Trout myomaker contains 14 minisatellites and two sequence extensions but retains fusogenic function

Received for publication, October 2, 2018, and in revised form, February 26, 2019 Published, Papers in Press, February 28, 2019, DOI 10.1074/jbc.RA118.006047

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Edited by Jeffrey E. Pessin

The formation of new myofibers in vertebrates occurs by myoblast fusion and requires fusogenic activity of the muscle-specific membrane protein myomaker. Here, using *in silico* (BLAST) genome analyses, we show that the *myomaker* gene from trout includes 14 minisatellites, indicating that it has an unusual structure compared with those of other animal species. We found that the trout *myomaker* gene encodes a 434–amino acid (aa) protein, in accordance with its apparent molecular mass (~40 kDa) observed by immunoblotting. The first half of the trout myomaker protein (1–220 aa) is similar to the 221-aa mouse myomaker protein, whereas the second half (222–234 aa) does not correspond to any known motifs and arises from two protein extensions. The first extension (~70 aa) apparently appeared with the radiation of the bony fish clade Euteleostei, whereas the second extension (up to 236 aa) is restricted to the superorder Protacanthopterygii (containing salmonids and pike) and corresponds to the insertion of minisatellites having a length of 30 nucleotides. According to gene expression analyses, trout *myomaker* expression is consistently associated with the formation of new myofibers during embryonic development, postlarval growth, and muscle regeneration. Using cell-mixing experiments, we observed that trout myomaker has retained the ability to drive the fusion of mouse fibroblasts with C2C12 myoblasts. Our work reveals that trout myomaker has fusogenic function despite containing two protein extensions.

Skeletal muscle is largely composed of myofibers: multinucleated cells whose formation depends on fusion of progenitor cells known as myoblasts. Myoblasts proliferate, differentiate into myocytes, fuse to form multinucleated myotubes, and finally mature into functional myofibers. The fusion process is a critical step in the formation and regeneration of muscle. In mammals, some proteins involved in myoblast fusion have been identified, but the complete molecular mechanisms that coordinate this process are not completely understood. Nephrin, a cell surface protein, has been shown to be essential for myocyte fusion in mice and normal muscle development in zebrafish (1). The protein Kirrel, the homolog of the *Drosophila* Kirre protein, is also necessary for proper fusion of myocytes in zebrafish (2), although its function in mammals has not yet been confirmed and remains a subject of debate (3). In zebrafish, a receptor ligand pair (Jam-b/Jam-c) has been reported to be involved in myocyte fusion (4).

Recently, the muscle-specific micropeptide myomixer has been shown to be essential for myoblast fusion in mice (5–7) and zebrafish (8). Another muscle-specific transmembrane protein of 221 aa,[2] called myomaker, was found to be necessary for myocyte fusion during mouse embryonic development (9) and muscle regeneration (10). In humans, the loss of myomaker activity can lead to disease (11). *In vitro*, mouse myomaker drives heterologous fusion between fibroblasts and myoblasts, but not between fibroblasts (9). However, when myomaker and myomixer are ectopically overexpressed together, they are sufficient to drive fusion between fibroblasts (5–7). A structure–function analysis demonstrated that the two last cysteines of the C-terminal end of myomaker are necessary for its fusogenic function (12, 13).

In adult mouse muscle, myomaker is not expressed except in response to injury, when it is up-regulated to promote regeneration (10). In zebrafish, the 221-aa myomaker protein shows high similarities with murine myomaker and is necessary for myocyte fusion during embryonic development (14, 15), and it also promotes the heterologous fusion between mouse fibroblasts and myoblasts (12). As shown in our previous study, *myomaker* expression in the zebrafish myotome is no longer detected just before hatching (14). However, no data are available on myomaker characteristics and function in nonmodel species.

In the present study, we characterized the trout *myomaker* gene, which encodes an unexpectedly longer 434-aa protein.

This work was supported by National Institutes of Health Grants AR-067294, HL-130253, DK-099653, and HD-087351. This study was also supported by French National Research Agency Grant ANR-12-JSV7–0001-01, by Brittany region funds (to A. L.), and by Robert A. Welch Foundation Grant 1-0025 (to E. N. O.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) KY563699.

This article contains Figs. S1–S4.

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[2] The abbreviations used are: aa, amino acid(s); nt, nucleotide(s); dpf, day(s) postfertilization; qRT, quantitative real-time; WGD, whole genome duplication; DM, differentiation medium.

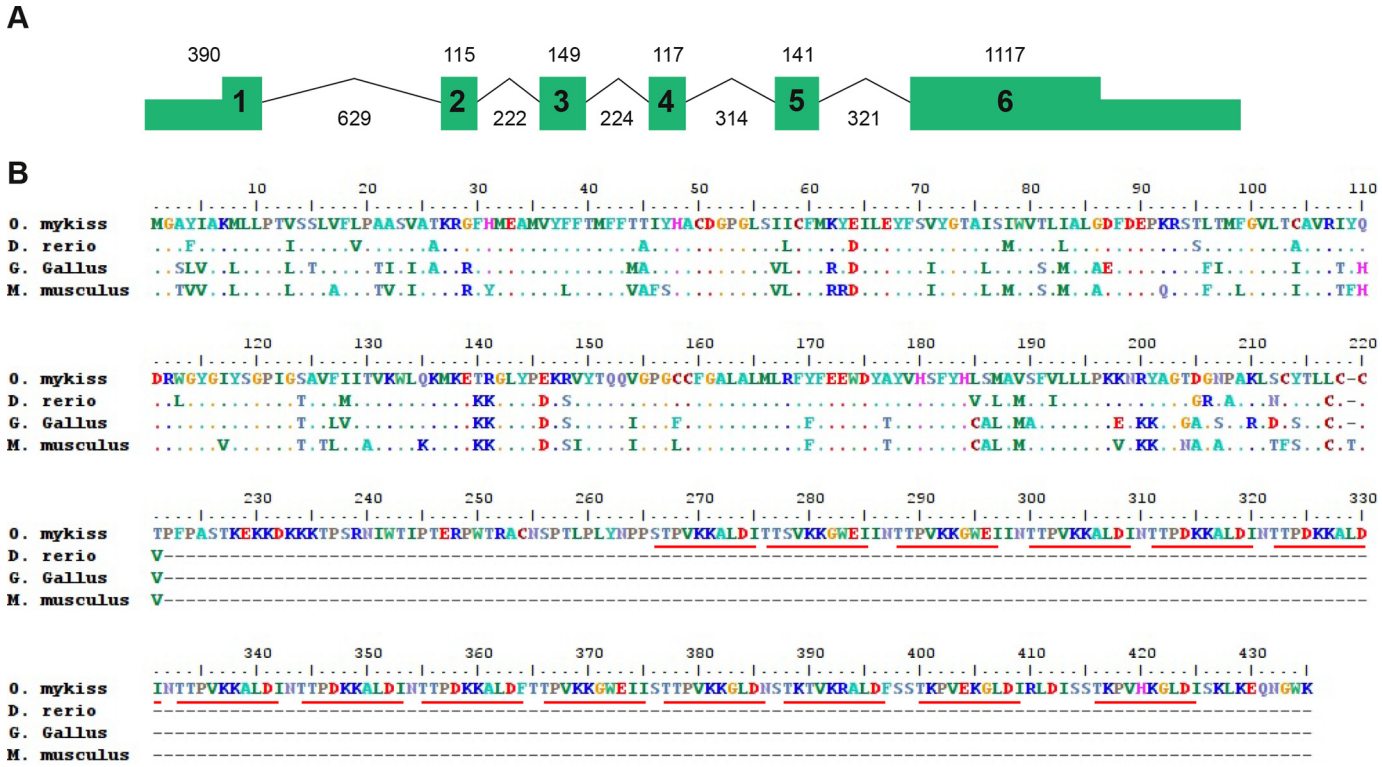

Figure 1. Structure of the trout myomaker gene. *A*, exon size (nt) is indicated above each exon, and intron size (nt) is indicated between exons. *B*, multiple alignment between the protein sequences of myomaker. *Red* underlines indicate minisatellites. Accession numbers are as follows: *O. mykiss*, ARM20036; *D. rerio*, NP_001002088.1; *Gallus gallus*, AJZ77002.1; and *Mus musculus*, NP_079652.1. The alignment was made from the complete protein sequences using ClustalW multiple alignment tool.

