HHS Public Access

Author manuscript

Science. Author manuscript; available in PMC 2017 July 09.

Published in final edited form as:

Science. 2017 April 21; 356(6335): 323–327. doi:10.1126/science.aam9361.

Control of muscle formation by the fusogenic micropeptide myomixer

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Abstract

Skeletal muscle formation occurs through fusion of myoblasts to form multinucleated myofibers. From a genome-wide clustered regularly interspaced short palindromic repeats (CRISPR) loss-of-function screen for genes required for myoblast fusion and myogenesis, we discovered an 84–amino acid muscle-specific peptide that we call Myomixer. Myomixer expression coincides with myoblast differentiation and is essential for fusion and skeletal muscle formation during embryogenesis. Myomixer localizes to the plasma membrane, where it promotes myoblast fusion and associates with Myomaker, a fusogenic membrane protein. Myomixer together with Myomaker can also induce fibroblast-fibroblast fusion and fibroblast-myoblast fusion. We conclude that the Myomixer-Myomaker pair controls the critical step in myofiber formation during muscle development.

Skeletal muscle is the largest tissue in the body, accounting for ~40% of human body mass. The formation of skeletal muscle begins with the specification of muscle cell fate by the myogenic transcription factors Pax7 and MyoD, followed by the expression of a vast number of genes that establish muscle structure and function (1, 2). A fundamental step in this process is the fusion of mononucleated myoblasts to form multinucleated myofibers (3–7). Similarly, in response to injury, myogenic progenitor cells within the adult musculature are activated and fuse to generate new myofibers (8–10). Whereas many of the initial steps of myoblast fusion are similar to those of other fusogenic cell types (11), the components and

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/356/6335/323/suppl/DC1

Materials and Methods

Figs. S1 to S7

References (*24*–*30*)

molecular basis of fusion of specific cell types, such as myoblasts, have not been fully defined.

To identify new regulators of myogenesis, we performed a genome-wide clustered regularly interspaced short palindromic repeats (CRISPR) loss-of-function screen for genes required for differentiation and fusion of C2C12 myoblasts, a mouse muscle cell line (fig. S1A). We infected 60 million C2C12 myoblasts with a lentiviral library comprising a pool of 130,209 single-guide RNAs (sgRNAs) and CRISPR-associated protein 9 (Cas9) for CRISPR gene editing (12). Lentivirus infection was performed at a multiplicity of infection in order to retain a 460-fold representation of the library. After puromycin selection for 2 days, myoblast cultures were switched to differentiation medium (DM) for 1 week so as to promote myotube formation. The cultures were subjected to a brief exposure to low trypsin (0.00625%), which promoted the detachment of myotubes, leaving mononucleated myoblasts attached to the dishes. Subsequent treatment with 0.25% trypsin allowed release of myoblasts. As a control for separation of the myotube and myoblast populations, we detected myosin heavy chain by means of Western blot analysis, which was highly enriched in the myotube population (fig. S1B).

We enumerated sgRNA representation in myoblast and myotube populations by means of high-throughput sequencing. Genes targeted by multiple myoblast-enriched sgRNAs were scored on the basis of their relative abundance. This analysis revealed numerous genes that were targeted by multiple independent CRISPR sgRNAs that were enriched in myoblasts. Because we sought to identify genes specifically required for myoblast differentiation or fusion, we narrowed down this list by comparing these genes with transcripts that are up-regulated during differentiation of C2C12 myoblasts (13), as well as $Pax7^+$ and $Twist2^+$ myogenic progenitors (fig. S1C) (14). Five genes fulfilled these criteria (fig. S1C). One previously unidentified gene on the list, annotated as *Gm7325*, was targeted by three independent sgRNAs in the initial screen and stood out as the most up-regulated gene of unknown function during differentiation of C2C12 myoblasts and $Pax7^+$ and $Twist2^+$ muscle progenitors (fig. S1C). This gene was also associated with conserved peaks for genomic binding of the myogenic transcription factors MyoD and Myogenin (fig. S1D) (15) and was identified as a putative target of MyoD (16).

There is a single publication describing *Gm7325* as a gene expressed in embryonic stem (ES) cells and germ cells, but the function was not elucidated (17). Here, we show that *Gm7325* is required for myoblast fusion and promotes the mixing of cell membranes; thus, we named this protein Myomixer. In the experiments that follow, we demonstrate the key role of this protein in the control of myoblast fusion and muscle formation.

