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Generation of MuRF-GFP transgenic zebrafish models for investigating *murf* gene expression and protein localization in Smyd1b and Hsp90α1 knockdown embryos

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Abstract

Muscle-specific RING-finger proteins (MuRFs) are E3 ubiquitin ligases that play important roles in protein quality control in skeletal and cardiac muscles. Here we characterized *murf* gene expression and protein localization in zebrafish embryos. We found that the zebrafish genome contains six *murf* genes, including *murf1a, murf1b, murf2a, murf2b, murf3* and a *murf2*-like gene that are specifically expressed in skeletal and cardiac muscles of zebrafish embryos. To analyze the subcellular localization, we generated transgenic zebrafish models expressing MurF1a-GFP or MuRF2a-GFP fusion proteins. MuRF1a-GFP and MuRF2a-GFP showed distinct patterns of subcellular localization. MuRF1a-GFP displayed a striated pattern of localization in myofibers, whereas MuRF2a-GFP mainly exhibited a random pattern of punctate distribution. The MuRF1a-GFP signal appeared as small dots aligned along the M-lines of the sarcomeres in skeletal myofibers. To determine whether knockdown of *smyd1b* or *hsp90α1* that increased myosin protein degradation could alter *murf* gene expression or MuRF protein localization, we knocked down *smyd1b* or *hsp90α1* in wild type, *Tg(ef1a:MurF1a-GFP)* and *Tg(ef1a:MuRF2a-GFP)* transgenic zebrafish embryos. Knockdown of *smyd1b* or *hsp90α1* had no effect on *murf* gene expression. However, the sarcomeric distribution of MuRF1a-GFP was abolished in the knockdown embryos. This was accompanied by an increased random punctate distribution of MuRF1a-GFP in muscle cells of zebrafish embryos. Collectively, these studies demonstrate that MuRFs are specifically expressed in developing muscles of zebrafish embryos. The M-line localization MuRF1a is altered by sarcomere disruption in *smyd1b* or *hsp90α1* knockdown embryos.

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Author contributions SD conceived the project. BL cloned the *murf* genes from zebrafish and generated the *Tg(ef1α:MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic zebrafish lines. SL and QH performed the subcellular localization and gene expression studies. SL and QH prepared figures for the manuscript. All authors contributed to the data analysis and writing of the manuscript.

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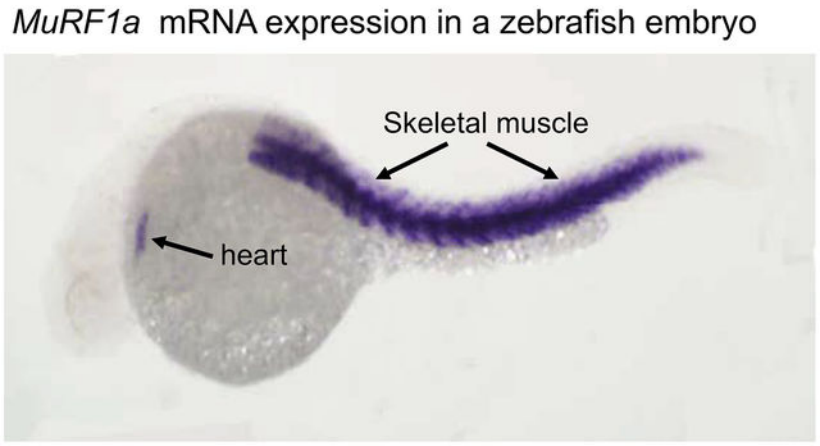
Declaration of interest: none

Graphical Abstract



Keywords

MuRF; sarcomere organization; transgenic zebrafish

1. Introduction

Skeletal muscle cells contain highly organized sarcomeres, the basic contractile units for muscle contraction. Sarcomeres are assembled together in a process called myofibrillogenesis during muscle cell differentiation and maturation, which involves large numbers of structural and regulatory proteins. Sarcomere assembly requires protein quality control that ensures proper protein folding and removal of misfolded proteins in skeletal and cardiac muscle cells (Kim et al., 2008; Willis et al., 2009a; Portbury et al., 2011; Carlisle et al., 2017; Hnia et al., 2019). When folding is not successful, the misfolded proteins are shuttled to the cellular degradation machinery for destruction by the Ubiquitin/Proteasome System (UPS), which is the primary system for muscle protein degradation (Lecker and Goldberg 2002; Lecker et al., 2004; Attaix et al., 2005; Coux et al., 1996; Solomon and Goldberg, 1996; Taillandier et al., 2004; Sandr, 2013; Sandri et al., 2013). UPS is necessary for maintaining muscle remodeling and homeostasis by balancing the protein syntheses and degradation. Malfunction of protein quality control pathways results in myopathies in skeletal and cardiac muscles (Kitajima et al., 2014; Fanzani et al., 2015; Mearini et al., 2008; Patterson et al., 2007; Willis et al., 2008; Willis et al., 2009a; 2009b; 2010).

E3 ubiquitin ligases play a vital role in determining the selectivity and specificity of the UPS. Three muscle RING finger proteins (MuRFs), namely MuRF1, MuRF2, and MuRF3, have been identified as E3 ligases that are specifically expressed in skeletal and cardiac muscles (Spencer et al., 2000; Centner et al., 2001). MuRF E3 ubiquitin ligases are required for skeletal muscle atrophy (Bodine et al., 2001; 2014; Bonaldo and Sandri, 2013), responsible for ubiquitination and degradation of myofibrillar proteins in skeletal and cardiac muscles, such as myosin-binding protein C, myosin light chain, myosin heavy chain, troponin and titin (Cohen et al., 2009; Kedar et al. 2004; McElhinny et al. 2004; Attaix and Baracos 2010; Mearini et al., 2010). It has been shown that mice lacking MuRF1, MuRF2 or

MuRF3 were phenotypically normal under unstressed physiological conditions (Fielitz et al., 2007; Bodine et al., 2001; Lange et al., 2005). However, mice lacking both MuRF1 and MuRF2 develope spontaneous cardiac hypertrophy and show a profound loss of type-II fibers (Witt et al., 2008; Moriscot et al., 2010). In addition, mice deficient for MuRF1 and MuRF3 develop skeletal muscle myopathies with myosin heavy chain accumulation, myofiber fragmentation, and impaired muscle performance (Fielitz et al., 2007). Genetic mutations in MuRF1 and MuRF3 have been implicated in causing protein aggregate myopathies in cardiac and skeletal muscles in human populations (Fletta et al., 2011; Chen et al., 2012; Gumucio and Mendias, 2013; Olive et al., 2015; Jokela et al., 2019).

