Single-nucleus RNA-seq and FISH identify coordinated transcriptional activity in mammalian myofibers

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Skeletal muscle fibers are large syncytia but it is currently unknown whether gene expression is coordinately regulated in their numerous nuclei. Here we show by snRNA-seq and snATAC-seq that slow, fast, myotendinous and neuromuscular junction myonuclei each have different transcriptional programs, associated with distinct chromatin states and combinations of transcription factors. In adult mice, identified myofiber types predominantly express either a slow or one of the three fast isoforms of Myosin heavy chain (MYH) proteins, while a small number of hybrid fibers can express more than one MYH. By snRNA-seq and FISH, we show that the majority of myonuclei within a myofiber are synchronized, coordinately expressing only one fast Myh isoform with a preferential panel of muscle-specific genes. Importantly, this coordination of expression occurs early during post-natal development and depends on innervation. These findings highlight a previously undefined mechanism of coordination of gene expression in a syncytium.

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**Results**

**SnRNA-seq identifies several cell populations in adult skeletal muscles.** To characterize the transcriptional profile of myonuclei within myofibers, we developed single nucleus RNA-seq (snRNA-seq) experiments using a droplet-based platform on purified nuclei from different skeletal muscles of adult mice (Fig. 1a and Supplementary Fig. 1a–d). By using Seurat software and Uniform Manifold Approximation and Projection (UMAP) clustering, we were able to detect a total of 19 different cell populations clusters present in the different muscles (Fig. 1b and Supplementary Fig. 2b, c). The origin of the nuclei was identified by specific markers (Fig. 1b and Supplementary Figs. 1e and 2a, Supplementary Data 1), with 68% of nuclei originating from myofibers, 19% from FAPs, and around 13% from other cell types, including tenocytes, endothelial, and lymphoid cells, smooth muscle cells, and myogenic stem cells (Supplementary Fig. 2d). To look at the heterogeneity of the myonuclei, we have then reclustered the myonuclei expressing Titin (Ttn) gene to remove all the non-fiber nuclei (Fig. 1c). Several populations of myonuclei where identified, among which MTJ myonuclei (expressing Col22a1), NMJ myonuclei (expressing Ach), a yet unknown myonuclei subpopulation (expressing Myh9, Flnc, Runx1), and body myonuclei accounting for 94% of all myonuclei (Figs. 1c and 2a). By comparing gene expression in body nuclei and specialized nuclei, we characterized numerous known (Ache and Etv5 for NMJ; Col22a1 for MTJ) and unknown genes specific to these specialized myonuclei (Etv4 for NMJ, Mamil2 for MTJ) (Fig. 2b). Unidentified, NMJ and MTJ myonuclei also expressed Myh genes (Fig. 2c). Slow myonuclei expressing Myh7 clustered independently from the fast Myh1/2/4 myonuclei. The fast myonuclei formed a large cluster that could be subdivided into several subpopulations, all expressing sarcomeric genes like Ttn and various Myh isoforms (Figs. 1c and 2a). Myh4 + myonuclei represented 51% of this fast myonuclei population. We also compared the expression of genes in slow and fast nuclei expressing the different types of Myh (Fig. 1d). Fast Myh2 and Myh1 nuclei showed a similar transcription program, contrasting with slow Myh7 and very fast Myh4 nuclei each showing a distinct transcription program (Fig. 1d). Interestingly, three main categories of Myh4 nuclei were identified, identifying transcriptional heterogeneity among these nuclei (Supplementary Fig. 3a–c). We observed higher expression of Mical2, Pde4d and Sorbs2 in the Myh4B myonuclei subgroup, of Taco1, Neat1 and of Kpn1 in Myh4A myonuclei subgroup, and of EssrY, Agbl1 and Ptn3 in Myh4C myonuclei subgroup.
**Fig. 1** SnRNA-seq analysis from adult slow and fast skeletal muscles.  

**a** Graphical scheme of the experiments used for snRNA-seq analysis of adult skeletal muscles.  

**b** Uniform Manifold Approximation and Projection (Umap) diagram from snRNA-seq from adult skeletal muscle. MuSC skeletal muscle stem cells, FAPs fibro-adipogenic progenitors, MTJ myotendinous junction, NMJ neuromuscular junction.  

**c** Umap diagram of snRNA-seq data with myonuclei only. Myonuclei are separated into five clusters: slow, fast, MTJ, NMJ, and unidentified myonuclei. The slow and fast myonuclei are called body nuclei and are surrounded by dotted lines. Five subcategories of fast myonuclei are clustered.  