The mouse *Myomixer* gene spans three exons with the open reading frame (ORF) in exon 3 (fig. S1E). To confirm that Myomixer is essential for myoblast fusion, we disrupted the gene in C2C12 cells and mouse primary myoblasts with lentiviruses that expressed Cas9 and a pair of sgRNAs that were separated by 122 base pairs (bp) in the Myomixer ORF (fig. S1E). Sequencing of genomic polymerase chain reaction (PCR) fragments generated with primers flanking the sgRNA target sequences from these puromycin stably selected cell cultures revealed numerous indel mutations that disrupted the Myomixer ORF (fig. S1F). Western

blot analysis with an antibody against amino acids 24 to 84 confirmed the absence of Myomixer protein in the knockout (KO) cultures (Fig. 1A and fig. S1I).

Disruption of the *Myomixer* gene prevented fusion of C2C12 cells and primary mouse myoblasts but did not affect expression of myosin heavy chain, a marker for differentiation (Fig. 1, A and B, and fig. S1, G to I). Quantification of the percentage of myonuclei in mono- versus multinucleated cells showed that ~89% of myoblasts targeted with the Myomixer sgRNAs were mononucleated after transfer to DM for 7 days (Fig. 1C). We never observed myotubes with more than five nuclei in the Myomixer KO cultures, whereas ~82% of myonuclei in wild-type (WT) cultures were contained in myotubes with more than five nuclei (Fig. 1C). MyoD and myogenin were expressed normally in differentiated Myomixer KO cells, as was Myh8 (myosin heavy chain 8) (fig. S1J), indicating a selective blockade to myoblast fusion independent of muscle differentiation.

The ORF of mouse Myomixer encodes an 84–amino acid micropeptide (Fig. 1D). Orthologs of Myomixer are conserved across diverse vertebrate species (Fig. 1D). In humans, a transcript encoding Myomixer is annotated as uncharacterized LOC101929726. Myomixer amino acid sequence is not annotated in fish or amphibians, presumably because ORFs smaller than 100 amino acids are not typically annotated. However, we identified putative Myomixer orthologs in turtle, frog, and fish genomes with conservation of several residues, especially many arginine, leucine, and alanine residues (Fig. 1D). The proteins from the various species contain an N-terminal hydrophobic segment, followed by a positively charged helix and an adjacent hydrophobic helix. Myomixer proteins from mammals and marsupials also contain a distinct C-terminal helix that is missing from other organisms (Fig. 1D).

Because the N terminus contains an extended hydrophobic segment, we postulated that it might serve as a membrane anchor. Indeed, immunostaining of intact C2C12 myoblasts expressing Myomixer with FLAG tag at the C terminus revealed the presence of the protein on the cell surface (Fig. 1E), suggesting that the N terminus is embedded in the membrane. Consistent with these findings, fractionation of C2C12 myotubes into membrane and cytosolic fractions showed the association of Myomixer with membranes (Fig. 1F). Similarly, in human kidney 293 cells infected with a Myomixer-expressing retrovirus, we found that the protein was preferentially localized to the membrane fraction, although weak staining was also seen in the cytosolic fraction (Fig. 1F).

Myomixer protein and mRNA expression were up-regulated during differentiation of C2C12 myoblasts and declined after myoblast fusion (Fig. 2A and fig. S2A). Similarly, Myomixer was strongly up-regulated during differentiation of isolated $Pax7^+$ and $Twist2^+$ adult muscle progenitor cells, which contribute to muscle regeneration (fig. S2B) (14). During pre- and postnatal muscle development, Myomixer transcripts showed a peak of expression at embryonic day 14.5 (E14.5) and declined thereafter (fig. S2C). Western blot analysis revealed the presence of the Myomixer protein in limb tissue from mouse embryos at E13.5 and in skeletal muscle at postnatal day 2, whereas the protein was undetectable in adult muscle of normal mice or in nonmuscle tissues (Fig. 2B). In situ hybridizations of mouse embryo sections at E12.5 and E15.5 showed intense expression of Myomixer exclusively in

developing skeletal muscles throughout the limbs and body wall (Fig. 2C). In response to cardiotoxin (CTX) injury of adult muscle, Myomixer expression was rapidly up-regulated, reaching a peak at day 3 after injury and declining thereafter (Fig. 2D). Immunostaining of CTX-treated muscle also showed robust expression of Myomixer in the myosin-positive muscle cells in the region of regeneration (fig. S2D). Consistent with the micropeptide's potential role in muscle regeneration, Myomixer mRNA expression was up-regulated in muscle of *mdx* mice, a model of muscular dystrophy that exhibits muscle degeneration and regeneration during disease progression (Fig. 2E).

