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Genetic mutations in *jamb*, *jamc* and *myomaker* revealed different roles on myoblast fusion and muscle growth

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Abstract

Myoblast fusion is a vital step for skeletal muscle development, growth and regeneration. Loss of Jamb, Jamc or Myomaker (Mymk) function impaired myoblast fusion in zebrafish embryos. In addition, *mymk* mutation hampered fish muscle growth. However, the effect of Jamb and Jamc deficiency on fish muscle growth are not clear. Moreover, whether *jamb;jamc* and *jamb;mymk* double mutations have a stronger effects on myoblast fusion and muscle growth remains to be investigated. Here we characterized the muscle development and growth in *jamb*, *jamc* and *mymk* single and double mutants in zebrafish. We found that although myoblast fusion was compromised in *jamb* and *jamc* single or *jamb;jamc* double mutants, these mutant fish showed no defect in muscle cell fusion during muscle growth. The mutant fish were able to grow into adults that were indistinguishable from the wild type sibling. In contrast, the *jamb*;*mymk* double mutants exhibited a stronger muscle phenotype compared to the *jamb* and *jamc* single and double mutants. The *jamb*;*mymk* double mutant showed reduced growth and partial lethality, similar to *mymk* single mutant. Single fiber analysis of adult skeletal myofibers revealed that *jamb, jamc* or *jamb*;*jamc* mutants contained mainly multinucleated myofibers, whereas *jamb*;*mymk* double mutants contained mostly mononucleated fibers. Significant intramuscular adipocyte infiltration was found in skeletal muscles of the *jamb*;*mymk* mutant. Collectively, these studies demonstrate that although Jamb, Jamc and Mymk are all involved in myoblast fusion during early myogenesis, they have distinct roles in myoblast fusion during muscle growth. While Mymk is essential for myoblast fusion during both muscle development and growth, Jamb and Jamc are dispensable for myoblast fusion during muscle growth.

Keywords

Jamb; Jamc; Myomaker; muscle fusion; zebrafish

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Conflict of Interest Statement

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Introduction

Skeletal muscles are composed of arrays of multinucleated myofibers. Each myofiber is assembled from the fusion of mono-nucleated myoblasts, the skeletal muscle precursor cells. The fusion of myoblasts with each other to make multinucleated myofibers or with an existing myofiber to increase the pool of myonuclei in a single fiber is central to muscle development, postnatal muscle growth and muscle repair (Chal and Pourquié, 2017). Defective myoblast fusion could lead to inherited muscle diseases such as congenital myopathies, as recently reported in the Carey-Fineman-Ziter syndrome resulting from defective myoblast fusion (Di Gioia et al., 2017).

Myoblast fusion is a highly regulated dynamic process which involves the migration of myogenic cells and alignment of their membranes close to each other to establish cell-cell recognition and adhesion (Krauss et al., 2017; Rochlin et al., 2010). This is followed by cytoskeleton rearrangements at the contact site, pore formation at the cell membrane, and ultimately exchange of cytoplasmic material leading to cell fusion (Kim et al., 2015). Genetic studies in *Drosophila*, mice and zebrafish have identified numerous genes that play vital roles in myoblast fusion (Abmayr and Pavlath, 2012; Chen and Olson, 2004; Dworak and Sink, 2002).

It has been recognized for over a decade that cell recognition and adhesion are mediated by immunoglobulin domain-containing cell adhesion molecules (CAMs) (Bazzoni, 2003; Ebnet, 2017; Ebnet et al., 2004). A recent study demonstrated that a pair of vertebrate-specific CAMs, Jamb and Jamc, are required for myoblast fusion in zebrafish embryos (Powell and Wright, 2011). Loss of Jamb or Jamc blocked myoblast fusion in zebrafish embryos, resulting in the formation of mononucleatd embryonic muscle fibers (Powell and Wright, 2011). The muscle phenotype in in adult *jamb* and *jamc* mutants has yet to be characterized. Moreover, it is not clear whether loss of *jamb* and *jamc* together in the double mutant could have a stronger effect on myoblast fusion and muscle growth in adult fish.

A major advance in the understanding of myoblast fusion in vertebrates was the recent identification of a transmembrane protein Mymk and its partner Myomixer (Bi et al., 2017; Millay et al., 2013; Quinn et al., 2017; Sampath et al., 2018; Shi et al., 2017; Zhang et al., 2017; Zhang and Roy, 2017). *Mymk* and *Myomixer* are specially expressed in muscle cells during myogenesis. Knockout of *Mymk* or *Myomixer* in mice resulted in defective myoblast fusion and compromised muscle regeneration (Bi et al., 2018; Bi et al., 2017; Millay et al., 2013; Millay et al., 2014; Quinn et al., 2017; Zhang et al., 2017; Zhang and Roy, 2017). The expression and function of Mymk and Myomixer are highly conserved during evolution (Landemaine et al., 2014; Luo et al., 2015). Loss of *mymk* or *myomixer* resulted in defective myoblast fusion in zebrafish (Di Gioia et al., 2017; Shi et al., 2017; Shi et al., 2018; Zhang and Roy, 2017). *mymk* mutant showed reduced muscle growth and increased adipocyte infiltration in the zebrafish skeletal muscle (Di Gioia et al., 2017; Shi et al., 2018). Given that Jamb, Jamc and Mymk play important roles in myoblast fusion in zebrafish, a question has been raised whether Jamb/Jamc and Mymk play similar or distinct roles in myoblast fusion during muscle development and growth.

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To determine Jamb and Jamc function in muscle development and growth and to assess the functional difference between Jamb, Jamc, and Mymk, we characterized the muscle phenotype in the single and double mutants of *jamb*, *jamc* and *mymk*. Our data showed although Jamb and Jamc were initially required for myoblast fusion in embryonic muscle fibers of early stage embryos, they are not essential for myoblast fusion during muscle growth. The single or double *jamb* and *jamc* mutants grew normally into adult fish with multinucleated myofibers. In contrast, the *jamb*;*mymk* double mutant showed reduced muscle growth and partial lethality as observed in the *mymk* single mutant. In addition, most of the skeletal myofibers were mononucleated in the *jamb*;*mymk* adult mutants. Moreover, *jamb*;*mymk* mutant showed increased intramuscular fatty infiltration. Collectively, these studies demonstrate that although Jamb, Jamc and Mymk are all required for myoblast fusion in early myogenesis, loss of Jamb, Jamc and Mymk had different effects on myoblast fusion during muscle growth. Jamb and Jamc are not required for myoblast fusion during muscle growth, whereas Mymk is essential for myoblast fusion during both muscle development and growth.

Results

1. The *jamb*$^{-/-}$ or *jamc*$^{-/-}$ mutant fish showed no visible defects in muscle growth

Muscle growth requires myoblast fusion. To determine whether muscle growth was compromised in *jamb*$^{-/-}$ or *jamc*$^{-/-}$ zebrafish mutants, we performed an in-cross of *jamb*$^{+/-}$ or *jamc*$^{+/-}$ heterozygous mutants and followed the growth performance of these two family populations. A typical Mendelian inheritance pattern was observed in the *jamb* mutant population containing the expected wild type (WT, 25%), *jamb* heterozygous (50%) and homozygous (25%) mutants. However, the *jamc*$^{-/-}$ homozygous mutant showed a lower survival rate. The *jamc*−/− homozygous mutant represented only 9% of the fish population (n=116), much lower than the expected 25% from the Mendelian inheritance. The *jamb*$^{-/-}$ or *jamc*$^{-/-}$ mutant adult fish were indistinguishable from their WT siblings with respect to fish and muscle size (Fig. 1). To determine whether subtle differences could be observed at the cellular levels in their skeletal muscle, the *jamb*$^{-/-}$ and *jamc*$^{-/-}$ mutants were subjected to histological analysis by hematoxylin and eosin (HE) staining. Compared with the WT control (Fig. 1D, G), no significant difference was detected in skeletal muscle tissues of *jamb*$^{-/-}$ (Fig. 1E, H) or *jamc*$^{-/-}$ mutants (Fig. 1F, I). Together, these data indicate that loss of *jamb* or *jamc* had no visible effect on fish muscle growth.

