Six Homeoproteins Directly Activate Myod Expression in the Gene Regulatory Networks That Control Early Myogenesis

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Abstract

In mammals, several genetic pathways have been characterized that govern engagement of multipotent embryonic progenitors into the myogenic program through the control of the key myogenic regulatory gene Myod. Here we demonstrate the involvement of Six homeoproteins. We first targeted into a Pax3 allele a sequence encoding a negative form of Six4 that binds DNA but cannot interact with essential Eya co-factors. The resulting embryos present hypoplastic skeletal muscles and impaired Myod activation in the trunk in the absence of Myf5/Mrf4. At the axial level, we further show that Myod is still expressed in compound Six1/Six4:Pax3 but not in Six1/Six4:Myf5 triple mutant embryos, demonstrating that Six1/4 participates in the Pax3-Myod genetic pathway. Myod expression and head myogenesis is preserved in Six1/Six4:Myf5 triple mutant embryos, illustrating that upstream regulators of Myod in different embryonic territories are distinct. We show that Myod regulatory regions are directly controlled by Six proteins and that, in the absence of Six1 and Six4, Six2 can compensate.

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Introduction

The Pax-Six-Eya-Dach genetic network was first identified in Drosophila as a key transcriptional regulator of compound eye development. Within this network, the Pax gene, Evedess, is an upstream regulator of genes for the Six transcription factor sine oculis and of its co-factor Eyes absent (Eya), with feedback regulation between these genes [1,2]. Vertebrate homologues are involved in eye development [3] but also in other developmental processes, suggesting that the mechanisms orchestrated by this genetic network are conserved and used for multiple types of organogenesis and tissue specification during embryonic development [4,5,6]. Indeed in Drosophila, Pax meso, dSix4 and Eya are involved in somatic myogenesis [7,8,9].

During vertebrate myogenesis, Pax3 and Pax7 are important upstream regulators of myogenic progenitor cell behaviour, survival and fate, as shown by genetic manipulations in the mouse embryo [5,10]. Skeletal muscles of the trunk and limbs are derived from progenitors present in the dorsal dermomyotome domain of the somites which segment from paraxial mesoderm and mature following an anterior/posterior gradient along the axis of the vertebrate embryo. Pax3 is expressed throughout the epithelial dermomyotome and Pax7 in its central domain that will give rise to the progenitor cells of the myostome [5]. Six1/4, together with the Six co-activators, are also present in the dermomyotome together with Eya1/2, expressed at a high level in the epaxial and hypaxial domains. These Six and Eya genes have been shown to control the myogenic progenitor cell population, particularly in the hypaxial domain, where Pax3 also plays a key role in the survival and delamination/migration of myogenic progenitors. Interactions between these genes in the myogenic context, were suggested by overexpression experiments in the chick embryo, in somite explants [11] and in cell culture [12]. Analysis of compound Six1/4 and Eya1/2 mutants show that these factors
Author Summary

The onset of skeletal muscle formation is controlled by complex gene regulatory networks. By manipulation of these genetic pathways in the mouse embryo, we have examined the interplay between genes encoding the transcriptional regulator Pax3; the major myogenic determination proteins Myf5, Mrf4, and Myod; as well as genes encoding homeodomain proteins Six1 and Six4. In the absence of Myf5 and Six1/4, Myod expression is compromised. We demonstrate that key regulatory elements of the Myod gene are directly targeted by Six factors, including Six2, which is unexpectedly upregulated in the absence of Six1 and Six4. This work therefore reveals new aspects of the gene regulatory networks that control myogenesis.

regulate Pax3 in the hypaxial dermomyotome, whereas Pax3 expression is increased in the posterior dermomyotome in the absence of Six transactivation [13,14,15]. In the head muscles, which form from anterior unsegmented paraxial mesoderm, Pax3 is not expressed in myogenic progenitors, and Pax3 only later, whereas Six1 and Eya1 co-factors are present and active [15,16,17], as well as Ptx2 which acts as an upstream regulator of craniofacial myogenesis [18].

Entry into the myogenic programme, both in the head and trunk, depends on the myogenic determination factors Myf5/Mrf4 and Myod. Another member of this family of basic-helix-loop helix transcription factors, Myogenin, intervenes at the level of myogenic differentiation [19]. During the onset of myogenesis in the mouse embryo, Myf3 is expressed before Myod and in the absence of Myf5 and Mrf4 the activation of Myod is delayed [20]. In Pax3;Myf3/Mrf4 double mutants, Myod is not activated and skeletal muscle does not form in the trunk and limbs. In the absence of Pax3, the onset of myogenesis in the epaxial somite, although perturbed [21] takes place, with Myf3/Mrf4 activation through Wnt, and Shh signalling pathways [22], acting on an early epaxial enhancer of Myf5. Later activation of Myf5 in the hypaxial somite and in myogenic progenitor cells that have migrated to the limb, depends on another enhancer element which is directly regulated by Pax3 [23] and by Six1/4 [24], illustrating the synergistic action of these upstream regulators in driving the expression of myogenic determination genes. Six1/4 mutant mice have separate functions at later stages. We showed already that the expression of Eya1 is maintained in Six1/4−/− mutants, and that the expression of Eya1 is preserved in the Pax3 mutant [15]. We now show that this is also the case for Eya2, which continues to be transcribed in the myogenic cells still present in somites of the Pax3 mutant (Figure 1M, 1N, 1Q, 1U). Activation of Eya1 and Eya2 is therefore independent of Six1/4 and Pax3. Furthermore, we note that the expression of Pax3 is only detected in Eya2 expressing cells of the somite, in the absence of Pax3 (Figure 1T, 1U, 1V), consistent with the proposed involvement of Eya co-factors acting with Six1/4, upstream of Myod during mouse embryogenesis [15].