MuRFs show dynamic patterns of gene expression and protein localization under normal and various myopathy conditions. MuRF1 is up-regulated during skeletal muscle atrophy and drug-induced muscle wasting (Bodine et al., 2001; Aniort et al., 2016), and downregulated during cardiac hypertrophy (Maejima et al., 2014). Mice lacking MuRF1 are resistant to skeletal muscle atrophy (Bodine et al., 2001; Baehr et al., 2011). Subcellular localization analysis reveals that MuRF proteins are not only identified within sarcomeric components such as the Z-band and the M-band but also found in the microtubule, cytoplasm, and nuclei (Spencer, et al, 2000; Centner et al., 2001; McElhinny et al., 2002; 2004; Pizon et al, 2002). Interaction of MuRF1 with titin plays an important role in M-line stability (McElhinny et al., 2002), whereas MuRF2 and MuRF3 appear to be important for microtubule stability and myofibrillogenesis during muscle differentiation (Centner et al. 2001; Chang et al. 2002; McElhinny et al., 2004). During atrophic stress, MuRF1 and MuRF2, and likely MuRF3, translocate in the nucleus and appeare to control muscle gene expression via polyubiquitination and degradation of transcription factors in cardiomyocytes and skeletal muscle (Pizon et al., 2002; Lange et al., 2005; Ochala et al., 2011). Thus, members of the MuRF family may have diverse functions based on their patterns of subcellular localization.

Zebrafish have become a model for studying muscle development and sarcomere assembly (Sanger et al., 2009; Keenan and Currie, 2019). Previous studies have demonstrated that molecular chaperon Hsp90α1 and lysine methyltransferase Smyd1 play vital roles in sarcomere assembly in zebrafish embryos (Tan et al., 2006, Etard et al., 2007; Du et al., 2008; Hawkins et al., 2008; Just et al., 2011; Li et al., 2013; Prill et al., 2016; Cai et al., 2019). *hsp90α1* and *smyd1* are specifically expressed in developing muscles of zebrafish embryos (Tan et al., 2006; Du et al., 2008; Hawkins et al., 2008; Cai et al., 2019). Loss of *hsp90α1* and *smyd1* result in defective sarcomere organization and increased myosin protein degradation (Bernick et al., 2012; Li et al., 2013; Cai et al., 2019). It has been suggested that MuRF ubiquitin-proteasome system (UPS) are likely to be involved in myosin protein degradation in the *hsp90α1* and *smyd1* deficient embryos (Du et al., 2008; Li et al., 2013). However, MuRF expression and protein localization are not well understood in zebrafish. In this study, we analyzed the expression profiles of six *murf* genes in zebrafish during embryogenesis and characterized their spatial patterns of expression by in situ hybridization. In addition, we generated transgenic zebrafish models expressing MuRF1a-GFP or MuRF2a-GFP fusion proteins and analyzed their subcellular localization in myofibers. We found that *murf* genes were primarily expressed in skeletal and cardiac muscles of zebrafish embryos. MuRF1a-GFP displayed a sarcomeric M-line localization, which was abolished in *smyd1b* or *hsp90α1* knockdown embryos. This was accompanied by an increased

punctuated pattern of MuRF1a-GFP distribution in myofibers of zebrafish embryos. Better understanding the MuRFs expression and subcellular localization may provide an insight into MuRF function in fish muscle development and growth.

2. Material and Methods

Zebrafish lines and maintenance

All zebrafish larvae and adult were maintained in a 28 °C recirculating aquatic system with a photoperiod of 14h light and 10h dark, in the Zebrafish Facility of the Aquaculture Research Center, Institute of Marine and Environmental Technology (Baltimore, MD). Zebrafish embryos over one day were anesthetized in 0.6 mM Tricaine (pH 7.0) before fixation in 4% paraformaldehyde (PFA) to ease pain and facilitate animal handling. This study was performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of Maryland Baltimore (Protocol Number: 0419010).

MuRF cDNAs cloning and expression analyses by RT-PCR

A BLAST search was conducted in zebrafish using the human MuRF1 cDNA as a query sequence (NM_032588.3). Six *murf* genes were identified in the zebrafish genome database. They represent MuRF1 (MuRF1a: NM_001002133.1, MuRF1b: NM_201095.1), MuRF2 (MuRF2a: NM_001002358.1, MuRF2b: NM_001039982.1, and MuRF2-like: NM_001003581.1), and MuRF3 (NM_001045025.2). The full-length sequences encoding all six *murfs* genes were cloned by RT-PCR from WT zebrafish embryos of 24 hpf. The PCR was carried out using the respective gene-specific MuRF-F1/R1 primers (Table 1). The PCR products were cloned into pGEM-T easy vector and confirmed by DNA sequencing. The gene expression was determined by RT-PCR in zebrafish embryos at 0h, 3h, 6h, 12h, 14h, 19h, 22, 26 hour-post-fertilization (hpf), and 2d, 3d, 4d, and 5 days-post-fertilization (dpf). The PCR was carried out using the respective gene-specific MuRF-F2/R2 primers (Table 1). The PCR products were analyzed on 1.5% agarose gel. The gel images were acquired and photographed by E-Gel Imager (Life Technologies).

Phylogenetic analysis of murf cDNAs

The phylogenetic tree was constructed using Phylogeny.fr (Dereeper et al., 2008 and 2010) on the website, http://www.phylogeny.fr/version2_cgi/index.cgi. The six zebrafish *murf* genes could be classified into three classes. Protein multiple alignments were analyzed using Multalin at http://multalin.toulouse.inra.fr/multalin//. The characteristic domains of these MuRFs were analyzed with the Scanprosite tool (http://prosite.expasy.org/scanprosite/).

Construction of Tg(ef1a:MuRF1α-GFP) and Tg(ef1α:MuRF2a-GFP) DNA constructs

Tg(*ef1α:MuRF1a-GFP)* and Tg(*ef1α:MuRF2a-GFP*) DNA constructs were generated by cloning the MuRF1a or MuRF2a coding sequence in-frame upstream of the EGFP coding sequence in the pTol2 vector (Urasaki et al., 2006). The full-length MuRF1a or the MuRF2a coding sequence without the stop codon was generated by PCR using Pfu Turbo DNA polymerase (Agilent) using MuRF 1 a-EGFP-F/R or MuRF2a-EGFP-F/R primers (Table 1).

A *BamHI* site was introduced at both 5' and 3' ends of the MuRF1a or MuRF2a coding sequences via the respective PCR primers. The PCR products were digested with *BamHI* and cloned into the *BamHI* site of T2A200R150G vector (Urasaki et.al, 2006). The DNA sequences at the MuRF1a-GFP or MuRF2a-GFP junctions were confirmed by sequencing.