2. The *jamb*$^{-/-}$ and *jamc*$^{-/-}$ mutant fish contained multinucleated myofibers

To determine whether myocyte fusion was compromised in the *jamb*$^{-/-}$ or *jamc*$^{-/-}$ adult mutant fish, we characterized the number of myonuclei in isolated single myofibers from 7 months old adult mutants. The results showed that similar to WT sibling (Fig. 2A), both *jamb*$^{-/-}$ and *jamc*$^{-/-}$ mutants contained multinucleated myofibers in their skeletal muscles (Fig. 2B, C, D), suggesting that although Jamb and Jamc are required for myoblast fusion in zebrafish embryos, they are dispensable for myocyte fusion during muscle growth into multinucleated myofibers in adult muscles.

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To exam when myocyte fusion occurred in *jamb*$^{-/-}$ or *jamc*$^{-/-}$ mutants during muscle development, we examined myoblast fusion in mutant zebrafish embryos expressing a nuclear mCherry. The pmCherry-NLS-C1 DNA construct expressing a nuclear mCherry under the control of the CMV promoter was microinjected into zebrafish embryos at 1–2 cell stages. The injected embryos were fixed at various developmental stages and stained with Hoechst 32258 for nuclei. The results showed that although majority of myofibers in *jamb*$^{-/-}$ or *jamc*$^{-/-}$ contained only one nucleus per fiber at the center of the myotome at 2 days-post-fertilization (dpf) (Fig. 3B, C), a few binucleated myofibers were observed in the *jamb*$^{-/-}$ or *jamc*$^{-/-}$ mutants (Fig. 3B, C). However, the number of fused myofibers was much less compared to WT control (Fig. 3A, G). By 5 dpf, the nuclear localization became broader in the myotome of the *jamb*$^{-/-}$ or *jamc*$^{-/-}$ mutants (Fig. 3E, F, H). Collectively, these studies demonstrated that myoblast fusion occurred relatively early in *jamb*$^{-/-}$ and *jamc*$^{-/-}$ mutant embryos around 2–5 dpf.

3. The *jamb*$^{-/-}$; *jamc*$^{-/-}$ double mutant showed no defective myocyte fusion and muscle growth in adult fish

Jamb and Jamc are closely related members of the JAM family that showed overlap pattern of expression in developing muscles of zebrafish embryos (Powell and Wright, 2011). It has been reported that the interaction between Jamb and Jamc are critical for myoblast fusion in zebrafish embryos (Powell and Wright, 2011). To test whether the lack of muscle defect in *jamb* or *jamc* adult mutant was due to their functional redundancy, we generated *jamb*$^{-/-}$;*jamc*$^{-/-}$ double mutant and characterized the myocyte in mutant embryos and adult myofibers. The results showed that majority of the myofibers were mononucleated myofibers in *jamb*$^{-/-}$;*jamc*$^{-/-}$ double mutant embryos. However, a small population of myofibers contained multiple nuclei were identified at 2 and 5dpf (Fig. 4B, D, E, F). Together, these data indicate that loss of Jamb and Jamc together did not completely block myocyte fusion during early development.

The *jamb*;*jamc* double mutants were viable and grew to similar size as the WT siblings (Fig. 5A, B). Single fiber analysis revealed that skeletal muscles in *jamb*$^{-/-}$;*jamc*$^{-/-}$ adult mutants were mainly multinucleated myofibers (Fig. 5D), which was similar to the WT siblings (Fig. 5C). Histological analysis by HE staining showed no visible difference in skeletal muscles between WT (Fig. 5E, G) and *jamb*$^{-/-}$;*jamc*$^{-/-}$ double mutant (Fig. 5F, H). Together, these data indicate that the loss of both Jamb and Jamc function had no significant effect on muscle growth, suggesting that the lack of muscle defect in *jamb* or *jamc* adult mutant was not due to their functional redundancy, arguing that other regulatory factors are likely to be involved in myoblast fusion during fish muscle growth in adult muscles.

4. The effect of *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutation on myoblast fusion and muscle growth

Recent studies demonstrated Mymk plays a vital role in myoblast fusion and muscle growth in zebrafish (Di Gioia et al., 2017; Shi et al., 2018). Mymk is a transmembrane protein specifically expressed in muscle cells of zebrafish embryos during myogenesis. To assess whether loss of *mymk* and *jamb* could have a stronger effect on myoblast fusion, we generated the *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutants and compared muscle phenotype between *mymk*$^{-/-}$ single and *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutants. The results showed that similar to

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mymk$^{-/-}$ mutant (Fig. 6A, C), *jamb*$^{-/-}$;*mymk*$^{-/-}$ mutant embryos contained exclusively monoucleated myofibers at 2 and 5 dpf (Fig. 6B, D, E), suggesting that loss of *jamb*;*mymk* resulted in similar defective myoblast fusion as *mymk* mutation.

The muscle phenotype was further characterized later in adult mutants. The *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutant fish showed poor growth and decreased survival rate. Compared to the WT siblings with an average weight of 0.391 g (n=10) at 7 month old (Fig. 7A), the *mymk*$^{-/-}$ single (Fig. 7B) and *jamb*$^{-/-}$;*mymk*$^{-/-}$ double (Fig. 7C) mutants weighed 0.170 (n=10) and 0.163 g (n=10), respectively. In addition, a craniofacial abnormality with an open lower jaw defect was observed in the *mymk*$^{-/-}$ or *jamb*$^{-/-}$;*mymk*$^{-/-}$ mutants (Fig. 7B, C). Single fiber analysis revealed that *jamb*$^{-/-}$ single and *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutants contained mostly mononucleated myofibers in 7 months old fish (Fig. 7E, F). Those developmental and growth defects were very similar, if not identical, to the phenotype we previously observed in the *mymk*$^{-/-}$ single mutant (Shi et al., 2018).

To further characterize the muscle phenotype in the mutant fish, we performed histological analysis by HE staining in adult fish at 7-month old. The results showed that the skeletal muscles were severely malformed in *mymk*$^{-/-}$ single (Fig. 7H, K) and *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutants (Fig. 7I, L). Moreover, the *mymk*$^{-/-}$ and *jamb*$^{-/-}$;*mymk*$^{-/-}$ mutant fish had an increased adipocyte infiltration in their skeletal muscles (Fig. 7K, I). Collectively, these data indicate that loss of *jamb* and *mymk* together gave rise to similar developmental and muscle defects as observed in *mymk* mutant, arguing that Mymk plays a dominant and essential role over Jamb in myoblast fusion and muscle growth.

5. The effect of *jamb* expression on fast muscle specific fusion

Zebrafish embryonic muscles can be divided into single nucleated slow fibers and multinucleated fast fibers (Devoto et al., 1996). It has been reported that *jamb and mymk* expression in zebrafish embryos was restricted to the fusion competent fast muscle precursor cells (Landemaine et al., 2014; Zhang and Roy, 2017). Our previous studies showed that overexpression of *Shh* in zebrafish embryos could block *mymk* expression in fast muscle precursors and inhibit myoblast fusion (Currie and Ingham, 1996; Du et al., 1997; Shi et al., 2018). To determine whether overexpression of *shh* could inhibit *jamb* gene expression in zebrafish embryos, *shh* mRNA was microinjected into zebrafish embryos. *Jamb* expression was analyzed by whole mount *in situ* hybridization. Compared with the un-injected control (Fig. 8A), overexpression of *shh* dramatically decreased *jamb* expression in fast muscles of zebrafish embryos (Fig. 8B), indicating that fast muscle specific expression of *jamb* may be involved in fusion competency of fast fibers.

To determine whether ectopic expression of *jamb* in slow muscle cells was able to induce myoblast fusion in slow muscles, *jamb* mRNA was microinjected alone or with pmCherry-NLS-C1 into *jamb* mutant or WT zebrafish embryos. Microinjection of *jamb* mRNA was able to rescue the fusion defect in fast muscles of the jamb mutant embryos (data not shown), indicating that the injected *jamb* mRNA was functional. Myonuclei in slow muscles were detected by anti-prox-1 antibody staining or nuclear mCherry localization at 24 and 48 hpf, respectively. Immunostaining revealed that slow myofibers of un-injected embryos were exclusively mononucleated fibers (Fig. 8C, D). Similar to the un-injected control, slow fibers

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in *jamb* mRNA injected embryos contained only one nucleus per fiber (Fig. 8E). Data from the mCherry nuclear localization further confirmed the lack of multinucleated fibers in slow muscles of embryos injected with *jamb* mRNA and pmCherry-NLS-C1 DNA construct (Fig. 8F).

