Development 138, 4399-4404 (2011) doi:10.1242/dev.070516

Prdm1a and miR-499 act sequentially to restrict Sox6 activity to the fast-twitch muscle lineage in the zebrafish embryo

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SUMMARY

Sox6 has been proposed to play a conserved role in vertebrate skeletal muscle fibre type specification. In zebrafish, *sox6* transcription is repressed in slow-twitch progenitors by the Prdm1a transcription factor. Here we identify *sox6* cis-regulatory sequences that drive fast-twitch-specific expression in a Prdm1a-dependent manner. We show that *sox6* transcription subsequently becomes derepressed in slow-twitch fibres, whereas Sox6 protein remains restricted to fast-twitch fibres. We find that translational repression of *sox6* is mediated by miR-499, the slow-twitch-specific expression of which is in turn controlled by Prdm1a, forming a regulatory loop that initiates and maintains the slow-twitch muscle lineage.

KEY WORDS: Sox6, Translational control, miR-499 (*mir499*), Muscle fibre type, Zebrafish, Prdm1

INTRODUCTION

During skeletal myogenesis in vertebrates, mesodermally derived muscle progenitor cells give rise to myoblasts that in turn differentiate into either slow-twitch or fast-twitch muscle fibres. In zebrafish embryos, slow-twitch fibres derive from paraxial mesodermal cells that lie immediately adjacent to the notochord, the so-called adaxial cells (Devoto et al., 1996; Blagden et al., 1997). The slow-twitch differentiation pathway is initiated in adaxial cells by the transcription factor Prdm1a (also known as Blimp1), expression of which is activated by notochord-derived Sonic hedgehog (Shh) protein (Roy et al., 2001; Baxendale et al., 2004). In the absence of Prdm1a activity, adaxial cells adventitiously activate transcription of fast-twitch-specific genes, such as those encoding the fast myosin heavy chains and fast troponin, as well as the transcription factor Sox6, the ectopic expression of which downregulates transcription of the slow-twitch-specific genes, including *slow myosin heavy chain 1* (*smyhc1*), *slow troponin* (*stnnc*; *tnnc1b* – Zebrafish Information Network) and *prox1* in *prdm1a* mutants (von Hofsten et al., 2008). Reciprocally, loss of Sox6 function in fast-twitch fibres results in ectopic expression of at least one slow-twitch-specific gene, *stnnc*, leading to the suggestion that Sox6 plays a key role in promoting fast-twitch-specific fate by repressing the slow-twitch differentiation pathway (von Hofsten et al., 2008).

A similar role was previously proposed for Sox6 during mammalian myogenesis, based on the excess of fibres with slow-twitch character that differentiate in *Sox6* mutant mice (Hagiwara et al., 2005; Hagiwara et al., 2007). Consistent with this, overexpression of Sox6 has been shown to repress transcription of the slow-twitch-specific β-MHC and troponin genes and to cause a concomitant increase in slow-twitch and decrease in fast-twitch muscle fibres in transgenic mice (van Rooij et al., 2009), and very recent studies have demonstrated that Sox6 binds to conserved cis-regulatory elements in slow-twitch fibre genes to represses their transcription in adult fast-twitch muscle (Quiat et al., 2011). Little is known about how the lineage-specific activity of Sox6 is regulated in mammals; however, a role for the microRNA miR-499 has been suggested, based upon the finding that the 3′UTRs of human, mouse and rat *Sox6* contain between 1 and 4 consensus recognition sites for the miR-499 SEED sequence and the demonstration that miR-499 reduces *Sox6* mRNA levels both in vitro and in vivo (McCarthy et al., 2009; van Rooij et al., 2009; Bell et al., 2010).

Here, through the in vivo analysis of *sox6* regulation using transgenic zebrafish carrying *sox6* reporter gene constructs, we identify 5′ cis-acting sequences that are sufficient to drive spatially restricted transcription in the fast-twitch muscle progenitors and 3′ cis-acting sequences that mediate translational repression in slow-twitch fibres. Our findings reveal a *prdm1a/mir499/sox6* gene regulatory network that underlies the establishment and maintenance of the slow-twitch muscle fibre lineage in response to Shh signalling.

