Mef2d Acts Upstream of Muscle Identity Genes and Couples Lateral Myogenesis to Dermomyotome Formation in Xenopus laevis

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Abstract

Xenopus myotome is formed by a first medial and lateral myogenesis directly arising from the presomitic mesoderm followed by a second myogenic wave emanating from the dermomyotome. Here, by a series of gain and loss of function experiments, we showed that Mef2d, a member of the Mef2 family of MADS-box transcription factors, appeared as an upstream regulator of lateral myogenesis, and as an inducer of dermomyotome formation at the beginning of neurulation. In the lateral presomitic cells, we showed that Mef2d transactivates Myod expression which is necessary for lateral myogenesis. In the most lateral cells of the presomitic mesoderm, we showed that Mef2d and Paraxis (Tcf15), a member of the Twist family of transcription factors, were co-localized and activate directly the expression of Mef2d, which acts upstream of Pax3 expression during dermomyotome formation. Cell tracing experiments confirm that the most lateral Meox2 expressing cells of the presomitic mesoderm correspond to the dermomyotome progenitors since they give rise to the most dorsal cells of the somitic mesoderm. Thus, Xenopus Mef2d couples lateral myogenesis to dermomyotome formation before somite segmentation. These results together with our previous works reveal striking similarities between dermomyotome and tendon formation in Xenopus: both develop in association with myogenic cells and both involve a gene transactivation pathway where one member of the Mef2 family, Mef2d or Mef2c, cooperates with a bHLH protein of the Twist family, Paraxis or Sx5 (Scleraxis) respectively. We propose that these shared characteristics in Xenopus laevis reflect the existence of a vertebrate ancestral mechanism which has coupled the development of the myogenic cells to the formation of associated tissues during somite compartmentalization.

Introduction

Embryonic and foetal muscle fiber development in vertebrates takes place by the appearance of successive myogenic waves. However, myotome formation presents some differences between mammals and anamniotes. In mammals, newly formed somites are a naïve tissue which subdivides into dorsal dermomyotome and ventral sclerotome [1]. Next myogenic cells arising from dermomyotomal lips give rise to myotome. So, dermomyotome formation is the initial event of myogenesis and all muscle cells of the trunk and limbs derive from the dermomyotome [2]. In anamniotes, somites are not a naïve tissue since the first myogenesis leads to early myotome formation [3–6]. The initial subdivision of the myotome between medial and lateral myogenic populations appears as a common scheme in anamniote myogenesis [3–6]. In Xenopus, medial and lateral myogenesis develops directly from presomitic mesoderm. During neurulation, the medial myogenic cells differentiate first before somite formation and gives rise to muscle fibers located near the notochord. Next, the lateral myogenic cells differentiate at the time of somitogenesis and gives rise to dorsomedial and ventrolateral cell populations [6]. The second myogenic wave which takes place at the tailbud stage arises from epaxial and hypaxial levels of the dermomyotome [7,8] like in amniotes. The Xenopus dermomyotome has been indeed described at the tailbud stage and is constituted by a cell layer on the dorsal surface of the somites [8]. However, the dermomyotome formation in Xenopus, probably takes place earlier in a particular context where presomitic cells are already subjected to myogenic signals.

Myogenesis is regulated by the four basic helix-loop-helix transcription factors, Myod, Myf5, Mrf4 (Myf6) and Myogenin (Mgn), known as the muscle regulatory factors (MRFs). Genetic circuitry regulating myogenesis has evolved depending on the location in the body, either through the myogenic program itself, or through the upstream regulators of the determination factors [9]. In zebrafish and mouse, Myf5 and Myod act as determination factors during myogenesis. Mrf4 has also been identified as a determination factor but only in mouse somites during embryonic myogenesis in Myf5/Myod double mutant [2,10]. In the same way, absence of Myf5 and Mrf4 affects specifically myogenesis in extraocular muscles during mouse craniofacial development [11]. Moreover, Myf5-dependent and -independent lineage was revealed by ablation of Myf5-expressing cells during mouse somitogenesis [12,13]. Distinct myogenic programs expressing only Myf5, only Myod or both have also been characterized during
somitic and craniofacial myogenesis in *Xenopus* [6]. Concerning the upstream regulators of myogenesis, *Pitx2* and *Tbx1* play a major role in activating myogenesis in craniofacial muscles [11], while *Pax3* and *Pax7* are expressed in myogenic precursors in mouse somites [9].

In this work, we focused on the molecular determinants specifying the lateral presomitic cells to a myogenic fate and we identified *Mef2d* as an upstream regulator of muscle identity genes. The myocyte enhancer factor 2 (Mef2) family of MADS (MCMI, agamous, deficiens, serum response factor) box transcription factors has four members in vertebrates, Mef2A, -B, -C and -D. Mef2 proteins form homo- and hetero-dimers and bind to a conserved A/T-rich sequence known as a MEF2 site. Mef2 acts through protein–protein interactions with other transcription factors, to either activate or inhibit specific sets of target genes. The Mef2 family proteins bind directly promoters or enhancers of the majority of muscle genes and interact with members of the *Myod* family of basic helix-loop-helix (bHLH) proteins to activate the skeletal muscle differentiation program [14–16]. The four *Mef2* genes exhibit overlapping but distinct expression patterns in embryonic and adult tissues in mice [17] and play a pivotal role in cell differentiation during myogenesis of skeletal, cardiac and smooth muscles. Mef2 proteins are expressed after the myogenic determination factors during mouse embryogenesis and do not seem involved in the initiating events of skeletal myogenesis [17,10]. Evidence for a role of the Mef2 transcription factors in skeletal myogenesis are essentially coming from cell culture experiments [14,19], mouse single gene invalidation experiments [17,18], and mouse embryonic and adult tissues in mice [17] and play a pivotal role in cell differentiation during myogenesis of skeletal, cardiac and smooth muscles. Mef2 proteins are expressed after the myogenic determination factors during mouse embryogenesis and do not seem involved in the initiating events of skeletal myogenesis [17,10]. Evidence for a role of the Mef2 transcription factors in skeletal myogenesis are essentially coming from cell culture experiments [14,19], mouse single gene invalidation experiments [17,18], and mouse embryonic and adult tissues in mice [17].

In *Xenopus*, using a series of gain and loss of function experiments of *Myod*, *Mef2d*, *Paraxis* and *Meox2*, we first showed that the lateral myogenesis appeared to be *Myod*-dependent. *Mef2d* is expressed before *Myod* at the beginning of neurulation in a large domain of the presomitic mesoderm and appears to be an upstream regulator of *Myod* expression in the lateral presomitic cells. Mef2d also drives dermomyotome formation, as evaluated by Pax3 expression at the tailbud stage. We next identified the dermomyotome progenitors at the beginning of neurulation in the most lateral part of presomitic mesoderm, at the border of lateral myogenic cells. *Mef2d* and *Paraxis* (*Tcf15*), a member of the Twist family of transcription factors, are colocalized in this region. Both are necessary for the expression of *Meox2*, a dermomyotome marker in *Xenopus* [23], which acts upstream of *Pax3* expression during dermomyotome formation. Although *Myod* function in lateral myogenesis is conserved between zebrafish and *Xenopus*, the early function of *Xenopus Myf2d* in coupling lateral myogenesis to dermomyotome formation is different from zebrafish and mammals. These results suggest that mechanisms driving somite compartmentalization had dramatically evolved in vertebrates. However, it seems that at least a part of this genetic regulatory network has been used in mammals since *Mef2* acts upstream of *Myod* during mouse skeletal muscle regeneration [24].

