

CRISPR/Cas9-mediated mstnb1 disruption enhances growth in rainbow trout

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Abstract

This study was conducted to determine the effects of knocking out the myostatin gene (*mstnb1*), which restricts muscle development in rainbow trout (*Oncorhynchus mykiss*), using CRISPR/Cas9 technology on growth performance and feed efficiency. Within the scope of this research, which aims to increase sectoral productivity in aquaculture, two different gRNAs targeting the first exon of the *mstnb1* gene were designed. Sanger sequencing analyses confirmed the successful creation of mutations in the F0 generation, involving a 5 bp deletion with gRNA1 and a 1 bp insertion and 6 bp deletion with gRNA2. During a 10-month follow-up period between May 2024 and March 2025, the growth parameters of mutant individuals were compared with a wild-type (WT) control group. At the end of the experiment, mutant fish reached an average weight of 825.0 ± 12.3 g, while the WT group remained at 464.1 ± 9.2 g. These results demonstrate that CRISPR/Cas9-mediated *mstnb1* disruption successfully induces the "double muscularity" phenotype and has high biotechnological potential in rainbow trout farming, potentially shortening production time and reducing unit costs.

1. Introduction

The global population is expected to reach 9.5 billion by 2050 (Madhuri et al., 2025). However, various risk factors—global warming, and limited freshwater sources—will make it harder for people to access nutritious and safe food in the future. Aquaculture is the fastest growing food production industry worldwide. Therefore, aquaculture products would become one of the primary animal protein and healthy animal fat sources to meet the increased food demand and to prevent nutritional deficiencies for humans (Pradeepkiran, 2019).

Myostatin (*mstn*) is a member of the transforming growth factor β (TGF- β) superfamily and negatively regulates skeletal muscle development in vertebrates (Yan et al., 2022). The *mstn* mutant fish, such as common carp (*Cyprinus carpio*) (Zhong et al., 2016), red sea bream (*Pagrus major*) (Ohama et al., 2020), yellow catfish (*Pelteobagrus fulvidraco*) (Zhang et al., 2023), and Nile tilapia (*Oreochromis niloticus*) (Wu et al., 2023), exhibited increased muscle growth, referred to as double muscling, compared to the wild-type (WT) fish. Double muscling was also determined in various *mstn* null domestic animals including cattle (Kwon et al., 2025), goats (Ni et al., 2014), chickens (Zhang et al., 2011), and sheep (Clop et al., 2006). Since knock down of the *mstn* gene plays a curious role to produce fast-growing domestic animals, recent research has focused on applying modern techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 to disrupt this gene.

Teleost fish underwent two whole genome duplications that occurred approximately 320 million years ago (mya) and 80 mya, namely teleost-specific whole genome duplication (ts-WGD) and salmonid-specific whole genome duplication (ss-WGD) (Lien et al., 2016; Sun et al., 2020). Both events led to the functional divergence of the genes (Richman et al., 2024). Rainbow trout (*Oncorhynchus mykiss*) genome contains four *mstn* duplicates: *mstna1* (*mstn1a*), *mstna2* (*mstn1b*), *mstnb1* (*mstn2a*), and *mstnb2* (*mstn2b*) due to the ss-WGD event (Garikipati et al., 2006 and 2007). However, genome editing techniques have not been applied in rainbow trout to knock down *mstn* ohnologs, most likely due to the difficulties arising from four copies of the *mstn* gene.

Fish *mstna* and *mstnb* genes were predominantly found in the muscle and brain, respectively (Maccatrozzo et al., 2001; Garikipati et al., 2006 and 2007; Uzun et al., 2025). We also determined that *mstna1* and *mstna2* were mostly found in the brain and *mstnb1* was preliminary expressed in the muscle in rainbow trout (Data not shown). The *mstna* has generally been targeted to create a double muscling phenotype using genome editing techniques, taking its high expression in the muscle as evidence that it is a negative regulator of muscle (Zhang et al., 2020a, b; Wu et al., 2023). The *mstnb* gene is thought to be involved in neurogenesis rather than growth because of its highest transcription levels in the brain (Østbye et al., 2007). However, Gao et al. (2016) in zebrafish (*Danio rerio*), Zhong et al. (2016) in common carp, and Sun et al. (2020) in blunt snout bream (*Megalobrama amblycephala*) have targeted the *mstnb* gene to produce a double muscling phenotype in fish. Therefore, we speculated that rainbow trout *mstnb1* might function in both neurogenesis and growth metabolism due to subfunctionalization, and then knocked down the *mstnb1* gene using CRISPR/Cas9 genome technology to produce fast-growing fish.

