Title: Six1 homeoprotein recruits the Linc MYH to control adult muscle fiber phenotype

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Abstract: The slow- and fast-type phenotypes of adult skeletal myofibers are established through the differential expression of numerous fiber type specific genes. Muscle specific Six1 knockout mice show enhanced fatigue resistance, resulting from increased number of slow type fibers. In

adult fast-muscle nuclei, Six1 binds at multiple clustered sites in the fast MyH genes locus, 50 kb upstream of the MYH2/1/4 genes, defining a potential MyH LCR. By 3C capture experiments we demonstrate direct interaction between the MyH2, MyH1 and MyH4 promoters and this LCR, which functions as an enhancer for these genes. We identify close to this LCR the long intergenic noncoding MyH (Linc-MyH) RNA, whose expression is under the control of Six1. Linc-MyH accumulates in a dozen of nuclear foci, and its knockdown in adult muscle fibers leads to upregulation of slow and downregulation of fast type muscle gene expression. This demonstrated that linc-MYH has a role for fine-tuning of fast type muscle phenotype.

One Sentence Summary: Linc-MYH participates in fine tuning of adult muscle phenotype.

Main Text: Thousands of long intergenic noncoding RNAs (lincRNAs) are encoded by the mammalian genome LincRNAs are reported to have various biological functions as transcriptional activators acting in cis (*1*) or in trans (*2*), as transcriptional repressors (*3*)(*4*) or as decoy of miRNAs(*5*)(*6*). However, the function of most LincRNAs has not yet been identified. Here, we identified a fast-type muscle specific lincRNA, linc-MYH, and analyzed its role in the adult muscle fiber.

Adult skeletal muscles are composed of slow- and fast-type myofibers, characterized by selective expression of fiber type specific genes required for the establishment of specific contraction activity and metabolic properties (*7*). Six homeoproteins are important transcription factors for the genesis of fast type myofiber during embryogenesis (*8*) (*9*), and Six1 accumulates to highest level in the nuclei of adult fast myofibers, as compared to slow myofibers (5). To analyze *Six1*

role in adult skeletal muscle physiology, and because *Six1 KO* animals die at birth (REF, Laclef et al), we bred *Six1*$^{flox/flox}$ mice and 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 c*Six1 KO*)(*10*)(*11*). Six1 mRNA and protein were no more detectable in fast-myofibers enriched gastrocnemius plantaris (GP) and slow-myofibers enriched soleus muscles of 12 weeks old cSix1 KO mice (Fig. 1, A and B). cSix1 KO mice have lower body weight and lower weight of fast type GP and tibialis anterior (TA) muscles (Fig. 1C), whereas no significant difference in the weight of other tissues (liver, kidney, epididymal fat and brown adipose tissue) was detected (fig. S1). This suggested that the lower body weight of cSix1 KO is mainly due to the lower weight of fast type muscles which form the majority of the mouse musculature. Next, we performed physiological tests on TA muscles of cSix1 KO by measuring their maximal force that was smaller, in accordance with the reduced TA muscle mass. We also observed that fatigue resistance of TA of cSix1KO mice was enhanced by 36% (Fig. 1D). To explain this increased endurance of cSIX1 KO TA, we analyzed its fiber-type composition by immunohistochemistry experiments for myosin heavy chain MYH7 (IIA), MYH2 (IIX) and MYH4 (IIB). TA muscles of cSix1 KO mice present a higher percentage of fiber expressing MYH7 and MYH2, whereas a lower percentage of fiber expressing MYH4 (Fig. 1E and fig. S2). qPCR analysis of MYH mRNA expression confirmed these results, showing increased MYH7 and MYH2 mRNA levels, and decreased MYH4 mRNA level (Fig. 1F). Expression levels of other specific fast and slow type genes was analyzed by qPCR experiments. Expression of fast type genes (Tnnt3, Tnni2, Tnnc2, Pvalb) is down-regulated, and slow type genes (Tnnt1, Tnni1, Tnnc1, Sarcolipin) was upregulated in cSix1 KO (Fig. 1F and fig. S3). These data indicated that

Six1 homeoprotein fine tunes the phenotype and the physiology of skeletal myofibers in adult animals.

We next analyzed cSix1 KO Soleus muscles and found that 100% of its fibers expressed MYH7 (I, β, slow), no fibers expressing MYH2, contrary to control Sol that present roughly 50% of MYH2 and 50% of MYH7 positive fibers. Consistent with immunohistochemistory data, MYH2 mRNA was no more detectable in Sol of cSix1 KO mice, and MYH7 mRNA level was increased by 2-fold as compared with Sol of control mice (Fig. 1G and H, and fig. S4). These data showed that Six1 is necessary to activate MYH2 expression in the slow Sol muscles, and required to activate MYH4 in the fast TA muscles.

To identify how Six1 controls the expression of fast MYH isoforms, we performed computational analysis to identify Six1 binding sites (MEF3) at the fast MYH locus. Fast MYH genes (MYH3, MYH2, MYH1, MYH4, MYH8, MYH13) are organized over 300kb as a gene cluster on mouse chromosome 11 (*12*). By this way, we identified six clustered MEF3 sites (TCAGGTTTC) conserved among human, rat and mouse genome and localized 50 kb upstream of MYH2 gene (Fig. 2a and fig. S5). Six1 binding on these six MEF3 sites was confirmed by ChIP experiments with Six1 antibodies on adult Gastrocnemius and TA muscles (Fig. 2B). Six1 binding on these MEF3 sites was also confirmed by EMSA assays (fig. S5). We hypothesized that this MYH intergenic region that binds Six1 *in vivo* may constitute an enhancer region that controls the spatio-temporal expression level of the different MYH genes at the locus. To test this possibility, we isolated a 2 kb fragment of the potential enhancer containing six MEF3 sites and 1kb upstream of transcription start site DNA from fast type MYH2/1/4 genes by PCR. This putative enhancer was ligated with each promoter of MYH genes in pGL3 basic to generate pGL3-Enhancer-MYH2/1/4. We next mutated all six MEF3 sites present in the enhancer to test

the involvement of Six binding in enhancer activation of MYH2, MYH1 and MYH4 promoters, and named these reporters pGL3-mtEnhancer-MYH2/1/4. The luciferase activity was tested after electroporation of reporter plasmids in adult TA muscles. The luciferase activity of pGL3-Enhancer-MYH2/1/4 was seven to twelve fold higher for each tested promoter, than luciferase activity observed with pGL3-MYH2/1/4. This enhancer activation was abolished by mutation of the MEF3 sites, whatever the MYH promoter tested (Fig. 2C). This demonstrates that the putative common cis element behaves as an enhancer, and that MEF3 sites are essential for this enhancer activity *in vivo.* To further analyze potential *in vivo* interactions between this enhancer and each MYH genes, we performed chromatin conformation capture (3C) assays with adult gastrocnemius muscles. These experiments revealed that in native chromatin of gastrocnemius myonuclei, the enhancer region interacts with the promoter of MYH2/1/4 genes (Fig. 2D). We observed stronger interactions between the enhancer and MYH1 and MYH4 promoters, consistent with the expression profile of these two genes in gastrocnemius muscles. These data demonstrated that Six1 can modulate fast type MYH isoforms expression by binding to a common 5' enhancer.