**Materials and Methods**

**Ethics Statement**

This work uses early *Xenopus* embryos. All experimental procedures used in this study followed the recommendations of the “Comité National de Réflexion Ethique sur l’Experimentation Animale” of the Ministry of Higher Education and Research and were approved by local Animal Care and Use Committees.

**Microinjection of Synthetic mRNA**

All pSP64T plasmids used for microinjection were linearized with Xba I. Capped mRNAs were produced *in vitro* from linearized plasmids using the SP6 message machine kit (Ambion). *Xenopus* embryos were injected unilaterally at the two-cell stage at the marginal zone level with 10 to 400 pg of synthetic mRNA and fixed at different stages for whole-mount in situ hybridization or extracted at stage 11 for western blot analysis.

**Morpholino Antisense Oligonucleotide (MO) Injections**

Several translation-blocking antisense morpholino oligonucleotides were purchased from Gene Tools (see table 1 and figure S1). In the pseudotetraploid *Xenopus laevis* species, two genes were cloned for each myogenic factor, *Myod* [25,26], *Mef2d* [27] and data not shown), *Myf5* [28], and *Meox2* [29,30]. In some cases, mixtures containing two morpholinos directed against each sequence were used: moMyod1 is the 1:1 mixture of moMyod1a and moMyod1b, moMyf5 is also the 1:1 mixture of morpholinos a and b. The sequence of moMrf4-1 and -2 is complementary to the two Mrf4 *Xenopus laevis* genes, moMrf4c, moMrf4c, moMrf4c and moMrf4c-1c are the mismatch controls of the corresponding oligomorpholinos. To assess the toxicity of MO injections, dose response experiments were performed by unilateral injection of 10 ng to 30 ng of control oligomorpholinos at the two-cell stage. We did not see any phenotypic variation in whole mount in situ hybridization following injection of 30ng control morpholinos per embryo. Injection of moMyod1 or moMyod2 gives rise to the same phenotype. All data presented herein derive from moMyod1. Two genes have also been identified for *Mef2d*, *Paraxis* and *Meox2*. Concerning *Mef2d*, moMef2d1c and moMef2d2c are respectively the mismatch controls of moMef2d1 and moMef2d2. All data presented herein derive from moMef2d1 since the same results were obtained with the two morpholinos. moMef2d1 is the 1:1 mixture of moMef2d1a and -1b. In the case of *Paraxis* and *Meox2*, all data derived from moParaxis1 and moMeox2-1. Concerning *Paraxis*, moParaxis2 is the 1:1 mixture of moParaxis2a and -2b. moParaxis1c and moParaxis2c are respectively the mismatch controls of moParaxis1 and moParaxis2. MoMeox2-1c and moMeox2-2c are respectively the mismatch controls of moParaxis1 and moParaxis2. We also used a morpholino XIMOF8 directed against *Fgf8* mRNA which has already been characterized [31].

**Constructs and Directed Mutagenesis**

For expression in *Xenopus* embryos, all constructs were subcloned into pSP64T-Flag to generate a C-terminal Flag tagged protein. The pSP64T-Myf5F, -Myf5F (F for Flag) plasmids have already been described [28]. This is also the case for Mef2dF and Mef2dGRF [32]. *Paraxis*F, *Paraxis*GRF, *Meox2*F and *Fgf8b* were cloned into pSP64T using “PCR-directed cloning”, a method developed in our laboratory [32] and by [33]. All these plasmids contain the 5'- and 3'-UTR of globin gene, the coding sequence of the corresponding gene and the coding sequence of Flag epitope (except for *Fgf8b*). To test the activity of morpholinos, pSP64T plasmids containing the 5'-UTR of *globin* gene were replaced by the 5'-UTR of *Myf5a* or b, *MyoD* or b, *Mrf4*, *Mef2da* or b, *Paraxis* and *Meox2* using PCR-directed cloning. These plasmids were named pSP64T-5utrMef2F, -5utrMef2F, -5utrMef2F and -5utrParaxis. pSP64T-5utrMyf5, Mefod and Mef2d are the 1:1 mixture of a and b forms. In order to construct rescue plasmids coding for a synthetic *Paraxis* mRNA which must not be recognized by moParaxis1, part of the sequence complementary to the morpholino was mutated using the quick change
Immunohistochemistry and Embryo Sections

Western Blot

Western blotting experiments with anti-Flag antibodies have already been described [35].

Whole-mount in situ Hybridization, Immunohistochemistry and Embryo Sections

cDNA for whole-mount in situ hybridization probes were prepared by RT-PCR (primers presented in table S1). Probes were carried out as previously described [29]. Double whole-mount in situ hybridizations were performed with fluorescein-labeled and digoxigenin-labeled RNA probes, and two successive color reactions (BCIP, Sigma-Aldrich, Saint-louis, MO and BM-purple, Roche applied science, Mannheim, Germany) separated by heat inactivation (65°C, 30 min) of alkaline phosphatase. For transverse sections, embryos were fixed 2 hours in MEMFA, embedded in 4% agarose, and sectioned at a thickness of 40 μm with a vibratome. For whole-mount immunohistochemistry, the concentrated muscle-specific 12/101 monoclonal antibody from Developmental studies hybridoma bank (University of Iowa) was used (1:2000). The primary antibody was either detected with an alkaline phosphatase-conjugated anti-mouse antibody (Vector Laboratories) diluted at 1:2000 followed by reaction with BM-purple or Magenta phosphate (Sigma-Aldrich) or with Alexa fluor donkey 488 anti-mouse secondary antibody (Life technologies). In cases where both in situ hybridization and 12/101 staining were carried out, embryos were first stained using in situ hybridization, immediately followed by immunohistochemistry.

Table 1. Sequence of oligomorpholinos.