Whole-mount *in situ* hybridization and quantitative real-time PCR analyses revealed that *myomaker* is expressed not only in hyperplasic zones of embryonic myotome but also in postlarval myotomal muscle. Our results clearly show that *myomaker* upregulation was associated with myotube formation during muscle regeneration and the *in vitro* fusion of trout myocytes. Furthermore, the 14 tandem repeats (minisatellites) in the coding region of the trout *myomaker* gene do not disrupt its fusogenic capacity.

Results

Identification of the trout myomaker gene

We performed a BLAST search in the trout genome (17) using the sequence of zebrafish myomaker protein (NP_001002088) to identify the trout myomaker gene, and we found a single gene (GSONMG00014531001) in scaffold_482 that contained six exons encoding a protein of 434 aa (Fig. 1). Although the number of exons was similar to the zebrafish gene, the length of the trout myomaker protein was twice as long as the zebrafish and the mouse orthologs, which only comprise 220 and 221 aa, respectively (9, 14). As shown in the protein sequence alignment, the first half (1–220 aa) of the trout myomaker protein sequence was well-conserved, sharing 88 and 71% identity with the zebrafish and mouse myomaker proteins, respectively. An analysis of the amino acid sequence showed that the two cysteines essential for myomaker fusogenic function were also present in the trout myomaker protein at positions 219 and 220. The second half of the protein (221–434 aa) was encoded by the sixth exon and exhibited no homology with the zebrafish or mouse myomaker protein. We amplified exons 5 and 6 from total trout cDNA and sequenced the PCR product to confirm our *in silico* results. The sequencing results confirmed the splicing site of the sixth exon of the myomaker cDNA, leading to an ORF encoding 434 aa. Moreover, using the sequence identified in the trout genome (GSONMG00014531001), we performed BLAST searches in the trout expressed sequence tag database (NCBI) and the PhyloFish database (16) that allowed us to identify a transcript of 2029 nt (GenBank™ accession number KY563699) that included exons 1–6. Furthermore, using a specific antibody against trout myomaker, we confirmed that the molecular mass of trout myomaker (~40 kDa) is double that of mouse myomaker by Western blotting (Fig. 2 and Fig. S4). The *in silico* analysis revealed the presence of three E-boxes (CANNTG) in the *myomaker* promoter.

According to the results of the synteny analysis, the trout myomaker gene is located in the *FAM163b–Adamtsl2–Tmem8c–TCC16–Slc2a8* locus (Fig. 3) in scaffold_482. Interestingly, a synteny conservation of this locus was observed within a region of chromosome 2 of the zebrafish genome and in the equivalent chromosomal region of the mouse genome (Chr 2). A whole genome duplication event occurred in salmonid genome, leading to the duplication of some genes in the trout genome. Indeed, we were also able to identify another myomaker syntenic group in scaffold_2354 of the trout genome (Fig. 3). Nevertheless, whereas complete copies of the *FAM163b*, *Adamtsl2*, and *Slc2a8* genes were identified in this scaffold, only a partial sequence homologous to trout *myo-*

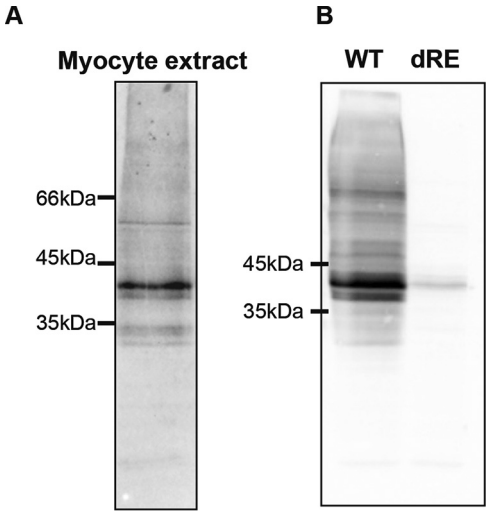

Figure 2. Myomaker protein has an apparent molecular mass of 40–42 kDa. *A*, Western blotting of trout myocytes extract with a custom trout myomaker antibody against the minisatellites. The antibody revealed one major band corresponding to a protein of ~40 kDa. *B*, Cos7 cells were transfected with the full-length (WT) or truncated (*dRE*) myomaker cDNA, and Western blotting was performed 48 h later. A band of similar apparent mass (~40 kDa) was observed with the full-length, and no band was observed with the truncated form of myomaker (aa 1–219) that did not contain the epitope.

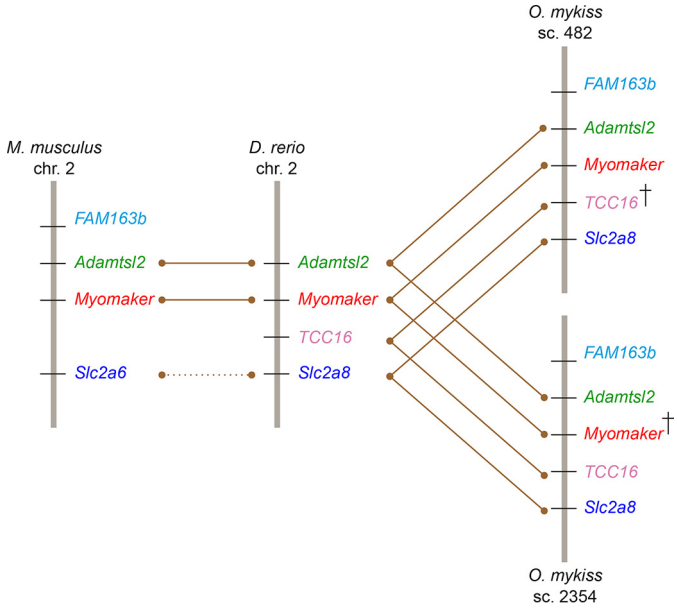

Figure 3. Myomaker gene is present in only one functional copy. Conserved synteny around myomaker locus was observed in mouse (*M. musculus*), zebrafish (*D. rerio*), and rainbow trout (*O. mykiss*). The Genomicus software program (http://www.genomicus.biologie.ens.fr/genomicus-trout-01.01/cgi-bin/search.pl; please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site) (37) was used to identify syntenic genes that were located near the myomaker gene. The *cross* indicates a pseudogene.

maker was identified between *Adamtsl2* and *TCC16*. This sequence contained several deletions and stop codons in the ORF, thus coding for an additional but nonfunctional *myomaker* gene (data not shown).

