Early transcriptional targets of MyoD link myogenesis and somitogenesis

Richard J. Maguire, Harry V. Isaacs, Mary Elizabeth Pownall *

Biology Department, University of York, Heslington, York, North Yorkshire YO10 5YW, United Kingdom

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Abstract

In order to identify early transcriptional targets of MyoD prior to skeletal muscle differentiation, we have undertaken a transcriptomic analysis on gastrula stage Xenopus embryos in which MyoD has been knocked-down. Our validated list of genes transcriptionally regulated by MyoD includes Esr1 and Esr2, which are known targets of Notch signalling, and Tbx6, mesogenin, and FoxC1; these genes are all known to be essential for normal somitogenesis but are expressed surprisingly early in the mesoderm. In addition we found that MyoD is required for the expression of myf5 in the early mesoderm, in contrast to the reverse relationship of these two regulators in amniote somites. These data highlight a role for MyoD in the early mesoderm in regulating a set of genes that are essential for both myogenesis and somitogenesis.

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Introduction

In vertebrates, the myogenic regulatory genes myoD, myf5, myogenin, and mrf4 code for bHLH transcription factors which are expressed specifically in the myogenic cell lineage. These MRFs (myogenic regulatory factors) act as developmental switches, committing cells to skeletal myogenesis. MRF expression provides the earliest marker of myogenic precursors (reviewed, Pownall et al., 2002) and are critical players in the establishment of this lineage, acting as dominant regulators of myogenesis (reviewed, Weintrub, 1993). While mouse knock-outs have shown that the functions of these genes are somewhat overlapping, it is clear that myoD, myf5 and mrf4 are essential for the early establishment of the myogenic lineage since mice deficient in these genes do not form any skeletal muscle, and also lack all skeletal muscle precursors (Rudnicki et al., 1993). Much work has been undertaken investigating the regulation of MRF gene expression (for instance, Carvajal et al., 2001) and the transcriptional mechanisms by which the MRFs drive skeletal muscle differentiation (Tapscott, 2005). However, very little is known about what genes these important transcription factors regulate prior to myogenic differentiation.

In Xenopus, XmyoD and Xmyf5 are expressed at high levels in the myogenic lineage prior to differentiation (Hopwood et al., 1989) and importantly, XmyoD protein is also present in gastrula stage embryos many hours before differentiation of skeletal muscle (Hopwood et al., 1992). A mesodermal cell from an early gastrula embryo requires continued cell signals, such as FGF4, to maintain a myogenic fate (Standley et al., 2001), however, a single cell taken from a late gastrula embryo behaves as a determined myoblast: when transplanted to a ventral region, it differentiates as skeletal muscle (Kato and Gurdon, 1993). These cells in the Xenopus gastrula therefore represent a population of XmyoD positive, myogenic progenitors. In amniotes, a similar pool of muscle progenitors is present in the medial edge of the dermomyotome (Denetclaw et al., 1997). In both cases, the myogenic progenitor cells express one or more MRFs and are committed to give rise to skeletal muscle, but do not yet express any differentiation markers such as the contractile protein genes (de la Brousse and Emerson, 1990; Emerson, 1990). When compared to amniotes, myogenic progenitor cells in the amphibian gastrula are more easily accessible and we have taken advantage of this to look for genes regulated by MyoD prior to myogenic differentiation in vivo.

A microarray expression analysis has been used to identify genes that require MyoD for their expression in the mesoderm during gastrula stages (NF 11.5). We have found that MyoD protein is required to activate transcription from a set of genes that include Esr1, Esr2, mesogenin (mespo), Tbx6 and FoxC1; all known to be important for normal somitogenesis (Hitachi et al., 2008; Ogimura et al., 2008; Tazumi et al., 2008; Topczewska et al., 2001). Esr1 and Esr2 are Hairy/enhancer of split (HES) related genes that are downstream effectors of Notch signalling, which is a central part of the segmentation clock that regulates the regular formation of somites from the presegmental plate mesoderm (Rida et al., 2004). All skeletal muscles in the vertebrate body are derived from the somites, and in amniotes the expression of the MRFs is closely associated with the segmentation of somites.
RNeasy expressing MyoD. RNA was further processed using the QIAgen kit (QIAgen) followed by lithium chloride precipitation. Some supernatant was used in a Western blot to confirm MyoD using the data throughout.

Multiple alignments were carried out using the tool MultiPIP-maker. The gapped BLASTZ alignments obtained were then scanned for the E-box consensus sequence “CANNTG”. Once a region containing a conserved E-box was found, the surrounding 200 base pairs were identified in each genome and Clustal-W alignment was performed on these shorter sequences to confirm the presence and the conservation of an E-box (Supplementary data).

Chromatin immunoprecipitation was performed as in Blythe et al. (2009). Xenopus tropicalis embryos were injected bilaterally with 500 pg of MyoD mRNA and crosslinking was carried out by fixing (1% formaldehyde/PBS) for 1 h. Embryos were homogenised on ice in the presence of protease inhibitors and sonicated before incubation with pre-blocked protein G beads (Sigma) followed by the anti-MyoD antibody D7F2 (DSHB) or goat anti-mouse unconjugated IgG (Sigma) overnight at 4 °C. Samples were then washed, eluted from beads, and reverse-crosslinked at 65 °C. Some supernatant was used in a Western blot to confirm MyoD pull down. After crosslink reversal and protease K treatment, nucleotides in the samples were purified by phenol:chloroform extraction and treated with 100 μg/ml RNase A and 4000 μg/ml RNase T1 (Ambion). Samples were then purified using the QiAquick™ PCR purification kit. The DNA samples were then subject to PCR targeted to genomic regions using the primers listed in the Supplementary data. An initial 25 cycles of PCR was followed by a second round of 25 cycles using one-fifth of the initial reaction. The resulting products were run on a 3% agarose gel.

