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There was an error published in Development 140, 1528-1536.

On p. 1532, the last line should state: the transcription of the NFATc4 isoform is dramatically increased after Nfix inhibition in murine fetal myoblasts (Messina et al., 2010).

The authors apologise to readers for this mistake.
Conserved and divergent functions of Nfix in skeletal muscle development during vertebrate evolution

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SUMMARY

During mouse skeletal muscle development, the Nfix gene has a pivotal role in regulating fetal-specific transcription. Zebrafish and mice share related programs for muscle development, although zebrafish develops at a much faster rate. In fact, although mouse fetal muscle fibers form after 15 days of development, in fish secondary muscle fibers form by 48 hours post-fertilization in a process that until now has been poorly characterized mechanically. In this work, we studied the zebrafish ortholog Nfix (nfixa) and its role in the proper switch to the secondary myogenic wave. This allowed us to highlight evolutionarily conserved and divergent functions of Nfix. In fact, the knock down of nfixa in zebrafish blocks secondary myogenesis, as in mouse, but also alters primary slow muscle fiber formation. Moreover, whereas Nfix mutant mice are motile, nfixa knockdown zebrafish display impaired motility that probably depends upon disruption of the sarcoplasmic reticulum. We conclude that, during vertebrate evolution, the transcription factor Nfix lost some specific functions, probably as a consequence of the different environment in which teleosts and mammals develop.

KEY WORDS: Nfix, Skeletal myogenesis, Zebrafish, Slow muscle fibers, Sarcoplasmic reticulum

INTRODUCTION

Muscles are formed by subsequent myogenic waves, regulated by intrinsic and extrinsic signals during prenatal mammalian development (Biressi et al., 2007; Buckingham, 2007; Cossu and Biressi, 2005). The myotome is the first differentiated skeletal muscle, formed by mononucleated myocytes that differentiate in a Pax3/7-independent manner. All subsequent myogenic populations depend upon Pax3/7 expression (Gros et al., 2005; Hutcheson et al., 2009; Relaxi et al., 2005). Embryonic myoblasts begin to fuse into primary multinucleated muscle fibers by E11, whereas fetal myoblasts fuse into secondary fibers between E14.5 and E17.5 (Cossu and Biressi, 2005). Once mature, skeletal muscles fibers differ in the expression of contractile protein isoforms and metabolic enzymes that confer a slow or fast twitching phenotype. Although zebrafish muscle development shares many features with amniotes, there are notable differences. The slow twitching fibers, derived from medial adaxial cells, are the first muscle fibers formed in zebrafish (Stickney et al., 2000). Adaxial originating cells are committed to a myogenic fate and express the myogenic regulator factors (MRFs) myoD and myf5 (Coutelle et al., 2001; Weinberg et al., 1996). After somite formation, adaxial cells migrate radially from the notochord, form a layer of superficial slow fibers (SSFs) on the myotomal surface and express slow myosin heavy chain (MyHC) (Bryson-Richardson et al., 2005; Devoto et al., 1996; Du et al., 1997). After slow fiber precursor migration, fast fibers are specified in the deeper part of the somite and express fast MyHC (Blagden et al., 1997; Henry and Amacher, 2004; Stellabotte et al., 2007). At 24 hours post-fertilization (24 hpf), segmentation is complete and a functional myotome is formed.

Following this primary myogenic wave in zebrafish, secondary slow twitching fibers differentiate in several body locations in a process called stratified hyperplasia or secondary myogenesis (48-72 hpf) (Devoto et al., 1996; Elworthy et al., 2008). In teleosts, muscle growth also occurs both by hypertrophy, owing to an increase in the size of pre-existing muscle fiber throughout life (Barresi et al., 2001), and hyperplasia, owing to the activity of Pax7-positive cells with typical muscle progenitor cell properties in the larval stage (Hollway et al., 2007; Seger et al., 2011).

Mouse embryonic and fetal myoblasts express distinct specific markers in the fibers they form (i.e. slow and fast MyHCs, MCK, β-enolase, PKCθ) (Biressi et al., 2007), indicating that embryonic and fetal myoblasts are intrinsically different populations of cells with distinct genetic programs. Among differentially expressed molecules, our group identified Nfix as a master switch regulator of the transcriptional transition from embryonic to fetal muscle (Messina et al., 2010).

Here, we report the identification of two zebrafish orthologs of Nfix, nfixa and nfixb, analyze their expression during development and use a loss-of-function approach to specifically abrogate the nfixa function in vivo and analyze the resulting muscle phenotype.

MATERIALS AND METHODS

Animals

Breeding wild-type fish of the AB strain were maintained at 28°C on a 14-hour light/10-hour dark cycle. Embryos were collected by natural spawning, staged according to Kimmel and colleagues (Kimmel et al., 1995) and raised at 28°C in fish water (Instant Ocean, 0.1% methylene blue) in Petri dishes, according to established techniques, approved by the veterinarian (OVSAC) and the animal use committee (IACUC) at the University of Oregon (USA), in agreement with local and national sanitary regulations. We express the embryonic ages in somites (s), hours post-fertilization (hpf) and days post fertilization (dpf). MyoDCre mice (Chen et al., 2005) and Nfixc/+ mice, their genotyping strategies and manipulation have been already described (Campbell et al., 2008).

RT-PCR

Total RNA from 12 samples (an average of 30 embryos per sample) was extracted with the TOTALLY RNA isolation kit (Ambion), treated with...
RQ1 RNase-Free DNase (Promega) and oligo(dT)-reverse transcription using SuperScript II RT (Invitrogen), according to manufacturers’ instructions. PCR products were loaded and resolved on 2% agarose gels.