| Oligomorpholino name          | sequence                                                                 |
|-------------------------------|--------------------------------------------------------------------------|
| moMyf5a                        | 5’-GATGTCTAGTGAGTGAAGCATC-3’                                           |
| moMyf5b                        | 5’-CTGATCTAGTGAGTGAAGCATC-3’                                           |
| moMyf5c                        | 5’-GATGTCTAGTGAGTGAAGCATC-3’                                           |
| moMyoD1a                       | 5’-GCGAAGGTCCCTAGAAGAACACCGG-3’                                        |
| moMyoD1b                       | 5’-GCGAAGGTCCCTAGAAGAACACCGG-3’                                        |
| moMyoD1c                       | 5’-GCGAAGGTCCCTAGAAGAACACCGG-3’                                        |
| moMyoD2a                       | 5’-GATCCAAATCGGGGGCTGACAGG-3’                                          |
| moMyoD2b                       | 5’-CAAAGTCTACACTCCACGGAAGTG-3’                                         |
| moMyoD2c                       | 5’-CAAATCAGACTGCAAGGAACGT-3’                                           |
| moMRF4-1                       | 5’-ATCGGTTCCATCATATGCTTGGCC-3’                                         |
| moMRF4-1c                      | 5’-ATCGGTTCCATCATATGCTTGGCC-3’                                         |
| moMef2dF1a                     | 5’-ATCTCTCATGACCCGAGAGACTT-3’                                          |
| moMef2dF1b                     | 5’-ATCTCTCATGACCCGAGAGACTT-3’                                          |
| moMef2dF1c                     | 5’-ATCGTGCTGGTCCAGATCTGCA-3’                                           |
| moMef2dF2                      | 5’-AGCTGCTGGTCCAGATCTGCA-3’                                            |
| moparaxis1                     | 5’-AACGGATCATGCTGAAAGGGACGTG-3’                                         |
| moparaxis2a                    | 5’-CCATGTTGACCCCTGGGCCAATC-3’                                          |
| moparaxis2b                    | 5’-CCATGTTGACCCCTGGGCCAATC-3’                                          |
| momeox2-1                      | 5’-CAGCTGCTGAGTACCCGAGATGTA-3’                                         |
| momeox2-1c                     | 5’-CAGCTGCTGAGTACCCGAGATGTA-3’                                         |
| momeox2-2                      | 5’-GTGTGCTGAGTACCCGAGATGTA-3’                                          |
| momeox2-2c                     | 5’-GTGTGCTGAGTACCCGAGATGTA-3’                                          |
| XIMOFl                        | 5’-GGAGGTGATTGAGTCTGCTGCTG-3’                                          |

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mutagenesis method (Stratagene, Agilent technologies). These constructs, named pSP64TParaxisF', present 10 mismatches in the nucleotide sequence recognized by the corresponding morpholinos. For immunoprecipitation experiments, Mef2d was subcloned into pcDNA3.1/V5/HIS (Invitrogen) and Paraxis into pBIND (Promega) vectors using PCR-directed cloning. For luciferase assay, Xenopus tropicalis Mef2 promoter (-907 to +80) [34] was cloned into pG5 vector (Promega) by PCR-directed cloning and named Pgl4p. These constructs, named Pgl4p were cloned into pGl4 vector (Promega) by PCR-directed cloning. For luciferase Paraxis into pcDNA3.1/V5/HIS (Invitrogen) and probe, probes were prepared by RT-PCR (primers presented in table S1). Probes were carried out as previously described [29]. Double whole-mount in situ hybridizations were performed with fluorescein-labeled and digoxigenin-labeled RNA probes, and two successive color reactions (BCIP, Sigma-Aldrich, Saint-louis, MO and BM-purple, Roche applied science, Mannheim, Germany) separated by heat inactivation (65°C, 30 min) of alkaline phosphatase. For transverse sections, embryos were fixed 2 hours in MEMFA, embedded in 4% agarose, and sectioned at a thickness of 40 μm with a vibratome. For whole-mount immunohistochemistry, the concentrated muscle-specific 12/101 monoclonal antibody from Developmental studies hybridoma bank (University of Iowa) was used (1:2000). The primary antibody was either detected with an alkaline phosphatase-conjugated anti-mouse antibody (Vector Laboratories) diluted at 1:2000 followed by reaction with BM-purple or Magenta phosphate (Sigma-Aldrich) or with Alexa fluor donkey 488 anti-mouse secondary antibody (Life technologies). In cases where both in situ hybridization and 12/101 staining were carried out, embryos were first stained using in situ hybridization, immediately followed by immunohistochemistry.

Lineage Tracing Experiments using Fluorescent Tracers

Stage 13 embryos were injected using pulled-glass capillaries either with WGA-rhodamine (dissolved at 200 μg/ml, Vector laboratories) to be analysed at stage 18 or with both WGA-rhodamine and -fluorescein (Vector laboratories) to be analysed at stage 23. Injections with WGA-rhodamine and -fluorescein were made at the most lateral level and in the lateral level of the trunk presomitic mesoderm respectively. All embryos were photographed at the beginning of the experiment, then incubated for 5 to 10 hours at 22°C until stage 18 or 23 and fixed in 4% formaldehyde. The fate of stained cells was directly analyzed on 50 μm transversal vibratome sections at the trunk level (stage 23) or after immunofluorescent experiment with the 12/101 antibody and Alexa fluor donkey 488 anti-mouse secondary antibody (stage 18). The experiment was repeated six times to verify that similar experimental results were obtained.

Microdissection Experiments

Dechorionated embryos were oriented and immobilized in 0.4XMMR 1% agarose gel slits. Using sharpened needles, anteroposterior incision in the ectoderm was realized at stage 14 on the dorsal side of the embryo at the mediolateral or lateral level. Ectoderm and mesoderm were separated from each other on the lateral incision side and the superficial part of the mesoderm was removed. The sham-operated embryos were treated without removing the mesoderm. Embryos were fixed immediately or following development until stage 19 or 30–32. Embryos were analysed by whole mount in situ hybridization.

Inhibition of Fgf Receptor Signalling at the End of Gastrulation

Normal embryos or embryos unilaterally injected at the two-cell stage with Mef2dF mRNA were cultured until stage 12. 20 nanoliters of DMSO solution or a 5 mM DMSO stock of the Fgf receptor kinase inhibitor SU5402 (Calbiochem) were injected into the archenteron. Embryos were cultured until stage 15/16 and were processed for in situ hybridization.

Cycloheximid and Dexamethasone Treatment

To induce glucocorticoid fusion variants, embryos were treated with 10 μM dexamethasone (DXM; Sigma) at stage 12.5–13 for 2 hours or until the tailbud stage. To block protein synthesis, embryos were incubated with 20 μg/ml cycloheximid (CHX; Sigma) in 0.4XMMR for 45 min [36,37] and then treated for hormone induction of glucocorticoid fusion variants with 10 μM DXM for 2 hours.
Transfections, Coimmunoprecipitation and Luciferase Assays

Cell culture of COS7 cells was described previously [30]. Transfections or co-transfections of pGFPmex2-luc, pBIND-Paraxis and pcDNA3-Mef2dV5/HIS were performed in 24-well plates using FuGENE 6 (Roche Applied Science) and luciferase activity was measured 48h after transfections using the dual luciferase system (Promega). Transfection assays were repeated 4 times. For co-immunoprecipitation assays, transfections or cotransfections of pBIND, pBIND-Paraxis and pcDNA3-Mef2dV5/HIS were realized in 6-well plates and protein were extracted 48h after transfections. V5/His tagged Mef2d constructs were immunoprecipitated using Ni-NTA beads (Invitrogen), followed by western blotting procedures. Antibodies used included mouse monoclonal anti-V5 (Invitrogen) and mouse monoclonal anti-GaH (Santa Cruz).

