Developmental Biology 423 (2017) 24–33

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Myomaker is required for the fusion of fast-twitch myocytes in the zebrafish embryo

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ARTICLE INFO

Keywords:
Skeletal muscle
Multinucleated muscle fiber
Myocyte fusion
Myomaker
Fast-twitch muscle
Zebrafish

ABSTRACT

During skeletal muscle development, myocytes aggregate and fuse to form multinucleated muscle fibers. Inhibition of myocyte fusion is thought to significantly derail the differentiation of functional muscle fibers. Despite the purported importance of fusion in myogenesis, *in vivo* studies of this process in vertebrates are rather limited. Myomaker, a multipass transmembrane protein, has been shown to be the first muscle-specific fusion protein essential for myocyte fusion in the mouse. We have generated loss-of-function alleles in zebrafish *myomaker*, and found that fusion of myocytes into syncytial fast-twitch muscles was significantly compromised. However, mutant myocytes could be recruited to fuse with wild-type myocytes in chimeric embryos, albeit rather inefficiently. Conversely, overexpression of Myomaker was sufficient to induce hyperfusion among fast-twitch myocytes, and it also induced fusion among slow-twitch myocytes that are normally fusion-incompetent. In line with this, Myomaker overexpression also triggered fusion in another myocyte fusion mutant compromised in the function of the junctional cell adhesion molecule, Jam2a. We also provide evidence that Rac, a regulator of actin cytoskeleton, requires Myomaker activity to induce fusion, and that an approximately 3 kb of *myomaker* promoter sequence, with multiple E-box motifs, is sufficient to direct expression within the fast-twitch muscle lineage. Taken together, our findings underscore a conserved role for Myomaker in vertebrate myocyte fusion. Strikingly, and in contrast to the mouse, homozygous *myomaker* mutants are viable and do not exhibit discernible locomotory defects. Thus, in the zebrafish, myocyte fusion is not an absolute requirement for skeletal muscle morphogenesis and function.

1. Introduction

There are instances during development, when cells relinquish their individual identities and fuse with each other. Perhaps the most important of such cell-cell fusion events occur during fertilization of the ovum by the sperm (Bianchi and Wright, 2016; Georgadaki et al., 2016). Subsequently, during tissue morphogenesis in the embryo, widespread cell-cell fusion occurs in the myogenic lineage (Demonbreun et al., 2015; Kim et al., 2015; Schejter, 2016). Here, the precursors of the skeletal muscles, the myocytes, aggregate at muscle formation sites, align with each other, and undergo fusion to make syncytial (multinucleated) muscle fibers. A large body of work with *Drosophila* have pioneered our understanding of the process of myocyte fusion at the genetic and cell biological levels. These studies have led to the discovery of several important aspects of the fusion process: a) fusion is initiated by the recognition of the fusing myocytes through the engagement of a set of transmembrane proteins expressed on their cell surfaces, b) once the membranes are closely apposed, fusion pores are generated *via* remodelling of the actin cytoskeleton that allow cytoplasmic continuity, and finally, c) the pores widen progressively, which culminate in the formation of a syncytial cell (Abmayr and Pavlath, 2012; Haralalka and Abmayr, 2010; Kim et al., 2015; Rochlin et al., 2010).

In contrast to all the information gained from *Drosophila* studies, our understanding of myocyte fusion in the vertebrates remains rather limited. The optical clarity of the early zebrafish embryo, as well as the amenability for genetic analysis, are advantages that have been exploited for evaluating the cellular events and molecular requirements for myocyte fusion. Here, precursors of a specific lineage of somitic muscle cells, the fast-twitch muscles, fuse with each other and differentiate into multi-nucleated fibers (Roy et al., 2001). In this system, there is evidence of some degree of conservation in the molecular components that drive myocyte fusion in *Drosophila*. Thus, zebrafish homologs of the Kirre family of immunoglobulin-containing transmembrane proteins, which

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http://dx.doi.org/10.1016/j.ydbio.2017.01.019
Received 30 November 2016; Received in revised form 18 January 2017; Accepted 27 January 2017
Available online 01 February 2017
0012-1606/

function to engage myocyte membranes for fusion in flies, have been implicated in the fusion of the fast-twitch myocytes (Sohn et al., 2009; Srinivas et al., 2007). In addition, Rac1, a small GTPase and an essential regulator of the actin cytoskeleton that acts downstream of the membrane proteins, also has a role in myocyte fusion in both flies and zebrafish (Chen et al., 2003; Hakeda-Suzuki et al., 2002; Luo et al., 1994; Srinivas et al., 2007). Furthermore, zebrafish embryos compromised in a number of proteins that function in regulating Rac activity, such as Dock1 and Dock5 (Moore et al., 2007), which are homologs of the *Drosophila* fusion protein Myoblast city, have also been shown to exhibit defects in myocyte fusion. In contrast to these findings, there are also emerging data that new components have been recruited to the fusion process in the zebrafish that do not function in *Drosophila*. For instance, biochemical and genetic analysis has led to the identification of a pair of transmembrane junctional adhesion molecules (Jams), Jam2a and Jam3b, as important players in the fusion of the fast-twitch myocytes (Powell and Wright, 2011).

Certain elements of the *Drosophila* and zebrafish myocyte fusion pathways also seem to be conserved during mammalian myocyte fusion *in vivo*, whereas others are not. For instance, Nephrin – a Kirre family protein, as well as the actin regulators Dock1, Rac1 and N-WASp, have been implicated in myocyte fusion in the mouse (Kestila et al., 1998; Laurin et al., 2008; Pajcini et al., 2008; Sohn et al., 2009; Vasyutina et al., 2009); on the other hand, there is no evidence yet of the involvement of the Jam proteins. Recently, a novel multipass transmembrane protein called Myomaker (aka Tmem8c) has been shown to play an important role in myocyte fusion in the mouse (Millay et al., 2016, 2013, 2014; Mitani et al., 2017). The Myomaker protein is highly conserved among the vertebrates, but a direct homolog does not exist in flies (Millay et al., 2013). The *Myomaker* gene is specifically expressed in the precursors of skeletal muscles of the mouse embryo, and most importantly, overexpression of the protein can result in hyperfusion of myocytes as well as the fusion of non-muscle cells to myocytes (Millay et al., 2016, 2013; Mitani et al., 2017). Additional studies have shown that *Myomaker* is not just required for myocyte fusion during embryonic muscle development, but also for the repair and regeneration of adult muscle fibers *via* the recruitment of satellite cells into fusion (Millay et al., 2014). Mechanistically, it remains unclear how Myomaker induces myocyte fusion. Structure-function studies using *in vitro* cultures of the C2C12 myoblast cell line have suggested that much of the protein is buried within the cell membrane with seven transmembrane regions, and that the intracellular C-terminal domain is crucial for the fusion process (Millay et al., 2016).