We next identified the presence of a lincRNA (2310065F04Rik) located next to the identified MYH enhancer and named it linc-MYH (Fig. 2A). To characterize its expression profile we tested its tissue distribution by qPCR experiments. Linc-MYH was expressed only in fast type skeletal muscle (gastrocnemius muscles, TA and EDL), and not in brain, kidney, heart or fat tissues. This expression pattern parallels the expression of MYH4 (Fig. 3A). Fast-type muscle fiber subtype specialization starts at the end of embryogenesis (REF, Richard). To analyze linc-MYH expression pattern during this period, we tested linc-MYH expression in back muscles of wild type control mice and c*Six1 KO* mice at embryonic day 18.5 (E18.5), and at several

postnatal stages; 2 weeks (P2W), P4W and P8W animals (Fig. 3B). By qPCR experiments, Linc-MYH was detected after birth in control samples, and its expression increases, in parallel with MYH4 expression (Fig. S7). Linc-MYH expression is strongly reduced in cSix1 KO mice, during postnatal development (Fig 3B), and in adult TA of cSix1 KO mice compared with Ctrl mice (Fig. 3C), indicating that linc-MYH expression is under the control of Six1. Linc-RNAs have been detected in the cytoplasm (REF), or in the nuclei as single (*1*) or multiple foci (*2*). To analyze linc-MYH localization in skeletal muscle fiber, we performed fluorescence *in situ* hybridization (FISH) with antisense RNA of linc-MYH in isolated muscle fiber from EDL. About 10 of linc-MYH foci were observed in nucleus, with perinuclear or intranuclear localization (Fig. 3D). We hypothesized that linc-MYH may have an important role to control gene expression in the fast myofibers, and may act in trans due to the number of nuclear foci observed (REF HOX Nature). To test this hypothesis, we developed shRNA against linc-MYH and introduced the shlinc-MYH into TA muscles by electroporation. Knock-down of linc-MYH was efficient, resulting in 90% reduction of its expression. To identify the consequences of Linc-MYH knock-down and precise its mode of action, RNA samples from shlinc-MYH transfected adult TA was analysed by Affymetrix microarrays (Fig. 3E and Table S1), and validated the results by qPCR experiments. We observed that the expression of many fast genes under the control of Six1 (MYH4, Tnnt3, Tnni2, Pvalb…) is downregulated in LincMYH knock down samples, and that the expression of many slow genes upregulated in cSix1 KO (Sln, Tnni1, Tnnt1…) are upregulated in LincMYH knock down samples (Fig. 3F). More particularly, it is striking to note that among the 25 genes whose expression is the most increased in knock down linc-MYH, 20 are also upregulated in cSix1 samples. These results lead to several hypothesis, the first is that linc-MYH controls positively the expression of fast-type genes and negatively

that of slow-type genes, suggesting the presence of both transcriptional positive and negative foci associated with linc-MYH in the same nucleus (REF, Rosenfeld), the second hypothesis suggests the presence of only negative linc-MYH nuclear foci, the third hypothesis the presence of only positive foci. To explain the phenotype of ok knock down linc-MYH in the second hypothesis, regulatory regions of slow type genes would be embedded in linc-MYH and associated with negative complexes that may composed of Sox6 (REF) and polycomb repressing complexes (REF Rosenfeld), absence of linc-MYH would then lead to the disruption of these structures and derepression of slow genes. In a third hypothesis, regulatory regions of fast type muscle genes, among which Pvalb, MYH4 and slow type muscle repressors among which Sox6 and specific mir would be trapped in active nuclear foci, allowing their efficient transcription. Active transcription of slow repressors would shut down slow muscle gene expression. To test between these hypothesis we immunoprecipitated formaldehyde cross-linked linc-MYH from adult muscle nuclei with specific biotynilated oligonucleotides and tested by dot blot experiments the presence in the foci of repressive (H3K27me3), or active (H3K4me3 and H3K9Ac) histone modifications with H3K27me3, H3K4me3 and H3K9Ac antibodies.

To analyze the mechanism of linc-MYH action, we performed Chromatin Isolation by RNA Purification (ChlRP)(*13*).

To rescue the phenotype, overexpression

In conclusion, we demonstrate that Six1 homeoprotein accumulation in fast-type myonuclei allows fine tuning of fast type MYH gene expression and is responsible for linc-MYH expression. We identify an enhancer element at the center of the fast MYH locus that activates MYH2/1/4 through direct connections with promoter regions as shown by 3C experiments. This

enhancer may act as a locus control region (LCR) and finely modulate the spatio-temporal expression of all fast MYH at the locus. We further show that Six1 directly controls the expression of long non coding RNA, named lincMYH, whose function *in fine* is to repress slow type skeletal muscle genes in fast type myofibers (Fig. 3G). Specific fast type myofibers specialization is achieved by the robust accumulation of Six1 in their nuclei, Six1 being involved in the direct activation of fast type muscle genes and involved in the upregulation of a long non coding RNA whose accumulation prevents slow type muscle gene expression. Thousands of lincRNAs have been identified. They seem to be expressed in a more tissue specifc manner as compared with protein coding genes (*14*). Each cell type has a specific gene expression pattern to achieve a specialized function, and our findings suggest that linc-RNA would be a suitable tool to further lock tissue-specific gene expression by suppressing non-specific gene expression.

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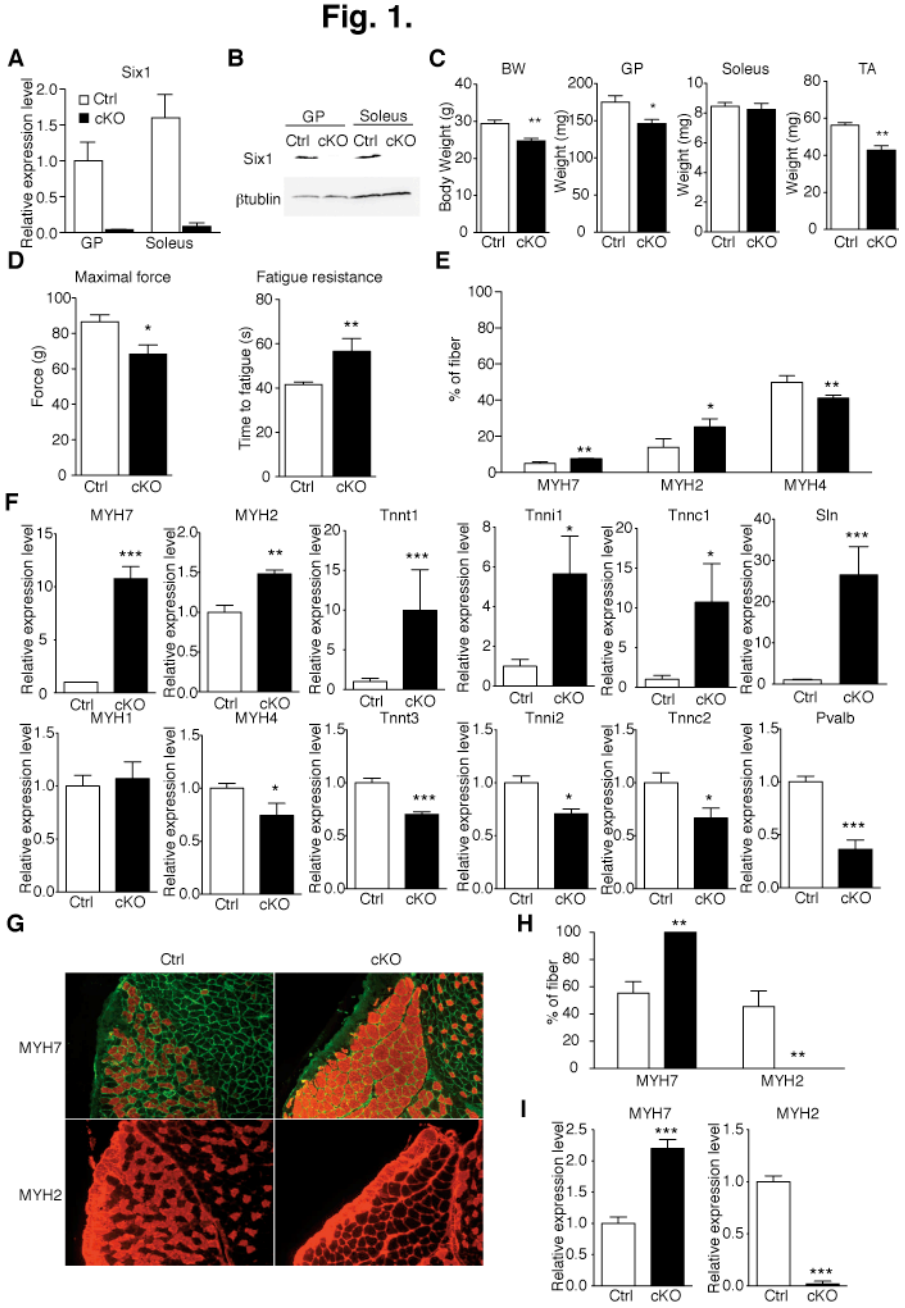


