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Prdm1- and Sox6-mediated transcriptional repression specifies muscle fibre type in the zebrafish embryo

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The zebrafish *u-boot* (*ubo*) gene encodes the transcription factor Prdm1, which is essential for the specification of the primary slow-twitch muscle fibres that derive from adaxial cells. Here, we show that Prdm1 functions by acting as a transcriptional repressor and that slow-twitch-specific muscle gene expression is activated by Prdm1-mediated repression of the transcriptional repressor Sox6. Genes encoding fast-specific isoforms of sarcomeric proteins are ectopically expressed in the adaxial cells of *ubo*[tp39] mutant embryos. By using chromatin immunoprecipitation, we show that these are direct targets of Prdm1. Thus, Prdm1 promotes slow-twitch fibre differentiation by acting as a global repressor of fast-fibre-specific genes, as well as by abrogating the repression of slow-fibre-specific genes.

INTRODUCTION

During vertebrate development, cells become committed to the myogenic fate through the activation of myogenic regulatory factors in the paraxial mesoderm. Subsequently, the committed cells or myoblasts differentiate into muscle fibres with distinct contractile speeds, the so-called slow- and fast-twitch fibres. Terminal differentiation of these different fibre types requires the expression of specific isoforms of sarcomeric proteins, such as the myosin light and heavy chains (MyLC and MyHC, respectively)

and troponins. In the zebrafish embryo, progenitors of the slow- and fast-twitch fibres can be identified on the basis of their morphology and positioning within the segmental plate before somitogenesis (Devoto *et al*, 1996). Paraxial mesodermal cells that lie in direct contact with the notochord—designated adaxial cells—differentiate into slow myoblasts that migrate out through the developing myotome to form a superficial layer of slow-twitch fibres. These slow fibres are mononuclear, span the entire length of each somite and express the homeodomain protein Prox1, as well as the slow myosin heavy chain 1 (*smyhc1*) and the slow-specific Troponin C (*stnnC*; Devoto *et al*, 1996; Roy *et al*, 2001; Xu *et al*, 2006). The fast-twitch fibres derive from more laterally located paraxial mesodermal cells that start to differentiate in the wake of the migrating slow myoblasts (Blagden *et al*, 1997). In contrast to the slow myoblasts, fast-twitch myoblasts undergo fusion to generate multinucleated fibres (Moore *et al*, 2007; Srinivas *et al*, 2007) and express fast MyLC and MyHC isoforms, as well as troponin T3a (*tnnt3a*) and troponin I2 (*tnni2*) (Xu *et al*, 2000; Hsiao *et al*, 2003).

The specification of adaxial cells to follow the slow-twitch fibre differentiation programme depends crucially on inductive signals from the notochord and floorplate mediated by members of the Hedgehog protein family (Currie & Ingham, 1996; Blagden *et al*, 1997; Du *et al*, 1997; Lewis *et al*, 1999; Barresi *et al*, 2000; Wolff *et al*, 2003). Reception of the Hedgehog signals by adaxial cells results in the activation of transcription of the *u-boot* (*ubo*) gene, the function of which is both necessary and sufficient to drive slow-twitch differentiation in myoblasts (Roy *et al*, 2001; Baxendale *et al*, 2004). In *ubo*[tp39] mutants, presumptive slow-twitch fibres lose sMyHC and Prox1 expression, and seem to differentiate into fast-twitch fibres (Roy *et al*, 2001). The *ubo* gene encodes the B-lymphocyte-induced maturation protein Blimp1 or Prdm1, a PR-domain-containing protein, which, in mammals, is involved in the terminal differentiation process of B lymphocytes, the response to viral infection and primordial germ cell specification (Keller & Maniatis, 1991; Turner *et al*, 1994; Ohinata *et al*, 2005;