Microinjection

DNA constructs of *Tg(ef1α-MuRF1a-GFP*) and *Tg(ef1α:MuRF2a-GFP)* were dissolved in sterile water to a final concentration of 50 or 100 ng/μl. For transient expression analysis, approximately 1–2 nl of DNA construct (50–100 pg) was injected into each embryo at one- or two-cell stage. For the generation of transgenic zebrafish lines, the DNA constructs were mixed with Tol2 transposase mRNA (50 ng/μl). Approximately 1–2 nl of mixed DNA construct/Tol2 transposase mRNA was injected into each embryo at one- or two-cell stage. For microinjection of Morpholino antisense oligos (MOs), the MOs were dissolved in 1× Danieau buffer (Nasevicius and Ekker, 2000) to a final concentration of 0.3–0.5 mM. The MOs were injected into *Tg(ef1α-MuRF1a-GFP)* or *Tg(ef1α:MuRF2a-GFP)* transgenic embryos at 1–2 cell stages. The sequences of the smyd1b ATG-MO, Hsp90α1-ATG-MO and standard control MO were listed in Table 1.

Transgenic fish screening

The *Tg(ef1α-MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic zebrafish founders (P1) were screened by examining GFP expression in their F1 embryos at 24 hpf under a fluorescence microscope (Axioplan 2, Zeiss). Adult F1 transgenic fish were identified by PCR using genomic DNA from individual caudal fin. The PCR was carried out using the EGFP-F and EGFP-R primers that specifically amplified the EGFP coding sequence (Table 1).

Whole-mount in situ hybridization

The whole-mount *in situ* hybridization was performed using digoxigenin-labeled RNA antisense probes as previously described (Du and Dienhart, 2001). The RNA antisense probes were synthesized by *in vitro* transcription using either T7 or Sp6 RNA polymerase and linearized *pGEM-T-zf-murf* plasmids as templates (Table 2). The in situ images were acquired using a Leica dissecting microscope MI12 equipped with a cool CCD digital camera (DX8, Olympus).

Whole-mount nuclear and immunostaining

Immunostaining was carried out on whole-mount zebrafish embryos as previously described (Cai et al., 2019). After fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature, the embryos were washed in PBST for 3×15 min and then digested with 1 mg/mL collagenase (28 hpf for 3.5 min, 30 hpf for 6 min) to increase permeability. Immunostaining was performed with anti-α-actinin (clone EA-53, #A7811, Sigma) and anti-myomesin (mMaC myomesin B4, DSHB) primary antibodies. The secondary antibody was TRITC-conjugated anti-mouse IgG secondary antibody (T7657, Sigma). Hoechst 33258 (Sigma B2883, 1ng/ml in PBST) was used to stain nuclei in fish embryos. After staining, the

trunk region was dissected from each embryo and mounted in Vectashield (Vector lab, H-1000) for confocal microscopy (Leica SP8).

3. Results

1. Identification and characterization of *murf* genes in zebrafish

Six *murf* genes were identified in the zebrafish genome sequence database. The six zebrafish *murf* genes could be classified into three classes, *murf1* (*murf1a, murf1b*), *murf2* (*murf2a, murf2b and murf2-like*), and *murf3* by phylogenetic analysis. The *murf1a, murf1b*, *murf2a, murf2b, murf2-like*, and *murf3* genes are located on chromosome 6, 9, 2, 24, 20 and 23, respectively. Sequence analysis revealed that the *murf1a* or *murf1b* genes had no introns. In contrast, *murf2a, 2b, 3, 2-like* genes contained 9, 8, 7, 9 exons, respectively. The full-length cDNAs of *murf1a, murf1b, murf2a, murf2b, murf3, and murf2-like* are predicted to encode MuRF proteins of 345, 348, 443, 366, 359 and 429 aa, respectively. The MuRF proteins show modest sequence identities with their orthologues from other vertebrates. For example, the protein sequence of zebrafish MuRF1a (CAQ15667.1) show 53% and 52% identities with chicken (XP_424369.3) and human MuRF1 orthologues (NP_115977.2), respectively.

All zebrafish MuRF proteins contain three characteristic structural and functional domains, including the ZF_RING_2 domain, ZF_BBOX domain and COS domain (Fig. 1B). The ZF_RING_2 domain, also known as the RING finger domain, contained a Cys3HisCys4 amino acid motif that binds two zinc cations (Borden and Freemont, 1996). The ZF_RING_2 domain contain approximately 40–60 amino acids involved in substrate binding. The B-box-type zinc finger domain is a short protein domain of approximately 40 amino acid residues in length. The COS, known as the C-terminal subgroup one signature domain, is predicted to form two coiled coils (Short and Cox, 2006). The three conserved structural domains make up the tripartite motif, which is essential for MuRF E3 ligase activity.

2. Characterization of *murf* gene expression in zebrafish embryos

The temporal expression of *murf* genes in zebrafish embryos was determined by RT-PCR (Fig. 2A). Members of the MuRF family exhibited different patterns of temporal expression, with a strong expression of *murf1a* starting around the time of myogenesis at 19 hpf. Nevertheless, all *murf* genes showed increased levels of expression during embryonic development and myogenesis (Fig. 2A). To characterize their spatial pattern of mRNA expression, we performed whole-mount *in situ* hybridization (Fig. 2B–G). The results showed that five *murf* genes were expressed in skeletal muscles of zebrafish embryos. In addition, *murf1a* expression was also detected in the heart primordium of zebrafish embryos at 24 hpf (Fig. 2B, C). Collectively, these data indicate that all five *murf* genes have an overlapping pattern of expression in skeletal muscles, except *murf1a* which also showed cardiac muscle expression.

3. Characterization of MuRF1a and MuRF2a subcellular localization in muscle fibers of zebrafish embryos

Previous studies have shown that MuRF1 and MuRF2 were localized at the peripheral region of M-lines in chick, mouse and rat cardiac myocytes and skeletal myocytes (Centner et al., 2001; Pizon et al., 2002; McElhinny et al., 2002; 2004). The subcellular localization of MuRF1a and MuRF2a in zebrafish skeletal muscles is not known. It is not clear whether they have similar or distinct patterns of subcellular localization. To analyze the subcellular localization of MuRF1a and MuRF2a in myofibers of zebrafish embryos, we generated *Tg(ef1α:MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic zebrafish models that express the EGFP tagged MuRF1a or MuRF2a fusion protein driven by the *elongation factor 1α (EF-1α)* gene promoter (Fig. 3A). Three *Tg(ef1α:MuRF1a-GFP)* and two *Tg(ef1α:MuRF2a-GFP)* transgenic lines were generated.

The protein localization of MuRF1a-GFP and MuRF2a-GFP was analyzed in the respective transgenic zebrafish embryos. MuRF1a-GFP showed a clear striated pattern of distribution in myofibers of zebrafish embryos at 28 hpf (Fig. 3B). In contrast, MuRF2a-EGFP fusion protein was mainly detected in a random punctate pattern (Fig. 3C). The distinct patterns of MuRF1a and MuRF2a localization was reproducible in multiple *Tg(ef1α:MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic lines. *Tg(ef1α:XBP1-GFP)* transgenic fish embryos expressing an XBP1-GFP fusion protein showed no such striated or punctate patterns of localization (Fig. 3D) (Li et al., 2015), suggesting that the localization was not caused by the presence of GFP tag.