MATERIALS AND METHODS

Zebrafish strains and husbandry

Adult fish were maintained on a 14-hour light/10-hour dark cycle at 28°C in the AVA (Singapore) certificated IMCB Zebrafish Facility. Previously described zebrafish strains used were *prdm1a*^{nrd} (Hernandez-Lagunas et al., 2005), *Tg(actin:GAL4)* (Scheer and Campos-Ortega, 1999), *Tg(PACprdm1:GFP)*^{i106} and *Tg(BACsmyhc1:gfp)*^{i108} (Elworthy et al., 2008).

Constructs and transgenics

The *sox6* transcription start site (TSS) was identified by 5′RACE (Ambion) following the manufacturer's protocol. Fragments of differing size were amplified by PCR from BAC DNA (BX537340.15) and cloned into a Gateway 5′ entry vector p5E-MCS (Kwan et al., 2007). Reporter constructs were generated by site-specific recombination with pME-EGFP or pME-mCherry, p3E-sv40 and pDestTol2pA. The 3 kb *sox6* 3′UTR was identified by 3′RACE, amplified from genomic DNA and cloned into the *sox6:EGFP tol2* vector replacing SV40 poly(A). Sequences identified in

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Accepted 10 August 2011

this analysis have been deposited in GenBank under accession numbers JN216841, JN216842. The *smyhc1:lyn-tdTomato*^{i261} construct utilised a 9.7 kb *smyhc1* promoter (Elworthy et al., 2008) to drive tdTomato fused to the minimal myristoyl-palmitoyl-membrane anchor sequence of Lyn kinase (Pyenta et al., 2001). To overexpress miR-499, its precursor (91 bp) was cloned into a *UAS:EGFPsv40* tol2 vector. All tol2 constructs were co-injected with *tol2* mRNA into one-cell stage embryos to produce transgenic lines or for transient expression analysis.

Site-directed mutagenesis

Mutation of cis-regulatory elements was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol, except that iProof DNA polymerase (BioRad) was utilised. The core sequence of the Prdm1a binding site GAAA was mutated into CTCT. The miR-499 SEED recognition site TCTTA in the *sox6* 3′UTR was changed to AAAAT.

Generation and purification of anti-Sox6 antibody

A short fragment of *sox6* cDNA 5′ to the HMG box was cloned into the His tag expression vector pET-21b. The encoded fusion protein was used to immunise rabbits by Absea-Antibody (Beijing). The resulting serum was affinity purified using the His-tagged Sox6 following standard protocols (Nishino et al., 1994).

In situ hybridisation and immunofluorescence

DIG-labelled miR-499 antisense locked nucleic acid (LNA) probe was purchased from Exiqon. Standard in situ hybridisation (ISH) was performed as described (Oxtoby and Jowett, 1993; Strähle et al., 1994). Fluorescence ISH was performed on sections using anti-DIG peroxidase and fluorescence substrate Cy5 tyramide signal amplification (TSA, Perkin Elmer) according to Brend and Holley (Brend and Holley, 2009). Antibody staining was performed as previously described (Elworthy et al., 2008) using mAb F59 (anti-myosin heavy chain 1; DSHB) 1:50; anti-zebrafish Sox6 1:500; and anti-EGFP (Abcam) 1:5000. Secondary antibodies comprised Rhodamine-coupled anti-mouse (Invitrogen) at 1:500 and FITC-coupled anti-rabbit (Invitrogen) at 1:1000. Images were acquired with an Olympus Fluoview confocal microscope using Olympus FV10-ASW software and analysed using ImageJ software (NIH).

Morpholino sequences and injections

MO1-myod1 (5′-ATATCCGACAACTCCATCTTTTTTG-3′) and MO1-myf5 (5′-TACGTCCATGATTGGTTTGGTGTTG-3′) were co-injected at 0.1 pm each per embryo (Lin et al., 2006). MO2-prdm1a (5′-TGGTGTCATACCTCTTTGGAGTCTG-3′) was injected at 0.2 pm per embryo (Baxendale et al., 2004). Morpholinos were supplied by Gene Tools (Philomath, OR, USA).