**d** Heatmap of genes upregulated (yellow) and downregulated (violet) in the myonuclei expressing the different slow and fast Myh isoforms. The values correspond to z-scores of normalized counts.
These Myh4 nuclei subpopulations were distributed differently in distinct muscles, with a higher percentage of the Myh4C population in the fast Tibialis compared to the fast Quadriceps and a higher percentage of Myh4B nuclei in the Quadriceps as compared with the Tibialis (Supplementary Fig. 3c). From these experiments, we concluded that Myh4 nuclei can have different transcription profiles, depending on the muscle, what was presently unsuspected.
SnATAC-seq identifies heterogenous transcriptional programs in myonuclei. Recent studies profiled the genome-wide chromatin accessibility of adult skeletal muscles. However, this global approach did not discriminate the chromatin status of each cell population present in skeletal muscles. Thus, we set up a protocol to perform single-nuclei ATAC-seq (snATAC-seq) with purified nuclei from adult soleus and quadriceps using a droplet-based platform. Briefly, nuclei were transposed using Tn5 transposase and then encapsulated into gel beads emulsion using 10X protocol. After quality assessments and filtering (Supplementary Fig. 4a–c), 132 966 peaks were identified from 6037 sequenced nuclei. To identify the different populations of nuclei, we classified nuclei from ATAC-seq based on snRNA-seq experiments from Fig. 1b: we used methods for cross-modality integration of snRNA-seq and snATAC-seq data and label transfer from Signac software (Fig. 3a and Supplementary Fig. 4d, e). This classification was robust, as shown by the chromatin accessibility of the Myh genes present only in myonuclei (Fig. 3b and Supplementary Fig. 5a, b). Using chromVar we characterized the transcription factor (TF) variations in motif accessibility between the different types of myonuclei (Fig. 3c). Knowing that this analysis frequently does not distinguish between related TFs of the same family (usually sharing similar motifs), we could characterize that the NFAT and SOX binding motifs accessibility are enriched in slow Myh7 myonuclei, ESRRβ and NRRA2A motifs in fast Myh2 and Myh1 myonuclei and SIX and NRL/MAF motifs in fast Myh4 myonuclei (Fig. 3c, d and Supplementary Fig. 3b). These distinct combinations of TF could participate in the genetic code controlling the expression of specific fast-subtypes and slow sarcomeric genes and of genes associated with myofiber specialization (Fig. 1d). Equivalently to Fig. 1d presenting genes differently expressed in slow and fast myonuclei (from snRNA-seq data), we observed specific chromatin accessibility between slow and fast myonuclei in genes differently expressed in slow and fast myonuclei. For example, the gene Povalb expressed only in fast Myh4 myonuclei (Fig. 1d) presented a strong chromatin accessibility only in fast Myh4 myonuclei (Supplementary Fig. 5d). At the opposite a strong chromatin accessibility in the Atp2a2 gene was observed in Myh7 nuclei only where this gene is expressed (Fig. 1d and Supplementary Fig. 5e). These results showed with a high-resolution how slow and fast-subtypes genetic programs are associated with different sets of TF controlling the expression and the chromatin accessibility of a set of co-expressed genes. Interestingly, a specific set of TF motifs is enriched in MTJ myonuclei as compared to body myonuclei, suggesting their participation in regionalized expression of specific genes such as Col22a1 (Fig. 3e and Supplementary Fig. 5c).

Most myonuclei express a single fMyh gene. Fast Myh genes are located side by side on the same locus and it is presently unknown whether they can be transcribed simultaneously (Fig. 4a). Using snRNA-seq, we found that only rare mixed nuclei co-expressed simultaneously Myh7 and Myh2 or Myh1 and Myh4 (Fig. 4b). Mixed nuclei co-expressing Myh2 and Myh1 were more numerous. Other combinations of co-expression were not detected (Supplementary Fig. 6a). This showed that the vast majority of adult myonuclei analyzed were pure and expressed only one Myh gene. To visualize the expression of fMyh genes, we performed fluorescence in situ hybridization (RNAscope) experiments using intrinsic probes to detect fMyh pre-mRNA in myofibers from EDL and soleus muscles (Fig. 4c–e). Myonuclei expressed fMyh genes in a mono or bi-allelic manner (Fig. 4c). Expression of two distinct fMyh genes from identical or distinct alleles was detected in some nuclei (Fig. 4d). Expression of two fMyh genes by the same allele (single bicolor dot) indicated that two Myh genes were transcribed simultaneously on the same locus (Fig. 4e–g). In some myonuclei, no fMyh pre-mRNA was detected. Quantification of RNAscope experiments is provided in (Fig. 4f, g); in EDL, the majority (>80%) of nuclei expressed only one Myh isoform and <5% co-expressed two isoforms. The number of nuclei co-expressing two isoforms of Myh was highest in the soleus, where ~25% co-expressed Myh1 and Myh2 (Fig. 4f). The co-expression was in most cases from the same allele (Fig. 4g), suggesting that Myh2 and Myh1 can be activated simultaneously from one allele. The results of RNAscope experiments thus confirmed snRNA-seq results, showing that almost all myonuclei were pure, expressing only one Myh isoform at a time, except in the soleus. Then we tested whether different populations of myonuclei express either metabolic, calcium handling or sarcomeric genes, or if all myonuclei co-express simultaneously these different categories of genes. SnRNA-seq data showed that most Myh4 myonuclei expressed the calcium handling gene Povalb and the glycolytic gene Pfkfb3, that a majority of fMyh myonuclei expressed Tbc1d1 involved in muscle glucose uptake, and most Myh7 nuclei expressed the sarcoplasmic reticulum Calcium transporting 2 gene Atp2a2 (Fig. 5a–d). These results were confirmed by RNAscope visualizing the localization of the pre-mRNA of AldolaseA, associated with glycolytic metabolism in myofiber, and of Idh2, associated with oxidative metabolism. Accordingly, more than 70% of Myh4 expressing nuclei also expressed the glycolytic enzyme AldolaseA pre-mRNA, while the TCA cycle isocitrate dehydrogenase Idh2 pre-mRNA was undetectable. At the opposite most Myh2 expressing nuclei expressed Idh2 pre-mRNA (Fig. 5e–h), while AldolaseA pre-mRNA was not detected. Thus, myonuclei co-expressed coordinated specialized sarcomeric, calcium handling and metabolic genes. Altogether these results showed that fMyh gene expression, and more generally muscle gene expression within the myofiber syncytium is not random but precisely controlled, each myonucleus showing a precise pattern of co-expressed genes.