2. Material and Methods

2.1. Artificial insemination of rainbow trout

Eggs from two female rainbow trout and sperm from three male trout were collected from the Freshwater Fish Research Center at Atatürk University on ice and transported to the Laboratory of Agricultural Biotechnology Department at the Faculty of Agriculture of Atatürk University in Erzurum, Türkiye, and stored at +4°C until the next day (January 18, 2024). The sperm was used to fertilize the eggs (1200 eggs for *mstnb1*- and 1200 eggs for WT) for in a microinjection solution composed of 6.01 g of NaCl, 1.21 g of Tris, 1.5 g of glycine, and 0.31 g of reduced glutathione (pH: 9). After fertilization, the eggs were rinsed in a 1 mM glutathione solution for 10 min. to activate development. Subsequently, they were washed with the microinjection solution

and kept in it until the microinjection process was complete. The experimental protocol was approved by the Committee for Laboratory Animal Experimentation at Atatürk University, with the number 2025/12, in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2. gRNA design and *in vitro* transcription

The sequences for the genomic and coding regions of rainbow trout *mstnb1* (LOC100136340, NM_001124576.1) were obtained from the National Center for Biotechnology Information. The CRISPR/Cas9 technology was utilized to knock out *mstnb1* by designing two *mstnb1*-specific guide RNAs (gRNA) targeting the first exon of the gene using the CRISPOR web tool (<https://crispor.gi.ucsc.edu/>; Concordet and Haeussler, 2018) (Table 1). A PCR template for gRNAs transcription was created by amplifying a Cas9 scaffold sequence with a universal reverse primer (scRev) and a three-part forward primer (*mstnb1*-F1 and *mstnb1*-F2) containing a T7 RNA polymerase promoter sequence, the gRNA target sites starting with GG followed by a sequence binding to the 5' end of the Cas9 scaffold sequences (Table 1). A PCR Master Mix was made containing 119.25 µL of H₂O, 15 µL of 10 × Taq buffer, 3 µL of dNTP (40 mM), 0.75 µL of *mstnb1*-F1 (25 µM), 0.75 µL of *mstnb1*-F2 (25 µM), 3 µL of scRev (25 µM), 6 µL of the 1ng/µL gRNA template, and 0.75 µL of One Taq Polymerase (New England Biolabs) for a total reaction volume of 150 µL. The Master Mix was vortexed and separated into six tubes, with each tube holding a volume of 25 µL. The PCR was conducted under the following conditions: an initial denaturation at 94 °C for 30 sec., followed by 29 cycles consisting of 20 sec. at 94 °C, 20 sec. at 60 °C, and 5 sec. at 68 °C, concluding with a final extension at 68 °C for 2 min. The gRNA DNA templates were purified using a column purification kit (GeneJet PCR Purification Kit, ThermoFisher Scientific). Afterwards, 1.2% agarose gel used to determine heteroduplex phenotyping.

Table 1
gRNA and primer sequences used in this study

gRNA1	Sequence
	3' TAAGTACACCTAAGAGTGTGTC 5'
mstnb1-F1	TTAATACGACTCACTATAGTAAGTACACCTAAGAGTGTGCGTTTTAGAGCTAGAAATAGCAA
gRNA2	3' GGTTCATTTCATCCCCA 5'
mstnb1-F2	TTAATACGACTCACTATAGGTTTCATTTCATCCCCAGTTTTAGAGCTAGAAATAGCAA
scRev	AAAAGCACCGACTCGGTGCC
gRNA template	GTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT
*: Underlined sequences are gRNA sequences.	

In vitro transcription of the CRISPR gRNA template was performed using the HiScribe® T7 High Yield RNA Synthesis Kit and the resulting transcribed RNA was then purified using a column-based RNA cleanup kit (NEB Monarch RNA Clean-Up Kit, New England Biolabs) according to the manufacturer's instructions. The Cas9 mRNA was dissolved in the provided buffer to create a solution with a concentration of 1 mg/mL. To reduce freeze-thaw cycles, the resulting solution in PCR tubes was stored as small injection-ready aliquots at -80°C. For creating a 5 µL injection solution, 2 µL of the 1 mg/mL Cas9 solution was utilized, allowing for the Cas9 to be dispensed into 2 µL aliquots using PCR strip tubes (Sorlien et al., 2018).