RESULTS AND DISCUSSION

Spatially regulated *sox6* transcription is controlled by myogenic regulatory factors and Prdm1a

Transcription of *sox6* in the zebrafish embryo is clearly detectable by in situ hybridisation (ISH) at the 10-somite stage in the lateral region of each somite, which is the location of fast-twitch muscle progenitors; however, no transcripts are detectable in the adaxial cells, which are the slow-twitch muscle progenitors (Fig. 1A). The levels of *sox6* transcript reach their peak in the somites 1 day post-fertilisation (dpf) and gradually diminish thereafter, although persisting at least until 6 dpf (see below). Transcripts are also detectable in the otic vesicle (Fig. 1E) and in the head muscle, pectoral fin, kidney and in specific regions of the brain and retina (see Fig. S1 in the supplementary material).

As previously shown, *sox6* transcription is derepressed in adaxial cells in the absence of Prdm1a function (von Hofsten et al., 2008). Ectopic transcripts are first readily detectable in adaxial cells at the 10-somite stage in embryos homozygous for the *prdm1a*^{nrd} null allele (Fig. 1B); at the same stage, expression of *stnnc* is markedly reduced (Fig. 1C,D) and continues to decline as embryogenesis proceeds, whereas the signal due to ectopic *sox6* expression persists and increases in intensity (not shown).

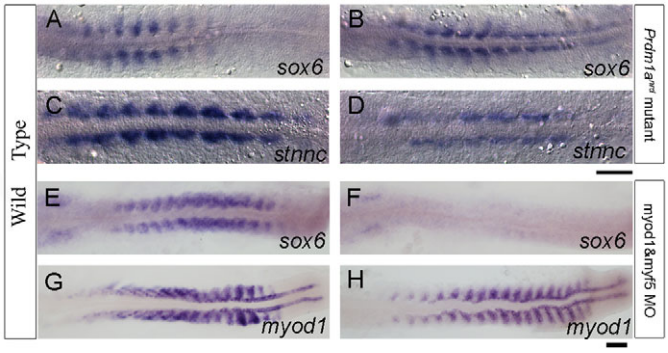

Fig. 1. Negative and positive regulation of *sox6* in the zebrafish myotome. (**A**,**B**) *sox6* expression is restricted to the fast muscle domain in a wild-type 10-somite stage embryo (A) but is ectopically expressed in adaxial cells in a *prdm1a*^{nrd} mutant at the same stage. (**C**,**D**) This derepression correlates with downregulation of *stnnc* expression in adaxial cells in the *prdm1a*^{nrd} mutant (D) relative to wild type (C). (**E**,**F**) *sox6* transcript persists in the fast-twitch muscle domain and also accumulates in the otic placode in the wild type at the 18-somite stage (E), whereas the *myod1;myf5* morphant shows loss of *sox6* expression specifically from the muscle progenitors. (**G**,**H**) Expression of *myod1* in wild-type (G) and *myod1;myf5* morphant (H) embryos. Scale bars: 50 μm.

The myogenic regulatory factors (MRFs) Myod1 and Myf5 are essential for the commitment of cells to the myogenic differentiation programme (reviewed by Pownall et al., 2002). Previous studies have shown that simultaneous morpholino-mediated knockdown of both genes results in the loss of myogenic gene expression in the somites (Hinits et al., 2009). Little, if any, *sox6* transcript was detected in the somites of *myod1;myf5* double-morphant embryos as compared with wild-type embryos (Fig. 1E,F); expression in the otic vesicle, which serves as a positive internal control, was unaffected. Morpholino-mediated knockdown of the Mef2 transcription factors (Hinits and Hughes, 2007), by contrast, had no effect on *sox6* transcription (see Fig. S2 in the supplementary material).

Identification of cis-acting *sox6* regulatory elements

To define the cis-acting sequences that mediate the spatiotemporal regulation of *sox6*, we first assayed the activity of sequences from the 5′ end of the gene in transgenic embryos. A 10.5 kb DNA fragment that includes 7.6 kb upstream of the transcription start site (TSS) and 2.9 kb extending downstream as far as the third exon in the 5′UTR region (see Fig. 2A) was cloned into an EGFPsv40 tol2 vector. Co-injection of this construct with *tol2* transposase mRNA resulted in strong transient expression of EGFP in the skeletal muscle fibres (see Fig. S1D in the supplementary material). Embryos from a stable line, *Tg(10.5 kb sox6:EGFPsv40)*^{i252}, showed expression specifically in the fast-twitch fibres of both trunk and head muscle, the intermediate cell mass, as well as in the otic vesicle (Fig. 2B, see Fig. S1E-G in the supplementary material).