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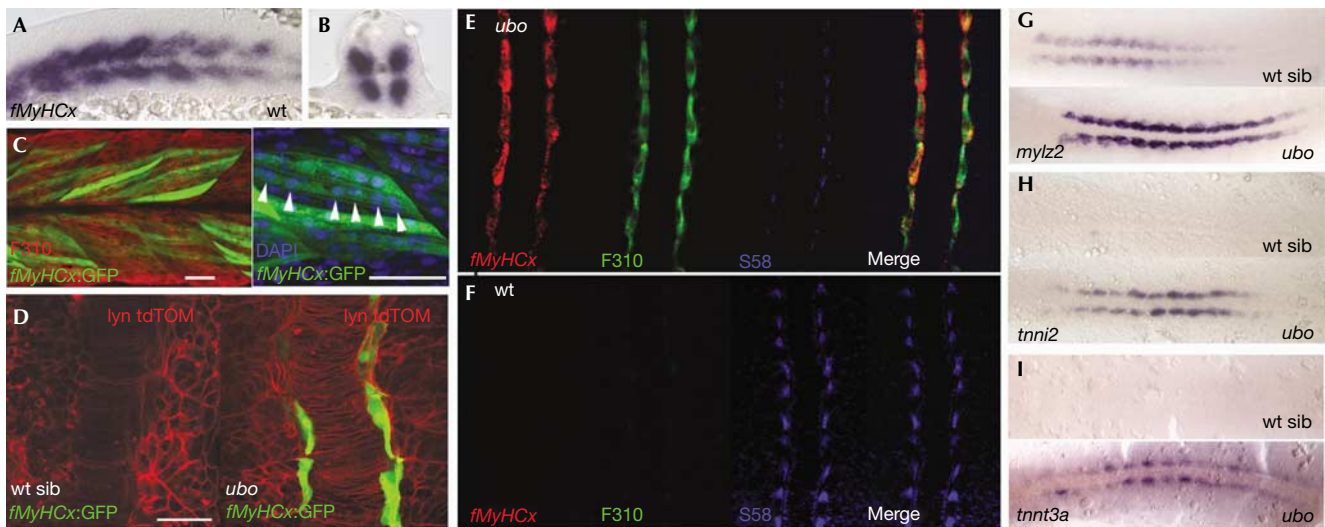E-mail: p.w.ingham@sheffield.ac.uk

Fig 1 | Fast-muscle-fibre-specific genes are ectopically expressed in the slow domain of *ubo*^tp39 mutant embryos. *In situ* hybridization showing fast-muscle-specific expression of the fast myosin heavy chain gene (*fMyHCx*) at the 18-somite stage in (**A**) lateral and (**B**) transverse views. (**C**) An *fMyHCx*:GFP construct expresses GFP specifically in the fast-muscle fibres in the trunk. Transient expression of *fMyHCx*:GFP in the fast fibres in a 30 hpf (hours post fertilization) embryo, as shown by the colocalization with the fast-fibre marker F310 antigen. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) to highlight the polynuclear arrangement (arrowheads) of the fast fibres expressing *fMyHCx*:GFP. (**D**) The construct *fMyHCx*:GFP is detected in the adaxial cells of *ubo*^tp39 mutants in transient assays. The *fMyHCx*:GFP construct is not expressed at the 12-somite stage in wild-type embryos, but is ectopically expressed in the adaxial cells of a 12-somite-stage *ubo*^tp39 embryo. Lyn tdTomato (red) was used to highlight cellular membranes and the outline of the notochord. (**E,F**) Ectopic expression of *fMyHCx* messenger RNA (red) and F310 (green) is detected in the adaxial cells of a 12-somite-stage *ubo*^tp39 embryo (100% of embryos assayed; $n > 30$). In wild-type siblings (wt sib), adaxial cells are only labelled with the slow MyHC S58 (blue) antibody. Genes that normally are expressed exclusively in the fast-muscle domain are ectopically expressed in the adaxial cells of the *ubo*^tp39 mutant: (**G**) *mylz2*, (**H**) *tnni2* and (**I**) *tnnt3a*. Scale bars, 25 μm. GFP, green fluorescent protein; *mylz2*, *myosin light chain 2*; *tnni2*, *troponin I2*; *tnnt3a*, *troponin T3a*; *ubo*, *u-boot*; wt, wild type.

Kallies & Nutt, 2007). In these contexts, Prdm1 has been shown to mediate transcriptional repression, acting as a scaffold that recruits co-repressors and chromatin-modifying enzymes to specific target genes (Yu *et al*, 2000; Gyory *et al*, 2004). Here, we investigate the nature of Prdm1 function and the regulatory networks underlying fibre type specification, and identify several direct targets of Prdm1.