Given that Myomaker is the first muscle-specific protein with a dedicated role in mammalian myocyte fusion (Millay et al., 2013), it is of interest to know whether it also functions in myocyte fusion in other vertebrate species. Evidence that this is indeed the case has come from the finding that siRNA-mediated inhibition of Myomaker in cultured chick myocytes inhibited their fusion (Luo et al., 2015). Moreover, *myomaker* has been shown to be expressed in the precursors of the fast-twitch muscles of the zebrafish embryo, and transient knockdown of the protein using antisense morpholinos impaired their fusion into multinucleated fibers ((Landemaine et al., 2014) and our unpublished observations). Here, we took an alternative approach, and made stable genetic mutations at the *myomaker* locus using the CRISPR/Cas9 technology. We found that the requirement and sufficiency of the gene in myocyte fusion, as well as the regulation of its expression in the myogenic lineage, is remarkably similar to that of the mouse. However, in contrast to *Myomaker* mutant mice and zebrafish *myomaker* morphants (*i.e.* the morpholino-injected zebrafish embryos) (Landemaine et al., 2014; Millay et al., 2013), *myomaker* mutant zebrafish are viable and exhibit no obvious defects in locomotion. Thus, our study uncovers conserved elements in the molecular pathway of myocyte fusion as well as evolutionary divergences in the requirement of the fusion process for muscle development and function across different groups of vertebrates.

2. Materials and methods

2.1. Ethics statement

All zebrafish experiments were approved by the Singapore National Advisory Committee on Laboratory Animal Research.

2.2. Zebrafish husbandry and strains

Zebrafish were reared at 28.5 °C unless otherwise stated. Embryos were obtained from natural mating and kept in egg water. The AB strain was used as the wild type strain for all experiments. The *Tg(mylpfa:H2B-GFP)* transgenic line expresses Histone 2B-GFP (H2B-GFP) fusion protein in all the nuclei of fast-twitch muscle cells (Zhang and Roy, 2016). The $jam2a^{hu3319}$ mutant allele was originally described by Powell and Wright (Powell and Wright, 2011), and the generation of both *myomaker* alleles is described below.

2.3. Generation of myomaker mutants with CRISPR/Cas9

The web tool CHOPCHOP (Montague et al., 2014) was used to design sgRNAs for targeting the first (sgRNA1) and last (sgRNA2) exons of *myomaker* gene. sgRNAs were synthesized according to (Bassett and Liu, 2014). In brief, PCR was performed with a unique forward oligonucleotide containing the T7 polymerase binding site and the sgRNA target sequence, and a common reverse oligonucleotide that encodes the remainder of the sgRNA sequence (Table 4). The PCR product was purified (ZYMO RESEARCH, D4005) and used for *in vitro* transcription with the MEGAshortscript T7 kit (Ambion, AM1354M). The sgRNA was then purified according to the manufacturer's instructions. An injection mixture of ~800 ng/μL of Cas9 protein (ToolGen) and ~1000 ng/μL of each sgRNA was incubated at 37 °C for 15 min, and 0.5–0.75 nL of the injection mixture was injected into one-cell stage AB embryos. For detection of fish with edited *myomaker* loci, PCR was performed on genomic DNA of injected fish with primers flanking the target sites of the sgRNAs (Table 4). The resulting PCR products were cloned into TOPO vector and sequenced to determine the mutations.

2.4. Genomic DNA (gDNA) extraction

gDNA extraction from embryos and caudal fins of adult fish was done using the alkaline lysis method (Yu et al., 2014) with some modifications. For 2 d.p.f. embryos, 8–10 embryos were lysed together in 40 μL of lysis buffer (25 mM NaOH, 0.2 mM EDTA) for pooled

Table 4
Primer list.

Primer name	Primer sequence	Notes
tmem8c	GAA ATT AAT ACG ACT CAC TAT AGG GTT TGT GCC TGC AGC	Oligonucleotide used to synthesize
sgRNA_1F	CAG CGG TTT TAG AGC TAG AAA TAG C	sgRNA1 targeting the first exon of myomaker. Underlined sequence is the target sequence.
tmem8c sgRNA_2F	GAA ATT AAT ACG ACT CAC TAT AGG TGC ATC ATA CAC AGC AGC AGG TTT TAG AGC TAG AAA TAG C	Oligonucleotide used to synthesize sgRNA2 targeting the last exon of myomaker. Underlined sequence is the target sequence.
tmem8c_F1	AGT CCA TCC AAG TTC TCC AG	Primers for detecting myomaker mutations
tmem8c_R2	GTC CCT ATC ATT CCC ATT TGC	Primers for detecting myomaker mutations
tmem8c_R3	CAT GCA CAA GCT GCA CTG TA	Primers for detecting myomaker mutations

gDNA extraction. For adult fish, the fish was first anesthetized with Tricaine (200 mg/L) before cutting off a small part of the caudal fin, which was then immersed in 40 μL of lysis buffer. The embryos or fin were lysed in lysis buffer by incubating at 95 °C for 30 min. The gDNA was then cooled down and 40 μL of neutralization buffer (40 mM Tris–HCl, pH 8) was added and mixed well. The gDNA was spun down for a few minutes to pellet the debris and 2 μL was used for PCR.

2.5. Genotyping assays for myomaker alleles

All genotyping assays can be performed from caudal fin or embryonic gDNA. For the *myomaker*^{sq36} allele, a combination of 3 primers (tmem8c_F1, R2 and R3) was used to amplify both the *myomaker*^{sq36} and wild-type alleles in a single PCR reaction. A single 541-bp band indicates a wild-type homozygote, a single 363-bp band indicates a mutant homozygote and amplification of both bands represents a heterozygote. For the *myomaker*^{sq35} allele, *Bgl*I (NEB, R0143L) digestion of an approximately 541-bp PCR product, amplified with tmem8c_F1 and tmem8c_R3, can differentiate between the different genotypes. An undigested 537-bp PCR band indicates a mutant homozygote, complete digestion to 109-bp and 432-bp bands indicates a wild-type homozygote and presence of all 3 bands represents a heterozygote (Table 4).

2.6. Cell transplantations

Cell transplantations were performed when embryos were at the high stage. For transplantations of *myomaker*^{sq36} mutant cells into wild-type AB hosts, we injected rhodamine dextran into progeny of *Tg(mylpfa:H2B-GFP);myomaker*$^{sq36/+}$ incross and transplanted blastomeres from individual donors into AB host embryos. Around 2 d.p.f., we confirmed by transgene expression which donors were mutants and then fixed the respective AB hosts (transplanted with *myomaker*^{sq36} mutant blastomeres) for immunofluorescence analysis. For transplantation of AB cells into *myomaker*^{sq36} mutant hosts, we injected rhodamine dextran into AB embryos and transplanted AB blastomeres into individual progeny of *Tg(mylpfa:H2B-GFP);myomaker*$^{sq36/+}$ incross. At 2 d.p.f., the hosts were sorted out according to transgene expression and *myomaker*^{sq36} mutant hosts were fixed for immunofluorescence analysis.

2.7. Molecular cloning

For cloning of *myomaker* promoter, we PCR-amplified a 3278-bp fragment upstream of the start codon of the *myomaker* coding sequence from AB gDNA and cloned it into pCRII-TOPO vector (Invitrogen, 452640). The *myomaker* promoter and eGFP cDNA (from pEGFP-1; Clontech) were then cloned into pBSKI2 vector (Amgen) by restriction enzymes, with the promoter placed upstream of the eGFP cDNA. To generate the *hsp70:myomaker* construct (for expression of Myomaker under the control of a heat shock promoter), *myomaker* cDNA was amplified from zebrafish embryo cDNA library and cloned into the hspIG vector, which also contains IRES2-eGFP cassette (which acts as a gratuitous reporter for cells expressing Myomaker), by restriction enzymes.