Interspecific sequence alignments revealed that this fragment contains a number of regions conserved between teleosts (conserved non-coding elements or CNEs), some of which are also conserved in mammals (highly conserved non-coding

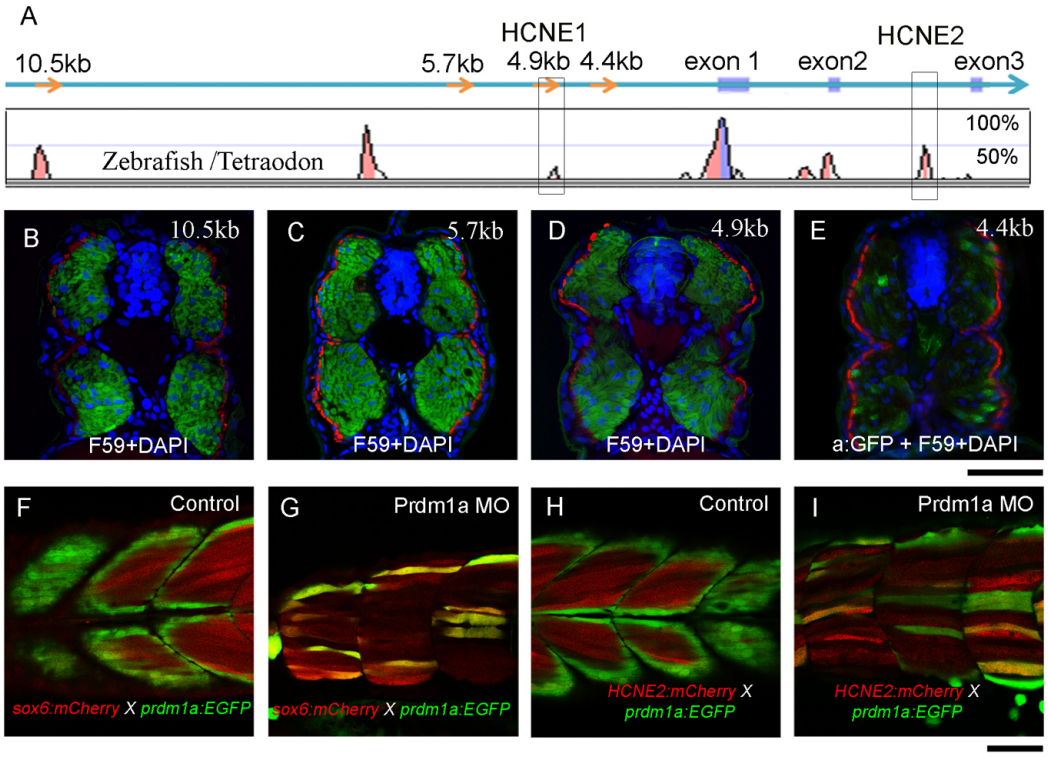

Fig. 2. Conserved cis-elements recapitulate the *sox6* expression pattern in the myotome. (**A**) Conserved non-coding elements (represented by peaks) upstream and downstream of the transcription start site revealed by alignment of the zebrafish and *Tetraodon sox6* genomic sequences. Several of these are also highly conserved in mammals (see Fig. S3 in the supplementary material), including HCNE1 and HCNE2 (red boxes). (**B-E**) Transverse sections through mid-trunk regions of 48 hpf embryos transgenic for *sox6* reporter genes; slow-twitch fibres are visualised with mAb F59 (red), nuclei by DAPI staining (blue). The 10.5 kb, 5.7 kb and 4.9 kb fragments drive bright and specific EGFP (green) expression exclusively in fast-twitch fibres; by contrast, the 4.4 kb fragment drives low-level mosaic expression of EGFP, detectable only in fixed specimens using anti-EGFP antibody (E). (**F-I**) Optical sagittal sections of 48 hpf *Tg(PACprdm1:GFP)*^{i106} embryos carrying the *sox6* 5.7 kb or HCNE2 mCherry reporter constructs. Both constructs are ectopically expressed (red) in fibres derived from adaxial cells (green) following injection of Prdm1a morpholino (G,I). Scale bars: 50 μm.

