Six Homeoproteins and a linc-RNA at the Fast MYH Locus Lock Fast Myofiber Terminal Phenotype

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Abstract

Thousands of long intergenic non-coding RNAs (lincRNAs) are encoded by the mammalian genome. However, the function of most of these lincRNAs has not been identified in vivo. Here, we demonstrate a role for a novel lincRNA, linc-MYH, in adult fast-type myofiber specialization. Fast myosin heavy chain (MYH) genes and linc-MYH share a common enhancer, located in the fast MYH gene locus and regulated by Six1 homeoproteins. linc-MYH in nuclei of fast-type myofibers prevents slow-type and enhances fast-type gene expression. Functional fast-sarcomeric unit formation is achieved by the coordinate expression of fast MYHs and linc-MYH, under the control of a common Six-bound enhancer.

Citation: Sakakibara I, Santolini M, Ferry A, Hakim V, Maire P (2014) Six Homeoproteins and a linc-RNA at the Fast MYH Locus Lock Fast Myofiber Terminal Phenotype. PLoS Genet 10(5): e1004386. doi:10.1371/journal.pgen.1004386

Editor: Dawn Cornelison, Univ. of Missouri, United States of America

Received October 7, 2013; Accepted April 2, 2014; Published May 22, 2014

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Funding: IS is supported by ANR, The Uehara Memorial Foundation and JSPS Postdoctoral Fellowships for Research Abroad. Financial support was provided by the Institut National de la Santé et la Recherche Médicale (INSERM), the “Association Française contre les Myopathies” (AFM), the Centre National de la Recherche Scientifique (CNRS), the Peps “Bio-Math-Info”, and the Agence Nationale pour la Recherche (ANR RFP09108XXA). We also acknowledge a contribution to the Institut Cochin animal care facility, made by the Région Ile de France. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Adult skeletal muscles are composed of slow and fast myofiber subtypes which selectively express the genes required for their specific contraction activity and metabolic properties [1–4]. These properties are acquired at the end of fetal development and during the neonatal period, when mixed skeletal myofibers expressing a panel of embryonic, fast and slow genes develop a specific slow or fast phenotype. The formation of efficient fast sarcomeric units, and Ca++ cycling and excitation/contraction relaxation coupling in fast-myofibers, is achieved through the coordinate control of fast MYH and associated fast sarcomeric genes (including Tm3, Tm2, Tnn1, Tri, Atp2a1 and Poldl) [2,4]. Myofibers can be classified by their MYH expression profile: slow-type myofibers in mice express MYH7 (also known as MYHCI, β or slow), and fast-myofibers express MYH2 (MYHCIIA), MYH1 (MYHCIIIX) or MYH4 (MYHCIIIB). Fast MYH genes found in developmental and adult stages (MYH3, MYH2, MYH1, MYH4 and MYH13) are organized as a cluster within a 300 kb region on mouse chromosome 11 [5]. The spatio-temporal expression of one specific fast MYH gene at the MYH locus is reminiscent of the organization and expression of Globin genes at the beta-globin locus [6]. However, we are yet to investigate potential enhancers or the MYH locus control region (LCR) that could be responsible for sequential and specific MYH gene expression in myofibers. The coordination of fast-type and slow-type gene expression in fast myofibers is not currently understood. Distinct intramyofibrillar calcium transients, evoked by slow tonic motor neuron firing, induce a cascade of downstream signaling involving Calcineurin and CamK. This results in the activation of selective transcription activators and repressors in slow myofibers. However, the signaling pathways operating in distinct MYH2, MYH1 and MYH4 myofiber subtypes, which coordinate the activation of the other fast-type genes and the repression of slow-type genes, is less well understood [1]. Better knowledge of the mechanisms controlling muscle specialization and plasticity is important to enable the understanding and modulation of muscle adaptations in pathophysiological conditions.

Six homeoproteins are major myogenic transcription factors which directly bind to DNA sequences (called MEF3s) to control myogenesis [7,8] and the genesis of fast-type myofibers during embryogenesis [9,10]. In adult skeletal muscle, Six1 accumulates at a higher level in the nuclei of adult fast myofibers than in of slow myofibers. Forced expression of Six1 and its Eya1 cofactor in slow myofibers causes adult slow-twitch oxidative fibers toward a fast-twitch glycolytic phenotype [11]. Animals with a Six1 KO present severe muscle hypoplasia and die at birth [12]. This prevents the in vivo analysis of the adult phenotype and the ability to investigate the direct or indirect involvement of Six1 in the spatio-temporal control of the expression of genes in the fast MYH cluster.

The mammalian genome encodes thousands of long intergenic non-coding RNAs (lincRNAs) which have multiple functions [13,14]. Some accumulate in the cytoplasm as miRNAs decoys [15,16]. Others accumulate in the nucleus and participate to gene regulation through chromatin remodeling and epigenetic modifications [14,17,18]. Here, they may act as cis [19] or trans [20] transcriptional activators, as transcriptional repressors [21,22] or through DNA-RNA triplex formation [23,24].