To define the striated distribution of MuRF1a-GFP with respective to the sarcomere structure, we performed immunostaining with anti-α-Actinin and anti-Myomesin antibodies that specifically label the Z-lines and M-lines, respectively. As expected, zebrafish embryos injected with the *Tg(ef1α:MuRF 1a-GFP)* or the *Tg(ef1α:MuRF2a-GFP)* transgene showed a mosaic pattern of MuRF1a-GFP and MuRF2a-GFP expression. Immunostaining of the injected embryos showed that MuRF1a-GFP was localized between Z-lines labeled with the anti-α-Actinin antibody (Fig. 4A, C). Immunostaining with anti-Myomesin antibody revealed a co-localization with MuRF1a-GFP at the M-line (Fig. 4D, F). Intriguingly, MuRF1a-GFP appeared as small dots aligned along the M-lines (Fig. 4F). In contrast, Murf2a-GFP displayed a diffused pattern of distribution with no clear sarcomeric localization (Fig. 4G–L). Collectively, these data indicate that MuRF1a-GFP and MuRF2a-GFP have different subcellular localization in myofibers of zebrafish embryos.

4. Knockdown of *hsp90ℓ1* or *smyd1b* had no effect on *murf* gene expression but altered MuRF1a-GFP or MuRF2a-GFP protein localization

It has been reported that loss of *hsp90α1* and *smyd1* resulted in defective sarcomere organization and increased myosin protein degradation (Bernick et al., 2012. Li et al., 2013). To determine whether knockdown of *smyd1b* or *hsp90α1* altered MuRF expression, we analyzed the *murf1a* and *murf2a* mRNA expression in *smyd1b* or *hsp90α1* knockdown embryos by RT-PCR and whole-mount in situ hybridization (Fig. 5). The data showed that knockdown of *hsp90ℓ1* or *smyd1b* had little or no effect on the mRNA levels of *murf1a* and *murf2a* transcripts in zebrafish embryos (Fig. 5).

To determine whether knockdown of *smyd1b* or *hsp90(1* altered MuRF1a-GFP and MuRF2a-GFP protein localization in myofibers of zebrafish embryos, we injected Smyd1b-MO or Hsp90α1-MQ into *Tg(ef1α:MuRF1a-GFP)* or *Tg(Tol2-MuRF2a-GFP)* transgenic zebrafish embryos and analyzed their subcellular distribution. The data showed that knockdown of *smyd1b* or *hsp90α1* abolished the M-line localization of MuRF1a-GFP in myofibers of zebrafish embryos (Fig. 6B, C). Intriguingly, this was accompanied by an increased random punctate distribution of MuRF1a-GFP and MuRF2a-GFP fusion proteins in myofibers of the knockdown embryos (Fig. 6B–C, E-F). Collectively, these results revealed that knockdown of *hsp90α1* or *smyd1b* had no effect on *murf1a* and *murf2a* mRNA expression, while it dramatically reduced the striated subcellular distribution on MuRF1a-GFP fusion proteins in myofibers.

4. Discussion

In this study, we characterized the expression and protein localization of MuRFs during muscle development in zebrafish embryos. We showed that the zebrafish genome contains six *murf* genes that are expressed specifically in skeletal and cardiac muscles of zebrafish embryos. By generating transgenic zebrafish models expressing MurF1a-GFP or MuRF2a-GFP fusion proteins, we were able to study the subcellular localization of MuRF1a and MuRF2a. Our data showed that MuRF1a-GFP or MuRF2a-GFP had distinct patterns of subcellular localization in skeletal muscles. MuRF1a-GFP displayed a sarcomeric pattern of distribution that co-localized with the M-line, whereas MuRF2a mainly appeared in a random punctate pattern. Knockdown of *smyd1b* or *hsp90α1* that induced myosin degradation had no effect on *murf* gene expression, but dramatically reduced the sarcomeric localization of MuRF1a-GFP and increased the random punctate formation in myofibers of zebrafish embryos. The *Tg(ef1α:MurF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic fish provide useful models for studying MuRF action in muscle cell differentiation.

Murf gene structure and expression

Our analyses identified six *murf* genes in the zebrafish genome, which doubles the number of *murf* genes *(murf1, murf2* and *murf3)* in mice and human genomes. Our results are in agreement with a previous report (Macqueen et al., 2014), and consistent with the theory of an extra teleost-specific whole-genome duplication in during evolution (Jaillon et al., 2004; Sato and Nishida, 2010). Sequence analysis indicates that *murf1a* and *murf1b* are the orthologues of *murf1* genes in mammals. However, the gene structure of zebrafish *muf1a* and *murf1b* differs significantly from the *murf1* genes in mice and humans. The zebrafish *muf1a* and *murf1b* genes contain no intron sequence, whereas the mice and human *murf1* genes contain 8 introns. Interestingly, all identified teleost *murf1* genes are intronless, whereas *murf1* genes from spotted gar, a ray-finned fish that split from teleost over 350 million years ago contain the same genomic organization as *murf1* in lobe-finned fish and tetrapods (Near et al., 2012). It has been suggested that the intronless gene might be generated by retrotransposition specifically within the teleost lineage, sometime before the common teleost-specific genome duplication occurred 320–350 million years ago (Glasauer and Neuhauss, 2014; Macqueen et al., 2014). Remarkably, the zebrafish *murf1a* and *murf1b* show a similar pattern of skeletal and cardiac muscle-specific expression as observed in

mice and other vertebrate embryos, suggesting that the *murf1* retrogene likely replaced the ancestral *murf1* gene at this same locus and thus retaining all the regulatory elements for muscle-specific expression.

In addition to the typical *murf1, murf2a* and *murf3* subfamilies, the zebrafish genome contains a novel *murf2-like* gene that cannot be found in mammals. Macqueen and colleagues named this novel *murf* gene as *murf4* because it could not be grouped into *murf1, murf2* or *murf3* subgroups (Macqueen et al., 2014). Our phylogenetic analysis suggest that this novel *murf4* gene is more closely related to *murf2*, thus named it *muf2-like* gene. The *murf2-like/murf4* gene has 9 exons and is expressed in skeletal muscles of zebrafish embryos although its function has yet to be determined.

MuRF-GFP transgenic zebrafish models

Members of the MuRF protein family show dynamic patterns of subcellular localization in skeletal and cardiac muscle cells, ranging from the nucleus to cytoplasm and sarcomeres (Spencer et al., 2000; Centner et al. 2001; Dai and Liew, 2001; McElhinny et al., 2002, 2004). The dynamic localization suggests that MuRFs may have distinct functions with respect to their subcellular localization. By using the unique *Tg(ef1α:MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic zebrafish models generated in this study, we analyzed the MuRF1a-GFP and MuRF2a-GFP protein localization in myofibers of zebrafish embryos. Our data showed that MuRF1a-GFP co-localized primarily with the M-lines in skeletal myofibers. This is consistent with previous findings in chick, mouse and rat cardiac myocytes and skeletal myocytes (McElhinny et al., 2002, 2004).