Fig. 1. Six1 deficiency impaired fast type muscle phenotypes. (**A**) Six1 mRNA expression levels of 3 months old Six1 KO mice in TA muscles. Ctrl (n=4), KO (n=3). (**B**) Western blots of Six1 and βtubulin in soleus and gastrocnemius/plantaris (GP) of cSix1 KO mice. (**C**) Body weight (BW) and muscle weight of GP, soleus, and Tibialis anterior (TA). Ctrl (n=4), KO (n=4). (**D**) maximal force and time to fatigue of TA muscles of cSix1 KO mice. Ctrl (n=4), KO (n=4). (**E**)

% of myofibers expressing MYH7, MYH2 or MYH4 in TA muscles of 3 months old cSix1 KO mice. Ctrl (n=4), KO (n=4). (**F**) mRNA expression levels of MYH7, MYH2, MYH1 and MYH4 in TA muscles of 3 months old cSix1 KO mice, Ctrl (n=4), KO (n=4). (**G**) Immunostaining of MYH7 and MYH2 in soleus muscles of 3 months old cSix1 KO mice. (**H**) % of myofibers expressing MYH7, MYH2 in soleus muscles of 3 months old cSix1 KO mice, Ctrl (n=3), KO (n=3). (**I**) mRNA expression levels of MYH7, MYH2 in soleus muscles of 3 months old cSix1 KO mice, Ctrl (n=4), KO (n=4). *P<0.05, **P<0.01, ***P<0.001.

Fig. 2.

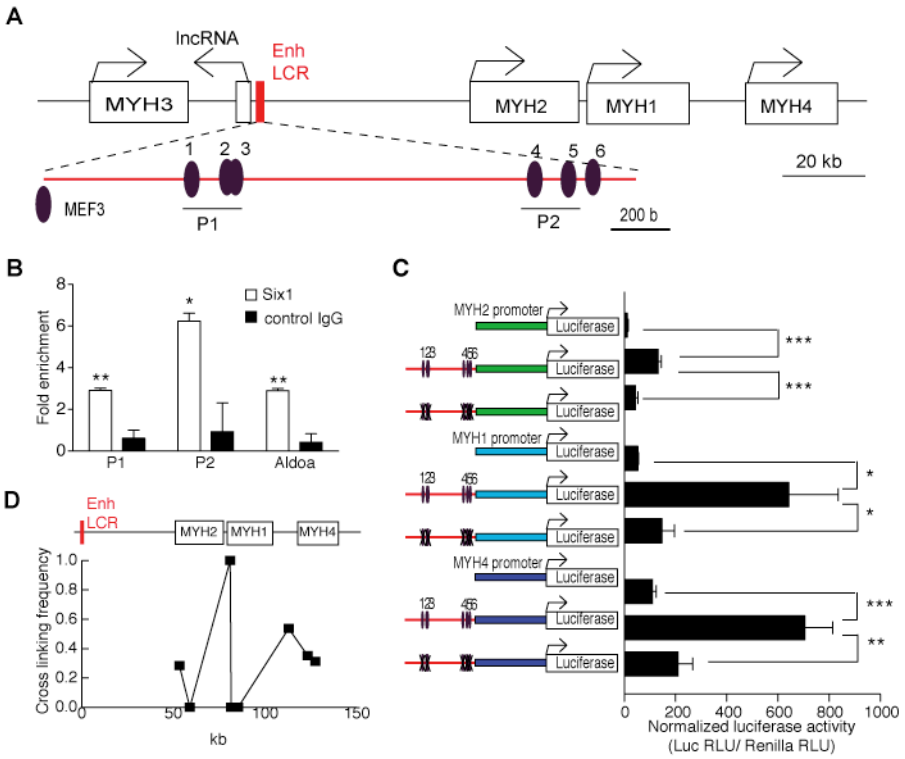


Fig. 2. Six1 directly binds at enhancer/LCR of MYH gene cluster. (**A**) Schematic representation of the fast MYH genes cluster. (**B**) qPCR values of ChIP experiments performed with Six1 antibodies or IgG on TA chromatin, and showing Six1 binding to P1 and P2 regions of the MYH enhancer, and to the muscle promoter of the AldolaseA. (**C**) Luciferase assays from adult TA muscles electroporated by indicated luciferase vectors and a TK-rennila luciferase vector allowing normalization. (**D**) qPCR experiments from 3C assays with soleus and EDL muscle of WT animals, showing the direct link of MYH2, MYH1 and MYH4 promoters with the MYH LCR/enhancer.

Fig. 3.