Western blots

For analyses of MyoD protein, 10 whole embryos at the stages indicated were homogenised in Phosphosafe homogenising buffer (Merck) and processed as described in Hopwood et al. (1992). Samples were run on an 11% SDS-PAGE gel and transferred onto a PVDF membrane. A 1:4 dilution of D7F2 tissue culture supernatant (DSHB) was used for MyoD and a 1:100,000 dilution of anti-GAPDH-71.1 antibody (Sigma) for GAPDH. A 1:3000 dilution of anti-mouse POD secondary antibody (Amersham) was used for both primaries. Proteins were visualised using an ECL detection kit (Roche).

**RNase protection**

RNase protections were carried out as described in Isaacs et al. (1994) using probes prepared from plasmids using the restriction enzymes and polymerases described in the Supplementary data. Probes were hybridized overnight at 45 °C and RNase digestion was carried out for 45 min at 37 °C.

**qPCR**

cDNA was synthesised from total RNA isolated from three experimental replicates using AMV reverse transcriptase cDNA synthesis kit (Roche) as per the manufacturer’s instructions with the supplied oligo (T) primers. Gene specific primers were designed using ABI Primer Express™ II software and are listed in Supplementary data. These primers were then used in a Power SYBR® Green (ABI) reaction using an ABI Prism 7000 sequence detection machine and software. The Ct values from three technical replicates and primer efficiency were then exported into REST™ 2008 software for statistical analysis, using the Pfaffl method of quantification and a pair-wise fixed reallocation randomisation test. ODC was used as the transcript for normalising the data throughout.

**Materials and methods**

**Microinjections**

Antisense morpholino oligos (AMOs) were designed to target both MyoD alleles in Xenopus laevis: XMMyoDa (Morpholino A: 5’ ACAGCTCCATAGCACCACCGCAGG 3’) and XMMyoDb (Morpholino B: 5’ GTCCCCAGGCAATACCGCAGTTG 3’). An effective dose of 10 ng Morpholino A and 30 ng Morpholino B was determined empirically and used throughout this study. For the controls, an equivalent 40 ng dose of GeneTools standard control oligo (CMO: 5’ CTTTACCTCAGTATATTATA 3’) was injected.

**Microarray sample preparation and analysis**

Total RNA was extracted from five whole embryos at NF stage 11.5 (Nieuwkoop and Faber, 1967) using TRI-Reagent (Sigma) as per the manufacturer’s instructions. Embryos were either experimental MyoD AMO or control CMO injected embryos; three separate samples were prepared from embryos from three different matings. In another experiment, total RNA was extracted from 50 animal caps, either from control embryos or embryos over-expressing MyoD. RNA was further processed using the QIAgen kit (QIAgen) followed by lithium chloride precipitation. About 5 μg of animal cap and 2 μg of whole embryo total RNA was amplified and biotin labelled using the MessageAmp™ II Biotin enhanced Single Round aRNA Amplification Kit (Ambion) as per the manufacturer’s instructions. Biotinylated aRNA was then hybridised to the array and the array scanned by the Genomics Laboratory at the University of York Technology Facility. Data were analysed using the BRB-ArrayTools plug in for Microsoft® Excel software. CEL files were imported and RNA normalisation performed. All genes which were called as absent by the software in more than 75% of samples were discarded. Class comparison analysis was carried out on the remaining genes in the case of triplicate data from knock-down embryos. For animal cap samples a 2-fold cut-off value was used to produce gene lists of up-regulated genes.

Whole mount in situ hybridisation

Antisense digoxigenin (DIG) labelled RNA probes were synthesized using the plasmids, restriction enzymes and polymerases described in the Supplementary data. In situ hybridisation was carried out as modified from Harland (1991). Briefly, de-membraned embryos were fixed in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO₄. 3.7% formaldehyde) and treated with 10 μg/ml Proteinase K before hybridising in 50% Formamide, 5 × SSC pH 7, 1 mg/ml total yeast RNA, 100 μg/ml Heparin, 1 × Denhardts, 0.1% Tween 20, 0.1% CHAPS, and 10 mM EDTA overnight at 60 °C. After extensive washes, embryos were incubated in a 1/2000 dilution of affinity purified anti-digoxigenin antibody coupled to Alkaline Phosphatase (Roche) at 4 °C overnight. Expression was visualised using BM purple (Roche).
Whole mount immunohistochemistry

*X. laevis* embryos were fixed for whole mount immunohistochemistry for 1 h in MEMFA and stored in methanol at –20°C. Embryos were rehydrated through a series of methanol/ PBS+1% Triton X-100 (Sigma) washes, blocked in PBST+0.1% BSA and then incubated at 4°C overnight in either a 1:2 dilution of 12/101 mouse monoclonal antibody tissue culture supernatant (DSHB), or 1:100 anti laminin rabbit polyclonal antibody (Sigma, L9393). After extensive washes, embryos were placed in either a 1:500 dilution of goat anti mouse F(ab) fragments coupled to horse radish peroxidase (Abcam, AB5880) or goat anti rabbit Alexa Flour® 488 (Invitrogen, A11034) and incubated overnight at 4°C. Antibody staining was visualised either by using 0.5 mg/ml DAB in PBST with 1:6000 hydrogen peroxide, or on a Zeiss LSM710 microscope using the appropriate filters. Embryos processed for confocal microscopy were cleared and mounted for imaging in 1:2 benzyl alcohol: benzyl benzoate (BA:BB).