To test whether co-expression of *jamb* with *mymk* was required for myoblast fusion, we co-injected *jamb* and *mymk* mRNAs into WT zebrafish embryos. The data revealed that similar to un-injected control (Fig. 8C, D), no multinucleated fibers were detected in the *jamb* and *mymk* co-injected embryos (Fig. 8G, H). Together, these data indicate that ectopic expression of *jamb*, *mymk* or both in slow muscle precursors was not sufficient to induce myoblast fusion in slow fibers of zebrafish embryos.

Discussion

In this study, we analyzed the effect of *jamb, jamc* and *mymk* single or double mutations on myoblast fusion and fish muscle growth. Our studies demonstrated that loss of *jamb* and *jamc* had a temporarily effect on myoblast fusion in early stage zebrafish embryos. The fusion defect quickly recovered in *jamb* and *jamc* mutant embryos. We provided new evidence that the lack of visible defects in *jamb* or *jamc* adult mutants was not due to functional redundancy between Jamb and Jamc because the *jamb*;*jamc* double mutants were able to grow into normal size adult zebrafish with multinucleated skeletal myofibers. In contrast to *jamb* and *jamc* mutants, loss of *mymk* alone or together with *jamb* had a long lasting effect on myoblast fusion and muscle growth. The *mymk* and *jamb;mymk* mutants were smaller in size and showed poor muscle growth. Together, these data indicate that although Jamb, Jamc and Mymk are all involved in initial myoblast fusion during early development, however, they have different requirement for myocyte fusion during muscle growth. This current study generated the unique *jamb*;*jamc* and *jamb*;*mymk* double mutants, thus further extended the previous findings in the *jamb*, *jamc* or *mymk* single mutants (Di Gioia et al., 2017; Powell and Wright, 2011; Shi et al., 2018; Zhang and Roy, 2017).

Cell adhesion and myoblast fusion

Myoblast fusion requires cell-cell interactions between myoblasts or myoblast with myofibers (Krauss et al., 2017). Cell-cell interactions are regulated by adhesion molecules of the immunoglobulin (Ig) superfamily (IgSF) (Bazzoni, 2003; Ebnet, 2017; Ebnet et al., 2004). Genetic analyses in *Drosophila* have shown that numerous members of the IgSF play important roles in mediating myoblast adhesion. These IgSF members include Sticks and stones (sns), Dumfounded (Duf)/Kin-of-IrreC (Kirre) and Irregular Chiasm-C (IrreC)/ Roughest (Rst) (Bour et al., 2000; Ruiz-Gómez et al., 2000; Strünkelnberg et al., 2001).

Jamb and Jamc are members of IgSF that are single transmembrane proteins specifically enriched at tight junctions (Ebnet, 2017; Ebnet et al., 2004). It has been shown that Jamb ad Jamc exist only in vertebrates (Ebnet, 2017; Williams and Barclay, 1988). The defective myoblast fusion in *jamb* and *jamc* zebrafish mutants is consistent with the importance of IgSF mediated cell adhesion in myoblast fusion (Powell and Wright, 2011). It has been shown by elegant transplantation experiments that the Jamb and Jamc must interact between neighboring cells (in trans) for fusion to occur (Powell and Wright, 2011). We showed here

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that *jamb*;*jamc* double mutant exhibited a similar muscle phenotype as the *jamb* or *jamc* single mutant, indicating the lack of functional redundancy. This is in agreement with the mode of trans-heterophilic interaction required for myoblast fusion (Powell and Wright, 2011).

We demonstrated in this study that loss of *jamb*, *jamc* or both had no effect on zebrafish muscle growth. The mutant fish contained multinucleated myofibers and were similar in size and weight compared with WT siblings. This differs from the size phenotype in *Jamc* mutant mice which showed mild growth retardation (Ye et al., 2009). Given that myoblast fusion involves several other members of the IgSF family, such as Duf/Kirre and IrreC/Rst, it will be interesting to test whether these genes play a redundant role as Jamb/Jamc in zebrafish. Interesting, a MO knockdown study indicated that Duf/Kirre was required for myoblast fusion (Srinivas et al., 2007). However, this was not observed in the *kirrel3l* mutant generated by CRISPR technology (Hromowyk, 2017). The *kirrel3l* mutants are indistinguishable from wild-type siblings with respect to embryonic myoblast fusion, muscle growth and repair (Hromowyk, 2017).

jamc$^{-/-}$ homozygous zebrafish mutant showed a reduced survival rate which has been observed in *Jamc* knockout mice, in which two-third of *Jamc* deficient mice died within 48 hours after birth (Imhof et al., 2007; Praetor et al., 2009; Ye et al., 2009). The cause of death is not clear although opportunistic infection or neuromuscular defect have been suggested as potential causes in mice (Scheiermann et al., 2007). Expression analysis revealed that unlike *jamb* which showed a restricted expression in zebrafish fast muscles, *jamc* was expressed in a broad pattern in many tissues of the fish embryos. It is likely that Jamc may function in other tissues or organs, the death could be due to multiple etiologic factors. Consistent with h tis idea, previous studies in humans revealed that JAM-3, the Jamc homolog, is a candidate gene for heart defects in Jacobsen syndrome, a 11q terminal deletion disorder (11q-) which could make a breakpoint to JAM-3. However, Jamc mutant mice that survived to adulthood have normal cardiac structure and function (Ye et al., 2009). It remains to be determined whether Jamc may function in heart development in zebrafish.

Myomaker function in myoblast fusion

We further showed in this study that loss of *mymk* and *jamb* together gave a similar muscle defect as the *mymk* mutant. No additive phenotype was detected in the *mymk*;*jamb* double mutant, suggesting that Mymk plays a dominant role in this process. It has been demonstrated that ectopic expression of *mymk* could rescue the myoblast fusion defects in *jamb* mutant embryos (Zhang and Roy, 2017), reinforcing the idea that Mymk may function downstream of Jamb and induce myoblast fusion directly without Jamb mediated cell-cell adhesion. Supporting this notion, we showed that loss of Jamb and Jamc only temporarily hampered myoblast fusion in fish embryos. It had not effect on myoblast fusion during muscle growth. In contrast, Mymk is essential for myoblast fusion during both embryogenesis and muscle growth, and Mymk can drive myoblast fusion to promote muscle growth without Jamb and Jamc.

At present, the molecular mechanism by which Mymk regulates myoblast fusion is not clear. It has been demonstrated that Mymk was able to induce fusion of fibroblasts with muscle

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cells, but not between fibroblasts themselves (Millay et al., 2013), suggesting that other factors may be required to work together with Mymk to promote myoblast fusion. Recent studies showed that *Myomixer*, also known as *Minion* or *Myomerger*, are specifically expressed in fusogenic muscles of mouse and zebrafish embryo, and plays a key role in myoblast fusion (Bi et al., 2017; Quinn et al., 2017; Shi et al., 2017; Zhang et al., 2017). Little is known, however, how Mymk and Myomixer are integrated in the other genetic pathways that regulate myoblast fusion. Previous studies showed that remodeling of the actin cytoskeleton is a critical step in the myocyte fusion. Disruption of the actin cytoskeleton in C2C12 cells using cytochalasin D or latrunculin B diminished the ability of Mymk to induce fusion (Millay et al., 2013). It has been reported that overexpression of a constitutively active form of human Rac1 (caRac), a regulator of the actin cytoskeleton, could stimulate myoblasts hyperfusion in zebrafish embryos to form large syncytia containing supernumerary nuclei (Srinivas et al., 2007). However, this hyperfusion activity of caRac was not observed in *mymk* mutant zebrafish embryos (Zhang and Roy, 2017). Collectively, these data indicate that the caRac-dependent hyperfusion is absolutely dependent on Mymk activity.