In this study we identify a new lincRNA, linc-MYH, and the mechanism of its control of adult muscle fast fiber-type specification
Author Summary

Adult skeletal muscles are classified into fast-type and slow-type, which display different resistance to muscle atrophy and metabolic protection against obesity. We identify in this manuscript a new mechanism controlling in vivo adult muscle fiber-type specification implicating a long intergenic non-coding RNA, linc-MYH. We demonstrate a three-element genetic partnership, where an enhancer under the control of the myogenic homeoprotein Six1 functions as a regulatory hub to control fibre phenotype. In this partnership, the enhancer controls positively the expression of both the adjacent fast myosin heavy chain (MYH) gene cluster and of linc-MYH. linc-MYH is present only in adult fast type skeletal myofibers and controls their phenotype by suppressing slow-type gene expression. The regulation of linc-MYH could provide a lead for new therapeutic approaches or drug development.

in vivo. We demonstrate a three-element genetic partnership, where an enhancer element under the control of the myogenic homeoprotein Six1 functions as a regulatory hub to control fibre phenotype. In this partnership, the enhancer positively controls the expression of both the adjacent fast Myh gene cluster and linc-MYH, suppressing slow-type gene expression and facilitating fast fiber-type specialization.

Results

Six1 binds directly to a newly identified enhancer of the Myh genes cluster

Our previous studies suggested that Six1 could be directly involved in the control of the expression of fast Myh genes, since higher levels of this transcription factor accumulate in the nuclei of adult fast myofibers than in slow myofibers [11]. To investigate how Six1 could control the expression of fast Myh isoforms, we used computational analysis to locate MEF3 sites at the fast Myh locus (see Materials and Methods). Six clustered MEF3 sites are conserved across human, rat and mouse genomes in an intergenic region located 50 kb upstream of the Myh2 gene (Figures 1A and S1) and 4 kb upstream of a lincRNA (2310065F04Rik); we refer to this lincRNA as linc-MYH (Figures 1A and S2). Six1 binding at these MEF3 sites was demonstrated in vivo by ChIP (Chromatin Immunoprecipitation) experiments with Six1 antibodies on adult fast gastrocnemius plantaris (GP) and tibialis anterior (TA) muscles (Figure 1B) but not on adult slow Soleus (data not shown), and confirmed for five of these sites (sites 1, 2, 3, 4 and 6) by EMSA assays (Figure S3A). We asked whether this Myh intergenic region could constitute an enhancer element, controlling the spatio-temporal expression of Myh genes in this locus. A 2 kb DNA fragment of this region, including the six identified MEF3 sites and 1 kb of DNA fragments upstream of the transcription start site of fast-type Myh2, Myh1 and Myh4 genes, was isolated. The putative enhancer was ligated to each Myh promoter using luciferase pGL3 basic plasmids to generate pGL3-Enhancer-Myh2/1/4 constructs. To test the involvement of Six binding in enhancer activation of the Myh2, Myh1 and Myh4 promoters, we mutated all six MEF3 sites present in the enhancer, and named these reporters pGL3-mutEnhancer-Myh2/1/4. Luciferase activity was tested two weeks after the electroporation of reporter plasmids in adult TA muscles. The luciferase activity of pGL3-Enhancer-Myh2/1/4 was seven to twelve times higher for either of the promoters, than with pGL3-Myh2/1/4. Enhancer activity was not observed in plasmids with mutated MEF3 sites associated with either of the Myh promoters (Figure 1C). Enhancer activity was neither observed with the promoters of the slow Shh (Figure S3B) or Tnni1 genes, or with the promoter of the ubiquitous β-actin gene (Figure S3C). A weak enhancer activity was observed with Myh4 promoter in primary embryonic fibroblasts, in which Six1 is expressed (Figure S3D and data not shown). These data showed that high MYH enhancer activity was only reached in vivo and required specific interactions with MYH promoter elements. To determine in vivo interactions between the enhancer and each Myh gene, we performed chromatin conformation capture (3C) assays of adult fast EDL (Extensor digitorum longus) myofibers. These experiments revealed that the enhancer interacts with the promoter of Myh2/1/4 genes in native chromatin of EDL myonuclei (Figure 1D). The strongest interactions were observed with the Myh1 and Myh4 promoters, consistent with the expression profile of these two genes in EDL muscles. The data demonstrates that the identified conserved cis-element acts as an enhancer for the Myh locus and that MEF3 sites are essential for its enhancer activity in vivo.

Loss of Six1 impairs fast muscle genes and linc-MYH expression during post-natal development

To further characterize the role of Six1 in the control of fast Myh gene expression, we bred Six1fl/flKO mice with transgenic mice expressing CRE recombinase under the control of the human skeletal actin (HSA) promoter and obtained Six1fl/fl-HSA-CRE conditional knockout mice [hereafter named cSix1 KO]. We analyzed the expression of fiber type specific genes in the back muscles of wild-type control mice and cSix1 KO mice at embryonic day 18.5 (E18.5) and at several post-natal stages (two weeks (P2W), four weeks (P4W) and eight weeks (P8W) animals (Figure 2), as muscle fiber fast-subtype specialization takes place from the end of embryogenesis [9]. Six1 mRNA was not detectable in back muscles of cSix1 KO mice (Figure 2). The expression of fast-type genes (Myh4, Tnni3, Tnnt2, Tnnc2 and Pvalb) increased during postnatal development in control mice but that of slow-type genes (Myh7, Tnn1, Tnn1, Tnnc1 and Ssl) decreased. The linc-MYH RNA was detected after birth in muscle samples and its expression increased in line with that of Myh4 (Figure 2). The induction of fast-type genes and linc-MYH and the suppression of slow-type genes, were impaired in cSix1 KO mice. Expression of linc-MYH was reduced by three to five times in cSix1 KO mice during postnatal development (Figure 2). These results show that Six1 controls the induction of linc-MYH and fast-type genes during postnatal development, and is required for the downregulation of slow type genes.