Cryosections

Cryosectioned embryos were prepared as in Roth et al. (2010) with the following modifications: prior to sectioning, embryos fixed in MEMFA were transferred to 15% fish gelatine/15% sucrose for 16 h followed by a second 16 h incubation in 25% fish gelatine/15% sucrose. Primary antibodies used were as above, but 12/101 was used at 1:10. Secondary antibodies were a 1:250 dilution of goat anti-rabbit Alexa Flour 488® and goat anti mouse Alexa Flour® 555 (Invitrogen, A21424).

Histology

Histological sectioning and counterstaining were carried out as in Winterbottom et al. (2011). Briefly, embryos were fixed in 4% paraformaldehyde (Sigma), and stained with borax carmine (10% borax carmine, 35% ethanol) before embedding in Paraplast (Sigma) and cutting 10 μm sections. The sections were counterstained with picro blue black (97.5% saturated picric acid, 2.5% of a 1% aqueous solution of naphthaleine blue black) and mounted in Histomount (National Diagnostics).

Results

Zygotic activation of *MyoD* expression occurs during early gastrulation, from approximately NF stage 10 (Hopwood et al., 1989) and *MyoD* protein is detectable very soon after this, from NF stage 10.5 (Fig. 1A) (Hopwood et al., 1992; Harvey, 1992). The expression of contractile protein genes and other genes that characterise myogenic differentiation is not detected until 3–4 h later at late gastrula/early neurula stages. *α-cardiac actin* is one of the first skeletal muscle genes expressed and it is not detected until late gastrula stage 12.5 (Fig. 1B). In order to determine the transcriptional targets of *MyoD* prior to myogenic differentiation, we have analysed gene expression in embryos depleted of *MyoD* protein at this time-point in development: after *MyoD* protein is present and prior to the expression of differentiation genes (NF stage 11.5). As part of our analysis we have also used a data set that allows temporal expression profiling of individual genes of interest (EMBL ArrayExpress accession number E-MEXP-2059; described in Branney et al., 2009). These data are useful for this study because the early transcriptional targets of *MyoD* will increase in expression soon after *MyoD* protein is detected, and prior to the increase in expression seen for *α-cardiac actin* (red line, Fig. 1C).

Fig. 1. Temporal expression profile of *MyoD* and *α-cardiac actin*. (A) A western blot using D7F2 shows the presence of *MyoD* protein at the stages indicated. *MyoD* protein does not become detectable until stage 10.5. A background band is present at all time points and runs at a size just below that of *MyoD*. The band corresponding to *MyoD* is indicated with an arrow head. (B) RNase protection assay for *α-cardiac actin* expression, an early marker of skeletal muscle differentiation. mRNA coding for *α-cardiac actin* is not detectable until stage 12.5. ODC expression serves as a loading control. (C) A microarray time course analysis was undertaken to determine the temporal profile of *MyoD* and *α-cardiac actin* gene expression. *MyoD* mRNA levels (blue) start to rise just before its protein becomes detectable, whereas *α-cardiac actin* mRNA levels (red) do not begin to rise until stage 12.5. The time window corresponding to when myogenic precursors that express *MyoD* protein are present, but not markers of differentiation is shown by the hatched grey area. (D) Three fertilisations from different matings were used to generate three biological samples for microarray analysis. Effective knock-down of *MyoD* in each experimental set was confirmed by Western blotting for D7F2.

Large-scale analysis of gene expression in embryos lacking *MyoD*

As *X. laevis* is an allotetraploid organism, both pseudoalleles, X*MyoDa* and X*MyoDb*, must be targeted to attain complete knockdown. Fertilisations were prepared from three different female–male pairings to provide three biological samples and translation blocking antisense morpholino oligomers (AMOs) targeting both alleles were injected into both blastomeres at the two-cell stage. Effective knock-down of *MyoD* protein in each sample was determined by Western blotting (Fig. 1D) using a well-characterised monoclonal antibody, D7F2, which recognises both X*MyoDa* and X*MyoDb* (Hopwood et al., 1992). Microarray analysis was carried out on NF stage 11.5 embryos using first-generation Affymetrix GeneChip X*laevis* arrays.

Data obtained were analysed using BRB ArrayTools and a class comparison between the control and injected sets of embryos was performed (Tusher et al., 2001). After the elimination of multiple probe sets representing the same gene, and using a significance
level of $p \leq 0.05$ and a fold change of $\geq 2$. 244 genes were found to be down-regulated and 59 genes up-regulated when MyoD is knocked-down (ArrayExpress accession number E-MEXP 3272). Fig. 2 shows a scatterplot of log$_2$ gene expression values in control morpholino injected embryos at NF stage 11.5 versus embryos injected with MyoD morpholino oligos A and B. Probe sets showing greater than 2-fold changes of expression in control versus experimental groups are indicated by red and green points. Analysis of gene ontology (GO) terms associated with down-regulated and up-regulated genes was undertaken so that where possible we have classified MyoD target genes based on the GO category molecular function (MF). An initial analysis was performed using GO:Slim categories and a 1.75-fold enrichment cut-off to provide an overview of gene functions present. In order to provide a robust view of which GO terms were over-represented or under-represented in the gene groups identified, a statistical analysis of the GO terms was undertaken using the online tool DAVID (http://david.abcc.ncifcrf.gov/home.jsp), which applies a modified version of Fisher’s exact test. A significance level of $p \leq 0.05$ was applied to the MF GO terms associated with the different groups of genes. This analysis shows that all significantly over-represented GO terms associated with genes positively regulated by MyoD are associated with DNA binding and transcriptional activity (Fig. 2B). In contrast, all significantly over-represented terms associated with genes negatively regulated by MyoD are associated with cell signal transduction and nucleotide metabolism and other metabolic processes (Fig. 2C).