To assess the potential involvement of Myomixer in muscle formation in vivo, we inactivated the gene during mouse embryogenesis through CRISPR-Cas9 mutagenesis using the same pair of sgRNAs that was shown to effectively target the gene in C2C12 cells and primary myoblasts. Fertilized zygotes were injected with Cas9 mRNA and sgRNAs, followed by implantation into pseudo-pregnant female mice. Embryos were harvested at E17.5 and analyzed. From 65 embryos analyzed, we obtained nine motionless embryos that appeared to lack skeletal muscle and were nearly transparent so that internal organs and bones were apparent (Fig. 3A).

Histological sections through limb, body wall, and diaphragm musculature revealed an absence of multinucleated myofibers in Myomixer KO embryos (Fig. 3B). Instead, presumptive muscle-forming regions were populated by mononucleated cells that stained for myosin expression (Fig. 3C). Tissues other than muscle appeared normal in these embryos. The cutting sites of the two sgRNAs were 122 base pairs apart in the third exon of the *Myomixer* gene. PCR with primers amplifying this region showed mutant mice with deletions that ranged from 163 to 470 bp, reflecting different indels (fig. S3, A and B). Western blot of hindlimbs confirmed the absence of Myomixer protein in the KO embryos (Fig. 3D).

The muscle phenotype of Myomixer mutant mice is reminiscent of that seen in mice lacking Myomaker, a fusogenic transmembrane muscle protein (18, 19), suggesting a functional relationship between these two regulators of myoblast fusion. To assess the functional relationship between Myomixer and Myomaker in myoblast fusion, we retrovirally expressed each protein in C2C12 myoblasts and monitored myotube formation. As shown in Fig. 4A, Myomixer markedly enhanced the fusion of C2C12 cells. Moreover, when Myomixer and Myomaker were overexpressed together in C2C12 cells, they promoted the formation of massive multinucleated myotubes, which is indicative of their synergistic activity (Fig. 4A and fig. S4).

Myomaker can induce fusion of 10T1/2 mouse fibroblasts with myoblasts (18, 19). In contrast, Myomixer expressing 10T1/2 cells did not fuse with C2C12 cells (Fig. 4B). To investigate whether Myomixer can synergize with Myomaker to promote heterologous cell fusion, we infected green fluorescent protein (GFP)–labeled 10T1/2 cells with Myomaker and Myomixer retroviruses and mixed them with mCherry-labeled C2C12 cells (fig. S5A). We found that expression of Myomixer and Myomaker together in 10T1/2 cells induced dramatic fusion to C2C12 myotubes so that only a few mononucleated GFP-labeled fibroblasts remained in the cultures (Fig. 4B). Moreover, when Myomaker and Myomixer

were coexpressed in two populations of 10T1/2 fibroblasts labeled with GFP or mCherry and the cells were mixed, fibroblast-fibroblast fusion was observed (Fig. 4C and fig. S5B). Multinucleated fibroblasts coexpressing GFP and mCherry, which appeared yellow, often resembled bird nests filled with eggs (Fig. 4C). Western blot confirmed expression of Myomaker and Myomixer in the appropriate cells (fig. S5C). We observed no effect of either protein on the level of expression of the other, indicating that their synergy does not reflect an effect on stability of either protein. A summary of the effects of Myomixer and Myomaker on fusion is shown in fig. S5D.

To further test the functional dependency of Myomaker on Myomixer for cell fusion, we mixed Myomaker-expressing 10T1/2 cells with WT or Myomixer-KO C2C12 myoblasts and switched them to DM for 1 week, after which we immunostained for myosin. Although Myomaker-expressing 10T1/2 cells fused with WT C2C12 cells, they were unable to fuse with Myomixer KO C2C12 cells (fig. S5E). This implies that Myomaker relies on Myomixer for cell fusion in trans. Indeed, Myomaker was expressed normally in Myomixer KO myoblasts and embryos, suggesting a dependency of Myomaker on Myomixer for normal muscle fusion and development (fig. S6, A and B).

