Fig. 3C), even though Mef2 protein was greatly reduced (Fig. S2B and (Hinits and Hughes, 2007)). At 48 hpf, *mef2ca* mutant embryos had normal gene expression and had undergone normal looping (Fig. S8A,B). Mutant inflow and outflow tracts (IFT and OFT) were well-formed and addition of new CMs in these locations occurred during the 2-3 dpf. At 5 dpf, *mef2ca* mutants had indistinguishable hearts from their siblings (data not shown). Moreover, we found no significant difference in heart rate between *mef2ca* mutants, *mef2ca* morphants and their respective control embryos (Fig. S8E). Taken together with *mef2ca* and *mef2cb* expression data and the dual loss of function results, these data suggest that either *mef2cb* is the main functional Mef2 gene in the heart, or that redundancy between Mef2ca and Mef2cb is sufficient to support a functional heart.

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Mef2cb mutant is viable and shows no heart defects

We next tested the function of Mef2cb in zebrafish development. Unlike *mef2ca* mutants, at 15ss, *mef2cb* morphants had no change of *myl7* or *vmhc* mRNA accumulation (Fig. 2D). Expression of *smyd1b* was also normal at both 13ss and 17ss (Fig. 2D). Considering *mef2cb* mRNA accumulation at both poles of 24 hpf hearts (Fig. 1I), we hypothesized an effect of lack of Mef2cb function on structures added to both poles. Indeed, Lazic and Scott, 2011 using an ATG-MO that has only a 4 bp overlap with ours, have reported that Mef2cb is necessary for ventricular development and for late CM addition at the arterial pole (Lazic and Scott, 2011). We also find that *mef2cb* morphants had smaller hearts at 24 hpf with both cardiac chambers appear defective (Fig. 3D). *By 48 hpf, Mef2cb* morphant hearts did not undergo looping, and their linear hearts showed mis-regulation various genes (Fig. S9A-C and data not shown). Other, non-myocardial derivatives, such as bulbus arteriosus that is marked by DAF-2 DA and Elastin (Grimes et al.; Grimes et al., 2006; Hami et al.; Lazic and Scott; Miao et al., 2007) were missing at 72 hpf (Fig. S9C,D). In order to test these results we analysed the TILLING mutant *mef2cb*^{fh288} (see earlier section). We did not observe any heart phenotype in embryos from various incrosses of *mef2cb*$^{+/fh288}$ fish. Moreover, progeny from incrosses that were allowed to grow to adulthood revealed close to expected numbers of adult homozygous mutants *mef2cb*$^{fh288/fh288}$ (5/14, 35% compared with 25% expected). These adults are healthy and able to breed. In agreement with these facts, genotyped *mef2cb*$^{fh288/fh288}$ homozygous mutants had no change in *myl7, vmhc* or *bmp4* mRNAs or MyHC protein accumulation at 15ss or 24 hpf (Figs 2E, 3E). Hearts were looped and expressed normal levels and pattern of *myl7, vmhc* and *bmp4* mRNAs (Fig. 7A,B). The bulbus arteriosus also formed correctly and expressed Elastin (Fig. 7C). Thus, *mef2cb*^{fh288} mutants have normal hearts, at least under normal growing conditions

Discussion

The current work makes five major points. First, Mef2c homologues are essential for heart formation in zebrafish. Second, early Mef2 function during heart development is in a specific step of CM differentiation that follows specification. Third, Mef2ca and Mef2cb function redundantly to drive differentiation of both early and late added CMs. Fourth, both *mef2ca* and mef2cb single mutants have a seemingly normal heart. Fifth, Mef2cb, if mis-expressed outside the cardiogenic region during early embryonic development, has the ability to override cell fates and convert cells to skeletal muscle. These findings provide experimental confirmation of the long-speculated view that Mef2 activity is essential for vertebrate CM differentiation.

Mef2 role in sarcomeric muscle differentiation

Our data show that Mef2 activity is required for differentiation of all CMs of both FHF and the putative SHF of zebrafish. Sarcomeric gene expression was lost when the two *Mef2c* paralogues in zebrafish, *mef2ca* and *mef2cb*, were knocked down by morpholinos or in genetic null mutants. Mef2c does not act alone; sarcomeric genes such as *myl7* and *vmhc* also require other transcription factors for their expression in CMs (Peterkin et al., 2007; Yelon et al., 2000). Nevertheless, to date, we have found no cardiac sarcomeric gene that is normally expressed without Mef2 activity. Expression of many other genes, including *smyd1b, nppa* and *bmp4*, that are involved in the maturation of CMs were also ablated after Mef2 loss of function. Mammalian orthologues of several of these genes are direct Mef2c targets, including *Bop* (*Smyd1*) and *Anf* (*Nppa*) (Morin et al., 2000; Phan et al., 2005; Zang et al., 2004). Bmp4 is suggested to function by increasing myofibrillar gene expression, turning cardioblasts into beating CMs (Tirosh-Finkel et al., 2010). Our findings place Mef2 function in CM differentiation at a step that precedes Bmp4 expression, although it is not clear if *bmp4* is a direct Mef2c target.