Tg(ef1α:MuRF1a-GFP) and *Tg(ef1α:MuRF2a-GFP)* transgenic zebrafish showed normal muscle development and growth. This is in contrast to the transgenic zebrafish model generated using the *Tg(myl7:MuRF1a-IRES-GFP)* transgene that expressed MuRF1a under the control of a myocardial-specific *myl7* promoter derived from the *myosin regulatory light chain* 7 (Shimizu et al., 2017). Shimizu and colleagues reported that upregulation of MuRF1a expression in cardiac myocytes led to myofibril disarray in zebrafish heart muscles, leading to dilated heart with severe edema in the transgenic zebrafish embryos (Shimizu et al., 2017). The reason for the phenotypic discrepancy is not clear. Given that our transgenic models were generated by different gene constructs, the phenotypic discrepancy could be caused by different levels of ectopic *murf1a* gene expression in the heart of different transgenic models used in these two studies. Shimizu and colleagues used a strong cardiac muscle-specific *myl7* promoter to drive the MuRF1a expression, whereas we used a moderate ubiquitous *ef1a* promoter to drive MuRF1a expression. Consistent with the idea that overexpression of MuRF1 is detrimental to sarcomere organization, it has been reported that overexpression of MuRF1 disrupted the integrity of titin's M-line region and the organization of thick filament components in chick cardiac myocytes (McElhinny et al., 2002).

Dynamic MuRF protein localization

We showed in this study that the sarcomeric localization of MuRF1a was abolished in *smyd1b* or *hsp90α1* knockdown zebrafish embryos. Smyd1b or Hsp90α1 are vital

regulators of sarcomere assembly. We and others have shown that loss of Smyd1b or Hsp90α1 increased myosin protein degradation leading to sarcomere disruption in skeletal and cardiac muscles of zebrafish embryos (Tan et al., 2006; Du et al., 2008; Hawkins et al., 2008; Just et al., 20121; Li et al., 2013; Prill et al., 2016; Cai et al., 2019). Given that MuRFs directly mediate muscle protein ubiquitination and degradation, we tested whether *murf* gene expression could be upregulated. Our data showed that knockdown of *smyd1b* or *hsp90α1* had no effect on *murf1a* and *murf2a* mRNA expression. However, the subcellular localization of MuRF1a-GFP was significantly altered. It appeared that the sarcomeric localization of MuRF1a-GFP disappeared and replaced with a dramatic increase of random punctate distribution of MuRF1a-GFP in defective myofibers of the *smyd1b* or *hsp90α1* knockdown embryos.

The molecular mechanism underlying the increased MuRF1a-GFP punctate distribution is not clear. The punctate MuRF1a-GFP signals were 1–2 micron in size that was scattered in the skeletal muscle fibers of *smyd1b* or *hsp90α1* knockdown embryos. Similar punctate distribution has been observed in chick and rat cardiac myocytes with GFP-MuRF1 or GFP-MuRF2 overexpression (McElhinny et al., 2002). It has been suggested that these punctate represent aggregates formed via the homo-oligomerization of MuRF proteins (Spencer et al., 2000; Centner et al., 2001). Interestingly, if GFP is only fused with the MuRF RING or tail domain, no aggregates were observed in the cytoplasm or assembled in the M-line region. In contrast, when the COS domain of MuRF1 was fused with GFP, majority of transfected cardiac myocytes were observed aggregated formed in the cytoplasm (McElhinny et al., 2002). The regulation of dynamic localization and the functional significance of forming the punctate aggregates are not clear. A recent study indicates that MuRF1 cellular localization is regulated by SUMO1 mediated post-translational modification (Heras et al., 2019). MuRF1 SUMOylation is essential for its nuclear translocation. It remains to be determined whether a post-translational modification is involved in regulating the sarcomeric distributions of MuRF proteins in myofibers of zebrafish.

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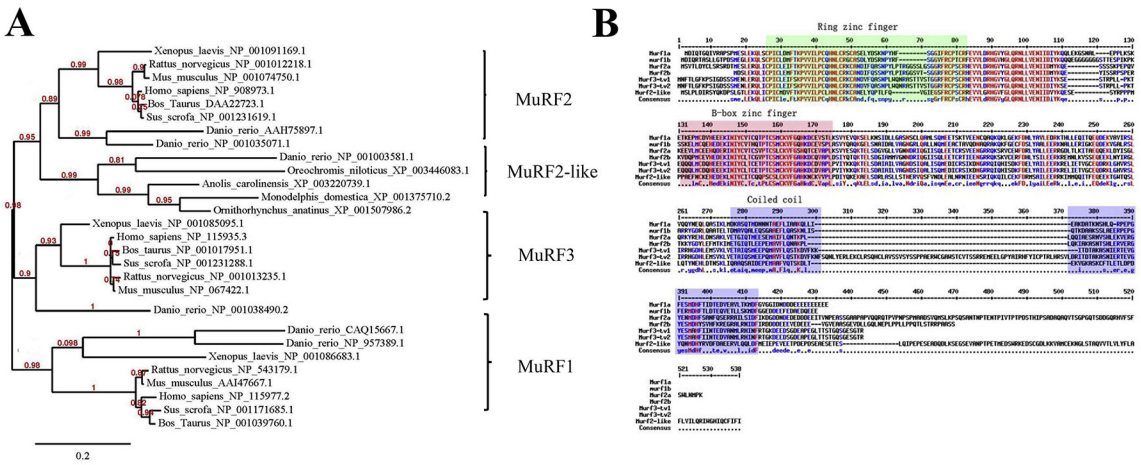

Figure 1. Phylogenetic analysis of vertebrate MuRFs and protein sequence alignment of MuRFs in zebrafish, *Danio rerio*..

A. A phylogenetic tree was developed based on MuRF protein sequences from fish to mammals. The MuRF proteins could be divided into three branches. The first branch is made up of the MuRF2 and MuRF2-like subgroup. The other two branches are MuRF1 and MuRF3, respectively.

B. The protein sequence alignment of six MuRFs in zebrafish. MuRF1a, CAQ15667.1; MuRF1b, NP_957389.1; MuRF2a, AAH75897.1; MuRF2b NP_001035071.1; MuRF2-like NP_001003581.1. MuRF3 has two isoforms from alternative splicing named MuRF3_tv1, NP_001038490.2 and MuRF3_tv2 CAI20953.1. The three conserved structural domains are indicated as ring zinc finger domain, B-box zinc finger domain and the coiled coil domain, respectively.

