

# Relation between skeletal muscle strength and ubiquitin ligase expression in muscle atrophy models in zebrafish larvae (*Danio Rerio*)

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## Research

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# Abstract

**Background** Skeletal muscle atrophy is often seen in patients with chronic diseases or muscle disuse. Upregulated expression of ubiquitin ligases such as Muscle Ring Finger 1 (MuRF-1) and Muscle Atrophy F-box (MAFbx) has been shown in different immobilization-induced atrophy models, which is believed to be responsible for the enhanced muscle protein proteolysis and thereafter results in muscle weakness. However, currently used immobilization animal models can include artefacts due to difficulty of food intake and stress. Zebrafish larvae 5-6 days after hatching do not require active movement for food intake, and it is possible to assess the muscle function of their trunk muscle. Therefore, we established two muscle atrophy models using zebrafish larvae and aimed to investigate the role of the MuRFs and MAFbx in relation with muscle function.

**Methods** An actin-myosin interaction blocker (BTS) was used to immobilize zebrafish larvae from 3-6 days after hatching; in another series, dexamethasone was fed to larvae from 3-5 days after hatching. Maximal active force of trunk muscles was examined and the distance between adjacent filaments in sarcomeric structure was measured using small angle x-ray diffraction. MuRF and MAFbx expression was determined using real time PCR. Two-way ANOVA was used to analyze the difference between groups.

**Results** We found a significant up-regulation of MuRF-1 and a lower active force generation in dexamethasone treated larvae. However, although the BTS immobilization induced muscle weakness, it was associated with decreased of MuRF-1 to 3 and MAFbx. After 3 days of immobilization, sarcomere became more compressed compared to the controls.

**Conclusions** Two kinds of muscle atrophy models were successfully established in zebrafish larvae. MuRFs and MAFbx was lowered in BTS treated model whereas MuRF-1 was up-regulated in dexamethasone treated model implicating the complex role of ubiquitin ligase in different muscle atrophy models.

## Background

Physical activity is essential for keeping muscle strength. In patients where active movement is impaired, e.g. after nerve/muscle injury or during intensive unit care, muscle degeneration is often observed [1]. Muscle atrophy, with reduction of muscle mass, is considered to be the consequence of alterations where the rate of muscle protein degradation exceeds the rate of synthesis. Several proteolytic systems are involved in protein degradation in cells and particularly, the ubiquitin-proteasome pathway has been found to be activated in several muscle wasting conditions, e.g. tumor-induced cachexia [2], starvation and denervation [3] and space travel [4]. Ubiquitin ligases are a group of enzymes responsible for attaching ubiquitin to target proteins making them susceptible for degradation [5]. The rate of ubiquitin conjugation increases in animal atrophy models [6] and several up-regulated ligase genes have been identified as transcriptional markers of muscle atrophy, such as Muscle Ring Finger 1 (MuRF-1) and

Muscle Atrophy F-box (MAFbx) alternatively named as atrogin-1 [1, 7–9]. MuRF-1 and MAFbx knock-out mice were shown to have lower muscle wasting compared to wild type animals following denervation [7]. MuRF-1 is considered to target proteins associated with thick myosin filaments for further degradation during muscle atrophy [10, 11], whereas MAFbx attacks several intermediate filament proteins including desmin and vimentin [12]. General hormonal changes can affect the gene expression. For example, glucocorticoids up regulate the expression of MuRFs and MAFbx in myotubes [10, 13, 14], by directly affecting transcriptional factors such as KLF15 and FoxO [15]. However, the factors affecting the expression of ubiquitin ligases and their role in inactivity-induced muscle atrophy are still unclear. Although studies have shown upregulation of MuRF-1 and MAFbx in different atrophy animal models, using this as an indicator of muscle protein breakdown could be problematic since they are also involved in muscle remodelling activity and metabolism [16].

To study mechanisms involved in immobilization-induced atrophy, animal models (mainly rodents) involving denervation, cast immobilization, or limb suspension have been applied [17]. However, artefacts may be introduced due to the difficulty of food and water intake when physical movement is restrained or other systemic changes induced by the intervention [18]. Denervation may induce other physiological consequences due to stress or wound recovery. Since these factors can upregulate the ubiquitin ligases expression through different pathways [16], it is difficult to ascertain the independent relationship between muscle inactivity and MuRF-1 and expression level under such circumstances. Zebrafish (*Danio Rerio*) has become an important model organism in developmental biology since most organ systems, including muscles, are fully developed in a few days after hatching and since several disease models are available [19]. The active force produced by the sarcomeres of the contractile system can be addressed using single larvae technique [20, 21]. It is thus possible to determine effects of different interventions on muscle function in larvae up to 5–6 days after hatching. Recently, it has also been shown that early immobilization [22] affects sarcomere assembly and that a chemical blocker of actin-myosin interaction BTS [23] can reversibly inhibit swimming activity for longer time and affect disease development in muscle dystrophy [24]. An important advantage of the zebrafish models is that the early larvae, up to 5–6 days after fertilization (dpf), live on the yolk sack and do not require active movement for food intake. BTS treatment inhibits the striated muscle and larval muscle movements, but does not affect the heart or blood circulation. The BTS immobilisation model can thus provide an ethically more attractive animal model compared to larger animal models, enabling full muscle immobilization for several days without effects on general physiology still enabling analysis of muscle function.