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Mef2c is clearly required at a later step than Nkx2.5, GATA and Hand2 transcription factors. Cardiac precursors lacking both Mef2ca and Mef2cb express *nkx2*.5 until the point of differentiation into contractile CMs. When differentiation fails, *nkx2.5* expression is lost, suggesting that CM precursors do not simply remain in an immature state. Other manipulations, that prevent differentiation of CMs, such as ectopic expression of dominant negative BMP receptors or *gata6* MO, also specifically block late *nkx2.5* expression (Peterkin et al., 2003; Shi et al., 2000). Combined loss of function of GATA factors prevents CM specification prior to *mef2ca* expression, whereas lack of GATA5 or GATA6 alone causes cardia bifida (Holtzinger and Evans, 2007; Peterkin et al., 2003, 2007). Loss of function of *hand2*, the only Hand factor in the current zebrafish genome, does not prevent *mef2ca* and *mef2cb* expression but results in cardia bifida with reduced CM number (Yelon et al., 2000). In contrast, heart cells lacking Mef2 are specified and migrate normally to the midline, but fail to differentiate. *Mef2ca;mef2cb* dual loss of function embryos retain high levels of *hand2* mRNA in the cardiogenic region, suggesting a developmental arrest at a pre-differentiation step involving Hand2 expression. Loss of Mef2c in the mouse, on the other hand, leads to a mild delay in *hand1* and absence of late *hand2* expression (Lin et al., 1997b; Vong et al., 2006). As far as we are aware, early *Hand2* expression remains to be investigated in *Mef2c* null mice. The up-regulation of both *hand2* and the endocardial marker *cdh5* in Mef2 loss of function zebrafish embryos suggests that failure of CM differentiation has effects beyond myocardium.

The *mef2d/c* morpholino, which targets *mef2d*, both *mef2c*s and possibly *mef2aa* and *mef2b* (Hinits and Hughes, 2007), causes a complete loss of cardiomyogenesis, showing that Mef2 is essential for CM differentiation. The stronger effect of *mef2d/c* MO when compared with other dual loss of function mutant or morphant combinations, which ablate most but not all CMs, strongly suggests that other Mef2 proteins are responsible for the residual cardiomyogenesis in *mef2ca;mef2cb* double mutants. Strong evidence supporting this view is that the residual CMs in double mutants have Mef2, but not Mef2c, immunoreactivity. Indeed, *mef2aa* mRNA is detected in the residual myocardial regions of double mutants (data not shown). We conclude that *mef2d/c* MO is likely to knock down Mef2aa activity sufficiently to prevent CM differentiation, despite six bases of mismatch. Moreover, Mef2aa appears, like the Mef2cs, to be able to drive CM differentiation.

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Mef2-dependency of zebrafish myocardium is remarkably similar to that in Drosophila. The single Mef2 factor in Drosophila, *D-Mef2*, is essential for expression of sarcomeric genes in the fly heart in the dorsal vessel, but is not required for expression of specification genes. Moreover, the dorsal vessel is more sensitive to the lack of *D-Mef2* than is body wall (i.e., skeletal) muscle (Lilly et al., 1995; Ranganayakulu et al., 1995). In zebrafish skeletal muscle, Myogenic Regulatory Factors (MRFs) drive expression of some sarcomeric genes after knockdown of Mef2 activity (Hinits and Hughes, 2007; Hinits et al., 2009; Hinits et al., 2011). Thus, cardiac and skeletal myocytes may differ in Mef2 dependency for terminal differentiation. Nevertheless, both in skeletal and cardiac muscle, Mef2c expression parallels differentiation of the contractile phenotype (Hinits and Hughes, 2007 ; Potthoff et al., 2007). Our findings suggest a deep conservation of function of Mef2 in heart development through animal evolution and contrast with the divergence of function of MRFs between vertebrates and Drosophila.

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Redundant roles for Mef2ca and Mef2cb during cardiac development

The FHF had early maturation defects in *mef2ca* mutants that were not observed in *mef2cb* morphants. Both *mef2ca* probable null alleles (Miller et al., 2007) and morphants showed a short developmental delay affecting some sarcomeric and maturation genes in the FHF.

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Mef2ca deficient hearts recovered well, but whether they are normal in all respects, for example under stress, remains to be determined.

The recovery of FHF cardiomyogenesis suggests that Mef2ca and Mef2cb are redundant during FHF development, but indicates a more prominent role for Mef2ca in FHF development. At 24 hpf, we detect strong *mef2cb* mRNA at both the arterial and venous poles, A similar pattern was observed in Atlantic cod, *Gadus morhua*, that has two *Mef2c* paralogues, each expressed strongly in a single cardiac pole at heart tube stage (Torgersen et al.). This is where SHF precursors reside (de Pater et al., 2009; Zhou et al., 2011). The *mef2cb* expression pattern suggests a more prominent role at the cardiac poles. Lazic and Scott (2011) described an arterial pole defect using *mef2cb* MO; we found the same result with our distinct *mef2cb* MO. However, the likely null *mef2cb*^{fh288} mutant has an apparently normal heart and grows well to a viable and fertile adult. This shows that although distinct regulatory elements cause distinct expression of each gene, a genetic redundancy of Mef2ca and Mef2cb function exist for both early/central FHF and later-added/polar SHF CMs.