The trout myomaker gene contains 14 minisatellites in its coding region

Trout myomaker protein is 214 aa longer than the zebrafish orthologs because of a long C-terminal extension. A BLAST

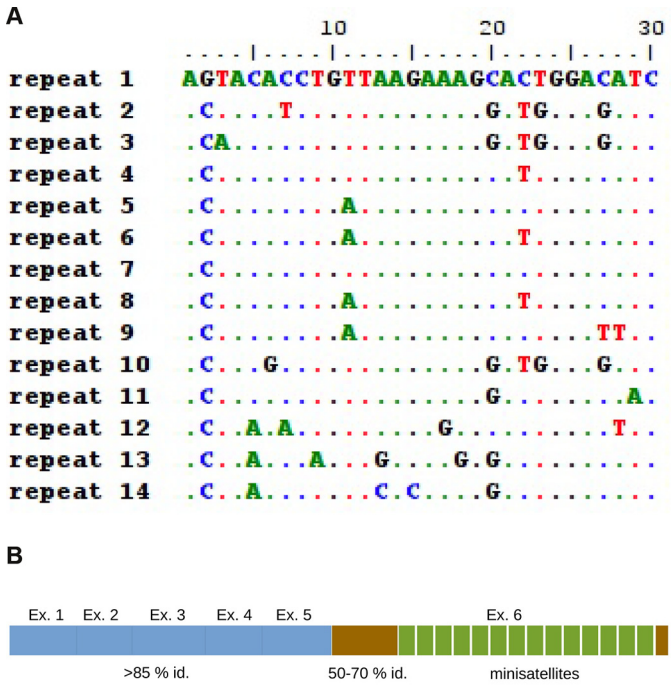

Figure 4. The sequence of the minisatellites are conserved. *A*, multiple alignment of the 14 minisatellites present in the trout myomaker protein. *B*, structure of trout myomaker protein. The mouse homologous part of the protein is represented in *blue*. The amino acids arising from the first extension are represented in *brown*, and the minisatellites are in *green*.

analysis of this protein extension revealed no homology with known motifs or other proteins. Surprisingly, a thorough analysis of the sequence encoding this extension revealed the presence of 14 tandem repeats of 30 nt coding amino acids 265–424 (Fig. 1). The sequences of these 14 repeats are very well-conserved with each other, with sequence identities ranging from 70 to 96% (Fig. 4*A*). These tandem repeats are therefore minisatellites, as defined in a previous study (18). We performed protein alignments and a phylogenetic analysis of myomaker proteins from several species to determine whether these minisatellites were widespread in teleost fish (Fig. 5). All tetrapod sequences were found to encode a myomaker protein of 220–221 aa. A protein of the same length (221 aa) was observed in ancestral nonteleost fish, such as the spotted gar (*Lepisosteus oculatus*), and teleosts that belong to the Otocephala lineage, such as zebrafish (*Danio rerio*), cave fish (*Astyanax mexicanus*), and herring (*Clupea harengus*). In sharp contrast, all teleosts examined that belonged to Euteleostei had a myomaker protein containing more than 220 aa. More specifically, in all considered Neoteleostei species, myomaker consisted of 283–289 aa, and the first 220 aa were highly similar (>85% identity) to the zebrafish myomaker protein. Within the Neoteleostei species, the extension of 63–69 aa was well-conserved (~70% identity) but showed no homology with zebrafish or mouse *myomaker*. Species belonging to the Protacanthopterygii lineage, such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and pike (*Esox lucius*) contained a *myomaker* gene encoding a protein with more than 434 aa. After sequence alignment, we discovered that all these species contained an insertion of minisatellites within the first extension of 63–69 aa specific to the Neoteleostei (Fig. 4*B*). Although the number of

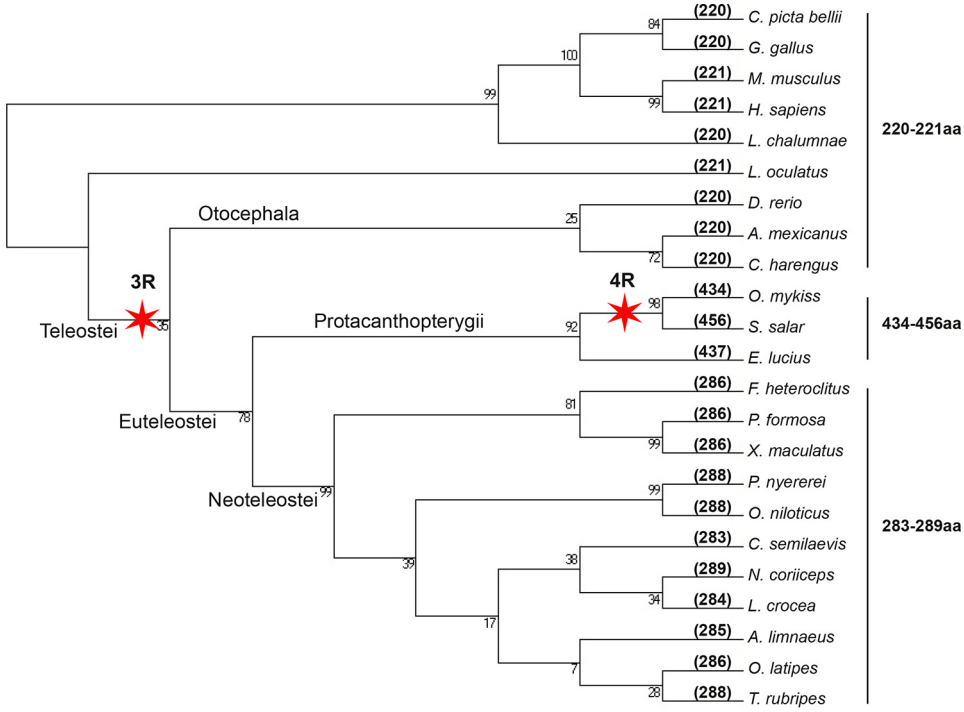

Figure 5. Phylogenetic analysis of myomaker in tetrapods and teleosts. The phylogenetic tree was constructed from a multiple alignment of the complete protein sequences using the neighbor-joining method. The *numbers* at the tree nodes represent percentage of bootstrap values after 1000 replicates. Full scientific names of species and respective accession numbers are detailed in Fig. S3. The *red star* represents a whole genome duplication (3R or 4R). The *numbers* between *brackets* indicate the number of amino acids encoded by myomaker in each species.

minisatellites varied by species (14 for trout, 17 for salmon, and 15 for pike), the minisatellite sequences were highly conserved (>70%) among these species. In contrast, only two minisatellites remain in the nonfunctional copy of myomaker.

Myomaker is expressed in embryonic and postlarval myotomal trout muscle

We performed whole-mount *in situ* hybridization to examine myomaker expression during embryonic myogenesis. The myomaker transcript was not detected during the early stage of somitogenesis (13 dpf, data not shown) but was readily detected at 17 dpf in all somites (Fig. 6*A*) when multinucleated fibers begin to form (19). Transverse sections (Fig. 6*B*) through the somites at 17 dpf showed that myomaker was expressed in the deep myotome, with stronger expression observed within the dorsal and ventral domains of the myotome. In contrast, the *myomaker* transcript was not detected in the undifferentiated myogenic dermomyotome-like epithelium surrounding the primary myotome.

In addition, we measured *myomaker* expression in white muscle from 4-, 8-, and 18-month-old fish weighing 15, 150, and 1500 g, respectively (Fig. 6*D*). Interestingly, at all three stages, *myomaker* expression was readily detected in trout muscle samples, although its expression decreased as body weight increased. We analyzed trout *myomaker* expression in several tissues by qRT-PCR to determine whether *myomaker* expression was restricted to muscle. As shown in Fig. 6*C*, the *myomaker* gene was only expressed in white and red muscle and was expressed at similar levels between both muscle types. In line with this observation, Western blotting analysis revealed the presence of myomaker protein only in myocyte extract and not in other tissues (Fig. S4).

