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.

Blurb

linc-MYH is transcribed at the LCR of fast *Myh* gene locus and control skeletal muscle slow/fast phenotypes by suppressing slow-type gene expression.

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 *Myhs* and associated fast sarcomeric genes (including *Tnnt3, Tnni2*, *Tnnc2, Atp2a1* and *Pvalb*) (Schiaffino et Regiani, 2011; Greising et al, 2012). 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* (*MYHCIIX*) or *MYH4* (*MYHCIIB*). Fast *Myh* genes found in developmental and adult stages *(Myh3, Myh2, Myh1, Myh4, Myh8* and *Myh13*) are organized as a cluster within a 300kb 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 intramyofibrillary 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 myofibres. 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 cytoplam 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 *in vivo*. We demonstrate a three-element genetic partnership, where a LCR under the control of the myogenic homeoprotein Six1 functions as a regulatory hub to control fiber phenotype. In this partnership, the LCR 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 and Discussion

Six1 binds directly to the enhancer/LCR of the *Myh* genes cluster.

We suggest that Six1 could be directly involved in the control of the expression of fast *Myh* genes as higher levels of this transcription factor accumulate in the nuclei of adult fast myofibers than in slow myofibers. We used computational analysis to locate MEF3 sites (at the fast *Myh* locus) to investigate how *Six1* could control the expression of fast *Myh* isoforms. 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 4kb upstream of a lincRNA (2310065F04Rik); we refer to this as *linc-MYH* (Figures 1A and S2). Six1 binding at these MEF3 sites was demonstrated *in vivo* by ChIP experiments with Six1 antibodies on adult fast gastrocnemius plantaris (GP) and tibialis anterior (TA) muscles (Figure 1*B*), and confirmed for five of these sites by EMSA assays (Figure S3). We asked

whether this *Myh* intergenic region could constitute an enhancer element, controlling the spatio-temporal expression of *Myh* genes in this locus. A 2kb DNA fragment of this region, including the six identified MEF3 sites and 1kb 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. We mutated all six MEF3 sites present in the enhancer, and named these reporters pGL3-mutEnhancer-*Myh2/1/4,* to test the involvement of Six binding in enhancer activation of the *Myh2*, *Myh1* and *Myh4* promoters. Luciferase activity was tested after the electroporation of reporter plasmids in adult TA muscles. The luciferase activity of pGL3-Enhancer-*Myh2/1/4* was between seven and twelve times higher with either of the promoters, than with pGL3-*Myh2/1/4*. Enhancer activity was not observed in plasmids with MEF3 mutated sites associated with either of the *Myh* promoters (Figure 1C). To determine *in vivo* interactions between the enhancer and each *Myh* gene, we performed chromatin conformation capture (3C) assays of adult EDL muscles. 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 $Six1^{flox/flox}$ mice with transgenic mice expressing CRE recombinase under the control of the human skeletal actin (HSA) promoter and obtained $Six1^{flox/flox}$*;HSA-CRE* conditional knockout mice (hereafter named *cSix1 KO*) [25,26]. 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 can be studied 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, Tnnt3*, *Tnni2*, *Tnnc2* and *Pvalb)* increased during postnatal development in control mice but that of slow-type genes (*Myh7, Tnnt1*, *Tnni1*, *Tnnc1* and *Sln*) decreased. The *linc-MYH* RNA was detected after birth in muscle samples and its expression increasing 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 between three and five times in *cSix1 KO* mice during postnatal development (Figure 2). These data show that *Six1* induces *linc-MYH* and fast-type genes during postnatal development.

***Six1* deficiency impairs adult muscle fast phenotype.**

We analyzed 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. 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: high levels of *Myh*7 and *Myh*2 mRNA, and lower levels of *Myh*4 mRNA levels, were observed (Figure 3E) in the fast TA muscles of *cSix1 KO*. Expression levels of other specific fast and slow-type genes were also tested. We found downregulation of fast-type genes (*Tnnt3*, *Tnni2*, *Tnnc2* and *Pvalb*) and between a five and to 25 times increase in the levels of slow-type genes (*Tnnt1*, *Tnni1*, *Tnnc1*

and *Sln*) (Figure 3E). The expression of *linc-MYH* expression was lower in the adult TA of *cSix1 KO* mice, than in control mice (Figure 3E). Our results indicate that the *Six1* homeoprotein can control the phenotype of fast skeletal myofibers in adult animals.

