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Defective cranial skeletal development, larval lethality and haploinsufficiency in Myod mutant zebrafish

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ABSTRACT

Myogenic regulatory factors of the *myod* family (MRFs) are transcription factors essential for mammalian skeletal myogenesis. Here we show that a mutation in the zebrafish *myod* gene delays and reduces early somitic and pectoral fin myogenesis, reduces miR-206 expression, and leads to a persistent reduction in somite size until at least the independent feeding stage. A mutation in *myog*, encoding a second MRF, has little obvious phenotype at early stages, but exacerbates the loss of somitic muscle caused by lack of Myod. Mutation of both *myod* and *myf5* ablates all skeletal muscle. Haploinsufficiency of *myod* leads to reduced embryonic somite muscle bulk. Lack of Myod causes a severe reduction in cranial musculature, ablating most muscles including the protractor pectoralis, a putative cucullaris homologue. This phenotype is accompanied by a severe dysmorphology of the cartilaginous skeleton and failure of maturation of several cranial bones, including the opercle. As *myod* expression is restricted to myogenic cells, the data show that myogenesis is essential for proper skeletogenesis in the head.

Introduction

As its name implies, skeletal muscle develops and functions in intimate contact with the skeleton. It is clear that cartilage and bone can develop in the absence of skeletal muscle (Chevallier, 1979; Christ et al., 1977; Kardon, 1998). However, abundant evidence from sports science and exercise physiology shows that there is mutual dependency of muscular and skeletal maintenance and growth (Robling, 2009). Indeed, skeletal defects have been observed early in development of mice genetically modified to lack skeletal muscle. For example, mice lacking the key myogenic regulatory transcription factors (MRFs) not only lack certain early muscle fibres but also show skeletal defects (Hasty et al., 1993; Rawls et al., 1998; Rudnicki et al., 1992; Valdez et al., 2000; Venuti et al., 1995; Zhang et al., 1995). As these MRF genes are expressed only in the skeletal muscle lineage, it has been suggested that skeletal defects arise due to altered signalling between early muscle cells and skeletal precursors (Vinagre et al., 2010; Vivian et al., 2000). Additionally, alteration of expression of adjacent genes caused by the genomic manipulations may have a role, as new knockout alleles have less severe skeletal consequences (Kassar-Duchossoy et al., 2004; Kaul et al., 2000). To compare musculoskeletal developmental interactions and the roles of individual MRFs across the vertebrates, we turned to the zebrafish, in which point mutations and/or antisense morpholinos minimise genetic complications.

Like mice, zebrafish have four MRF family members: Myod, Myf5, Myogenin and Mrf4/Myf6. Previously, we analysed *myf5* and *myf6* mutants and found rather mild defects, less pronounced at early stages than those in mice lacking these MRFs individually (Hinits et al., 2009; Kassar-Duchossoy et al., 2004; Kaul et al., 2000; Zhang et al., 1995). Mice lacking Myod also have a surprisingly mild phenotype (Rudnicki et al., 1992). In contrast, we observed severe defects in somitic and cranial myogenesis in *myod* morphant embryos (Hinits

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et al., 2009). These differences raise the possibility that the functions of *myf5* and *myod* have diverged during vertebrate evolution. On the other hand, the phenotype of double *myf5;myod* loss of function appears quite similar in mice and fish; most, but not all, early myogenesis is ablated (Hinits et al., 2009; Kassar-Duchossoy et al., 2004). Thus, the extent of divergence in role of MRF genes is unclear.

Here we report an analysis of *myod* and *myog* mutant alleles which reveals that zebrafish Myod is essential for viability. Myod is required for most early cranial myogenesis and some early pectoral fin and somitic myogenesis. Mutants lack the ability to feed and die during the post-hatch larval period. Myotome size is reduced in *myod* mutants, prior to independent feeding. There is also a transient reduction in somite bulk in *myod* heterozygotes. Unlike mice, *myf5; myod* double mutant zebrafish lack all myogenesis at least until 5 days post fertilisation (dpf). Most strikingly, the lack of cranial muscle in *myod* mutants leads to a severe deformation and failure of maturation of cranial skeletogenesis. The data indicate that early myogenesis is essential for normal head patterning.

Materials and methods

Zebrafish lines and maintenance

Wild type and transgenic lines *Tg(acta1:GFP)zf13* (Higashijima et al., 1997), *Tg(−2.2mylz2:GFP)i135* (Moore et al., 2007), *Tg(βactin:HRAS-EGFP)vu119* (Cooper et al., 2005), and *myf5*^{hu2022} (Hinits et al., 2009) were maintained on King's wild type background and staging and husbandry were as described (Westerfield, 1995). *Myod*^{fh261} and myog^{fh265} mutant alleles were identified by TILLING (Draper et al., 2004) and were maintained on the *AB background and genotyped by sequencing of PCR products amplified from fin clip or embryo genomic DNA using primers 5′GGACCCCAGGCTTGTTC3′ and 5′GTTGGATCTCGGACTGGA3′ for *myod*, 5′AACCGGGCCATTGTCTCCA3′ and 5′CATCGGCAGGCTGTAG-TAGTTCTC3′ for *myf5* and 5′TGACAGCTTTACCATCGCGCTTGA3′ and 5′ GTCAGTTCACTCAACCAGCAGGAGCATGA**C**3′ for *myog* (the last contains a single base mutation for the dCAPS method, http://labs.fhcrc.org/moens/Tilling_Mutants/index.html).