The following primers were used: nfxa sense, 5'-AGGCTGTC- AAACACTGCA-3'; nfxa antisense, 5'-TCATTAAATACAGAGT- TGATG-3'; nfxb sense, 5'-CCACCTACGAGACTGAGA-3'; nfxb antisense, 5'-GCCAGCTGCTCTGACAATC-3'; beta-actin sense, 5'-TTGTTTCCCCCTATGTTG-3'; beta-actin antisense, 5'-TTTCCCTATGTCAGGAGA-3'; flil sense, 5'-AAATTTGCTGGGGCCCT- ACTG-3'; and flil antisense, 5'-CTTGGTGAAGACGACGAC-3'.

Primers for myod were used according to Amali and colleagues (Amali et al., 2004).

**In situ hybridization, histological analysis, immunohistochemistry and transmission electron microscopy**

Whole mount in situ hybridization experiments were carried out as described previously (Thiese et al., 1993). Immunohistochemistry and immunofluorescence analyses were carried out as described previously (Panzer et al., 2005). The following primers were used for PCR reactions to clone the probes: nfxa 300 bp sense, 5'-AGCCTGTTACGAGAATCTGC-3'; nfxa 300 bp antisense, 5'-TACATTAAATACAGAGTGTGATG-3'; nfxb 3' UTR sense, 5'-CCACCTACGAGACTGAGA-3'; nfxb 3' UTR antisense, 5'-CCACCTACGAGACTGAGA-3'; nfxa sense, 5'-CCACCTACGAGACTGAGA-3'; and nfxb sense, 5'-CCACCTACGAGACTGAGA-3'.

**Quantitative real-time RT-PCR**

Reverse transcriptions (RTs) were performed using 2 μg of DNA-treated (DNA-free, Ambion) total RNA in the presence of random hexamers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen). Real-time PCRs were carried out in a total volume of 15 μl containing 1× SYBR Green Super Mix (BioRad), using 1 μl of the RT reaction. PCRs were performed using the BioRad iCycler iQ Real Time Detection System (BioRad Laboratories). For normalization purposes, efla RNA levels were tested in parallel with the gene of interest. The following primers were used: nfxa sense, 5'-CCCCGTAGTCTGGTGACACA-3'; nfxa antisense, 5'-GCTTGTGTTGTGAGACTGCGG-3'; smyhel sense, 5'-TGCCAAAGACCTACAGAAGT-3'; smyhel antisense, 5'-CACACCAATGTTGATTGC-3'; nfact4 sense, 5'-CAGAGGAACACCTACGGCTATC-3'; nfact4 antisense, 5'-GGTTTCAATATCTCTTGGACCA-3'; eflalpha sense, 5'-GGTACCTTCTACGGCTACTG-3'; and eflalpha antisense, 5'-CAGAGGTACCTTCTACGGCTACTG-3'.

**Injection**

To repress nfxa mRNA translation, an ATG-targeting morpholinon (nfxa-MO) and an nfxa-splice-MO were synthesized (Gene Tools) (nfxa-MO, 5'-CCGGTCTCAAAGGGAAATGATT-3'; nfxa-splice-MO, 5'-AAGAGGAGTGTAAACCACCTGAC-3') and used at the concentration of 1 pmole in 1× Danieau buffer (pH 7.6) as previously reported (Nasevicius and Ekker, 2000). As a control, we injected a standard control morpholino oligonucleotide (ctrl-MO). The p53 morpholinon has been designed (Gene Tools) and used as described previously (Robu et al., 2007).

The in vivo test of the specificity was carried out as described previously (Del Giacco et al., 2010). In brief, 300 pg/embryo of the CS2~nfxa-MO-GFP sensor plasmid have been injected alone or co-injected with 8 ng/embryo of nfxa-MO. The presence/absence of the GFP signal has been monitored under a fluorescent microscope from 24 to 48 hpf. nfxa-MO cDNA fragments inserted in the BomHI site were obtained using the following complementary oligos: nfxa MO sense, 5'-gateATCTT- ATTTCCCGCTTIGAGACCGCTGACGT-3'; and nfxa MO antisense, 5'-gateATCTGACGGCTCTAAAGGGAAATGATT-3'.

For the specificity of phenotype, 8 ng of nfxa-MO were injected together with 200 pg/embryo of murine HA-Nfx2 mRNA, 150 pg/embryo of MeF2aFlag mRNA and 150 pg/embryo MeF2aFlag mRNA, prepared as described previously (Briessi et al., 2007). The same results were obtained with the injection of 300 pg/embryo of the endogenous nfxa mRNA.

To repress nfact4 mRNA translation, an nfact4-splice-MO was synthesized (Gene Tools) (nfact4-splice-MO, 5'-CAGAAAATGTTGATTGC-CTTCCACCTGACT-3') and used at the concentration of 8 ng/embryo. To validate nfact4-splice-MO and verify the skipping of the second exon, following primers were used: nfact4-E1 sense, 5'-ACACGAAACCCAGGATCTGC-3'; and nfact4-E3 antisense, 5'-ACCGATGAATCCACGCAAGG-3'.

**Sorting**

Alpha-actin-GFP transgenic embryos (50-100) at 24 and 48 hpf were incubated in trypsin solution (0.5% trypsin and 1 mM EDTA) for 2 hours with gentle pipetting to dissociate the cells. Cells were resuspended in PBS (Gibco)/20% fetal calf serum (FCS; BioWhittaker)/20 mM HEPES and 2 mM EDTA and filtered through 40 μm cell strainers (Falcon) before sorting using a Vantage Sorter SE (Becton Dickinson) at a flow rate of 3000 cells per second. GFP was exited at 488 nm using an argon laser. Cells dissociated from wild-type embryos were used to set the gating to exclude green autofluorescence. After sorting, the GFP+ cells were collected and RNA was extracted with the micro-RNAeasy kit (Qiagen). RNA was directly retro-transcribed with the iSCRIPTtm cDNA synthesis kit (BioRad) and the obtained cDNA was used for RT-PCR reactions.