RESULTS AND DISCUSSION

Adaxial cells transform from slow into fast in *ubo*^tp39 mutants

In wild-type embryos, adaxial cells are characterized by their expression of a slow isoform of the MyHC—detected by the S58 antibody—but are devoid of staining with F310—a fast MyLC isoform-specific antibody. Differentiation of adaxial cells into slow-twitch muscle starts several hours earlier than that of the fast-twitch fibres. Previous studies of *ubo* mutants have shown that loss of Prdm1 expression causes adaxial cells to transform from slow- to fast-twitch character (Roy *et al*, 2001). Consistent with this, we found that adaxial cells were labelled with F310 in *ubo*^tp39 homozygotes well before the normal onset of fast-muscle differentiation. To confirm that this represents the precocious differentiation of adaxial cells into fast-twitch fibres, we constructed a reporter gene, using sequences upstream from the previously uncharacterized *fMyHC* gene, which drives green fluorescent protein (GFP) expression strictly in fast-muscle cells (Fig 1A–C). When injected into *ubo*^tp39 mutant embryos, this

fMyHCx:GFP transgene was ectopically expressed in adaxial cells (Fig 1D), indicative of their transformation from slow to fast character (Fig 1E,F). By using *in situ* hybridization (ISH), we found that *tnnt3a* and *tnni2*, encoding fast-specific isoforms of troponin, were also ectopically expressed in the adaxial cells of *ubo*^tp39 mutants, whereas expression of the gene encoding MyLC (*mylz2*) was significantly elevated above the levels found in wild-type adaxial cells (Fig 1G–I). Thus, Prdm1 acts to repress fast-specific genes, as well as to promote expression of slow-specific genes in adaxial cells.

Prdm1 acts as a repressor to promote slow twitch fibre type

To investigate whether Prdm1 acts as an activator or repressor of transcription during slow-twitch muscle development in zebrafish, constructs in which the Prdm1 DNA-binding domain is fused to either the Engrailed (Eng) repressor or the VP16 activator domain (Kessler, 1997) were tested for their ability to substitute for the wild-type protein. Sequences encoding the fusion constructs were cloned downstream from a heat-shock-inducible promoter that simultaneously drives expression of the fluorescent protein tdTomato. Transient expression of the Eng–Prdm1 protein induced by heat shock was sufficient to rescue the expression of Prox1 in *ubo*^tp39 mutant embryos (Fig 2C). By contrast, transient expression of the Vp16–Prdm1 fusion protein was unable to rescue Prox1 expression or suppress myoblast fusion (Fig 2D,E), but was sufficient to activate fast-myosin expression. Taken together, these
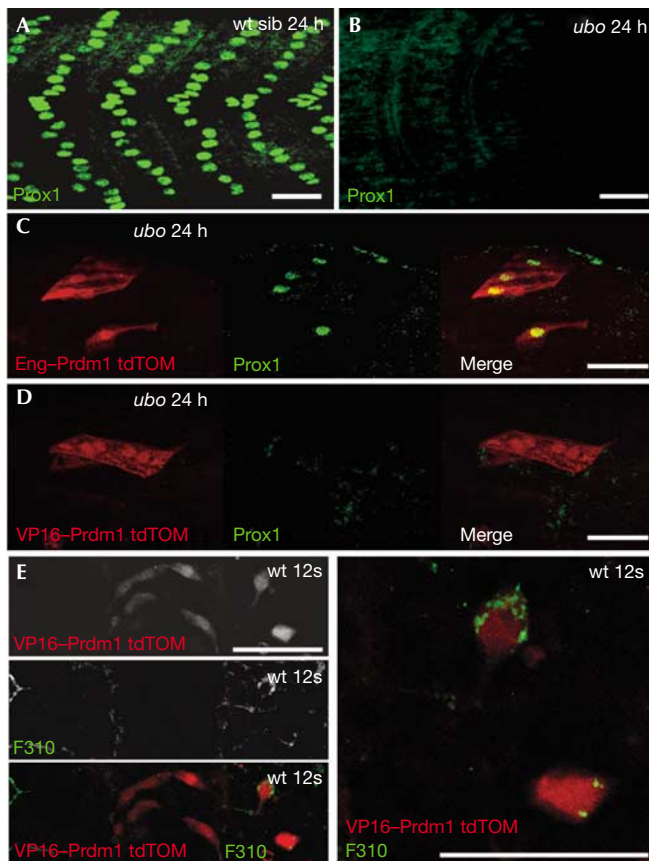
Fig 2 | Slow-fibre differentiation requires the repressive function of Prdm1. (**A**) Prox1 expression in slow-muscle fibres of wild-type (wt) embryos at 24 h. (**B**) ubo^{tp39} mutant embryos lack Prox1-expressing slow fibres at 24 h. (**C**) Eng–Prdm1 expression, marked by tdTomato (tdTOM), rescues mononucleate fibre differentiation and Prox1 expression in ubo^{tp39} homozygotes (49 out of 94 Eng–Prdm1-expressing fibres were Prox1 positive; $n = 8$). (**D**) VP16–Prdm1 expression, marked by tdTOM, was found exclusively in multinucleate fibres lacking Prox1 staining in ubo^{tp39} homozygotes (none of the 134 Vp16–Prdm1-expressing fibres was Prox1 positive; $n = 11$). (**E**) Precocious labelling with the fast-specific F310 antibody in VP16–Prdm1-expressing muscle precursors at the 12-somite stage (12s; 12 out of 30 F310-positive cells; $n = 4$). Scale bars, 25 μm. Eng, Engrailed; Prox1, prospero-related homeobox gene 1; sib, sibling; ubo, u-boot.