Results

Pax3 and Six1 act through a common genetic pathway

In order to investigate whether Pax3 and Six1 act in the same genetic pathway, we analysed Pax3/Six1 double mutants. Comparison of Six1+/−/− mutant embryos from the same litter shows that somite defects are similar at E11.5 and E13.5 with more cell dispersion of Six1−/− cells, notably hypaxially, in the Pax3 mutant (Figure 1A−1I). The somitic phenotype of Six1/−/− is similar to that of the Pax3 mutant, but with more somite truncation at E11.5 (Figure 1J−1K), consistent with partially overlapping function of Pax3 and Six1 at this stage. At E13.5 however, the phenotype of Pax3/Six1 double mutant embryos is clearly more pronounced than either single mutant (Figure 1F, 1I, 1L), indicating that Pax3 and Six1 have separate functions at later stages. We had shown already that the expression of Eya1 and Eya2 is maintained in Six1−/−/− mutants, and that the expression of Eya1 is preserved in the Pax3 mutant [15]. We now show that this is also the case for Eya2, which continues to be transcribed in the myogenic cells still present in somites of the Pax3 mutant (Figure 1M, 1N, 1Q, 1U). Activation of Eya1 and Eya2 is therefore independent of Six1/4 and Pax3. Furthermore, we note that the expression of Myod is only detected in Eya2 expressing cells of the somite, in the absence of Pax3 (Figure 1T, 1U, 1V), consistent with the proposed involvement of Eya co-factors acting with Six1/4, upstream of Myod during mouse embryogenesis [15].

Targeting of a dominant negative form of Six4 (Six4Δ) into the Pax3 locus

To investigate the role of the Pax3-Six-Eya network in vivo while bypassing both functional compensation between genes in the same family, and potential problems of cell loss due to the function of Pax3 in cell survival, we adopted a dominant negative approach. We selected a Six coding sequence mutated in the Eya interaction domain, but nevertheless able to bind specifically to the Six (ME3) binding site. Nuclear translocation of the co-activator Eya depends on the Six-Eya interaction which requires the N-terminal Six domain [29,30], however this domain is also required for DNA binding specificity [31]. We therefore used a sequence encoding an alternative splice variant of Six4, Six4Δ (isolated from a mouse muscle cDNA library), which is divergent in the N-terminal-region of the conserved Six binding domain (Figure 2A). The truncated Six protein encoded by Six4Δ is still able to bind DNA, but has lost the capacity to associate with Eya2, as shown in gel mobility shift analyses (GMSA) (Figure 2B). While full length Six4 protein synergizes with Eya2 to activate ME3 reporter activity in transient transfection assays, Six4Δ is unable to synergize with Eya2, and increasing amounts of added Six4Δ competes with the Six4-Eya2 transcription complex, leading to decreased transcriptional activation (Figure 2C).

We targeted the Six4Δ sequence into an allele of Pax3, to evaluate the function of the Six-Eya interaction during myogenesis in vivo. To avoid potential problems of lethality, we used a conditional strategy (see Figure 2D−2G), similar to that previously reported [32], with an IRESGal4 reporter following the Six4Δ sequence, to monitor expression. X-Gal staining revealed correct expression of the reporter at E10.5 when compared to embryos where an nlacZ reporter is targeted into an allele of Pax3 (Pax31Dscm/nlacZ, abbreviated Pax31DC-lacZ) (Figure 3A−3B). This was also the case after Pax3 in situ hybridization, compared to wild type Pax3;Six1/4 mutant embryos from the same litter shows that somite defects are similar at E11.5 and E13.5 with more cell dispersion of Six1−/− cells, notably hypaxially, in the Pax3 mutant (Figure 1A−1I). The somitic phenotype of Six1−/− is similar to that of the Pax3 mutant, but with more somite truncation at E11.5 (Figure 1J−1K), consistent with partially overlapping function of Pax3 and Six1 at this stage. At E13.5 however, the phenotype of Pax3/Six1 double mutant embryos is clearly more pronounced than either single mutant (Figure 1F, 1I, 1L), indicating that Pax3 and Six1 have separate functions at later stages. We had shown already that the expression of Eya1 is maintained in Six1−/−/− mutants, and that the expression of Eya1 is preserved in the Pax3 mutant [15]. We now show that this is also the case for Eya2, which continues to be transcribed in the myogenic cells still present in somites of the Pax3 mutant (Figure 1M, 1N, 1Q, 1U). Activation of Eya1 and Eya2 is therefore independent of Six1/4 and Pax3. Furthermore, we note that the expression of Myod is only detected in Eya2 expressing cells of the somite, in the absence of Pax3 (Figure 1T, 1U, 1V), consistent with the proposed involvement of Eya co-factors acting with Six1/4, upstream of Myod during mouse embryogenesis [15].