For our purposes, a list of genes down-regulated in MyoD knock-down embryos was compiled by comparing gene expression in MyoD AMO injected embryos to CMO injected embryos. The criteria of a 2-fold decrease in expression and a significance level of $p \leq 0.05$ were used as an initial selection tool; however, the fold increase of expression in response to MyoD overexpression in animal caps was also considered when selecting the putative targets to investigate further. Table 1 shows a curated shortlist of some of the most highly down-regulated targets in MyoD knock-down embryos and also the fold increase of each gene when MyoD is overexpressed in animal caps. The putative MyoD targets highlighted on this shortlist were analysed further in this study.

The change in gene expression shown on Table 1 is the output from Affymetrix array analysis; we have validated these data using qPCR. Data from three separate experiments (Supplementary Fig. S1A) confirm that MyoD is essential for the normal expression of the putative target genes, Esr1, Esr2, FoxC1, Msgn1, Myf5, Seb4 and Tbx6. Loss of MyoD has no effect on Xbra expression, which is included in these analyses as a control. We conclude that the expression of Esr1, Esr2, Msgn1, Tbx6, FoxC1, Seb4 and Myf5 requires MyoD during gastrula stages. To assess whether MyoD is sufficient to activate these genes in a naive tissue, mRNA coding for XrMyoD was injected into the animal hemisphere of a Xenopus embryo at the 2-cell stage. Animal cap explants were taken at blastula stages, cultured until mid-gastrula stage 11.5 and cDNA was prepared for analysis by qPCR. The graph in Supplementary Fig. S1B shows that the expression of some genes (Esr1, FoxC1, Msgn, Tbx6 and Seb4) is dramatically up-regulated by MyoD in animal caps, while the expression of others is not affected. As previously demonstrated, MyoD is not sufficient to activate the expression of Myf5 in animal caps (Fisher et al., 2003; Hopwood et al., 1991). The expression of Xbra is included as a control and its expression is not affected by the over-expression or knock-down of MyoD.

Fig. 2. Microarray analysis of MyoD knock-down embryos at NF stage 11.5. (A) A scatterplot of log$_2$ gene expression values in control morpholino injected embryos at NF stage 11.5 versus embryos injected with MyoD morpholino oligos A and B. Probe sets showing greater than 2-fold changes of expression in control versus experimental groups are indicated by red and green points. Gene ontology (GO) terms associated with down- and up-regulated genes were used to classify MyoD target genes based on the GO category molecular function (MF). (B) A table of GO terms associated with genes down-regulated in MyoD knock-down embryos. (C) A table of GO terms associated with genes up-regulated in MyoD knock-down embryos.
neural tissue in the gastrula (arrows), and in the neurogenic columns at neurula stages, and in large parts of the CNS at tailbud stages. Esr2 shows stronger expression in the mesoderm during gastrula and neurula stages than Esr1 and is also expressed in the CNS at tailbud stages. At later stages, some level of expression for each of these genes is found in the tailbud mesoderm that will generate the somites of the extending tail and give rise to skeletal muscle (Davis and Kirschner, 2000; Tucker and Slack, 1995). The expression of Tbx6, Myf5 and Seb4 is also found in the somites during tailbud stages; Seb4 is also expressed in the heart at later stages (Fetka et al., 2000).

Table 1
Fold change in gene expression in MyoD knock-down relative to control embryos as assayed by microarray. Fold change in gene expression in MyoD AMO injected embryos relative to control at gastrula stage is shown in the fourth column. The results from over-expressing MyoD in animal caps are shown in the fifth column. The targets selected for further analysis in this paper are highlighted in blue.

<table>
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<tr>
<th>Name</th>
<th>GenBank</th>
<th>Unigene</th>
<th>MyoD knock-down (fold change)</th>
<th>P-value</th>
<th>MyoD overexpression in animal caps (fold change)</th>
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<tr>
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</table>

In order to validate these putative transcriptional targets of MyoD identified in our microarray screen, the effective morpholino combination was injected unilaterally into one blastomere at the 2-cell stage and the embryos were allowed to develop to NF stage 11.5 when they were fixed and assayed by ISH for the expression of the selected target genes (Fig. 4, second column). For all target genes analysed, between 67 and 76% of the embryos analysed showed reduced expression on the antisense morpholino injected side. The number of embryos analysed in these experiments is shown in the Supplementary data (Supplementary Table S1) and the results are shown to be statistically significant (Supplementary Table S2).

To assess the ability of MyoD over-expression to up-regulate the expression of the putative target genes in developing embryos, miRNA coding for MyoD was injected into one cell at the 2-cell stage and the resulting embryos were analysed at NF stage 11.5 (Fig. 4, third column). MyoD overexpression has no effects on the expression of Myf5, while the expression of all the other targets is increased in the mesoderm in at least 83% of the embryos analysed. It is interesting that distinct target genes respond differently to MyoD overexpression; the expression of Seb4 is dramatically up-regulated throughout the injected side, while other targets including Esr1, Esr2, FoxC1, mesogenin and Tbx6 are up-regulated by MyoD but their expression is restricted to the mesodermal ring around the blastopore, suggesting that important co-factors are only present in the mesoderm or that powerful repressors are present outside this region. Xenopus Seb4 has previously been shown to be regulated by MyoD in a study using the overexpression of MyoD-GR and MyoD-EnR (Li et al., 2010). Myf5 is not up-regulated by MyoD overexpression, consistent with previous reports (Fisher et al., 2003; Hopwood et al., 1991) suggesting that Myf5 expression requires additional regulators that are limited and tissue specific. The ability of MyoD to activate the expression of these targets in naive animal cap explants was also tested (Supplementary data Fig. S1B) and largely consistent with these findings.