**Western blot**

Fish embryos and mouse fetuses were lysed in RIPA buffer (5 μl for each zebrafish embryo) and homogenized. Samples were boiled for 10 minutes at 95°C. Zebrafish protein samples (20 μl) were size-fractionated by gel Precast (Invitrogen) and transferred with iBlot (Invitrogen). The nitrocellulose membranes were blocked with 5% non-fat dry milk in PBST (PBS containing 0.1% Tween 20) for 30 minutes at room temperature and subsequently incubated with the primary antibody: mouse anti-slow myosin heavy chain (F59, DSHB), mouse anti-sarcomeric (MF20, DSHB), mouse anti-ryanodin receptor (RyR:34C Sigma), mouse anti-syt2 (zm1, ZIRC), mouse anti-alcama (zn-5, ZIRC), mouse anti-s2v (DSHB), mouse monoclonal anti-Pax7 (DSHB) and rabbit anti-Nfix (kindly provided by Richard Gronostajski, State University of New York at Buffalo, USA), then treated with biotinylated or fluorescent secondary antibody (Vector Laboratories). Postysaptic AChRs staining was performed as described previously (Devoto et al., 2005) (BTX, Molecular Probes, Eugene, OR). F59, MF20, H3P and Pax7 staining were performed as described previously (Devoto et al., 2006) and Seger and colleagues (Seger et al., 2011). Regarding the wild-type and skeletal muscle Nfix-null mice at E16.5/P3, immunofluorescence and transmission electron microscopy were carried out as described previously (Messina et al., 2010). Mouse anti-Ry-R was diluted 1:2000 and incubated for 1 hour at 37°C. Secondary antibodies used were Alexa Fluor 488 or 594-conjugated donkey anti-mouse. DAPI was used to stain the nuclei.

**Cyclosporine (CsA) treatment**

Controls and nfxa-MO-injected embryos were dechorionated at shield or at 8-10 somite stage and incubated in CsA for 12 hpf. CsA was used at 100 mM and dissolved in 0.1% DMSO in egg water. Vehicle control treatment consisted of 0.1% DMSO in egg water.

**RESULTS**

**Identification of zebrafish nfixa and nfixb genes**

As a first step, we sought to identify the ortholog(s) of Nfix in zebrafish. Ensembl search using human Nfix as a bait, identified two candidates referred to as nfixa gene (assembly Zv9, and nfixb gene (assembly Zv10, respectively). As a second step, we investigated the expression pattern of these two genes in zebrafish using in situ hybridization (ISH) experiments. The nfixa and nfixb genes were expressed in different tissues and organs, with a distinct spatial and temporal expression pattern.

**Figure 1.** In situ hybridization of nfixa and nfixb genes in zebrafish embryos. (A) nfixa expression in the CNS, heart and lateral plate mesoderm. (B) nfixb expression in the CNS, heart and lateral plate mesoderm.
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chromosome 1, nucleotide position 52,397,904-52,595,159) and nfixb (assembly Zv9, chromosome 3, nucleotide position 14,397,904-14,663,156). Analysis of the amino acid sequence homology between human, murine and the two zebrafish Nfix orthologs revealed high degree of conservation among them but did not reveal which zebrafish gene was the functional ortholog (supplementary material Table S1). Analysis of the amino acid sequence homology between human, murine and the two zebrafish Nfix orthologs revealed high degree of conservation among them but did not reveal which zebrafish gene was the functional ortholog (supplementary material Table S1). Analysis of the amino acid sequence homology between human, murine and the two zebrafish Nfix orthologs revealed high degree of conservation among them but did not reveal which zebrafish gene was the functional ortholog (supplementary material Table S1). Analysis of the amino acid sequence homology between human, murine and the two zebrafish Nfix orthologs revealed high degree of conservation among them but did not reveal which zebrafish gene was the functional ortholog (supplementary material Table S1).

RT-PCR analyses of nfixa and nfixb showed expression of both transcripts in the developing embryo, larva and in adult muscle (Fig. 1C). Therefore, to determine whether one or both orthologs are expressed during muscle formation, we examined the spatial and temporal distribution of nfixa and nfixb transcripts by whole-mount in situ hybridization. The nfixa-specific probe (nfixa) revealed a hybridization signal in the notochord, in the unsegmented paraxial mesoderm at the 5-somite stage (Fig. 1D) and in the segmented mesoderm at 8- to 10-somite stage (Fig. 1D'). By contrast, we did not detect any specific labeling for nfixb in already formed somites (supplementary material Fig. S1A). We also noted that by 3 days post-fertilization (3 dpf) the labeling of both nfixb