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embryos at E10.5 shows that extent of the somite where $\text{Sy}^{\text{Shen}}\alpha\beta\alpha\gamma\beta\alpha\gamma\beta$ is expressed, particularly hypaxially at E11.5 (E,H). Disorganisation and loss of hypaxial muscle fibers is observed at E13.5 (F,I) in the interlimb level. These phenotypes are more severe in $\text{Pax}3^{\text{Sp/Sp}}$ : $\text{Six}1^{+/+}$ double mutants (JK), notably at E13.5 (L). B,E,H,K and C,F,I,L show enlargements in the interlimb region. M-N, Whole mount in situ hybridization using an $\text{Eya2}$ probe on $\text{Pax}3^{\text{nLacZ/}}$ (M) and $\text{Pax}3^{\text{nLacZ/nLacZ}}$ (N) embryos at E10.5 shows that $\text{Eya2}$ expression is independent of $\text{Pax3}$. O-V, co-immunohistochemistry with $\text{Eya2}$ (Q,R,U,V) and $\text{Myod}$ (P,T,R,V) antibodies on interlimb sections of $\text{Pax}3^{\text{nLacZ/}}$ and $\text{Pax}3^{\text{nLacZ/nLacZ}}$ embryos confirms continuing expression of $\text{Eya2}$ in the absence of $\text{Pax3}$. Reduction of $\text{Eya2}$ expression, notably in hypaxial lips of the somites, is due to dermomyotome reduction in the $\text{Pax3}$ mutant. O,S, DAPI staining.

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Expression of $\text{Six}4.1$ in vivo specifically impairs the $\text{Pax3}$-mediated myogenic pathway

In order to determine if $\text{Six}-\text{Eya}$ lies in the $\text{Pax3}$$\text{Myod}$ myogenic pathway, we crossed the $\text{Pax}3^{\text{Sp/Sp}}$ (abbreviated $\text{Pax}3^{\text{Sp}}$) mice with $\text{Myf5}^{\text{nLacZ/}}$ (abbreviated $\text{Myf5}^{\text{nLacZ}}$) mice [33]. In wild type embryos, $\text{Myod}$ expression is initiated around E10 in the hypaxial domain of thoracic somites [34]. However, in $\text{Myf5}$ mutant embryos, $\text{Myod}$ expression is delayed by about 24 h. Muscle formation is normal at later stages, indicating that $\text{Myod}$ is able to rescue myotome formation in the absence of $\text{Myf5}$ after E11.5 [20]. In $\text{Myf5}^{+/+}$ and $\text{Myf5}^{+/}$ : $\text{Pax}3^{\text{nLacZ/}}$ embryos, $\text{Myod}$ is activated normally and by E11.5 $\text{Myod}$ expression is seen throughout the myotome (Figure 4A–4B, 4E–4F). As previously shown (Tajbakhsh et al., 1997), in $\text{Myf5}$ mutant embryos ($\text{Myf5}^{\text{Sp/Sp}}$), Figure 4C, 4G) $\text{Myod}$ is activated later in the muscle precursor cells which are blocked in the epaxial and hypaxial somite, and at E11.5 the hypaxial part of the myotome is partially rescued (Figure 4G, arrowheads). In contrast, in $\text{Myf5}^{+/+}$ : $\text{Pax}3^{\text{nLacZ/}}$ embryos, $\text{Myod}$ expression is significantly reduced in epaxial and, notably, in hypaxial muscle precursor cells at E11.5 (Figure 4D, 4D', 4H', 4K'). This impaired $\text{Myod}$ expression leads to only partial rescue of myotome development; the cells that are expressing $\text{Myod}$ in the hypaxial domain, despite activation of myogenic differentiation genes, like $\text{Myogenin}$, remain restricted to this part of the somite (data not shown). At E12.5, trunk muscles still show some disorganisation in $\text{Myf5}^{+/+}$ embryos, but this is more pronounced in $\text{Myf5}^{+/}$ : $\text{Pax}3^{\text{nLacZ/}}$ embryos (Figure 5A–5D, 5A–5H). By E14–E14.5, myogenesis is rescued in $\text{Myf5}^{+/}$ fetuses (Figure 5G–5H', 5K–5K'). In contrast, $\text{Myf5}^{+/+}$ : $\text{Pax}3^{\text{nLacZ/}}$ fetuses display a reduction in trunk muscles (Figure 5L–5H') which is more severe than in $\text{Myf5}^{+/+}$ $\text{Pax}3^{+/+}$ embryos at this stage (Figure 5L–5L'). These results indicate that $\text{Six}/\text{Eya}$ intervene in the $\text{Pax3}$-dependent pathway of $\text{Myod}$ activation.

$\text{Myf5}$ is required for $\text{Myod}$ activation in the absence of $\text{Six}1$ and $\text{Six}4$

We had previously shown that $\text{Myod}$ expression is severely compromised in $\text{Six}1^{+/-}$/$\text{Six}4^{+/-}$ double mutant embryos [13]. In these embryos, $\text{Pax3}$ expression is maintained in anterior and posterior domains of the dermomyotomes, while impaired in the epaxial and hypaxial domains. Early $\text{Myf5}$ expression is detectable,
although decreased [13]. To test whether the remaining expres-
sion of Pax3 and/or Myf5 bind the MEF3 site, but that only Pax3 can interact with Eya2 protein to form a larger complex. C, Transfection experiments performed in primary cultures of chick myoblasts show that Six4 and Eya2 display no functional synergy, and increasing amounts of Six4Δ compete for Six4-Eya2 transcriptional activation. Y axis, ratio between Luciferase and Renilla activities in arbitrary units. D-G, Strategy for targeting the Six4Δ coding sequence into an allele of Pax3. The probes and restriction enzymes (EcoRV; RV) are indicated, with the size of the resulting wild-type and recombined restriction fragments. The targeting construct (E) contains 2.4 kb and 4 kb of 5’ and 3’ genomic flanking sequences of the mouse Pax3 gene. A floxed puromycin-pA selection marker (Puro), replaces the coding sequence in exon 1 of Pax3 (D), followed by a di-cistronic cassette containing the murine Six4Δ cDNA comprising the whole coding region, followed by an IRES-LacZ cassette and by a final pA signal. The IRES-LacZ allows easy detection of Six4Δ expression [32]. A counter-selection cassette encoding the A subunit of Diptheria Toxin (DTA) was inserted at the 5’ end of the vector. After homologous recombination in embryonic stem (ES) cells, Six4Δ-IRES-LacZ expression from the Pax3(Six4Δ-IRES-LacZ) allele is blocked by the floxed puromycin-pA cassette (F) and is therefore conditional to removal by crossing with a PGK-Cre mouse [52]. This generates the Pax3(Six4Δ-IRES-LacZ) allele (abbreviated Pax3Six4Δ-IRES-LacZ) (G). doi:10.1371/journal.pgen.1003425.g002