***linc-MYH* is exclusively expressed 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, like in the nuclei of *MYH4* myofibers [11], and that the less robust nuclear accumulation of Six1 observed in SOL myonuclei did not induce *linc-MYH* expression. We used luciferase reporter transfection assays (as described previously) to test the requirement for Six binding on the *MYH* enhancer to activate *linc-MYH* expression. These transient transfection assays, performed in adult TA, show that the *MYH* enhancer activates *linc-MYH* expression in a Six-dependent manner (Figure 4B). *lincRNAs* can localize in cytoplasm [16] or as a single focus [19] or multiple foci [20] in nuclei. We performed fluorescent *in situ* hybridization (FISH), using *linc-MYH* antisense RNA and isolated myofibers from fast EDL, to analyze *linc-MYH* localization in skeletal muscle fiber. Intranuclear localization of *linc-MYH* was observed, with approximately 10 *linc-MYH* foci per nucleus (Figure 4C).

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

Following these observations, we hypothesized that *linc-MYH* 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-MYH* (sh*linc-MYH*) in TA muscle and analyzed the transfected samples after fourteen days. This method yielded the efficient knockdown of *linc-MYH*, with a 90% reduction of its expression (Figure 5A). RNA samples from sh*linc-MYH* transfected adult TA were analyzed by Affymetrix microarrays (Figure 5B and Table S1), and

validated by qPCR experiments, to identify the consequences of *linc-MYH* knockdown and understand its mode of action. The expression of *linc-MYH* was significantly lower in the absence of *Six1* but, *Six1* expression was not affected by the absence of *linc-MYH.* Knockdown of *linc-MYH* produced a phenotype with robust gene expression phenotype; this downregulated the expression of several fast genes, (including *Tnnt3*, *Tnni2* and *Pvalb*), and upregulated the expression of numerous slow genes (such as *Sln*, *Tnni1*, *Tnnc1* and *Tnnt1*) (Figure 5B). Contrary to what was observed in the muscles of *cSix1* KO mice, slow *Myh*7 expression level did not change. Following *linc-MYH* knockdown, expression levels of the neighboring genes *Myh2* and *Myh1* did not change but the expression of the more distant *Myh4* gene was downregulated; this suggests a specific requirement of *linc-MYH* for *MYH4* expression. We found a strong qualitative and quantitative correlation in the expression of specific genes between *linc-MYH* knockdown and *cSix1* mutant myofibers. The expression of slow muscle genes was between 3 and 10 times greater in *linc-MYH* knockdown samples, and between 5 and 25 times greater in c*Six1*KO samples, than in the wildtype. This suggested 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 *Myh*4), and the upregulation of slow-type genes, was weaker in the *linc-MYH* knockdown than in the *Six1*cKO line. *Six1* may control several inhibitory pathways, including the *linc-MYH* pathway, to prevent slow-type genes expression in adult fast myofibers. During fetal development, at a stage where *linc-MYH* expression is not yet activated, *Six1/4* increases the nuclear accumulation of the slow muscle repressors Sox6 and HDAC4 to repress slow muscle gene expression [9,27,28]. In accordance with this, the expression of the slow genes *Myh7, Sln*, *Tnni1* and *Tnnt1* is upregulated in the muscle-specific *cSox6* mutant [29]. This demonstrates that *linc-MYH* and Sox6, both lying downstream of Six1, directly participate in the downregulation of *Sln*, *Tnni1* and *Tnnt1* in fast myofibers. However, the repression of slow

Myh7 in fast myofibers has a Six1-Sox6 dependent, but *linc-MYH* independent, repression mechanism. In this study, we observed that levels of fast muscle gene expression decreased by between a factor of 2 and 3 in the *linc-MYH* knockdown, with the highest decrease found for *Myh*4 expression. Their expression decreased by a factor of 1.3 to 2.5 in c*Six1*KO, with the highest decrease in levels of *Pvalb* expression (Figures 4E 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 c*Six1* 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.

Transcriptomic analysis of *cSix1* and *linc-MYH* knock down.

We compared the networks of genes under the control of *linc-MYH* and of Six1 homeoprotein in adult muscles by the transcriptomic analysis of c*Six1* and *linc-MYH* knockdown lines (Figure 5A 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). Two genes, *Ankrd1* and *Peg10*, were more severely upregulated in the *linc-MYH* knockdown line (10 and 8 times, respectively) than in c*SIX1* mutant myofibers (by 2.8 and 1.5 times, respectively). These non slow-type genes may be exclusively repressed by *linc-MYH* in adult fast myofibers as there was stronger downregulation of *linc-MYH* accumulation after its knockdown than in c*Six1* mutant myofibers. The accumulation of *linc-MYH* transcripts in the nuclei of fast myofibers seem to facilitate the regulation of a network of genes that drive myofiber specialization via the same pathway as *Six1* and downstream of this transcription factor.