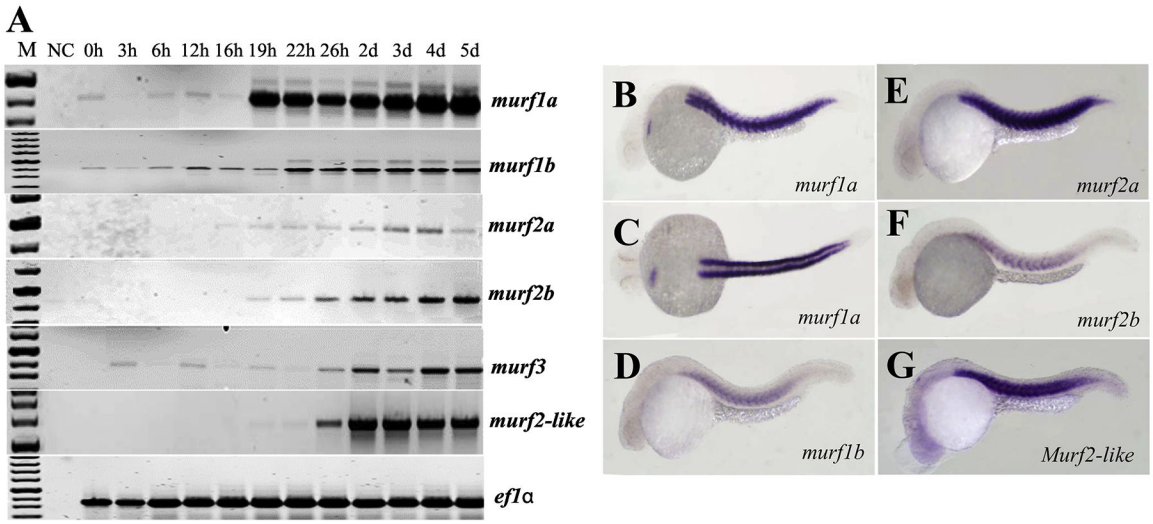

Figure 2. The temporal and spatial patterns of *murf* gene expression in zebrafish (*Danio rerio*) embryos.

A. RT-PCR showed the temporal expression of 6 zebrafish *murf* genes during embryonic development at various stages from newly fertilized eggs to 5 day old larvae. *EF1α* was included as an internal reference control.

B-G *In situ* hybridization showed the restricted pattern of expression for each member of the 6 *murf* genes in zebrafish embryos at 24 hpf. All 6 *murf* genes were expressed in skeletal muscles of the zebrafish embryos. In addition, *murf1a* also showed a cardiac muscle expression (Fig. B, C).

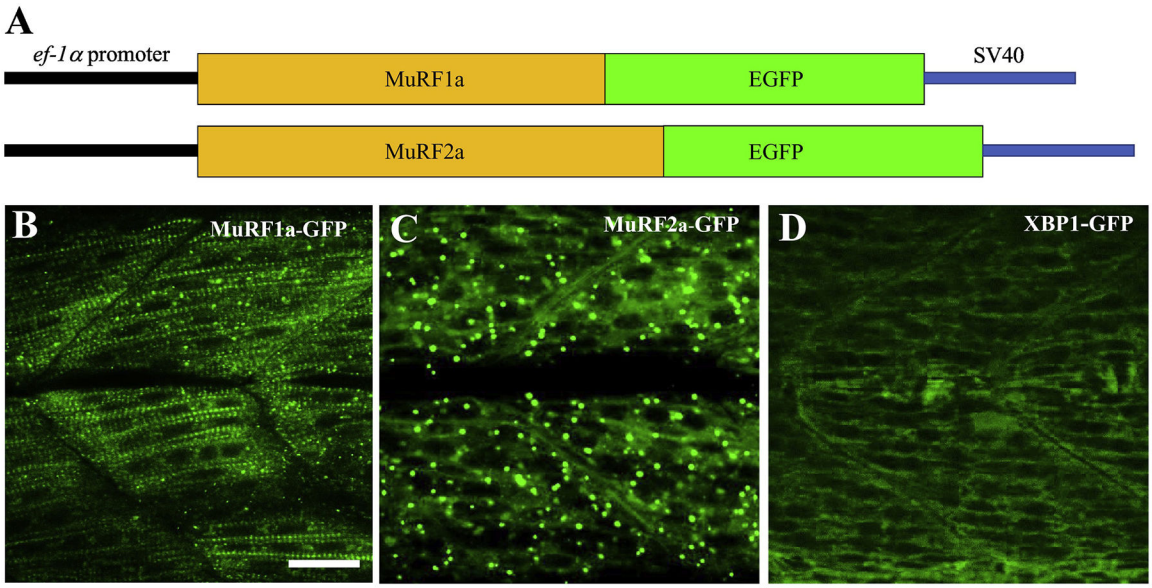

Figure 3. Generation of *Tg(ef1α:MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenic zebrafish, *Danio rerio*.

A: Diagram of the *Tg(ef1α:MuRF1a-GFP)* and *Tg(ef1α:MuRF2a-GFP)* transgenes. The full-length coding sequences of zebrafish *murf1a* or *murf2a* were cloned in frame upstream of the EGFP coding sequence in the pT2AL200R150G vector. The pT2AL200R150G vector contained an *elongation factor-1 alpha* (*EF-1α*) gene promoter. Transgenic zebrafish were generated using these two transgenes.

B-D: Subcellular localization of MuRF1a-GFP (B), MuRF1a-GFP (D), or XBP1-GFP fusion protein in myofibers of the respective transgenic zebrafish embryos at 28 hpf. Scale bar: 30 μm.

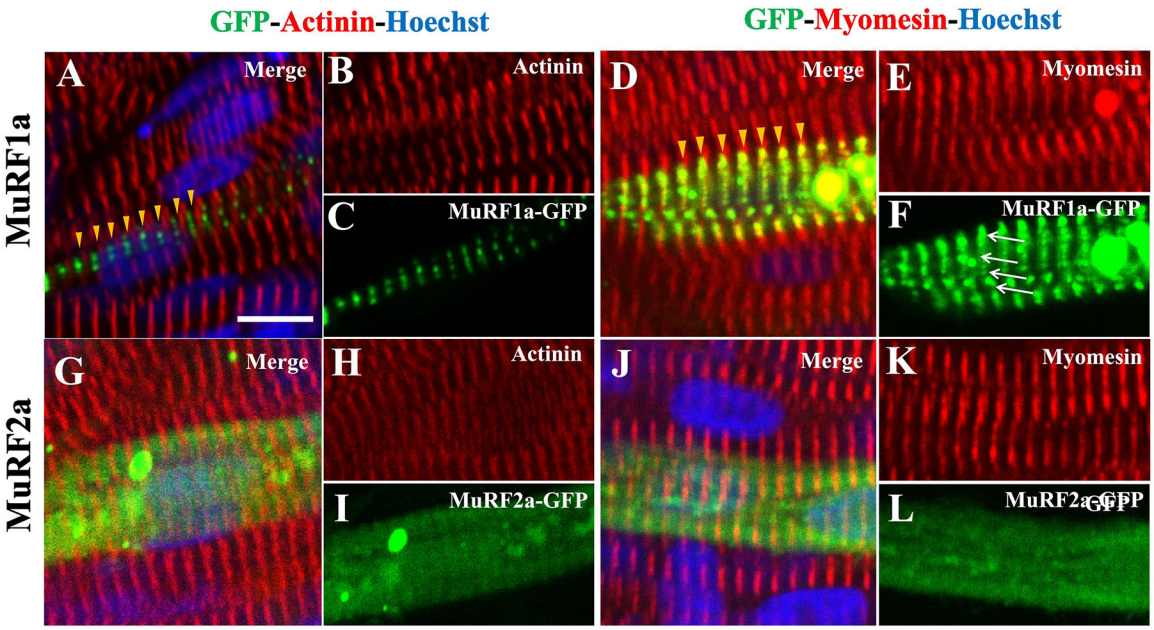

Figure 4. Subcellular localization of MuRF1a-GFP and MuRF2a-GFP in myofibers of zebrafish (*Danio rerio*) embryos.