How can one explain the cardiac pole-specific defects in *mef2cb* morphants? Clearly, the MOs work to knockdown Mef2cb protein, as Mef2c immunreactivity is ablated when they are used in combination with the *mef2ca* mutant. Moreover, the absence of Mef2c protein and the early stop codon in the *mef2cb* mutant make it highly likely to be null. We hypothesise that the cardiac pole defects arise from a non-specific effect of the MO added to a loss of Mef2cb function that preferentially sensitizes CMs at the cardiac poles.

Given time, the residual CMs in double *mef2ca;mef2cb* mutants can reach their normal position, aggregate, take on ventricular or atrial character, form small tubular structures, beat and even recruit non-myocardial cells into bulbus arteriosus. As discussed above, Mef2aa may contribute towards this residual differentiation. *Mef2aa* is expressed later than the two Mef2cs in zebrafish heart, and we detected *mef2aa* mRNA only in differentiated CMs. Consistent with this, Mef2aa knockdown causes late cardiac defects relating to contractility (Wang et al., 2005). So late recovery of CMs in double mutants may reflect independent activation of *mef2aa*. Alternatively, other factors such as GATAs and Hand2 may be sufficient to slowly rescue a few cells to CM differentiation. In mouse, *Mef2a* (and *Mef2d*) expression begins only at the heart tube stage, whereas *Mef2c* (and *Mef2b*) are expressed in pre-cardiac mesoderm, (Edmondson et al., 1994). We speculate that redundancy between *Mef2c* and *Mef2b* may explain the ability of CMs in early heart development of *Mef2c* knockout mice. *Mef2a* null mice exhibit perinatal lethality from an array of cardiovascular defects (Ewen et al., 2011; Naya et al., 2002). Thus, expression and function of zebrafish *Mef2c* and *Mef2a* orthologues appear conserved between fish and mammals.

Mef2cb can drive ectopic skeletal myogenesis

Our data show that over-expression of Mef2cb leads to ectopic expression of both cardiac and skeletal muscle genes. Extra Mef2cb in its normal location, induced by BAC injection, leads to ectopic CMs within the heart field, but not elsewhere. Strikingly, however, the entire cardiogenic region appears able to up-regulate *bmp4* expression in response to Mef2cb, when it is introduced early by *mef2cb* mRNA injection, but does not undergo extensive ectopic cardiomyogenesis. This finding suggests that the ability of Mef2 to drive CM differentiation is under tight additional controls.

Elsewhere in the embryo, particularly, but not exclusively, in head mesoderm, *mef2cb* mRNA over-expression causes high levels of conversion of cells to skeletal muscle. This effect is efficient, comparable to that of *myod* or *mrf4* mRNA injection (Hinits et al., 2009; Osborn et al., 2011), and can lead to loss of myocardial markers, possibly through conversion of early cranial mesoderm to skeletal muscle before it attains the character of the

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cardiogenic region. The distinct action of Mef2cb over-expression in the cardiogenic and other regions of the embryo suggests that the activity of Mef2cb within a cell is strongly influenced by other signals/molecules involved in head mesoderm patterning ((Tzahor and Evans, 2011) and others).

Our findings indicate a potential role for Mef2 as a skeletal muscle determination factor, and is reminscent of the observation that MEF2A can initiate skeletal myogenesis in some circumstances (Kaushal et al., 1994). However, other studies have shown that Mef2c alone is unable to activate the myogenic program, but requires Myod or another MRF (Black et al., 1998; Molkentin et al., 1995). As no MRFs are expressed in the zebrafish head region prior to 24 hpf, it appears that Mef2cb can trigger the MRF expression required for efficient enhancement of terminal muscle differentiation by Mef2s (della Gaspera et al., 2009; Molkentin et al., 1995). Indeed, Xenopus Mef2a can activate the Myod promoter (Wong et al., 1994). In Drosophila, Dmef2 is a key regulator of body wall striated myogenesis and does not require on MRF (Gunthorpe et al., 1999; Lin et al., 1997a). In C. elegans, by contrast, Mef2 is dispensible for myogenesis (Dichoso et al., 2000). It remains to be established whether the ability of Mef2cb is shared by other Mef2 factors and/or plays a role during development of any vertebrate skeletal muscle in the wild type situation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Mef2c homologues are essential for heart formation in zebrafish.
2. Mef2ca and Mef2cb function redundantly to drive differentiation of FHF and SHF CMs.
3. *mef2ca* and *mef2cb* single mutants have a seemingly normal heart.
4. *Mef2cb* overexpression during early embryonic development converts cells into skeletal muscle.