The aim of the present study was to investigate the role of the regulatory factors MuRFs and MAFbx in muscle atrophy. Two models were applied: glucocorticoid treatment and pharmaceutical immobilization using zebrafish larvae. Maximal active force of the trunk muscles was examined and correlated with studies of sarcomeric structure using small angle x-ray diffraction and with expression analysis of the ubiquitin kinases using real time PCR. We demonstrate that whereas growth/development and glucocorticoid-induced atrophy was associated with increased ubiquitin ligase expression, the immobilization-induced atrophy was associated with decreased of ubiquitin kinases, showing that atrophy can be associated with both increased and decreased expression of ubiquitin kinases.

# Methods

## 1. Zebrafish larvae

Zebrafish larvae (*Danio Rerio*, AB strain) were obtained from the Zebrafish Facility at Karolinska Institutet and kept in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, and 0.33 mM MgSO<sub>4</sub>, pH 6.8–6.9, with 200 µl of 0.05% methylene blue in 1 L) at 28 °C to 6 dpf in the laboratory. The larvae were anesthetized in E3 medium containing 0.0017% MS-222 tricaine (3-amino benzoic acid ethylester) prior to mechanical analysis, small angle x-ray (SAXS) analysis and fixation for mRNA extraction. The maintenance and mating of adult fish at the zebrafish facility are approved by the local authorities and follow required routines.

## 2. Pharmacological immobilization and glucocorticoid treatment

BTS (N-benzyl-p-toluene sulphonamide), a small-molecule inhibitor of actin-myosin interaction in muscle [23, 25], was added to E3 medium from 3 to 6 dpf to keep the larvae completely immobilized [24]. In one series, BTS was added at 48 hours post fertilization after dechoriation to ensure that these larvae would never move. The lowest BTS dose required for full immobilization (when larvae did not react to gentle touch) of 10 larvae in 5 ml of E3 medium was titrated to 40–50 µM, and the medium was replaced every day. Control larvae that were actively swimming from 3–6 dpf, were treated with solvent control (0.1% DMSO) in E3 medium. To study the recovery process of larvae that were immobilized 1–2 days from 3 dpf, these larvae were transferred to BTS-free E3 medium for recovery of 1 or 2 days. Prior to analysis, DMSO and BTS treated larvae were kept in regular E3 medium for 1 h to wash out BTS and DMSO. In a series where acute effects of immobilization were studied, BTS was only introduced to larvae at 5 dpf. Zebrafish larvae were thereafter immobilized for 1, 2 and 4 h and immediately sampled for further mRNA extraction. In a separate series where the effect of glucocorticoids was investigated, 300 µM dexamethasone was added to 10 larvae in 5 ml of E3 medium from 3 to 5 dpf.

## 3. Mechanical analysis

The maximal active force generated by zebrafish larvae was analysed at each day following the protocol by Dou et al, 2008 [20]. Anesthetized larvae were transferred and held in a physiological buffer solution at 22 °C (composition in mM: 118 NaCl, 24 MOPS, 5 KCl, 1.2 MgCl<sub>2</sub>, 1.2 Na<sub>2</sub>HPO<sub>4</sub>, 1.6 CaCl<sub>2</sub> and 10 glucose at pH 7.4). The larva was anesthetised with 0.017% tricaine (3-amino benzoic acid ethylester) and then the head was crushed. A segment of 1-1.5 mm including the mid portion of the trunk muscles was prepared and attached with aluminium clips. The preparation was thereafter mounted between a force transducer and a fixed hook attached to a micro manipulator, enabling the muscle length to be varied. The preparation was held in the physiological buffer solution at 22 °C and stimulated with 0.5 ms electrical pulses at supra-maximal voltage at 2 min intervals. The muscles were mounted at slack length (when passive force is just noticeable) and stimulated 2–3 times to ensure reproducible responses. Thereafter the preparations were stretched in steps of 10% of the initial length between contractions. At each length step the passive and active force were recorded to determine the length-force relationships.

The maximal active force at optimal stretch (at muscle length about 1.3 to the slack length) was taken as a measure of the muscle function of the larvae.