2.3. Incubation of embryos and maintenance of rainbow trout larvae

The embryos were placed in six incubators with 400 embryos each (three for microinjected and three for WT embryos) supplied with 10.0 ± 1.0 °C well-water at the Aquaculture Research Unit, Faculty of Fisheries, Atatürk University (Erzurum, Türkiye). Following hatching, 282 larvae from each group were distributed into six 55-L fiberglass tanks (94 fish per tank; three tanks for *mstnb1*⁻ fish and three tanks for WT) within a recirculating aquaculture system (RAS). Although 1051 WT fish were available, their number was reduced to 282 to match the *mstnb1*⁻ rainbow trout. Water temperature of the RAS was initially set 11 °C with continuous aeration and 8.0 ± 0.5 ppm dissolved oxygen. The water temperature was gradually raised by 1 °C per month until reaching a constant 15 °C for the remainder of the study. Both the WT (*mstnb1*⁺) and the CRISPR/Cas9-modified mutant fish (*mstnb1*⁻) were fed four times a day *ad libitum* with a commercial trout feed at 08:00, 12:00, 16:00, and 20:00. They were bulk weighed each month to calculate growth parameters.

2.4. Detection of CRISPR/Cas9-mediated *mstnb1* mutations in rainbow trout in the F0 generation

Genomic DNA (gDNA) was extracted from the caudal fins of both *mstnb1*⁺ and *mstnb1*⁻ rainbow trout employing the phenol-chloroform extraction technique (Kishimoto et al., 2018). Amplification of DNA fragments that encompass the target site of the gRNAs was performed on samples from both *mstnb1*⁺ and *mstnb1*⁻ phenotypes (Table 1). The analysis of the mutated sequences was conducted through heteroduplex-based genotyping and Sanger sequencing described by Wu et al. (2023).

2.5. Statistical analyses

The experimental design followed a completely randomized block design (n = 3). Before analysis, the Shapiro-Wilk normality test and Levene's homogeneity of variances test was applied. One-way one-way analysis of variance (ANOVA) was applied to determine growth parameters using

SPSS 25.0 software for Windows (SPSS, 2017). The significant differences of the three replicates were determined using Duncan's multiple range test (p < 0.05) (Duncan, 1955).

3. Results

3.1. Survival rates at different stages

A total of 792 of injected embryos (66.0%) reached to eyed stage compared to 92.0% in the WT fish. Transition from the eyed stage to yolk-sac larvae yielded survival rates of 64.9% for injected and 95.2% for WT eggs. By the swim-up stage, survival rates were 23.5% (282 fry) and 87.6% (1051 fry) for the injected and WT groups, respectively.

3.2. *mstnb1* disruption in rainbow trout F0

We used the CRISPR/Cas9 technique to verify negative effects of the *mstnb1* gene in rainbow trout muscle. The gRNA positions targeting rainbow trout exon 1 were shown in Fig. 1. Mutations were determined in the F0 *mstnb1* mutants using Sanger sequencing from 50 fish. We found that gRNA1 and gRNA2 produced different mutant F0 lines. While gRNA1 produced a mutant line containing 5-bp deletions, gRNA2 produced another mutant line with 1 bp insertion and 6-bp deletions (Figs. 2 and 3). Heteroduplex amplicons were identified in the mutant F0 fish (Supplementary file 1).

3.3. Double muscling and growth parameters of *mstnb1* deficient fish

We calculated growth parameters for *mstnb1*⁻ and WT rainbow trout monthly for 10 months, from May 2024 to March 2025. While *mstnb1*⁻ rainbow trout reached 825.0 ± 12.3 g individual weights (n = 50; Fig. 4) with $43.841,4 \pm 1212.4\%$ weight gain (WG) for this period, WT fish reached 464.1 ± 9.2 g with $36.685,9 \pm 1000.9\%$ WG during the same period. Specific growth rate (SGR) of *mstnb1*⁻ and WT rainbow trout was 2.2 ± 0.6 and 2.1 ± 0.5 , respectively. Feed conversion ratio (FCR) was 0.93 ± 0.2 for *mstnb1*⁻ and 0.94 ± 0.1 for WT fish (p < 0.05; Table 2).