Fig. 1. Identification and expression analysis of nfixa and nfixb. (A) Each zebrafish nfix gene is shown as a reference locus. Genes annotated as paralogs (no surrounding line) or orthologs (with a surrounding line) by the Ensembl database share the same color; blue lines beneath individual tracks indicate that orientations of gene blocks and are inverted with respect to their genomic annotation. Analysis of chromosomal organization for zebrafish nfixa (chromosome 1), nfixb (chromosome 3), and the human (chromosome 19) and mouse (chromosome 8) Nfix genes. Syntenous human and mouse genes are labeled. The figure was derived from the output of the Genomicus website (version 57.01). (B) Phylogenetic relationship of Nfi genes. Evolutionary comparison of different members of Nfi protein family represented in a Maximum-likelihood bootstrap consensus tree. The zebrafish nfixa and nfixb are highlighted. Branch lengths are measured in terms of amino acid substitutions, with the scale indicated over the tree. Numbers at nodes indicate percent of bootstrap probabilities. Ce, Caenorhabditis elegans; Ci, Ciona intestinalis; Dr, Danio rerio; Ga, Gasterosteus aculeatus; Gg, Gallus gallus; Hs, Homo sapiens; Mm, Mus musculus; Oi, Oryzias latipes; Ti, Tetraodon nigroviridis; Tr, Takifugu rubripes; Xt, Xenopus tropicalis. (C) RT-PCR performed at different developmental stages: ladder (L), one- to two-cell stage (lane 1), 30% epiboly (lane 2), 50% epiboly (lane 3), tail bud (lane 4), eight somites (lane 5), 15 somites (lane 6), 24 hpf (lane 7), 48 hpf (lane 8), 3 dpf (lane 9), 5 dpf (lane 10), adult muscle (lane 11) and negative control (lane 12) in the absence of cDNA. (D,D') Whole-mount in situ hybridization with nfixa-specific probe at the 5-somite stage and 8-10 somite stage. (D) The transcript is present in the unsegmented paraxial mesoderm and in the notochord (boxed region). (D') nfixa signal is present in the already segmented somites (arrow) and in the central nervous system (arrowhead). (E) RT-PCR performed on cDNA of muscle cells sorted from α-actin GFP transgenic embryos at 24 and 48 hpf. White line marks lanes run on a separate gel but using the same samples and amounts of input RNA. (F) Quantitative real-time PCR (qRT-PCR) of nfixa mRNA expression normalized to ef1a.
and nfixa probes is restricted to the telencephalon and mesencephalon (supplementary material Fig. S1B,C). To further analyze nfixa and nfixb expression specifically in muscles, we FACs sorted muscle cells from α-actin transgenic embryos at 24 and 48 hpf. cDNA from sorted cells was analyzed by RT-PCR and, as shown in Fig. 1E, only nfixa is expressed in muscle cells. myod was used as a positive control for the enrichment of muscle cells, whereas the vascular marker flt1 was used as a negative control (Thompson et al., 1998). On the basis of these results, we focused our study on nfixa. Expression of nfixa was therefore quantified by qRT-PCR on cDNA obtained exclusively from tails (to exclude expression in the CNS) (Fig. 1F). nfixa expression is high during somitogenesis and at 48 hpf, but lower at 24 hpf and close to background after 48 hpf. Interestingly, this expression pattern correlates with the first and second myogenic waves that occur during somitogenesis and from 48 hpf (Barresi et al., 2001; Stellabotte et al., 2007).

**Nfixa loss of function in zebrafish leads to muscle disorganization and impairs fish motility**

To achieve insight into the role of nfix during zebrafish muscle development, we specifically knocked down the nfixa gene by injecting an antisense oligonucleotide morpholino (MO, Gene Tools) designed against the start site of the nfixa transcript (nfixa-MO). In all experiments, nfixa-MO-injected embryos were compared with embryos injected with the same amount of a non-specific control MO (ctrl-MO) at the same developmental stage.

To test the in vivo efficiency of nfixa-MO, the nfixa-GFP sensor plasmid was co-injected with nfixa-MO or ctrl-MO, respectively (Fig. 2). The presence/absence of the GFP was monitored under a fluorescent microscope from 24 to 48 hpf. Most (90% n=30) of the embryos injected with the sensor plasmid alone were positive for the GFP (Fig. 2A, A'). This percentage decreased to 25% (n=80) when the plasmid was co-injected with nfixa-MO, indicating that nfixa-MO specifically binds to its target region (Fig. 2B, B'). Notably, embryos injected with a splice-morpholino designed to exon 2-intron 2 junction (nfixa-MO2, 1 pmol/embryo), showed the same phenotype as the nfixa-MO. Nfix protein reduction in embryos injected with both AUG-nfixa-MO and splice-site nfixa-MO2 was also confirmed by western blot analysis (Fig. 2C, C'). Therefore, for all the following results, we used indistinguishably nfixa-MO or nfixa-MO2, and referred to both as nfixa-MO.

We observed that, up to 3 dpf, nfixa-MO-injected embryos showed normal somite number and size, as well as normal muscle formation and movements (data not shown). Moreover, nfixa knockdown did not affect any of the myogenic markers examined (myod and myog), suggesting that the segmentation process and the first myogenic wave take place properly (supplementary material Fig. S2). However, from 3 dpf, the majority of nfixa-MO-injected embryos (80%, n=150) appeared partially or completely immotile, failed to hatch from their chorion and to respond to external stimuli (touch response defects) (see supplementary material Movies 1, 2). All the nfixa-MO-injected embryos died at 6 dpf.

To exclude that motility impairment could be due to motoneuron defects, we sorted islet1-positive motoneurons from the transgenic line islet1-GFP (Higashijima et al., 2000) and verified by RT-PCR that nfixa is not expressed in motoneurons (supplementary material Fig. S3A). Moreover, islet1 expression was not changed at 48 hpf (supplementary material Fig. S3B, B') and axonal projections of primary and secondary motoneurons (visualized by znp1 and zn-5 antibodies, respectively) were correctly formed in nfixa-MO-injected embryos at 3 dpf (supplementary material Fig. S3C-D').