Six1 deficiency impairs adult muscle fast phenotype

We next analyzed adult 12 week-old cSix1 KO mice to further characterize the role of Six1 in adult muscle. Six1 mRNA and protein were not detectable in GP enriched with fast-myoﬁbers or soleus (SOL) muscle enriched with slow-myoﬁbers (Figure 3A and B), and fatigue resistance of TA muscle was 35% higher (Figure 3C) in the cSix1 KO mice. We used immunohistochemistry to analyse the composition of MYH7, MYH2 and MYH4 in cSix1 mutant myoﬁbers. Mutant TA muscles had a higher percentage of ﬁbers containing MYH7 and MYH2, but a lower percentage of ﬁbers containing MYH4 (Figures 3D and S4). We found consistent results during qPCR analysis of Myh mRNA i.e., higher levels of Myh7 and Myh2 mRNA and lower levels of Myh4 mRNA levels were observed in the fast TA muscles of cSix1 KO (Figure 3E). Expression levels of other speciﬁc fast and slow-type genes were also tested. We found in mutant TA muscles a downregulation of fast-type genes (Tnni1, Tnni2, Tnnc2 and Pvalb) and a ﬁve and to 25 fold increase in the levels of slow-type genes (Myh7, Tnn1, Tnn1,

PLOS Genetics | www.plosgenetics.org 2 May 2014 | Volume 10 | Issue 5 | e1004386
Nevertheless expression of slow Myh7 is increased more than ten fold at the mRNA level in cSix1 mutant TA myofibers, while by immunohistochemistry the number of MYH7 positive myofibers is increased less than two fold. This showed that there is no major phenotype switch in cSix1 mutant TA myofibers. This observation could be explained either by the higher amount of Myh7 mRNA accumulating in MYH7 positive fibers or by a general increase of Myh7 mRNA in TA myofibers, mRNAs that would not be translated efficiently and leading to the absence of increase of MYH7 positive fibers. The expression of linc-MYH expression was lower in the adult TA of cSix1 KO mice, than in control mice (Figure 3E). These results indicate that the Six1 homeoprotein can control the phenotype of fast skeletal myofibers in adult animals.

**linc-MYH is expressed exclusively in adult fast-type muscles**

We found that linc-MYH is expressed in fast-type skeletal muscles (GP, TA and EDL), but not in SOL, brain, kidney, heart or fat tissues, an expression pattern which parallels that of the fast-fiber Myh4 (Figure 4A). This suggested that linc-MYH is only expressed following robust nuclear accumulation of Six1, as takes place in the nuclei of MYH4 myofibers [11], and that the weaker nuclear accumulation of Six1 observed in SOL myonuclei does not allow efficient Six1 binding on the MIH enhancer and linc-MYH expression. We used luciferase reporter transfection assays (as described above) to test the requirement for Six binding on the MIH enhancer to activate linc-MYH expression. These transient transfection assays, performed in adult TA, show that the MIH enhancer activates linc-MI expression in a Six-dependent manner, as measured two weeks after electroporation (Figure 4B).

**linc-MYH coordinates fiber-type gene expression**

Due to the number of linc-MI foci observed in fast type nuclei, we hypothesized that linc-MI could act in trans [17] to control gene expression in fast myofibers. To test this theory, we used electroporation to introduce a shRNA against linc-MI (shlinc-MI) in TA muscle and analyzed the transfected samples after fourteen days. This method yielded the efficient knockdown of linc-MI, with a 90% reduction of its expression (Figure 5A). To identify the consequences of linc-MI knockdown and understand its mode of action, RNA samples from shlinc-MI transfected...
The expression of fast-type genes and linc-MYH is impaired in cSix1 KO mice during postnatal development. mRNA expression level of Six1, linc-MYH, Myh3, slow-type genes (red) and fast-type genes (blue) in back muscles of cSix1 KO mice at E18.5, P2W, P4W and P8W, as determined by qPCR experiments, (n = 3 to 6 for each point). *P<0.05, **P<0.01, ***P<0.001. doi:10.1371/journal.pgen.1004386.g002

Transcriptomic analysis of cSix1 and linc-MYH

We compared the networks of genes under the control of linc-MYH and of Six1 homeoprotein in adult muscles by the transcriptomic analysis of cSix1 and linc-MYH knockdown (Figure 5D and Table S2). We found that the six genes whose expression was the most increased in the linc-MYH knockdown were also significantly upregulated in cSix1 KO muscles (Figure S5).
Besides slow muscle genes, two genes, Ankrd1 and Peg10, were more severely upregulated in the linc-MYH knockdown line (10 and 8 times, respectively) than in cSIX1 mutant myofibers (by 2.8 and 1.5 times, respectively). These non slow-type genes could be exclusively repressed by linc-MYH in adult fast myofibers since there is a stronger downregulation of linc-MYH accumulation after its knockdown than in cSix1 mutant myofibers. Transcriptomic analysis of adult myofibers deprived of either Six1 or of linc-Myh identified a strong qualitative and quantitative correlation in the expression of specific genes between linc-MYH knockdown and cSix1 adult mutant myofibers. The expression of slow muscle genes was 3 to 10 fold higher in linc-MYH knockdown samples, and 5 to 25 fold higher in cSix1 KO samples, than in the wild type. We further showed that linc-MYH lies downstream of Six1 in the Six myogenic pathway and helps to repress slow muscle genes in fast myofibers. The downregulation of all fast-type genes (other than Myh4), and the upregulation of slow-type genes, was weaker in the linc-MYH knockdown than in the cSix1 cKO line. Six1 may control several inhibitory pathways, including the linc-MYH pathway, to prevent slow-type gene expression in fast myofibers.