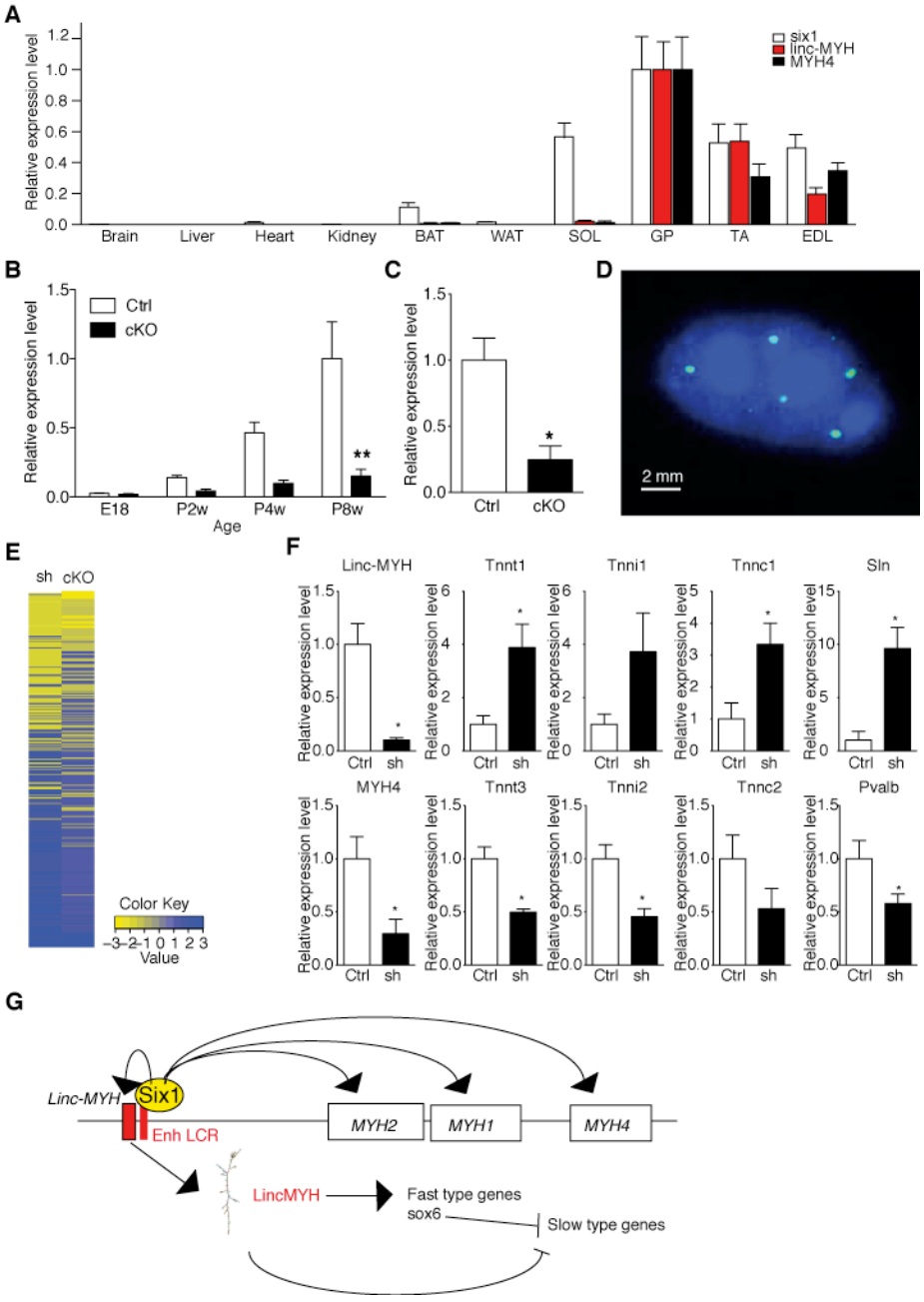


Fig. 3. LincRNA accumulation in the myonuclei of fast fibers suppresses slow type gene expression. (**A**) Tissue distribution of linc-MYH RNA. BAT, brown adipose tissue; WAT, white adipose tissue. (n=4 for each tissue). (**B**) Linc-MYH RNA expression pattern in back muscles of cSix1 KO mice at E18.5, P2W, P4W and P8W. (n=3 to 6 for each point). (**C**) Linc-MYH RNA expression levels in 3 months old cSix1 KO mice in TA muscles Ctrl (n=4), KO (n=3). (**D**) FISH

of linc-MYH in isolated EDL myofiber. (**E**) Heat map of genes up-regulated more than 3-fold identified by microarray analysis of TA muscles transfected by shRNA against linc-MYH. (**F**) qPCR experiments revealing mRNA expression levels of linc-MYH, Tnni1, Tnnt1 and Sarcolin/Sln in TA muscles expressing a shRNA directed against linc-MYH or a shCtrlRNA, shCtrl (n=3), shlinc-MYH (n=3). (**G**) ChLRP (**H**) model

Supplementary Materials:

Materials and Methods

Figures S1-S7

Tables S1-S2

References (##-##)

Supplementary Materials:

This section includes the actual text of the Supplementary Materials, which can include any or all of the preceding items, and figure captions and tables that can easily be incorporated into one supplementary material file. Please edit the list above as appropriate and include it at the end of your main paper. If there are additional files that cannot be easily accommodates (e.g., movies or large tables), please include captions here.

Materials and Methods:

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. Six1KO mice were obtained by crossing the Six1-LoxP mice (*11*) and the transgenic mice, expressing a CRE recombinase under the control of the human skeletal actin promoter (HSA) (*10*).

Muscle contraction test

Skeletal muscle function was evaluated by measuring in situ muscle contraction, as described previously (*15*). 12 weeks old male mice were anesthetized (ip, pentobarbital sodium, 50 mg/kg). 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). The duration corresponding to a 20% decrease in force was noted. Body temperature was maintained at 37°C using radiant heat.

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 (1x PBS, 1.5% goat serum, 0.1% triton X-100). Rabbit poly-clonal antibodies directed against Laminin (Z0097, Dako) (1/100 dilution), and monoclonal antibodies against MYH7 (NOQ7.5.4D) (1/1000 dilution), MYH2 (SC-71) (1/20 dilution) and against MYH4 (BF-F3) (1/20 dilution) were applied overnight at 4 °C to the treated sections. The next day, after three washes of 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 of 1× PBS containing 0.05% tween 20, samples were mounted in Vectashield mounting medium.

RNAs prepation

TA, back, soleus and gastrocnemius muscles were collected from cSix1 KO and control mice. Total RNAs were extracted by Trizol Reagent (Invitrogen) according to manufacture'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. Gene expression levels were normalized by the housekeeping gene TBP. Sequences of the oligonucleotides used in this study are shown in table S3.

microarray

EMSA

EMSA was carried out with Six1 and Six4 full-length mouse cDNA cloned into the pCR3 vector (Clontech) vector as previously described (*16*). Recombinant mouse Six1 and Six4 proteins were obtained with a T7 transcription/translation kit (Promega). Labeled double-stranded myogenin MEF3 site was incubated with recombinant proteins. Competition experiments were performed with a ten fold and hundred fold molar excess of unlabelled MEF3, or Myogenin promoter NFI site (Table S3).

ChIP experiment

ChIP experiments were performed on GP and TA muscles of 2 months old mice with Six1 antibodies (HPA001893, Sigma), using EZ-Magna ChIP G (millipore) according to manufacture's instruction. Briefly, GP and TA muscles of 2 months old mice were minced by scissors just after sampling and fixed by 1% formaldehyde for 10 minutes. The formaldehyde was quenched by 0.125 M glycine, and muscles were washed twice by 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 homogenized in a mortar and, subsequently, in a Dounce homogenizer. The nucleus 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 by Bioruptor (Diagenode). The debris was removed by a centrifugation. The sonicated DNA was incubated with 1 μg of Six1 antibodies (HPA001893, Sigma) with rotation at 4 °C overnight. 20 μl of Dynabeads protein G (invitrogen) was added to the tubes and incubated with rotation at 4 °C for 1 hour. The beads were washed by low salt buffer (2 mM EDTA, 20mM Tris-HCl pH 8, 150 mM NaCl, 1% TitonX-100, 0.1% SDS), high salt buffer (2 mM EDTA, 20mM Tris-HCl pH 8, 500 mM NaCl, 1% TitonX-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). DNA was eluted by elution buffer (1% SDS, 0.1 M $NaHCO_3$) with proteinase K (invitrogen) at 62 °C for 2 hours, then, proteinase K was inactivated by incubation at 95°C for 10 minutes. DNA was purified with MinElute PCR purification kit (Qiagen).

3C

3C experiment was performed on adult EDL muscles as described (*17*) with the following modifications. Single myofibers were obtained from EDL muscles as previously described (*11*) and were fixed.

RNA-FISH

Fluorescently-labelled anti-sense linc-MYH probes were synthesized according to manufacture's instruction (FISH Tag RNA kit, invitrogen). FISH experiments were performed with isolated EDL myofiber, and the image was taken by Leica SP2 confocal microscope.

Generation of shRNA against mouse linc-MYH

Five distinct shRNAs targeting mouse linc-MYH were designed, shLincMYH, and inserted into the psiSTRIKE hMGFP system (Promega). The efficiency of each shRNA was examined by linc-MYH transcripts levels in TA muscles transfected by each shLincMYH. The shRNA against 5'-TTCTGCTCACCACCTACAATT-3' sequence was selected for the knock down experiment.