Defective skeletal muscle growth and fatty infiltration in *jamb*;*mymk* double mutant

We showed here that zebrafish *jamb*;*mymk* double mutants were able to survive into adult although at a decreased survival rate comparable with the WT siblings. However, the survival rate was similar to the *mymk* single mutant, suggesting that *jamb* mutation had no additive effect on *mymk* mutant survival. The *jamb*;*mymk* double mutants were smaller in size and showed poor muscle growth. Increased adipocyte infiltration was detected in skeletal muscles of *jamb*;*mymk* double mutants. This is not surprising given that similar muscle defects were observed in the *mymk* zebrafish mutants (Addison et al., 2014; Di Gioia et al., 2017; Shi et al., 2018). The characteristic muscle defect referred as intermuscular adipose tissue (IMAT) accumulation has been observed in CFZS patients with *Mymk* mutations and also in variety of myopathies (Addison et al., 2014). The mechanism underlying the fatty infiltration is not clear. It is generally believed that adipose replacement is an unspecific response to defective muscle growth due to pathological processes from muscle diseases or chronic-disuse (Dulor et al., 1998; Goodpaster et al., 2000, Manini et al., 2007).

The effect of *jamb* and *mymk* expression pattern in fiber type specific fusion

It is known that zebrafish embryonic slow fibers are single nucleated fibers, whereas the fast muscles are multinucleated myofibers generated from myoblast fusion (Devoto et al., 1996). The molecular mechanism underlying the fast muscle-specific fusion is unclear. It has been suggested that distinct patterns of gene expression involved in myoblast fusion might be involved (Powell and Wright, 2011; Zhang and Roy, 2017). Consistent with this idea, several fusion regulators such as *jamb*, *kirrel* and *mymk*, are exclusively expressed in fusion competent fast muscle precursors in zebrafish embryos (Powell and Wright, 2011; Srinivas et al., 2007; Zhang and Roy, 2017). Our previous studies demonstrated that overexpression of *shh* inhibited *mymk* expression in fast muscle precursor cells (Shi et al., 2018). Moreover, overexpression of *shh* blocked myoblast fusion in zebrafish embryos (Shi et al., 2018). Together with our finding that Shh inhibited *jamb* expression, these studies indicate that the

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fiber type specific myoblast fusion may be determined in part by the fast muscle specific expression of these fusion regulators in zebrafish embryos. However, we showed in this study that overexpression of *jamb*, or *mymk and jamb* together was unable to induce myoblast fusion in slow fibers, suggesting that additional factors are likely to be involved in determining the fiber type specific fusion.

Materials and methods

Zebrafish lines and maintenance

The *jamb*^{HU3319} and *jamc*^{sa0037} zebrafish mutants were obtained from Dr. Gavin Wright's laboratory at the Wellcome Trust Sanger Institute, UK. The *jamb*$^{-/-}$;*jamc*$^{-/-}$ double mutant was generated in our laboratory by crossing *jamb*^{HU3319} with *jamc*^{sa0037}. The *mymk*^{mb14} mutant was generated by CRISPR-Cas9 in our laboratory (Shi et al., 2018). The *jamb*$^{-/-}$;*mymk*$^{-/-}$ double mutant was generated in our laboratory by crossing *jamb*^{HU3319} with *mymk*^{mb14}. All fish were raised at the Zebrafish Facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology (Baltimore, MD). The fish were maintained at 28°C with a photoperiod of 14 h light and 10 h dark in 8-gallon aquaria supplied with freshwater and aeration. All animal studies were carried out in accordance to the guideline for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland (Permit Number 0516005).

DNA isolation from fish embryos and caudal fin clips for genotyping by PCR

For genotyping, genomic DNA was isolated from zebrafish embryos or caudal fin clip of adult fish using the alkaline method (Cai et al., 2018; Shi et al., 2018). 1 μl of the DNA lysate was used for each PCR reaction. The PCR was carried out using the Jamb-F2/R2 or Jamc-F2/R2 primers (Table 1), followed by Sanger sequencing or digestion with SfaNI, respectively for Jamb and Jamc PCR products.

Single muscle fiber isolation and staining

Adult zebrafish of 7 months old were euthanized in 0.6 mM Tricaine and then fixed in 4% paraformaldehyde (1×PBS) for 1 hour at room temperature. Single fiber isolation was performed as described (Li et al., 2017; Shi et al., 2018). The dissected myofibers were placed on a slide and stained with 50ng/ml of Phalloidin-TRITC conjugate (P1951, Sigma) and 1μg/ml of Hoechst 32258 (Shi et al., 2018). The fibers were mounted in Vectashield (Vector lab, H-1000), and photographed using a Leica SP8 confocal microscope.

Whole mount Phalloidin-TRITC and Hoechst 32258 staining

Whole mount staining with Phalloidin-TRITC and Hoechst 32258 was carried out on zebrafish embryos at 24–120 hpf. Embryos were anesthetized in a 0.6 mM Tricaine solution and fixed in 4% paraformaldehyde for 1 h at room temperature. After washed with PBS containing 0.1% Tween-20 (PBST) for three times (10 min each), the embryos were digested with collagenase (Sigma C9891) and treated with cold acetone to increase the permeability. The embryos were washed with PBST for 30 min and stained with Phalloidin-TRITC and Hoechst 32258 for 20 min. After washed with PBST three times (30 min each), trunk

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regions of fish embryos were dissected and mounted in Vectashield (Vector lab, H-1000). The trunk muscles were photographed using a Leica SP8 confocal microscope.

Isolation of *jamb* and *jamc* cDNA clones and construction of *jamb* expression vector

The full length cDNA coding sequence of zebrafish jamb (NM_001098264.1) and jamc (NM_001082863.3) were cloned from zebrafish by RT-PCR. Total RNA was isolated from zebrafish embryos at 24 hpf using the Trizol reagents (Invitrogen). The PCR was carried out using zfjamb-F1/R1 or zfjamcF1/R1 primers (Table 1), respectively. The high fidelity Phusion DNA polymerase (ThermoFisher Cat#F530S) was used. The DNA products were treated with Taq DNA polymerase to add an extra A at the 3' end, and followed by cloning into the pGEM-T easy vector to generate the pGEM-T-zfjamb and pGEM-T-zfjamc plasmid constructs, respectively. To generate *jamb* mRNA for microinjection, the pGEM-T-zfjamb plasmid construct was linearized with EcoRI enzyme and followed by purification with QIAquick Gel Extraction Kit (QIAGEN). The purified *jamb* DNA insert was then subcloned into the EcoRI site of the $CS2^+$ vector to generate the expression $pCS2^+$-zfjamb vector.

In vitro transcription of mRNA for microinjection

The *myomaker* mRNA was synthesized by *in vitro* transcription using the mMESSAGE mMACHINE Sp6 kit (Ambion #1340) (Shi et al., 2018). The $pCS2^+$-zfjamb vector linearized with NotI was used as template for *in vitro* transcription using the mMESSAGE mMACHINE Sp6 kit (Ambion #1340). The capped *mymk* and *jamb* mRNAs were purified using the MEGAclear™ Transcription Clean-Up Kit (Ambion #1908).

Microinjection in zebrafish embryos

The pmCherry-NLS-C1 was obtained from Shengyun Fan's laboratory at University of Maryland. The plasmid construct (50 ng/μl) was microinjected into zebrafish embryos at one-cell stage. Each embryo was injected with approximately 2 nl plasmid (~100pg DNA) (Qiu et al., 2014; Guan et al., 2014) The *jamb* or *mymk* mRNAs were injected into the zebrafish embryos at one-cell stage. The final concentration of the *jamb* mRNA was 350 ng/μl, and the final concentration of *mymk* mRNA was 350 ng/μl. For co-injection of *jamb* and *mymk* mRNAs, the *jamb* mRNA (350ng/μl) was mixed with equal volume of *mymk* mRNA (350 ng/μl). Approximately 2nl of the mRNA mix was microinjected into each embryo. For co-injection with pmCherry-NLS-C1, the pmCherry-NLS-C1 plasmid was added to above *jamb* and *mymk* mRNAs to the concentration of 50 ng/μl. The DNA/mRNA mixture was microinjected into zebrafish embryos as described above.

Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was carried out using digoxigenin-labeled antisense probes as previously described (Du et al., 2008). The digoxigenin-labeled probes of *Smyhc, fmyhc* and *mymk* have been described previously (Li et al., 2013; Shi et al., 2018; Xu et al., 2012). The pGEM-T-zfjamb and pGEM-T-zfjamc were linearized with NcoI. The linearized DNAs were purified using the gel extraction kit (QIAGEN). The purified DNAs were used as template for *in vitro* transcription with Sp6 RNA polymerase to synthesize digoxigenin-

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labeled antisense RNA probes. The antisense digoxigenin-labeled probes wer4e purified using the MEGAclear™ Transcription Clean-Up Kit (Ambion #1908).

Whole mount immunostaining

Immunostaining was carried out using whole-mount zebrafish embryos at 24 and 48 hours post-fertilization (hpf) as previously described (Tan et al., 2006; Xu et al., 2012). Embryos were anesthetized in a 0.6 mM Tricaine and fixed in 4% paraformaldehyde for 1 h at room temperature. After washed with PBS containing 0.1% Tween-20 (PBST) for three times (20 min each), embryos of 48 hpf were digested with 1 mg/ml collagenase for 45 min at room temperature. Immunostaining was performed with the anti-MyHC (F59) and anti-prox-1 antibodies, and followed by Alexa Fluor® 488 Goat Anti-Mouse or Alexa Fluor® 552 Goat Anti-Rabbit antibodies (A-11001, A31630, ThermoFisher Scientific). Trunk regions of fish embryos were dissected and mounted in Vectashield (Vector lab, H-1000). The trunk muscles were photographed using a Leica SP8 confocal microscope.

Histology analysis

Adult fish of 7 months old were euthanized in 0.6 mM Tricaine solution. The trunk region of ~5 mm thick were dissected from the trunk region spanning the entire dorsal fin and fixed in Bouin's solution for 24 h. The fixed specimen were dehydrated in ethanol gradient and embedded in paraffin. The paraffin was sectioned on a HM340 microtome (MICROM) to produce 7 μm cross sections. The sections were stained with hematoxylin/eosin and photographed using a DP70 Olympus camera.

Statistical analysis

All the results were expressed as mean ± SEM. Differences between WT and mutants were analyzed by One-Way Analysis of Variance (ANOVA) followed by Student-Newman-Keuls' test, or by "t" test between two groups. A p value of <0.05 was considered as the level of significance.

Acknowledgments

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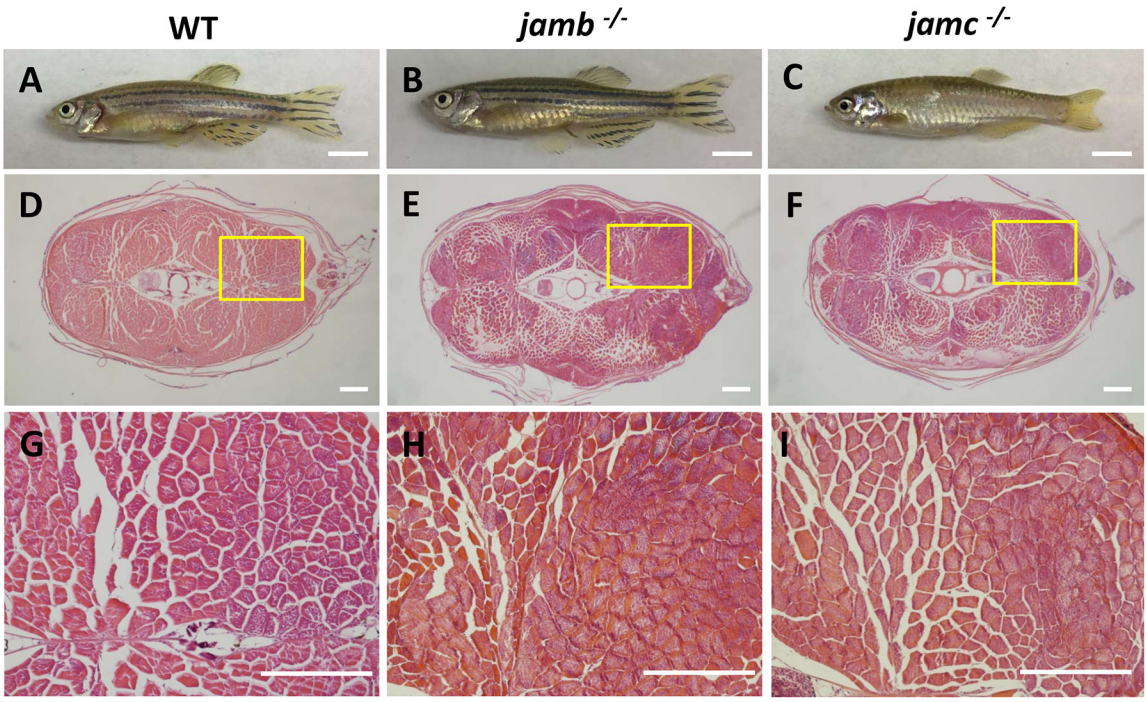

Fig. 1. Loss of *jamb or jamc* had no effect on fish muscle development and growth

A-C: Morphological comparison of WT control (A), *jamb* (B) *or jamc* (C) mutant fish at 7 months old. Scale bar (500 mm).

D-I: HE staining showing cross sections of trunk skeletal muscles in WT control (D, G), *jamb* (E, H) or *jamc* (F, I) mutant fish of 7 months old. Scale bar (250 μm).

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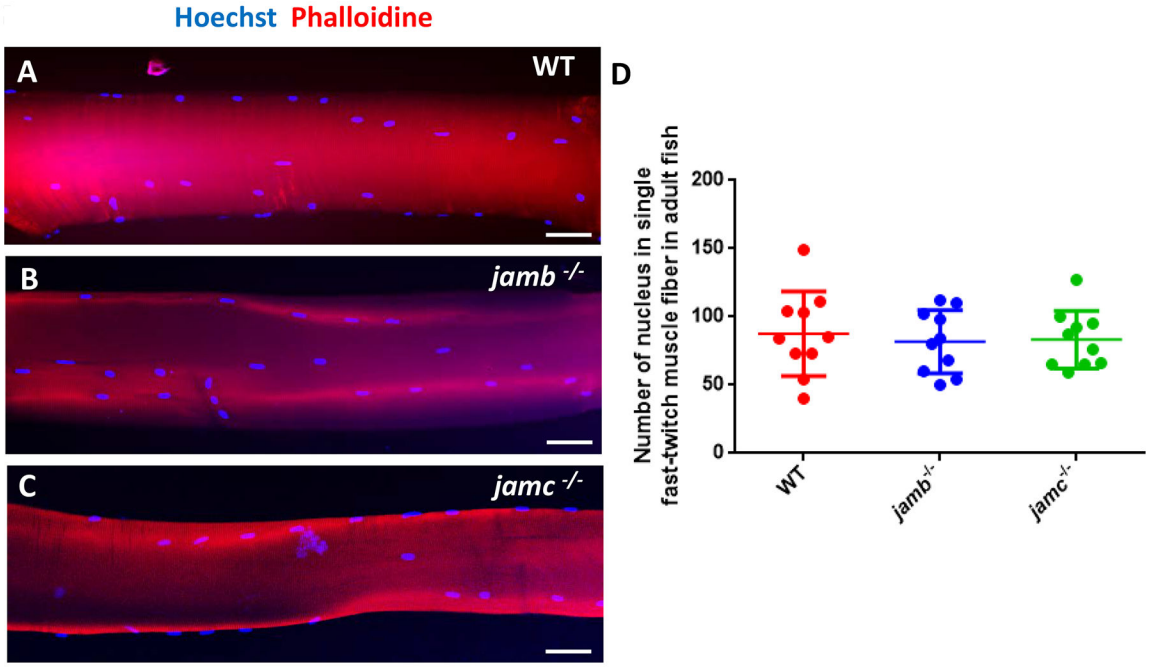

Fig. 2. Single fiber analysis of myonuclei in skeletal muscles from adult WT, *jamb* and *jamc* mutants

Single fibers were isolated from skeletal muscles of WT adult fish (A), *jamb* (B) or *jamc* (C) mutants at 7 months old. The isolated single fibers were stained with Hoechst 32258 and phalloidin-TRITC, and photographed under a confocal microcopy. Statistical analysis was performed to compare the numbers of nucleus in multiple myofibers from the three different groups. No significant difference was detected in myofibers from these three groups of fish. Scale bar (50 μm).