elements or HCNEs) (Fig. 2A, see Fig. S3 in the supplementary material). To test their functional significance, we generated deletion derivatives of the 10.5 kb fragment lacking two or three of the upstream CNEs and assayed their activities in stable transgenic lines. *Tg(5.7 kb sox6:EGFPsv40)*^{i253} embryos showed an essentially identical pattern of expression to that of *Tg(10.5 kb sox6:EGFPsv40)*^{i252}, indicating that the two most distal elements that are conserved between fish species are dispensable for expression in the myotome (Fig. 2C). *Tg(4.4 kb sox6:EGFPsv40)*^{i255} embryos, by contrast, showed much lower and highly mosaic EGFP expression, albeit restricted to fast-twitch fibres, as well as ectopic expression in the notochord (Fig. 2E), indicating a crucial requirement for the more proximal HCNE1, which is highly conserved between zebrafish and mammals. Addition of a 0.5 kb fragment of this HCNE, which includes the single Myod1 consensus binding site upstream of the TSS, to generate *Tg(4.9 kb sox6:EGFPsv40)*^{i254}, was sufficient to restore the wild-type expression pattern driven by the longer (10.5 and 5.7 kb) fragments (Fig. 2D).

To confirm that these *sox6* cis-regulatory elements respond to Prdm1a repression, we generated an additional line, *Tg(5.7 kb sox6:mCherrysv40)*^{i256}, in which the 5.7 kb fragment drives mCherry expression and crossed this to fish carrying the previously described *Tg(PACprdm1:GFP)*^{i106} line that drives GFP expression specifically in the slow-twitch lineage (Elworthy et al., 2008). As expected, embryos derived from this cross show mutually exclusive patterns of EGFP and mCherry expression in the myotome. Knockdown of Prdm1a activity by morpholino injection, however, resulted in ectopic mCherry expression in EGFP-expressing cells. Thus, transcription driven by the 5.7 kb fragment is repressed by Prdm1a. Notably, we identified nine consensus binding sites for Prdm1a within the 5.7 kb sequence. To test whether Prdm1a-dependent repression is mediated by these binding sites, lines carrying a modified 5.7 kb:EGFP reporter, in which all nine sites had been mutated by site-directed mutagenesis, were generated. Surprisingly, no difference in expression was seen between the wild-type and the mutated reporter constructs (data not shown). Thus, Prdm1a-mediated repression is independent of the consensus binding sites flanking the TSS, suggesting that it might be an indirect effect.

Highly conserved sequences within the second intron of *sox6* are sufficient for fast-twitch-specific transcription

The interspecies sequence alignment revealed a second, short (219 bp) HCNE within the second intron, the deletion of which abolishes the activity of the 5.7 kb:EGFP reporter in stable lines (not shown). To determine whether HCNE2 acts as a muscle enhancer, we cloned it into the EGFP vector with a β-*globin* minimal promoter. A stable transgenic line, *Tg(sox6*

HCNE2:mCherry)^{i257}, carrying this construct showed EGFP expression exclusively in the myotome, which unexpectedly was restricted to fast-twitch fibres (Fig. 2H). As with the *Tg(5.7 kb sox6:mCherry)*^{i256} reporter, *Tg(sox6HCNE2:mCherry)*^{i257} was ectopically expressed in slow muscle cells in *prdm1a* morphant embryos (Fig. 2I). Analysis of the HCNE sequence using MatInspector software (Cartharius et al., 2005) identified a single Prdm1a consensus binding sequence, as well as sites for various transcription factors including Mef2, Srf, Dmrt2 and Maf (see Fig. S4 in the supplementary material). Mutation of any of these sites resulted in complete loss of reporter gene expression in stable lines (data not shown).

Transcription of *sox6* is derepressed in slow-twitch fibres at postembryonic stages

Although expression of the *Tg(5.7 kb sox6:EGFPsv40)*^{i253} reporter gene is initially restricted to fast-twitch fibres (Fig. 3A), this restriction starts to break down by 4 dpf (Fig. 3B), and, by 6 dpf, most slow-twitch fibres are also EGFP positive (Fig. 3C). To confirm that this expression profile reflects that of the endogenous gene, we assayed *sox6* mRNA distribution in *Tg(BACsmyhc1:gfp)*^{i108} larvae using fluorescent ISH. At 1 dpf, the red *sox6* signal generated by tyramide Cy5 substrate was localised to fast-twitch fibre cells and excluded from the EGFP-expressing slow-twitch fibres (Fig. 3D). At 6 dpf, by contrast, *sox6* transcript could be detected in all the slow muscle cells (Fig. 3F). Despite this accumulation of endogenous *sox6* mRNA, no Sox6 protein could be detected in slow-twitch fibres, even by 6 dpf (Fig. 3E,G).