Transcription is coordinated in myonuclei along the myofiber. We next evaluated the coordination of fMyh genes expression in distinct myonuclei of the same fiber. In EDL, coordinated Myh expression was detected in all myonuclei in a majority of fibers (Fig. 6a, upper panel). In contrast, a large number of uncoordinated fibers was detected in soleus (Fig. 6b). Different nuclei within the same soleus fiber were detected expressing different fMyh isoforms, while some nuclei co-expressed two isoforms. Quantification of these experiments is provided in (Fig. 6c, d). We never observed hybrid coordinated myofibers: meaning that we did not observe that all nuclei of a given fiber express the same combination of 2 or 3 Myh genes. In quadriceps and EDL, most myofibers (90%) showed Myh gene expression coordination between their myonuclei expressing only Myh4 (Fig. 6c, d). Myh4 mRNA accumulated homogenously in the coordinated fibers expressing Myh4 pre-mRNA (Supplementary Fig. 7a). Interestingly, half of the fibers from the soleus were hybrid (Fig. 6c, d). In hybrid Myh4 and Myh1 fibers, Myh4 mRNA accumulated less efficiently, and did not spread far from the nuclei expressing Myh4 pre-mRNA (Supplementary Fig. 7a), demonstrating that mRNA diffusion was confined within myonuclear domains. The transcriptional program of Myh4-positive myonuclei may differ in hybrid myofibers from pure myofibers, explaining the heterogeneity of Myh4 myonuclei observed by snRNA-seq (Supplementary Fig. 3b, c). Myh4 pre-mRNA was detected also in NMJ myonuclei, as identified by AchE mRNA expression (Supplementary Fig. 7b).
Innervation controls myonuclei coordination along the myofiber. To understand the basis behind pure and mixed myonuclei and their coordination along the myofiber, we tested whether innervation, known to modulate adult muscle fiber type\textsuperscript{14,15,30}, was involved in the coordination process. At the protein level, 1 week after denervation, we observed a preferential atrophy of fast MYH4 and MYH1-positive fibers and 4 weeks after denervation a fast to slow transition in all muscles (Supplementary Fig. 8a). In agreement, 1 week after denervation, the number of nuclei negative for fast Myh\textsuperscript{7} genes was increased (Figs. 4f and 6a). The percentage of uncoordinated myofibers after 1-week denervation in the EDL was
increased more than five-fold but did not significantly differ in soleus (Fig. 6d). These observations showed that innervation is absolutely required to coordinate fast Myh genes expression in the nuclei within myofibers in EDL (Fig. 6a–d and Supplementary Fig. 8d). Shown in Supplementary Fig. 8e, a strong increase of Myh2 and Myh1 pre-mRNAs was detected in the MTJ myonuclei of EDL 1 week after denervation, suggesting that in absence of innervation or muscle contraction, MTJ myonuclei expressed a distinct Myh gene than body myonuclei. These data support an important role of innervation in the activation of fMyh genes transcription and coordination in myonuclei along the myofiber. Altogether these experiments identified that in normal conditions myonuclei of fast myofibers (Quadriceps and EDL) are coordinated and that denervation induced an increased number of de-coordinated hybrid fibers associated with a shift of Myh expression toward slower isoform. In the soleus more hybrid fibers are present, and denervation did not induce drastic changes of fMyh...
expression like the ones observed in fast muscles. This suggests that the expression of Myh1 and Myh2 in the soleus is less under the control of innervation than the expression of Myh4 in fast muscles, according to published data. Knowing that in adult myofibers the homeoprotein SIX1 controls the expression of many fast/glycolytic muscle genes among which Myh genes and that SIX binding motifs accessibility are enriched in Myh4 myonuclei (ref. 26 and Fig. 3c), we looked at the nuclear accumulation of this protein in fast myonuclei along EDL myofibers. As shown in Fig. 6e we observed robust SIX1 accumulation in all myonuclei from an extremity to the other of the fiber. To further test the involvement of SIX1 in Myh gene
coordination along the myofiber we tested the consequences of its absence in mutant myofibers from muscle-specific Six1 conditional knock-out (Fig. 6f, g). In absence of Six1, EDL myofibers appeared slower and presented an increased number of non-coordinated hybrid fibers (Fig. 6f, g), showing that SIX transcription complexes participate to coordinate the expression of Myh genes in adult myonuclei.