Table 2

Growth parameters in *mstnb1*⁻ and wild-type rainbow trout for ten months

Month	Initial Weight (g)		Final weight (g)		WG (%) ¹		SGR ²		FCR ³	
	<i>mstnb1</i> ⁻	WT	<i>mstnb1</i>	WT	<i>mstnb1</i> ⁻	WT	<i>mstnb1</i> ⁻	WT	<i>mstnb1</i> ⁻	WT
May 2024	1.9 ± 0.1	1.3 ± 0.1	3.4 ± 0.2	2.1 ± 0.1	-	-	-	-	-	-
June 2024	3.4 ± 0.2	2.1 ± 0.1	8.1 ± 0.6	5.4 ± 0.5	81.8 ± 5.4	78.1 ± 2.2	2.6 ± 0.7	2.5 ± 0.6	0.85 ± 0.1	0.90 ± 0.0
July 2024	8.1 ± 0.6	5.4 ± 0.5	19.0 ± 1.4	12.2 ± 1.2	135.9 ± 6.7	161.9 ± 5.1	3.9 ± 0.5	3.2 ± 0.5	0.83 ± 0.1	0.85 ± 0.1
August 2024	19.0 ± 1.4	12.2 ± 1.2	46.0 ± 2.4	28.7 ± 1.9	135.7 ± 6.1	126.1 ± 4.0	2.8 ± 0.6	2.6 ± 0.7	0.86 ± 0.1	0.89 ± 0.1
September 2024	46.0 ± 2.4	28.7 ± 1.9	91.2 ± 3.2	57.6 ± 2.0	142.3 ± 6.7	135.2 ± 4.8	3.2 ± 0.7	3.1 ± 0.6	0.89 ± 0.1	0.95 ± 0.1
October 2024	91.2 ± 3.2	57.6 ± 2.0	166.3 ± 4.4	101.2 ± 3.9	98.1 ± 4.6	100.6 ± 3.7	2.4 ± 0.8	2.5 ± 0.5	0.87 ± 0.1	0.86 ± 0.0
November 2024	166.3 ± 4.4	101.2 ± 3.9	254.2 ± 5.7	152.0 ± 5.2	82.5 ± 4.8	75.7 ± 1.9	2.2 ± 0.5	2.0 ± 0.4	0.86 ± 0.0	0.88 ± 0.0
December 2024	254.2 ± 5.7	152.0 ± 5.2	391.9 ± 7.4	218.0 ± 6.6	52.8 ± 2.1	50.2 ± 1.2	1.5 ± 0.3	1.5 ± 0.4	0.85 ± 0.1	0.85 ± 0.0
January 2025	391.9 ± 7.4	218.0 ± 6.6	617.5 ± 10.5	338.0 ± 8.4	54.2 ± 1.7	43.4 ± 0.7	1.6 ± 0.3	1.3 ± 0.2	0.96 ± 0.0	1.03 ± 0.1
February 2025	617.5 ± 10.5	338.0 ± 8.4	825.0 ± 12.3	464.1 ± 9.2	57.6 ± 2.7	55.1 ± 1.9	1.6 ± 0.4	1.5 ± 0.3	0.95 ± 0.1	0.97 ± 0.1
March 2025	825.0 ± 12.3	464.1 ± 9.2			33.6 ± 1.1	37.4 ± 1.1	1.0 ± 0.1	1.1 ± 0.1	0.93 ± 0.1	0.91 ± 0.1
From May 2024 to March 2025	1.9 ± 0.1	1.3 ± 0.1	825.0 ± 12.3	464.1 ± 9.2	43.841,4 ± 1212.4	36.685,9 ± 1000.9	2.2 ± 0.6	2.1 ± 0.5	0.93 ± 0.2	0.94 ± 0.1
¹ : WG: Final Weight (g) - Initial Weight (g)/Initial Body Weight (g) * 100, ² [LN Final Weight (g) - LN Initial Weight (g)/Days]*100, ³ : Given Feed (g)/Weight Gain (g)										