The absence of Sox6 protein implies that *sox6* mRNA is subject to post-transcriptional regulation in slow-twitch fibres. Previous studies have implicated *Mir499*, a microRNA gene residing in the nineteenth intron of *Myh7b*, in regulating *Sox6* transcript levels in mammalian myofibres, but not in direct repression of *Sox6* translation (McCarthy et al., 2009; van Rooij et al., 2009; Bell et al., 2010). MicroRNAs are known to regulate both mRNA stability and translation by targeting sequences in the 3′UTRs of mRNAs (Huntzinger and Izaurralde, 2011). Analysis of the *sox6* 3′UTR in the miRBase database (http://www.mirbase.org) identified two recognition sequences for miR-499; a further three potential target sites were identified by manual alignment with the miR-499 SEED sequence alone (see Fig. S5 in the supplementary material). None of these is a perfect match, implying that they might mediate translational repression rather than transcript degradation (Huntzinger and Izaurralde, 2011).

Repression of Sox6 activity in slow-twitch fibres is maintained by miR-499

To determine the functional significance of these miR-499 target sequences, we replaced the SV40 3′UTR sequences in *Tg(5.7 kb sox6:EGFPsv40)*^{i253} with the endogenous *sox6* 3′UTR and used this construct to generate the stable line *Tg(5.7 kb sox65.7:EGFPsox63′UTR)*^{i258}. As in the original transgenic line, expression of EGFP driven by this modified transgene was restricted to fast-twitch fibres from 48 hours post-fertilisation (hpf). In contrast to the original SV40 3′UTR line (Fig. 3H), however, expression remained restricted to the fast-twitch fibres in larvae after 4 dpf (Fig. 3I), consistent with the miR-499 sites mediating translational repression of the reporter. To confirm this, we mutated all five potential miR-499 target sites and used this construct to generate the stable line *Tg(5.7 kb sox6:EGFPsox63′UTR-M499)*^{i259}. Larvae from this line similarly showed EGFP expression restricted to the fast-twitch fibres at 2 dpf, but by 6 dpf most slow muscle also showed ectopic EGFP expression (Fig. 3J). We also