#### **4. Small angle x-ray scattering**

Lattice spacing of contractile filaments at 6 dpf was determined by small angle x-ray scattering (SAXS) using synchrotron radiation. as previously described [20, 26, 27] at beamline PO3 at Petra III, Deutsches Elektronen-Synchrotron (DESY) synchrotron facility in Hamburg Germany [28]. Larvae muscle preparations with aluminum clips were mounted between two hooks in a Kapton cuvette in the physiological solution as described (Sect. 3 above) at 22 °C. The preparations were stretched to optimal length. The equatorial diffraction patterns were recorded with 0.1 to 1 s exposures using a PILATUS detector (DECTRIS Ltd.). A beam size of  $16 \times 22 \mu\text{m}^2$ , a camera length of 5.1 m and a wavelength of 0.95 Å were used. Slightly stretched, air-dried rat tail tendon was used to calibrate the diffraction signal in both directions using the collagen reflections (periodicity 65 nm).

#### **5. Quantitative PCR analysis**

Quantitative real time PCR (qPCR) was used to follow the expression of Muscle Ring Finger protein 1–3 and MAFbx/atrogin-1 at different time points during zebrafish larvae development, immobilization and glucocorticoid treatment. For each analysis at different time points, 10 larvae were anesthetized and stored in 1 ml of RNAlater solution (Qiagen) at 4 °C for further preparation. For mRNA extraction, 10 larvae from the RNAlater solution were homogenized with a pestle homogenizer and mRNA was extracted using the RNeasy mini kit (Qiagen). The purity and concentration of the obtained mRNA was controlled (NanoDrop 1000, 3.6.0 program). Following reverse transcription (QuantiTect Reverse Transcription kit; Qiagen), cDNA was used for amplifications with primers for the candidate genes (listed in Table 1). Before quantitative PCR analysis, the PCR products of each pair of primers were analysed with 3% agarose gel electrophoresis and confirmed to have a single band by using GelRed Nucleic Acid Gel Stain (Biotium) and visualized on a Bio-Rad system (Bio-Rad Laboratories). cDNA was thereafter mixed with Fast SyBR Green Master (Qiagen) mix according to the protocol provided by the company and

qPCR was performed using Step One Plus™ Real-Time PCR System (Thermo Fischer).

Table 1  
Primers used for quantitative PCR.

mRNA target (NCBI Accession No.)	Forward-Primer 5 – 3	Reverse-Primer 3–5	Length (bp)
Beta actin (NM_131031)	CCCAGACATCAGGGAGTGAT	TCTCTGTTGGCTTTGGGATT	200
MuRF-1(trim63a) (NM_001002133.1)	TTCCGATGCCCTACTTGTCTG	TTTCAAAGGGGGCTCAAGGG	108
MuRF-2(trim55a) (NM_001002358.1)	AGCGCAAGTACAGAGAGCAT	GACACATTCCGGCTTTCTGC	103
MuRF-3(trim54) (NM_001045025)	AAATCAGAATCCACCAGGCC	GCCCCAAACACTTTGCACAT	100
MAFbx (fbxo32) (NM_200917.1)	CACCAAAGAGCGTCATGGAT	AGAAGGCAGTTGGGACTTGG	139

## 7. Statistical analysis

All data are presented as mean  $\pm$  SEM. Statistical analysis was performed using Sigma plot 8.0 for Windows and SigmaStat for Windows 3.0 (Systat Software, Inc.). Difference between two groups was compared using Student's t-test. Two-way ANOVA was performed for multiple group comparison and Holm-Sidak test was used for pairwise comparison.

## Results

The first step was to examine the dynamics of MuRF expression in the zebrafish larvae in relation to contractile function, using dexamethasone treatment known to up-regulate MuRF expression in skeletal muscle [10]. We treated larvae with 300  $\mu$ M dexamethasone from 3 to 5 dpf, and found a significant up-regulation of MuRF-1 and a lower active force generation as shown in Fig. 1. To explore if pharmacological immobilization affected MuRF-1 expression and active force we immobilized zebrafish larvae from 3 to 6 dpf using an actomyosin inhibitor (BTS) to inhibit contractile function and active swimming completely. Although the larvae stopped swimming and become unresponsive to gentle touch, normal heart beating and blood circulation were observed by microscopy. After removal of BTS prior to mechanical analysis, larvae swimming recovered in all the groups within 40 min, and therefore a 60 min BTS-wash out time period was used to ensure that no residual BTS could influence subsequent mechanical analysis. Maximal active force at optimal length was determined at different developmental and BTS immobilisation time. Immobilization had significant effect on active force (Table 2).

Table 2  
Summary of p values from Two-way ANOVA

	<b>Maximal active force</b>	<b>MuRF-1</b>	<b>MuRF-2</b>	<b>MuRF-3</b>	<b>MAFbx</b>
Days	0.458	0.143	0.197	0.229	< 0.001
Immobilization	< 0.001	0.016	0.001	0.034	0.022

As shown in Fig. 2, active force increased gradually with development from 4 to 6 dpf in DMSO-treated controls. In the immobilized group, active force was slightly lower but not significantly different compared to the controls after 1 day of immobilization (at 4 dpf). However, the difference became bigger after 2 days of immobilization (at 5 dpf). Immobilized larvae were significantly weaker compared to the controls at 5–6 dpf as illustrated in Fig. 2. To see if the effect of immobilization on active force is reversible, larvae treated with BTS for 1 day were transferred to BTS-free medium for recovery. After 2-day recovery (from day 4 to 6 dpf), active force was similar to that in the controls at 6 dpf (hatched bar compared to open bar at 6 dpf, Fig. 2). In contrast, larvae immobilized for 2-days and recovered only for 1-day did not recover (grey bar compared to open bar at 6 dpf, Fig. 2).