Moreover, to analyze neuromuscular junctions, we labeled acetylcholine receptor synapses with bungarotoxin (BTX, postsynaptic AChRs) and synaptic vesicle 2 with anti-SV2 synaptotagmin antibodies (Panzer et al., 2005). The co-staining of both signals in control and nfixa-MO injected embryos at 3 dpf showed mature neuromuscular synapses (supplementary material Fig. 3E-F'), thus providing evidence that the phenotype observed in the nfixa-MO injected embryos is specifically due to muscular defects.

It is known that morpholino molecules could elicit undesirable off-target effects such as activation of the p53 protein (Robu et al., 2007). Therefore, as an additional control we performed the coinjection of the nfixa- and the p53-MO that did not recover the defects in motility, sarcoplasmic reticulum development and the slow MyHC levels at 3 dpf, indicating that possible p53 activation is not responsible for the muscle defects due to nfixa loss of function (data not shown).

To further validate a cause-effect relationship between lack of nfixa and the subsequent phenotype in the nfixa-MO-injected embryos, we performed phenotypic/functional rescue experiments. We have previously described that, in mouse, Mef proteins participate in Nfix activity (Messina et al., 2010). Therefore, to rescue the nfixa-MO phenotype we co-injected mouse Nfix2 (one of the splicing isoforms of Nfix), together with its co-factors Mef2a...
and Mef2c mRNAs (Messina et al., 2010). We observed that only the co-injection of Nfix2 with Mef2a/Mef2c rescues the immotile phenotype in 35% of the injected embryos (n=70), whereas the injection of the single Nfix2 transcript did not, recapitulating the murine mechanism (see supplementary material Movie 3). A rescue of the phenotype was also obtained by injecting the zebrafish nfixa mRNA (data not shown).

To obtain a detailed insight of the immotile phenotype, we made histological semi-thin sections of control and nfixa-MO-injected embryos but no evident morphological changes were observed at 48 hpf (data not shown). However, sections from 3 dpf nfixa-MO-injected embryos showed disorganized, centrally nucleated fibers, which are characteristic of an immature hypertrophic state (Fig. 3A,B). In addition, myofibers of 3 dpf nfixa-MO-injected embryos appeared less dense than control in transmission electron microscopy (TEM): in a given area of 0.4 μm², 46±5 fibers were present in control and only 24±4 fibers in nfixa-MO (n=10 different fields scored in three different control and treated embryos, Fig. 3D,E). Notably, fibers of nfixa-MO injected embryos showed an extremely reduced, close to absent, sarcoplasmic reticulum (SR) between the sarcomeres (Fig. 3G,H), thus explaining the impairment to move and swim. As specific marker of SR, we looked at the levels of the calcium-channel ryanodin receptors (RyRs) in control and nfixa-MO-injected embryos. Immunofluorescence (Fig. 3J-K) and western blot analyses (Fig. 3M) showed a strong inhibition of RyRs following nfixa loss of function. In addition, in Nfix2-Mef2a/Mef2c rescued embryos, fiber organization and the levels of RyRs were partially recovered (Fig. 3C,F,I,L,M). The absence of SR was not documented before in the muscle-specific skeletal muscle Nfix-null mouse ( Messina et al., 2010). For this reason, we investigated more thoroughly the SR phenotype both in mouse fetal E16.5 and in postnatal P3 developmental stages. In mouse, the SR is present (supplementary material Fig. S4A-D) and levels of RyRs are comparable between wild-type and Nfix-null E16.5 fetuses and postnatal P3 mice (supplementary material Fig. S4C-E”). Therefore, the absence of the SR appears as a specific feature for zebrafish nfixa loss-of-function.

**In the absence of nfixa, primary slow MyHC levels remain elevated and the second myogenic wave is impaired**

We have previously demonstrated that mis-expression of Nfix in mouse causes an altered expression of slow MyHC: both transcript and protein levels decrease when Nfix is overexpressed, while the increase when Nfix is inhibited (Messina et al., 2010).

As in zebrafish smyhc1 is a marker of primary slow fibers (Elworthy et al., 2008; Henry and Amacher, 2004), we compared its expression during development in control and nfixa-MO injected fish. By qRT-PCR and whole-mount in situ hybridizations analyses, we observed that in controls the physiological expression of smyhc1 peaks at 48 hpf, at the onset of the second myogenic wave, and decreases after 48 hpf. By contrast, the level of smyhc1 in nfixa-MO injected embryos remains elevated even after 48 hpf (Fig. 4A-C). Moreover, by using F59 antibody (which recognizes the primary slow fibers (Devoto et al., 1996), we observed that not only the transcript of primary slow myosin, but also slow MyHC protein levels are still high in the nfixa-MO-injected embryos (Fig. 4D-F). Interestingly, in rescued larvae the levels of F59 slow myosin decreases to levels comparable with control siblings (Fig. 4D). To confirm that this effect is specific for slow myosin and is not dependent on a developmental delay after nfixa loss of function, we checked that vessel formation was comparable in control and nfixa-MO fltl-GFP transgenic injected embryos (Thompson et al., 1998). As shown in supplementary material Fig. S5, even though the morphology of nfixa morphants is altered, the vascular tree is correctly formed and does not show developmental delay.

It has previously been demonstrated that NFAT family members regulate the transcription of slow MyHC in adult muscle (Calabria et al., 2009) and that the transcription of the NFATc4 isoform is dramatically decreased after Nfix inhibition in murine fetal.
myoblasts (Messina et al., 2010). The nfatc4 ortholog is present in zebrafish (ENSDARG00000054162 chromosome 2: 37,496,488-37,524,447), and, by qRT-PCR, we verified that its level decreased during the second myogenic waves, suggesting a possible nfatc4-mediated regulation of primary slow MyHC in fish as well as in amniotes (Fig. 5A). We therefore tested whether nfixa could repress slow MyHC expression by inhibiting the transcription of nfatc4; as shown in Fig. 5A, the level of nfatc4 remains elevated in nfixa-MO injected embryos.