data indicate that Prdm1 acts as a repressor rather than an activator to promote slow-muscle differentiation, and show that activation of *prox1* and *smyhc1* expression is an indirect effect of Prdm1 activity. As the PR domain is absent from the Eng–Prdm1 protein fusion, it also follows that this domain is not essential for Prdm1 function, at least in this context. Consistent with this, previous studies have shown the PR domain to be dispensable for Prdm1 function in the context of β-interferon repression (Gyory *et al*, 2004).

Prdm1 regulates Sox6—a repressor of slow fibre identity

In mice, the transcription factor Sox6 acts as a repressor of fetal slow-twitch fibre differentiation (Hagiwara *et al*, 2005, 2007). We

analysed expression of the zebrafish *sox6* gene and found that it was expressed in the fast-muscle progenitor domain of the somites but excluded from adaxial cells (Fig 3A). In ubo^{tp39} mutant embryos, by contrast, *sox6* is ectopically expressed in adaxial cells, indicating that Prdm1 represses *sox6* expression in slow-muscle progenitors. Forcing ectopic expression of *sox6* in the adaxial cells of wild-type embryos caused an inhibition of Prox1 expression (Fig 3B). Conversely, morpholino-mediated knockdown of *sox6* in ubo^{tp39} embryos partly rescued Prox1 expression and restored expression of *smyhc1* to normal levels in adaxial cells (Fig 3C). Although neither Prox1 nor *smyhc1* was expressed ectopically in fast fibres in response to *sox6* knockdown, robust expression of *stnnC* was induced in the fast muscle of both wild-type and smoothened (*smo*) mutant (that lack all Hedgehog signalling activity) embryos injected with the *sox6* morpholino (Fig 3E,F). This disparity might reflect a differential sensitivity of these slow-twitch-specific genes to *sox6* activity, revealed by incomplete knockdown by the morpholino. Taken together, these data indicate that Sox6 acts as a repressor of slow-twitch-specific gene expression and suggest that Prdm1 activates such expression by repressing transcription of *sox6*.

Fast-twitch-specific genes are direct targets of Prdm1

The coordinated repression of multiple genes encoding fast-twitch isoforms of sarcomeric proteins could be accomplished by Prdm1-mediated repression of a fast-specific global transcriptional activator; alternatively, Prdm1 might itself act directly to repress transcription of these genes. To distinguish between these two scenarios, we used chromatin immunoprecipitation (ChIP) to test for binding of the protein to upstream regulatory regions of the putative targets. A polyclonal antibody was raised against 186 amino acids from the Prdm1 PR domain; as expected, this labelled adaxial cell nuclei during early- and mid-myogenesis (Fig 4A), and bound to Prdm1 specifically in immunoprecipitation assays (Fig 4B). DNA from chromatin immunoprecipitated with this antibody was amplified by using primer pairs specific for sequences proximal to the transcription initiation sites of the *mylz2*, *fMyHCx*, *tnnt3a* and *tnni2* genes. These sequences were all found to be enriched in the Prdm1-precipitated chromatin; by contrast, none of the slow-muscle-specific genes *smyhc1*, *stnnC* or *Prox1* was enriched (Fig 4C). These data indicate that Prdm1 selectively binds to putative regulatory regions of fast-fibre-specific genes *in vivo*, suggesting that Prdm1 acts as a direct repressor of their transcription.