2.8. Heat shock treatment

For embryos injected with the *hsp70:myomaker* construct, heat shock was performed twice in a 37 °C water bath for 1 h at 10–12 somites-stage and 18–20 somites-stage. Embryos were fixed at around 36 h.p.f. for immunofluorescence analysis. For analysis of slow-twitch muscle cells, the embryos were fixed at around 21 h.p.f. instead.

2.9. Antibody staining

Embryos of various developmental stages were fixed in 4% paraformaldehyde (in PBS) for 2 h at room temperature, washed with PBS and then kept in 100% methanol overnight at −20 °C. Fixed embryos were rehydrated in a gradient of methanol/PBS and then permeabilized with acetone. Next, the embryos were blocked with 3% sheep serum in PBDT (PBS, 1% BSA, 1% DMSO and 1% Triton X-100) for an hour at room temperature, followed by primary antibody incubation overnight at 4 °C. The embryos were washed in PBDT and then incubated with secondary antibody for 3 h at room temperature. After PBDT washes, the embryos were stored in 70% glycerol until they are dissected and mounted on slides for microscopy. The following antibodies were used: chicken anti-GFP (1:500, Abcam, ab13970), mAb F310 (1:20, which recognizes fast myosin; Developmental Studies Hybridoma Bank), mAb F59 (1:5, which recognizes slow myosin; Developmental Studies Hybridoma Bank), rabbit anti-β-catenin (1:200, Abcam, ab6302), Alexa Fluor-conjugated secondary antibodies (1:500, Molecular Probes). DAPI (4′,6-diamidino-2-phenylindole, Molecular Probes, #D1306) was used to label cell nuclei.

2.10. Microscopy

Slides with fixed and stained preparations were imaged with an Olympus confocal microscope (Fluoview FV1000). Unless otherwise stated, all images are single confocal sections. For nuclei counts, Z-stacks of 2 μm sections were taken.

2.11. Microinjections

Microinjections were all performed on freshly fertilized zebrafish embryos at the one-cell stage and then kept in egg water until desired developmental stages. For the *hsp70:myomaker* construct, 0.75–1 nL of linearized plasmid (~60 ng/uL) was injected into embryos of various genotypes. For injections of *hsp70:myomaker* into *myomaker*^{sq36} mutants, the mutants were a mixed population, either containing or lacking the *Tg(mylpfa:H2B-GFP)* transgene. For the *myogenin:GFP-caRac* construct, 0.5 nL of plasmid (~50 ng/μL) was injected into embryos from an incross of *Tg(mylpfa:H2B-GFP);myomaker*^{sq35} heterozygotes, and 2 d.p.f. embryos were collected for immunofluorescence analysis. For the *myomaker* promoter reporter construct, AB embryos were injected with 0.5 nL of an injection mix consisting of the plasmid construct (50 ng/μL) and the *I-SceI* meganuclease (7.5 units) in 1X CutSmart Buffer (NEB). The injection mix was incubated at room temperature for 30 min before being used for microinjections.

2.12. Swimming behavior assay

The fish to be tested was first placed into the swim tunnel respirometer (Loligo Systems, Mini Swim-170), with no current flowing, for 15 min to be acclimatized. After acclimatization, the current velocity, which is controlled by a motor, was increased to a motor speed of 500 rpm for 10 min, followed by an incremental increase of 100 rpm every 5 min. The final speed was 900 rpm and successful completion of the whole swimming course would take 30 min. When a fish became tired and unable to continue swimming against the current, it was swept by the current to the downstream screen of the swim tunnel, and remained stuck on the screen. The time was then recorded as its swimming time. For fish that were able to maintain their swimming positions throughout the entire assay, their swimming times were recorded as 30 min.

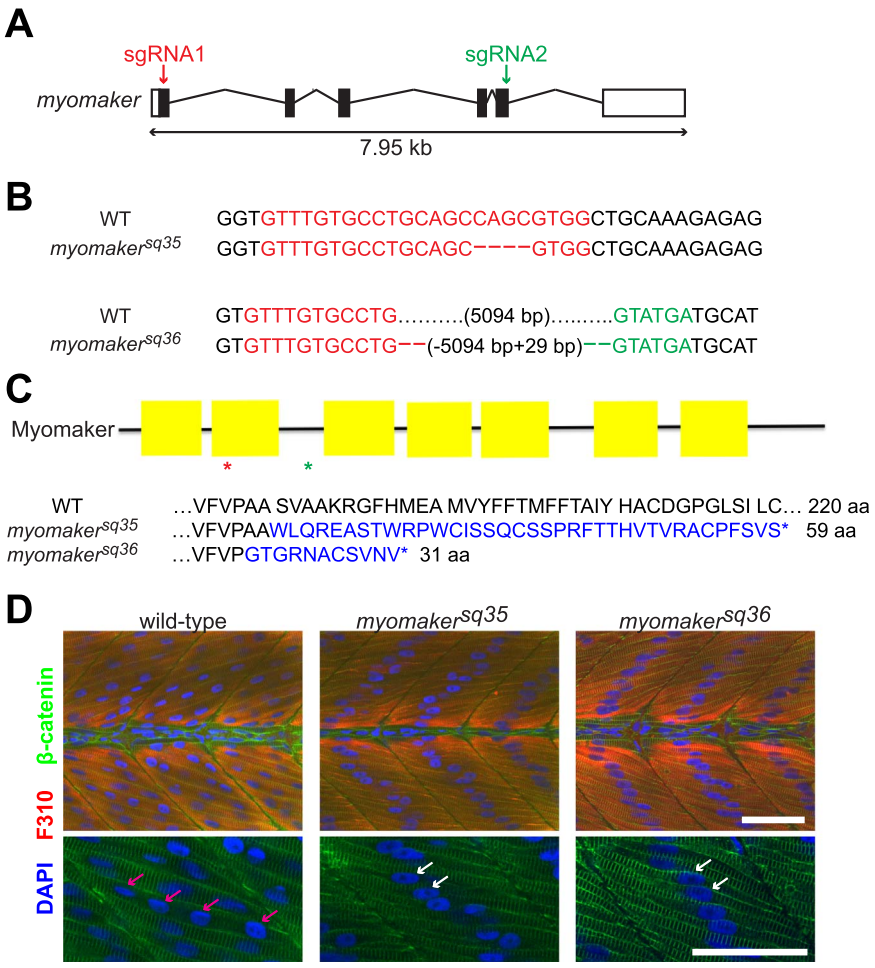

Fig. 1. Myomaker mutants are defective in myocyte fusion. A: Schematic diagram of *myomaker* gene structure. White and black boxes represent UTRs and exons respectively, and the lines indicate introns. The arrows indicate regions targeted by sgRNAs. B: Sequences of wild-type and *myomaker* alleles showing the mutations. Deleted bases are shown as dashes, while sgRNA1 and sgRNA2 target regions are shown in red and green respectively. C**:** Diagram of Myomaker protein structure and predicted protein sequences of *myomaker* alleles. The yellow boxes in the diagram represent the transmembrane domains, while the red and green asterisks indicate the premature STOP codon positions of the *myomaker*^{sq36} and *myomaker*^{sq35} mutant proteins respectively. For the protein sequences, the sequences in blue are those that are different from wild-type sequence. D**:** Fast-twitch muscle cells of 2 d.p.f. *myomaker* mutant embryos are mostly mononucleated, unlike the multinucleated fast-twitch muscle cells of wild-type embryos. Fast-twitch muscle cells are labeled with F310 antibody, with the nuclei and cell membranes stained with DAPI and anti-β-catenin antibodies, respectively. The bottom panel shows magnified images of a few muscle cells from the top panel. The pink arrows indicate the multiple nuclei within a wild-type muscle cell, while the white arrows indicate the single nuclei of mononucleated mutant muscle cells. Scale bar=50 μm.