To begin to define the mechanistic basis of the cooperativity between Myomixer and Myomaker, we examined whether the two proteins physically interact. Indeed, we found that in coimmunoprecipitation assays, FLAG-tagged Myomaker coimmunoprecipitated with Myomixer in 10T1/2 fibroblasts and differentiated C2C12 cells (Fig. 4D). Replacement of arginines at amino acid position 34, 38, or 46 with glutamic acid residues within the charged segment of Myomixer diminished association with Myomaker but did not affect membrane association (Fig. 4E and fig. S7A). The EEEAA mutant failed to synergize with Myomaker to stimulate fusion of C2C12 myoblasts (Fig. 4F and fig. S7B), as well as heterologous fusion of C2C12 myoblasts with 10T1/2 fibroblasts (fig. S7C), indicating a correlation between the association of Myomixer with Myomaker and their synergistic fusogenic activity. Although mutation of cysteine 52 to alanine in the second hydrophobic region disrupted the fusogenic activity of Myomixer (Fig. 4F and fig. S7B), this mutation did not prevent membrane localization (fig. S7A) or interaction with Myomaker in coimmunoprecipitation experiments (Fig. 4E). This suggests that the second hydrophobic domain of Myomixer may mediate the fusogenic function after its binding with Myomaker. A summary of the effects of Myomixer mutations is shown in fig. S7D. Given the cross-species homology of Myomixer orthologs, we tested whether the ortholog from zebrafish (*Danio rerio*) could also promote heterologous fusion of cells. As shown in fig. S7E, this zebrafish peptide of only 75 amino acids induced cell fusion when overexpressed with mouse Myomaker. Consistent with this finding, deletion of the C terminus of mouse Myomixer (Myomixer$^{\Delta C}$) did not abolish its fusogenic function (fig. S7E). Thus, we conclude that Myomixer is an evolutionarily conserved regulator of myoblast fusion and that the C-terminal region that is specific to higher vertebrates is dispensable for function, at least in this assay.

The requirement of Myomixer for fusion of myoblasts in vivo and in vitro, its ability to synergistically promote fusion together with Myomaker, and the physical and functional interaction between these proteins indicate that they govern the critical step of

multinucleation of skeletal muscle. We hypothesize that Myomixer activates the fusogenic activity of Myomaker to drive membrane mixing, perhaps by establishing a fusion pore.

The small size of Myomixer places it in the category of micropeptides, characterized by unprocessed ORFs of less than 100 amino acids (20). A majority of micropeptides identified to date are embedded in membranes (21, 22). There are some striking similarities between Myomixer and the heart-specific micropeptide DWORF (Dwarf open reading frame), which localizes to the sarcoplasmic reticulum of cardiomyocytes where it associates with the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) calcium adenosine triphosphatase (ATPase) and stimulates its activity (23). We speculate that the activities of many membrane proteins may be governed by association with micropeptides that are as yet unidentified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to J. Cabrera for graphics, B. Chen for CRISPR library data processing, W. Ye for chromatin immunoprecipitation sequencing data analysis, and N. Grishin and J. Pei for bioinformatic analysis of Myomixer orthologs. We thank K. White in the Histopathology Core for technical assistance. We also thank H. Zhou, L. Amoasii, A. Bookout, Z. Wang, and N. Liu for biological materials and other members of the Olson laboratory for technical advice. This work was supported by grants from the NIH (AR-067294, HL-130253, DK-099653, and HD-087351) and the Robert A. Welch Foundation (grant 1-0025 to E.N.O.).