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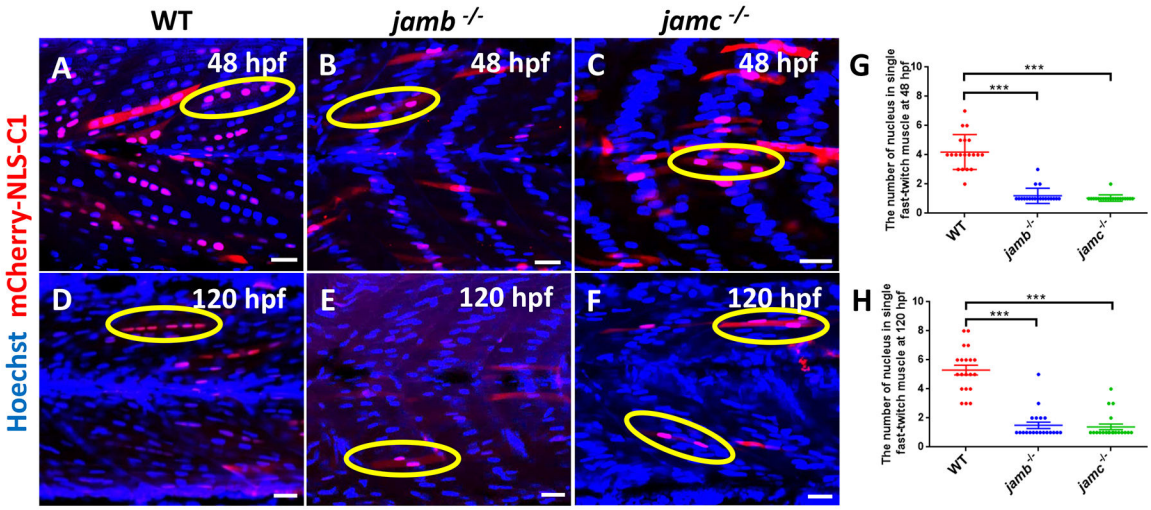

Fig. 3. Characterization of myoblast fusion in *jamb* or *jamc* mutant embryos

Myoblast fusion was analyzed in WT, *jamb* or *jamc* mutants by nuclear labelling with Hoechst 32258 (nuclei, blue) and nuclear mCherry (mCherry-NLS-C1; red) at 48 (A-C) and 120 (D-F) hpf. **A:** WT control contained mostly multinucleated fast myofibers at 48 hpf. **B, C:** Myofibers in *jamb* (B) or *jamc* (C) mutant were mostly mononucleated fibers with a central nuclear localization within the myotome. However, a small number of binucleated myofibers were observed in the *jamb* and *jamc* mutants. **D:** WT control contained mostly multinucleated fast myofibers at 120 hpf. **E, F:** A broader distribution of myonuclei was detected in the myotome of *jamb* (E) and *jamc* (F) mutants at 120 hpf. **G, H**: Statistical analysis was performed to compare the numbers of nucleus in myofibers from multiple embryos for each genotype (n=20). Significant difference ($p < 0.05$) was observed between WT and *jamb* or *jamc* mutant, whereas no significant difference was detected between *jamb* and *jamc* mutants. Scale bar (25μm).

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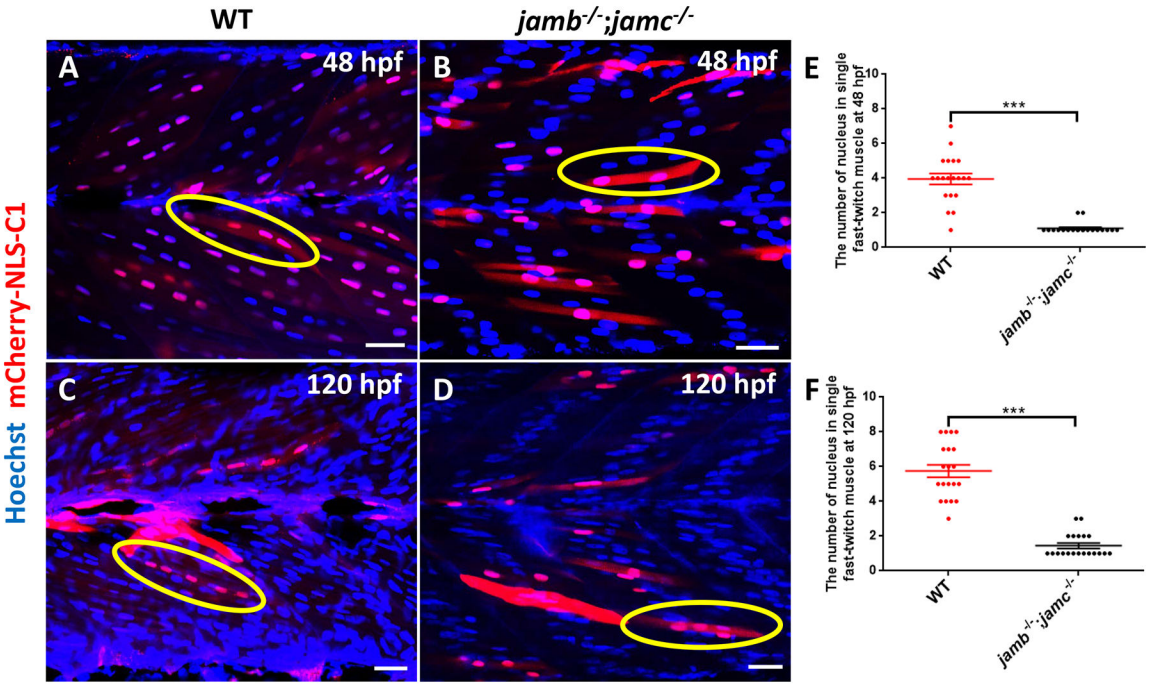

Fig. 4. The effect of *jamb* and *jamc* double mutation on myoblast fusion in zebrafish embryos

The mCherry-NLS-C1 plasmid was injected into WT control and *jamb*;*jamc* double mutant embryos at 1–2 cell stages. The injected embryos were fixed at 48 and 120 hpf. Myonuclei were labelled with Hoechst 32258 (blue) and mCherry-NLS-C1 (red). **A, C:** Myofibers in WT embryos contained multiple nuclei per fiber at 48 (A) and 120 (C) hpf. **B, D:** Myofibers in *jamb*;*jamc* double mutants were mostly mononucleated fibers at 48 (B) and 120 (D) hpf. However, a few myofibers containing 2–3 myonuclei were detected in the *jamb*;*jamc* double mutants at 48 and 120 hpf. **E, F:** *** showed the significant statistical difference between WT and *jamb*;*jamc* double mutant when comparing the number of nucleus in single fast-twitch muscle fiber at 48 (E) and 120 (F) hpf ($p < 0.05$, n=20). Scale bar (25μm).