Figure 2. Targeting of a sequence encoding dominant negative Six4 into the Pax3 locus. A, Alignment of Six protein sequences shows conservation of the N-terminal-most regions of the Six-domain. This region is absent in the Six4Δ mRNA splicing variant. B, Bandshift assays show that Six4 and Six4Δ bind the MEF3 site, but that only Six4 can interact with Eya2 protein to form a larger complex. C, Transfection experiments performed in primary cultures of chick myoblasts show that Six4 and Eya2 synergistically activate transcription of a luciferase reporter driven by the multimerized MEF3 sequence. In contrast, Six4Δ and Eya2 display no functional synergy, and increasing amounts of Six4Δ compete for Six4-Eya2 transcriptional activation. Y axis, ratio between Luciferase and Renilla activities in arbitrary units. D-G, Strategy for targeting the Six4Δ coding sequence into an allele of Pax3. The probes and restriction enzymes (EcoRV; RV) are indicated, with the size of the resulting wild-type and recombined restriction fragments. The targeting construct (E) contains 2.4 kb and 4 kb of 5’ and 3’ genomic flanking sequences of the mouse Pax3 gene. A floxed puromycin-pA selection marker (Puro), replaces the coding sequence in exon 1 of Pax3 (D), followed by a di-cistronic cassette containing the murine Six4Δ cDNA comprising the whole coding region, followed by an IRES-LacZ cassette and by a final pA signal. The IRES-LacZ allows easy detection of Six4Δ expression [32]. A counter-selection cassette encoding the A subunit of Diptheria Toxin (DTA) was inserted at the 5’ end of the vector. After homologous recombination in embryonic stem (ES) cells, Six4Δ-IRES-LacZ expression from the Pax3(Six4Δ-IRES-LacZ) allele is blocked by the floxed puromycin-pA cassette (F) and is therefore conditional to removal by crossing with a PGK-Cre mouse [52]. This generates the Pax3(Six4Δ-IRES-LacZ) allele (abbreviated Pax3Six4Δ-IRES-LacZ) (G). doi:10.1371/journal.pgen.1003425.g002

Six proteins directly activate Myod regulatory elements

Myod expression has been shown to be under the control of at least three separate DNA elements, a promoter region, a distal regulatory region (DRR), 6 kb 5’ of the transcription start site [35], and a core enhancer (CE) region, located 20 kb 5’ of the transcription start site (TSS) [36]. Both the CE and the DRR drive expression of a LacZ reporter to sites of myogenesis in transgenic embryos, where the CE shows a higher and more precocious activity [35,37]. A specific deletion of either enhancer by the CREloxP system indicates functional redundancy [38,39]. Both CE and DRR elements have been shown to bind Six1 and Six4.
transgene. C–D, X-Gal staining of homozygotes Pax3ILZ/ILZ embryos at E10.5 demonstrates correct expression of the Six4 gene. A–B, X-Gal staining of Pax3IRESnLacZ/+ embryos (Pax3ILZ/+). (A) and Pax3Six4/+ (B) embryos at E10.5 demonstrates correct expression of the Six4 transgene. C–D, X-Gal staining of homozygotes Pax3ILZ/ILZ (C) and Pax3Six4/+ (D) embryos at E10.5 demonstrates that the Six4A sequence does not rescue deficiencies due to the absence of Pax3 (Encephaly, spina bifida, limb defects and neural crest cell deficiencies). E–F, Whole mount in situ hybridization using a Myod probe on Pax3ILZ/+ (E) and Pax3Six4/+ (F) embryos at E11.5 shows that the Six4A sequence does not overtly perturb Myod expression. G–H, Whole mount in situ hybridization using a Myod probe on homozygote Pax3ILZ/ILZ (G) and Pax3Six4/+ (H) embryos at E10 shows that the onset of Myod expression is similar to that of a Pax3 mutant, in the absence of Pax3 but in the presence of a dominant negative Six4A (H).

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Figure 3. Expression of Six4A does not perturb normal embryonic development nor rescue Pax3 mutant deficiencies. A–B, X-Gal staining of Pax3MD5.8-LacZ+ (Pax3ILZ/+). (A) and Pax3MD5.8-LacZ− (B) embryos at E10.5 demonstrates correct expression of the Six4A transgene. C–D, X-Gal staining of homozygotes Pax3MD5.8-LacZ+ (C) and Pax3MD5.8-LacZ− (D) embryos at E10.5 demonstrates that the Six4A sequence does not rescue deficiencies due to the absence of Pax3 (Encephaly, spina bifida, limb defects and neural crest cell deficiencies). E–F, Whole mount in situ hybridization using a Myod probe on Pax3MD5.8-LacZ+ (E) and Pax3MD5.8-LacZ− (F) embryos at E11.5 shows that the Six4A sequence does not overtly perturb Myod expression. G–H, Whole mount in situ hybridization using a Myod probe on homozygote Pax3MD5.8-LacZ+ (G) and Pax3MD5.8-LacZ− (H) embryos at E10 shows that the onset of Myod expression is similar to that of a Pax3 mutant, in the absence of Pax3 but in the presence of a dominant negative Six4A (H).