3. Results

3.1. Myomaker mutants are compromised in fast-twitch myocyte fusion

We generated *myomaker* mutant zebrafish with the CRISPR/Cas9 system, using a pair of short guide RNAs (sgRNAs) targeting the first and last exons of the *myomaker* locus (Fig. 1a). After screening the F0 fish, we obtained 2 different germline-transmitting founders. One founder carried a 4-bp deletion within exon 1, and the other had a large deletion of 5094 bp and small insertion of 29 bp between the first and last exons of *myomaker* (Fig. 1b). We refer to these two distinct alleles as *myomaker*^{sq35} and *myomaker*^{sq36}, respectively. The founders were each outcrossed to wild-type fish to generate the F1 generation, and the F1 fish were then screened for heterozygotes that inherited the *myomaker* mutations. The identified F1 heterozygotes were then incrossed to produce F2 embryos, from which we identified homozygous mutants that were used for subsequent analysis. Zebrafish Myomaker is a 220-aa protein with 7 putative transmembrane domains, and both mutant alleles lead to predicted translation of truncated proteins with only 59 or 31 aa, respectively (Fig. 1c). Therefore, both alleles are likely to be strong loss-of-function.

Consistent with the morpholino data ((Landemaine et al., 2014) and our unpublished observations), when we stained 2-days old embryos with mAb F310, an antibody that recognizes fast-twitch muscle myosin, as well as antibodies to β-catenin, which stain the cell membrane, we found that the fast-twitch muscle cells in homozygous mutant embryos for both alleles of *myomaker* are predominantly mononucleated, unlike the multinucleated fibers in their non-mutant siblings (Fig. 1d). Similar to what has been described for zebrafish embryos deficient in the Jam proteins (Powell and Wright, 2011), even though the fast-twitch muscles in the *myomaker* mutants are mononucleated, their size, shape and arrangement within the myotome are indistinguishable from the multinucleated fast-twitch fibers of wild-type embryos. Thus, Myomaker is necessary for fast-twitch myocyte fusion in the zebrafish embryo.

3.2. Myomaker is not absolutely required for fusion

To test if Myomaker is required cell autonomously for myocyte fusion, we generated chimeric embryos using cell transplantation experiments, in which donor *myomaker*^{sq36} mutant blastomeres were transplanted into wild-type embryos. Unlike in transplantation experiments involving wild-type donors and wild-type hosts, where 96.9% of donor cells fated to be fast-twitch myocytes can fuse with host fast-twitch myocytes (Powell and Wright, 2011), we found that an average

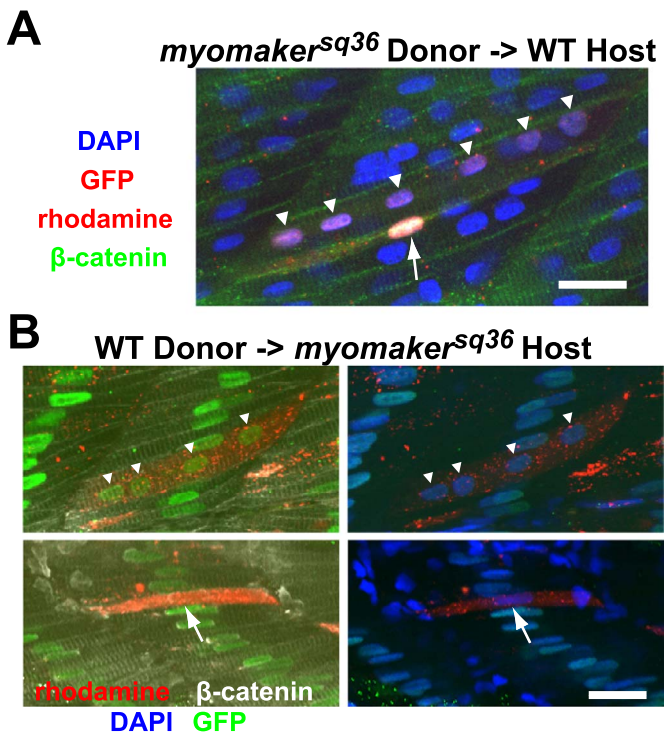

Fig. 2. Myomaker is not required cell autonomously for myocyte fusion. A: A portion of donor *myomaker*^{sq36} mutant cells transplanted into wild-type host can undergo myocyte fusion. The image shows a rhodamine-labeled donor mutant cell, which also expresses H2B-GFP in its nuclei, that underwent fusion to form a multinucleated cell with 6 nuclei (indicated by triangles), and a rhodamine-labeled donor mutant cell that did not undergo fusion and remained mononucleated (indicated by an arrow). All nuclei and cell membranes are stained with DAPI and anti-β-catenin antibodies respectively, while H2B-GFP is labeled with anti-GFP staining. The image is a projection of 2 Z-sections. Scale bar=20 μm. B: Some donor wild-type cells are able to fuse with *myomaker* ^{sq36}mutant cells when transplanted into the *myomaker* ^{sq36} mutant host. Top panel shows a rhodamine-labeled multinucleated chimeric cell expressing H2B-GFP in all its nuclei (indicated by triangles). The chimeric cell resulted from fusion between rhodamine-labeled donor wild-type cell and host mutant cells that express H2B-GFP in the nuclei. The bottom panel shows a rhodamine-labeled donor wild-type cell that did not undergo fusion and remained mononucleated (indicated by an arrow). Nuclei and cell membranes are stained with DAPI and anti-β-catenin antibodies respectively, while H2B-GFP is labeled with anti-GFP staining. The top and bottom panel images are projections of 5 and 3 Z-sections respectively. Scale bar=20 μm.

Table 1
Fusion of donor fast-twitch muscle cells in transplantation experiments. Unfused refers to mononucleated cells and fused refers to multinucleated cells (which includes both donor-donor and donor-host fusion).

Percentage of fused and unfused donor fast-twitch muscle cells				
Embryo #	*myomaker*^{sq36} - > WT		WT- > *myomaker*^{sq36}	
	Unfused	Fused	Unfused	Fused
1	57.1	42.9	74.4	25.6
2	77.3	22.7	83.7	16.3
3	71.4	28.6	78.6	21.4
4	90.0	10.0	100.0	0.0
5	81.8	18.2	64.3	35.7
6	81.8	18.2	81.8	18.2
7	90.3	9.7	100.0	0.0
8	100.0	0.0	63.5	36.5
9	92.9	7.1	100.0	0.0
10	100.0	0.0	85.7	14.3
11	94.7	5.3	85.7	14.3
12	84.4	15.6	100.0	0.0
Average	85.1	14.9	84.8	15.2
Total # of donor muscle cells analyzed	320		380	

of only 14.9% of mutant donor cells that differentiated into fast-twitch myocytes underwent fusion in the wild-type hosts (Fig. 2a, Table 1). Conversely, in the reciprocal experiment in which wild-type donor blastomeres were transplanted into *myomaker*^{sq36} mutant host embryos, an average of only 15.2% of wild-type donor myocytes underwent fusion in the *myomaker*^{sq36} mutant hosts (Fig. 2b, Table 1). These experiments suggest that Myomaker is not absolutely required cell autonomously within a myocyte for it to be able to fuse with wild-type myocytes. However, compared to wild-type donor into wild-type host transplants described previously (Powell and Wright, 2011), the low levels of fusion that occurred in our experiments imply that Myomaker is indeed needed in both fusing myocytes for fusion to take place efficiently.