The *mylz2* promoter has functional Prdm1 binding sites

Expression of a GFP reporter gene containing 2.3 kb of the *mylz2* promoter sequence is specifically repressed in adaxial cells by Prdm1 activity (Fig 4D). Although the consensus binding site for Prdm1 has not been determined in zebrafish, we identified five putative Prdm1-binding sites in this fragment, containing the GAAAG core of the sequence (A/C)AG(T/C)GAAAG(T/C)(T/G) that has been defined as mediating Prdm1-dependent gene regulation in mammals (Kuo & Calame, 2004). The introduction of point mutations in each of these five potential Prdm1-binding sites in this construct led to ectopic adaxial GFP expression in wild-type embryos, similar to that seen with the wild-type construct in *ubo* morphants (Fig 4D). This finding is consistent with Prdm1 acting directly to repress the *mylz2* gene in adaxial cells at the 12-somite stage.
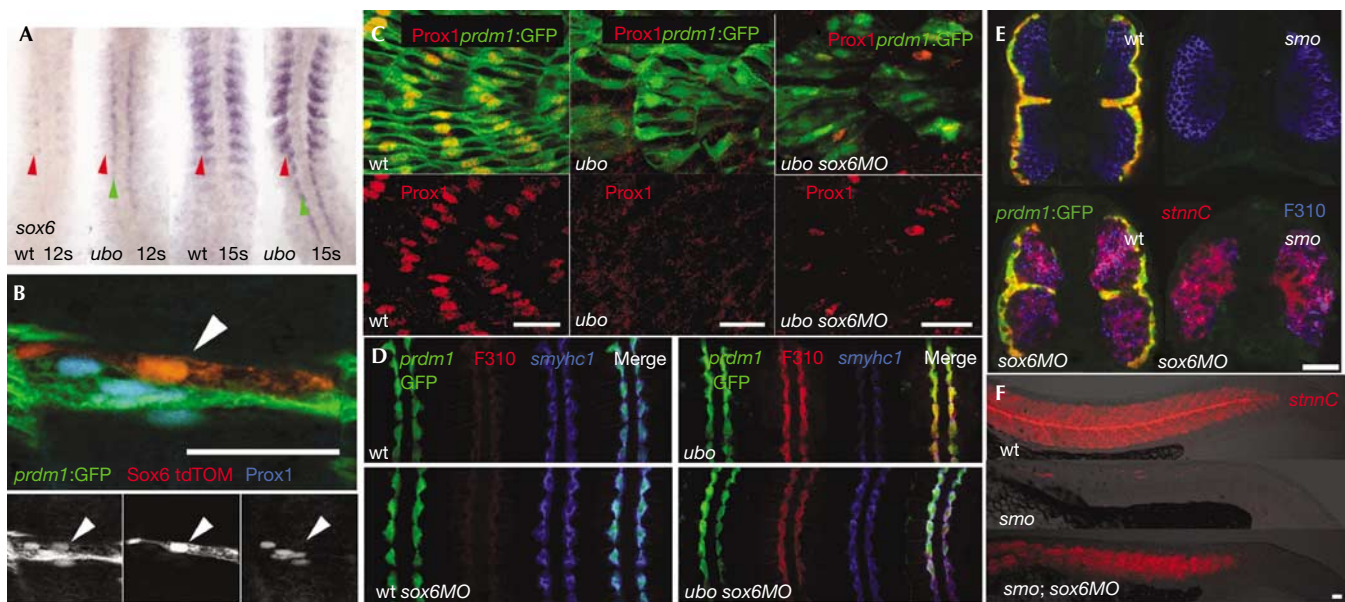
Fig 3 | Slow-specific gene expression is upregulated in *sox6* morphant embryos. **(A)** *sox6* is expressed in the fast-muscle precursors (red arrowheads) in wild-type (wt) embryos and ectopically expressed in the adaxial cells (green arrowheads) in *ubo*[tp39] embryos at the 12-somite (12s) and 15-somite (15s) stage. **(B)** Heat-shock-induced expression of Sox6 (marked with tdTomato (tdTOM)) inhibits Prox1 expression at 24 hpf (hours post fertilization) in adaxially derived cells marked by *prdm1*:GFP (arrowhead; 100% of the adaxially derived cells lacked Prox1 when ectopically expressing Sox6 (from the analysis of 14 fibres in 4 embryos)). **(C)** Lateral views of the tail at 24 hpf showing partial rescue of Prox1 expression in *ubo*[tp39] embryos injected with *sox6MO* (*ubo*[tp39]: 0 rescued fibres in 20 embryos; *ubo*[tp39]; *sox6MO*: mean of 58 rescued fibres per embryo, $n = 5$, s.d. $= 29$). **(D)** At 12s, *ubo*[tp39] mutants have reduced expression of *smyhc1* and ectopic expression of fast-MyLC (recognized by the F310 antibody) in the adaxial cells marked with *prdm1*. *sox6MO* causes rescue of the *smyhc1* expression in *ubo*[tp39] mutants but does not prevent ectopic fast-MyLC expression. **(E)** Transverse views of 24 hpf wt and *smoothened* (*smo*) embryos carrying the *prdm1*:GFP reporter gene (upper panel) and siblings of the same genotypes that have been injected with the *sox6* morpholino (*sox6MO*; lower panels), showing that loss of *sox6* causes Hedgehog-independent ectopic expression of *stnnC* (red) in fast muscle (identified by F310 antigen (blue)). **(F)** Lateral views of *stnnC* expression (red) in wt, *smo* and *smo;sox6MO* embryos at 24 hpf (9 out of 9 *smo;sox6MO* embryos, and 15 out of 15 wt embryos showed strong ectopic *stnnC* expression). Scale bars, 0.25 μm. GFP, green fluorescent protein; Prox1, prospero-related homeobox gene 1; *smyhc1*, slow myosin heavy chain 1; *sox6MO*, sox6 morpholino; *stnnC*, slow-specific Troponin C; *ubo*, u-boot.