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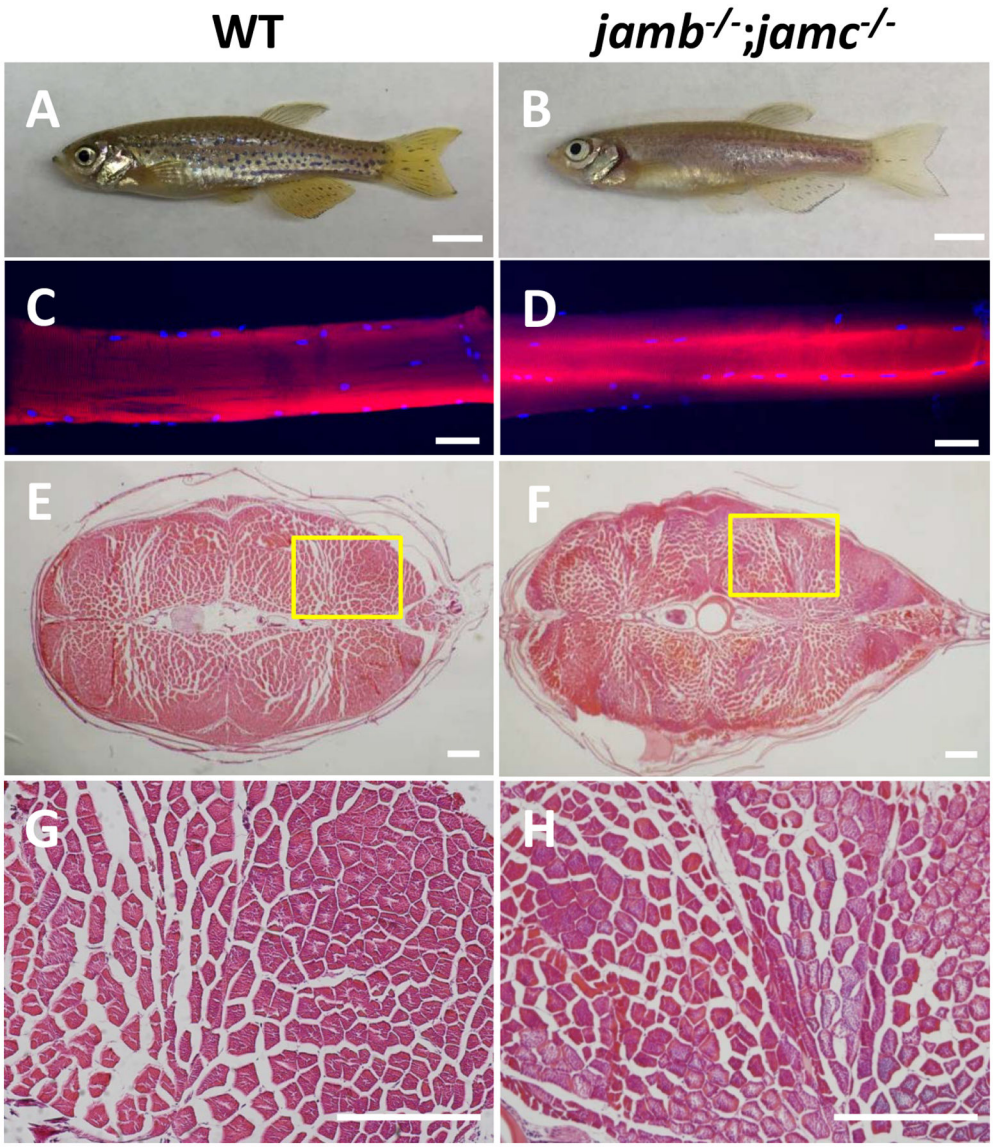

Fig. 5. Characterization of myonuclei and muscle structure in adult WT and *jamb*;*jamc* double mutant fish

A, B: Morphological comparison of WT control (A), *jamb*$^{-/-}$;*jamc*$^{-/-}$ mutant fish (B) at 7 months old. Scale bar (500 mm). **C, D:** Single fibers were isolated from adult WT (C) and *jamb*;*jamc* double mutant (D) of 7 months old. The isolated fibers were stained with Hoechst 32258 and phalloidin-TRITC, and photographed by a confocal microcopy. Scale bar (25 μm). **E-H:** Hisological analysis of trunk muscles of WT (E, G) and *jamb*;*jamc* double mutant (F, H) at 7 months old. G and H are higher magnification regional images of E and F, respectively. Scale bars (250μm).

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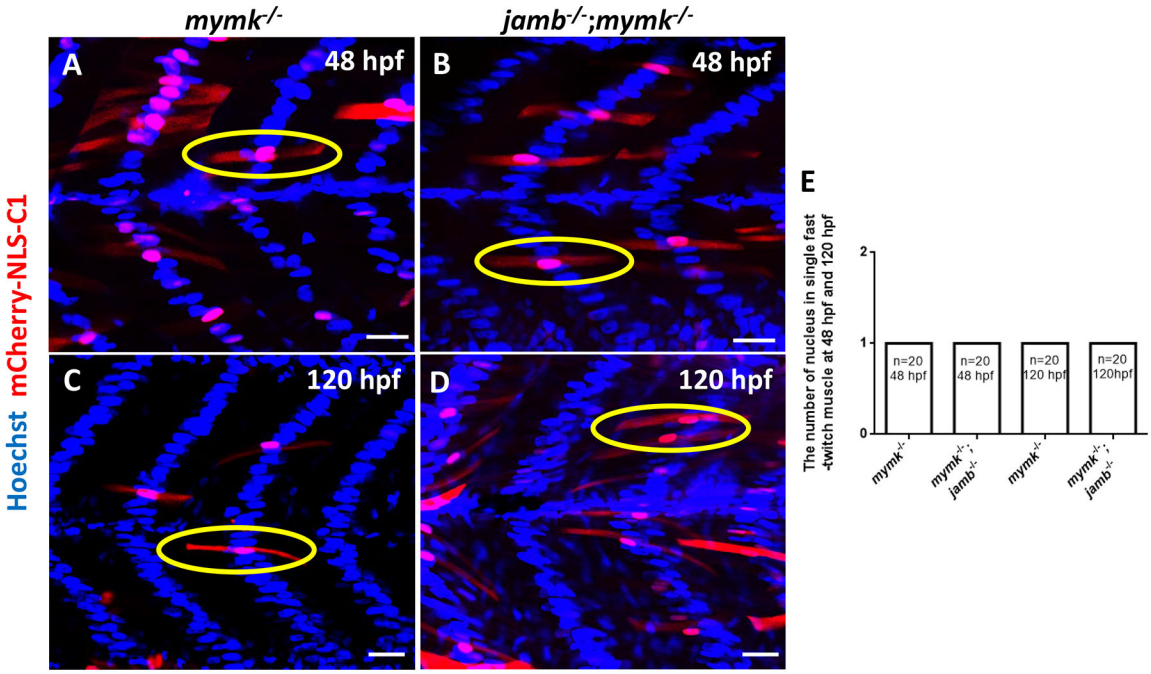

Fig. 6. The effect of *jamb*;*mymk* double mutation on myoblast fusion in zebrafish embryos

The mCherry-NLS-C1 plasmid was injected into fertilized eggs from *mymk*$^{+/-}$ or *jamb*$^{-/-}$;*mymk*$^{+/-}$in-crosses. The injected embryos were genotyped using DNA isolated from the head at 48 and 120 hpf. The trunk regions were labelled with Hoechst 32258 (blue). **A, C:** Center localization of myonuclei in the myotome of *mymk* mutant and mononucleated mCherry-NLS-C1 positive myofibers at 48 (A) and 120 (C) hpf. **B, D:** Center localization of myonuclei in the myotome of *jamb*;*mymk* double mutant and exclusive mononucleated mCherry-NLS-C1 positive myofibers at 48 (B) and 120 (D) hpf. **E:** Statistical analysis showed no significant difference between *mymk* single mutant and *jamb*;mymk double mutant (n=20). Scale bar (25μm).