Results

Gene Knock Down Experiments Reveal that Myod is Critical for Xenopus Lateral Myogenesis

In Xenopus, lateral myogenesis is characterized by a myogenic program expressing Myod at a strong level, Myogenin at moderate levels and Myf5. In contrast, the medial cell population expresses a different combination of MRF: Myf5, Myod and Myf4 [6]. We first studied the role of the three determination MRFs using gene knockdown experiments to identify essential factors involved in lateral myogenesis. Translation was efficiently inhibited by injections of the blocking MO (moMyod1, moMyf5, moMrf4-1) and the corresponding mRNA coding for the flag-tagged MRF (Fig. 1A, lane 3). Then, embryos were unilaterally injected with 20 ng of MO at the two-cell stage and differentiation of the lateral myogenesis was analyzed at the beginning of the neurulation (stage 14) (Fig. 1B). The three MRF morphants showed little variation in Actc (Cardiac β-actin) expression, whereas Myod morphants displayed a slight decrease in Desmin expression in 50% of the analyzed embryos (n = 62). At later stages, only Myod morphants displayed a sharp decrease in Desmin (73.3%, n = 70), MyhE3 (70%, n = 50) and Myogenin (71.4%, n = 56) expression (Fig. 1B). Desmin, MyhE3, and Myogenin expression at this stage correspond to the differentiation of lateral myogenesis [6]. Transverse sections of somites at stage 26 indicated that the myotome development is specifically affected in Myod morphants (Fig. 1C). Staining with the muscle specific 12/101 antibody on transverse sections at stage 22 confirmed that only the lateral myogenesis is affected in Myod morphants (70%, n = 10) since the first differentiated myogenic cells of medial myogenesis, located near the notochord [6] remains strongly stained (Fig. 1D). Mismatch control MOs had no effect (see supporting information). Furthermore, the specificity of Myod oligomorpholino was corroborated by a partial rescue of Myod mRNA expression (85.3%, n = 60) when co-injected with Myod mRNA (Fig. 1E) and was confirmed by another set of translation blocking MO against Myod (moMyod2) and Myf4 (moMrf4-2) (see supporting information). Previous knock down experiments carried out with another Myf5 translation blocking MO also showed no drastic effect on myogenesis [39]. Altogether these results showed that Myod is required for the lateral myogenesis in Xenopus, as supported by previous works in zebrafish [3].

Mef2d is Required for Lateral Myogenesis by Activating Myod Expression

Next, we were interested in the members of the Mef2 family of transcription factors which cooperate with Myod during myogen-esis. Mef2a and Mef2d are expressed during the first wave of Xenopus myogenesis [32]. Mef2a expression has the profile of a differentiation gene as Desmin or Myf5 [6]; it begins to be expressed at stage 13 in paraxial mesoderm (data not shown). In contrast, Mef2d expression [32] is similar to Myod one [6,40] and it was early detected by whole-mount in situ hybridization in presomitic mesoderm in gastrula at stage 11 [32] suggesting that Mef2d could play an early role. Therefore, we compared Mef2d expression with Myod one. During gastrulation, Myod expression is concentrated around the blastopore and in the medial region of paraxial mesoderm (Fig. 2A, st. 11 to 12). At the gastrulation-neurulation transition time, the residual expression of Myod in the posterior region disappears (Fig. 2A, st. 12.5–13, brackets) before its new extension in the lateroposterior region (Fig. 2A, st. 13–14). These results were in accordance with the previously described Myod mRNA expression pattern at stage 12.5/13 [41] and 13/14 [42]. At mid-gastrulation Mef2d expression is faint and localized inside the anterior Myod expression domain (Fig. 2A, st. 11 to 12). At the gastrulation-neurulation transition time, Mef2d expression begins to be strong and extends lateroposteriorly before Myod. (Fig. 2A, st. 13–14, arrows and Fig. 2B, arrows). Myod has been identified as a Mef2 target gene in Xenopus [43,44], therefore we investigated Mef2d function in the lateral myogenesis using gain and loss of function experiments. Injection of Mef2d mRNA (Mef2dF) induced a lateral enlargement of the Myod expression domain (68.4%, n = 38) at stage 15 (Fig. 2C, brackets) and enhanced Desmin expression (70.3%, n = 37) at stage 18 (Fig. 2C). For knockdown experiments, we used the same procedure as for MRF oligomorpholinos to test the specificity and the efficiency of translation inhibition (Fig. 2D). The Mef2d morphants displayed no Myod mRNA expression (67.3%, n = 62) in the lateral presomitic mesoderm at stage 16 (Fig. 2E, brackets). Differentiation of the lateral myogenic wave was also strongly affected by the moMef2d1 morpholino injection since Desmin (Fig. 2E) and MyhE3 (data not shown) were not expressed at stage 18, (70.2%, n = 57) although Desmin expression of the medial myogenesis remained unaffected (72.1%, n = 49) at stage 16 (Fig. 2E). Rescue experiments totally restored Myod expression (75%, n = 60) in the lateral presomitic mesoderm and partially (70.6%, n = 68) Desmin (Fig. 2F) and MyhE3 mRNA (data not shown) expression in somites. Since a feed-forward loop initiated by Myod appears to be involved in the regulation of Mef2 expression [45,46], we tested whether Myod may induce Mef2d expression. Myod mRNA (MyodF) injection resulted in a strong ectopic expression of Actc (78.3%, n = 35), without significantly altering Mef2d (100%, n = 40) expression (Fig. 2G). Likewise, moMyod1 had no effect on Mef2d expression (70%, n = 44) in presomitic mesoderm (Fig. 2G). In conclusion, Mef2d controls Myod expression during Xenopus lateral myogenesis.

Mef2d is Necessary for the Activating Effect of Fgf on Lateral Myod Expression at the Early Neurula Stage

In Xenopus, Fgf is a major player in the mesoderm maintenance [47,48]. In addition, Fgf induces Myod expression in an animal cap assay [49] and, like Mef2d, leads to a lateral enlargement of Myod expression domain [31,50]. In particular, Fgf8 is strongly expressed in the posterior mesoderm during neurulation [31,51] and is involved in lateral last myogenesis in zebrafish [4]. Here, unilateral injections of Fgf8 at the two-cell stage not only induced Xbra (T) (85.5%, n = 35) and Myod overexpression (73.7%, n = 38), as already reported [31], but also that of Mef2d (69.4%, n = 56) (Fig. 3A and B). In the presence of moMef2d1, Fgf8 activation of Myod transcription was abolished (60%, n = 40); it was rescued (69.2%, n = 39) by co-injection with Mef2dF (Fig. 3A). Fgf8
Figure 1. Myod is required for lateral myogenesis. (A) Western blot with anti-flag and anti-tubulin antibodies of gastrula embryos injected bilaterally at the two-cell stage with 300 pg of SutrMyf5F, SutrMyodF or 70 pg of SutrMrf4F synthetic mRNAs alone or with oligomorpholinos. Arrows point out the specific signal. Lane 1: synthetic mRNA alone, 2: synthetic mRNA+moControl, 3: synthetic mRNA+specific mo (moMyf5, moMyod1 or moMrf4-1 with SutrMyf5F, SutrMyodF or SutrMrf4F respectively). (B) Whole-mount in situ hybridization of embryos unilaterally injected with 20 ng of moMyf5, moMyod1 or moMrf4-1 and fixed at stages 14 or 18/19. β-galactosidase mRNA (blue) was co-injected to identify the injected side, indicated by an asterisk (*). Dorsal views. The anterior side of the embryos is on the left; st., stage. (C) Transverse sections of the morphants at stage 26. (D) Transverse section of the Myod morphant submitted to whole-mount immunohistochemistry with the 12/101 antibody. (E) Rescue experiments: Embryos were injected unilaterally with 20 ng of moMyod1 alone or co-injected with synthetic mRNA coding for MyodF (150 pg) and probed with MyhE3. Nc, notochord. Probes are in a framed box and indicated for each panel. For complete statistical data, see supporting information, figure S2. doi:10.1371/journal.pone.0052359.g001
induction of Mef2d was not affected (75%, n = 48) by moMyod1 (Fig. 3A). Conversely, the Fgf8 morphants, injected unilaterally with the characterized oligomorpholino XIMOF8 [31], showed not only a loss of Myod (69%, n = 42) expression (Fig. 3C, brackets), but also a loss of Mef2d expression (63.8%, n = 58) in the lateral presomitic mesoderm (Fig. 3C). The Myod expression domain was rescued by co-injection (68.2%, n = 44) with Mef2dF (Fig. 3C). Moreover, the injection of a Fgf receptor inhibitor (SU5402) in the archenteron cavity at stage 11.5/12 results in the loss of lateral expression of Myod (73.6%, n = 53) and Mef2d (63.8%, n = 52) at stage 16 (Fig. 3D). A previous unilateral injection of Mef2dF at the two-cell stage partly rescued Myod (68.7%, n = 48) expression (Fig. 3D). Altogether, these data demonstrate that Mef2d is necessary for the activating effect of Fgf on the lateral Myod expression domain at the neurula stage.