Myomaker is strongly up-regulated during the regeneration of trout muscle

In vertebrates, the formation of new muscle fibers occurs during both embryogenesis and muscle regeneration. We studied the kinetic of muscle regeneration in adult trout following mechanical muscle injury to determine whether *myomaker* is up-regulated during regeneration of trout muscle. The histological analysis (Fig. 7*A*) showed that 16 days after muscle injury (Fig. 7*B*), a large number of myofibers was degraded, with many immune cells infiltrating into the injury site. After 30 days, all the injured fibers disappeared and were replaced with connective tissue containing small (<20 μm) round cells labeled in *green* (Fig. 7*C*). Immunocytofluorescence staining revealed that these cells expressed myosin and that their nuclei was often centrally positioned (Fig. 7*D*). These observations point out the presence of newly formed muscle fibers on day 30, showing that muscle regeneration had occurred. Importantly, uninjured control muscle did not contain small nascent myofibers (Fig. 7*A*), indicating that myofiber formation had ceased at this stage, consistent with the results reported by Rescan *et al.* (20). According to the qRT-PCR analysis, *myogenin* and *myomaker* gene expression in muscle did not change until day 16 postinjury (Fig. 7, *E* and *F*). In contrast, 30 days after injury, a sharp increase in both *myomaker* and *myogenin* expression was observed in the injured muscle. Indeed, *myogenin* and *myomaker* were expressed at 10- and 15-fold higher levels, respec-

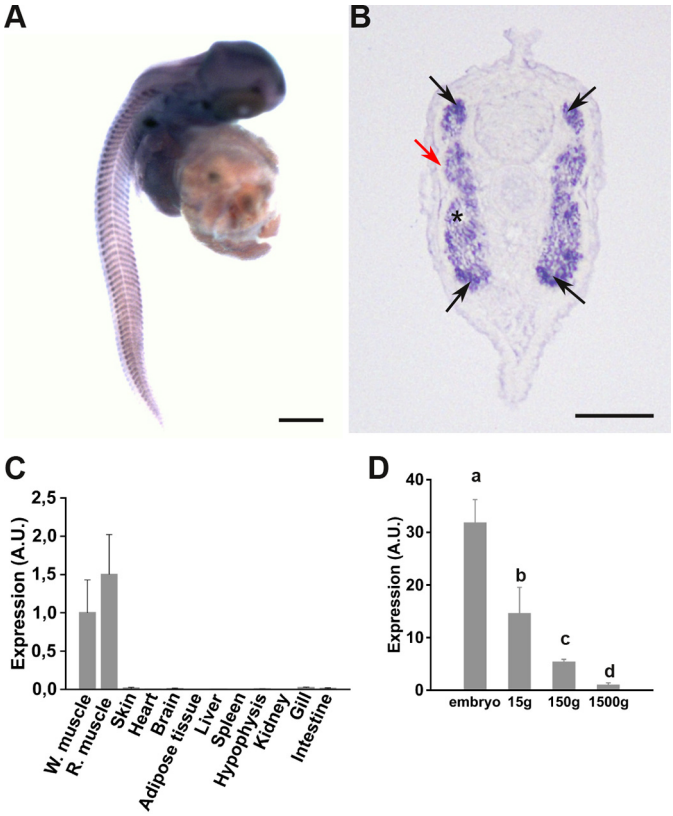

Figure 6. Muscle-specific expression of myomaker starts in the embryo and persists after hatching in trout. *A* and *B*, analysis of myomaker expression during trout embryonic development by *in situ* hybridization. Embryos were analyzed at stage 20. The *scale bars* correspond to 500 μm (*A*) and 50 μm (*B*). *C* and *D*, qRT-PCR analysis of myomaker expression in several tissues of trout (150 g; *C*) and in muscle of trout of different weight (*D*). A *black star* indicates the deep myotome where primary myogenesis takes place, and *black arrows* indicate dorsal and ventral domains of the myotome where stratified hyperplasia takes place. The *red arrow* indicates the dermomyotome-like epithelium. Total RNA was extracted from three different fish (n = 3). The qRT-PCR results are presented as a ratio of myomaker expression and eF1a expression, and the *bars* represent the standard error. The *letters* (*a*–*d*) in *D* indicate the significant differences between means ($p < 0.05$; Kruskal–Wallis rank test followed by the post hoc Dunn test). *W.*, white; *R.*, red.

tively, in injured muscle compared with control muscle (Fig. 7, *E* and *F*).

Myomaker is up-regulated during myotube formation in vitro

After extracting trout satellite cells from white muscle, we induced the differentiation and fusion of trout satellite cells *in vitro* (22). Quantitative PCR analysis showed an increase (2-fold) in *myomaker* expression soon after satellite cell differentiation was induced (Fig. 8*A*). By performing immunofluorescence staining with an anti-myosin antibody, we quantified the number of small myotubes (2 < nuclei ≤ 4) and large myotubes (5 ≤ nuclei) during differentiation (Fig. 8*B*). Small myotubes began to form 1 day after the induction of differentiation and were strongly increased up to day 3 of differentiation, whereas large myotubes appeared on day 2. These results showed that the maximum level of myocyte fusion occurred on days 2 and 3 and correlate with highest *myomaker* expression.

Trout myomaker drives heterologous cell fusion

We performed cell-mixing experiments using myoblasts (C2C12) and fibroblasts expressing GFP infected with a vector