To confirm that the reduced expression seen in the knockdown embryos is specifically due to the lack of MyoD, mRNA coding for MyoD was co-injected with the morpholino to assess its ability to rescue gene expression (Fig. 4, fourth column). In at least 71% of the embryos analysed, gene expression was rescued by co-injecting the AMOs with mRNA coding for XitMyoD, a transcript that lacks the morpholino target sequences. Xenopus brachyury (Xbra) is expressed throughout the early mesoderm (Smith et al., 1991) and its expression was analysed as a control (Fig. 4H). The finding that Xbra expression is not affected by overexpression or knock-down of MyoD shows that the mesoderm in general is not being disrupted and supports the argument that
These experiments have identified a specific set of genes that are transcriptionally regulated by MyoD.

**MyoD interacts directly with regulatory regions in the Esr1, Esr2, Seb4, FoxC1, and Myf5 genes**

In order to activate transcription, MyoD forms heterodimers with E-proteins and binds a consensus sequence (CANNTG) called an E-box (Blackwell and Weintraub, 1990). E-boxes have been identified in the regulatory regions of many muscle-specific genes (Weintraub et al., 1991). We used multiple genome alignments to identify conserved sequences around the putative target genes, outside of coding regions, as the presence of conserved sequences would indicate that the region is under selection pressure and could be involved in the regulation of gene expression. Human, chicken, zebrafish and mouse genomes were compared to *X. tropicalis* genomic sequences in order to provide a good coverage of evolutionary distance (alignments shown in Supplementary data). E-boxes were found in regions around Esr1, Esr2, FoxCl, Myf5 and Seb4, including two in the 3’ UTR of Seb4 and one in the coding region of FoxCl (Fig. 5 and Supplementary data). No conserved E-boxes were found in the proximal promoters of mesogenin or Tbx6. Furthermore, because of their position at the end of the *X. tropicalis* genome scaffolds and a relatively poor degree of synteny it was not possible to perform a longer range analysis than this. In a recent study by Li et al. (2010) five E-boxes were found within 650 bp of the transcriptional start site of *X. tropicalis* Seb4 and found to be responsive to MyoD. However, these sequences are not conserved in other vertebrates and as such were not included in our study.

It has recently been shown that MyoD binds to many promoters and modifies chromatin throughout the genome in mice (Cao et al., 2010) and invertebrates (Lei et al., 2010). We have investigated the chromatin conformation flanking our putative MyoD targets using data available through www.xenbase.org. Work from Gert Veenstra’s lab using ChIP-seq identifies regions of the *X. tropicalis* genome that feature Histone 3 Lysine 4 and Lysine 27 tri-methylation.

**Fig. 3.** Expression patterns of shortlisted targets. In situ hybridisation analysis of Esr1, Esr2, FoxCl, mesogenin (MSGN1), Myf5, Seb4 and Tbx6 expression patterns at gastrula, neurula and tailbud stages. All the putative target genes are expressed circumblastoporally at gastrula stages. After gastrula stages, many of the targets are still expressed in the posterior mesoderm in the tailbud region of the embryo, which is the source of myogenic progenitors at this stage. On the right, a temporal expression profile, based on microarray data, is shown for each putative target genes depicting relative expression levels from NF stage 8 to stage 15. The expression of MyoD is shown in the three bottom panels for comparison.
Fig. 4. Validation of the effect knocking down MyoD on shortlisted targets. Embryos were injected unilaterally with 10 ng of Morpholino A and 30 ng of Morpholino B (knock-down), 2.5 ng XtMyoD mRNA (overexpression) or both (rescue). Control embryos were injected with the equivalent amount of control MO. The injected side is shown by an asterisk. Numbers and percentages indicate the number of embryos with the phenotype shown. Numbers are from several experiments. The knock-down of MyoD protein causes the loss of expression of all shortlisted targets. Overexpression of MyoD up-regulates the expression of most targets, with the exception of Myf5. Target expression is rescued effectively for all targets when XtMyoD mRNA is co-injected with morpholino. Xbra is expressed in all mesoderm and is included as a control. Embryos have been analysed at mid-gastrula Nieuwkoop stage 11.5 and are viewed vegetally. For numbers and statistical analysis see Supplementary data Table S1.
modifications (H3K4me3 and H3K27me3) at gastrula stages. These modifications indicate transcriptionally active regions of DNA, or regions of potentially localised gene expression (Akkers et al., 2009). The H3K4me3 is associated with active promoters and enhancers (Santos-Rosa et al., 2002) and we have found that this mark is present in regions overlapping or close to the E-boxes identified in our analyses of the putative gene targets regulated by MyoD (see Supplementary Fig. S3).

In order to determine whether MyoD directly interacts with the identified E-boxes Chromatin immunoprecipitation (ChIP) was carried out using XtMyoD injected X. tropicalis embryos and the D7F2 Xenopus MyoD antibody. Primers were designed to the relevant X. tropicalis genome sequence and ChIP was carried as in Blythe et al. (2009). An unconjugated mouse IgG was used as a negative control for immunoprecipitation. We found that each of the putative target genes analysed had at least one E-box that interacted with MyoD protein (Fig. 5). The E-box pair in Myf5.b was the only region identified in our Myf5 alignment to be pulled down with MyoD. This region is located within a 785 bp fragment known as the MRR that was previously shown to contain elements required for the normal regulation of Myf5 expression in embryos (Polli and Amaya, 2002).