3.3. Overexpression of Myomaker is sufficient to drive ectopic fusion among the fast-twitch as well as the slow-twitch muscle cells

Overexpression experiments with the zebrafish, mouse and human Myomaker proteins have shown that it is not only sufficient to drive hyperfusion among myocytes, but also the fusion of non-muscle cells, which are not normally fusogenic, with myocytes (Millay et al., 2016, 2013; Mitani et al., 2017). To test the sufficiency of Myomaker function to induce fusion in an *in vivo* context in zebrafish, we generated a construct that allows expression of Myomaker under the control of a heat shock promoter. To aid identification of the cells expressing Myomaker, the construct also expresses eGFP from an IRES2 element. We injected the construct into wild-type embryos and then subjected them to heat shock treatment. We found that overexpression of Myomaker using this strategy was sufficient to drive a proportion (20.9%) of fast-twitch myocytes to undergo excessive fusion, and form muscle cells with an abnormally large number of nuclei (Fig. 3a, Table 2). However, when we used the same construct to express Myomaker in the *myomaker*^{sq36} mutant embryos, only about 8.2% of Myomaker-expressing cells were able to undergo fusion (Fig. 4a, Table 2). Thus the myocyte fusion defects in *myomaker* mutants cannot be fully rescued by expression of Myomaker in a mosaic manner in some fast-twitch myocytes. This is consistent with the notion that Myomaker is required in both myocytes undergoing fusion for the process to occur in an efficient fashion. Besides fast-twitch muscle cells, the zebrafish embryo differentiates a layer of slow-twitch muscles on the surface of the myotome that, unlike their fast-twitch counterparts, do not undergo fusion and remain mononucleated. We also examined whether overexpression of Myomaker in the slow-twitch muscles is sufficient for ectopic fusion of these cells. Strikingly, we found that about 5.8% of GFP-positive slow-twitch muscle cells underwent fusion under this condition; however, all of the fused cells differentiated as binucleated syncytia (Fig. 3b, Table 2). Even though the numbers are small and fusion was limited to the production of only binucleate cells, this result is nevertheless significant given that the slow-twitch muscles in wild-type embryos always differentiate as mononucleate fibers.

3.4. Overexpression of Myomaker can rescue fusion in jam2a mutants

Using the overexpression strategy described above, we next investigated whether overexpression of Myomaker would be sufficient to restore fusion in mutants for the Jam proteins. Fusion is compromised in these mutants presumably because the proper recognition and adhesion among the fast-twitch myocytes, an important prerequisite for fusion, is affected (Powell and Wright, 2011). We found that about 11.2% of the Myomaker-expressing cells underwent fusion in *jam2a*^{hu3319} mutants, and they were mostly binucleated (Fig. 4b, Table 2). Thus, even in a situation where fast-twitch myocyte fusion is significantly compromised due to improper cell-cell adhesion, overexpression of Myomaker is sufficient to bypass this block.

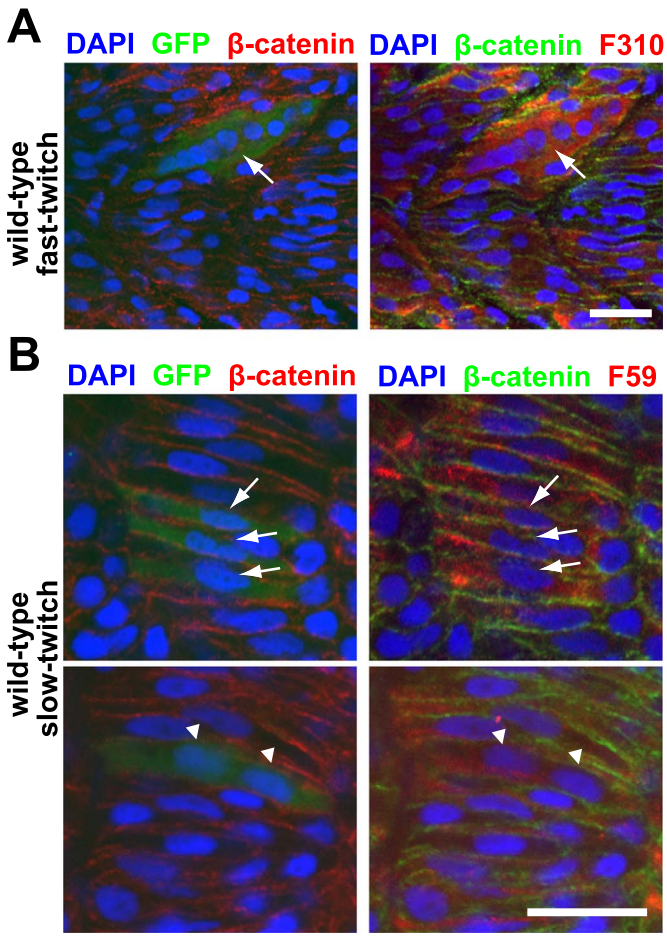

Fig. 3. Myomaker overexpression is sufficient to drive ectopic fusion in both fast-twitch and slow-twitch muscle cells. A: A 36 h.p.f. embryo with a Myomaker-expressing (as reported by anti-GFP staining) fast-twitch muscle cell displaying hyperfusion (arrow). Fast-twitch muscle cells are labeled with F310 staining, and nuclei and cell membranes are labeled with DAPI and anti-β-catenin staining respectively. Scale bar=20 μm. B: A small percentage of Myomaker-expressing slow-twitch muscle cells can fuse. Top panel shows 3 mononucleated Myomaker-expressing slow-twitch cells (indicated by arrows), while bottom panel shows a binucleated Myomaker-expressing slow-twitch cell (indicated by triangles). The embryos are 21 h.p.f. and stained with anti-GFP (to report Myomaker expression) and F59 antibody (to detect slow-twitch muscle cells). Nuclei and cell membranes are labeled with DAPI and anti-β-catenin staining respectively. Scale bar=20 μm.

Table 2
Fusion phenotype of cells expressing heat-inducible Myomaker in different genetic backgrounds.