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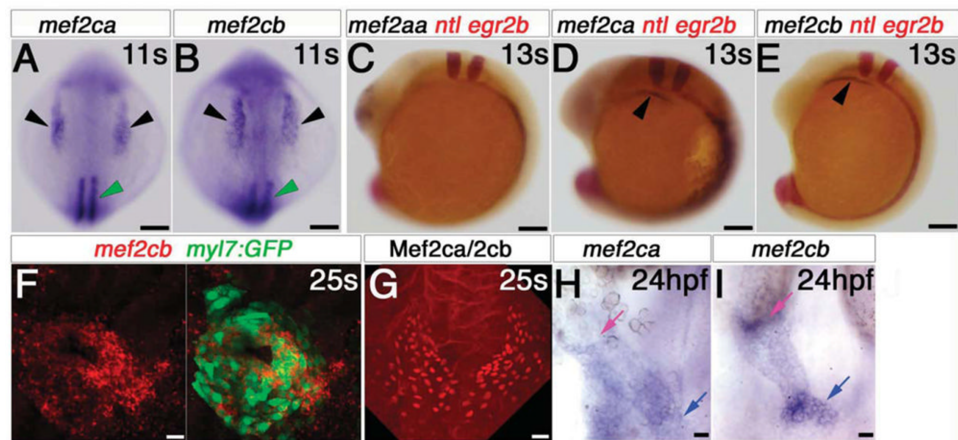

Figure 1. *Mef2ca* and *mef2cb* expression during early cardiogenesis

In situ mRNA hybridisation of *indicated genes*, or immunodetection of Mef2ca/cb protein (G) for wild type (A-E, G-I) and *Tg(myl7:EGFP)* (F) in dorsal (A,B) or lateral (C-E) views of wholemount embryos or in flatmounts of dorsal views of the cardiac region, anterior to top (F-I). **A,B.** *Mef2ca* and *mef2cb* mRNAs accumulate in the bilateral heart fields in ALPM (arrowheads) and adaxial cells (green arrowhead). **C-E.** *Egr2b* expression in rhombomeres 3 and 5, and *ntl* expression in the notochord positions the row of ventral cells in the ALPM (black arrowheads; D,E) that contain *mef2ca* and *mef2cb*, but not *mef2aa* mRNA (C). **F.** Confocal stack of *Tg(myl7:EGFP)* heart at 25ss showing co-localisation of *mef2cb* mRNA (Fast Red) and EGFP. **G.** Mef2c protein in nuclei of a similar crescent of CMs spanning the midline. **H,I.** By 24 hpf, both *mef2ca* (H) and *mef2cb* (I) mRNAs are detected weakly in the heart tube, but *mef2cb* also accumulates strongly in the venous (blue arrow) and arterial (pink arrow) poles of the heart. Scale = 100 μm (A-F), 20 μm (G-I).

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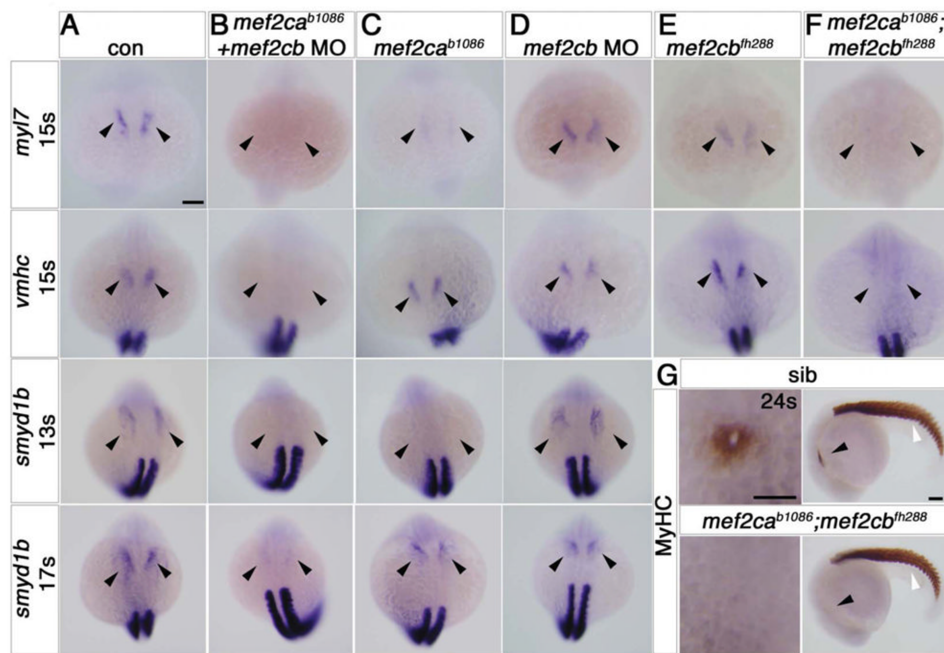

Figure 2. Early cardiomyocytes fail to differentiate after loss of Mef2c function

A-F. In situ mRNA hybridisation for *myl7, vmhc* and *smyd1b* in wild type control (A), *mef2ca*^{b1086}+*mef2cb* MO (B), *mef2ca*^{b1086} (C) *mef2cb* MO (D), *mef2cb*^{fh288} (E) and *mef2ca*^{b1086};*mef2cb*^{fh288} (F) embryos, shown in wholemounts in dorsal view, anterior to top. Loss of both *mef2ca* and *mef2cb* function greatly reduces *myl7, vmhc* and *smyd1b* mRNAs in the bilateral heart fields (arrowheads; A,B,F). *Mef2ca*^{b1086} mutant embryos have weak *myl7* and *smyd1b* mRNAs early, but recover later, and show no change in *vmhc* (C). *Mef2cb* single morphants or *mef2cb*^{fh288} mutant show no changes (D). **G.** Immunostaining for MyHC (A4.1025) in 24ss *mef2ca*^{b1086};*mef2cb*^{fh288} embryos and their siblings, shown in wholemounts in dorsal view, anterior to top (left panel) and lateral view, anterior to left (right panel). No MyHC is detected in the heart, whereas somitic muscle appears normal (white arrowheads). Scale = 100 μm.