Fig. 5. MyoD binds directly to genomic sequences of target genes. Chromatin immunoprecipitation (ChIP) analysis of conserved E-boxes in target genes. Alignments of several vertebrate genomes were used to identify conserved E-boxes proximal to the putative target genes (see Supplementary data) and primers were designed to amplify these regions of genomic DNA. Embryos were analysed at NF stage 11.5 by ChIP using the MyoD antibody D7F2. Above shows that MyoD is capable of binding to conserved E-boxes found in the genome near Myf5 (Myf5.b, ~1.2 Kb), ESR1 (ESR1.b, ~212 bp), ESR2 (ESR2.a, ~6.3 Kb), FoxC1 (FoxC1.a, ~1.3 Kb), and SEB4 (SEB4.a + 8746, SEB4.b + 9411). MyoD does not bind to distant E-boxes found in the 5’genome upstream of SEB4 and Myf5 (SEB4.d, SEB4.e, Myf5.c), or the 5’upstream region of ESR1 (ESR1.a). PCR products present in the ChIP lane that are indistinguishable from the negative control lane are considered a negative result (SEB4.b). All diagrams are to scale (note the doubled scale for the SEB4 outline). Distances given are from transcriptional start sites. Red triangles represent E-boxes bound by MyoD, black triangles E-boxes found not to be bound by MyoD.

As several of the early transcriptional targets of MyoD identified are known to be important for somitogenesis (Cha et al., 2007; Kume et al., 2001; Oginuma et al., 2008; Tazumi et al., 2008; Topczewska et al., 2001; Wang et al., 2007; White et al., 2003; Yoon and Wold, 2000), we directly assessed the morphology of somites in MyoD knock-down embryos. We used immunohistochemistry and histology to examine somite structure at swimming tadpole stages (NF stage 40). We used the antibody 12–101 to detect skeletal muscle and anti-laminin to detect somite boundaries. We have also used DAPI staining to mark the nuclei which are known to align neatly down the middle of the mononucleated myofibres that span the length of a somite in Xenopus embryos.

Unsurprisingly, 12/101 staining shows that differentiated skeletal muscle is present in embryos lacking MyoD, consistent with findings in mice (Kassar-Duchossoy et al., 2004; Rudnicki et al., 1993). Anti-laminin staining shows that somite boundaries form (Fig. 6A and B), but can be irregular and reveal that the overall shape of some somites is more rounded (Fig. 6B) and unlike the sharp chevron shape typical of somites (Fig. 6A). By analysing both 12–101 to detect skeletal muscle and anti-laminin to detect somite boundaries. We have also used DAPI staining to mark the nuclei which are known to align neatly down the middle of the mononucleated myofibres that span the length of a somite in Xenopus embryos.

Somite morphology is disrupted in Xenopus embryos lacking MyoD

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embryo lacking MyoD (Fig. 6E and bracket in Fig. 6E'). This does not occur all along the axis, but only in a few anterior somites. It has previously been shown that overexpressing a dominant negative form of Delta2 can cause a similar, but more widespread disruption of somites (Jen et al., 1997); the effects caused by knocking-down MyoD are milder and localised to only a few early forming somites.

**Hatching is delayed in Xenopus embryos lacking MyoD**

Functional analyses of the myogenic regulatory genes in mice have established redundant roles for MyoD and Myf5 in the determination of myogenic cell lineage, where the presence of one or two alleles of either regulator can support myogenesis (Rudnicki et al., 1993). It is not surprising that when MyoD is knocked-down in *Xenopus*, skeletal muscle develops and by NF stage 40 tadpoles swim normally. *Xenopus* embryos hatch from the vitelline membrane at about NF stage 28 using strong coordinated twitching movements. We found that embryos in which MyoD is knocked-down (n=34) the average hatching time was delayed by a period of approximately 12 h at 23 °C compared to embryos injected with control morpholino (n=28). A Wilcoxon rank sum test on this data shows that this result was statistically significant (P=0.05). From these observations we conclude that although skeletal muscle differentiates in these embryos, there is a significant delay before it becomes fully functional.
A transient requirement for MyoD

To determine when MyoD is required for the expression of these genes during early development, we have analysed gene expression in sibling embryos at NF stage 11 (gastrula) and NF stage 14 (neurula) when MyoD is knocked-down as compared to embryos injected with control morpholino oligo. Mesogenin and Tbx6 are expressed in the presegmental mesoderm (PSM) during neurula stages when somites begin to segment from the posterior mesoderm and we have analysed the effects of MyoD knockdown on the expression of these genes at this stage. Fig. 7(A–F) shows embryos unilaterally co-injected with mRNA coding for beta-galactosidase together with morpholino oligos, such that the injected side in each panel is apparent by the presence of pale blue nuclei and is labelled with an asterix. In contrast to the dramatic down regulation of Mesogenin and Tbx6 expression on the MyoD knock-down side at gastrula stages (Fig. 7A and D; also see Fig. 4), at neurula stages the expression of these genes in the posterior mesoderm has largely recovered (Fig. 7C and F). qPCR data also indicate that this set of genes require MyoD for the activation of expression in the mesoderm during gastrula stages (stage 11), but at later stages (stage 14) the expression of these genes is independent of MyoD (Fig. 7G).

To assess the earliest formation of somites we used Delta2 expression as a marker (Fig. 7H–O). At open neural plate stages, Delta2 is expressed during the earliest segmentation of the paraxial mesoderm (arrows Fig. 7H). When MyoD is knocked-down unilaterally (Fig. 7I and J; knock-down side marked with an asterix), this early expression of Delta2 is lost. In contrast, at later neurula stages, Delta2 continues to be expressed on the side of the embryo lacking MyoD (asterix side Fig. 7L and M) but its expression indicates that segmentation is shifted slightly to the anterior (note the position of the arrows). By tailbud stages (Fig. 7N and O), Delta2 is expressed in two stripes (see arrows) in the posterior paraxial mesoderm in embryos injected with control morpholino (Fig. 7N) as well as embryos injected with morpholinos targeted against MyoD (Fig. 7O). This suggests that the effects of MyoD on somitogenesis are transient, as the expression of Delta2, and the other genes analysed are sensitive to the loss of MyoD at early stages but not later. This transient effect is also apparent from the somite phenotype that is restricted to anterior region of the trunk. In Fig. 7O some disruption of somite structure can be seen by the failure of the blue nuclei to align in anterior somites (see also Supplementary Fig. S5).