Forced expression of linc-MYH in adult slow Soleus

To test the possibility that linc-MYH could modulate the expression of specific muscle genes, we forced its expression in myogenic C2 cells and in primary myoblasts, where endogenous muscle-specific cSox6 mutant [30]. This demonstrates that linc-MYH and Sox6, lying both downstream of Six1, directly participate in the downregulation of Sln, Tnn1, and Tnt1 in fast myofibers. However, the repression of slow Myh7 in fast myofibers acts by a Six1-Sox6 dependent, but linc-MYH independent, repression mechanism. In this study, we observed that the levels of fast muscle gene expression decreased by 2-3 folds in the linc-MYH knockdown, with the highest decrease found for Myh4 expression. The expression of these genes decreased by a factor of 1.3 to 2.5 in cSix1KO, with the highest decrease found for Pvalb expression (Figures 3E and 5A). The presence of Six4 and Six5 proteins in adult myofibers [11], which have the same DNA binding specificity as Six1, could compensate its absence in cSix1 KO animals and enable the activation of downstream fast muscle targets. In this case, linc-MYH expression could be preferentially dependent upon Six1, rather than on Six4 or Six5. Altogether, these experiments suggest that the accumulation of linc-MYH transcripts in the nuclei of fast myofibers facilitates the regulation of a network of genes that drive myofiber specialization via the same pathway as Six1 and downstream of this transcription factor.

Figure 3. Six1 deficiency impairs the adult phenotype of fast muscle. (A) Six1 mRNA expression levels in GP and Sol muscles of three month-old Ctrl (n = 4) and cSix1 KO (n = 3) mice. (B) Western blot analysis of Six1 and βtubulin expression in Sol and GP of Ctrl and cSix1 KO mice. (C) Time to fatigue ratio of TA muscles of Ctrl (n = 4) and cSix1 KO (n = 4) mice. (D) Percentage of myofibers expressing MYH7, MYH2 and MYH4 in TA muscles of three month-old Ctrl (n = 4) and cSix1 KO (n = 4) mice. *P<0.05, **P<0.01, ***P<0.001. doi:10.1371/journal.pgen.1004386.g003
lin-c-MYH expression was faintly detectable even in myotubes four days after their differentiation (data not shown). Transfection of a 15 kb genomic fragment encompassing the whole lin-c-MYH gene lead to efficient lin-c-MYH RNA accumulation in myotubes, but after this forced expression, we were unable to detect any modification in the expression of slow or fast type genes (data not shown). These results suggest that specific cofactors of lin-c-MYH required for its appropriate functioning are lacking in cultured myotubes in culture, in agreement with the expression of lin-c-Myh only in adult fast type fibers. To circumvent the limitations of cultured cells, we turned to in vivo experiments in the Soleus in which lin-c-Myh is weakly expressed. Two weeks after lin-c-MYH gene transfection in the Soleus we observed that lin-c-MYH RNA accumulates up to approximately 80% of its expression level in TA (Figure 5E). We observed a selective upregulation of Myh4 and Pvalb mRNAs which were increased to approximately one-third their expression level observed in TA, while mRNA for slow genes remained unchanged (Figure 5E). To test whether the increase in Myh4 and Pvalb mRNA was due to an increased transcription of their genes, and potentially exclude a mechanism implicating mRNA stabilization, we measured pre-mRNA accumulation. As can be seen in Figure 5F, the transcription of these two fast genes is upregulated in the Soleus samples expressing lin-c-MYH proportionally to their mRNA accumulation, demonstrating that lin-c-Myh can work in trans and allow efficient activation of the transcription of specific fast genes. Absence of down regulation of the expression of slow genes in Soleus myofibers expressing lin-c-Myh suggests that, like in cultured myotubes, lin-c-Myh RNA needs specific protein-binding partners to achieve its function. Such specific protein-binding partners may be absent in Soleus as well as in cultured myotubes. Nuclear long non coding RNA are known to guide chromatin modifiers to specific gene loci, and by recruiting histone modifiers or DNA methyltransferase to modulate their transcription rate [31]. Potential lin-c-Myh protein partners expressed differentially in fast and slow adult myofibers may explain how lin-c-Myh efficiently represses the transcription of slow genes and activates the transcription of fast genes in fast myofibers, while in slow myofibers its forced expression is only able to activate the transcription of fast genes.