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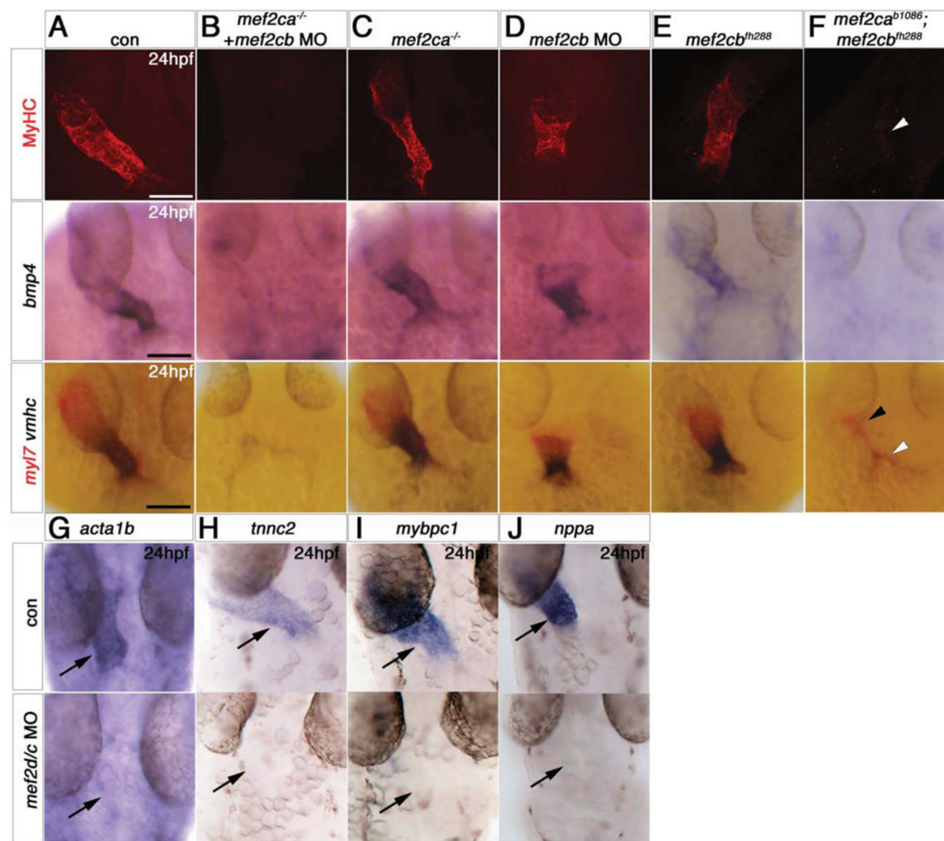

Figure 3. Redundant and specific functions of Mef2ca and Mef2cb drive cardiomyogenesis and heart tube formation

Immunodetection of MyHC (confocal stacks, top panels) or in situ mRNA hybridisation for indicated genes (A-F, lower panels and G-J) in hearts of 24 hpf zebrafish embryos shown in a dorsal view, anterior to top. **A,B,F.** Loss of both *mef2ca (tn213* allele, in MyHC and *b1086* allele, *in bmp4* and *myl7+vmhc)* and *mef2cb* function (B,F) led to lack of all markers, compared with control (A). Note the few cells expressing *myl7* only (black arrowhead) or both *myl7* and *vmhc* (white arrowhead). **C.** *Mef2ca* mutants have a normal heart. **D,E.** *Mef2cb* morphants have a shortened heart with substantial loss of both atrial and ventricular volume, yet *mef2cb*^{fh288} mutants have a normal heart. **G-I.** Loss of Mef2c function with *mef2d/c* MO ablated all actin (*acta1b*, G), *tnnc2* (H), *mybpc1* (I) and *nppa* (J) mRNAs. Scale = 100 μm.