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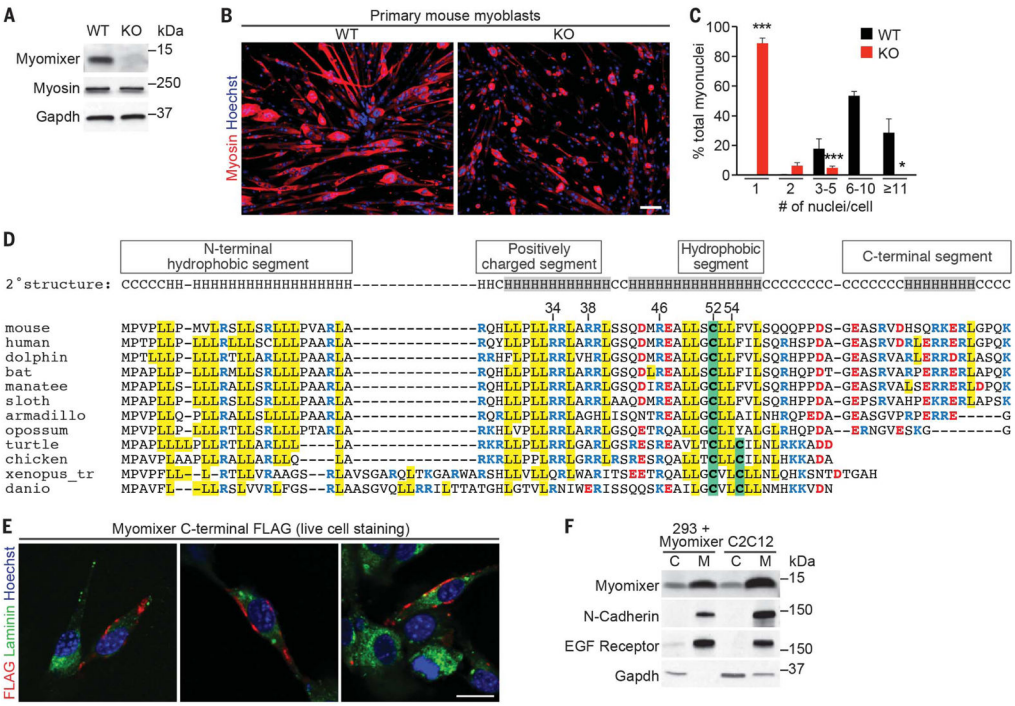

Fig. 1. Myomixer is a small-membrane protein that is essential for myoblast fusion

(**A**) Western blot showing Myomixer, Myosin, and Gapdh expression in WTor Myomixer KO primary myoblast cultures after 4 days in DM. (**B**) WT and Myomixer KO primary myoblasts differentiated for 1 week and stained with MY32 antibody (myosin) and Hoechst show the requirement of Myomixer for fusion. Scale bar, 50 μm. (**C**) Quantification of fusion in WT and Myomixer KO primary myoblast cultures ($n = 3$ pairs). (**D**) Amino acid sequence of Myomixer and cross-species homology. Basic residues are blue, acidic residues are red, cysteines are green, and leucines are yellow. H, helix; C, coil. (**E**) Live-cell staining of Myomixer-transfected C2C12 myoblasts showing cell-surface localization of Myomixer with a C-terminal FLAG tag. Laminin was stained after FLAG staining and permeabilization. (**F**) Western blot analysis of cytosolic (C) and membrane (M) fractions of retrovirus-Myomixer–infected 293 cells and WT C2C12 cells (day 4 after differentiation). Gapdh blot was used as a positive control of cytosolic proteins. N-Cadherin and epidermal growth factor (EGF) receptor blots were used as positive controls of membrane proteins. $*P < 0.05$, $***P < 0.001$, Student's t test. Data are mean ± SEM.