Discussion

The commitment and maintenance of muscle fiber fast sub type specialization relies on the specific expression of one of the fast Myosin heavy chain gene present at the fast Myh locus, and of specific isoforms of sarcomeric genes [1,2,4]. Myosin heavy chains are the primary determinant of the efficiency of muscle contraction. In this manuscript, we identified a novel mechanism for the specialization of the fast-myofiber subtype. We show that the long intergenic non-coding RNA lin-c-MYH and fast MYH genes, both of which are essential for myofiber specification, share a common enhancer which is regulated by Six1 homeoproteins. The lin-c-MYH specifically accumulates in nuclei of adult fast myofibers. Its function, as revealed here by in vivo knockdown and transcriptome-wide analysis, is to prevent slow-type muscle gene transcription and increase fast-type muscle gene expression in fast-type myofibers. We found lin-c-MI1H downregulates the transcription of genes associated with slow muscle contractile properties like the slow genes Tnn and Sln (a known repressor of Serca1/Atp2a1 protein [32,33] involved in Ca++ reuptake by the sarcoplasmic reticulum). These genes, which belong to the muscle contractile machinery and are repressed in adult fast myofiber, are positively controlled by Six1 in myogenic C2 cells [34], where lin-
Figure 5. Slow-type gene expression is suppressed by linc-MYH. (A) qPCR experiments revealing mRNA expression levels of linc-MYH, Six1, slow-type genes (red) and fast-type genes (blue) in TA muscles expressing a shRNA directed against linc-MYH or LacZ (n = 5). (B) qPCR experiments revealing pre-mRNA expression of the Tnnt1 and Sin slow genes in TA muscles expressing a shRNA directed against linc-MYH or LacZ (n = 5) (C) Luciferase activity of pGL3-Enhancer-M4 in TA muscles expressing a shRNA directed against linc-MYH or LacZ (n = 4). (D) Microarray analysis of TA muscles transfected by shRNA against linc-MYH: a heat map of genes (red) upregulated to more than double the levels observed in cSix1KO. (E) qPCR experiments revealing mRNA expression levels of linc-MYH, Six1, slow-type genes (red) and fast-type genes (blue) in Soleus muscles expressing linc-MYH or the empty vector pSF (n = 4). (F) qPCR experiments revealing pre-mRNA expression of the Myh4 and Pvalb fast genes in Soleus muscles expressing linc-MYH or the empty vector pSF (n = 4) (G) A model of Six1 controlling the expression of the different MYH and of linc-MYH at the fast Myh locus in fast myofibers. Below, the hypothesis explaining the linc-MYH mode of action, as supported by the transcriptomic-wide analysis performed after linc-MYH knockdown in fast TA. *P < 0.05, **P < 0.01, ***P < 0.001.

doi:10.1371/journal.pgen.1004386.g005
MYH expression is not detected. This suggests that their expression in adult fast myofiber may be restricted by an additional level of regulation involving the Six1-linc-MYH axis. As a result of our study we suggest that Six1 controls the acquisition of fast-type myofiber mechanical properties by binding to a single enhancer region of the fast Myh locus. It promotes the coordinated expression of fast Myhs and that of a strong repressor of genes controlling slow contractile properties. The modulation of Six activity (depending on fiber-type) facilitates changes in the expression levels of the fast genes Myh and Tam; these changes are required for the formation of efficient sarcomeric units and the appropriate Ca\(^{2+}\) cycling and excitation/contraction/relaxation coupling [1–4]. The Myh enhancer element therefore connects distinct regulatory hubs to achieve ultimate muscle fiber specialization. In this context, linc-MYH functions as an end-of-the-chain control element, conveying information on the state of fast Myh enhancer activity to repress slow-type specific genes and coordinates a finer level of regulation. This genomic organization at the fast Myh locus is reminiscent of the slow Myh7 locus where two microRNA miR-208b and miR-199 involved in fast myofiber program repression are co-regulated with Myh7 [35]. The precise molecular interactions between linc-Myh and higher order chromatin modifying complexes remains to be identified, to explain how linc-Myh coordinates the activation of target genes at specific sites in the nucleus, and the repression of others.

**Materials and Methods**

**Mice, ethics statement**

Animals were bred and handled as recommended by European Community guidelines. Experiments were performed in accordance with the guidelines of the French Veterinary Department. cSix1 KO mice were obtained by breeding the Six1-LoxP mice [26] and transgenic mice expressing a CRE recombinase under the control of the human skeletal actin promoter (HSA) [25].

**ChIP experiments**

GP and TA muscles of 2 months old mice were minced with scissors just after sampling and fixed in 1% formaldehyde for 10 minutes. Formaldehyde was quenched by addition of 0.125 M glycine, and muscles were washed twice in PBS. The muscles were incubated on ice in lysis buffer (10 mM Tris-HCl pH 7.9, 85 mM KCl, 0.5% NP40, protease inhibitors (Complete, Roche)) for 10 minutes and were reverse transcribed with Superscript III kit (Invitrogen) according to manufacturer's instruction. The data was performed by quantitative real-time PCR using the Lightcycler 480 probe master (Roche Diagnostic). The sequences of the oligonucleotides used in this study are given in Table S3.

**RNA preparation**

TA, back, soleus and GP muscles were collected from cSix1 KO and control mice. Total RNAs were extracted by Trizol Reagent (Invitrogen) according to manufacturer's instruction.

**cDNA synthesis and qPCR**

RNAs were treated with DNase I (Turbo DNA-free, Invitrogen) and were reverse transcribed with Superscript III kit (Invitrogen) according to manufacturer’s instruction. Reverse transcription was performed with 1 µg of total RNA. Quantitative real-time PCR (Light Cycler 480, Roche) was performed using Light Cycler 480 SYBR Green I Master Kit (Roche) according to the manufacturer’s protocols. PCR was performed for 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. Genes expression level was normalized by the expression level of the housekeeping gene Actb. The sequences of the oligonucleotides used in this study are given in Table S4. Pre-mRNA qPCR experiments to measure RNA transcription rate were performed in the same conditions. Reverse oligonucleotides were complementary to intronic sequences, while forward oligonucleotides were complementary to exonic sequences. Samples without reverse transcription were used as controls, and signal due to contaminating DNA was subtracted to the values obtained with cDNA. We noticed that genomic DNA contamination was very low (less than hundred fold level of qPCR value observed with cDNA).