The expression of MuRF-1 to 3, and MAFbx/atrogen-1 were altered due to immobilization during 4–6 dpf as summarized in Table 2. As shown in Fig. 3A, MuRF-1 had the highest expression among these four genes, whereas MuRF-3 expression was the lowest in general. In the controls, the expression of the MuRF-1 and MAFbx increased gradually during muscle development from 4–6 dpf. After 1-day immobilization, no difference was noticeable in any of the examined ligases expression. There is a tendency that immobilization reduced ubiquitin ligase expression as illustrated in Fig. 2A. After 1-day immobilization (at 5 dpf), compared to the controls, MuRF-1 and -2 expression became significantly lower. After 3-day immobilization, MuRF-2, -3 and MAFbx expression was significantly lower in treated groups compared to the controls. To examine if immobilization would induce more rapid (within hours) changes in MuRFs and MAFbx, control larvae at 5 dpf were immobilized with BTS for only 1, 2 and 4 hours. As seen in Fig. 3B, no changes were observed in these genes in this shorter time perspective. In a separate series we dechorionated the larvae at 18 h post fertilization, and thereafter immobilized them until 4–6 dpf. In this circumstance where larvae never moved until being analysed, the changes of ligase expression were similar compared to the ones immobilized from 3 dpf. In the controls, MAFbx expression increased significantly from 4–6 dpf (Table 2), which correlated with muscle development.

To explore that if the immobilization affected the contractile filament structure, we performed x-ray scattering experiments. Strong scattering patterns were recorded from 6 dpf larvae with clear 1.1 and 1.0 spacings of about 25 and 43 nm respectively as shown in Fig. 4. Panel A and B show the equatorial x-ray pattern from 6 dpf controls and 6 dpf larvae immobilized from 3 dpf. A clear outward movement of 1.0 and 1.1 reflections was observed after 3-day immobilization, demonstrating the shrinkage of the filament

lattice. The average spacing of the 1.0 reflection (d1.0) from multiple analysis was significantly smaller in the BTS treated group (Fig. 4C).

## Discussion

Zebrafish muscle developed their size and active force during the first days after hatching, most likely reflecting an interplay between mechanical activity (swimming) and the growth of muscles. It has been previously shown that immobilization affects muscle contractile function in muscle injury and dystrophic zebrafish larvae models [21, 22]. Our study builds on these observations. We provided evidence that freely-swimming zebrafish larvae development during 4–6 days after fertilization is associated with an increase of active force of the trunk muscles, but immobilization could inhibit this effect. In the immobilization group, we showed that the inhibitory effect of BTS in muscle contraction is fully reversible and did not significantly affect the cardiac function. However, immobilization led to a significant loss of active force, demonstrating that muscle contraction is required for muscle strength development. After 1-day immobilization and 2-day swimming active force could be fully restored, but after 2-day immobilization, a full recovery of contractile force was not achieved within 1 day although the larvae were actively swimming and alive. This model could be of interest for further studies for movement recovery after immobilization-induced muscle weakness. Dexamethasone treatment for 2 days successfully lowered the active force of zebrafish larvae, suggesting muscle atrophy probably had occurred although active swimming was observed all the time. Glucocorticoids stimulate MuRF-1 up-regulation, accelerating proteolysis degradation of myosin heavy chain [10]. The effect of glucocorticoid is mediated by direct targeting on transcriptional factors KLF15 and FoxO [15]. We observed almost two-fold of increase in MuRF-1 in the dexamethasone-treated larvae. This finding confirmed with previous studies that proteolysis due to ubiquitin ligase activation may be involved in glucocorticoid-induced muscle atrophy [10, 29, 30].