Mef2d Drives the Formation of the Dermomyotome

The size of the muscle compartment stained with the 12/101 antibody at stage 25–26 was enlarged in the dorsal somitic region of embryos injected with Mef2dF (66.6%, n = 9) and decreased in Mef2d morphants (73%, n = 8), particularly in the hypaxial region, confirming that Mef2d promotes myogenesis (Fig. 4A). As the most lateral cells of the paraxial mesoderm express Mef2d at the beginning of neurulation (Fig. 2A) and give rise later to the most dorsal somitic structures [6], we hypothesized that Mef2d could also be involved in dermomyotome formation, which was analyzed by Pax3 expression at tailbud stages 28–32. Pax3 [8] like Pax7 [52]
Figure 3. Mef2d is necessary for the activating effect of Fgf on Myod expression at the neurula stage. (A) Embryos were injected unilaterally either with Fg8b synthetic mRNA alone (5 pg), or with moMyod1 or with moMef2d1, fixed at the gastrula stage and submitted to in situ hybridization for Xbra, Myod or Mef2d. Co-injection of Mef2dF mRNA with moMef2d1 was able to rescue the phenotype of moMef2d1 embryos (+Mef2dF). (B) Unilateral injection of Fg8b mRNA induced the lateral expansion of Mef2d expression domain at stage 16. (C) Embryos were injected unilaterally with 20 ng of XIMOF8 and fixed at stage 16. Co-injection of Mef2dF mRNA with XIMOF8, a MO that co-localizes with Xbra, Myod or Mef2d. (D) Embryos were injected at stage 11.5/12 with DMSO or 5 mM SU5402, an inhibitor of Fgf signaling, and fixed at stage 16. A first unilateral injection of Mef2dF mRNA at the two-cell stage was able to rescue the phenotype of treated embryos (+Mef2dF). Injected side (*) at the bottom except in (A), on the left. Bracket indicates the position of lateral myogenic cells. Probes are in a framed box and indicated for each panel. For complete statistical data, see supporting information, figure S2.

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begins to be expressed in the most dorsolateral region of the somites from stage 17/18 (Fig. 4B). At stage 22/23, Pax3 expression is restricted to dermomyotome (Fig. 4C), especially in the hypaxial region. A Mef2dF injection induced an increase of Pax3 expression (66%, n = 94) in the dorsal somitic region and, in one third of the embryos, a faint ectopic Pax3 expression in the lateral mesoderm (Fig. 4D, arrows). Conversely, Mef2d morphants exhibited a decrease of Pax3 expression (71.4%, n = 49) that was restored in rescue experiments (67.3%, n = 50). In addition, one third of the rescued embryos showed an increase of Pax3 expression in somites and an ectopic expression in the lateral mesoderm (Fig. 4D, arrows). Altogether, these results demonstrate that Mef2d promotes Pax3 expression during dermomyotome formation.

Mef2d Cooperates with Paraxis to Promote Dermomyotome Formation

As injections of Mef2dF only slightly affect Pax3 expression, we looked for a partner which could cooperate with Mef2d to induce Pax3 expression. Paraxis, a bHLH transcription factor of the Twist family, is a good candidate for several reasons. First, Paraxis is necessary for hypaxial myogenesis and dermomyotome formation in mouse [53]. Second, we have shown previously by gain of function experiments that another member of the Twist family, Sex (Scleraxis), cooperates with another member of the Mef2 family, Mef2i, to induce the expression of the tendon structural genes Tgfi (beta-IgH3) and Tnc (tenasin-c) [31]. In Xenopus, Paraxis is expressed in somites [54], particularly in the most lateral part of paraxial mesoderm (Fig. 5A and B, st. 13, rounded brackets), co-localizing with Mef2d at stage 13 (Fig. 5A and B, st. 13). At stage 17/18, Paraxis is expressed in the most dorsolateral part of the somites (Fig. 5 B, st. 17/18), a region where Pax3 and Pax7 are also expressed (Fig. 4B). This observation is confirmed at stage 23 (Fig. 5C). To identify the role of Paraxis, loss of function experiments were realized. The efficiency of translation inhibition was demonstrated by successive injections of a blocking MO (moparaxis1) and the mRNA coding for flag-tagged Paraxis (Fig. 5D). An injection of moparaxis1 induces a decrease of Pax3 expression (71.7%, n = 68) at the tailbud stage, and this phenotype was rescued by injection of ParaxisF (79%, n = 61) (Fig. 5E). Overexpression experiments resulted in an increase of Pax3 expression. While only 20% of the embryos displayed this phenotype with ParaxisF (data not shown), an inducible ParaxisGRF injection resulted in Pax3 overexpression in 75% of the embryos (n = 62) (Fig. 5F). As both transcription factors, Mef2d and Paraxis, modulate Pax3 expression, we tested a putative cooperative effect and found that Pax3 expression was strongly enhanced in dermomyotome and extended to the ventral region in 20% of the embryos co-injected with Mef2dF and ParaxisF and in 70% of the embryos co-injected with Mef2dF and ParaxisGRF (n = 40) (Fig. 5F). As expected, injections of moparaxis1 abolished Pax3 overexpression (11.3%, n = 62) induced by a ParaxisGRF injection (Fig. 5F).