Coordination of myonuclei during development. We next addressed how fMyh expression coordination appears during development. A sequential transition from embryonic to neonatal and to adult myosin heavy chain isoforms has already been characterized in skeletal muscles during mouse post-natal development. We further analyzed the expression pattern of mRNAs or pre-mRNAs of all sarcomeric Myh (embryonic, neonatal, slow and adult fast) on isolated forelimbs myonuclei, and their number increased drastically after E18.5 (Fig. 7b, c). Myh7 and Myh4 nuclei were coordinated from their onset. We showed that this coordination is dependent on innervation and is established early during development. Coordination of myonuclei was restricted to slow fibers, which homogeneously accumulated this mRNA specifically (Supplementary Fig. 10a). However, some nuclei expressed Myh4 without AldoA (red arrowhead), and some nuclei are negative for both genes. Percentage of the nuclei co-expressing Myh4 and AldoA or expressing only Myh4 or AldoA and negative nuclei in EDL fibers. RNAseq on isolated fibers from EDL showing the localization of Myh4 (red) and AldoA (green) pre-mRNAs. Most myonuclei expressed at the same time Myh4 and AldoA (white arrowhead). However, some nuclei expressed Myh2 without Idh2 (red arrowhead) and other nuclei are negative for both genes. Percentage of the nuclei co-expressing Myh2 and Idh2 or expressing only Myh2 or Idh2 and negative nuclei in soleus fibers. For e.g., scale bar: 100 μm. For f, h the graphs show data pooled from three animals and fifteen fibers per animal. Numerical data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Source data for f, h are provided in the Source Data file.

Discussion
By combining single-nucleus RNA-seq/ATAC-seq and FISH experiments, we established an atlas of the gene expression pattern and chromatin landscape of the different cell populations of adult skeletal muscles, including mononucleated cells and multinucleated myofibers. From our experiments, we estimated that myonuclei constitute ~68% of the nuclei present in adult skeletal muscles of 2 months old mice at rest. Recent single-cell RNA-seq studies of skeletal muscle have characterized the gene expression pattern of mononucleated cells of adult skeletal muscle, but failed to identify the transcription signature of multinucleated myofibers. The composition and gene expression of cell types identified by these reports is in agreement with our data, providing the same global signature of skeletal muscle at rest, identifying distinct populations of FAPs, tenocytes, immune cells, pericytes, adipocytes, smooth muscle cells, and myogenic stem cells. Exploring these studies, our results showed that myofibers are composed of three different types of myonuclei localized differently and expressing distinct sets of genes (Fig. 2), namely the MTJ, NMI, and body myonuclei. The full transcription of these nuclei was to this day unknown, and we characterized numerous genes expressed specifically in these specialized myonuclei. We also characterized an unidentified population of Titin + myonuclei expressing Runx1, Dysf, Ehkd4, Fluc, and Myh9. As Filamin C is involved in early stages of myofibrillar remodeling and repair, this myonuclei population, which had never been described could correspond to myonuclei in area of myofiber damage to support fast repair. Our results demonstrated that most myonuclei express a single fMyh gene, and that in the majority of myofibers myonuclei coordinately express fMyh genes in a specific set of genes, including this Myh gene (Fig. 8). Our RNAseq experiments also identified a feature of transcriptional control in myonuclei of adult myofibers. In fast skeletal muscles, the transcriptional activity of nuclei within each myofiber is finely coordinated. We showed that this coordination is dependent upon innervation and is established early during development. Several reports have shown that apparently homogenous populations of cells present an important variability of gene expression. This variability can be the consequence of stochastic gene expression, different stages of cell cycle, or distinct sub-categories of cells in the studied population. In synctyia like osteoclasts and syncytiotrophoblasts, gene expression can be heterogeneous in nuclei that share the same cytoplasm. We found that the transcription of adult fMyh genes in myonuclei along given myofibrils was finely controlled during development, allowing the coordinated expression in their nuclei of a single fMyh gene. This transcriptional coordination could be a consequence of various processes. In Drosophila, one founder myonucleus was shown to reprogram newly fused myonuclei, inducing coordination of expression between nuclei of the same fiber. Alternatively, distinct populations of preprogrammed...
myogenic stem cells with distinct potential could fuse (homotypic fusion), activating a single Myh gene along the myofiber. Moreover, extracellular signals from surrounding cells may also intervene. The de-coordination of myonuclei after denervation observed in our study supports the importance of innervation in the coordination process during development. A better understanding of the matching between motoneuron subtypes and myofiber subtypes that operates during development may help understand how neuromuscular specialization contributes to the onset of coordination.