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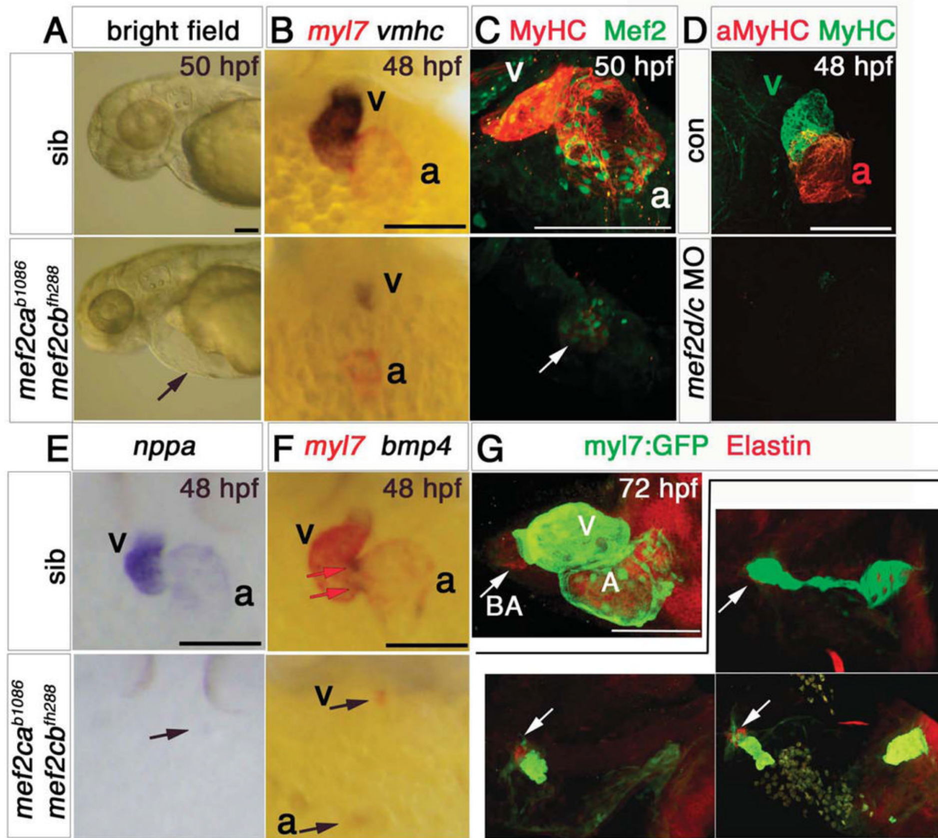

Figure 4. Loss of Mef2c function abolishes sarcomeric gene expression

Hearts of 48-50 hpf (A-F) or 72 hpf (G) *mef2ca*^{b1086};*mef2cb*^{fh288} mutant embryos and their siblings in bright field (A), after immunodetection (C,D,G) or in situ mRNA hybridisation (B,E,F) shown in lateral view, anterior to left (A,G) or ventral (B-F) view, anterior to top. **A.** *mef2ca* ^{b1086};*mef2cb*^{fh288} embryos had a tiny residual heart (arrow) and cardiac chamber edema. **B,E,F.** Double mutant embryos lacked almost all *myl7, vmhc, bmp4* and *nppa* mRNAs as well as MyHC and Mef2 proteins in ventricle (v), atrium (a) or AV canal (red arrows in F) except for one (arrows in C,E) or two small heart structures expressing these markers. **C.** Confocal stack showing genotyped double mutant expressing low levels MyHC and nuclear Mef2 in a residual myocardial tissue. Sibling presented is *mef2ca*$^{+/b1086}$;*mef2cb*$^{+/+}$. **D**. No MyHC or atrial MyHC is detected in *mef2d/c* morphants. **G.** Immunodetection for GFP (atrium and ventricle, green) and Elastin (bulbus arteriosus, ba) in hearts of three different genotyped *mef2ca*^{b1086};*mef2cb*^{fh288};*Tg(myl7:EGFP)twu26* showing variation in residual differentiated myocardial and bulbus tissue, compared to the normal heart of a *mef2ca*$^{+/b1086}$;*mef2cb*$^{+/fh288}$;*Tg(myl7:EGFP)twu26* sibling. Scale = 100 μm.

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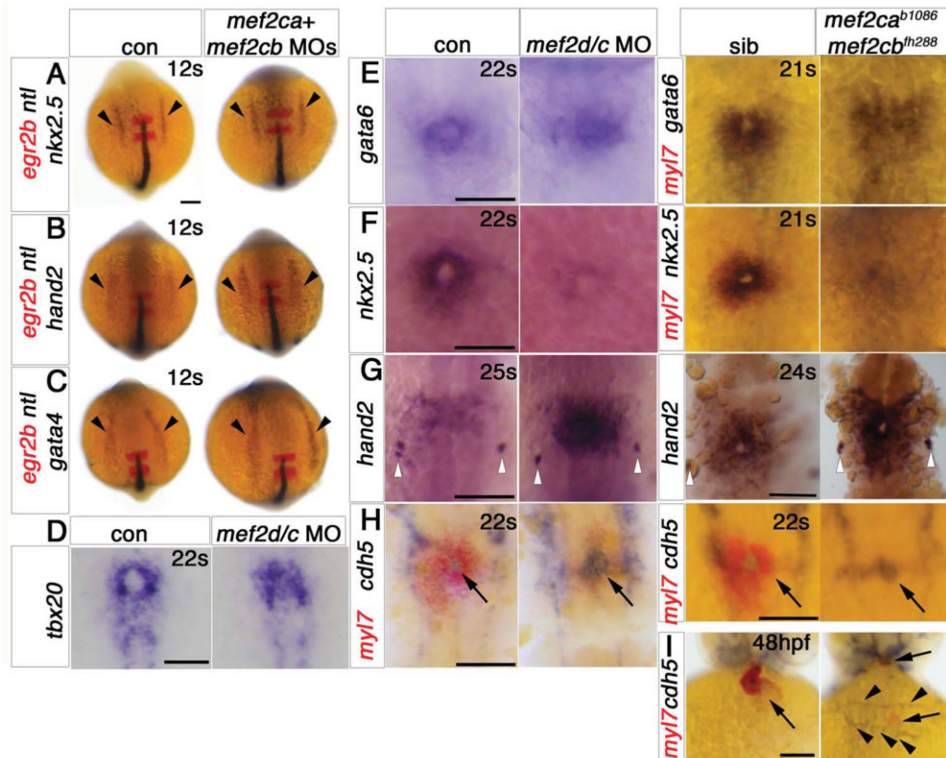