We also looked directly at the formation of somites from the paraxial mesoderm of late neurula/early tailbud (NF stage 21) using immunohistochemistry (Fig. 7P). The MyoD knock-down side (asterix) is labelled with RFP (red), and the cell morphology is revealed by staining with anti-beta catenin (green) which labels cell membranes and DAPI (blue) which labels nuclei. On the right (control) side, we have used an imaging tool to label a single cell in the pre-segmental mesoderm (PSM) which shows a typically elongated cell oriented perpendicular to the notochord. As somites form, the cells rotate to a position parallel to the axial structures (note the highlighted cells in S0, SI, and SII on the control side of Fig. 7P).

In the absence of MyoD (side labelled with an asterix), we were unable to find any elongated cells to highlight in the PSM. In more
anterior positions on this side we have identified and highlighted some elongated cells in the process of rotating; these cells tend to be thinner and restricted to medial positions. In addition the nuclei are delayed in aligning on the MyoD knock-down side (arrows) while the contralateral somitic nuclei are aligned by SIII. This analysis suggests that in the early tailbud (stage 21) the mechanism of somitogenesis is disturbed in the absence of MyoD, and the effects of this are apparent later as malformed anterior somites.

**Discussion**

*A set of genes transcriptionally activated by MyoD in the early mesoderm*

One interesting finding from our analysis is that *Myf5* requires MyoD for its early expression in the gastrula stage *Xenopus* embryo. This is in contrast to the relationship of these genes in the mouse, where *myf5* is the first MRF gene expressed in the epaxial part of the somite as a response to Shh signalling (Borycki et al., 1999; Ott et al., 1991) and Myf5 is required to activate the expression of *myoD* (Tajbaksh et al., 1997). It is implicit that additional *Myf5*-independent mechanisms exist to activate *myoD*, as the expression of *myoD* is only delayed and not absent in *myf5* mutant mice. In light of our results it is interesting to note that the formation of the myotome is delayed in *myf5* null mice (Kaul et al., 2000). In zebrafish, *myoD* and *myf5* are essential, but redundant, for the earliest, medial myogenesis (Hinits et al., 2009). However, in contrast to our findings, *myf5* is expressed normally in fish myotoblast morphants (Hinits et al., 2009). The timing of the analysis in the zebrafish study was at somite stages, while our work analysed gene expression at gastrula stages and it is possible that the regulatory relationships among the MRFs are different at earlier stages. A single element upstream of *myf5*, containing the E-box identified in Fig. 5, has been shown to be necessary and sufficient for correct *myf5* expression during gastrulation stages in *Xenopus* (Polli and Amaya, 2002), while in mouse there are multiple interdigitated control sequences required for *myf5* expression during somite stages (Summerbell and Rigby, 2000).

Another gene we found to require MyoD for its expression is *Seb4*, which has previously been recognised as having a role in myogenesis (Li et al., 2010). *Seb4* codes for a RNA binding protein and its *Caenorhabditis elegans* orthologue, Sup-12, was shown to be a muscle specific splicing factor (Kuroyanagi et al., 2007). The expression of *Seb4* in frogs is remarkably similar to that of *myoD*, with additional domains of expression in the heart and retina (Fetka et al., 2000).

*Tbx6* is also known to be important for skeletal muscle development (Chapman et al., 2003; Mitani et al., 1999; White et al., 2003). Injection of mRNA coding for *Xenopus* Tbx6 induces the expression of α-cardiac actin in animal cap explants (Uchiyama et al., 2001). When Tbx6 is knocked down in frogs, the expression of both *XWnt8* and *FGF8* are markedly reduced and when Tbx6 is injected into animal caps, both are up-regulated (Li et al., 2006; Lou et al., 2006). Since both Wnt8 and FGF8 are known to activate MyoD expression (Fletcher and Harland, 2008; Hoppler et al., 1996), and we have shown that MyoD regulates Tbx6 expression, these data point to a model where MyoD acts in a feed-forward circuit, in addition to auto-regulating its own expression (Cao et al., 2010; Thayer et al., 1989).

**Effectors of notch signalling are transcriptional targets of MyoD**

*Esr1* and *Esr2* code for effectors of the Notch signalling pathway and we have shown here that these genes require MyoD for their expression during gastrula stages. In addition, there is a weight of evidence that *Tbx6*, *mesogenin* and *FoxC1* act genetically upstream of Notch signalling during somitogenesis. In mouse, a hypomorphic *Tbx6* mutant expresses significantly lower levels of the Notch target gene and ligand, *Delta1* (Beckers et al., 2000). Furthermore, Tbx6 binding sites upstream of *Delta1* are required for its normal expression (White and Chapman, 2005). Previous experiments in *Xenopus* found that MyoD transcriptionally activates the expression of the Notch ligand *Delta1* (Wittenberger et al., 1999) at the same early time point analysed in our study. *Delta1* was not identified in our analyses as it is not present on the Affymetrix GeneChip *Xenopus* leavis 1.0 array.