The present study shows that ubiquitination ligase signalling pathway is involved in the zebrafish larvae muscle developmental stage and can be altered by muscle immobilization. The mechanistic process could be that the active contraction of sarcomeres stimulates the signalling pathway via the mechanical load sensing elements such as titin, which acts like a link between thick filaments and z-disk [31]. MAFbx expression correlated positively with muscle development in the controls. A tendency of MuRF-1 and – 3 up-regulation was also observed although was not significant. This finding most likely reflects the essential role of ubiquitin ligases in muscle development. As reported previously, MuRF-1 and – 3 double knockout mice exhibited skeletal myopathy due to abnormal MHC accumulation and subsequent fibre degradation during growth [32]. Despite the role in muscle atrophy, MAFbx was increasingly expressed during the postnatal stages after hatching in chicken [33], indicating its important role in muscle growth. MAFbx activates the degradation of transcriptional factors MyoD and myogenic that controls myogenic stem cell function [34–36]. In addition, ubiquitin ligase plays an important role in microtubules stabilization thus affects sarcomere assembly [37]. Therefore, the current study is consistent with the view that protein degradation via the activation of the ubiquitin-proteasome system is required for sarcomeric protein turnover and maintenance of skeletal muscle function during muscle development. In

contrast, the expression of all the investigated ubiquitin ligases reduced significantly after immobilization for 2 days accompanied with a loss of active force. Opposite to studies finding an up-regulation of MuRF-1 and MAFbx in muscle disuse animal models including rat [38] and mouse [9, 39] as well as in humans [40], our findings suggest that up-regulation of ubiquitin ligases are not mandatory for observing a loss of active force in all the circumstances. This is in a line with previous observations showing non-consistent responses of MuRFs and MAFbx to muscle disuse in different animal models (see review [17]).

As revealed by our SAXS data, the lack of active contraction not only negatively influence the contraction force, but also resulted in smaller distance between thick and thin filaments. The loss of cytoskeletal components affects sarcomeric structure which correlates with contractile dysfunction. Several muscular atrophies have been shown associated with alterations in filament lattice as demonstrated in zebrafish models [26, 27]. However, the extent of lattice compression itself was suggested not be a major component explaining the loss of active force. Most likely it is a marker for the loss of sarcomeric components that in turns influence the force production [22]. Ubiquitin ligases located within sarcomere and targets on important skeletal proteins. MuRF-1 associates with the Z-disks and M-line whereby MuRF-3 is found at the Z-disks [41–43]. MuRF-1 targets proteins associated in thick filaments in muscle atrophy such as titin and myosin heavy chain [10, 11, 44]. It has been shown that MuRF-3 binds to, and has a stabilizing effect, on microtubules [41]. Intermediate filament proteins including desmin and vimentin are substrate for MAFbx [12]. As shown by us, the down-regulation of ubiquitin ligases expression appears to be related to the shrinkage of interfilamental space, it implicates that ubiquitination process may affect proteins responsible for interfilamental space enlargement.

## Conclusion

In summary we demonstrate that it was possible to establish muscle atrophy model by BTS-induced immobilization and glucocorticoids treatment in zebrafish larvae. We have observed significantly reduced active force of trunk muscle in both models during 3–6 dpf. Ubiquitin ligase MuRF-1 expression was elevated in glucocorticoid treated larvae whereas MuRF 1–3 and MAbx expression was lowered in immobilized larvae compared to the controls, suggesting that ubiquitin ligase expression play a complex role in different muscle atrophy expression. MAFbx expression increases with muscle strengthening during larvae early development stage in the controls.

## List Of Abbreviations

MuRF: Muscle Ring Finger; MAFbx: Muscle Atrophy F-box; dpf: days post fertilization;

## Declarations

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### **Authors' contributions**

JL, EL and AA designed the study, performed most of the experiments and conducted data analysis. JL, AA and MS accomplished x-ray scattering experiment. JL and AA wrote the manuscript, and all the authors participated during result discussion and manuscript revision before the submission.

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### **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### **Ethics approval**

The experiments were performed according to European guidelines for animal research, complied with national regulations for the care of experimental animals. Experiments on zebrafish larvae up to 6 dpf are not required to be approved by ethics committee.

### **Consent for publication**

Not applicable

### **Competing interests**

The authors declare that they have no competing interests.