Identification of Prdm1 target genes by ChIP on chip

To confirm and extend the findings of our candidate gene analysis, we used a recently constructed zebrafish promoter array, consisting of 60-mer probes for more than 11,000 genes within the zebrafish genome (Wardle *et al*, 2006), to probe the DNA isolated by ChIP of myoblast extracts (supplementary information online, accession code GSE10883 at GEO). By setting the gene array threshold for enrichment to the level of *mylz2*, which was at the level of significant *P*-value 0.0078 and *P*[Xbar] 0.0075, we identified 381 putative target genes (supplementary information online). Gene ontology analysis showed various genes enriched in the ChIP-on-chip sample: 11% were documented transcription factors, 24% were new genes or genes without known function, 15% were genes encoding proteins with enzyme activity, such as kinases and phosphatases, whereas others had gene ontology terms linking them to the immune and haematopoietic systems, cell-cycle regulation or apoptosis. Significantly, we found several genes encoding fast-fibre-specific isoforms of sarcomeric proteins, including those encoding fast MyHC and troponins described above, whereas no genes encoding slow-specific sarcomeric proteins or Prox1 were identified (supplementary information online). Surprisingly, *sox6* was not among the transcription factor-encoding genes identified in this analysis. However, we note that representation of regulatory regions on the gene array is restricted to sequences 9 kb upstream from the 5′ end of the complementary DNAs used in its design (Wardle *et al*, 2006). We have identified additional *sox6* sequences 30 kb upstream from the transcription start site used in the array (J.v.H., S.E. & P.W.I., unpublished data); whether *sox6* is a direct target of Prdm1 remains to be determined.