Conclusion. We identified a novel mechanism for the specialization of the fast-myofiber subtype. The long intergenic non-coding RNA *linc-MYH* and fast *MYH* genes, both of which are essential for myofiber specification, share a common enhancer which is

regulated by Six1 homeoproteins. The RNA *linc-MYH* specifically accumulates in nuclei of adult fast myofibers. Its function, as revealed by *in vivo* knockdown and transcriptome-wide analysis, is to prevent slow-type muscle gene expression and increase fast-type muscle gene expression in fast-type myofibers. *linc-MYH* was found to downregulate genes associated with slow muscle contractile properties like the slow genes *Tnn* and *Sln* (a known repressor of Serca1/Atp2a1 protein [30,31] 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 [32]. 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 these observations 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 *Tnn*; these are required for the formation of efficient sarcomeric units and the appropriate Ca^{++} cycling and excitation/contraction/relaxation coupling. The enhancer element therefore connects distinct regulatory hubs to achieve ultimate muscle fiber specialization. *linc-MYH* functions as an end-of-the-chain control element, conveying the state of fast *Myh* LCR activity to repress slow-type specific genes and coordinate a finer level of regulation.

Materials and Methods

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 homogenized with a mortar and, subsequently with a Dounce homogenizer. The nuclei were obtained by centrifugation, incubated in SDS lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, protease inhibitors) for 10 minutes, and sonicated in a Bioruptor apparatus (Diagenode). The debris were removed by centrifugation. The sonicated DNA was incubated with 1 μg of *Six1* antibodies (HPA001893, Sigma) under agitation at +4 °C overnight. 20 μl of Dynabeads protein G (Invitrogen) were added to the samples and incubated under rotation at +4 °C for 1 hour. The beads were washed with low-salt buffer (2 mM EDTA, 20mM Tris-HCl pH 8, 150 mM NaCl, 1% TritonX-100, 0.1% SDS), high salt buffer (2 mM EDTA, 20mM Tris-HCl pH 8, 0.5M NaCl, 1% TritonX-100, 0.1% SDS), LiCl buffer (1 mM EDTA, 10mM Tris-HCl pH 8, 0.25M LiCl, 1% NP40, 1% deoxycholate) and TE buffer (1 mM EDTA, 10mM Tris-HCl pH 8). The DNA was eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$) containing 0.1mg/ml proteinase K (Invitrogen) at 62 °C for 2 hours, and, proteinase K was inactivated by incubation at 95°C for 10 minutes. The DNA was finally purified with MinElute PCR purification kit (Qiagen). The amount of specific amplified DNA is normalized by *beta-Actin* promoter amplification. The sequences of the oligonucleotides used in this study are as follows. Enh LCR 1F, ATCTCCACCTCCCTCCAACT; Enh LCR 1R, ACCCCCTAGCTTTGACAGGT; Enh LCR 2F, AATCTGACGACAGGGTGAGC; Enh LCR 2R, GGTCGCCTGACCTGATAGAG; AldoaF, CTCTCAAGGCAAACCAAAGC; AldoaF, CCAGTGTCCCAGACCTTCTC; ActbF, TGTTACCAACTGGGACGACA; ActbR, ACCTGGGTCATCTTTTCACG.

3C. 3C experiments were performed on adult EDL muscles as described [33]. Single myofibers were obtained from adult EDL muscles as previously described [26] and fixed. The sequences of the oligonucleotides used in this study are given in Table S3.

Mice. 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].

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 manufacture'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.

Muscle contraction test. Skeletal muscle function was evaluated by measuring *in situ* muscle contraction, as described previously [34]. 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 50–143 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 (sub-maximal 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 myofibres 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 sh*lincMYH*, 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 sh*lincMYH*. The shRNA against 5'-TTCTGCTCACCACCTACAATT-3' sequence was selected for the knockdown experiment.

Electroporation. 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 dissected under a Nikon SMZ1500 stereo microscope and frozen in liquid nitrogen before processing.

Immunohistochemistry. TA, soleus and gastrocnemius muscles were embedded in cryomatrix and quickly frozen in isopentane cooled with liquid nitrogen. Cryostat sections (10 mm) were fixed in 4% PFA, washed in PBS, permeabilized with 0.1% Triton X-100 and left

for 1 hour in blocking solution (1x 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*7 (NOQ7.5.4D, Sigma) (1/1000 dilution), *MYH*2 (SC-71, Santa Cruz biotechnology) (1/20 dilution) and against *MYH*4 (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 (using Agilent RNA6000 nano chip kit), 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 mg of single strand DNA was used for generation of Sens Target DNA using Ovation Exon Module kit (Nugen). 2.5 mg 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. RMA normalization was performed using R and normalized data was subjected to statistical tests.