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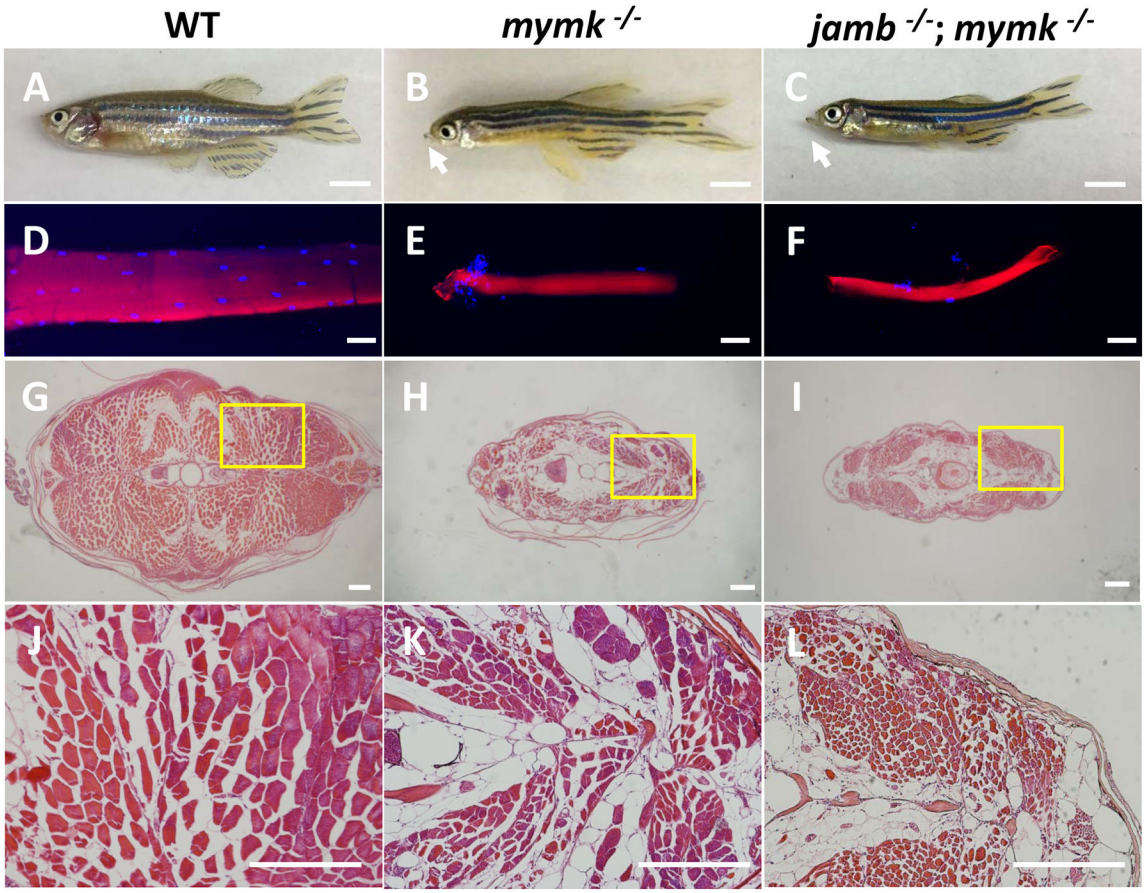

Fig. 7. Morphological and single fiber analyses of skeletal muscles in adult WT, *mymk* and *jamb*;*mymk* mutants

A-C: Photographs of WT (A), *mymk mutant* (B) and *jamb*;*mymk* mutant (C) at 7 months old. The *myomaker* and *jamb*;*mymk* mutants were skinnier and exhibited craniofacial deformities indicated by the white arrows (B, C). Scale bar (500 mm). **D-F:** Single fibers were isolated from trunk muscles of WT (D), *mymk* mutant (E) and *jamb*;*mymk* double mutant (F) at 7 months old. The isolated fibers were stained with Hoechst 32258 and phalloidin-TRITC, and photographed by a confocal microcopy. Myofibers from *mymk* and *jamb*;*mymk* mutants were smaller and mainly mono- or bi-nucleated fibers. Scale bar (25 μm). **G-L**: HE staining showing cross sections of trunk skeletal muscles in WT control (G, J) and *mymk* mutant fish (H, K)and *jamb*;*mymk* mutant fish (I, L) at 7 months old. Noticeable adipocyte infiltration was observed in skeletal muscles of *mymk* mutant (H, K) and *jamb*;*mymk* mutant (I, L). Scale bar (250μm).

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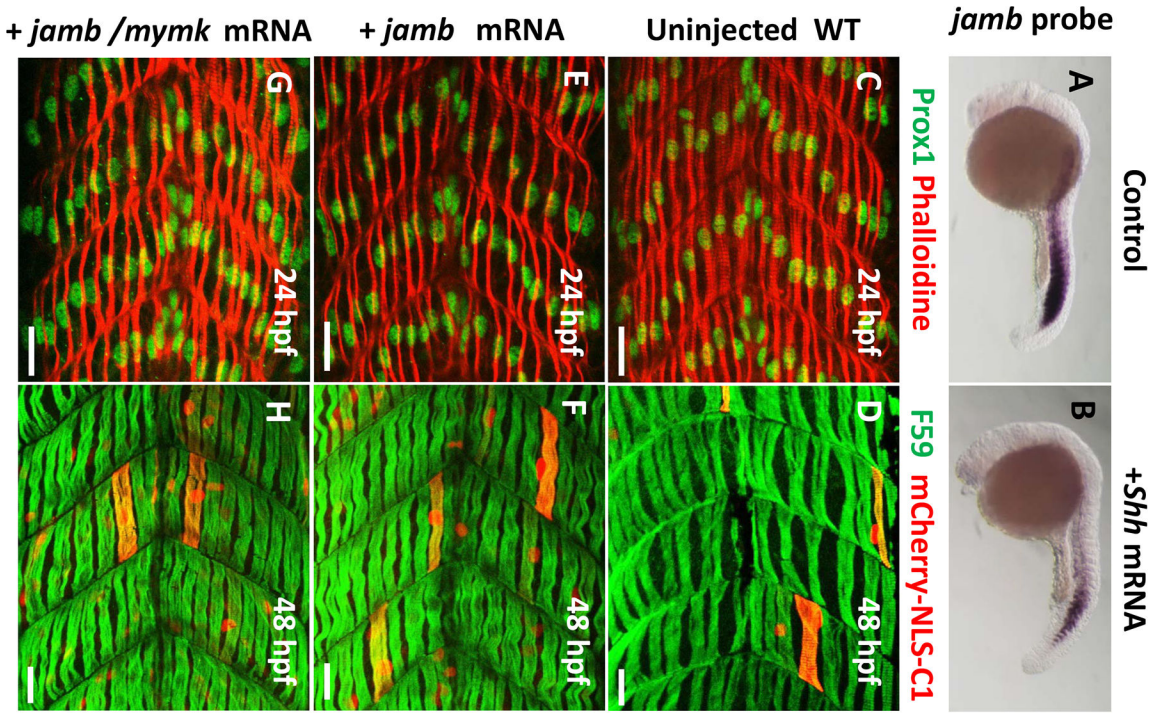

Fig. 8. The effects of *jamb* and *mymk* ectopic expression on slow muscle fusion

The mRNAs encoding Shh, JamB or Mymk was injected into WT fertilized zebrafish eggs at 1–2 cell stage. The injected embryos were subjected for *in situ* hybridization to analyze the effect of Shh overexpression on *jamb* expression, or the effect of Jamb and Mymk ectopic expression on slow fiber fusion by immunostaining with anti-prox-1 antibody or nuclear mCherry localization. **A, B:** Whole mount *in situ* hybridization shows *jamb* expression in un-injected control (A) or *Shh* mRNA injected embryos (B) at 20 hpf. *Shh* injection inhibited *jamb* expression. **C, D:** Mononucleated slow fibers revealed by anti-prox-1 antibody and Phalloidin-TRITC staining (C) or F59 antibody staining and nuclear mCherry localization (D) in control WT embryos at 24 (C) and 48 (D) hpf. **E, F:** Mononucleated slow fibers revealed by anti-prox-1 and Phalloidin-TRITC staining (E) or F59 antibody staining and nuclear mCherry localization (F) in *jamb* mRNA injected WT embryos at 24 (E) and 48 (F) hpf. Ectopic expression of Jamb was unable to induce myoblast fusion in slow muscles. **G, H:** Mononucleated slow fibers in *jamb* and *mymk* mRNAs co-injected WT embryos at 24 (G) and 48 (H) hpf. Ectopic expression of Jamb was unable to induce myoblast fusion in slow muscles. Scale bars (25μm).

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Table 1:

Primers for PCR analyses

Primers	Sequences
ZF-jamb-F1	5’- ATGCTGGTGTGCGTGTCTCTGCTGA-3’
ZF-jamb-R1	5’- TCACAGCATGAAGGACTGCGTGTGC-3’
ZF-jamc-F1	5’- ATGTACAGCCAAACAGAACATTTCA-3’
ZF-jamc-R1	5’-TCAGATGACAAATGAGGATTTGTGC-3’
ZF-jamb-F2	5’- GCGTCCCTGTGTCTCCCGTCACCGT-3’
ZF-jamb-R2	5’- CTACGAATCGTTCTCCATAGTAGAC-3’
ZF-jamc-F2	5’- GAGCAGTGTGTTCAGCTGTAGACTG-3’
ZF-jamc-R2	5’- CAGCGTGCCCATTTCCCCATCTAGGG-3’