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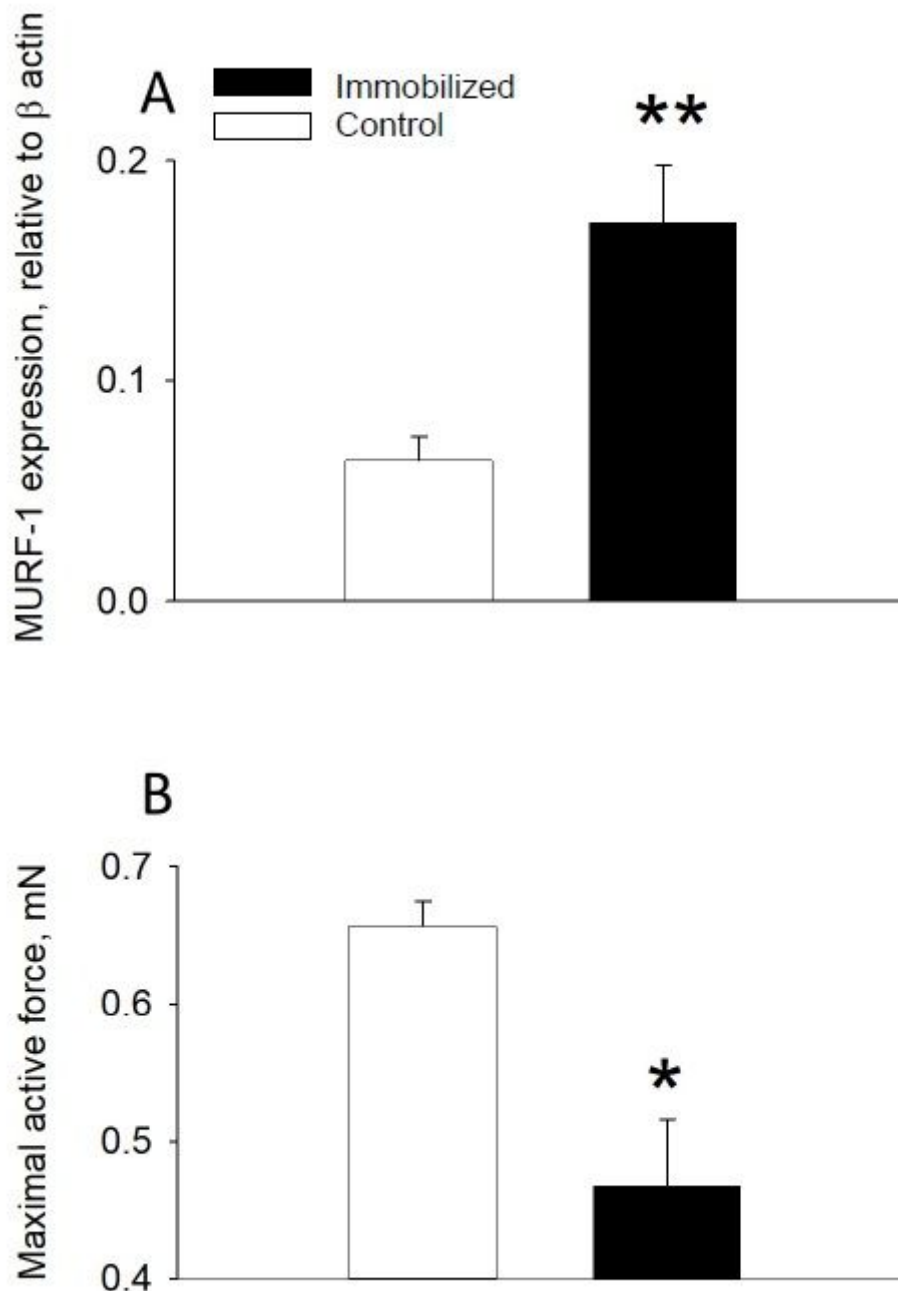
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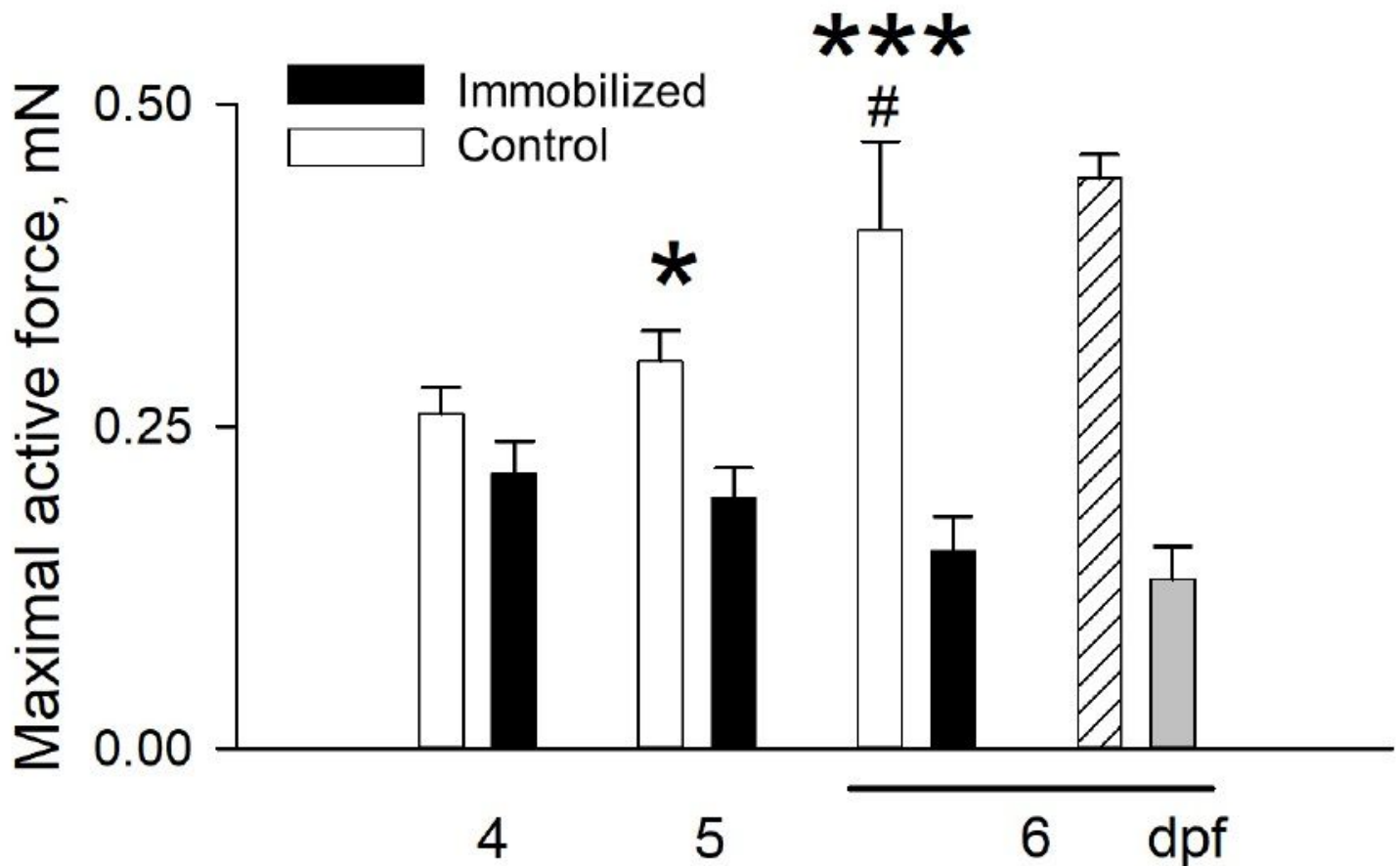
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## Figures