EMSA. EMSA was carried out with *Six1* and Six4 full-length mouse cDNA cloned into the pCR3 vector (Clontech) as previously described [35]. Recombinant mouse *Six1* and Six4 proteins were 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 Myogenin promoter NFI or MEF3 sites. The sequences of the oligonucleotides are as follows;

Enh1F CTCTTGGGTAACTGGAGCCCCTC

Enh1R GAGGGGCTCCAGTTACCCAAGAG

Enh2R GGTTGACTTAGATTTCCTTATGA

Enh2F TCATAAGGAAATCTAAGTCAACC

Enh3F TGTAAGAGAAACTGAAATAAAAT

Enh3R ATTTTATTTCAGTTTCTCTTACA

Enh4F GGGGTAAGAAATCTGACGACAGG

Enh4R CCTGTCGTCAGATTTCTTACCCC

Enh5F CTATCAGGTCAGGCGACCTCAGT

Enh5R ACTGAGGTCGCCTGACCTGATAG

Enh6F CGTCAAGGAAACCTTATTCCATC

Enh6R GATGGAATAAGGTTTCCTTGACG

MyogF TGGGGGGGCTCAGGTTTCTGTGGCGT

MyogR ACGCCACAGAAACCTGAGCCCCCCCA

NF1F TATCTCTGGGTTCATGCCAGCAGGG

NF1R CCCTGCTGGCATGAACCCAGAGATA

Western blot. Western blots were performed with protein extracts of GP and soleus muscles from c*Six1*KO mice and control mice as previously described [9]. 1:1000 dilutions of anti-Six1 antibodies (HPA001893, Sigma) or anti-b-tubulin antibodies (2128, Cell Signaling) were used.

Statistical analysis. All graphs represent mean values ± SEM.

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Figure 1. *Six1* binds directly the enhancer/LCR of the fast *Myh* gene cluster. (A) Schematic representation of the fast *Myh* gene cluster. (B) qPCR values of ChIP experiments performed with Six1 antibodies, or IgG on GP and TA chromatin, showing Six1 binding to P1 and P2 regions of the fast *Myh* enhancer and to the muscle promoter of *AldoA* (n=3). (C) Luciferase assays from adult TA muscles electroporated with luciferase vectors (indicated) and a TK-Rennila luciferase vector allowing normalization (n=4). (D) qPCR experiments from 3C assays of wild type EDL muscle, showing the direct interactions of *Myh2*, *Myh1* and *Myh4* promoters with the fast *Myh* LCR/enhancer. *P<0.05, **P<0.01, ***P<0.001.

Figure 2. 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.

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 control (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. (E) mRNA expression levels of slow-type genes (red), fast-type genes (blue) and *linc-MYH* 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.

Figure 4. *linc-MYH* is expressed in adult fast-type skeletal muscles and accumulates in their nuclei. (A) Tissue distribution of *Six1, Myh4* and *linc-MYH* RNAs. BAT, brown adipose tissue; WAT, white adipose tissue. (n=4). (B) Luciferase assays of adult TA electroporated with *linc-MYH* promoter luciferase vectors (indicated) and a TK-Renilla

luciferase vector (allowing normalization). *P<0.05. (C) FISH of isolated EDL myofiber with a *linc-MYH* antisense RNA fluorescent-labeled probe (green) and Dapi staining (blue).

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* (n=3), or a sh*CtrlRNA* (n=3). (B) 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 c*Six1*KO. (C) A model of *Six1* controlling the expression of the different *MYH* and of *linc-MYH* at the fast *Myh* locus. 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.

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.

Figure S2. Predicted linc-MYH structure. Predicted minimum free energy (MFE) structure of the 1050nt long linc-MYH, as determined by RNAfold [36]. The color encodes base-pair probabilities.

Figure S3. Competitive Electromobility shift assays. Competitive Electromobility shift assays performed with recombinant Six1 and Six4 proteins and labeled Myogenin MEF3 oligonucleotide and 10 or 100 fold molar excess of unlabelled oligonucleotides containing Myogenin MEF3 or NF1 site, or with MYH MEF3 sites (1, 2, 3, 4, 5, 6) whose sequence is presented on Figure S1.

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.

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* muscles. ($p=10^{-17}$ as given by a hypergeometric test). (*B*) Scatter plot of mRNA expression fold change (log2) as determined by microarray analysis of shlinc-MYH and *cSix1*. Genes that are up-regulated more than five fold in shlinc-MYH and two fold in cSix1 muscles 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), shCtrl (means ± SEM; n=3), shlinc-MYH (means ± SEM; n=3). (*D*) mRNA expression level of *Ankrd1*, *Zdbf2* and *Peg10* and 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).

Table S1. Microarrays analysis of TA muscles electroporated by shRNA

Table S2. Microarrays analysis of GP muscles of cSix1

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

Table S4. Sequence of the oligonucleotides for qPCR.