4. Discussion

Various studies have been conducted to clarify the negative effects of *mstn* gene on muscle growth in teleost fishes (Zhong et al., 2016; Yan et al., 2022; Ohama et al., 2020; Zhang et al., 2023; Wu et al., 2023), as well as in other vertebrates (Cloop et al., 2006; Zhang et al., 2011; Ni et al., 2014). They all reported that the *mstn* null genome improved muscle mass in animals. The number of the target genes for CRISPR/Cas9 studies in fish is two- or four-fold (in salmonids and some cyprinid species) compared to tetrapods due to ts-WGD or ss-WGD events. Therefore, the first step in these studies is to determine the target gene in the fish genome. While numerous studies in fish have knocked down the *mstna* gene (Kim et al., 2019; Zhang et al., 2020a, b) to produce fast-growing fish using CRISPR/Cas9 technology, others have targeted the *mstnb* gene (Gao et al., 2016; Sun et al., 2020; Wu et al., 2023), as described earlier. It was generally suggested that the *mstnb* (or *mstn2*) gene is involved in neurogenesis rather than growth. Based on this hypothesis, it might be suggested that *mstnb1* (not *mstnb2*, as it is a pseudogene) only functions in neurogenesis in rainbow trout. However, we identified a syntenic relationship between human chromosome 2, where *MSTN* is located, and rainbow trout chromosome 7, where *mstnb1* is located, for the *cnot9*, *plcd4*, *mmadh*, *rnd3*, *r3hdm1* genes, and more (data not shown). Therefore, we speculated that rainbow trout *mstnb1* gene might function in both neurogenesis and growth metabolism due to subfunctionalization, where functions of the ancestral gene are subdivided between duplicated genes (Venkatachalam et al., 2009), and then knocked down the *mstnb1* gene using the CRISPR/Cas9 technology to produce fast-growing rainbow trout.

Survival rate of WT fish was determined much higher (87.6%) than that of *mstb1*⁻ fish (23.5%) ($p < 0.05$). This result is similar to previous studies describing much lower survival rate in CRISPR/Cas9-based gene editing in fish (Tao et al., 2021; Coogan et al., 2022; Ou et al., 2022). We determined that body weight increased by about 56% in the *mstnb1*-distributed rainbow trout compared to WT fish at the end of 10 months. As this is the first report on knocking out the *mstnb1* gene in salmonids, we could not compare this result with previous studies in salmonids. However, common carp (*C. carpio*) underwent a fourth-round WGD (Erbaş et al., 2025), and its genome contains both *mstnb1* and *mstnb2* genes. In their study, Zhong et al. (2016) reported that disruption of *mstnb1* in common carp resulted in a double-muscling phenotype similar to that in our study. Moreover, the body weight of *mstna*-/ *mstnb*-deficient blunt snout bream, *mstnb*-deficient zebrafish, and *mstnb*-deficient Nile tilapia were 8.0%, 6.8%, 21%, and 49.45% higher, respectively, than those of WT fish (Gao et al., 2016; Sun et al., 2020; Wu et al., 2023). Therefore, our results are supported by previous research indicating that the fish *mstnb1* gene functions as a negative regulator in growth metabolism.

Surprisingly, SGR and FCR between *mstnb1*⁻ and WT rainbow trout were not different (Table 2). A study was conducted in Nile tilapia to determine the effects of *mstnb* deficiency on muscle growth and growth parameters. In this study, Wu et al. (2023) reported better SGR in *mstnb*^{-/-} than in WT. Similar results were reported in red sea bream for SGR and FCR (Ohama et al., 2020). Differences between this study and previous studies can be explained in a species-specific manner. We also determined that the *mstnb1*-deficient rainbow trout were 1.46-, 1.62-, 1.49-, 1.55-, 1.60-, 1.58-, 1.64-, 1.67-, 1.80-, 1.83-, and 1.78-fold heavier than WT fish between May 2024 and March 2025 (Table 2). This result clearly indicates that following the hatchery period, *mstnb1* null rainbow trout grow faster than WT fish, and this difference between the two groups is maintained in later periods.

The aquaculture industry aims to produce fast-growing fish for economic reasons, which is also crucial for sustainably feeding the growing global population. Rainbow trout is the second most widely farmed salmonid species in the world after Atlantic salmon (*Salmo salar*) (FAO, 2022). The commercial size of rainbow trout ranges from 300 g to 600 g globally (Skalecki et al., 2013), which between 200 g and 300 g in Türkiye (Anonymous, 2025). The *mstnb1*-deficient rainbow trout reached 254 g of weight in December 2024, whereas WT reached the same weight around two months later (February 2025), when *mstnb1*-null rainbow trout were approximately 620 g (Table 2). These results indicate that CRISPR/Cas9-mediated *mstnb1* knock-out rainbow trout reaches the market size around two months earlier than WT fish, suggesting more profitable commercial rainbow trout farming, especially with respect to fish feed accounting for 50%-60% of total expenses in aquaculture (IAFFD, 2025).

In this study, we determined that rainbow trout *mstnb1* functions in growth metabolism as a negative regulator of muscle growth in the first time in salmonids. Therefore, CRISPR/Cas9-mediated *mstnb1*-deficient rainbow trout can be used in the commercial farming for more profitable and sustainable purposes.

Declarations

Funding Declaration

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Data Availability Statement

Data available on request from the authors.