Adult myofibers are plastic and can adapt their contractile and metabolic properties depending on external stimuli by activating...
or repressing a set of specific genes, such as the Myh genes. These changes occur in a sequential order recapitulating the organization of the genes at the fast Myh locus (Myh4-Myh1-Myh2). In this report, we showed that most myonuclei expressed only one Myh isoform at a given time (Fig. 4). This robust and exclusive expression is reminiscent of the control of the β-Globin genes by a locus control region. The regulation of the fast Myh locus is not fully understood and could be controlled like the β-Globin genes by a locus control region, such as a super enhancer, activating the expression of associated embryonic, fetal and adult Globin genes in a temporal order. Transient transfection of adult mouse TA and soleus with GFP reporters under the control of 1 kb upstream of each Myh2, Myh1 and Myh4 promoters recapitulate their specific expression in the corresponding MYH2, MYH1 and MYH4 myofibers, respectively. This suggests that each fast Myh promoter possesses its own fiber specific cis-regulatory elements. Accordingly, in Myh1 + nuclei, we observed in snATAC-seq experiments a higher chromatin opening in Myh4 than in Myh1 and Myh2 genes. In Myh4 + nuclei, we failed to detect chromatin opening at the Myh1 and Myh2 promoter regions, suggesting the absence of TF binding. In soleus myofibers with a high proportion of Myh1 and Myh2 co-expression (where Myh1 and Myh2 pre-mRNAs are detected in many myonuclei), we mainly observed Myh1 and Myh2 pre-mRNAs as two dots in the myonuclei, suggesting that each allel transcribed a single gene, and that these two adjacent fast Myh genes could not be transcribed simultaneously. Whether this exclusive expression reflects the competition for a shared enhancer at the locus, as already illustrated for the β-Globin genes, is an interesting possibility.

The coordinated expression of a single isoform of Myh could be due to a similar combination of transcription activators and repressors concentration in these myonuclei, affecting all the genes that they express, or only those with high expression, excluding stochastic expression as previously proposed. In support of this, snRNA-seq experiments showed that specific genes are co-expressed with Myh4, such as genes coding for other sarcomeric proteins, proteins associated with metabolism or associated with calcium handling. The snRNA-seq results showed harmonious non-random gene expression pattern in each nucleus of the myofiber, with some variability between muscles with distinct subprograms among Myh4 + myonuclei. In the mouse, denervation induces muscle atrophy, associated with a fast to slow transition of Myh at the protein level. These changes occur also at the pre-mRNA level, as shown in the present study. We observed that transitions after denervation were sequential, Myh4 + fibers expressing Myh1 but not Myh2, and some nuclei switching their Myh expression profile earlier than others. Thirty days after denervation, myofibers remained uncoordinated showing that the increased number of hybrid fibers observed in EDL 7 days after denervation was not due to a transitional state but that innervation was required to maintain the coordination of fast Myh expression. The atrophy of myofibers induced by denervation involves the activation of protein degradation pathways and the repression of protein synthesis. We observed an increased number of fast Myh-negative nuclei as well as a decreased bi-allelic expression of fast Myh after muscle denervation, suggesting that downregulation of fast Myh expression may participate as well in this atrophy.