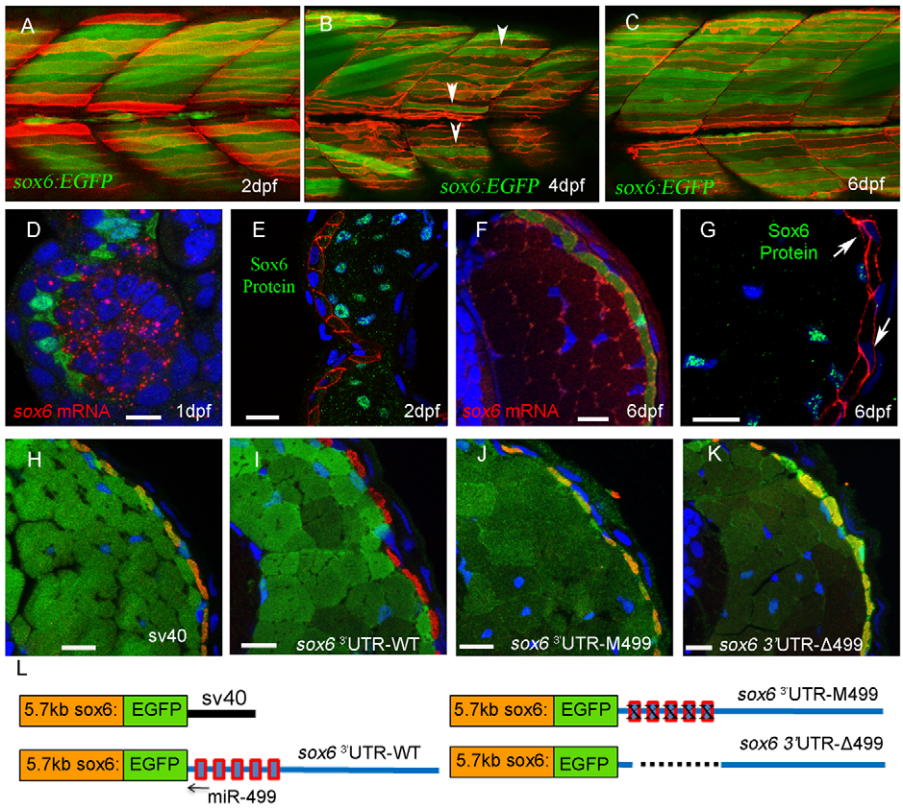

Fig. 3. Derepression of *sox6* transcription but not translation in slow-twitch fibres. (**A-C**) Optical sagittal sections of posterior trunk region of *Tg(5.7 kb sox6:EGFP)*^{i253}*; Tg(9.7 kb smyhc1:lyn-tdTomato)*^{i261} zebrafish larvae. At 2 dpf (A), EGFP expression is restricted exclusively to fast-twitch fibres (green) lying beneath the superficial slow-twitch fibres (red). At 4 dpf (B), EGFP expression is detectable in a few slow-twitch fibres (arrowheads). By 6 dpf (C), all slow-twitch fibres express EGFP. (**D-G**) Transverse cryostat sections of *Tg(BACsmyhc1:GFP)*^{i108} (D,F) or *Tg(9.7 kb smyhc1:lyn-tdTomato)*^{i261} (E,G) embryos/larvae hybridised with a probe for *sox6* mRNA (red) or stained with anti-Sox6 antibody (green), respectively. Arrows indicate slow-twitch fibre nuclei. (**H-L**) Transverse cryostat sections of 6 dpf larvae carrying the 5.7 kb *sox6:EGFP* reporter constructs with differing 3′UTRs, as illustrated in L. (H) Expression of EGFP driven by the 5.7 kb reporter construct with the SV40 3′UTR in slow-twitch fibres, as identified by mABF59 staining (red), is repressed by replacement of the SV40 3′UTR with the endogenous *sox6* 3′UTR (I). Mutation (J) or deletion (K) of the five putative miR-499 target sites restores ectopic EGFP expression in slow-twitch fibres. Note the increased levels of EGFP expression caused by deletion of the miR-499 sites (K). Scale bars: 10 μm.