**Muscle contraction test**

Skeletal muscle function was evaluated by measuring in situ muscle contraction, as described previously [37]. 12 week-old male
mice were anesthetized (intraperitoneal injection of pentobarbital sodium, 50 mg/kg). Body temperature was maintained at 37°C using radiant heat. The distal tendon of the TA muscle was attached to an isometric transducer (Harvard Bioscience) using a silk ligature. The sciatic nerves were proximally crushed and distally stimulated by a bipolar silver electrode using supramaximal square wave pulses of 0.1 ms duration. Responses to tetanic stimulation (pulse frequency 50–145 Hz) were successively recorded. Maximal forces were determined at optimal length (length at which maximal force was obtained during the tetanus). Fatigue resistance was then determined after a 5-minutes rest period. The muscle was continuously stimulated at 50 Hz for 2 minutes (submaximal continuous tetanus), and the duration corresponding to a 20% decrease in force was recorded.

RNA-FISH

Fluorescent-labeled antisense linc-MYH probes were synthesized according to manufacturer’s instruction (FISH Tag RNA kit, Invitrogen). FISH experiments were performed on isolated EDL myofibers and images acquired on a Leica SP2 confocal microscope.

Generation of shRNA against mouse linc-MYH

Five distinct shRNAs targeting mouse linc-MYH were designed, called shlinc-MYH, and inserted into the psiSTRIKE hMGFP system (Promega). The efficiency of each shRNA was established by determination of linc-MYH transcript levels in TA muscles transfected by each shlinc-MYH. The shRNA against 5'-TTG TGC TCA CGA CCT ACA ATT-3' sequence was selected for the knockdown experiment. For knockdown experiments using shlinc-MYH, a plasmid coding for shLacZ was electroporated in the contra-lateral TA as a negative control.

Electroporation

In vivo transfections were also carried out on 10-weeks old C57Bl/6 mice. For each experimental conditions, three to five Tibialis anterior (TA) or Soleus (Sol) muscles belonging to different mice were used. Under isoflurane anesthesia, legs were shaved and muscles were pre-treated by injection of a sterile 0.9% NaCl solution containing 0.4 U of bovine hyaluronidase/μl two hours before plasmid injection. Ten μg of shRNA-expressing vector were introduced into TA muscles of 8-week-old mice by electroporation as previously described [11]. Two weeks following electroporation, TA myofibers expressing GFP were disected under a Nikon SMZ1500 stereo microscope and frozen in liquid nitrogen before processing for Luciferase assays or RNA purification.

Immunohistochemistry

TA, soleus and gastrocnemius muscles were embedded in cryomatrix and quickly frozen in isopentane cooled with liquid nitrogen. Cryostat sections (10 μm) were fixed in 4% PFA, washed in PBS, permeabilized with 0.1% Triton X-100 and left for 1 hour in blocking solution (1× PBS, 1.5% goat serum, 0.1% Triton X-100). Rabbit polyclonal antibodies directed against Laminin (Z0097, Dako) (1/100 dilution), and monoclonal antibodies against Myh (NOQ7.5.4D, Sigma) (1/1000 dilution), Myh2 (SC-71, Developmental Studies Hybridoma Bank) (1/20 dilution) and against Myh (BF-F3, Developmental Studies Hybridoma Bank) (1/20 dilution) were applied overnight at 4°C to the treated sections. The next day, after three washes with 1× PBS containing 0.05% Tween-20, cryosections were incubated for 1 h with appropriate fluorescent secondary antibodies (Alexa Fluor 488 goat anti-rabbit IgG 1/1000 dilution, Alexa Fluor 594 goat anti-mouse IgG 1/1000 dilution, Invitrogen). After three washes with 1× PBS containing 0.05% Tween 20, samples were mounted in Vectashield mounting medium.

Microarray

After validation of RNA quality with the Bioanalyzer 2100, 50 ng of total RNA were reverse transcribed following the Ovation PicoSL WTA System (Nugen). Briefly, the resulting double-strand cDNA was used for amplification based on SPIA technology. After purification according to Nugen protocol, 5 μg of single strand DNA was used for generation of Sens Target DNA using Ovation Exon Module kit (Nugen). 2.5 μg of Sens Target DNA were fragmented and labelled with biotin using Encore Biotin Module kit (Nugen). After control of fragmentation using Bioanalyzer 2100, the cDNA was then hybridized to GeneChip Mouse Gene 1.0 ST (Affymetrix) at 45°C for 17 hours. After overnight hybridization, the ChiPs were washed using the fluidic station FS450 following specific protocols (Affymetrix) and scanned using the GCS3000 7G. The scanned images were then analyzed with Expression Console software (Affymetrix) to obtain raw data (cel files) and metrics for Quality Controls. The analysis of some of these metrics and the study of the distribution of raw data show no outlier experiment. RNA normalization was performed using R and normalized data was subjected to statistical tests.