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Six4 is expressed in myogenic territories in the embryo

To explain the discrepancies observed between mut3Mef3-CE-MD5.8-LacZ+ and the expression of Myod in Six1/Six4 mutant embryos, we looked for other Six genes expressed in myogenic territories during embryogenesis [40] that could be responsible for the rescue of Myod expression observed in Six1/Six4 embryos at the epaxial and craniofacial levels. Six2 [41] and Six5 [42,43,44] are the two other Six genes expressed in myogenic cells. By whole mount in situ hybridization, we further show that Six2 is

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To explain the discrepancies observed between mut3Mef3-CE-MD5.8-LacZ+ and the expression of Myod in Six1/Six4 mutant embryos, we looked for other Six genes expressed in myogenic territories during embryogenesis [40] that could be responsible for the rescue of Myod expression observed in Six1/Six4 embryos at the epaxial and craniofacial levels. Six2 [41] and Six5 [42,43,44] are the two other Six genes expressed in myogenic cells. By whole mount in situ hybridization, we further show that Six2 is
expressed in the first branchial arch of E9.5 embryos, and also in the dorsal regions of newly formed somites, where early epaxial Myf5 is first activated (Figure 8A). Both Six2 and Six5 bind efficiently to the three Myod MEF3 elements, as determined by gel mobility shift experiments (Figure 8B). We next isolated chromatin for ChIP experiments to check if Six2 binds in vivo on Myod regulatory elements. With wild type embryos we did not observe significant binding. However with chromatin from E12 Six1/Six4 mutant embryos we observed efficient binding of Six2 on Myod CE and DRR elements, demonstrating that Six2 can bind to Myod regulatory elements in the embryo (Figure 8C). We examined Six2 protein in the masseter muscle of Six1/Six4/Six5−/− and Myf5−/−/Six1−/−/Six4−/− mutant embryos by immunocytochemistry at E12.5 and show that it co-localizes with Myod protein (Figure 8D). These results indicate that Six2 is a good candidate for the activation of Myod expression in the absence of Six1 and Six4. They also suggest that Six2 is upregulated under these conditions (Figure 8C, 8D).

Figure 4. Six4Δ affects Myod expression and myogenesis in the absence of Myf5. A–D’, Whole mount in situ hybridization experiments using a Myod probe on Myf5+/− (A, A’), Myf5−/−: Pax3+/− (B, B’), Myf5−/− (C, C’) and Myf5−/− : Pax3+/− (D, D’) embryos at E11.5. At this stage, in Myf5−/− embryos (C, C’), Myod is activated and begins to rescue the formation of the myotome (arrowheads in C’). However, in Myf5 deficient embryos which express Six4Δ under the control of Pax3 regulatory elements, Myf5−/−: Pax3+/− (D, D’), Myod expression is reduced, affecting the rescue of myotome formation (D’, arrowheads). In contrast, in thoracic somites of Myf5−/−: Pax3+/− (B, B’) Myod expression is not altered compared to Myf5+/− embryos (A, A’). A’-D’, show enlargements in the interlimb region of A–D, E–H’, co-immunohistochemistry on transverse sections of hypaxial somites from Myf5+/− (E, E’), Myf5−/−: Pax3+/− (F, F’), Myf5−/− (G, G’) and Myf5−/− : Pax3+/− (H, H’) embryos at E11.5 using anti-β-Galactosidase (β-Gal) (green, E–H) and anti-Myod (red, E’–H’) antibodies confirms the severe reduction of Myod expression in Myf5−/− : Pax3+/− (H, H’) embryos. Arrowheads indicate examples of cells in which the β-Gal reporter from the Myf5nLacZ allele is expressed and which co-express Myod.

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Figure 5. Impaired myogenesis in the presence of \textit{Six4}, in the absence of \textit{Myf5}. \(\text{A--L}^{;} ; \) X-Gal staining of E12.5 (\(\text{A--D}^{;} \)), E14.5 (\(\text{E--H}^{;} \)) or E14 (\(\text{I--L}^{;} \)) \textit{Myf5}^{+/−} (A, A′, E, E′, I, I′), \textit{Myf5}^{−/−} : \textit{Pax3}^{+/+} (B, B′, F, F′), \textit{Myf5}^{−/−} (C, C′, G, G′, K, K′) and \textit{Myf5}^{−/−} : \textit{Pax3}^{Six4Δ+} (D, D′, H, H′), \textit{Myf5}^{−/−} : \textit{Pax3}^{−/−} (J, J′),
Discussion

We show that Six1/4 play an essential role in the Pax3/Myod genetic pathway that regulates the onset of myogenesis [20]. This is revealed on a Myf5 mutant background. Since the Myf5 mutation that we use also affects Myod, entry into the myogenic program depends entirely on the myogenic determination factor Myod in the absence of Myf5/Mrf4 [45]. We illustrate with the Six4A sequence that Eya co-activators are required for Six transactivation, as previously shown [15,30]. Furthermore our results show that key enhancer sequences of the Myod gene are directly regulated by MEF3 sites that are required in vivo at all sites of myogenesis to control Myod expression through the recruitment of Six1, Six2 and Six4 transcription factors.