The loss of coordination in myonuclei of adult myofibers after denervation observed in the present study is consistent with the role of motoneurons in controlling adult muscle physiology. After denervation observed in the present study is consistent with the role of motoneurons in controlling adult muscle physiology. Resting Ca2+ concentration differs in fast and slow fibers and is modulated by slow or fast myofiber firing. Slower transients following slow motoneuron stimulation activate specific signaling pathways among which Calcineurin phosphatase that controls the nuclear-cytoplasmic localization of NFATc1 in slow myofibers important for the expression of slow muscle genes. MyoD activity as well is modulated by myonucleus coordination. Several proteins controlling the fast-muscle subtypes phenotype and myonuclei coordination is presently unknown. Whether Ca2+ concentrations are similar along the myofiber and how denervation may perturb this basal intracellular Ca2+ concentrations are potential important issues to understand myonuclei coordination. Several proteins controlling intracellular Ca2+ concentration in myofibers (Calsequestrin 1 et 2, Serca1 and 2, Parvalbumin, Calmodulin, Phospholamban, Myozenin 1 and 2, Sarcalumenin, IP3R) are differentially expressed in fast and slow myofibers, and induce a variety of local Ca2+ concentration gradients that may modulate the activity of different transcription factors. Here, we showed that the transcription factor SIX1 accumulates in all myonuclei of EDL myofibers and participates in the coordination of fast Myh expression. The mechanisms controlling SIX1 nuclear accumulation specifically in fast myofibers remain to be established. We observed by snRNA-seq an increased expression of the SIX cofactor EYA4 in Myh4 + myonuclei. Whether the SIX1-EYA4 transcription complex activity may be controlled by motoneuron firing frequency and participates in
myonuclei coordination is an interesting possibility that may explain its role in the control of the adult fast phenotype. The genome-wide chromatin landscape of adult muscles had not been evaluated previously at the myofiber level, due to the lack of adapted experimental protocols. We show here that snATAC-seq is a powerful tool to overcome this limitation and that it allows to detect all the cell populations identified by snRNA-seq in adult skeletal muscles, including myofibers. We identified chromatin accessibility domains coherent with the defined patterns of gene expression, specific of fast-subtypes or slow myonuclei, of MTJ or body myonuclei, as well as of other cell types in skeletal muscle. This analysis also allowed to identify enriched TF-binding sites in ATAC-peaks specific for each myonuclei population. We identified TF-binding motifs known to participate in muscle genes regulation in slow and fast myofibers, among which the NFATc, MEF2 and SIX families, and...
nuclear receptors of the ERR-β/γ79 and the NR4A180 families. The differential TF ATAC-peaks enrichment observed between fast-subtypes myonuclei sheds some light on the basis of their specialization. We identified TF DNA binding motifs, which had never been implicated in muscle genes regulation, such as those recognized by the b-ZIP family of NRL/MAF transcription factors81. Accordingly, we identified c-Maf mRNAs enriched in the Myh4 + myonuclei in our snRNA-seq, suggesting that this TF may contribute to the fine-tuning of muscle gene expression in specialized fast myonuclei, in synergy with SIX homeoproteins. Serria et al showed earlier that c-Maf expression is upregulated in myogenic cells during their differentiation in culture, but the role of c-Maf in adult muscle physiology is presently unknown82.

Last, in addition to defining the exact transcriptional landscape within skeletal muscle, the snRNA-seq and snATAC-seq methods presented here open new perspectives to characterize in the future the crosstalk between myonuclei and their environment, such as with associated myogenic stem cells in pathophysiological conditions, to date poorly investigated due to technical limits.

Methods

Animals. Animal experiments were carried out in strict accordance with the European STE 123 and the French national charter on the Ethics of Animal Experimentation. Protocols were approved by the Ethical Committee of Animal Experiments of the Institut Cochin, CNRS UMR 8104, INSERM U1016, and by the Ministère de l’éducation nationale, de l’enseignement et de la recherche, no. APAFIS#15699-2018021516569195. We used 6- to 8-week old C57black6N mouse females for most of our experiments. Mice were anesthetized with intraperitoneal ketamine and xylazine and with subcutaneous buprecare injections of A-tailing, SPRIselect cleanup, adaptor ligation, SPRIselect cleanup, sample libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, fragment size estimation with a Bioanalyzer (Agilent), the libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, and subsequent washing. After a cleanup with SPRIselect Reagent Kit and fragment size estimation with a Bioanalyzer (Agilent), the libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, SPRIselect cleanup, adaptor ligation, SPRIselect cleanup, sample index PCR, and SPRIselect sample size selection. The fragment size estimation of the resulting libraries was assessed with the High-SensitivityTM HS DNA kit run on 2100 Bioanalyzer (Agilent), and quantified using the QubitTM double stranded DNA (dsDNA) High-Sensitivity HS assay (ThermoFisher Scientific). Libraries were then sequenced by pair with a HighOutput flowcell using an Illumina Nextseq 500.

Single-nucleus RNA-seq from skeletal muscle. After FACS sorting, nuclei were centrifuged 5 min at 500 × g at +4 °C, resuspended in 15 μl of wash buffer and counted with a hemocytometer. The concentration of nuclei was then adjusted to 1000 nuclei/μl with wash buffer. We loaded around 4000 nuclei per condition into the 10x Chromium Chip. We used the Single-Cell 3′ Reagent Kit v3 according to the manufacturer’s protocol. GEM-Reverse Transcription (GEM-RT) was performed in a thermal cycler: 53 °C for 45 min, 85 °C for 5 min. Post GEM-RT Cleanup using DynaBeads MyOne Silane Beads was followed by cDNA amplification (98 °C for 3 min, cycled 12 × 98 °C for 15 s, 67 °C for 20 s, 72 °C for 1 min). After a cleanup with SPRIselect Reagent Kit and fragment size estimation with High-SensitivityTM HS DNA kit run on 2100 Bioanalyzer (Agilent), the libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, SPRIselect cleanup, adaptor ligation, SPRIselect cleanup, sample index PCR, and SPRIselect sample size selection. The fragment size estimation of the resulting libraries was assessed with the High-SensitivityTM HS DNA kit run on 2100 Bioanalyzer (Agilent) and quantified using the QubitTM double stranded DNA (dsDNA) High-Sensitivity HS assay (ThermoFisher Scientific). Libraries were then sequenced by pair with a HighOutput flowcell using an Illumina Nextseq 500.

