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► **To cite this version:**

Amaury Korb, Shahrugim Tajbakhsh, Glenda Comai. Functional specialisation and coordination of myonuclei. *Biological Reviews*, 2024, 99 (4), pp.1164-1195. 10.1111/brv.13063 . pasteur-04779558

HAL Id: pasteur-04779558

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Submitted on 13 Nov 2024

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Functional specialisation and coordination of myonuclei

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ABSTRACT

Myofibres serve as the functional unit for locomotion, with the sarcomere the fundamental subunit. Running the entire length of this structure are hundreds of myonuclei, located at the periphery of the myofibre, juxtaposed to the plasma membrane. Myonuclear specialisation and clustering at the centre and ends of the fibre are known to be essential for muscle contraction, yet the molecular basis of this regionalisation has remained unclear. While the ‘myonuclear domain hypothesis’ helped explain how myonuclei can independently govern large cytoplasmic territories, novel technologies have provided granularity on the diverse transcriptional programs running simultaneously within the syncytia and added a new perspective on how myonuclei communicate. Building upon this, we explore the critical cellular and molecular sources of transcriptional and functional heterogeneity within myofibres, discussing the impact of intrinsic and extrinsic factors on myonuclear programs. This knowledge provides new insights for understanding muscle development, repair, and disease, but also opens avenues for the development of novel and precise therapeutic approaches.

Key words: sn-RNAseq, myonuclear heterogeneity, Myh coordination, neuromuscular junction, myotendinous junction, nuclear communication, mRNA transport.

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I. INTRODUCTION

Large cells, such as neurons and skeletal muscle fibres, have evolved different adaptations to ensure that sufficient levels of transcripts and proteins are distributed throughout their extensive cytoplasm. Neurons actively regulate the transport of proteins produced in the cell body along axons to their site of action in the synapse (Guedes-Dias & Holzbaur, 2019). In the parasitic nematode *Ascaris lumbricoides*, mononucleated muscle cells rely on a single nucleus for transcription, contributing both to the neuromuscular junction (NMJ) and the anchor point of the cell (Rosenbluth, 1965). By contrast, skeletal myofibres in most other metazoans contain multiple nuclei dispersed along one cell formed through fusion events (Petraný & Millay, 2019). Skeletal myofibres are the longest cells of the human body, reaching up to 60 cm in the sartorius muscle (Harris *et al.*, 2005) and contain more than 100 myonuclei/mm fibre length (Ohira *et al.*, 1999). Skeletal muscle is arguably the most plastic structure in mammalian organisms, undergoing changes in mass, cross-sectional area, metabolism, and contractile properties dynamically during growth, upon training, and during ageing and disease (Braun & Gautel, 2011; Schiaffino *et al.*, 2013; Murgia *et al.*, 2017; Jorgenson, Phillips & Hornberger, 2020).

The understanding of why muscle requires multiple nuclei stems from foundational studies defining the ‘myonuclear domain’ concept, where each myonucleus behaves independently within the syncytium by controlling a finite volume of cytoplasm (reviewed by Bagley *et al.*,

2023). Pioneer studies using interspecies heterokaryons or stable cell lines carrying a reporter gene showed that the intracellular distribution of transcript, organelle, membrane, and structural proteins derived from individual nuclei was limited to a local area surrounding the encoding nucleus and adjacent nuclei (Pavlath *et al.*, 1989; Ralston & Hall, 1989*a,b*, 1992; Blau & Blakely, 1999). Moreover, while cytoplasmic proteins could be widely distributed in the cytoplasm, their messenger RNA (mRNA) was restricted to the perinuclear space in which they were synthesised (Pavlath *et al.*, 1989; Ralston & Hall, 1992). The myonuclear domain concept is consistent with the notion that the capability of a nucleus to sustain a given cytoplasmic volume cannot be without disrupting cellular processes (Demidenko & Blagosklonny, 2008; Miettinen & Björklund, 2016; Neurohr *et al.*, 2019). Accordingly, recent studies underscored the role of scaling of nuclear numbers and their spatial arrangement in the regulation of myofibre size (Cramer *et al.*, 2020; Hansson *et al.*, 2020; Hansson & Eftestøl, 2023).

Transcriptional independence within the syncytium was originally supported by several findings. First, by the observation of stochastic gene expression among myonuclei despite the presence of a uniform contractile apparatus (Newlands *et al.*, 1998). Second, by the identification of specialised myofibre regions at the interface with adjacent cell types, i.e. the NMJ at the synaptic plate, and the myotendinous junction (MTJ) at the tendon interface (Fig. 1). These regions, at the centre or tips of the myofibres, respectively, have smaller myonuclear domains due to the presence of myonuclear clusters (Englander & Rubin, 1987; Sanes *et al.*, 1991; Rosser, Dean & Bandman, 2002; Bruusgaard *et al.*, 2003; Grady *et al.*, 2005; Hastings *et al.*, 2020) and accumulate specific proteins (Chiquet & Fambrough, 1984; Englander & Rubin, 1987; Fontaine & Changeux, 1989; Tidball & Lin, 1989; Dix & Eisenberg, 1990; Sanes *et al.*, 1991; Carlsson *et al.*, 1999). Finally, by the identification of hybrid fibres expressing more than one myosin heavy chain (MyHC) as the result of

heterogeneity in gene expression among myonuclei of the same fibre (Schiaffino & Reggiani, 2011; Medler, 2019). Altogether, these observations illustrate the need for coordination, and disruption of coordination, between syncytial myonuclei. Given that myogenesis occurs in a lineage continuum from stem/progenitor cells that differentiate and fuse to form multinucleated myofibres, this raises the question of how much information is brought by the fusing cells and to what extent transcriptional reprogramming takes place in postmitotic nuclei. The identification of stromal fusogenic populations (Liu *et al.*, 2017a; Lima *et al.*, 2021; Yaseen *et al.*, 2021; Flynn *et al.*, 2023) recently added another layer of diversity to the syncytium.