Electroporation

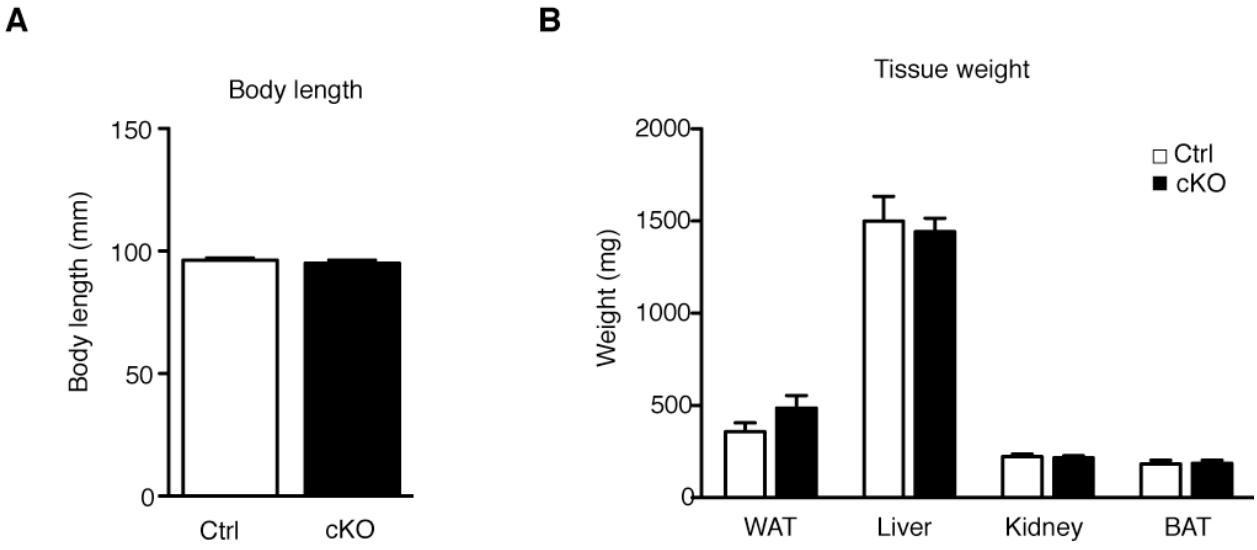
Ten μg of shRNA expressing vector was introduced into TA muscles of 8 weeks old mice by electroporation as previously described (*18*). Two weeks after electroporation, TA muscles expressing GFP were dissected under a Nikon SMZ1500 stereo microscope and were frozen by liquid nitrogen.

Western blot

Western blots were performed with protein extracts of GP muscles and soleus muscles from cSix1KO mice and control mice as previously described(*8*). 1:1000 dilution of anti-Six1 antibodies or anti-β−tublin antibodies () was used.

RNA folding

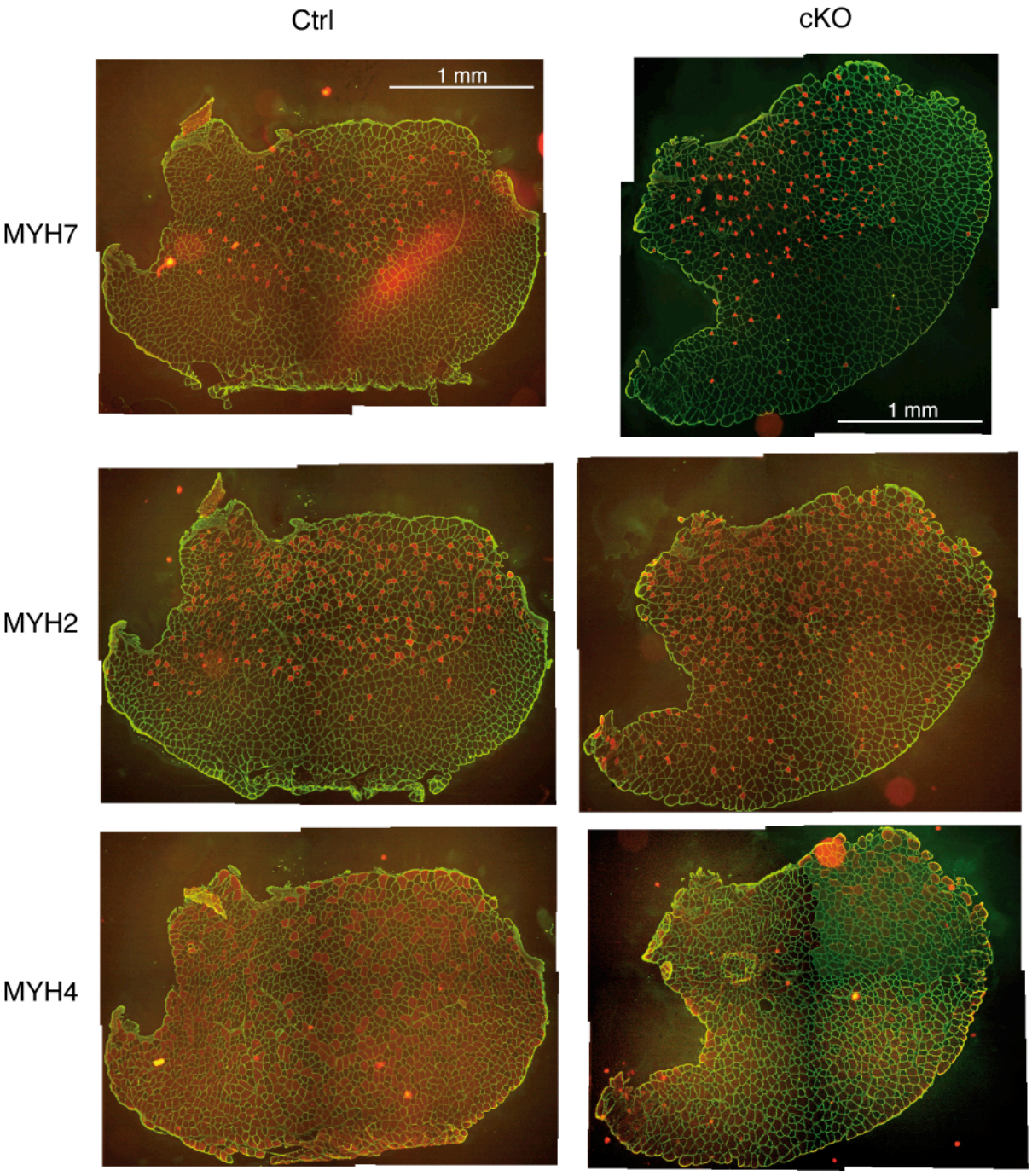
fig. S1



Supplementary Figure S1.

(A) Adult body length of control and cSix1KO animals. (B) Weight of liver, kidney, epididymal fat (WAT) and brown adipose tissue of 12 weeks old male mice, Ctrl (n=4), cSix1KO (n=4).