FACS sorting of nuclei from adult skeletal muscle for single-nucleus RNA-seq. For the mix condition 6 tibialis, 6 gastrocnemius, 6 soleus, 6 plantaris, and 6 EDL were pulled together. For the soleus condition, 24 soleus, for the tibialis condition 6 tibialis and for the quadriceps condition 6 quadriceps were pulled together. Muscles were dissected and pulled in cold phosphate-buffered saline (PBS) with 0.2 U/μl RNase inhibitor. PBS was removed and muscles were minced in 1 ml cold lysis buffer (10 mM Tris-HCl pH7.5, 10 mM NaCl, 3 mM MgCl2, and 0.1% NonidetTM P40 in Nuclease-Free Water) with scissors. After 2 min, 4 ml of cold lysis buffer were added and muscles were lysed for 3 min at +4 °C. Nine milliliter of cold wash buffer (PBS, BSA 2% and 0.2 U/μl RNase inhibitor from Roche) was added and the lysate was dounced with 10 strokes of loose pestle avoiding too much pressure and air bubbles. The homogenate was filtered with 70 and 40 μm cell strainers. Nuclei were pelleted by centrifugation for 5 min at 500 × g at +4 °C. Nuclei were washed to remove ambient RNAs in 1 ml of cold wash buffer, transferred in a 1.5 ml Eppendorf tube and centrifuged 5 min at 500 × g at +4 °C. The pellet was resuspended in 250 μl of wash buffer and stained during 15 min in the dark at +4 °C with DAPI (10 μg/ml). Then the nuclei were washed 1 ml of wash buffer, centrifuged 5 min at 500 × g at +4 °C, resuspended in 300 μl of wash buffer and filtered with 30 μm cell strainers. Nuclei were then FACS sorted to exclude debris with a BD FACSaria III and the BD FACSDIVA software. More than 200,000 nuclei were collected in a tube containing 200 μl of wash buffer.

Single-nucleus RNA-seq from skeletal muscle. After FACS sorting, nuclei were centrifuged 5 min at 500 × g at +4 °C, resuspended in 15 μl of wash buffer and counted with a hemocytometer. The concentration of nuclei was then adjusted to 1000 nuclei/μl with wash buffer. We loaded around 4000 nuclei per condition into the 10x Chromium Chip. We used the Single-Cell 3′ Reagent Kit v3 according to the manufacturer’s protocol. GEM-Reverse Transcription (GEM-RT) was performed in a thermal cycler: 53 °C for 45 min, 85 °C for 5 min. Post GEM-RT Cleanup using DynaBeads MyOne Silane Beads was followed by cDNA amplification (98 °C for 3 min, cycled 12 × 98 °C for 15 s, 67 °C for 20 s, 72 °C for 1 min). After a cleanup with SPRIselect Reagent Kit and fragment size estimation with High-SensitivityTM HS DNA kit run on 2100 Bioanalyzer (Agilent), the libraries were constructed by performing the following steps: fragmentation, end-repair, A-tailing, SPRIselect cleanup, adaptor ligation, SPRIselect cleanup, sample index PCR, and SPRIselect sample size selection. The fragment size estimation of the resulting libraries was assessed with the High-SensitivityTM HS DNA kit run on 2100 Bioanalyzer (Agilent) and quantified using the QubitTM double stranded DNA (dsDNA) High-Sensitivity HS assay (ThermoFisher Scientific). Libraries were then sequenced by pair with a HighOutput flowcell using an Illumina Nextseq 500.

Fig. 8 Diagram of Myh expression in pure and hybrid myonuclei and myofiber. a Diagram showing the different possibilities of Myh isoforms expression in a myonucleus from the results of our experiments. b Diagram showing the different possibilities of Myh isoforms expression in a myofiber from the results of our experiments.
with the following mode: 26-base-pairs (bp) (10x Index + UMIs), 8 bp (index Index), and 57 bp (Read 2).