Percentage of cells with different nuclear number								
Genotype	# of nuclei per cell							
	1	2	3	4	5	6–10	11–14	15–20
WT (fast-twitch muscle cells)	15.5	23.7	23.0	10.8	6.1	18.2	2.7	0.0
WT (slow-twitch muscle cells)	94.2	5.8	0.0	0.0	0.0	0.0	0.0	0.0
myomaker^{sq36}	91.8	5.2	2.6	0.0	0.5	0.0	0.0	0.0
jam2a^{hu3319}	88.8	10.7	0.5	0.0	0.0	0.0	0.0	0.0

Genotype	Total # of cells counted	Total # of embryos analyzed
WT (fast-twitch muscle cells)	148	16
WT (slow-twitch muscle cells)	86	17
myomaker^{sq36}	194	14
jam2a^{hu3319}	206	10

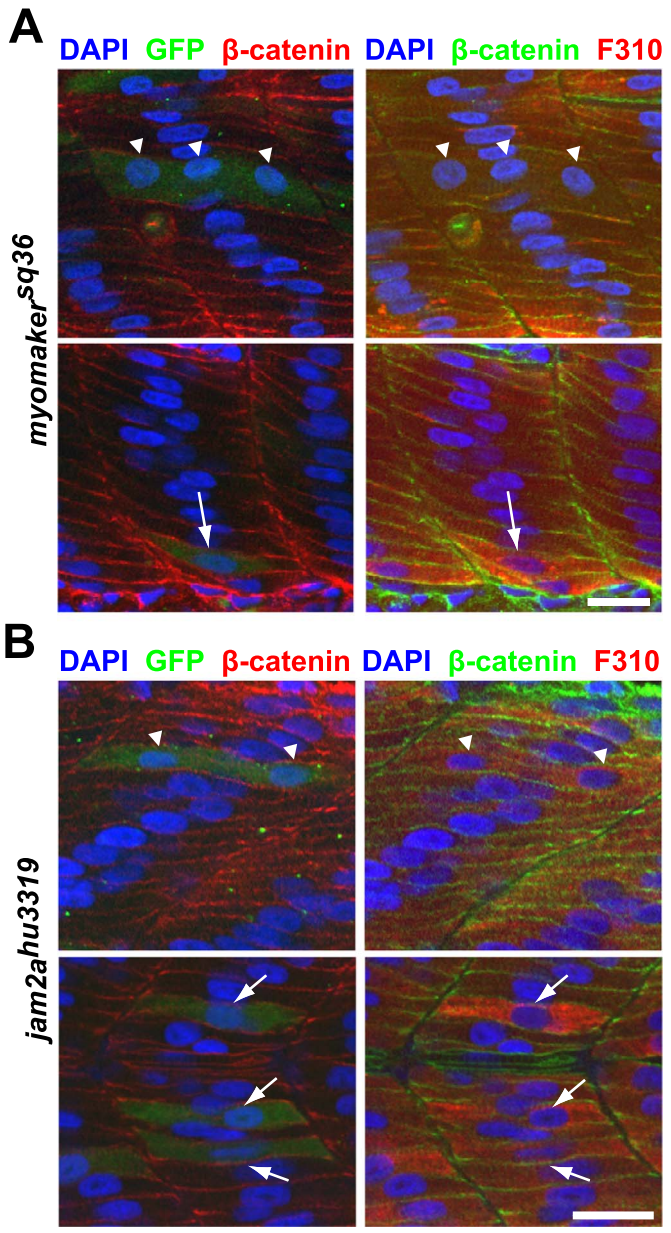

Fig. 4. Myomaker overexpression is sufficient to partially rescue myocyte fusion in *myomaker*^{sq36} and *jam2a*^{hu3319} mutants. A: Myomaker-expressing cells (as reported by anti-GFP staining) in *myomaker*^{sq36}mutant embryos can undergo fusion to form multinucleated fast-twitch muscle cells (Top panel; triangles indicate 3 nuclei within a Myomaker-expressing cell), or remain unfused (Bottom panel; arrow indicates a Myomaker-expressing mononucleated cell). Embryos are 36 h.p.f. and stained with DAPI, anti-β-catenin and F310 antibodies to label nuclei, cell membranes and fast-twitch muscle cells, respectively. Scale bar=20 μm. B: Myomaker overexpression can partially rescue myocyte fusion in *jam2a*^{hu3319} mutants. Top panel depicts a binucleated Myomaker-expressing (reported by anti-GFP staining) fast-twitch muscle cell (nuclei indicated by triangles), and the bottom panel shows 3 mononucleated Myomaker-expressing fast-twitch muscle cells (indicated by arrows). All embryos are 36 h.p.f. *jam2a*^{hu3319} mutants that have also been stained with DAPI, anti-β-catenin and F310 antibodies to label nuclei, cell membranes and fast-twitch muscle cells, respectively. Scale bar=20 μm.

3.5. Constitutively active Rac1 is unable to induce robust ectopic fusion in myomaker mutants

Remodelling of the actin cytoskeleton is an important step in the myocyte fusion pathway. In mammalian myocytes, disruption of the actin cytoskeleton using cytochalasin D or latrunculin B impairs the ability of Myomaker to induce fusion (Millay et al., 2013). As discussed

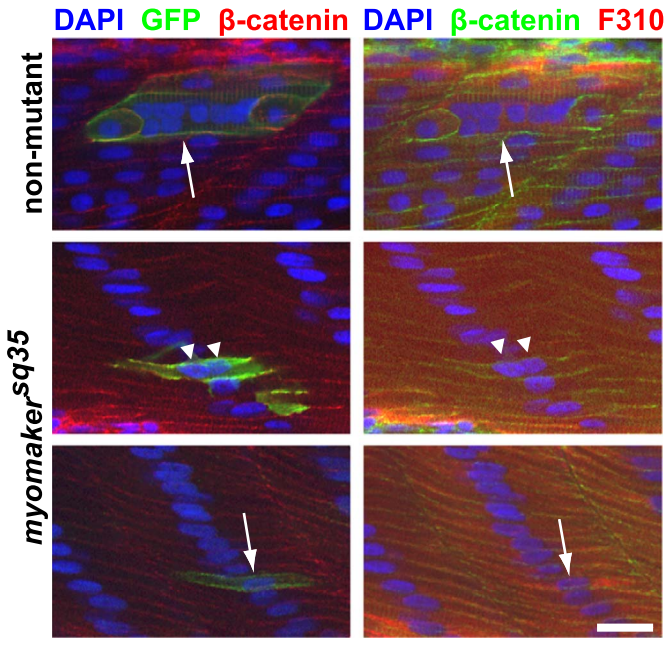

Fig. 5. caRac is unable to induce robust ectopic fusion in *myomaker*^{sq35} mutants. caRac can induce hyperfusion in non-mutant embryos (Top panel; arrow indicates a hyperfused caRac-expressing cell (labeled by anti-GFP staining)), but not in *myomaker*^{sq35} mutants. Most caRac-expressing fast-twitch muscle cells in *myomaker*^{sq35}mutants are mononucleated (Bottom panel; arrow indicates an unfused cell), and a few cells are binucleated (Middle panel; triangles indicate the nuclei of a binucleated cell). All embryos are 2 d.p.f. and also stained with DAPI, anti-β-catenin and F310 antibodies to label nuclei, cell membranes and fast-twitch muscle cells, respectively. The bottom panel images are projections of 2 Z-sections. Scale bar=20 μm.

Table 3
Fusion phenotype of cells expressing caRac in different genetic backgrounds.