To confirm that nfatc4 is negatively regulated by nfixa and that, in the absence of nfixa, nfatc4 allows the persistent expression of genes involved in primary slow fiber differentiation, we designed a splice-nfatc4-MO to inactivate zebrafish nfatc4 function. We validate the splice-nfatc4-MO by RT-PCR, showing the skipping of the second exon (150 bp) in the nfatc4-MO injected embryos at 24 hpf. RT-PCR primers were designed in exon 1 and exon 3, respectively; the amplification product, which contains the second exon, was 326 bp in control embryos, whereas two bands at 326 bp and 174 bp, respectively, were detected in splice-nfatc4-MO injected embryos, confirming the partial skipping of the second exon (Fig. 5B).

We co-injected the nfixa and the nfatc4 morpholinos and observed a reduction of the primary slow fibers by F59 immunohistochemistry.

Fig. 4. Slow myosin remains expressed in nfixa-MO injected embryos after 48 hpf. (A) Quantitative real-time PCR (qRT-PCR) of smyhc1 mRNA expression normalized to ef1a. The expression of smyhc1 in control embryos presents a peak at 48 hpf and is then downregulated at 3 and 5 dpf. In nfixa-MO-injected embryos, the expression of smyhc1 remains elevated even after 48 hpf. (B–C) Whole-mount in situ hybridization with smyhc1 probe replicates qRT-PCR results: at 3 dpf, nfixa-MO-injected embryos (C,C') still present a stronger signal of smyhc1 than controls (B,B'). B' and C' are transverse sections of the tail. (D) Western blot: slow myosins are recognized by F59 antibody; all sarcomeric myosins are recognized by MF20 antibody. At 3 and 5 dpf in nfixa-MO injected embryos, slow and all myosin protein levels are higher than the control, whereas protein levels were recovered in rescued embryos at 3 dpf. Additional lower molecular weight bands in the 3 dpf nfixa-MO MyHC lane correspond to degradation products of the protein. (E–F') Immunostaining with F59 antibody (slow fibers): at 3 dpf, nfixa-MO-injected embryos (F,F') present a stronger F59 signal than the control (E,E'). Insets in E' and F' show a higher magnification of the tail region. In all figures, lateral views are shown, anterior is always towards the left. Scale bars: 100 μm in B,B',C,C',E,F; 200 μm in E',F'.

Fig. 5. Evolutionarily conserved inhibition of nfatc4 by nfixa and regulation of primary slow fibers. (A) Quantitative real-time PCR (qRT-PCR) of nfatc4 mRNA expression normalized to ef1a. The expression of nfatc4 remains elevated in 48 hpf, 3 and 5 dpf nfixa-MO injected embryos, compared with controls. (B) RT-PCR on control and splice-nfatc4-MO-injected embryos at 24 hpf. RT-PCR primers were designed in exon 1 and exon 3, respectively; the amplification product, which contains the second exon, was 326 bp in control embryos, whereas two bands at 326 bp (arrowhead) and 174 bp (arrow) were detected in splice-nfatc4-MO injected embryos, confirming the partial skipping of the second exon (150 bp). (C–E) Immunostaining with F59 antibody (slow fibers); at 3 dpf, nfixa-nfatc4-MO injected embryos (E) present a F59 signal comparable with controls (C), whereas F59 remains elevated in nfixa-MO-injected embryos (D).
Similar results were obtained by blocking Calcineurin/Nfatc4 signaling with cyclosporine A (CsA) treatment (Chang and Mun, 2004) in nfixa-MO-injected embryos (data not shown).

These data unequivocally demonstrate an evolutionarily conserved role of nfixa in primary slow fiber regulation through a mechanism that involved the Mef2a-Mef2c machinery and that negatively regulated the nfatc4 expression.

The Nfix-deficient mouse presents a block in fetal myogenesis that leads to a persistence of slow embryonic fibers. We therefore wondered whether, as in mammals, new secondary fiber development does not occur in fish because primary slow MyHC fibers remain predominant. First, we analyzed the rate of proliferation in muscles at 3 dpf: in comparison with control (Fig. 6A), nfixa-MO-injected larvae presented an increased number of proliferating H3P muscle cells (Fig. 6B), whereas rescued larvae presented a number of H3P-positive cells that was comparable with controls (Fig. 6C). Then, to check specifically secondary fiber formation, we looked at the dorsal and ventral extremities of the superficial muscle monolayer, as described by Barresi and colleagues (Barresi et al., 2001). As clearly shown by histological semi-thin sections (Fig. 6D,E) and also confirmed by TEM analysis (data not shown) and by confocal images of all sarcomeric MyHCs expression (Fig. 6G-I), these secondary fibers do not form in nfixa-MO-injected embryos at 3 dpf, whereas they are present in rescued embryos. Moreover, we injected embryos with nfixa-MO and treated them with cyclopamine, which inhibits Hh signaling and blocks primary slow fiber formation (Biressi et al., 2008; Chen et al., 2002). Cyclopamine-treated control embryos normally developed secondary slow muscle fibers at the dorsal and ventral extremes of the myotome, whereas in nfixa-MO-injected embryos the secondary fibers were not formed. This phenotype was partially rescued by the injection of the murine Nfix2 transcript (Fig. 6J-L). Several lines of evidence present Pax7 as an expression marker of muscle progenitor cells that generate fibers during a secondary period of zebrafish larval muscle growth (Hollway et al., 2007; Seger et al., 2011). We thus investigated whether in nfixa-MO-injected embryos secondary fibers do not form owing to the absence of Pax7-positive-progenitor cells. Immunofluorescence (Fig. 7A-F) and western blot analyses (Fig. 7G) performed on protein extracted presented an increased number of proliferating H3P muscle cells (Fig. 6B), whereas rescued larvae presented a number of H3P-positive cells that was comparable with controls (Fig. 6C). Then, to check specifically secondary fiber formation, we looked at the dorsal and ventral extremities of the superficial muscle monolayer, as described by Barresi and colleagues (Barresi et al., 2001). As clearly shown by histological semi-thin sections (Fig. 6D,E) and also confirmed by TEM analysis (data not shown) and by confocal images of all sarcomeric MyHCs expression (Fig. 6G-I), these secondary fibers do not form in nfixa-MO-injected embryos at 3 dpf, whereas they are present in rescued embryos. Moreover, we injected embryos with nfixa-MO and treated them with cyclopamine, which inhibits Hh signaling and blocks primary slow fiber formation (Biressi et al., 2008; Chen et al., 2002). Cyclopamine-treated control embryos normally developed secondary slow muscle fibers at the dorsal and ventral extremes of the myotome, whereas in nfixa-MO-injected embryos the secondary fibers were not formed. This phenotype was partially rescued by the injection of the murine Nfix2 transcript (Fig. 6J-L). Several lines of evidence present Pax7 as an expression marker of muscle progenitor cells that generate fibers during a secondary period of zebrafish larval muscle growth (Hollway et al., 2007; Seger et al., 2011). We thus investigated whether in nfixa-MO-injected embryos secondary fibers do not form owing to the absence of Pax7-positive-progenitor cells. Immunofluorescence (Fig. 7A-F) and western blot analyses (Fig. 7G) performed on protein extracted