Figure 5. Cardiomyocytes of *mef2ca;mef2cb* dual loss of function are specified but developmentally arrested

In situ mRNA hybridisation for indicated genes (wholemounts in dorsal view, anterior to top, except J, ventral view). **A-C.** ALPM expression (arrowheads) of *nkx2.5* (A), *hand2* (B) and *gata4* (C) at 12ss is unaffected by lack of Mef2ca and Mef2cb. Notochord and rhombomeres 3 and 5 are marked by *ntl* (blue) and *egr2b* (red), respectively. **D,E.** *Tbx20* and *gata6* mRNAs are present but disorganised in the heart region of *mef2d/c* morphants and $mef2ca^{b1086};mef2cb^{fh288}$ mutant embryos (lacking myl7 expression in red, E, right panel) compared to the typical ring-shape in control and sibling embryos. **F.** During heart cone stage (22ss), *nkx2.5* mRNA is abolished. **G**. *hand2* mRNA is enhanced in a sheet of cells spanning the cardiac region but not elsewhere. Pharyngeal pouch expression is unchanged (white arrowheads). **H,** Whereas myocardial cells are undifferentiated in *mef2d/c* morphants and $mef2ca^{b1086};mef2cb^{fh288}$ mutants (*myl7*, red), the endocardium is expanded and *cdh5* is up-regulated. **I.** Endocardium (*cdh5*, arrow) lines the myocardium (*myl7*, red) in the normal looped heart of a sibling embryo. In *mef2ca;mef2cb* mutant embryos, little endocardial makrer is co-localised with the residual myocardium (arrows). Extra *cdh5*-expressing tissue is detected in the cardiac region (arrowheads). Scale = 100 μm.

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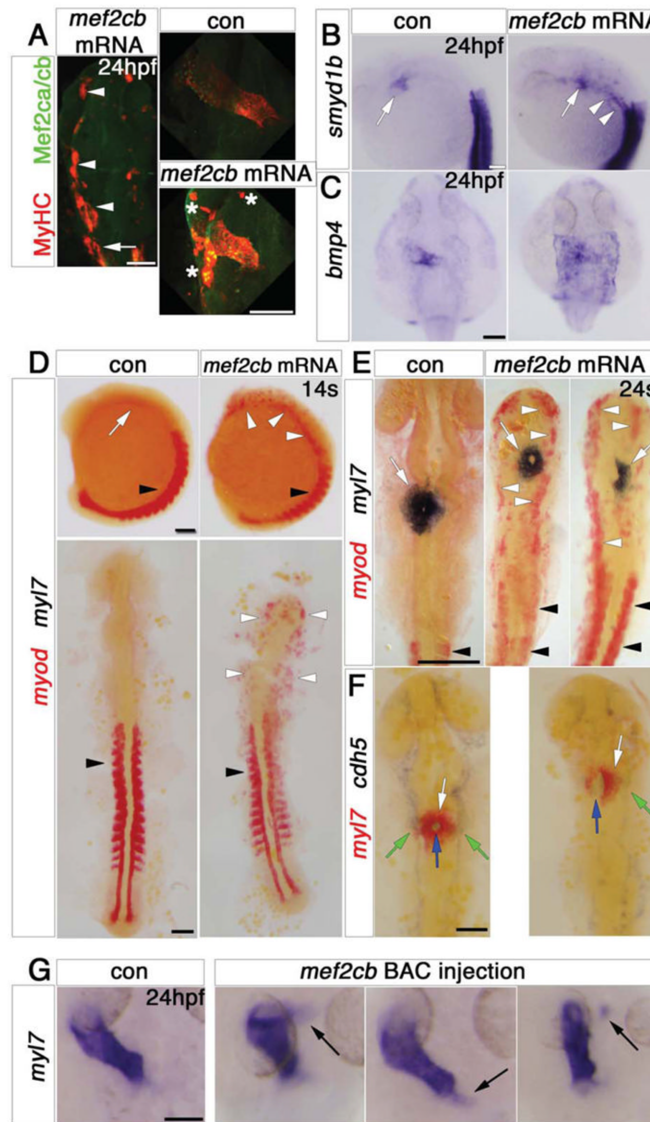