Percent of cells with different nuclear number								
Genotype	# of nuclei per cell							
	1	2	3	4	5	6–10	11–14	15–20
myomaker^{sq35}	99.3	0.7	0.0	0.0	0.0	0.0	0.0	0.0
Non-mutant	28.7	25.6	23.3	7.8	6.2	7.8	0.0	0.8

Genotype	# of cells counted	# of embryos analyzed
myomaker^{sq35}	450	8
Non-mutant	129	11

earlier, Rac, a regulator of the actin cytoskeleton, is an intracellular effector of myocyte fusion in all organisms examined (Chen et al., 2003; Hakeda-Suzuki et al., 2002; Luo et al., 1994; Srinivas et al., 2007; Vasyutina et al., 2009). We have previously shown that overexpression of a constitutively active form of human Rac1 (caRac) in zebrafish muscle cells using the *myogenin* promoter can lead to hyperfusion and formation of large syncytia containing supernumerary nuclei (Srinivas et al., 2007). We expressed caRac in *myomaker*^{sq35} mutants, and did not observe any hyperfusion amongst the fast-twitch myocytes that expressed the protein (Fig. 5, Table 3), suggesting that the caRac-dependent hyperfusion is absolutely dependent on Myomaker activity. In addition, there was very little fusion (0.7%) even amongst the caRac-expressing cells themselves (Fig. 5, Table 3), further indicating that caRac is insufficient to drive robust ectopic fusion without wild-type Myomaker function.

3.6. A myomaker promoter fragment with multiple E-boxes is sufficient to direct reporter expression in the fast-twitch muscle lineage

Myomaker expression in the chick and the mouse embryo has been shown to be controlled by MyoD and Myogenin (Luo et al., 2015; Millay et al., 2014), which are muscle-specific transcription factors that bind to E-box elements (CANNTG) in the promoter regions of their target genes. We analyzed the 5′ region of the zebrafish *myomaker* gene and found 23 putative E-box elements within a 3.278 kb region upstream of the start codon of the coding sequence (Fig. 6a). We generated an eGFP reporter construct with this promoter fragment, and when this construct was injected into wild-type embryos, GFP fluorescence was observed specifically in the fast-twitch muscle cells Fig. 6b). This is in keeping with the restricted expression of the *myomaker* gene exclusively in the fast-twitch muscle lineage as observed using *in situ* hybridization ((Landemaine et al., 2014) and our unpublished observations). Thus, not only the sequence, predicted structure and function of the Myomaker protein is highly conserved across the vertebrates, the regulation of *myomaker* gene expression is also highly conserved.

3.7. myomaker mutants are viable without a discernible locomotory defect

Myomaker mutant mice die perinatally, with little muscle movement (Millay et al., 2013). In line with this, zebrafish embryos affected in muscle development exhibit absent or reduced twitching movements during the first few days of embryogenesis, and subsequently show a failure or reduced ability to hatch from their chorions and swim freely (Granato et al., 1996; Naganawa and Hirata, 2011). In contrast, we did not find any twitching, hatching or swimming defects among mutant embryos obtained from incross of *myomaker* heterozygotes (unpublished data). A similar observation has been made for *jam2a*^{hu3319} and *jam3b*^{sa37} mutant embryos in which, despite a similar inhibition of fast-myocyte fusion, locomotory ability does not seem to be affected (Powell and Wright, 2011). To analyse locomotion in our *myomaker* mutants in a more detailed and quantitative manner, we investigated swimming performance of approximately 1 month-old *myomaker*^{sq36} juveniles by placing the fish in a water tunnel with an adjustable current velocity. This is an established experimental paradigm for the quantification of swimming performance in zebrafish (Palstra et al., 2010). In order to maintain its position in the water tunnel, the fish must swim against the controlled water current, whose velocity is increased at fixed intervals for a total duration of 30 min. Both the *myomaker*^{sq36} mutants and their non-mutant siblings were able to successfully maintain their positions in the swim tunnel during most of the experiment, with *myomaker*^{sq36} mutants lasting an insignificantly shorter average time of 27.0 min. This demonstrates that despite the lack of multinucleated fast-twitch muscles, *myomaker* juveniles do not have a major problem in their swimming performance compared to their wild-type siblings, particularly with respect to exertion tolerance (Fig. 7, Student's *t*-test, P=0.25).

4. Discussion

In this paper, we have provided definitive genetic evidence for the requirement of the membrane protein Myomaker in the fusion of fast-twitch myocytes into syncytial muscle fibers in the zebrafish. Using chimeric embryos, we also found evidence that Myomaker activity is necessary in both fusing cells for fusion to progress optimally. Besides the requirement in fusion, we have also demonstrated that Myomaker overexpression is sufficient to trigger hyperfusion among the fast-twitch myocytes, and that it can also instigate fusion in slow-twitch myocytes – a cell type that is normally fusion-incompetent. In addition, we have found that an E-box rich regulatory sequence of the *myomaker* gene is

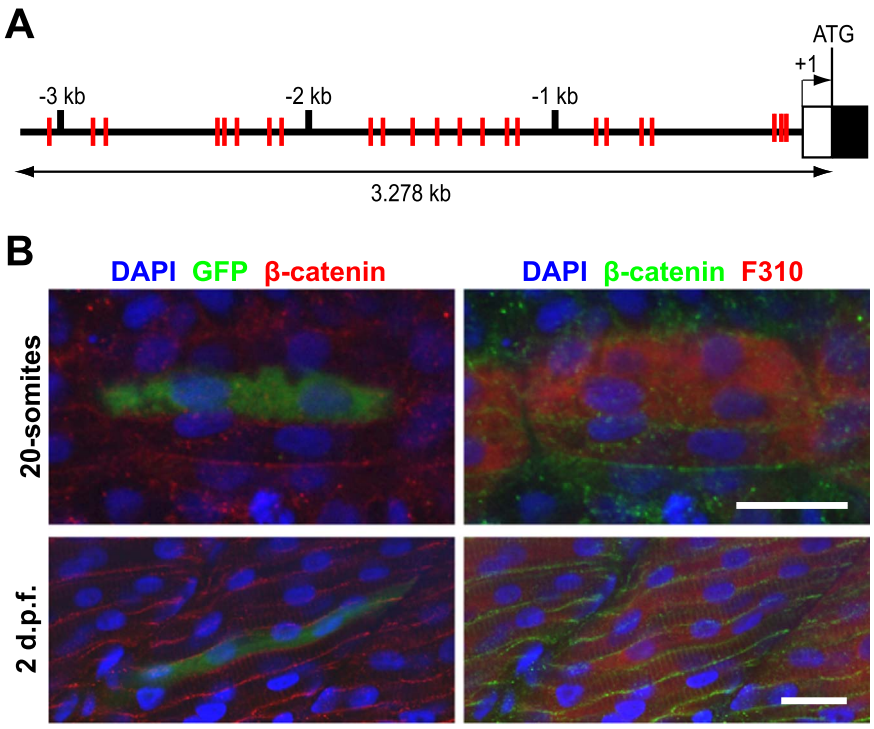

Fig. 6. A *myomaker* promoter fragment with multiple E-boxes is sufficient to direct reporter expression in the fast-twitch muscle lineage. A: Schematic diagram of 5′ genomic region of *myomaker*. The black and white boxes represent Exon 1 and 5′ UTR respectively, while the red lines indicate locations of the 23 E-box sequences. B: The 3.278 kb promoter fragment is sufficient to drive GFP expression (detected with anti-GFP staining) in fast-twitch muscle cells (labeled with F310 antibody), as observed in both 20-somites (Top panel) and 2 d.p.f. embryos (Bottom panel). Nuclei and cell membranes are identified by DAPI and anti-β-catenin antibodies. Scale bar=20 μm.