fig. S2

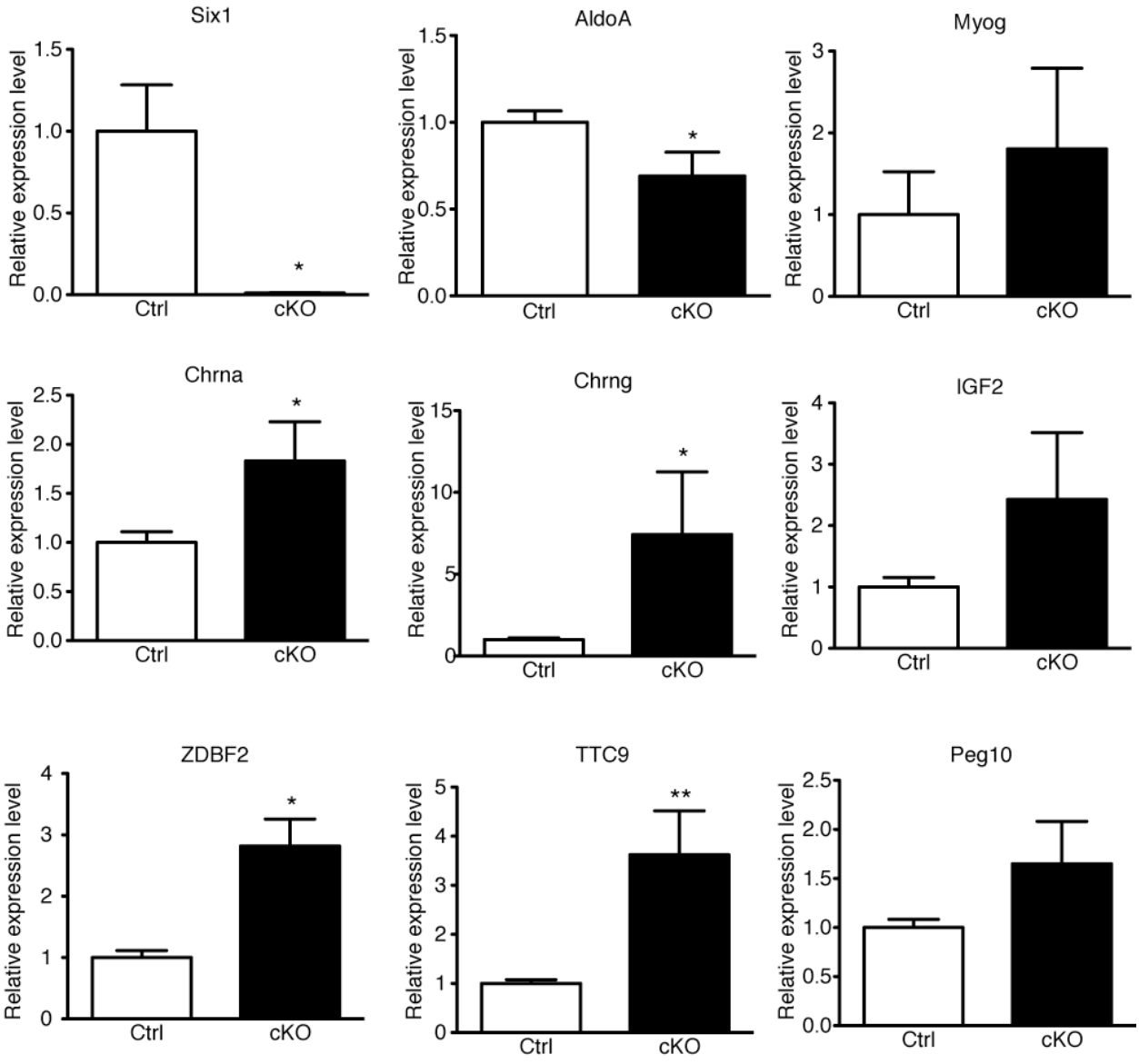


Supplementary Figure S2.

Immunostaining of MYH7, MYH2 and MYH4 in TA of 12 weeks old control and cSix1KO male

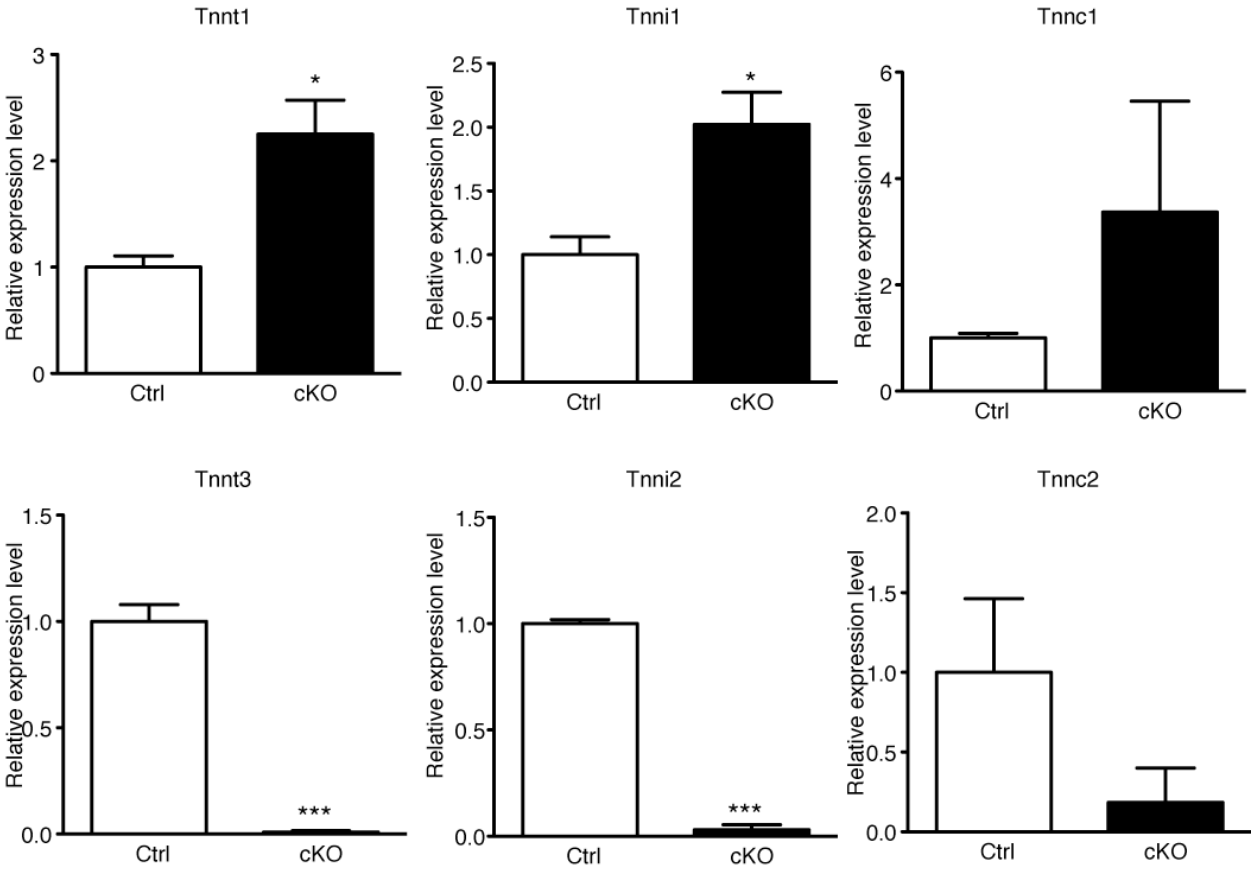
mice.

fig. S3



Supplementary Figure S3. mRNA expression levels in TA muscles of 3 months old cSix1 KO and control mice, as measured by qPCR experiments. Ctrl (n=4), KO (n=4).