Mef2d and Paraxis Target Meox2 Gene in the Dermomyotome Progenitors at the Beginning of Neurulation

At the beginning of neurulation, Mef2d co-localizes with Paraxis in the most lateral part of paraxial mesoderm, a region preferably corresponding to the precursors of dermomyotome (Fig. 5A and B, st. 13). From stage 17/18, Mef2d expression profile (Fig. 5B, st. 17/18) is no longer expressed in the most dorsolateral part of the somites where Pax3 begins to be expressed (Fig. 4B). Therefore, the
The effect of Mef2d on Pax3 expression should be indirect. Thus, we looked for a direct target gene of Mef2d and Paraxis in the progenitors of dermomyotome. Among genes involved in somite formation, Meox2 is expressed in these Mef2d/Paraxis expressing cells at stage 14 (Fig. 6A, st. 14, rounded brackets). Later, at stage 17/18, Meox2 is present in the most lateral region of the somites (Fig. 6A, st. 17/18) where Pax3 and Pax7 begin to be expressed dorsally, and at stage 23 in the most dorsal part of the somites corresponding to the dermomyotome (Fig. 6A, st. 23). Meox2 has already been reported as a marker of dermomyotome in Xenopus [23] and the double KO mice for the two Meox genes, Meox1 and 2, display drastic anomalies in all somitic derivatives, dermomyotome included with severely reduced pax3 expression [55]. To confirm that the Meox2 expressing cells at stage 14 give rise to the dermomyotome, we realized cell tracing experiments by injecting fluorescent dyes at stage 13 in the most lateral part of presomitic mesoderm. The location of the fluorescent dye (red) at stage 18 in the lateral part of the somitic domain near the muscle fibers stained by 12/101 antibody corresponds to the Meox2 expression domain (Fig 6B, stage 18). At stage 23, the comparison of the fates of the lateral cells (WGA-fluorescein/green) to the most lateral cells (WGA-rhodamine/red) indicated that the most lateral cells of presomitic mesoderm give rise to the most dorsal cells of the somites, corresponding to the dermomyotome (Fig 6B, stage 23). To support this view, we proceeded to ablation of the most lateral cells of presomitic mesoderm at stage 14, checked that Meox2 expression was decreased at stage 19 (87%, n = 18) (Fig. 6E) and showed that absence of these cells dramatically affected dermomyotome formation evaluated by Pax3 expression at the tailbud stage (87.8%, n = 33) (Fig. 6E). Neither the sham-operated embryos (73.7%, n = 19) (Fig. 6C), nor the ablation of less lateral presomitic mesoderm (85%, n = 20) (Fig. 6D) or lateral plate mesoderm (data not shown) leaded to a decrease in Pax3 expression. These data confirm that dermomyotome comes from the most lateral cells of the presomitic mesoderm. By gain and loss of function experiments, we tested the effect of Paraxis and Mef2d on Meox2 expression during neurulation. Meox2 expression decreased in both Mef2d (60%, n = 30) and Paraxis (66.7%, n = 33) morphants (Fig. 7A). The phenotype was rescued by injection of Mef2dF (67.1%, n = 45 without CHX, 69%, n = 58 with CHX) or ParaxisF (66.7%, n = 33) mRNA respectively (Fig. 7A). To test if Meox2 could be a direct target gene of Paraxis and Mef2d, ParaxisGRF and Mef2dGRF were induced to translocate into nuclei by dexamethasone, after translation inhibition by cycloheximide. Induction of ParaxisGRF (71.1%, n = 45 without CHX, 69%, n = 58 with CHX) or MEF2GRF (61.2%, n = 55 without CHX, 61.2%, n = 62 with CHX) led to a moderate increase of Meox2 expression, with or without cycloheximide treatment (Fig. 7B). The strongest effect was achieved when both (61.5%, n = 65 without CHX, 73.6%, n = 55 with CHX) were co-injected (Fig. 7B). These experiments suggest a combined action on Meox2 promoter, which was verified by luciferase assays on the proximal promoter of Xenopus tropicalis Meox2 gene, fused to a luciferase reporter (pmeo2-luc). Luciferase activity of pmeo2 was induced in COS7 cells transiently.
immunoblotting against Gal4, indicating that Mef2d physically interacts with Paraxis, co-immunoprecipitation assays were realized by using extracts from COS7 cells overexpressing a V5/His tagged full length Mef2d and Paraxis on a transverse section at stage 23. (D) Western blot with anti-flag and anti-tubulin antibodies of late gastrula embryos injected bilaterally either with 300 pg of 5utrParaxis synthetic mRNA alone (lane1) or with oligomorpholinos: moControl (lane 2) or moParaxis1 (lane3). (E) Embryos injected unilaterally with 20 ng of moParaxis1 were submitted to in situ hybridization with Pax3 antisense probe at the tailbud stage (lateral view or transverse section). A co-injection of ParaxisF' mRNA (150 pg) with moParaxis1 was able to rescue the phenotype of moParaxis1 injected embryos (lateral view). (F) Unilateral injection of ParaxisGRF (150 pg) induced an increase of Pax3 expression after co-injection of ParaxisF-Mef2d or ParaxisGRF-Mef2d. ParaxisGRF injection with moMef2d1 had no effect on Pax3 expression. (*) Injected side. Probes are in a framed box and indicated for each panel. Nc, notochord. For complete statistical data, see supporting information, figure S2.

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cotransfected with Mef2d, Paraxis or both (Fig. 7C). Combined transfections of Mef2d with Paraxis were required to get a maximal induction of luciferase activity, confirming the cooperative effect of Mef2d and Paraxis on Meox2 expression (Fig. 7C). To test whether Mef2d could physically interact with Paraxis, co-immunoprecipitation assays were realized by using extracts from COS7 cells overexpressing a V5/His tagged full length Mef2d with either Gal4 alone or full length Paraxis fused to Gal4 (Fig. 7D). Mef2d-V5/His was immunoprecipitated with Ni-NTA beads, specific for the C-terminal His tag of Mef2d. Gal4-Paraxis was detected by immunoblotting against Gal4, indicating that Mef2d physically interacts with Paraxis (Fig. 7D).

Meox2 Promotes Dermomyotome Formation
Downstream of Paraxis and Mef2d

The role of Meox2 on dermomyotome formation was investigated by gain and loss of function experiments. The efficiency of translation inhibition was demonstrated by injections of a blocking MO (moMeox2-1) and the mRNA coding for flag-tagged 5utrMeox2 (Fig. 8A). Unilateral injection of Meox2 morpholinos (moMeox2-1) at the two-cell stage induced a decrease of Pax3 expression (68.3%, n = 82) at stage 30 which was restored (68.9%, n = 61) in rescue experiments (Fig. 8B). Conversely, a Meox2 injection at the two-cell stage induced an extension of Pax3 (61.5%, n = 81) expression domain at stage 30 (Fig. 9C). As mentioned previously, the timing of Mef2d accumulation during Xenopus embryogenesis does not support a direct activation of Pax3 gene in dermomyotome. Moreover, the lateral extension of Pax3 expression domain observed after co-injection of Paraxis and Mef2d could be a consequence of the combined action of Paraxis and Mef2d on Meox2 expression in the most lateral part of the presomitic mesoderm at the beginning of neurulation. So, we examined the hierarchical relationship existing between Mef2d/Paraxis and Meox2 on Pax3 expression. At stage 30, Mef2d (76.3%, n = 72) morphants (Fig. 8D) as well as Paraxis (76.2%, n = 84) morphants (Fig. 8E) exhibited a decrease of Pax3 expression domain in the injected side that was restored (62.5%, n = 80 for Mef2d morphants, 67.6%, n = 68 for Paraxis morphants) by the injection of Meox2 (Fig. 8D, 8E). These data clearly demonstrate that Meox2 acts downstream of Paraxis and Mef2d on Pax3 expression during dermomyotome formation.

Discussion

This report reveals a previously unrecognized regulatory network within myogenesis and points out an unexpected and pivotal role of one member of the Mef2 family, Mef2d, as a coupling factor of lateral myogenesis and dermomyotome formation in Xenopus embryos.