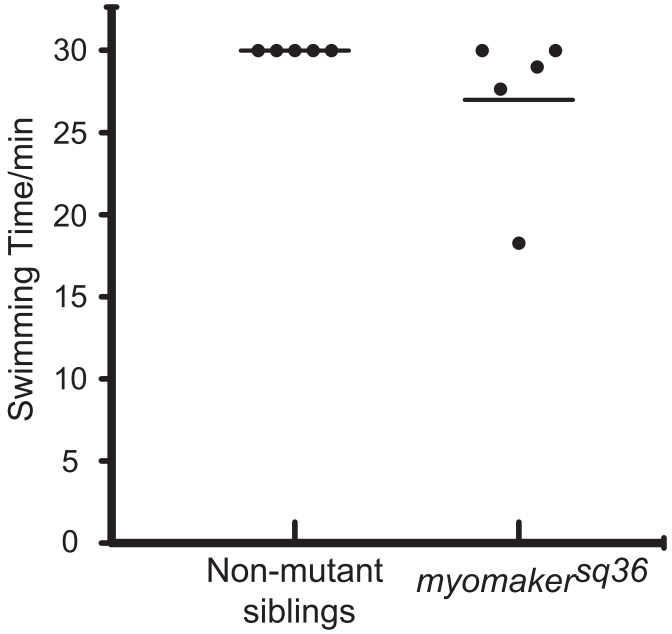

Fig. 7. Swimming performance of *myomaker*^{sq36}mutant juveniles. Swimming times of *myomaker*^{sq36} mutants and their non-mutant siblings in the swim tunnel. 5 juveniles were assessed for each genotype and the average swimming time of *myomaker*^{sq35} mutants (represented as a horizontal line) is not significantly lower than that of the non-mutant siblings (Student's *t*-test, P=0.25).

sufficient to direct expression of the GFP reporter exclusively within the fast-twitch muscle lineage. All of these data are highly congruent with the existing evidence of Myomaker function in the mouse and chick embryo (Landemaine et al., 2014; Luo et al., 2015; Millay et al., 2013, 2014, 2016; Mitani et al., 2017). Based on these observations, we conclude that Myomaker has a highly conserved role in organizing myocyte fusion across vertebrate species, including fishes and mammals.

We have also exploited the ease of genetic manipulation of the zebrafish embryo to further extend our understanding of Myomaker function in myocyte fusion. First, we have been able to show that Myomaker activity is able to bypass the requirement of myocyte adhesion for myocyte fusion, since overexpression of the protein was able to rescue fusion in embryos mutant for the Jam2a junctional adhesion molecule, which has previously been implicated in zebrafish myocyte fusion (Powell and Wright, 2011). Secondly, we have shown that constitutively active Rac1, which can induce hyperfusion among the fast-twitch muscles in wild-type embryos (Srinivas et al., 2007), is unable to do so in the *myomaker* mutants. Both of these findings, together with earlier evidence, strongly implicate Myomaker in having a critical activity in the membranes of the fusing cells. The precise molecular changes brought about by Myomaker on the membranes of fusing myocytes, however, remains an unresolved question.

Based on sequence homology, it has been proposed that Myomaker may belong to a family of transmembrane hydrolases, although it should be noted that a histidine residue that is critical for enzymatic function of these proteins is not conserved in Myomaker (Millay et al., 2013). Structure-function studies have also shown that the C-terminal intracellular domain of Myomaker is required for its activity (Millay et al., 2016). It is likely that this region of the protein links alterations in the membrane to intracellular changes such as the reorganization of the actin cytoskeleton. Further biochemical work will help to elucidate the precise molecular activity of the Myomaker protein with regard to cell-cell fusion. In this connection, it is important to revisit the intriguing ability of Myomaker to induce ectopic fusion. Non-muscle cells expressing Myomaker display enhanced fusion with myocytes but not with each other (Millay et al., 2013, 2016; Mitani et al., 2017). Furthermore, this ability of Myomaker to confer fusion capability to non-muscle cells appears to be limited to the context of skeletal muscles as fusion with cardiomyocytes was not detected in Myomaker-expressing mesenchymal stromal cells transplanted into cardiac tissue in mice (Mitani et al., 2017). Similarly, in our *in vivo* overexpression experiments with the zebrafish embryo, although the

heat-inducible promoter overexpressed Myomaker indiscriminately in multiple cell lineages, we found that the hyperfusion phenotype was restricted to the context of muscle tissue. Together, all of these data reinforce the view that involvement of one or more additional myogenic factor/s is vital for fusogenic activity of Myomaker. Identification of this factor/s will be crucial to fully unravel the function of Myomaker in regulating myocyte fusion.

Finally, our analysis of Myomaker function in the zebrafish has brought into question the biological significance of myocyte fusion. One conceivable advantage for multinucleation is that it allows the differentiation of a highly structured cell, such as a muscle fiber, which needs an amplified transcriptional program to assemble a complex, contractile machinery. It has also been shown that individual nuclei within the muscle fiber syncytium can have distinct transcriptional programs (Bursztajn et al., 1989; Duca et al., 1998; Newlands et al., 1998). This allows the fiber to multitask: for example, transcription of synaptic components in nuclei closer to nerve endings, and transcription of attachment molecules in nuclei in the vicinity of attachment sites. In line with these views, a large body of work on the *Drosophila* larval body wall muscles has shown that fusion is indeed absolutely important for muscle formation and function (Abmayr and Pavlath, 2012; Haralalka and Abmayr, 2010). In mutants that show a strong inhibition of fusion, one class of myoblasts, the “founder cells”, can mature into single-celled fibers whereas the other class of myoblasts, the “feeder cells” that normally fuse with the founders, are unable to differentiate and die by apoptosis (Haralalka and Abmayr, 2010). In *Myomaker* mutant mouse embryos, block in fusion does not inhibit myocyte differentiation, but similar to flies, the single-celled muscles are non-functional and many die by apoptosis (Millay et al., 2013). In contrast to flies and mice, zebrafish embryos deficient in fusion are able to fully differentiate all of the fast-twitch myocytes into single-celled muscles, whose size, shape and organization within the myotome seem indistinguishable from wild-type multinucleated fibers (Powell and Wright, 2011; Rochlin et al., 2010). Given this, and as has been proposed previously, the segregation of fast-twitch myocytes into “founders” and “feeders” is unlikely to be operative in this system. Moreover, the single-celled fast-twitch muscles appear to be fully functional. Thus, uniquely in this organism, fusion is not an absolute requirement to build a functional muscle fiber, although at this point we cannot definitively rule out subtle defects in muscle differentiation and contractility that went undetected in our assays. Interestingly, it has been reported that reduced myocyte fusion of the *Drosophila* smooth-like testes muscles (multinucleated muscles that surround the adult testes) do not affect testes development or fertility, leading to the proposal that the fusion defects are compensated by the other kinds of testes muscles (Kuckwa et al., 2016). A similar compensatory mechanism could potentially also be activated in zebrafish skeletal muscles, in which any contractile deficit arising from the lack of fusion in the fast-twitch lineage is somehow functionally compensated by the slow-twitch muscles. In any case, the zebrafish *myomaker* mutant will be a very useful system to further explore the developmental and physiological significance of myocyte fusion, and how this process may have shaped the myogenic program through evolution in different groups of animals.

Acknowledgments

This work was supported by funds from the Agency for Science, Technology and Research (A*STAR) of Singapore to S. R.

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