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**Fig. 6. Secondary slow fibers do not form in nfixa-MO-injected embryos.** (A-C) Immunohistochemistry experiments with the H3P antibody that recognizes proliferating cells. nfixa-MO-injected larvae (B) presented an increased rating of proliferation at 3 dpf in comparison with controls (A) and rescued larvae (C). (D-I) Secondary fibers at the dorsal and ventral extremities of the superficial muscle monolayer did not form in 3 dpf nfixa-MO-injected embryos (E, H, asterisks) in comparison with controls (D, G, arrows and arrowheads), whereas they are recovered in rescued embryos (F, I, arrows and arrowheads). (D-F) Histological semi-thin sections, inset shows higher magnification. (G-I) Confocal images of MF20 immunostaining. (J-L) Lateral views of F59 labeling, which is maintained in the extreme dorsal and ventral regions of embryos treated with cyclopamine (arrows) but absent in nfixa-MO-injected embryos (K, asterisks). (L) In rescued embryos treated with cyclopamine, the formation of secondary fibers is partially recovered in small areas (arrows). (A-F) Transverse sections, dorsal on top. (G-L) Lateral views, anterior is always towards the left. Scale bar: 100 μm.

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**Fig. 7. The absence of Pax7-positive progenitor cells in nfixa-MO-injected embryos determines the loss of secondary fibers.** (A-F) Immunohistochemistry experiments with the Pax7 antibody that recognizes secondary fiber precursors. Pax7-positive secondary fiber progenitors at the dorsal and ventral extremities of the superficial muscle monolayer did not form in 48 hpf and 3 dpf nfixa-MO-injected embryos (B, E, arrow) in comparison with controls (A, D, arrow), whereas they are recovered in rescued embryos (C, F, arrowhead). (G) Western blot: secondary fiber precursors were recognized by Pax7 antibody; all sarcomeric myosins MyHC were recognized by MF20 antibody. In isolated tails of nfixa-MO-injected larvae, Pax7 protein levels were higher than the control whereas they were recovered in rescued embryos at 3 dpf. All myosin protein levels were higher in nfixa-MO-injected larvae, as expected. (A-C) 48 hpf transversal sections, dorsal on top. (D-F) 3 dpf longitudinal sections, anterior towards the left. Scale bar: 100 μm.
from the tails (to exclude the CNS component) showed a strong
downregulation of Pax7 following nfixa loss of function at 3 dpf. In
addition, in rescued embryos, Pax7-positive secondary progenitors
fibers in the lateral surface of the myotome and Pax7 protein levels
in the trunk were partially recovered (Fig. 7A-F).

Together, these data show the existence of a functional conservation
of the role of nfixa in the regulation of the second myogenic wave: its
absence determines persistent expression of primary slow fibers and
prevents the switch towards the second myogenic wave owing to the
absence of the Pax7-positive precursor cells.

DISCUSSION
The four nuclear factor one (NFI) genes Nfixa, Nfixb, Nfic and Nfix are
highly conserved in vertebrates and are expressed in different tissues
both in the embryo and in the adult. In mouse, specific knockdown of
each gene demonstrated their essential role for the proper
development of the embryo (Nasevicius and Ekker, 2000; Steele-
Perkins et al., 2003; Steele-Perkins et al., 2005). In particular, the Nfix
gene is involved in the development of brain, spinal cord and skeletal
muscle, in which it regulates the transition from embryonic to fetal
gene expression, a role with a profound evolutionary significance in
amniotes (Campbell et al., 2008; Driller et al., 2007; Messina et al.,
2010). Fetuses that lack Nfix in skeletal muscle are smaller and have
fibers with a reduced diameter, whereas embryos prematurely
expressing the isoform Nfix2 are larger, reflecting more robust
transcription of fetal muscle genes (Messina et al., 2010). In particular,
we demonstrated that Nfix represses the expression of slow myosin
heavy chains, which are responsible for slow twitching contractile
activity. Although the mechanisms that regulate the secondary fiber
formation in zebrafish have been poorly characterized until now, our
data shed light on conserved and divergent function of Nfix in this
process, leading to the hypothesis of the conserved role for this gene
may reflect the existence of a parallelism between the two myogenic
waves in zebrafish and mouse.