DNA construct expressing the MuRF1a-GFP or MuRF2a-GFP fusion protein was microinjected into zebrafish embryos. The injected embryos were stained with anti-α-Actinin or anti-Myomesin antibodies that specifically labeled Z-line and M-line, respectively. The nuclei were labeled with Hoechst 33258 (blue).

A-C: Confocal analysis of MuRF1a-GFP and α-Actinin localization in myofibers of zebrafish embryos at 30 hpf. The yellow arrowheads in (A) indicate the striated distribution of MuRF1a-GFP (green) between Z-lines (red). Scale bar: 8 μm.

D-F: Confocal analysis of MuRF1a-GFP and Myomesin localization in myofibers of zebrafish embryos at 30 hpf. The yellow arrowheads in (D) indicate the co-localization of MuRF1a-GFP (green) with M-lines (red). The white arrows in (F) indicate MuRF1a-GFP positive dots aligned along the M-lines (Fig. 4F).

G-L: Confocal analysis of MuRF2a-GFP localization in myofibers stained with anti-α-Actinin (G, H) or anti-Myomesin (J, K) antibodies.

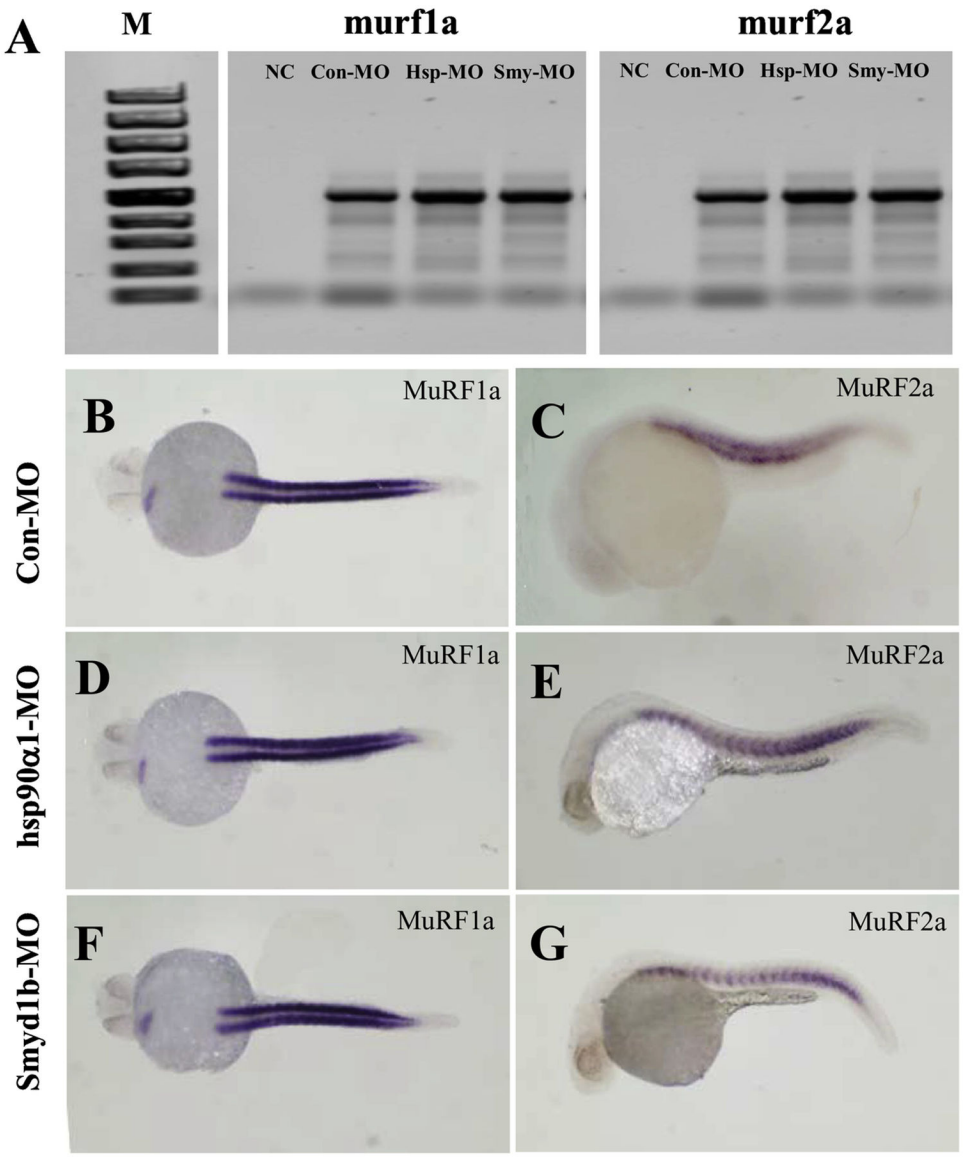

Figure 5. The effect of *hsp90α1* and *smyd1b* knockdown on *murf1a* and *murf2a* gene expression in zebrafish (*Danio rerio*) embryos.

The *hsp90α1* and *smyd1b* genes were knocked down in zebrafish embryos by gene specific MO injection. *murf1a* and *murf2a* gene expression was analyzed by RT-PCR (A) and whole mount in situ hybridization (B-G). A. RT-PCR analysis showed the levels of *murf1a* and *murf2a* gene expression in knockdown and control embryos. B-G: Whole mount *in situ* hybridization showed the *murf1a* and *murf2a* transcript signals in control (B and C), *hsp90α1* (D and E) or *smyd1b* (F and G) knockdown embryos at 24 hpf, respectively.