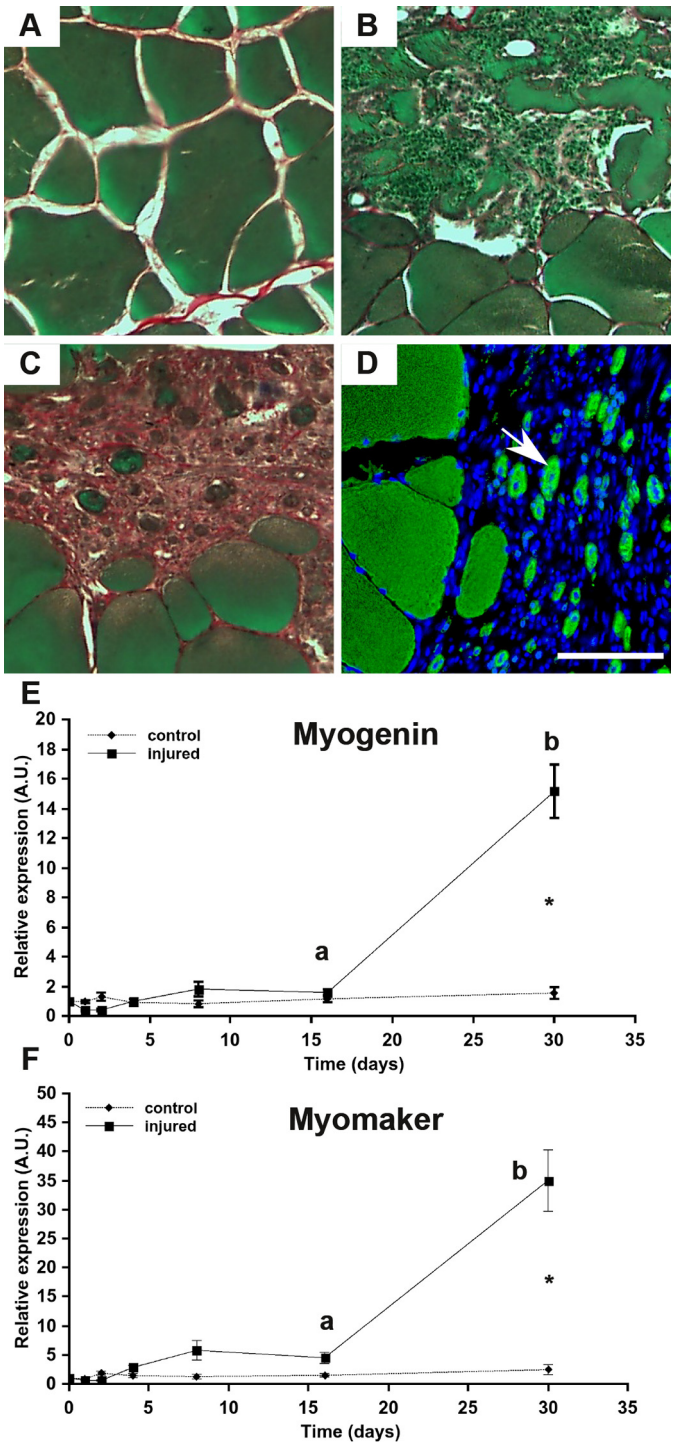

Figure 7. Myomaker is induced during trout muscle regeneration. *A*–*D*, histological analysis during muscle regeneration in rainbow trout. Muscle sections (10 μm) were stained with Sirius Red (connective tissue), Fast Green (muscle fibers), and hematoxylin (nuclei). *A*–*D*, uninjured white muscle (*A*) and white muscle at days 16 (*B*) and 30 (*C* and *D*) after muscle injury. Immunocytofluorescence detection of myosin heavy chain (MyHC) was performed at 30 days (*D*). A *white arrow* indicates newly formed myofibers. The *scale* corresponds to 100 μm. *E* and *F*, gene expression profile of myogenin (*E*) and myomaker (*F*) during muscle regeneration in rainbow trout. The expression of myogenin and myomaker were normalized with eF1a expression, and *bars* represent the standard error. The *letters* (*a* and *b*) in *E* and *F* indicate the significant differences between means within the same treatment (control or injured). The *asterisk* indicates significant differences between treatments at a given time. Statistical significance ($p < 0.05$) was determined using the Kruskal–Wallis rank test followed by the post hoc Dunn test.

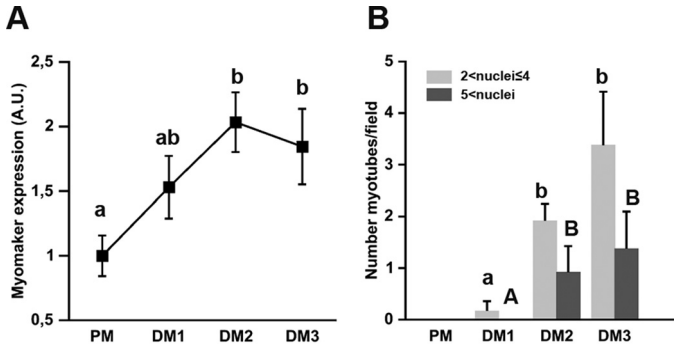

Figure 8. Myomaker expression is induced during trout satellite cell differentiation. *A*, analysis of myomaker gene expression during satellite cell differentiation. The cells were cultivated in proliferative medium (*PM*) and then in differentiation medium for 1, 2, and 3 days (*DM1*, *DM2*, and *DM3*). The qRT-PCR results are presented as a ratio of myomaker and eF1a expression, and *bars* represent the standard error. *B*, myocyte fusion quantification during the satellite cell differentiation. The cells were cultivated in proliferative medium (*PM*) and then in differentiation medium for 1, 2, and 3 days (*DM1*, *DM2*, and *DM3*). The number of myotubes with 2–4 nuclei or with >5 nuclei was determined by immunocytofluorescence analysis of MyHC. Different letters (*a–c* or *A* and *B*) indicate significant differences between means. Statistical significance ($p < 0.05$) was determined using the Kruskal–Wallis rank test followed by the post hoc Dunn test.

expressing trout myomaker cDNA to determine whether trout myomaker is able to drive cell fusion. We first showed that the infection of fibroblasts with trout myomaker construct actually resulted in the production of trout myomaker protein using immunofluorecence staining (Fig. 9*A*). After 5 days of co-culture, we failed to observe any fusion between C2C12 and the GFP-empty fibroblasts (Fig. 9*B*). In contrast, GFP-myomaker–infected fibroblasts were able to fuse with C2C12 myoblasts. We implemented a dual split luciferase assay previously used to monitor cell–cell fusion to accurately quantify the fusion between fibroblasts and myoblasts (21). In this assay, luciferase activity is only reconstituted when fusion between fibroblasts and myoblasts occurs (Fig. 9*C*). Fibroblasts were infected with full-length or truncated trout myomaker along with mouse myomixer to increase the basal fusion level (5). This assay confirms that the full-length myomaker protein is able to drive the fusion of fibroblasts with myoblasts. Surprisingly, deletion of trout myomaker C terminus resulted in a significant reduction in cell fusion. Together, our results confirmed that trout myomaker drove heterologous cell fusion, although to a lesser extent than mouse myomaker (Fig. 9*D*).

Discussion

Given the unique structure of the trout myomaker protein, we aimed in this study to determine whether the expression and function of myomaker are conserved in this nonmodel fish. Based on sequence alignments and phylogenetic and syntenic analyses, we identified the unique functional *myomaker* gene in the trout genome. We also identified a *myomaker* pseudogene containing long deletions in the coding region. This pseudogene probably results from the salmonid-specific whole genome duplication (WGD) that occurred ~96 ± 5 million years ago (17). After the WGD, the salmonid genome underwent a process of gene pseudogenization that resulted in the loss of half of the duplicated genes in the trout genome (17). Therefore, we hypothesize that the second identified *myomaker* gene originated from the WGD and became a pseudogene through deletions and mutations.

The trout myomaker protein consists of 434 aa with an apparent molecular mass of 40 kDa and is nearly twice the size of the mouse and zebrafish myomaker proteins. As shown in the sequence alignment, the first 220 aa of trout myomaker are 71–88% similar to the mouse and zebrafish orthologs. Importantly, trout myomaker contains the two conserved cysteines essential for mouse myomaker function (12). Furthermore, the hydrophobicity analysis (data not shown) strongly suggested the presence of seven transmembrane domains, similar to the mouse myomaker protein (9). Together, these results highlight the strong evolutionary conservation of the first section of the trout myomaker protein.

The additional amino acid stretch in trout myomaker mainly consists of 14 tandem repeats of 30 nucleotides in the coding region. A tandem repeat is a short sequence (unit) that is repeated several times in a head-to-tail orientation (18). Repeats with units less than 9 nucleotides in length are known as microsatellites, and those with 10 nucleotides or more are known as minisatellites. Therefore, the identified tandem repeats in trout *myomaker* correspond to minisatellites. Tandem repeats are preferentially located in noncoding regions, but when tandem repeats are present in protein coding regions, they most commonly occur in multiples of three nucleotides to avoid frameshifts (18). Accordingly, the trout myomaker minisatellites consist of 30 nucleotides that preserve the ORF of *myomaker*. An analysis of the human genome indicates that tandem repeats are present in only 17% of the genes and that 14 repeated minisatellites (>30 nt) in the ORF are very rare (18).

The phylogenetic analysis and sequence alignments showed that the minisatellites detected in trout *myomaker* are also present and conserved in other Protacanthopterygii species such as salmon (*S. salar*) and pike (*E. lucius*). For the minisatellites to be present through the Protacanthopterygii group, they must have appeared before the salmonid-specific WGD. Tandem repeats are unstable (18), and thus the minisatellites in *myomaker* must have undergone duplications and deletions at different rates in salmonids, leading to differing numbers of minisatellites in this group. The phylogenetic analysis also revealed that teleost myomaker sequences were classified into three groups according to myomaker protein length. Teleost belonging to Otocephala contain a *myomaker* sequence encoding 220–221 aa, as observed in all tetrapods. The Protacanthopterygii contain minisatellites in the myomaker gene that are translated into a protein of 434–456 aa. Surprisingly, teleost of the third group (Neoteleostei) do not contain minisatellites, and the length of the myomaker protein ranges from 283 to 289 aa. The first 220 aa of that myomaker sequences are highly similar to mouse myomaker, whereas residues 221–289 have no homology with any known protein motif. Interestingly, this sequence of ~70 aa is well-conserved (~70% identity) in Neoteleostei and is also present in Protacanthopterygii, in addition to the minisatellites. This phylogenetic analysis allowed us to determine the evolutionary history of the *myomaker* gene. The ancestral *myomaker* gene consisted of 220–221 aa and evolved by maintaining the same sized protein in tetrapods and some teleost fish (Otocephala). With the appearance of Euteleostei, a