Figure 6. Mef2cb overexpression induces skeletal muscle at the expense of myocardial and endothelial cells

In situ mRNA hybridisation (or immunodetection, A) for indicated genes, shown as wholemounts in dorsal (G,C) or lateral (B, D, top panel) views, or as flatmounts in dorsal view (A, D, bottom panel, E,F). **A.** Injection of *mef2cb* RNA results in many ectopic muscle cells expressing strong Mef2ca/cb and MyHC at 24 hpf (white arrowheads) anterior to the first somite (white arrow). In the heart, MyHC and Mef2ca/cb are mosaically stronger than in control embryos. Asterisks = ectopic muscle. **B.** *Smyd1b* is upregulated in CMs (white arrow), and in ectopic muscle in the head region (white arrowheads). **C.** Expression of *bmp4* is upregulated in much of a sheet of cardiogenic cells but not elsewhere in the embryo. **D.** In 14ss control embryos, *myod* mRNA is expressed in somites (black arrowheads) and *myl7* is expressed weakly in CMs (white arrow). Embryos injected with *mef2cb* RNA express no ectopic *myl7*, but have ectopic *myod* mRNA in the head region (white arrowheads). **E.** By 24ss, high levels of ectopic *myod* correlated with reduction or lack of *myl7*-expressing CMs (right panel) and defective brain development. **F.** At 24ss, *mef2cb* RNA-injected embryos that had fewer *myl7*-expressing CMs (white arrows) also had less *cdh5* expression in

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vascular endothelium (green arrows) and disorganised endocardium (blue arrows). **G.** Compared to wild type control (leftmost panel), three examples of embryos injected with *mef2cb* BAC DNA show ectopic *myl7* mRNA either contiguous with (left panels) or detached from (right panel) the arterial (flanking panels) or venous (middle panel) poles. Scale = 100 μm.

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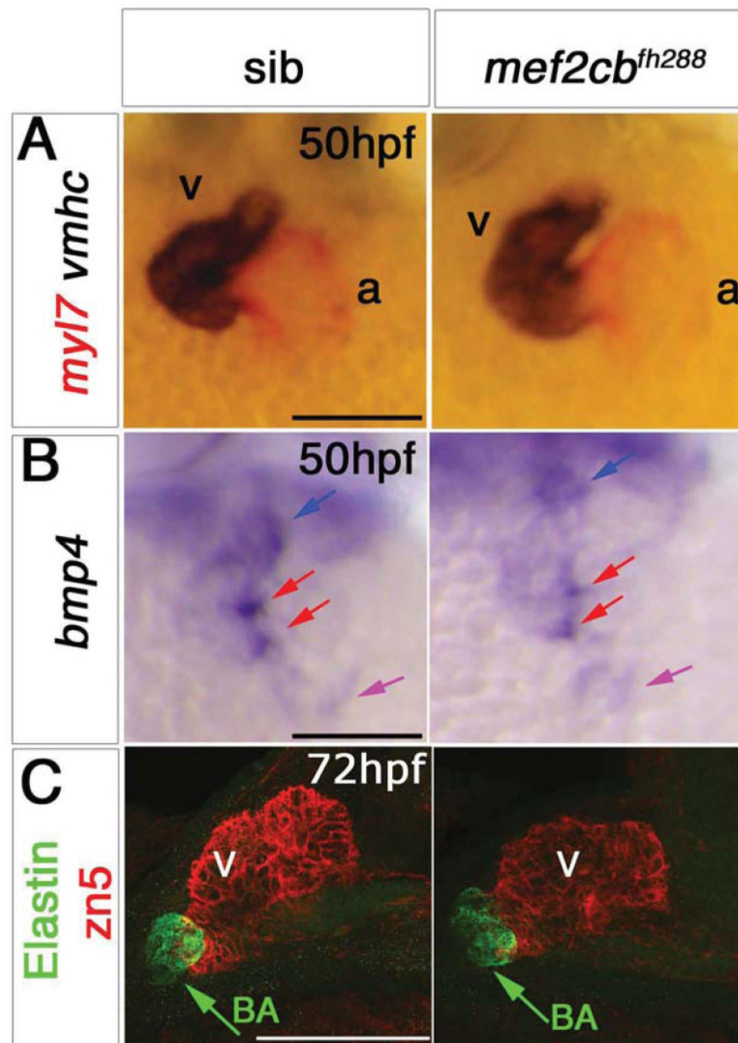

Figure 7. Loss of *mef2cb* function results in no heart phenotype

In situ mRNA hybridisation for *myl7, vmhc* and *bmp4* (A,B, ventral view, anterior to top) and immunodetection of DM-GRASP (zn5) and Elastin (C, lateral view, anterior to left) of hearts at indicated stage of genotyped *mef2cb*^{fh288} mutants and their siblings. A,B. *mef2cb*^{fh288} mutants had a normal looped heart and normal expression of chamber markers, and *bmp4* mRNA in OFT (blue arrow), IFT (purple arrow) and AV canal (red arrows). C. Confocal stacks of genotyped *mef2cb*^{fh288} mutants and siblings with a normal looped heart with developed chambers and bulbus arteriosus. Scale = 100 μm.