Notch signalling is important for maintaining a population of myogenic precursors, and MyoD may be involved in this process by regulating *Esr1* and *Esr2*, as well as *Delta1* (Wittenberger et al., 1999) in early determined myoblasts. Notch signalling has been shown to be important in regulating the choice between differentiation and self-renewal in myogenic progenitors; a mouse with a hypomorphic *Delta* allele shows reduced amounts of skeletal muscle due to the premature differentiation of progenitor cells (Schuster-Gossler et al., 2007). Similar effects are seen in mice where the effector of Notch signalling, RBP-J, has been knocked-out specifically in muscle cells (Vasyutina et al., 2007). Together, these data suggest that Notch plays a role in myogenesis analogous to its well established role in neurogenesis, where activation of the Notch signalling pathway prevents differentiation and maintains populations of precursor cells. Given the targets identified in this paper, it is apparent that MyoD plays a role in the transcriptional network regulating the expression of some genes important for Notch signalling in the skeletal muscle cell lineage.

The Notch signalling pathway is also a key regulator of somitogenesis (Dequeant et al., 2006). Cyclic expression of HES genes in the anterior PSM is a key feature of the somitotic clock, and *chirality* was first of these genes found to cycle during chick somitogenesis (Palmeirim et al., 1997). It is now known that the specific HES genes that show cyclic expression vary among vertebrates (Krol et al., 2011). In *Xenopus*, only the expression of *Esr9* (Li et al., 2003) and *Esr2* (Blewitt, 2009) has been shown to cycle in the posterior mesoderm at neurula stages. In a recent transcriptomic analysis Hes5 and Hes1 orthologues were found to be uniquely conserved as cycling genes in chick, mouse and zebrafish (Krol et al., 2011) The *X. tropicalis* orthologues of *X. laevis* *Esr1* and *Esr2* are annotated as *Hes 5.1* and *Hes 3.3*, respectively. Our in silico analyses suggest *Esr2* (*Hes3.3*) is closely related to *Esr1* (*Hes5.1*) and these genes group together with *Esr9* and with human *Hes5* on the same branch of an unrooted phylogenetic tree of Hes genes (Supplementary Fig. 54). This suggests that consistent with what has been described for chick, mouse and zebrafish, the cycling genes in frogs are *Hes5* related.

*Hes6* has been found to inhibit cell cycle withdrawal and skeletal muscle differentiation in both *Xenopus* and *C2C12* cells (Cossins et al., 2002). These activities of *Hes6* were found to be independent of its ability to bind DNA, but require the WRP domain, suggesting an important role for protein–protein interaction. *Hes6* is known to interact with other transcriptional regulators via this domain, in particular Groucho/TLE4 (Murai et al., 2007) which has been shown to inhibit MyoD expression in gastrula stage *Xenopus* embryos (Burks et al., 2009). MyoD is activated in the *Xenopus* mesoderm by FGF signalling (Fletcher and Harland, 2008; Fisher et al., 2002), which can inhibit the repressive activity of Groucho and *Hes6* (Murai et al., 2007; Burks et al., 2009). Together these studies put *Hes6* regulation of myogenesis upstream of MyoD; interestingly, overexpression of *Hes6* leads to disrupted somite formation similar to those described in our study (Cossins et al., 2002).

Notch maintains progenitor populations in many cell lineages, including skeletal muscle (Brack et al., 2008) and MyoD is essential for the establishment of myogenic progenitors (Rudnicki et al., 1993). Our finding that MyoD regulates components of the Notch pathway, *Esr1* and *Esr2*, establishes a previously unsuspected link
between these two key regulatory pathways involved in progenitor specification. It is interesting that several MyoD targets, in addition to Esr2, are known to be important for the segmentation of somites. The mouse knockout of Msgn1 exhibits a complete failure of somitogenesis (Yoon and Wold, 2000). Foxc1 is essential for somitogenesis in zebrasfish (Topczewska et al., 2001), while Msgn1 and Tb6x have been found to interact during somite development in Xenopus (Oginuma et al., 2008; Tazumi et al., 2008). It is worthwhile noting that other studies have used large-scale gene analyses to investigate targets of MyoD in cultured muscle cells using forced expression of MyoD (Bergstrom et al., 2002; Wyzykowski et al., 2002), and analysing primary myoblasts from MyoD knock-out mice (Ishibashi et al., 2005). None of the genes we describe here were identified in these studies using cultured cells; it is unlikely that the set of genes described here could have been identified using anything but an in vivo approach.

A MyoD-like transcription factor co-ordinated myogenesis with somitogenesis?

Somites are transient embryonic structures that are characteristic of vertebrates. They arise progressively in the posterior of the embryo from the paraxial mesoderm and first appear as blocks of epithelial cells adjacent to the neural tube and notochord. Signals from the midline axial tissues are essential for initiating myogenesis (Emerson, 1993). All the skeletal muscles in the vertebrate body are derived from somites, while other somite cell gives rise to skeletal structures such as the vertebrae and ribs, and others go on to form the dermis of the skin (Brand-Saberi et al., 1996; Christ and Ordahl, 1995). In addition, the somites also provide cues for migrating axons and thereby impart segmental pattern to the peripheral nervous system (Keynes and Stern, 1984).

In this way, the coordinated development of segmented mesodermal derivatives of the somite promotes the maturation of an exquisitely patterned musculoskeletal system. Drosophila, C. elegans and sea urchin each have a single myoD related gene, while lower chordates have two MRFs: Branchiostoma floridae and Ciona related gene, while lower chordates have two MRFs: an exquisitely patterned musculoskeletal system. Mesp genes (Schubert et al., 2003). There are four MRF genes in most vertebrate genomes and these genes are redundantly essential for development in zebrafish (Topczewska et al., 2001), while Msgn1 exhibits a complete failure of somitogenesis in Xenopus embryos. Dev. Cell 17, 425–434.

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