Conclusion

Our data underline the pivotal role of Prdm1 in switching between alternative muscle fibre type programmes in the zebrafish embryo. We have shown that it accomplishes its function in two ways: first, by repressing the transcription of a repressor of slow-specific gene transcription, *sox6*, in a manner analogous to its repression of *Pax5* in B cells (Lin *et al*, 2002) and, second, by acting directly as a global repressor of fast-specific differentiation genes. Although Prdm1 is expressed in the myotome of the mouse (Chang *et al*, 2002; Vincent *et al*, 2005), at present it is unclear whether it has an analogous role in fibre type specification in amniotes. Our finding that Sox6 suppresses slow-twitch fibre specification in zebrafish, however, establishes that at least some aspects of the
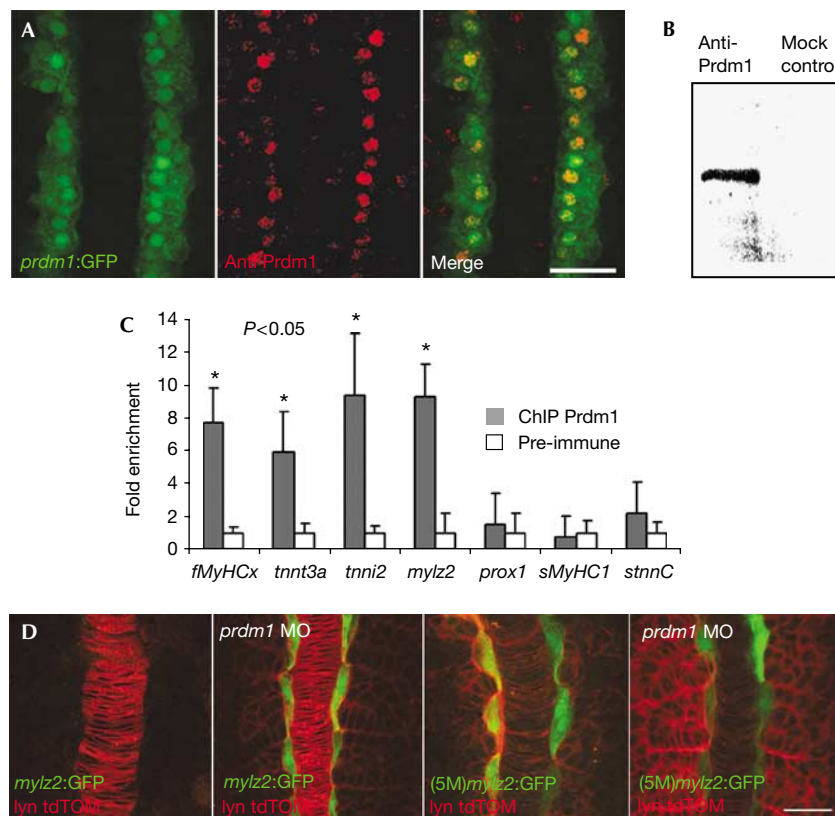
Fig 4 | Prdm1 acts as a direct repressor of fast-fibre-specific gene expression in adaxial cells. (**A**) *prdm1*:GFP expression is specific to adaxial cells in the somite area at the eight- to ten-somite stage. A Prdm1-specific polyclonal antibody detects Prdm1 protein in adaxial cells, consistent with the *prdm1*:GFP expression pattern. Scale bar, 0.25 μm. (**B**) Prdm1 is specifically immunoprecipitated using the Prdm1 antibody. (**C**) Amplification of the fast-fibre-specific genes *fMyHCx*, *tnnt3a*, *tnni2* and *mylz2* using Taqman real-time PCR showed significant enrichment compared with the pre-immune controls. Three slow-muscle-specific genes, *prox1*, *sMyHC1* and *stnnC*, were tested as negative controls and were not enriched in the chromatin immunoprecipitated (ChIP) chromatin. (**D**) The *mylz2*:GFP promoter fusion is not expressed in wild-type embryos at the 12-somite stage, but is expressed in the adaxial cells in *prdm1* morphants at the 12-somite stage. Mutation of the Prdm1-binding sites (5M) resulted in ectopic adaxial GFP expression in both control and *prdm1* morphant embryos. Scale bar, 0.25 μm. *fMyHC, fast myosin heavy chain*; GFP, green fluorescent protein; MO, morpholino; *mylz2, myosin light chain 2*; *prox1, prospero-related homeobox gene 1*; *sMyHC1, slow myosin heavy chain 1*; *stnnC, slow-specific Troponin C*; *tnni2, troponin I2*; *tnnt3a, troponin T3a*.

regulatory network underlying fibre type specification (Hagiwara *et al*, 2005, 2007) have been conserved in evolution.

METHODS

Fish strains, cloning of gene promoters and injection of embryos. Zebrafish mutants *ubo*[tp39] and *smo*[b641], and the transgenic line *Tg(actal:GFP)*[zf13] have been described previously (van Eeden *et al*, 1996; Higashijima *et al*, 1997; Barresi *et al*, 2000; Chen *et al*, 2001; Roy *et al*, 2001; Baxendale *et al*, 2004). The *Tg(prdm1:gfp)*[i111] and *Tg(prdm1:gfp)*[i106] are described by Elworthy *et al* (2008). The *mylz2*:GFP promoter construct was generated by using 2,239 bp of the *mylz2* upstream region (Ju *et al*, 2003; Moore *et al*, 2007) to generate a stable line *Tg(mylz2:GFP)*[i135]. The *mylz2*:GFP plasmid was also used as a template for *in vitro* mutagenesis of the five sites containing the Prdm1 GAAAG core sequence using the QuikChange® Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions to generate the *Tg((5M)mylz2:GFP)*[i130] transgenic line. A 6.8 kb fragment upstream