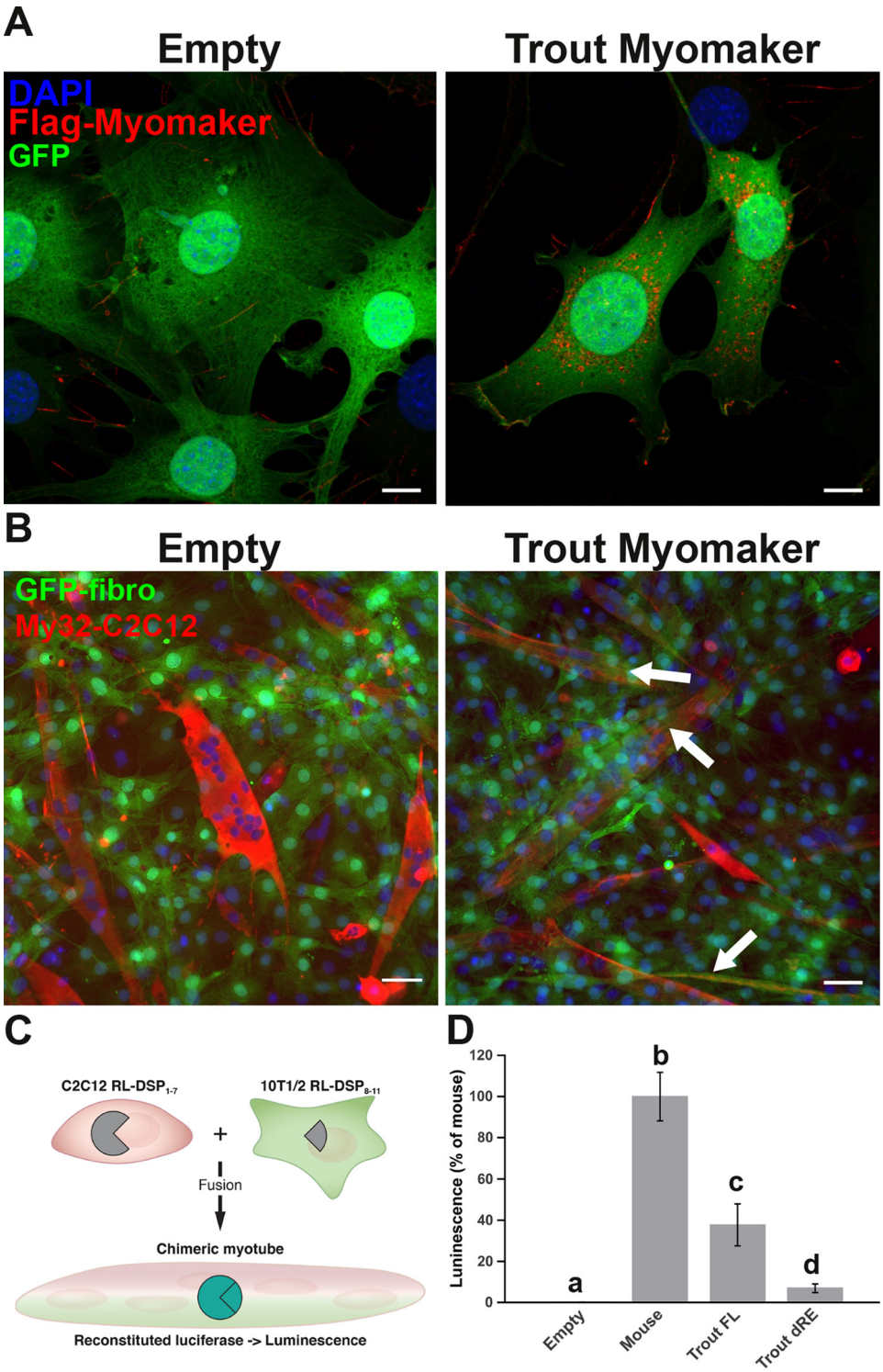

Figure 9. Trout myomaker is fusogenic *in vitro*. *A*, expression of trout myomaker in 10T1/2 mouse GFP fibroblasts after infection with trout Myomaker vector or empty vector. A strong red signal was observed only in cells infected with the trout myomaker vector. *B*, cell-mixing experiments with GFP-fibroblast and myoblasts (C2C12). The cultures were differentiated for 4 days and then immunostained with myosin antibody as a marker for myotubes. *White arrows* indicate fusion between fibroblasts and myoblasts (*orange labeling*). *Scale bar* corresponds to 50 μm. *C*, schematic representation of the dual split luciferase assays. The assay measures reconstituted luciferase activity from fusion between myoblasts expressing pRluc8155–156-DSP1–7 (RL-DSP1) and fibroblasts expressing pRluc8155–156-DSP8–11 (RL-DSP2) and infected with myomaker construct. Myotube formation was then induced for 5 days in DM, and luminescence was measured. *D*, quantification of fusion by luciferase assay. Fusion activity of mouse full-length (Trout FL) and truncated (Trout dRE) myomaker was quantified using a split luciferase assay system. Different letters (*a–d*) indicate significant differences between means. Statistical significance ($p < 0.05$) was determined using the Kruskal–Wallis rank test followed by the post hoc Dunn test. *DAPI*, 4′,6′-diamino-2-phenylindole.

first extension (60–70 aa) of the myomaker protein occurred that has been conserved to the present day. Later, minisatellites appeared in this extension in Protacanthopterygii, further lengthening the protein.

Given the unique evolution of trout *myomaker* gene sequence, we examined whether its expression pattern was different from that of the mouse or zebrafish. Using whole-mount *in situ* hybridization, we observed that *myomaker* was expressed in the myotome at the end of somitogenesis (stage 20) when myoblasts differentiate and start to fuse (19, 23). This result is reminiscent of our previous observation in zebrafish (14), which showed that *myomaker* expression was induced at 20 h postfertilization, at the inception of embryonic myocyte fusion (24). Consistent with this observation, the trout dermomyotome, a somitic external epithelium that contains undifferentiated muscle progenitors, did not express *myomaker* (25). Furthermore, vibratome sectioning of stage 20 trout embryos revealed that *myomaker* was expressed in the deep myotome, which is formed during the primary wave of myogenesis. Interestingly, *myomaker* was expressed at the highest level in the dorsal and ventral domains of the myotome, indicating that it is particularly associated with the secondary wave of myogenesis known as stratified hyperplasia (19, 27).

In contrast to mammals, salmonids have undergone an additional third wave of fiber formation (mosaic hyperplasia) that is responsible for the large increase in the muscle mass of larvae and juveniles, as well as the sustained muscle growth in adults (19). Based on our results, *myomaker* is expressed in the muscle of fry, juvenile, and, to a lesser extent, mature fish. Thus, *myomaker* expression persists in growing fish, in contrast to adult mice where *myomaker* expression in muscle is only observed during regeneration (9, 10). The persistence of *myomaker* expression in trout white muscle is associated with the persistence of new fiber formation from mosaic hyperplasia. Accordingly, the lowest expression of *myomaker* was observed in mature fish, when hyperplasia is reduced (20, 28).