EMSA

EMSA was carried out with Six1 full-length mouse cDNA cloned into the pCR3 vector (Clontech) as previously described [38]. Recombinant mouse Six1 protein was obtained with a T7 transcription/translation kit (Promega). The oligonucleotide containing double-stranded myogenin MEF3 site was incubated with recombinant proteins. Competition experiments were performed in the presence of a ten-fold and hundred-fold molar excess of unlabeled identified Myh enhancer MEF3 sites (Enh1 to Enh6) or mutated Myh MEF3 sites (mtEnh1 to mtEnh6), or Myogenin promoter NFI or MEF3 sites. The sequences of the oligonucleotides used are as follows, the MEF3 consensus sequence is underlined; Enh1F 5'-CTG TGTC AAC TGG AGG CCC TC-3'. Enh1R 5'-GAG GGG CTC CAG TTA CCC AAC AG-3'. Enh2R 5'-GGT TGA CCT AGT TTA CCA ATC-3'. Enh3F 5'-TGG TGA AAG AGC TIA AAT ATC-3'. Enh3R 5'-ATT TTA TTT CAG TTT CCT TTA CA-3'. Enh4F 5'-GGA GTA AGA ATG CTC ACC AAC AGG-3'. Enh4R 5'-CTT GTC TGA AGA TTT ACC CCC CC-3'. Enh5F 5'-CTT GTC TGA CCT ACC CCC CC-3'. Enh5R 5'-TTA TTT CAG TTT CCT TTA CA-3'. Enh6F 5'-GGG GTA AGA ATG CTC ACC AAC AGG-3'. Enh6R 5'-CTT GTC TGA AGA TTT ACC CCC CC-3'. Enh7F 5'-CTT GTC TGA CCT ACC CCC CC-3'. Enh7R 5'-TTA TTT CAG TTT CCT TTA CA-3'. Enh8F 5'-GGG GTA AGA ATG CTC ACC AAC AGG-3'. Enh8R 5'-CTT GTC TGA AGA TTT ACC CCC CC-3'.

ChIP-seq

ChIP-seq was performed on 16 weeks old C57Bl/6 mice. The distal tendon of the TA muscle was continuously stimulated at 50 Hz for 2 minutes (submaximal continuous tetanus), and the duration corresponding to a 20% decrease in force was recorded. RMA normalization was performed using R and normalized data was subjected to statistical tests.

Linc-MYH Locks Fast Muscle Phenotype
mtEnh6F 5′-CGT CAA GGA AGG ATC CCT TCG CCA TC-3′.
mtEnh6R 5′-GAT GGA AGG ATC CCT CTC TGT TGA CG-3′.

Western blot
Western blots were performed with protein extracts of GP and soleus muscles from c57bl6N mice and control mice as previously described [9]. 1:1000 dilutions of anti-Six1 antibodies (HPA001893, Sigma) or anti-β-tubulin antibodies (2120, Cell Signaling) were used.

Statistical analysis
All graphs represent mean values ± SEM. Significant differences between mean values were evaluated using two-tailed, unpaired Student’s t test (when two groups were analyzed) or one-way ANOVA followed by Student Newman-Keuls test (for three or more groups).

Plasmids construction
For the construction of the pGL3-Myh2/1/4, e57bl6N mouse DNA was first used as a template to clone 1.1 kb promoters of Myh2/1/4 with forward KpnI/SacI 5′-TTC AGC AAC TGC ATC ACT TAA A-3′ and reverse MluI, 5′-GCA CCT CGG GCA GTG GCC AGT GT-3′, forward KpnI/SacI 5′-CAT ATC TGC ATC TCT AGA TAC C-3′ and reverse MluI, 5′-GGG AGC AGC AGG CAT GAT GTG T-3′, forward KpnI/SacI 5′-ACC GCT AGC CTT GAG CCT TTG-3′ and reverse MluI, 5′-ATA GCG AGA GCC CTT TGT TCT C-3′, respectively. Myh2/1/4 promoter fragments were subsequently inserted into KpnI-SacI site of pGL3 basic plasmid. For the construction of the pGL3-mutEnhancer-Myh2/1/4, mouse DNA was first used as a template to clone the enhancer with forward KpnI/SacI 5′-GCG TTT CTA ATT CGG CCT GAA C-3′ and reverse SacI, 5′-CAT TTT CCT CTT CTA AAG GCT TTT TTT C-3′. This enhancer fragment was subsequently inserted into KpnI-SacI digested pGL3-Myh2/1/4 plasmids. For the construction of the pGL3-mntEnhancer-Myh2/1/4, the six MEF3 sites of the enhancer were mutated as follows; MEF3 1: 5′-GAAACTGAA to 5′-CCAACTGAA; MEF3 2: 5′-GAAA-AGGATCC; MEF3 3: 5′-GAAAACCTGA to 5′-GAAACCCTGA; MEF3 4: 5′-GAAATCTGA to 5′-GAGGATTCC; MEF3 5: 5′-GTCAGCGGGA to 5′-GTGGCGATCC; MEF3 6: 5′-GAAAGCCTTA to 5′-GAAAGGATCC. All plasmids sequence was confirmed by sequencing.

For the construction of the pSf-pa-CMV-linc-MYH, genomic DNA fragment containing linc-MYH was obtained from digestion of a BAC clone containing (RP23-61C14) by BsaWI and AvrII. The 13.3 kb DNA fragment was subsequently inserted into a SpeI-Xmal digested pSf-pa-CMV plasmid. For linc-MYH gain of function experiments, the empty pSf-pa-CMV plasmid was electroporated in the contra-lateral Soleus as a negative control.

For the construction of the pGL3-Actb, pGL3-Sln and pGL3-TnnI1, mouse DNA was first used as a template to clone the promoters of Actb, Sln, and TnnI1 with forward 5′-TCT CTC TAT CTA TAG GTA CTT TCT TCT GAG CAG GGG GTA AGG AGC TGG AAA GAA GCT G-3′, forward 5′-TCT CTC TAT CTA TAG GTA CTT TCT TCT GAG CAG GGG GTA AGG AGC TGG AAA GAA GCT G-3′, and reverse MluI, 5′-GAG CCG GTA CTA GGC GTA AGG GTA TGG AAA GAA GCT G-3′, then all nucleotide changes were confirmed by sequencing.