The Pax/Six/Eya pathway to tissue specification is therefore important for the formation of skeletal muscle in the mouse embryo. However this network appears to be more complex than the Eyeless/sine oculis/Eyes absent cascade that leads to eye formation in Drosophila [1,5]. As we show here for Six1 and Eya2, their activation takes place in the absence of Pax3, whereas Eyeless initiates the cascade in Drosophila. In the mouse somite, Pax7 is also expressed in the central domain of the dermomyotome and may compensate. However, prior to the extensive cell death seen in the hypaxial somite in the absence of Pax3, Six1/4 genes are transcribed. Furthermore during craniofacial myogenesis, the Six1 gene and genes for Eya co-factors are expressed [41,46] and the polyMEF3-LacZ reporter of Six transcriptional activity is high [13], in the absence of Pax3 that is not expressed during head myogenesis [5]. In Six1−/−/Six2−/− or Eya1−/−/Eya2−/− double mutants, Pax3 expression is compromised in the hypaxial domain [15] indicating that Six/Eya can also regulate Pax3. Our analyses of Six1−/− and Pax3−/− mutants shows that they have overlapping but not identical myogenic phenotypes, confirmed by the double mutant phenotype which is more severe, particularly at later stages.

The Six4A sequence, which we targeted to an allele of Pax3, encodes a protein that still binds DNA, but does not bind Eya and is transcriptionally inactive, thus acting as a dominant-negative factor. The effectiveness of its action will depend on competition with wild type Six factors present in Pax3 expressing cells. By diminishing the effects of Six factors (Six2 and Six3), also expressed at sites of myogenesis [41] [42], as well as Six1 and Six4, it serves as a probe, under conditions that are less radical than double mutants. This type of strategy, with a Pax3Pax3Eya−/ mouse line had previously proved valuable for probing Pax3 function [23]. In the absence of Myf5/Mrf4, when Six4A is present, down-regulation of Myod expression is clearly observed, under conditions in which somites are less perturbed, at E11.5-12.5. Later, the failure of skeletal muscles to develop leads to severe perturbations at sites of myogenesis in the trunk. Head musculature on the other hand appears normal, as do the forming limb muscles. In Six1−/−/Six4−/− double mutants, in the absence of Myf5/Mrf4, Myod is not transcribed in the trunk and limbs and myogenesis does not occur, whereas Myod transcripts are detectable in head muscle progenitors and muscle markers are present. These observations show that head myogenic progenitors, that are not derived from the somites, activate Myod and form head muscles in the absence of Six1 and Six4 [13]. This is in contrast to a report on zebrafish where Six1a was found to be essential for craniofacial myogenesis.

![Figure 6. Axial Myod expression is lost in Myf5−/−:Six1−/−:Six4−/− embryos.](http://example.com/figure6.png)

A–H, Whole mount in situ hybridization using a Myod probe, I–L, X-Gal staining, and i–l, i–l immunohistochemistry on sagittal sections of I–L embryos at the interlimb somites or head level using Desmin antibodies, at E11.5 (A–H) or E12.5 (I–L) with Pax3−/−:Six1−/−:Six4−/− (A), Pax3−/−:Six1−/−:Six4−/− (B), Pax3−/−:Six1−/−:Six4−/− (C), Pax3−/−:Six1−/−:Six4−/− (D), Myf5−/−:Six1−/−:Six4−/− (E, I), Myf5−/−:Six1−/−:Six4−/− (F, J), Myf5−/−:Six1−/−:Six4−/− (G, K), Myf5−/−:Six1−/−:Six4−/− (H, L) embryos, showing the role of Pax3/Six proteins and Myf5 acting upstream of Myod during trunk myogenesis. Desmin expression in E12.5 compound embryos at the axial level (i–l) and at the head level (i–l) is not detected in Myf5−/−:Six1−/−:Six4−/− embryos at the axial level (i) but at the head level (l), showing that craniofacial myogenesis can take place in this compound mutant. e: eye. White arrow in L shows the presence of craniofacial muscles.

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Six1;Eya1 mutant mice [17]. In addition to Six1 and Six4, Six2 and Six5 genes are also transcribed in myogenic cells in the mouse embryo [41,42], and we now show that Six2 can regulate Myod expression. Other transcriptional regulators, such as Pitx2, play an important upstream role in head myogenesis. Pitx2 has been shown to activate Myod in the trunk [47], where Pitx2 lies genetically downstream of Pax3. However in the head, where Pax3 is not expressed at the onset of myogenesis [18], Pitx2 acts independently. In keeping with this, Pax3/Mrf4/Myf5 triple mutants do not have defects in Myod activation and myogenesis in the head. However Myod is not activated in the trunk where skeletal muscles do not form [20]. In this context, Six genes do not rescue the phenotype.

Activation of Myod relies on two enhancer elements at 5 kb (DRR) and 20 kb (CE) upstream of the gene, as well as on the
proximal promoter [35,36]. In adult myogenic cells, Pax7 activates the promoter [48] and Pax3/7 have been shown to bind the CE in myogenic cell cultures [49], but there are no data on such a role of Pax3/7 in the embryo. The CE is an important regulator of embryonic Myod expression, but the DRR is also implicated in this activity. When the CE is deleted, delayed Myod expression is still observed, notably in the branchial arches and limb buds [39]. Deletion of the DRR does not abolish embryonic Myod expression [38], in keeping with the important role of the CE. We identify three separate DNA elements in the CE and DRR of [38], in keeping with the important role of the CE. We identify three separate DNA elements in the CE and DRR of Myod that are bound by Six1 and Six4 [25], and bound in vivo by Eya. In a transgene controlled by the proximal promoter, DRR and CE, we show expected expression of the nLacZ reporter at all sites of myogenesis in E12.5 embryos. When the Six/MEF3 binding sites are mutated, this activity is mainly lost, with low level expression at sites of myogenesis, in a few Myod-positive cells. These results show that Six transactivation is required for the function of these regulatory elements. Residual activity may be due to Myf5 activation of Myod regulatory sequences, through E-boxes that are also known to play an important role [37,50]. Indeed our genetic experiments, which show that a major effect on Myod activation in the Six1/2 double mutant is only seen in the absence of Myf5, are in keeping with this. In Myf5/Myf4 mutant embryos, Pax3-dependent rescue of CE enhancer activity is observed [39], potentially due to Six transactivation acting in the Pax3/Myod pathway. In the linker scanning experiments where human MYOD CE elements were sequentially mutated [37], box 4 was found to be essential for expression in all skeletal muscle lineages. This sequence contains the Six binding site CE1. In contrast mutation of box 16 which contains our box CE2 did not lead to loss of activity, demonstrating that CE1 is the main functional MEF3 site [37].