In zebrafish, there are two ortholog of Nfix that we named nfixa
and nfixb; however, only nfixa is expressed in embryonic muscles.
The loss-of-function approach led us to identify a defined role for
nfixa during muscle development. Until 48 hpf, nfixa loss-of-
function embryos are indistinguishable from control siblings and
proper expression of myoblast-specific transcription factors myod
and myog confirms that commitment and myogenic differentiation
of somitic cells is not affected. However, after 48 hpf, embryos
show reduced motility and larvae do not survive to adulthood. These
phenotypes are consistent with defects in skeletal muscle, as already
shown in several mutants with reduced touch response or motility
(Granato et al., 1996). Indeed, when nfixa is downregulated, muscle
structure is altered and not properly organized, as in the Nfix-null
fetus (Messina et al., 2010). As nfixa is also expressed in the CNS
by 3 days post-fertilization, we verified that motoneurons, their
axonal projections and neuromuscular junctions were correctly
formed in nfixa-MO-injected embryos, excluding any defects
resulting from to alterations in motoneuron development.

To our surprise, the sarcoplasmic reticulum is reduced or absent
in nfixa-MO-injected zebrafish embryos, thus explaining defects of
contraction and motility. This Nfix function appears not to be
conserved between fish and mammals, as in the fetus and newborn
(P3) skeletal muscle Nfix-null mice the sarcoplasmic reticulum
developed properly. This suggests that development in a protected
environment relieves the evolutionary pressure in amniotes to
develop the early motility that, in fish, is essential for escaping from
predators. Obviously complex functions such as locomotion are
likely to depend on numerous genes and their biochemical pathways
related to muscle energy metabolism and calcium handling. On the
basis of these considerations, we can speculate that zebrafish have
developed different strategies from mammals for sarcoplasmic
reticulum development.

Nevertheless, we demonstrate that the role of the zebrafish nfixa in
regulating the proper switch towards the secondary myogenic wave
is conserved during vertebrate evolution: its absence determines the
persistent expression of primary slow MyHC when, naturally, their
level should decrease. According to these data, we also observed an
increased proliferation of primary muscle cells in nfixa-MO injected
larvae at 3 dpf when normally in wild-type fish the significant slow
fibers deposition occurs before 3 dpf (Barresi et al., 2001). This
evolutionary Nfix conserved mechanism is also supported by
evidence that indicates, as in mouse, the involvement of MeF2 and
Nfatc4. In fact, we have demonstrated that, in zebrafish, Nfix2 acts
only with its co-factors MeF2a and MeF2c, as the rescue happens only
with the co-injection of all the transcripts together. Moreover as in
mouse, nfixa negatively modulates the expression of nfatc4 which, in
turn, is responsible for the physiological expression of primary slow
MyHC. In nfixa loss-of-function larvae, nfatc4 transcript levels are
increased, leading to a persistent expression of primary slow MyHC,
whereas the downregulation of nfatc4 restore normal primary slow
MyHC levels in the nfixa-MO injected zebrafish embryos.

Moreover, we observed that the persistent expression of slow-
MyHC in primary fibers following nfixa loss-of-function, causes
the absence of the second myogenic wave. But at variance with mouse,
we observed that the Pax7-positive precursors of secondary
fibers at the external layers of the myotome are absent in nfixa-MO
larvae at 3 dpf. These pax7-positive cells in zebrafish are similar to
those found in amniotes, and participate in hypertrophic growth
during larval stages, as well as in muscle repair after injury or in
dystrophic conditions (Seger et al., 2011). In our previous work, we
demonstrated that the expression of the murine Pax7 does not depend
on Nfix but rather is required for Nfix expression at the onset of the
fetal myogenesis (Messina et al., 2010). The results we obtained in
zebrafish might be explained with a feedback loop exerted by nfixa on pax7 expression; alternatively, the action of
nfixa on pax7 expression might be indirect. Further experiments
will be needed to elucidated this pathway.

Although an embryonic and fetal myogenesis in zebrafish have not
been yet described in great detail, our experimental evidence provides
a clue for comprehending the mechanisms that regulate this process.
Previous work from several laboratories demonstrated the existence
of mechanisms that specifically regulate the development of slow
muscle in the early embryo. For example, the specification of some
secondary slow fibers is independent of Hh signaling, which is rather
essential for the development of primary slow fibers (Buckingham,
2007; Devoto et al., 1996). The nfixa involvement in the second
myogenic wave correlates well with its expression peak at 48 hpf,
because, during somitogenesis, nfixa knockdown does not alter the
initial formation of slow muscle precursors in zebrafish embryos.
Interestingly, the loss of function of other genes expressed during
somitogenesis, such as smyhc1, also produces a phenotype only
during the second myogenic wave (Codina et al., 2010).

In conclusion, our data suggest that, at least for this molecular
pathway, the second myogenic wave in fish may correspond to fetal
myogenesis in mammals through a mechanism that is mainly
conserved. Indeed, we clearly demonstrate that the role of Nfix in
driving the second myogenic wave and in the regulation of primary
slow myosin embryonic fibers is strongly conserved during evolution.
Unfortunately, our knowledge of morphological and biochemical
differences between the primary and secondary myogenesis in fish is
still modest when compared with what already known in mammals. This prevents us from achieving an in-depth understanding of the similarities and differences between these myogenic waves. Further work will hopefully address this issue in the future.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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