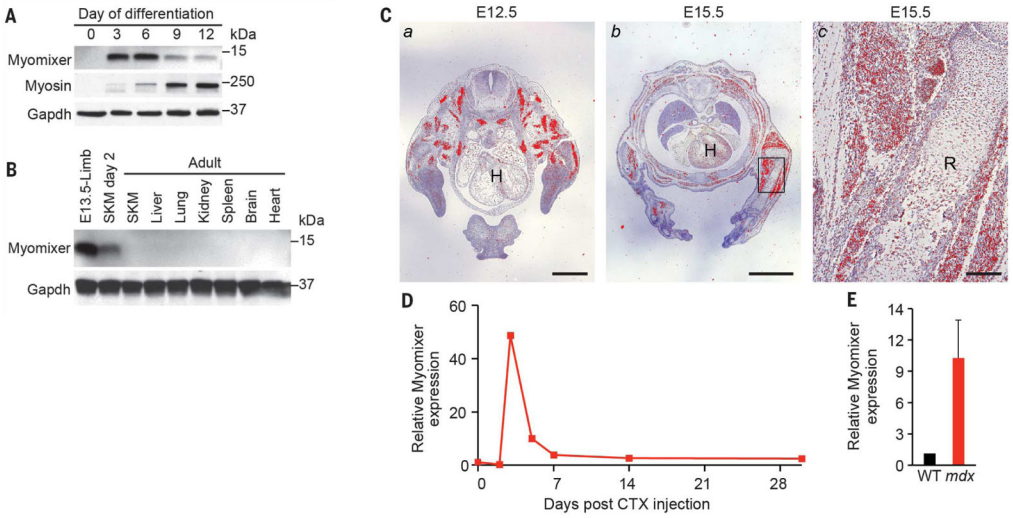

Fig. 2. Myomixer expression during muscle development and regeneration in mice

(**A**) Western blot showing Myomixer, Myosin, and Gapdh expression during C2C12 differentiation. (**B**) Western blot showing Myomixer and Gapdh expression in the indicated tissues. SKM, skeletal muscle. (**C**) In situ hybridization showing Myomixer transcript expression in transverse sections of mouse embryos at E12.5 and E15.5. Scale bar, 250 μm (*a*), 500 μm (*b*), and 200 μm (*c*). Image *c* is a magnification of the boxed area shown in image *b*. H, heart; R, radius. (**D**) Myomixer mRNA expression in CTX-injured skeletal muscle from 1-month-old WT mice, as determined with quantitative PCR. ($n = 3$ mice/time point). (**E**) Up-regulation of Myomixer mRNA expression in *mdx* compared with WT muscle, as detected with quantitative PCR ($n = 4$ pairs). Data are mean ± SEM.

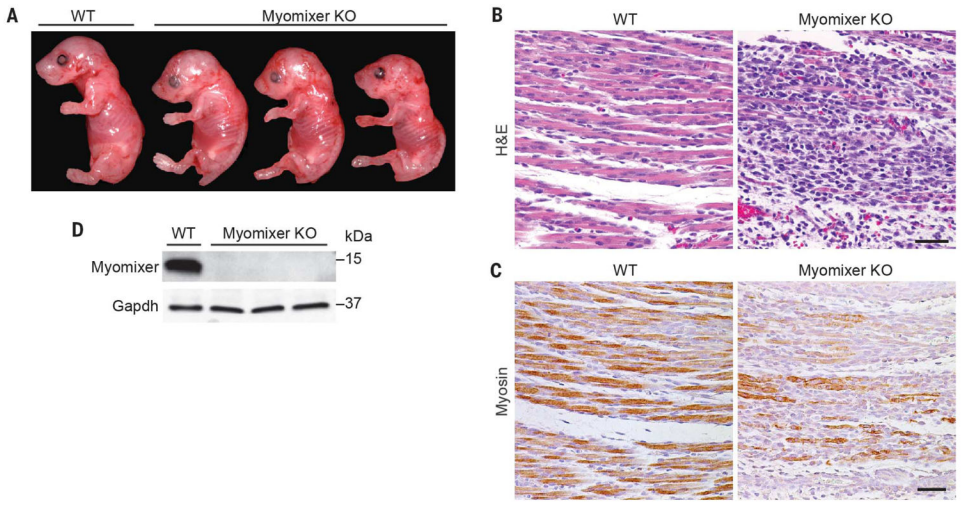

Fig. 3. Myomixer is essential for muscle development in mice

(**A**) WT and Myomixer KO embryos at E17.5 dissected and skinned to reveal the lack of muscle in Myomixer KO limbs. (**B**) Hematoxylin and eosin (H&E) staining of E17.5 limb muscles shows lack of muscle fibers in Myomixer KO embryos. Scale bar, 50 μm. (**C**) Immunohistochemistry of E17.5 limb muscles using MY32 (myosin) antibody. WT myofibers show multinucleation, which is absent in Myomixer KO sections. Scale bar, 50 μm. (**D**) Western blot analysis of Myomixer and Gapdh in forelimb tissues of E17.5 WT and Myomixer KO embryos.