Mef2d Acts as an Upstream Regulator of Myod Expression during Lateral Myogenesis in Xenopus

We have previously shown that during myotome formation, the medial myogenesis gives rise to the first differentiated fibers located near the notochord and the lateral myogenesis gives rise to dorsomedial and ventrolateral cell populations [6]. The lateral myogenesis is characterized by a myogenic program with strong

Figure 5. Paraxis and Mef2d have a cooperative effect on dermomyotome formation. (A) Expression of Paraxis mRNA during neurulation. Costaining of Mef2d (blue) and Paraxis (purple) mRNA at stage 13 in comparison with Mef2d expression alone. Rounded brackets indicate the region of colocalization of Paraxis and Mef2d. Dorsal views. The anterior side of the embryos is on the left; st., stage. Vertical lines define the limit between anterior and trunk region. (B) Transverse sections of the costained embryos compared to Mef2d staining at stage 13 (upper panels). Expression of Paraxis and Mef2d at stage 17/18 (lower panels). Dotted lines indicate the position of the medial and lateral population of myogenic cells. (C) Expression of Paraxis mRNA on a transverse section at stage 23. (D) Western blot with anti-flag and anti-tubulin antibodies of late gastrula embryos injected bilaterally either with 300 pg of 5utrParaxis synthetic mRNA alone (lane1) or with oligomorpholinos: moControl (lane 2) or moParaxis1 (lane3). (E) Embryos injected unilaterally with 20 ng of moParaxis1 were submitted to in situ hybridization with Pax3 antisense probe at the tailbud stage (lateral view or transverse section). A co-injection of ParaxisF' mRNA (150 pg) with moParaxis1 was able to rescue the phenotype of moParaxis1 injected embryos (lateral view). (F) Unilateral injection of ParaxisGRF (150 pg) induced an increase of Pax3 expression at the tailbud stage. Pax3 expression after co-injection of ParaxisF-Mef2d or ParaxisGRF-Mef2d. ParaxisGRF injection with moMef2d1 had no effect on Pax3 expression. (*) Injected side. Probes are in a framed box and indicated for each panel. Nc, notochord. For complete statistical data, see supporting information, figure S2.

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Discussion

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Myod expression and without sustained Myf5 expression [6]. Here, we showed by loss of function experiments, that Myod is necessary for lateral myogenesis as observed in zebrafish [5]. Myf2d expression extends in a large paraxial domain and precedes Myod expression in lateral presomitic mesoderm. By gain and loss of function experiments, we showed that Myf2d is required for Myod expression in lateral presomitic mesoderm. A Myf2 binding site has been indeed described on Xenopus MyoD promoter, and was shown as necessary for Myod expression in cell culture [43,44]. However, in our experiments, Myf2d alone is not able to induce ectopic expression of Myod, which means that other factors probably participate to the Myf2 transactivation activity on the Myod promoter. Moreover, Myf2 morphants seem essentially affected in the hypaxial region of the myotome and express normal level of Myogemin in the epaxial region during the second myogenic wave (data not shown), suggesting that signals emanating from the neural tube and the dorsal ectoderm might be sufficient for the development of the epaxial region.

The function of Me2d seems not conserved in anamniote, as MeF2 proteins are expressed after Myod and is not involved in the control of Myod expression during lateral myogenesis in zebrafish [56]. The early activity of MeF2 is actually similar in Drosophila and Xenopus, as Drosophila MeF2 regulates several steps of muscle differentiation, from regulation of muscle identity genes to terminal muscle differentiation process [57,58]. It should also be noted that a recent work [24] showed that Me2F protein cooperates with the coactivator MASTR to regulate the transcription of Myod during mouse skeletal muscle regeneration. MASTR is also expressed in the Xenopus lateral presomitic mesoderm [59]. Although the function of Me2F protein seems not conserved during embryonic myogenesis between Xenopus, zebrafish and mouse, at least part of this genetic circuitry has been used in mammals in the context of muscle regeneration.

Fgf plays various roles during early development of Xenopus, including the development of dorsal mesodermal structures [60]. In particular, Colas et al. [50] have described an inhibitory effect of Fgf on the development of intermediate mesoderm to the benefit of muscle development, during gastrulation. Moreover, Fgf can induce Myod expression in cap animal assays [61] or during development [31] either directly [61] or indirectly [62]. In zebrafish, lateral Myod-dependent myogenesis [5] develops in an Fgfg dependent way. In Xenopus, gain and loss of function experiments showed that Fgfg modulates Myod expression in the lateral paraxial mesoderm essentially [31], suggesting that Fgfg constitutes an important signal for lateral myogenesis. The treatment of Xenopus embryos with the inhibitory drug SU5402 from stage 11.5/12 (this work) suggests that the Fgf effect could continue until the beginning of neurulation where Fgf7 is expressed in the posterolateral region [31]. While Myod is clearly expressed in the lateral marginal zone at the beginning of gastrulation [40] and could be directly activated by Fgf, its expression seems to decrease afterwards in this region. In this report, we showed that the later Fgf effect on lateral Myod expression is mediated by Me2F, as suggested by the appearance of Me2F promoter-binding activity in the beginning of neurulation [44]. Moreover, two important works from Harland laboratory [40,41] are consistent with this shift in Myod gene regulation at the gastrulation-neurulation transition. First, the initial expression of Myod in the ventralateral domain in gastrula is not sufficient to give rise to muscle tissue in culture [40] suggesting that the initial Myod expression is transient in this region. Second, Myod gene is the target of a new inductive signal from neural plate during gastrulation, beginning probably at the end of gastrulation and preceding during early neurulation [41]. Indeed, Me2F seems to be a good candidate to be the relay of the neural plate signal in mesoderm, but the relationship between the neural plate signal on one hand, Me2F and Fgfg on the other hand, needs to be determined. Finally, other transcription factors could be involved, such as Egr1 that controls Myod expression in an Fgf dependent way [62]. The relation between Egr1 and Me2F in lateral myogenesis remains also to be established.

Identification of Dermomyotome Precursors in Xenopus

Even if we cannot totally exclude that some cells outside of the presomitic mesoderm could be mixed with presomitic mesoderm to participate in dermomyotome construction, altogether, gene expression data, cell lineage tracing, ablation and gain-and-loss of function experiments strongly suggest that dermomyotome precursors are localized in the most lateral part of the presomitic mesoderm (Fig. 9A). To support our data, it has already been shown in Xenopus that satellite cells originate from lateral mesoderm [63] but the authors concluded that they originate “from the dorsolateral plate rather than from the paraxial mesoderm”. Considering that satellite cells come from the dermomyotome in amniotes [64,63] and in the light of our results showing lateral expression of Meox2, it could be proposed that most satellite cells originate from dermomyotome precursors rather than from dorsolateral plate mesoderm. Concerning the epaxial region of somites, its development seems independent from the formation of lateral dermomyotome precursors since it develops almost normally in Me2F morphants. The compartmentalization of the presomitic and somitic mesoderm in Xenopus presents some characteristics that could impose specific constraints and explain the existence of dermomyotome precursors. First, it is well known that paraxial mesoderm, notochord and neural tube are subjected to extension convergent movements during neurulation leading to an anteroposterior elongation of the embryo [66–68]. These extension movements are particularly strong in paraxial mesoderm. As cells are specified early, it seems necessary to
couple cellular movements to the cellular fate to have the right tissue at the right place. Second, the presomitic mesoderm and the newly formed somites are not naive tissue due to the presence of specified and differentiated muscle cells. In this context, some cells of the somites may be protected from myogenic signals to remain competent to form the dermomyotome. Would Meox be able to maintain the competence of these cells? In this case, since the two Meox genes are expressed in the entire naive somites of amniotes [55,69] and are involved in dermomyotome and sclerotome formation [55], the function of Meox genes could be similar in amniotes and anamniotes.