Because *myomaker* expression decreases as fish mature, we wondered whether its expression in aged trout was reinduced during muscle regeneration. Few data on muscle regeneration in fish are available, but some studies have successfully used mechanical injury to induce muscle regeneration in zebrafish (29), sea bream (29), trout (20), and salmon (30). Our histological and immunocytofluorescence analyses clearly indicated that mechanical muscle injury induced the formation of new myofibers on day 30. At the molecular level, the appearance of new fibers coincided with the peak of *myogenin* expression, thus confirming the resumption of myogenesis at this time, consistent with the findings reported by Rescan *et al.* (20). As expected, the expression of *myomaker* was also up-regulated at day 30. The parallel expression of *myogenin* and *myomaker* suggested that myogenin directly regulates *myomaker* expression, as reported in mice (10). In keeping with this, an analysis of the trout proximal promoter revealed the presence of several E-boxes (CANNTG) that are known to be binding sites for myogenic transcription factors such as MyoD and Myogenin (26). Our results from the muscle regeneration experiment indicated that *myomaker* up-regulation is associated with the appearance of new myofibers when myocyte fusion occurs. Accordingly, trout satellite cell cultures exhibited increased *myomaker* expression when the fusion of trout myocytes occurred. Based on these results, myomaker appears essential for fiber formation during muscle regeneration in trout and in mice (9, 10).

Because the coding sequence of trout *myomaker* contains 14 minisatellites, we examined whether the protein conserved its fusogenic function. Although tandem repeats are generally located in noncoding regions, some tandem repeats in coding regions alter protein activity and lead to disease (18). We tested the fusogenic activity of trout myomaker using heterologous fibroblast–myoblast fusion experiments in mouse cells (9). Trout myomaker was sufficient to induce fusion of mouse fibroblasts with myoblasts, although at lower efficiency than with the mouse orthologs. Furthermore, deletion of the expanded C terminus impaired myomaker activity, which suggests that minisatellites are required for full trout myomaker activity. Thus, despite the presence of 14 minisatellites, trout myomaker has preserved its fusogenic function, similar to mouse and zebrafish orthologs (12).

In conclusion, we identified the unique *myomaker* gene in the trout genome and discovered 14 minisatellites of 30 nucleotides in length at the end of the coding region. Surprisingly, this long insertion did not abolish the fusogenic activity of myomaker. Furthermore, the formation of new fibers was constantly accompanied by an up-regulation of *myomaker* showing that this gene should be considered a marker of muscle hyperplasia.

Experimental procedures

Animals and experimental design

The experiments were performed in accordance with legislation governing the ethical treatment of animals (décret no. 2001-464, May 29, 2001) and the Institut National de la Recherche Agronomique PEIMA (Pisciculture Expérimentale INRA des Monts d'Arrée) Institutional Animal Care and Use Committee (B29×777-02), which specifically approved this study. Investigators were certified by the French government to conduct animal experiments (agreement no. 35-47). The fish facility was approved by the Ministère de l'Enseignement Supérieur et de la Recherche (authorization no. A352386).

Muscle regeneration experiment

Muscle regeneration experiments were performed at the Institut National de la Recherche Agronomique facility PEIMA (Sizun, Britany, France). Rainbow trout (*O. mykiss*) with a mean weight of 1530 ± 279 g were anesthetized with MS-222 (50 ml/liter). Using a sterile 1.2-mm needle, muscle injuries were done on the left side, posterior to the dorsal fin and above the lateral line. Prior to sampling of muscle tissue, fish were sacrificed with an overdose of MS-222. Muscle sampling was performed at 0, 1, 2, 4, 8, 16, and 30 days postinjury using a sterile scalpel. White muscle was collected from the site of injury, and noninjured muscle tissue from the opposite side of the fish was used as control. During the experiment, all fish remained alive, and no infection was observed. The samples were stored in liquid nitrogen until RNA extraction or fixed with Carnoy's solution (6:3:1 absolute ethanol, chloroform, and acetic acid)

for 24 h at 4 °C, dehydrated with 95% alcohol and alcohol/butanol (50/50), and then embedded in paraffin. Transverse muscle sections (10 μm) were cut using a microtome (Microm HM 355; Microm Microtech, Francheville, France), stained with Sirius Red and 0.1% Fast Green in saturated picric acid, and counterstained with hematoxylin. This staining marks the muscle fibers in *green*, the connective tissue in *red*, and the nuclei in *black*.

Trout satellite cell culture

Satellite cells from trout white muscle (5–10 g) were cultured as previously described (22, 33). Briefly, after several enzymatic digestions and cell filtration steps, the cells were seeded on glass coverslips at a density of 160,000 cells/cm^2 and incubated for 40 min. The cells were cultured in F10 medium (nutrient mixture Ham's F10, Sigma, N6635) supplemented with 10% fetal bovine serum to stimulate cell proliferation. The medium was changed to Dulbecco's modified Eagle's medium (Sigma, D7777) containing 2% fetal bovine serum to stimulate cell differentiation.

The cells on glass coverslips were briefly washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. For permeabilization, the cells were incubated with 0.1% Triton X-100 in PBS for 3 min. After three washes, the cells were incubated with 3% BSA and 0.1% Tween 20 in PBS (PBST) for 1 h. The cells were incubated with the primary anti-myosin antibody (catalog no. MF20; Hybridoma Bank) diluted in blocking buffer for 3 h. The secondary antibody (catalog no. A11001, Molecular Probes) was diluted in PBST and applied for 1 h. The cells were mounted with Mowiol 4-88 (catalog no. 475904, Calbiochem) containing 4′,6′-diamino-2-phenylindole (10 μg/ml). The cells were photographed using a Nikon digital camera coupled to a Nikon 90i microscope.

Phylogenetic analysis

The amino acid sequences were aligned using Clustal X software (31). A phylogenetic tree was generated using the sequences of vertebrates myomaker proteins listed in Fig. S1. The phylogenetic tree was created using the neighbor-joining method with MEGA 7 software (32). The robustness of the nodes of the phylogenetic tree was tested using bootstrapping methods.

RNA extraction, cDNA synthesis, and quantitative PCR analyses

Total RNA was extracted from cell cultures or from 100 mg of muscle using TRI reagent (Sigma–Aldrich, catalog no. T9424). Extracted RNA was quantified by measuring the absorbance at 260 nm (NanoDrop ND-1000 spectrophotometer), and 0.5 μg of total RNA was used for reverse transcription (Applied Biosystems kit, catalog no. 4368813). Trout myomaker primers (forward, 5′-AATCACTGTCAAATGGTTACAGA-3′; and reverse, 5′-GTAGTCCCACTCCTCGAAGT-3′) were designed at exon–exon boundaries to avoid genomic DNA amplification. The sequences amplified were tested for secondary structure formation using mFOLD (34). The amplification conditions were optimized before the expression analysis. Quantitative PCR analyses were performed with 5 μl of cDNA using a real-time PCR kit that contained a SYBR® Green fluorophore (Applied Biosystems), according to the manufacturer's instructions, with a final concentration of 300 nM of each primer. The amplification was performed using the following cycle: 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The relative abundance of target cDNAs within the sample set was calculated from a serial dilution (1:1–1:256) (standard curve) of a cDNA pool using StepOne^{TM} software V2.0.2 (Applied Biosystems). Subsequently, real-time PCR data were normalized using elongation factor-1α (*eF1*α) gene expression as previously detailed (35).

Whole-mount in situ hybridization

Embryos were fixed with 4% paraformaldehyde overnight and stored in methanol at −20 °C until use. Digoxigenin antisense RNA probes were synthesized from PCR-amplified templates using appropriate RNA polymerases. Whole-mount *in situ* hybridization was performed using standard protocols (36) with an INSITU PRO VS automated instrument (INTAVIS AG). For the histological examination of sections, the samples were embedded in 2.5% gelatin and 2% agar in distilled water. Blocks were sectioned at 35 μm on a Leica vibratome. Images of the sections were obtained using a Nikon 90i microscope.