In our transgenic analysis, mutation of Six/MEF3 sites leads to loss of transgene expression in most embryos, at sites of myogenesis in the head, as well as in the trunk. This contrasts with our findings with Six1−/−/Six4−/− and Myf5−/−:Six1−/−/Six4−/− mutants. An explanation for these discrepancies is that other Six proteins known to be expressed in myogenic cells compensate in some embryonic territories for the lack of Six1 and Six4. We provide evidence that Six2 may play such a role since it is expressed at key determinant for activation (Figure 9). Our analysis of the Six1−/−/Six4−/− double mutant, Six2 is expressed in Myod-positive cells and binds the Six regulatory elements. We have not been able to examine Six3 in this context due to the lack of appropriate antibodies.

We conclude that during skeletal muscle formation in the trunk the Pax3 genetic cascade that leads to Myod activation functions through Six genes and that in the absence of Myf5/Mrf4, the Six transactivation complex plays a key role in the activation of myogenesis. During the onset of craniofacial myogenesis, where Pax transcription factors do not play a role, Six expression is also a key determinant for Myod activation (Figure 9). Our analysis of the Pax3/Six/Eya genetic cascade in the context of myogenesis has implications for the derivation of skeletal muscle from stem cell populations [44] and also, more generally, for other examples of tissue specification and organogenesis in vertebrates that also employ this genetic network [5].
Materials and Methods

Cloning, targeting vectors, and mice

Six4 and Six4ΔA CDNA were obtained by screening a λgt11 library from adult mouse muscle (Clontech) [26]. The Pax3\(^{Six4ΔA/+}\) construct is derived from a construct previously reported [32]. The Pax3\(^{Six4ΔA-L\mu\gamma}\) allele contains 2.4 kb of 5′ Pax3 genomic region, in which the coding sequence of exon 1 is replaced by targeted sequences and 4 kb of 3′ sequence containing exons 2–4. The genomic sequences surround a Floxed Paromycin (Para) cassette followed by 2.6 kb of Six4ΔcDNA, then an IRES\(\mu\gamma\)-lac\(\gamma\) cassette. In addition, a PGK-DTA cassette encoding the A subunit of the Diptheria toxin gene (DTA) was inserted at the 5′ end of the construct to allow negative selection in ES cells. The targeting vector was electroporated into C535 ES cells [51]. ES cells were selected and screened for recombination events by Southern blot analysis using EcoRV (RV in Figure 2) digests and a 5′-flanking probe (Figure 2E). Targeted ES cells were recovered with a 0.5–1% frequency and injected into blastocystos to generate chimaeras.

Germline transmitted alleles were identified by the classical \(\mu\gamma\)-lac\(\gamma\) in situ hybridization, immunohistochemistry, and whole-mount \(\mu\gamma\)-lac\(\gamma\) staining.

X-Gal staining, immunohistochemistry, and whole-mount \(\mu\gamma\)-lac\(\gamma\) in situ hybridization

We collected mouse embryos after natural overnight matings; for staging, embryonic day (E) 0.5 corresponded to midday assuming that fertilization had taken place at 6 a.m. Genotyping was carried out by X-Gal staining in X-Gal, with 0.2% PAF for 30 minutes following 1–2 h fixation in 4% PAF, on ice. When a light blue color had developed, embryos were rinsed in PBS and post-fixed overnight in 4% PAF. Whole mount in \(\mu\gamma\)-lac\(\gamma\) hybridization with digoxigenin-labelled riboprobes was performed as described [20]. The \(\mu\gamma\) riboprobe has also been previously described [20].

Fluorescent co-immunohistochemistry was carried out according to [32], using the following antibodies: polyclonal anti-\(\beta\)-Gal (MolecularProbe, diluted 1:200), monoclonal anti-Myc (Dako, 1:200), monoclonal anti-Desmin (Abcam, 1:100), polyclonal anti-\(\gamma\)tubulin (ProteinTech, 1:200) and monoclonal anti-Mycogynin (Dako, 1:200). Secondary antibodies were coupled to Alexa 488 1/250 and 546 1/1000 (MolecularProbes).

DNA binding assays and transactivation

Gel mobility shift assays (GMSA) were performed essentially as previously described [26], with a labeled probe corresponding to the MEF3 site of the Myogin promoter or Myod DRR and CE MEF3 elements and with Six1, Six2, Six4, Six3, Six4Δ and Eya2 proteins produced using the TNT T7 Coupled Reticulocyte Lysate System (Promega). The DNA templates used for \(\mu\gamma\)-transcription of mouse transcripts Six1, Six2, Six4, Six4Δ, Six4 and Eya2 were cloned in the pCR3 vector (Invitrogen). Because of the high molecular weight of Six4 and Six4Δ (about 90 kDa) we used a 3% acrylamide gel, run overnight at +4°C. To ensure that proteins were appropriately translated, parallel reactions were performed in the presence of \([\text{\textsuperscript{35}}S]\)methionine, separated on SDS-PAGE gels and visualized using autoradiography.