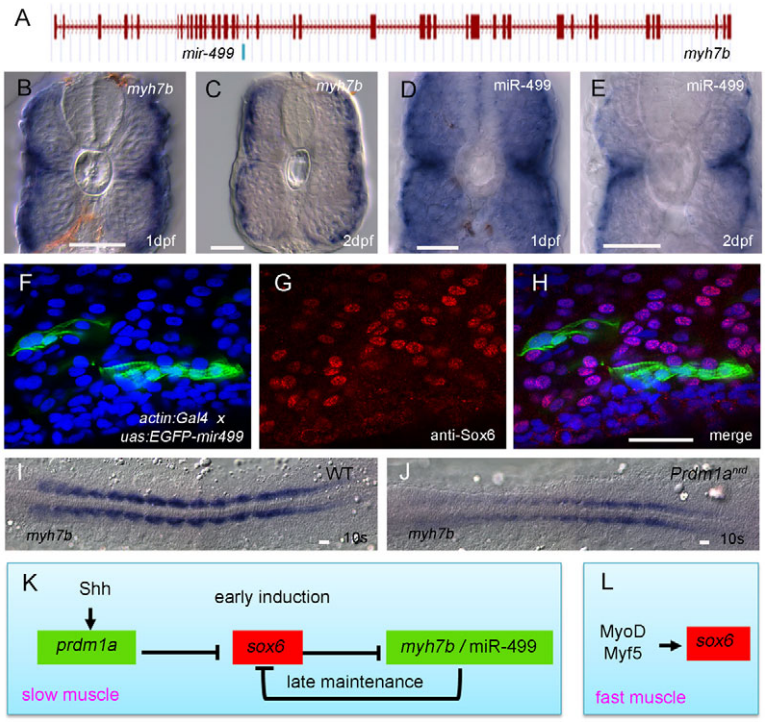

Fig. 4. Prdm1a promotes the spatially restricted expression of *myh7b* and *mir499*, which is sufficient to repress *sox6* translation. (**A**) Exon-intron structure of the *myh7b* gene showing the location of *mir499* in intron 18. (**B-E**) Transverse sections of wild-type embryos hybridised with probes for *myh7b* or miR-499 transcripts, both of which are restricted to the superficially located slow-twitch fibres and the medially located muscle pioneers, a subset of the slow-twitch lineage. (**F-H**) Sagittal optical sections of a 24 hpf *Tg(actin:GAL4)* embryo injected with an *EGFP-mir499* construct under UAS control; fibres expressing the construct (F, green) show significant reduction in nuclear accumulation of Sox6 protein (G, red) as revealed by the merged image (H); nuclei are stained with DAPI (blue). (**I**,**J**) Flat mounts of 10-somite stage wild-type and *prdm1a*^{nrd} mutant embryos hybridised with probe for *myh7b*, showing strong downregulation in the absence of Prdm1a function. (**K**,**L**) Gene regulatory network underlying the specification and maintenance of slow-twitch and fast-twitch muscle lineages in zebrafish. Scale bars: 20 μm.

generated a 1 kb internal deletion of the *sox6* 3′UTR that removes all five miR-499 target sites and used this to generate a stable *Tg(5.7 kb sox6:EGFPsox63′UTR-Δ499)*^{i260} line. Such transgenic larvae also showed ectopic expression of EGFP in slow-twitch fibres by 6 dpf; interestingly, the EGFP expression levels within the slow-twitch fibres were significantly higher than in the fast-twitch fibres (Fig. 3K).

Our findings imply that lineage-restricted expression of miR-499 is sufficient to suppress *sox6* translation in slow-twitch fibres. To explore this hypothesis, we generated probes for zebrafish miR-499 and its host gene *myh7b* (GenBank accession JN216840), and used these to analyse the spatial distribution of their transcripts by ISH. As predicted, both transcripts were found to accumulate specifically in slow muscle fibres (Fig. 4B-E). To confirm that expression of miR-499 is sufficient to repress *sox6* translation, we next forced its expression in fast-twitch fibres by injecting a *UAS:EGFP-mir499* construct into embryos transgenic for a muscle-specific *actin:GAL4* driver line: such ectopic expression coincided with a loss of Sox6 protein accumulation in the nuclei of fast-twitch fibres (Fig. 4F-H).

These findings suggest a mechanism whereby suppression of Sox6 activity in the slow-twitch lineage is established by Prdm1a-mediated transcriptional repression and maintained by miR-499-mediated translational repression. This begs the question as to how the lineage-restricted expression of miR-499 itself is established. Previous studies in mammalian systems have shown that *Mir499* transcription can be repressed by Sox6 (van Rooij et al., 2009; Bell et al., 2010), which would be consistent with slow-lineage-specific *mir499* transcription being promoted by Prdm1a activity in zebrafish. To test this possibility, we analysed expression of the *mir499* host gene *myh7b* in wild-type and *prdm1a* mutant embryos. At the 10-somite stage, *myh7b* is expressed in the adaxial cells of wild-type embryos (Fig. 4I), but this expression is significantly diminished in *prdm1a*^{nrd} homozygotes (Fig. 4J), consistent with its transcription being promoted by the repression of Sox6.

Our data reveal a Shh-activated gene regulatory network that acts through both transcriptional and post-transcriptional mechanisms to establish and maintain lineage-specific gene expression patterns in the skeletal muscle of the zebrafish (Fig. 4K,L). Notably, the involvement of miR-499 and Sox6 in this network has been conserved from fish to mammals, although a role for miR-499 in inhibiting *sox6* translation had not previously been demonstrated. The role of Prdm1a in this network, by contrast, appears to be teleost specific, as there is no evidence implicating it in mammalian fibre type specification. Although our data confirm the role of Prdm1a in repressing *sox6* transcription, they imply that this function is likely to be indirect, indicating that other key components of the network remain to be discovered.

Acknowledgements
We thank Stone Elworthy for the His-tagged Sox6 fusion construct, Augustine Cheong Weng Onn for affinity purification of the anti-Sox6 antibody, Byrappa Venkatesh and Ravi Vydianathan for advice on multi-species sequence alignments and members of the P.W.I. laboratory for helpful comments and advice.

Funding
This work was supported by the Agency for Science Technology and Research (A*STAR).

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.070516/-/DC1

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