fig. S4



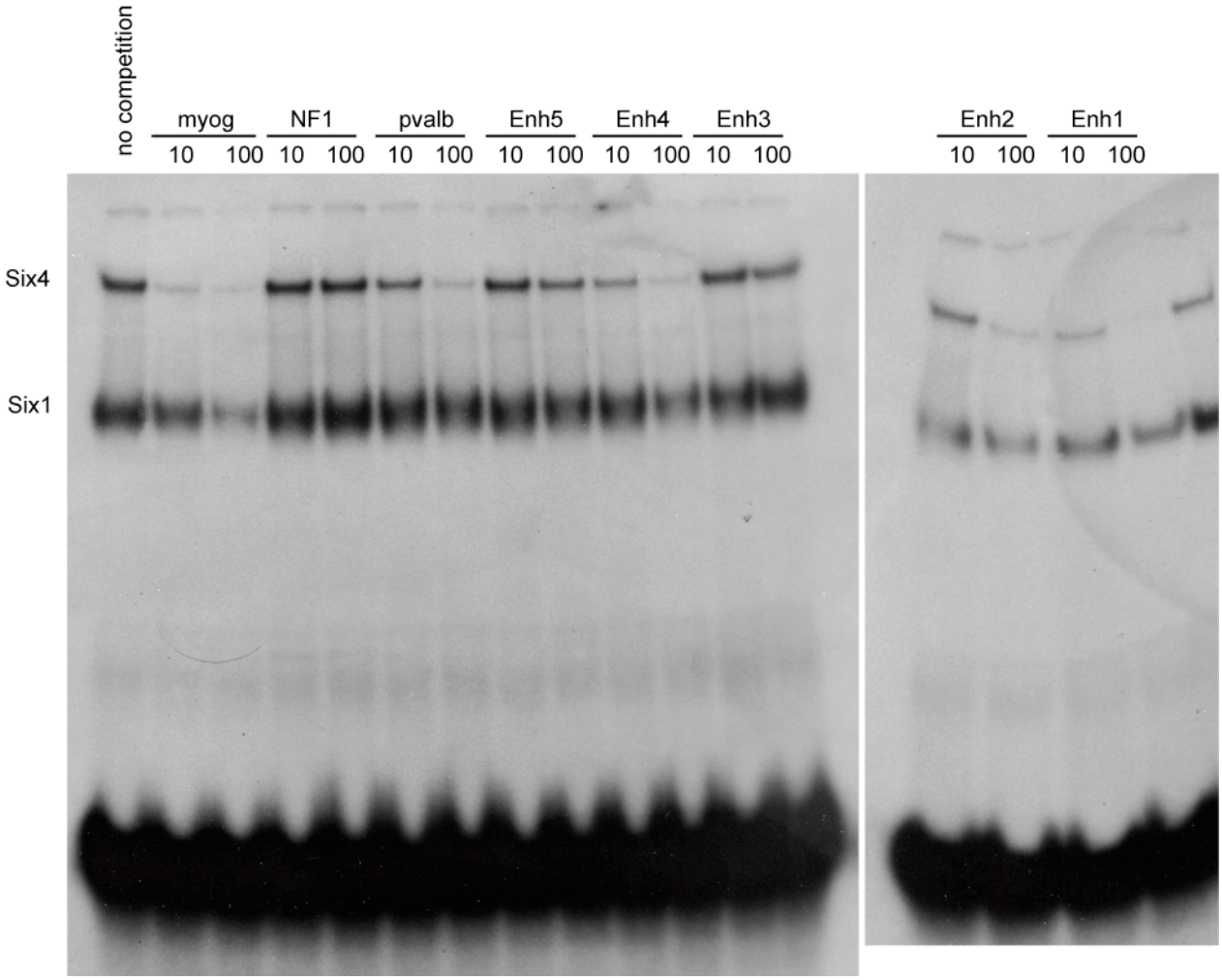
Supplementary Figure S4. mRNA expression levels in soleus muscle of 3 months old cSix1 KO and control mice. Ctrl (n=4), KO (n=4).

fig. S5

Supplementary Figure S5.

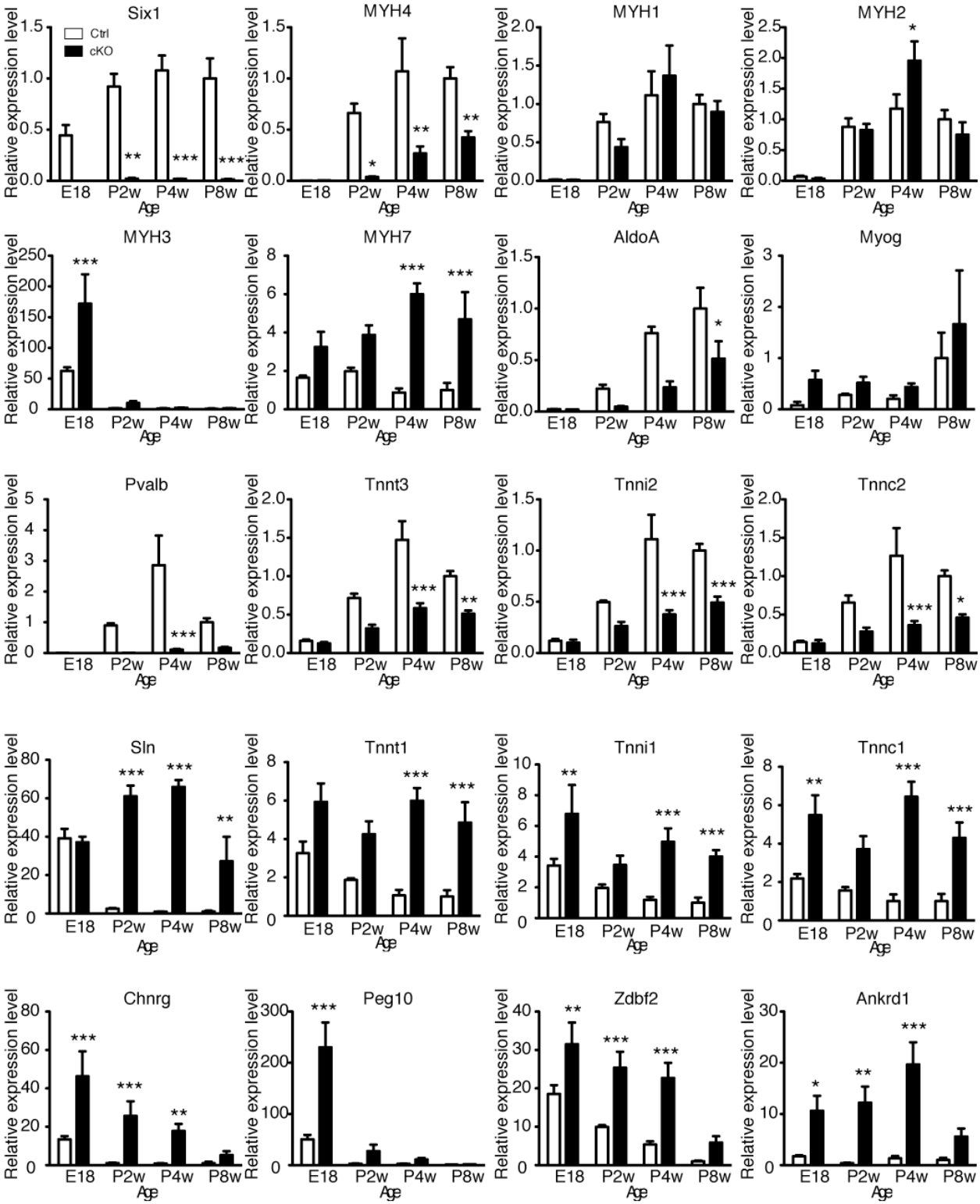
Sequences of P1 and P2 boxes of the MYH enhancer in mouse, rat, human, bovine and equinides species, and showing the sequence conservation of the six MEF3 sites and E boxes.

fig. S6



Supplementary Figure S6. Competitive Electromobility shift assays with a 30-bp labelled oligonucleotide containing the myogenin MEF3 site with 10 or 100 fold molar excess of unlabelled oligonucleotide containing for Myogenin MEF3 or NF1 site, or with MYH MEF3 sites (Enh1, 2, 3, 4, 5, 6) whose sequence is presented on Figure S5, incubated with Six1 and Six4 recombinant proteins.