Single-nucleus RNA-seq analysis. A minimum of 50,000 reads per nucleus were sequenced and analyzed with Cell Ranger Single-Cell Software Suite 3.0.2 by 10x Genomics. Raw base call files from the Nextseq 500 were demultiplexed with the cellranger mkfastq pipeline into library-specific FASTQ files. The FASTQ files for each library were then processed independently with the cellranger count pipeline. This pipeline used STAR21 to align reads to the Mus musculus genome.

Single-nucleus ATAC-seq from skeletal muscle. We used the 10x genomic nuclei Isolation for Single-Cell ATAC Sequencing protocol (CG000169|Rev B) with the following modifications: 12 quadriiceps and 12 soles were dissected and pulled in cold PBS. PBS was removed and muscles were minced 2 min in 1 ml of cold ATAC-lysis buffer (10 mM Tris-HCl pH7.4, 10 mM NaCl, 3 mM MgCl2, 1% BSA and 0.1% Tween-20 in Nuclease-Free Water). Six milliliter of cold ATAC-lysis buffer were added and muscles were lysed on ice. After 3 min the lysate was dounced with 10 strokes of loose pestle avoiding too much pressure and air bubbles. After douncing, 8 ml of wash buffer were added and the homogenate was filtered through 70 μm cell strainers. Nuclei were pelleted by centrifugation for 5 min at 500 x g at +4 °C. Next, we used the Chromium Single-Cell ATAC kit according to the manufacturer’s protocol. Nuclei were resuspended in nuclei buffer from the kit, transposed 1 h at 37 °C. We loaded around 6000 nuclei into the 10X Chromium Chip. GEM incubation and amplification were performed in a thermal cycler: 72 °C for 5 min, 98 °C for 30 s, and 12 repeated cycles of 98 °C for 10 s, 59 °C for 30 s, and 72 °C for 1 min. Post GEM Cleanup using DynaBeads MyOne Silane Beads was followed by library construction (98 °C for 45 s, cycled 12 × 98 °C for 10 s, 59 °C for 30 s, and 72 °C for 1 min). The libraries were constructed by adding sample index PCR, and SPRIselect size selection. The fragment size estimation of the resulting libraries were done by high sensitivity/HT HS DNA kit runned on 2100 Bioanalyzer (Agilent) and quantified using the QubitTM dsDNA High-Sensitivity HS assay (ThermoFisher Scientific). Libraries were then sequenced by pair with a HighOutput flowcell using an Illumina Nextseq 500.

Single-nucleus ATAC-seq analysis. A minimum of 10,000 reads per nucleus were sequenced and analyzed with Cell Ranger Single-Cell Software Suite 3.0.2 by 10x Genomics. Raw base call files from the Nextseq 500 were demultiplexed with the cellranger-atac mkfastq pipeline into library-specific FASTQ files. The FASTQ files for each library were then processed independently with the cellranger count pipeline. This pipeline used STAR21 to align reads to the Mus musculus genome. Once aligned, barcodes associated with these reads—cell identifiers and Unique Molecular Identifiers (UMIs), underwent filtering and correction. The subsequent visualizations, clustering and differential expression tests were performed in R (v 3.4.3) using Seurat (v3.0.2)25. Quality control on aligned and counted reads was performed keeping cells with >200 and ≤2500 nFeature RNA and ≤5% mitochondrial genes. We get 6477 nuclei from the mix of tibialis, EDL, gastrocnemius, plantaris and soleus, 1335 nuclei from quadriceps, 4838 nuclei from tibialis, 2317 nuclei from soleus. We detected the expression of approximately one thousand genes per nucleus in each of these muscles (Supplementary Fig. 1).

Immunohistochemistry. For immunostaining against MYH4, MYH2, MYH7, and Laminin, adult legs without fixation and without skin were embedded with TissueTek OCT, and directly frozen in cold isopentane cooled in liquid nitrogen. Muscles were conserved at −80 °C and cut with Leica cryostat 3050s with a thickness of 10 μm. Cryostat sections were washed three times 5 min with PBS and then incubated with blocking solution (PBS and 10% goat serum) 30 min at room temperature. Sections were incubated with primary antibody solution at +4 °C, then washed three times for 5 min with PBS and incubated with secondary antibody solution 1 h at room temperature. Sections were further washed three times for 5 min and mounted with Mowiol solution and a glass coverslip. Images were collected with an Olympus BX63F microscope and a Hamamatsu ORCA-Flash 4.0 camera. Images were analyzed with ImageJ program. The references of the antibodies used are listed in Supplementary Table 1.

Statistical analysis. The graphs represent mean values ± SEM. Significant differences between mean values were evaluated using two-way ANOVA with Graphpad 6 software and student’s t test for Fig. 6g.

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Author contributions

M.D.S., A.S., F.R., and P.M. conceived the study. M.D.S., S.B., F.L., B.I., B.S., M.A., and A.S. performed experiments. M.D.S. and P.M. wrote the manuscript with input from A.S. and F.R.

Competing interests

The authors declare no competing interests.

Additional information

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