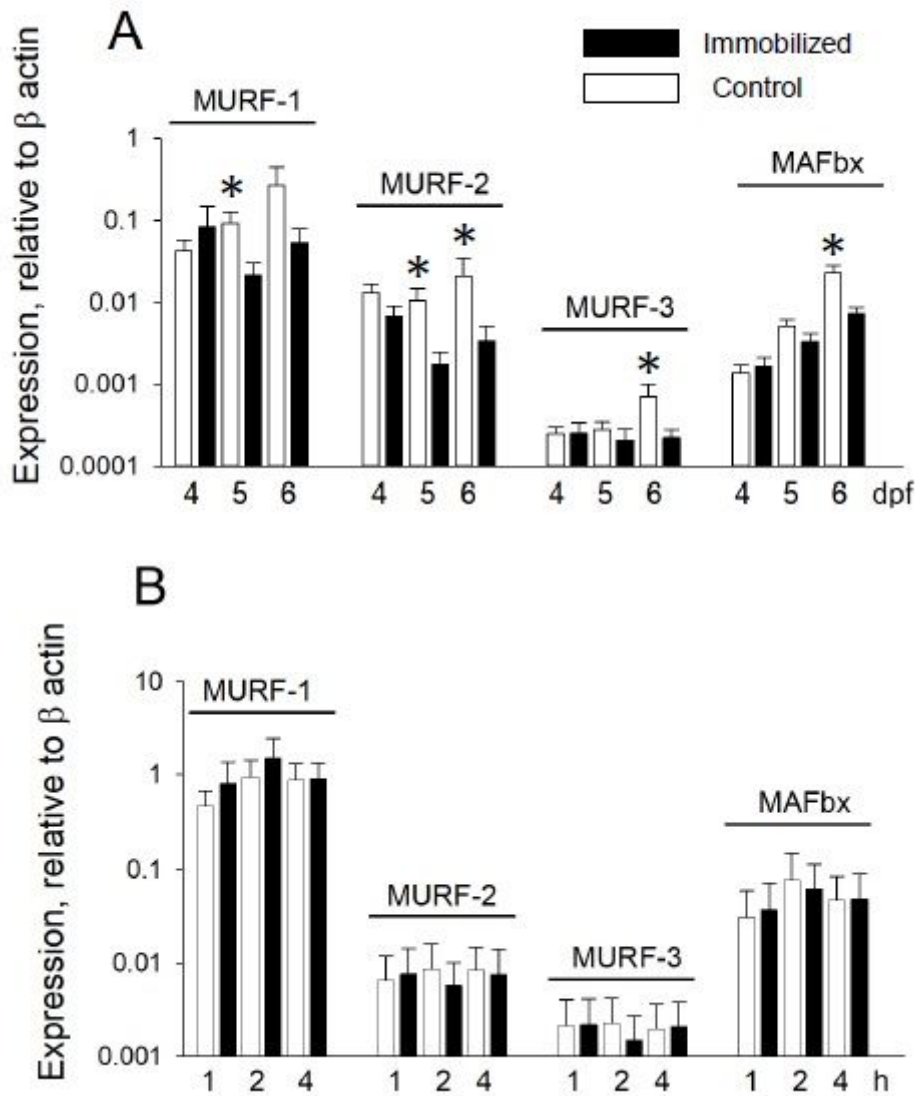
**Figure 1**

Effects of dexamethasone (300  $\mu$ M, introduced at 3 dpf) on MuRF-1 expression (Panel A) and maximal active force (Panel B) at 5 dpf. Open bars: DMSO treated controls; filled bars: dexamethasone treated. Panel A: N=6 (analysis number, each analysis contained mRNA from 10 larvae); Panel B: N=3-4 (number of larvae). \* =  $P < 0.05$ , \*\* =  $P < 0.01$  (Student's t-test).