In situ mRNA hybridisation, immunohistochemistry and histology

In situ mRNA hybridisation and immunohistochemistry were performed as described (Hinits and Hughes, 2007). Fluorescein- or digoxigenin-tagged probes used were *myog* (Weinberg et al., 1996), *eng2a* (Ekker et al., 1992), *mylz2* and *myhz1* (Xu et al., 2000), *smyhc1* (Bryson-Richardson et al., 2005), actin (*acta1b*, IMAGE 7284336), *dlx2a* (Akimenko et al., 1994) and *klf2b* (Oates et al., 2001). Dual-digoxigenin-labelled miR-206 specific LNA probes were obtained from Exiqon and hybridised and washed at 50 °C as described in (Sweetman et al., 2008). Antibodies were against Myod and Pax3/7 (Hammond et al., 2007), Myog (Hinits et al., 2009), striated muscle myosin heavy chain (MyHC; A4.1025 (Blagden et al., 1997) or MF20 (DHSB)), slow MyHC (F59; Devoto et al., 1996) and smooth muscle myosin (Myh11, Biomedical Technologies). Staining with alcian blue for cartilage and alizarin red for ossified bone was as described (Walker and Kimmel, 2007), followed by dissection to separate upper and lower head skeleton. 4,5-diaminofluorescein diacetate (DAF-2DA, Santa Cruz) staining of bone was as described (Grimes et al., 2006).

Embryo manipulation

Myod MO (Gene-Tools, 5′-ATATCCGACAACTCCATCTTTTTTG-3′) was injected into 1–2 cell stage embryos at 4 ng/embryo. Cyclopamine (100 μM in fish medium) or vehicle control was added at 50% epiboly to embryos whose chorions had been punctured with a 30 G hypodermic needle.

Muscle size analysis

Tg(βactin:HRAS-EGFP)vu119 fish were bred onto *myod*^{fh261} and live 0.2 mM 1-phenyl-2-thiourea-treated embryos hemizygous for ßactin: GFP were embedded in 1.5% low melting point agarose in fish water containing MS222, and imaged by confocal microscopy on a Zeiss Exciter using a 20×1.0 W dipping objective on successive days. Fish were released from the agarose each day. A single lateral image and three equi-spaced transverse images within somite 17 were collected from each embryo at each stage. The somite was outlined in Zen software and an average transverse area was calculated. This area was multiplied by the somite length, measured at the horizontal myoseptum, to yield an estimate of somite volume for each fish at each stage. After analysis, individual embryos were genotyped by sequencing of genomic PCR. Data are presented as mean ± s.e.m. with the number of embryos at each developmental stage indicated. Somite volume of fixed MF20-stained 72 hpf embryos was calculated similarly, and genotyped as mutants or sibs according to head muscle immunostaining. Data was analysed by two way ANOVA showing significant effect of both age and genotype using Graphpad Prism 4.0. Adult (15 months) fish from a single tank were anaesthetised, weighed and length measured, then tail-clipped for genotyping by sequencing of genomic PCR.

Results

Myod mutation delays slow and reduces fast myogenesis

TILLING (Draper et al., 2004) was used to isolate a zebrafish mutant in *myod* with a stop codon at residue 126 early in the second helix, thereby generating a predicted null mutation (Fig. 1A; Davis et al., 1990; Ma et al., 1994). One quarter of embryos from a carrier in-cross of *myod*$^{fh261/+}$ lacked Myod immunoreactivity. DNA sequencing of individual stained embryos confirmed that those lacking Myod were mutants and that heterozygotes had less staining than wild types (Fig. 1B; quantification of all experiments is presented in Table S1). *Myod* mRNA was reduced in heterozygotes and greatly diminished in mutants at 10 somite stage (10s), suggesting nonsense-mediated decay of the mutant mRNA (Fig. 1C). Consistent with the result of morpholino (MO) knockdown of Myod, slow myogenesis was delayed in mutants (Fig. 1B) (Hinits et al., 2009). In adaxial precursors of superficial slow fibres, *myog* mRNA and other muscle differentiation markers examined were expressed, although delayed (Fig. 1D). However, *eng2a*, a marker of the specialised muscle pioneer cells, was diminished (Fig. 1E). Thus, this mutant confirms that Myod contributes to, but is not essential for, early slow myogenesis (Hinits et al., 2009).

Loss of Myod caused more dramatic defects in fast myogenesis in the somite. Early *myog* and later *mylz2* and *myhz1* myosin mRNAs were diminished in fast muscle (Fig. 1D,F). Conversely, Pax3/7, a marker of dermomyotome was up-regulated in the lateral somite at 24 hpf, with more Pax3/7-immunoreactive nuclei in sections of mutants (Fig. 1F). In summary, fast myogenesis was reduced in *myod*^{fh261} mutants, extending results from MO knockdown studies (Hammond et al., 2007; Hinits et al., 2009; Maves et al., 2007).

Myogenin cooperates with Myod in fast myogenesis

Myogenin is a Myod target gene. We previously suggested that Myogenin cooperates with Myod in somitic fast myogenesis (Hinits et al., 2009). We isolated a *myog*^{fh265} mutant by TILLING, which has a stop codon in place of residue 167, just downstream of the helix–loop–helix domain (Fig. 2A). As with *myod*, *myog* mRNA was reduced in heterozygotes and greatly diminished in mutants at 10s, suggesting nonsense-mediated decay of the mutant mRNA (Fig. 2B). Although Myog immunoreactivity was lost in mutants, no defect was observed either in in-crosses from heterozygous *myog*$^{fh265/+}$ carrier fish or in genotyped *myog*$^{fh265/+}$ mutants: pectoral fin, jaw and somite morphology and