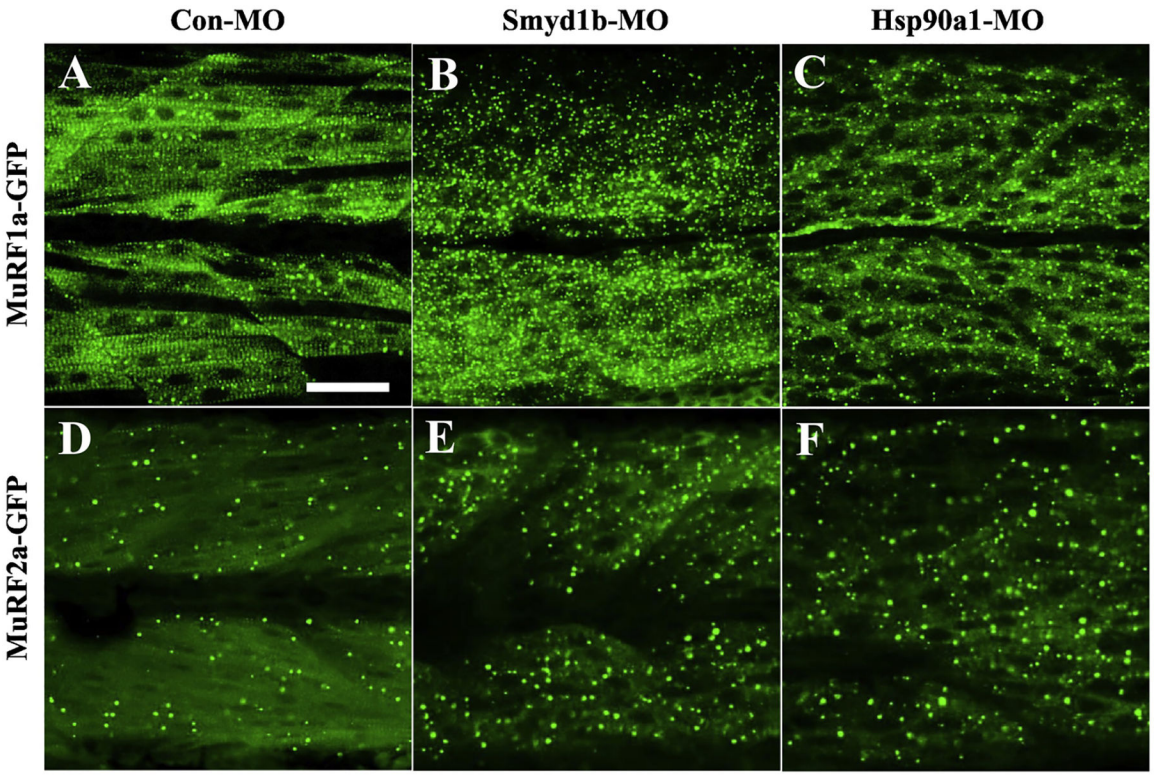

Figure. 6. The effect of *hsp90α1* or *smyd1b* knockdown on the subcellular localization of MuRF1a-GFP and MuRF2a-GFP in skeletal muscle fibers of zebrafish (*Danio rerio*) embryos.

hsp90α1 and *smyd1b* expression was knocked down in *Tg(ef1a:MuRF1a-GFP)* and *Tg(ef1a:MuRF2a-GFP)* transgenic fish embryos. The MuRF1a-GFP and MuRF2a-GFP subcellular localization was determined by confocal microscopy in the *hsp90α1* and *smyd1b* knockdown and control embryos.

A-C: MuRF1a-GFP localization in myofibers of zebrafish embryos injected with control-MO (A), Smyd1b-MO (B) or Hsp90α1-M0 (C) at 30 hpf.

D-F: A-C: MuRF2a-GFP localization in myofibers of zebrafish embryos injected with control-MO (D), Smyd1b-MO (E) or Hsp90α1-MQ (F) at 30 hpf.

Scale bar: 50 μm.

Table 1.

Sequences of PCR primers and MOs

Gene name	Primer sequence
MuRF1a	F1:ATGGACATCCAAACGGGTCAAATA R1:TTATTCCTCCTCCTCTTCTTCTTC
MuRF1b	R1: TTACTCCTCTTCTTGATCTTCAG F1: ATGGACATTCAGCGAACTGCCTC
MuRF2a	F1: ATGAGCGTGACTTTGGATTACTG R1: TTACTTTGGCATATTTAACCAAGA
MuRF2b	F1: ATGGACAGCTTGGAGAAGCAACT R1: TTAGGATGATGCGGCGGGTCTGCGT
MuRF3	F1: ATGAACTTCACTTTGGGCTTCAAA R1: TTAGCGCGTCCCTGATTCACCAC
MuRF2-like	F1: ATGTCTCTTCCACTGGATATACG R1: CTGACCTGCTGAATGTGTCTTCA
MuRF1a	F2: CATCTACAAGCAGCAGTTGGA R2: CTCAGACTTCTGGACCTCATA
MuRF1b	F2: AACTTGTCGATTTGAAGTGGT R2: TATTGGACTTTCAGTGGTGCT
MuRF2a	F2: TATCTTCCTGCAGAACACGAA R2: TCATCATCCTCATCCTCATCA
MuRF2b	F2: AGGAATACAGACACTGGAAGA R2: CATCATCATCATCTCTGATGA
MuRF3	F2: CACAACCAACAGTCTCGATTA R2: CTTACTGAGAAGGTTGGTAAA
MuRF2-like	F2: CTTACTGAGAAGGTTGGTAAA R2: TCTGCTTCAGAATCTGGTTCA
EF-1α	F: GCATACATCAAGAAGATCGGC R: GCAGCCTTCTGTGCAGACTTTG
MuRF1a-EGFP	F: GCGGGATCCATGGACATCCAAACGGGTCAAAT R: GCGGGATCCAATTCCTCCTCCTCTTCTTCTTCCT
MuRF2a-EGFP	F: GCGGGATCCATGAGCGTGACTTTGGATTACTG R: GCGGGATCCAGCTTTGGCATATTTAACCAAGAGA
EGFP	F: GCACAAGCTGGAGTACAACTACAAC R: GTACAGCTCGTCCATGCCGAGAGT
Hsp90α1 ATG-MO	5’-CGACTTCTCAGGCATCTTGCTGTGT-3’
Smyd1b ATG-MO	5’-ACTTCCACAAACTCCATTCTGGATC-3’
Standard cont-MO	5’-CCTCTTACCTCAGTTACAATTTATA 3’

The sequences of PCR primers used to isolate respective *MuRF* genes from zebrafish (*Danio rerio*) and the MOs used to knock down *hsp90α1* or *smyd1b* expression in zebrafish embryos.

Table 2.

DNA plasmids for antisense RNA probes

DNA plasmids	RNA polymerase	Restriction Enzymes
Murf1a	T7	NarI
Murf1b	SP6	HindIII
Murf2a	T7	StuI
Murf2b	SP6	NarI
Murf3	SP6	EcoRV
Murf2-like	SP6	StuI

The DNA plasmids used to generate the anti-sense probes for in situ hybridization in zebrafish (*Danio rerio*) embryos. Each plasmid was digested with a specific restriction enzyme and used as template for RNA synthesis using respective T7 or Sp6 RNA polymerase.