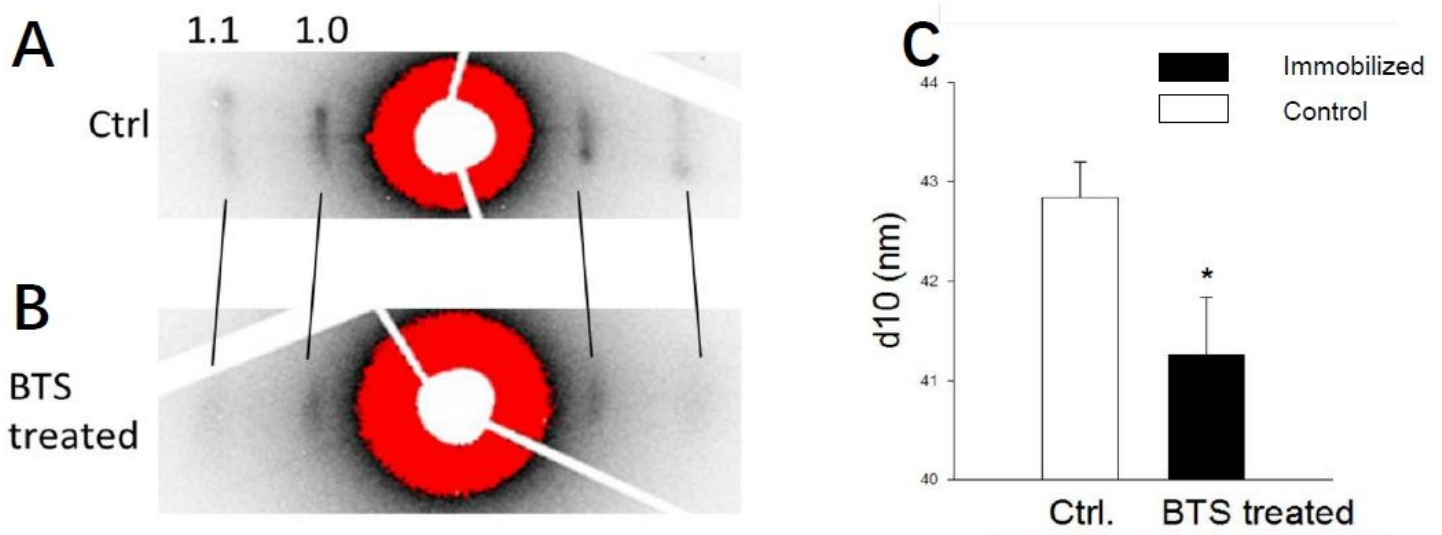
**Figure 2**

Maximal active force in DMSO controls and BTS immobilized larvae (initiated at 3 dpf) at 4- 6 dpf. N=9-14 (number of larvae). Open bars: DMSO treated controls; filled bars: BTS immobilized; hatched and grey bars indicate larvae where BTS was removed at 4 and 5 dpf (recovered for 2 and 1 days) respectively, and analysed at 6 dpf. N= 3-9. \* =  $P < 0.05$ , \*\*\* =  $P < 0.001$ : compared to respective BTS group on the same day; # =  $P < 0.05$ : compared to 4-dpf controls (Two-way ANOVA, Holm-Sidak method for pairwise comparison).



**Figure 3**

Panel A shows MuRF-1, 2, 3 and MAFbx/atrogin-1 expression in DMSO controls and BTS immobilized larvae (initiated at 3 dpf) at 4-6 dpf. N = 6-7 (analysis number, each analysis contained mRNA pool extracted from 10 larvae). Panel B shows expression of these genes after 1, 2 and 4 h of BTS immobilization at 5 dpf. Open bars: DMSO treated controls; filled bars: BTS immobilized. N=3 (analysis number). \* =  $P < 0.05$  compared to the respective BTS group (Two-way ANOVA, Holm-Sidak method for pairwise comparison)



**Figure 4**

Small angle x-ray scattering (SAXS) of 6 dpf control (DMSO treated, Panel A) and BTS immobilized larvae (Panel B). The equatorial 1.0 and 1.1 reflections are indicated. Panel C shows the mean values for the spacing of the 1.0 reflections (d1.0) in controls (open bars) and BTS treated (filled bars). N=6 (larvae number); \* =  $P < 0.05$  (Student's t-test).