Western blotting

After 3 days of culture in differentiation medium, the cells were washed with cold PBS, and proteins were extracted with radioimmune precipitation assay buffer supplemented with 5 mM NaF,1 mM $NaVO_4$, and a protease inhibitor mixture (Roche). The samples were subjected to 12% SDS-PAGE and Western blot analysis. The membranes were saturated with 5% nonfat milk in 25 mM TBST and subsequently incubated with a rabbit antibody against the trout myomaker protein overnight. This antibody (GenScript, Piscataway, NJ) was produced using a synthetic peptide (CTTPDKKALDINTTPPVKK) located in the tandem repeats of trout myomaker. After several washes, the membranes were incubated with a horseradish peroxidase–conjugated secondary antibody (1/15,000) (Jackson Immunoresearch) for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence, and images were obtained with an image acquisition system (Fusion FX7, Vilbert Lourmat).

Amplification, cloning, and sequencing of myomaker sequences

Reverse transcription (Applied Biosystems kit, catalog no. 4368813) was performed with 10 μg of total RNA extracted from trout embryos in a total volume of 100 μl. After a 10-fold dilution, we performed PCR (Promega GoTaq, catalog no. M7122) with primers (forward, 5′-TGGGACTACGCCTATGTCCACA-3′; and reverse, 5′-CCCATCCTTTCTTAACAGGCGTA-3′) that amplified exons 5 and 6. A single band of 595 bp was obtained, purified, and sequenced (Eurofins).

We first produced a synthetic gBlocks DNA fragment (Integrated DNA Technologies, Coralville, IA) of the 5′ part of the cDNA (1–561 nt) and inserted a FLAG tag (GATTACAAGGATGACGACGATAAG) into exon 2 to clone the full-length cDNA (12). The second part of the cDNA (562–1348 nt) was obtained by PCR using the following primers: 5′-GGGACTA-

CGCCTATGTCCACA-3′ and 5′-TCACTTCCACCCATTC-TGTTCTTTG-3′. Then the two DNA fragments were ligated and inserted into the pGEM vector. The full-length *myomaker* cDNA was then sequenced to validate the cDNA insert. We also produced a truncated form of myomaker (from 1 to 219 aa) that did not contain the repetitive elements, by conventional PCR with the following primers: 5′-GAGATCTAGAGAATTCCG-CCACC-3 and 5′-GAGAGAATTCTCATGTGCAGCA-GAG-3. Untagged constructs for all trout plasmids were generated by mutagenesis (200519, QuikChange Agilent) with the primers 5′-CTCTCCATCATATGTTTCATGAAGTATGA-GATCCTGGAGTAC-3 and 5′-GTACTCCAGGATCTCAT-ACTTCATGAAACATATGATGGAGAG-3. The vectors containing the mouse myomaker cDNA and myomixer cDNA were prepared as previously reported (5, 9).

In vitro fusion assay

For retroviral infection, Platinum-E cells (Cell Biolabs, catalog no. RV-101) were transfected with 10 μg of plasmid DNA using FuGENE 6 (Promega, catalog no. E2692) in a 10-cm cell culture dish. Two days after transfection, viral medium was filtered through a 0.45-μm cellulose syringe filter and mixed with Polybrene (Sigma) at a final concentration of 6 μg/ml. Then 10T1/2 mouse fibroblast cells that had been plated on the day before infection were incubated with the viral medium for 24 h before the cells were mixed. C2C12 mouse myoblasts were mixed with virus-infected 10T1/2 fibroblasts at equal amounts of 3×10^5 cells of each type and plated on a 35-mm dish. Twelve hours after plating, the cells were switched to myoblast differentiation medium (Dulbecco's modified Eagle's medium supplemented with 2% horse serum and 1% penicillin/streptomycin) and incubated for 4 days with a medium change on day 2 of differentiation.

Immunocytochemistry was performed by fixing cells with 4% paraformaldehyde in PBS, permeabilization with 0.2% Triton X-100 in PBS, blocking with 3% BSA in PBS, incubation with the primary antibody for 2 h, and incubation with Alexa Fluor–conjugated secondary antibodies for 1 h. The anti-mouse M2 flag antibody (Sigma) and myosin antibody (MY32; Sigma) were used as primary antibodies at 1:500 and 1:200 dilutions, respectively. The nuclei were stained with Hoechst (Invitrogen). These cultures were visualized under a Zeiss LSM 800 confocal microscope. ImageJ software was used to merge images.

Dual split luciferase assays

We developed a dual split luciferase/GFP assay to accurately quantify the fusion efficiency. The reporter system consists of a pair of chimeras (RL-DSP1 and RL-DSP2) encoding the N- or C-terminal portions of a fusion protein of *Renilla* luciferase and GFP protein (24). The original pRluc8155-156-DSP1–7 (RL-DSP1) and pRluc8155-156-DSP8–11 (RL-DSP2) sequences were kindly provided by Zene Matsuda (Institute of Medical Science, University of Tokyo, Tokyo, Japan) and subcloned into pMXs-puro using conventional PCR. Initially, two different cell populations expressing either RL-DSP1 or RL-DSP2, which are catalytically inactive, were generated. When both populations are mixed and fusion occurs, each fragment of the reporter protein spontaneously interacts. Thus, in chimeric myotubes, the reporter becomes active, and luciferase activity is used as a surrogate for fusion efficiency. Luciferase readings were performed after 5 days of culture in differentiation medium using a CLARIOstar microplate reader (BMG Labtech) and the cell-permeable ViviRen substrate (Promega). Medium was replaced with 50 μl of 60 μM ViviRen, and the cells were incubated for 12 min at room temperature before measuring the luciferase activity.

We first validated whether luciferase readings were able to quantitatively measure fusion. We initially generated a calibration curve using C2C12 myoblasts and expected luciferase activity to increase linearly with the amount of cells expressing the reporters. For this experiment, we generated three stable C2C12 cell lines expressing one component of the split system (C2C12-RL-DSP1 and C2C12-RL-DSP2) or only the puromycin resistance cassette (C2C12-Empty). Those populations were then mixed as follows: the amount of C2C12-RL-DSP2 was kept constant at 9,000 cells/well, whereas the amount of C2C12-RL-DSP1 was gradually increased up to 9,000 cells. Myotube formation was then induced for 5 days in differentiation medium (DM), and luminescence was measured. The luciferase signal was linearly proportional to the amount of C2C12-DSP1 cells throughout the range of cells used (Fig. S1). Then we generated an equivalent calibration curve for heterologous myoblast–fibroblast fusion by mixing C2C12-RL-DSP2 cells with increasing amounts of 10T1/2-RL-DSP1 fibroblasts infected with either the empty vector (no fusion) or mouse myomaker plasmid. As expected, we also observed a high linearity within the range of cells used (Fig. S2). For luciferase assays comparing mouse and trout myomaker, we used untagged myomaker constructs and co-expressed mouse myomixer to increase fusion basal levels.

Statistical analyses

The data were analyzed using the nonparametric Kruskal–Wallis rank test followed by the post hoc Dunn test. All analyses were performed using the R statistical package (3.5.1 version).

Author contributions—A. L. data curation; A. L., A. R.-M., O. M., and E. N. O. formal analysis; A. L., A. R.-M., O. M., and N. S. methodology; A. R.-M. and J.-C. G. investigation; A. R.-M., P.-Y. R., E. N. O., and J.-C. G. writing-review and editing; P.-Y. R., E. N. O., and J.-C. G. conceptualization; E. N. O. and J.-C. G. validation; J.-C. G. supervision; J.-C. G. funding acquisition; J.-C. G. writing-original draft; J.-C. G. project administration.

Acknowledgments—We particularly thank F. Borel and C. Duret for trout rearing and egg production and the fish facility PEIMA (Pisciculture Expérimentale INRA des Monts d'Arrée) for muscle regeneration experiments. We also thank Dr. Li for assistance with dual split luciferase assays.

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