The sequences of the double stranded oligonucleotides containing MEFS3 sites used for bandshift assays are: DRR: 5′ AGT TGG ATC CGG TTT CCA GAG GC, CE1: 5′ TGA GAC GAT AAT TTT ATC CTG CT, CE2: 5′ GGT GCT CTC CGG CGT TTC CTG TAG CT, Myogin MEF3: 5′ TGG GGG GCC TCA GGT TTC TCC GTG GGT GT, Myogin NFI: TAT CTC TGG GTT CAT GCC AGC AGG G. The TCAGGTTTC MEF3 sequence is underlined.

Chick primary myoblasts were grown and transfected as previously described [26], using RSV-Remilla as a control for transfection efficiency. Eya2, Six4 or Six4Δ expression was driven by the CMV promoter-enhancer present in pCR3, with the luciferase reporter gene under the control of a multimerized MEF3 element cloned upstream of the human Aldolase A minimal −35 to +45 bp promoter [54]. Two days after transfection, luciferase activity was measured using standard procedures.

Generation and analysis of transient transgenic embryos

For the construction of the CE-MD5.8-lac\(\gamma\) sequences, mouse DNA was first used as a template to clone the core enhancer (CE) of Myod [36] with forward Apal/Not1 5′ GGCG-GCC-GGC-GGC-GCT-GAG-CCC-CAC-AGG-ATT-TGG and reverse 5′ GAA-TTC-CTC-CAG-CGG-CAG-GCC-TGA-GGT oligonucleotides; the MEF3 sequence is underlined. This 262 bp CE fragment was subsequently inserted into an Apal-Prim1 site (position −5792 to −5652) of the pMD6.8-lac\(\gamma\) linearized plasmid [35], 340 bp upstream of the distal regulatory region (DRR) lying at −5310 bp from the Myogin gene. The sequence of the pCE-MD6.8-lac\(\gamma\) reporter vector was verified by sequencing. To obtain mutated pCE-MD6.8-lac\(\gamma\), one MEF3 site in the DRR (position −5176 to −5167) and two MEF3 sites in the CE (at position 53 and position 229) were mutated by substitution with a Hind III site (TCCGGTTTC- >AAGCTTTTC), a Xhol site (GTAATTCTT- >CCTAGGGTATA) or a BglIII site (TCCGGTTTC- >TCTCAAGTT), respectively. The pMutCE-MD6.8-lac\(\gamma\) reporter vector was verified by sequencing. The plasmid was digested with Not1. Migration on an agarose gel allowed removal of plasmid sequences. Transgenic mice were generated by microinjection of the purified construct into fertilized F2 eggs from C57BL/6jJsJ mice, at a concentration of approximately 1 ng/µl using standard techniques. Injected eggs were reimplanted the same day or the day after the injection into outbred pseudo-pregnant foster.
mothers. Transient transgenic embryos were dated taking the day of reimplantation into the pseudo-pregnant foster mothers as E0.5. Embryos were dissected in PBS, fixed in 4% paraformaldehyde for 15 minutes, rinsed 5 times in PBS and stained in X-gal solution [35] at 37°C overnight. DNA was prepared from the vitelline membrane from each embryo and analyzed by PCR, using nlaZ primers, and Myod primers in the DRR and in the CE.

ChIP experiments

Pax3GFP/+ males were crossed with C57Bl6N females to obtain Pax3GFP/+ embryos. Somites were collected from E11.5 embryos by removing heads, neural tubes and internal organs. These samples were enzymatically digested with collagenase and dissociated cells were fixed with 1% formaldehyde at room temperature for 15 min. The GFP-positive cells were sorted by flow cytometry (BD FACs ARIA III). The gates for positive and negative GFP cells were determined using an equivalent sample isolated from wild type embryos and from Pax3GFP/+ heterozygous embryos. About 7.5×10⁶ cells were collected for ChIP experiments from nine embryos. E12 Six1/-/-/Six4/-/- embryos were collected and enriched myogenic tissues were pooled after removing limbs, neural tube and internal organs. Dounce dissociated cells were fixed with 1% formaldehyde at room temperature for 15 min.

The chromatin immunoprecipitation procedure was performed according to the manufacturer’s protocol (EZ-Magna ChiP G Kit; Merck Millipore) with antibodies recognizing all Eya proteins (Santa Cruz), Six2 protein (Proteintech), and Normal Mouse IgG (BD Transduction Laboratories). DNA was extracted from enriched myogenic tissues and diluted 1:10 to amplify the product. PCR reactions were performed using Myod primers to verify enrichment of Myod DNA in the ChIP samples. Immunoprecipitated DNA was quantified by the Qubit dsDNA HS Assay Kit (ThermoFisher). The % Myod enrichment was calculated as the percentage of Myod DNA in the ChIP sample relative to the input DNA.

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Supporting Information

Figure S1 Immunohistochemistry with Desmin antibodies on sagittal sections of Myf5+/–Six1+/–Six4+/– (A). Myf5+/–Six1+/–Six4–/– (B). Myf5–/–Six1+/–Six4+/– (C). Myf5–/–Six1–/–Six4–/– (D) embryos at E12.5 at the masseter level, with DAPI staining.

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Author Contributions

Conceived and designed the experiments: FR MB PM. Performed the experiments: FR JD CL JP MS CN ML DR PM. Analyzed the data: FR MB PM. Wrote the paper: FR MB PM.

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