Figure 7. Paraxis and Mef2d targets Meox2 gene in the most lateral part of the presomitic mesoderm. (A) Embryos injected unilaterally with 20 ng of moParaxis1 or moMef2d1 were submitted to in situ hybridization with Meox2 antisense probe at the neurula stage. Injection of either ParaxisF’ mRNA with moParaxis1 or Mef2dF with moMef2d1 was able to rescue the phenotype. (B) Unilateral injection of either ParaxisGRF or Mef2dGRF induced an increase of Meox2 mRNA expression at the neurula stage after induction by dexamethasone (DXM) at stage 12.5. A cooperative effect was observed after co-injection of ParaxisGRF and Mef2dF. A treatment by cycloheximid (CHX) followed by induction by dexamethasone (DXM) at stage 12.5 indicated that Meox2 is a direct target gene of Paraxis and Mef2d. (*) Injected side. Probes are in a framed box and indicated for each panel. (C) COS7 cells were transfected with pmeox2-luc alone, or co-transfected with either Paraxis, Mef2d-V5/His or both and luciferase activity was determined 48 h after transfection. * P<0.01 (D) Protein extracts from COS7 cells transfected with Mef2d-V5/His alone or with either empty vector (Gal4) or Gal4-Paraxis construct were immunoprecipitated (IP) with Ni-NTA beads and subjected to Western blot (WB) using an anti-Gal4 antibody (upper panel). Input control experiments with anti-V5 (lower panel) or anti-Gal4 (mid panel) antibodies. For complete statistical data, see supporting information, figure S2.

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Mef2d Couples Dermomyotome Formation and Lateral Myogenesis in Xenopus

In amniote myogenesis, Paraxis is necessary for the development of the hypaxial region of the somites [53] and has been involved in the regulation of somite epithelialization and the maintenance of the epithelial state of dermomyotome [70]. Here, we identified a new and early function of Paraxis in activating Meox2 expression in the dermomyotome progenitors which develop in association with lateral myogenic cells in Xenopus (Fig. 9A). Mef2d is also involved in dermomyotome formation. It co-localizes with Paraxis in the most lateral part of presomitic mesoderm, interacts with Paraxis by co-immunoprecipitation and activates the MyoD gene.

Figure 8. Meox2 acts downstream of Paraxis and Mef2d on dermomyotome formation. (A) Western blot with anti-flag and anti-tubulin antibodies of late gastrula embryos injected bilaterally either with 300 pg of 5utrMeox2F synthetic mRNA alone (lane1) or with oligomorpholinos: moControl (lane 2) or moMeox2-1 (lane3). (B) Embryos injected unilaterally with 20 ng of moMeox2-1 were submitted to in situ hybridization with Pax3 antisense probe at the tailbud stage (lateral view or transverse section). A co-injection of Meox2F mRNA with moMeox2-1 was able to rescue the phenotype of moMeox2-1 (lane 5). Arrows indicate hypaxial expression of Pax3. (*) Injected side. Nc, notochord. For complete statistical data, see supporting information, figure S2.

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Figure 9. Schematic representation of myotome and dermomyotome formation in Xenopus. (A) The most lateral Meox2 expressing cells of the paraxial mesoderm give rise to the dermomyotome (De) whereas the lateral myogenic cells give rise to a dorsomedial (DoMe) and a ventrolateral (VeLa) cell population. The medial myogenic cells differentiate first and remain associated with the notochord [5]. Arrows design the movement of lateral paraxial mesoderm. Dotted arrows design the movement of invagination of neur ectodermal cells. NT, neural tube. Nc, notochord. (B) Mef2d couples lateral myogenesis to dermomyotome formation: In lateral presomatic cells, Mef2d transactivates the MyoD gene and in the most lateral presomatic cells Meox2 and Paraxis transactivates the Meox2 gene.

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assay and cooperates with Paraxis either to enhance \textit{Mox2} expression in the dermomyotome progenitors or to activate \textit{Xenopus tropicalis Mox2} promoter in luciferase assays. Moreover, an evolutionary conserved Mef2 binding site on the mouse \textit{Mox2} promoter is necessary for \textit{Mox2} expression in cultured cells [34]. However, the effect of Mef2d alone is relatively moderate and is time-limited to the beginning of neurulation since Mef2d is not coexpressed with \textit{Mox2} afterwards. Interaction of Me2f2d with histone modifying enzymes that introduce epigenetic modifications [71] without interacting directly with the RNA polymerase II complex could explain this transitory role of Mef2d.

Finally, Mef2d can directly regulate muscle identity genes (Fig. 9B). Mef2d function in coupling lateral myogenesis to dermomyotome formation does not seem to be conserved during evolution. This is not the case in zebrafish, another anamniote species, where Mef2d is not required for lateral myogenesis [5,56] nor for dermomyotome formation. The compartmentalization of somites presents some differences between \textit{Xenopus} and zebrafish. In zebrafish, lateral myogenesis and dermomyotome formation take place after somitogenesis. Their positioning results from the coordinated rotation of two somitic populations: the anterior one expressing \textit{Pax3} gives rise to the most exterior dermomyotome and the posterior one expressing \textit{Myod} gives rise to the more interior fast lateral fibers [72]. In this case, the early coupling function of Mef2 protein in presomitic mesoderm might have been lost. In mammals, it is well established that trunk and limb muscles derive from the dermomyotome [9], which formation is the initial event of myogenesis, whereas in \textit{Xenopus}, the first medial and lateral myogenesis appears before the dermomyotome. Mef2d-null mice are viable and present no obvious skeletal muscle development defects [18,20]. Although we cannot exclude that the function of Mef2d could be compensated by other members of the Mef2 family, early coupling function of Mef2d has probably been lost during evolution since the dermomyotome precursors are no longer associated with lateral myogenesis during their formation. In mammals, Mef2 proteins are able to enhance transcription of the transfected \textit{Mox2} promoter, particularly in C2C12 myoblasts [34], therefore we cannot exclude that Mef2 might control \textit{Mox2} expression in one of the mouse myogenesis.

**Supporting Information**

**Figure S1** Sequences targeted by oligomorpholinos. 5’utr sequence of the two genes coding for Myod, Myf5, Mrf4, Mef2d, Paraxis and Meox2 with sequences recognized by oligomorpholinos. (DOCX)

**Figure S2** Statistical analysis of injected or treated embryos. Histograms showing percentage of embryos with enhanced, decreased or unchanged expression. (DOCX)

**Table S1** Primers used for cloning of in situ hybridization probes. Forward and reverse primers used for the cloning of Paraxis, Mmeox2, Pax7 and Xbra probes. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: BDG CC. Performed the experiments: BDG SL ASA. Analyzed the data: BDG CC. Contributed reagents/materials/analysis tools: BDG ASA SL. Wrote the paper: BDG FC CG.

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