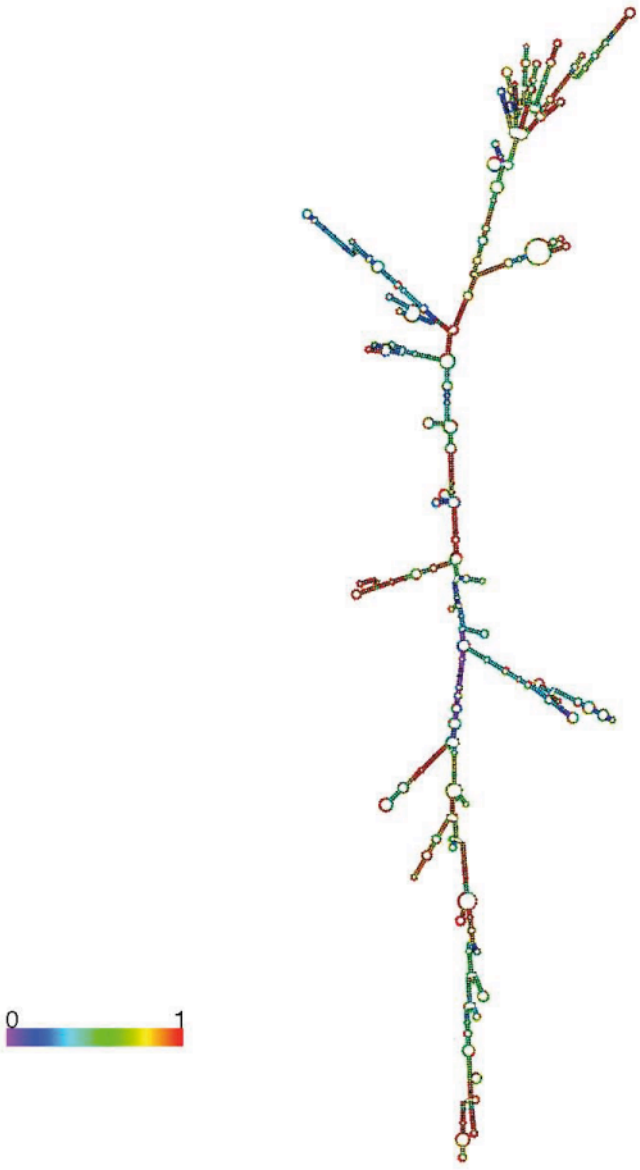
fig. S7



Supplementary Figure S7. mRNA expression level 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).

fig. S8



Supplementary Figure S8. folding of linc-MYH

Table S1: Microarray analysis of TA muscles electroporated by shRNA

Table S3. Sequence of the oligonucleotides for QPCR.

gene name	Forward (5'- 3')	Reverse (5'- 3')
Six1	CTTTAAGGAGAAGTCTCGGG	TTCCAGAGGAGAGAGTTGAT
MYH7	AGGGCGACCTCAACGAGAT	CAGCAGACTCTGGAGGCTCTT
MYH2	CCAAGAAAGGTGCCAAGAAG	CGGGAGTCTTGGTTTCATTG
MYH1	CGGTGGTGGAAAGAAAGG	CAGGAGTCTTGGTTTCATT
MYH4	GCTTGAAAACGAGGTGGAAA	CCTCCTCAGCCTGTCTCTTG
MYH3	GCAAAGACCCGTGACTTCACCT CTAG	GCATGTGGAAAAGTGATACGTG G
Tnnt1	CCCCCGAAGATTCCAGAAGG	TGCGGTCTTTTAGTGCAATGAG
Tnnt2	CAGAGGAGGCCAACGTAGAAG	CTCCATCGGGGATCTTGGGT
Tnnt3	GGAACGCCAGAACAGATTGG	TGGAGGACAGAGCCTTTTTCTT
Tnni1	ATGCCGGAAGTTGAGAGGAAA	TCCGAGAGGTAACGCACCTT
Tnni2	AGAGTGTGATGCTCCAGATAGC	AGCAACGTCGATCTTCGCA
Tnni3	TCTGCCAACTACCGAGCCTAT	CTCTTCTGCCTCTCGTTCCAT
Tnnc1	GCGGTAGAACAGTTGACAGAG	CCAGCTCCTTGGTGCTGAT
Tnnc2	ATGGCAGCGGTACTATCGACT	CCTTCGCATCCTCTTTCATCTG
Sln	GGTCCTTGGTAGCCTGAGTG	CGGTGATGAGGACAACTGTG
Pvalb	ATCAAGAAGGCGATAGGAGCC	GGCCAGAAGCGTCTTTGTT
Linc-MYH	GTGCAGCCAGAACAAGACAG	CAAGATGGGAGGCTCTCAAA
Myog	GAAAGTGAATGAGGCCTTCG	ACGATGGACGTAAGGGAGTG
Aldoa	ACTCTCTGCTGACCGGGCTCT	AATGCTTCCGGTGGACTCAT
Chrna	ACCAATGTACGTCTGAAACAGC	TTTTCCGAGGGGATGTGAATTTT
Chrng	GGCCAGAGACCTCATCTCCT	GGGGTCGTAGTTTCGCATCA
IGF2	CAGTTTGTCTGTTCGGACCG	ACGTCCCTCTCGGACTTGG
Zdbf2	TAGCGGCTCTTTCGAGAGAC	CCCTGATCTGGGGAGTCAA
TTC9	CCAGGCCGAGCTGGTAAATTA	CCTTCAGGCAATACTCCTTGACT
Peg10	TGCACAACTACACTGCCTTTATG	CTGGGCAATCATCTGGAATGC
Ankrd1	TGCGATGAGTATAAACGGACG	GTGGATTCAAGCATATCTCGGA A

Table S4. Sequence of the oligonucleotides for ChIP.

Target	Forward (5'- 3')	Reverse (5'- 3')
Enh LCR 1	ATCTCCACCTCCCTCCAACT	ACCCCCTAGCTTTGACAGGT
Enh LCR 2	AATCTGACGACAGGGTGAGC	GGTCGCCTGACCTGATAGAG
Aldoa	CTCTCAAGGCAAACCAAAGC	CCAGTGTCCCAGACCTTCTC
Bactn	TGTTACCAACTGGGACGACA	ACCTGGGTCATCTTTTCACG

Table S5. Sequence of the oligonucleotides for EMSA.

Target	Forward (5'- 3')	Reverse (5'- 3')
Enh1	CTCTTGGGTAACTGGAGCCCCTC	GAGGGGCTCCAGTTACCCAAGAG
Enh2	GGTTGACTTAGATTTCCTTATGA	TCATAAGGAAATCTAAGTCAACC
Enh3	TGTAAGAGAAACTGAAATAAAAT	ATTTTATTTCAGTTTCTCTTACA
Enh4	GGGGTAAGAAATCTGACGACAGG	CCTGTCGTCAGATTTCTTACCCC
Enh5	CTATCAGGTCAGGCGACCTCAGT	ACTGAGGTCGCCTGACCTGATAG
Enh6	CGTCAAGGAAACCTTATTCCATC	GATGGAATAAGGTTTCCTTGACG
Myog	TGGGGGGGCTCAGGTTTCTGTGGCGT	ACGCCACAGAAACCTGAGCCCCCCCA
NF1	TATCTCTGGGTTCATGCCAGCAGGG	CCCTGCTGGCATGAACCCAGAGATA
Pvalb	AGTACCTGACACCGGAAGGGGAG	CTCCCCTTCCGGTGTCAGGTACT

Table S6. Sequence of the oligonucleotides for 3C.

name of oligonucleotides	Sequence (5'- 3')
probe	FAM- TCAGCTGCCCAGGGTGACCA-Tamra
Enh_3CF	CCAGCCTGTTCTGGGTACAT
MYH1_3CR	ACCCCTTGGAATGAGAGTGA
MYH2_3CR	TGAAGCAGTGTGGAACAAGC
MYH4_3CR	CCAAATTGGTTGATGCTCATT
78038_3CR	CCTGACGCACCATGTCTAAA
104838_3CR	CAGGATTTGGTAGGGGATGA
106285_3CR	CGCACAGCCTAATGAAGACA
134682_3CR	CCCTCTCATATGGTGCCAGT
148019_3CR	GGTCACTGGAGGGATCTGAA

Any Additional Author notes: I.S. performed and analyzed 3C, qPCR, immunofluorescence, ChIP, luciferase assay and RNA FISH. R.D. and E.P. helped expression analysis of Six1 KO mice. F.A. performed muscle contraction tests. M.S. and V.H. performed computational analysis. P.M. performed EMSA assay. I.S. and P.M. wrote the manuscript.