from the *fMyHCx* ATG was isolated by PCR from bacterial artificial chromosome zCR392328 by using a left primer containing an *Asc*I restriction site (TGGCGCGCCTGCATGGTGTTTGACA) and a right primer containing *Nco*I (ACCCATGGTGGCGGCTTACCGT). The promoter DNA was in all cases subcloned into a GFP vector with flanking I-sceI sites. One-cell-stage embryos were microinjected with 4–8 nl of plasmid at a concentration of 40 ng/nl. Embryos were kept at 28.5 °C and analysed for GFP at the 12-somite stage. *Ubo* morpholino-mediated knockdown of Prdm1 activity was carried out as described previously (Baxendale *et al*, 2004). The *sox6* translation targeted morpholino (GTGGCTTGCTTGGAAGACATGATTC) was injected into one-cell-stage embryos at 0.9 mM. All fish were raised, staged and maintained as described previously (Kimmel *et al*, 1995; Westerfield, 2000).

Prox1 rescue assay. Eng–Prdm1 and VP16–Prdm1 fusion constructs (gifts from Dr Johaness Bischof; for details, see the supplementary information online) were used in attempts to rescue Prox1 expression. Both constructs and the complete *sox6* cds (EU532205) were subcloned into a pSGH2 vector containing

a bidirectional heat-shock promoter (Bajoghli *et al*, 2004) that drives expression of both the fusion protein and the fluorescent protein tdTomato. One-cell-stage embryos were microinjected with plasmid at a concentration of 40 ng/μl. At the three- to four-somite stage, the injected embryos were heat shocked by incubation at 39 °C for 2 h. Injected embryos were fixed in 4% paraformaldehyde at 24 hpf (hours post fertilization) and analysed by using confocal microscopy.

Antibodies, immunohistochemistry and ChIP. A Prdm1 polyclonal antibody was raised against a fragment of the protein corresponding to amino acids 161–346 expressed as a His-tag fusion protein using the pET19b vector (Novagen, Darmstadt, Germany). Immunoprecipitation using the Prdm1 antibody was carried out according to Link *et al* (2006) using crude protein extracts from zebrafish embryos at the ten-somite stage. The precipitated proteins were analysed on SDS–polyacrylamide gel electrophoresis gel after Coomassie staining.

A Prox1 antibody was raised against recombinant zebrafish Prox1 purified from *Escherichia coli* (A.M. Taylor & P.W.I., unpublished data). Whole-mount immunohistochemical analysis using F310 fast MyLC (1:50, DSHB), S58 slow MyHC (1:10, DSHB), Prox1 (1:5,000) and Prdm1 (1:15,000) antibodies was performed on embryos fixed in 4% paraformaldehyde (for protocols, see the supplementary information online). For ChIP analysis, α-*actin*:GFP embryos were injected with dominant negative Protein Kinase A (dnPKA) at the one- to two-cell stage and were kept in embryonic medium until the 12- to 14-somite stage. The chorions were removed using pronase and cells were fixed for 15 min in 1.85% formaldehyde. For the ChIP-on-chip assay (supplementary information online), the embryos were dissociated using collagenase and GFP-positive cells were isolated. In addition to the ChIP on chip, three replicates of 300 embryos were used in the ChIP assay, which was performed as described previously (Wardle *et al*, 2006) using the Prdm1 antibody or rabbit preserum. Precipitated chromatin was analysed using Custom TaqManR Assays (Applied Biosystems, Foster City, CA, USA), specific to regions within the first 1 kb of upstream sequences from the first codon of *mylz2* (NM_131188), *fMyHCx* (EU115994), *tnnt3a* (NM_131565), *tnni2* (NM_205742), *stnnC* (AF281003), *prox1* (NM_131405) and *sMyHC1* (NM_001020507; for oligonucleotides and probes, see the supplementary information online). For whole-mount ISH, antisense digoxigenin (DIG) probes for *mylz2*, *tnnt3a*, *tnni2*, *fMyHCx*, *sox6* and *stnnC* (subcloned from IMAGE_6899234, zgc:86932; Thisse *et al*, 2001; Xu *et al*, 2006) were generated and the ISH was performed as described previously (Thisse & Thisse, 1998). Fluorescent ISH used anti-dig POD (Roche, Basel, Switzerland) at 1:10,000 with tyramide signal amplification (TSA) Cyanine 3 (Perkin Elmer, Waltham, MA, USA).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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