Luciferase assays
Two μg of Luciferase-expressing vector and one hundred ng of pRL-TK vector (Promega) were introduced into TA muscles of 8 week-old mice by electroporation as previously described [11]. Two weeks following electroporation, the TA muscles were dissected and frozen in liquid nitrogen before processing. The TA muscles were homogenized in Passive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega) and rotated for 15 minutes. The homogenate were centrifuged to remove debris, and the supernatant was used for measurement of Luciferase activity according to manufacturer’s instruction (Dual-Luciferase Reporter Assay System, Promega).

Computational analysis
In order to computationally identify MEF3 binding sites, we built a PWM (position-specific weight matrix) for MEF3, starting from a list of 15 binding sites (see Table S5) that were previously tested by Electrophoretic Mobility Shift Assay on the basis of their proximity to the MEF3 Myogenin consensus GAAACCTGA [10]. The PWM (shown in Figure S6 and Table S6) was generated by the de novo motif finder Imogene [39] on small DNA fragments (see Table S5) that contained these binding sites. Imogene used phylogeny to enrich mouse set of DNA fragments with orthology in 11 other mammalian sequenced genomes and to produce a refined PWM. The information content of the PWM (the genmot Sg parameter of Imogene) was set to 8.7 bits. Binding sites were predicted using a prediction threshold (the scangen Ss parameter of Imogene) of 9 bits and requiring conservation, as explained in [39].

Supporting Information
Figure S1 Sequences of P1 and P2 boxes of the Myh enhancer. Sequences of P1 and P2 boxes of the Myh enhancer in mouse, rat, human, bovine and equinides species, and showing the sequence conservation of the six MEF3 sites and E boxes. Coordinates are in mm9 assembly. (TIF)

Figure S2 Predicted linc-MYH structure. Predicted minimum free energy (MFE) structure of the 1050 nt long linc-MYH, as determined by RNAfold [40]. The color encodes base-pair probabilities. (TIF)

Figure S3 (A). Competitive Electromobility shift assays. Competitive Electromobility shift assays performed with recombinant Six1 proteins and labeled Myogenin MEF3 oligonucleotide and 10 or 100 fold molar excess of unlabelled oligonucleotides containing Myogenin MEF3 or NF1 site, with MYH MEF3 sites (1, 2, 3, 4, 5, 6) or with mutated MYH MEF3 sites whose sequence is presented on Figure S1 and in the Materials and Methods section. (B). qPCR experiments measuring the relative levels of Sln mRNA in adult wt and transgenic mouse. (C). Luciferase assays of adult TA transfected with Sln, TnnI1 and Actb promoters with or without the Myh enhancer. TA sampling was performed two weeks after the electroporation, (n = 4). (D). Primary embryonic fibroblasts were transfected with Luciferase plasmids under the control of Myh4 promoter or Myh4

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promoter linked with the Myh enhancer. After transfection, fibroblasts were cultured two days before sampling, (n = 3). *P<0.05, **P<0.001.

**(TIF)**

**Figure S4** Immunostaining of MYH proteins. Immunostaining of MYH7 (red), MYH2 (red), MYH4 (red) and laminin (green) in TA of 12 weeks old control and cSix1KO male mice. (TIF)

**Figure S5** Comparison between cSix1 mice and shlinc-MYH treated mice. (A) Venn diagram showing the overlap between genes that are up-regulated more than five fold in shlinc-MYH and two fold in cSix1. Genes that are up-regulated more than five fold in shlinc-MYH and two fold in cSix1 are indicated in red. (C) mRNA expression levels in shlinc-MYH knock-down and cSix1 TA muscles, as measured by qPCR experiments. Ctrl (means ± SEM; n = 4), cSix1 (means ± SEM; n = 4), shLacZ (means ± SEM; n = 5), shlinc-MYH (means ± SEM; n = 5). (D) mRNA expression level of Ankrd1, Zdbf2 and Pdgfr in back muscles of cSix1 KO mice at E18.5, P2W, P4W and P8W, as determined by qPCR experiments, (means ± SEM; n = 3 to 6 for each point). *P<0.05, **P<0.001.

**(TIF)**

**Figure S6** MEF3 PWM used in this study.

**(XLS)**

**Table S1** Microarrays analysis of TA muscles electroporated by shRNA.

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**Table S2** Microarrays analysis of GP muscles of cSix1.

**Table S3** Sequence of the oligonucleotides used for 3C.

**Table S4** Sequence of the oligonucleotides used for qPCR.

**Table S5** Coordinates (mm9) of the fragments used to learn the MEF3 PWM.

**Table S6** MEF3 frequency matrix.

**Acknowledgments**

We thank V. Moncollin at ENS Lyon for help with the adult muscle ChIP experiments, the imaging facility at Institute Cochin for technical assistance, the sequencing and genomic platform at Institute Cochin for microarray experiments and F. Dumont and S. Jacques for advice. We thank P. Bilharz for the shRNA expression vector, F. Le Grand for teaching isolated myofibers isolation, E. Pilet for his help with gene expression analysis, D. Blanchot for advice on the FISH experiment and S. Gautron, C.Rougeulle, I. Daudelo, F. Le Grand and A. Sotiroupolous for reading the manuscript.

**Author Contributions**

Conceived and designed the experiments: IS MS AF VH PM. Performed the experiments: IS AF PM. Analyzed the data: IS MS AF VH PM. Contributed reagents/materials/analysis tools: IS PM. Wrote the paper: IS PM.
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