Author Contribution Statement

A.B. Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Supervision, Funding acquisition. **M.B.** Methodology, Investigation, Resources, Writing - Review & Editing. **W.T.** Methodology, Software, Validation. **C.W.** Formal analysis, Investigation. **S.T.** Investigation, Data Curation. **Burcu Naz Uzun:** Investigation, Formal analysis. **G.A.** Investigation, Resources. **H.A.** Investigation, Validation. **D.W.** Conceptualization, Methodology, Supervision, Writing - Review & Editing

Conflict of Interest Statement

The authors declare no conflict of interest.

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Figures

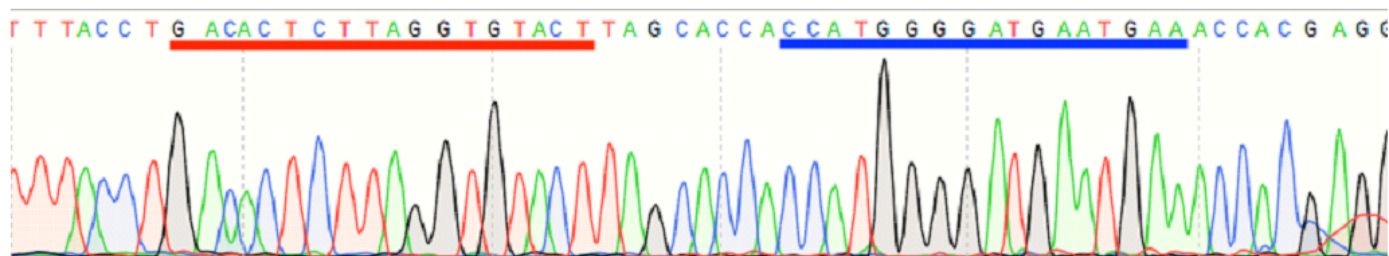


Figure 1. Genomic organisation and gRNA position of rainbow trout *mstnb1* gene (nt: nucleotides). gRNA was given in red capital letters and PAM sequence was highlighted in turquoise.

Figure 1

Genomic organisation and gRNA positions of rainbow trout *mstnb1* gene (nt: nucleotides). gRNAs were given in red capital letters and PAM sequence was highlighted in turquoise.

a)



b)

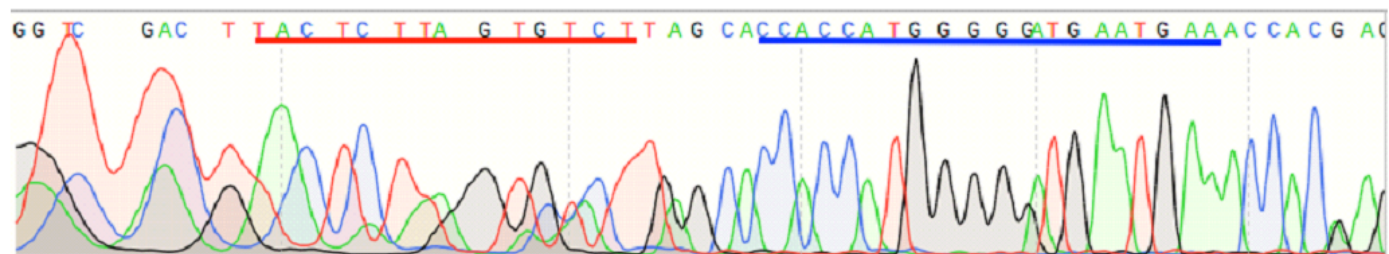


Figure 2
Sanger sequencing results for WT (a) and *mstnb1*-mutated (b) rainbow trout. gRNA1 was highlighted in blue.

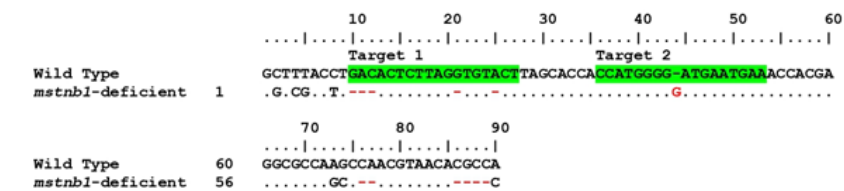


Figure 3
CRISPR/Cas9-mediated mutations in rainbow trout *mstnb1* gene. gRNA1 (Target 1) and gRNA2 (Target 2) were highlighted in green.



Figure 4

Double muscling in ten-months old *mstnb1*-deficient rainbow trout. a represents the mutated fish and b shows the WT fish.

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