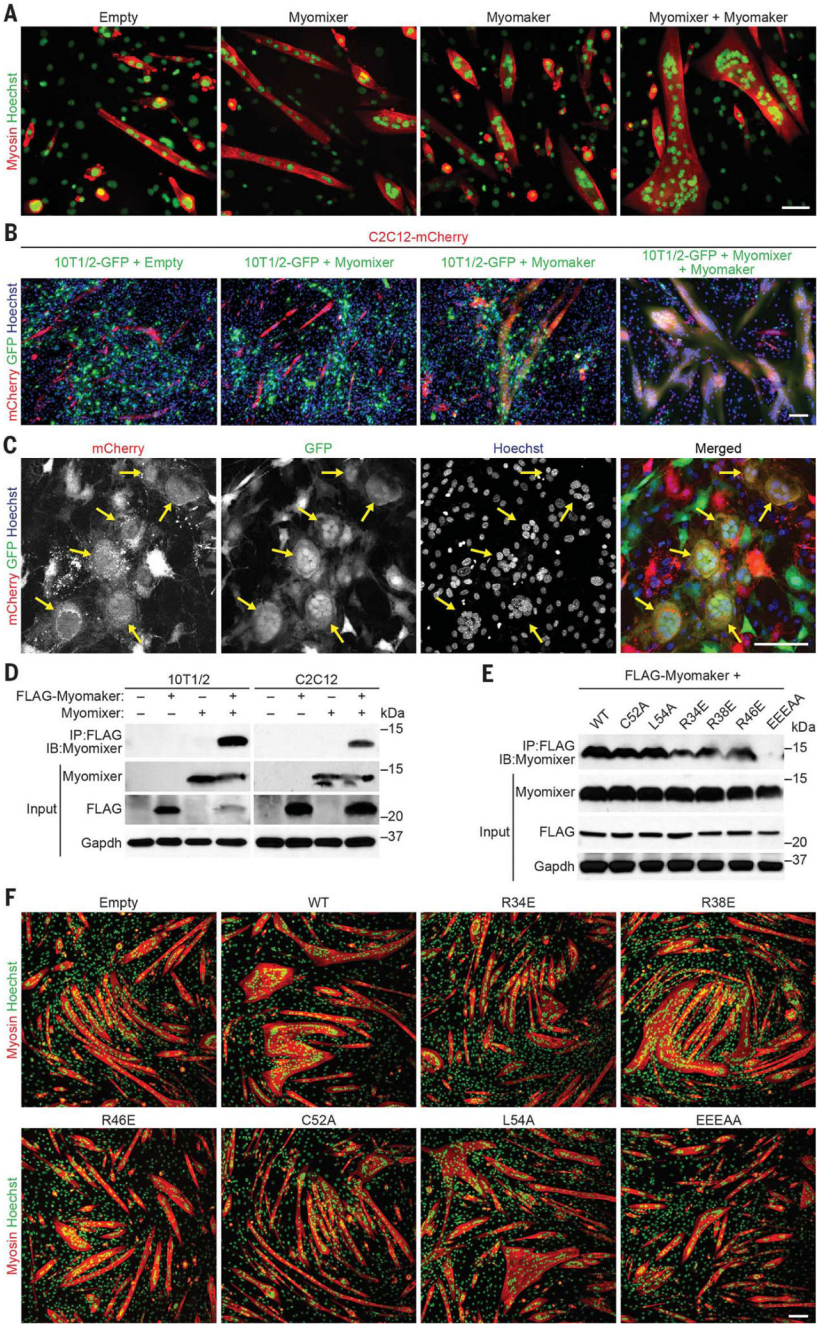

Fig. 4. Myomixer binds Myomaker and synergizes to induce cell fusion

(**A**) MY32 (myosin) immunostaining of C2C12 cells infected with retroviruses expressing Myomaker, Myomixer, or both and differentiated for 4 days. Nuclei were counterstained with Hoechst and pseudocolored green. Scale bar, 50 μm. (**B** and **C**) Fluorescence images of GFP, mCherry, and Hoechst to counterstain nucleus. Scale bars, 100 μm (B), 50 μm (C). Arrows point to multinucleated GFP^{+}/$mCherry^{+}$ cells. (**D** and **E**) Coimmunoprecipitation assays were performed by using 10T1/2 cells or C2C12 myoblasts infected with retroviruses expressing Myomixer and/or FLAG-Myomaker. IP, immunoprecipitation; IB, immunoblot.

(**F**) MY32 (myosin) immunostaining of C2C12 cells infected with retroviruses expressing Myomaker, together with WTor mutated versions of Myomixer, differentiated for 1 week. Nuclei were counterstained with Hoechst and pseudocolored green. Scale bar, 50 μm. R, arginine; E, glutamic acid; C, cysteine; L, leucine; A, alanine; EEEAA, R34E-R38E-R46E-C52A-L54A.