In this review, we provide insights into the regulation of myonuclear coordination and specialisation, emphasising intrinsic (nuclear origin) and extrinsic (nuclear environment) factors shaping myonuclear subpopulations. We highlight the remarkable transcriptional heterogeneity of myonuclear subpopulations present during postnatal development, homeostasis, and disease conditions identified by innovative omics approaches. We also describe in detail the postsynaptic endplates and muscle–tendon junctions as the links between the spatial tissue background and functional specialisation are most striking at these sites. We also discuss other mechanisms that confer myofibre regionalisation and coordination of gene expression, such as those regulated by protein and mRNA transport. Finally, we discuss how maintaining or disrupting coordination can be either beneficial or detrimental, depending on the physiopathological context.

II. NOVEL METHODS TO STUDY COORDINATION AND FUNCTIONAL SPECIALISATION

Initial studies on myonuclear transcriptional heterogeneity, communication, and coordination between myonuclei relied mostly on the use of heterokaryons (i.e. multinucleated cells that

contain nuclei from diverse origins), combined with immunostaining, *in situ* hybridisation, nuclear reporters, or metabolic incorporation of ^3H -thymidine into DNA (Pavlath *et al.*, 1989; Ralston & Hall, 1989*a,b*; Hall & Ralston, 1989; Blau & Blakely, 1999).

While single-cell RNA-sequencing and mass cytometry studies have helped to identify the major mononuclear cell types present in skeletal muscle (Schaum *et al.*, 2018; Dell'Orso *et al.*, 2019; Giordani *et al.*, 2019; Barruet *et al.*, 2020; Micheli *et al.*, 2020; Rubenstein *et al.*, 2020; Xi *et al.*, 2020; McKellar *et al.*, 2021), mature myofibres could not be captured despite their predominance within the tissue, owing to their large size which is incompatible with microfluidics-based single-cell methods (Zeng *et al.*, 2016). Moreover, whereas single-fibre sequencing and single-fibre proteomics revealed inter-fibre differences (Blackburn *et al.*, 2019; Chemello *et al.*, 2019), the comprehensive interrogation of transcriptional heterogeneity within syncytial myofibres has been challenging due to the presence of a shared cytoplasm. These problems were recently overcome by the development of single-molecule fluorescent *in situ* hybridisation (sm-FISH) and single-nucleus RNA-sequencing (snRNA-seq) protocols in mouse, human and *in vitro*-generated myotubes (Williams, Yokomori & Mortazavi, 2022*b*) (Fig. 2). snRNA-seq has the advantage of using a rapid mechanical dissociation protocol to isolate nuclei and thus minimises the induction of stress-response genes upon dissociation (Machado *et al.*, 2021; Santos *et al.*, 2021).

Besides capturing resident populations, these studies identified the transcriptional signature of specialised myonuclear subpopulations during postnatal development, adulthood, ageing and in disease conditions (Table 1). smFISH, which incorporates signal amplification methods, allows for sensitive and quantitative detection of multiple transcripts at high resolution (Wang *et al.*, 2012). The optimisation of this method for skeletal muscle has enabled the spatial visualisation of specialised transcriptional programs in muscle stem cells (MuSCs) and myonuclei on isolated myofibres and muscle tissue sections (Kann & Krauss, 2019; Kim *et*

al., 2020; Santos *et al.*, 2020, 2023). Moreover, the use of intronic probes, which are retained in the nucleus, also permitted the study of coordination of expression between individual nuclei from the same myofibre to examine, for example, mono- *versus* biallelic gene expression (Santos *et al.*, 2020). This method provides steady-state snapshots of mRNA distribution and it is generally used when a limited number of known transcripts needs to be assessed.

To identify the gene regulatory networks underlying myonuclear coordination and specialisation, single nucleus transcriptome methods need to be related to chromatin accessibility assays. Some studies have combined snRNA-seq and single-nucleus ATAC-sequencing (snATAC-seq) information (Santos *et al.*, 2020) or performed multiomic assays to profile gene expression and open chromatin simultaneously from the same nuclei (Orchard *et al.*, 2021; Santos *et al.*, 2023).

III. ORIGIN OF NUCLEI AS SOURCE OF HETEROGENEITY IN THE SYNCYTIUM

In this section, two potential mechanisms that might be responsible for heterogeneity among syncytial myonuclei are discussed: (i) heterogeneity induced by fusion of different types of myogenic progenitors, including heterogeneity induced by the microenvironment, and (ii) heterogeneity resulting from fusion of other cell types.

(1) Heterogeneity of myogenic precursors and MuSCs

Myonuclear accretion from prenatal development to adulthood depends on related, but distinct populations of stem/progenitor cells (reviewed by Murphy & Kardon, 2011). During fetal and neonatal myogenesis, myofibre growth occurs by a rapid increase in myonuclear numbers upon fusion of myoblasts (Schultz, 1996; White *et al.*, 2010; Bachman &

Chakkalakal, 2022). While the total myofibre number is established prior to birth (Rowe & Goldspink, 1969; White *et al.*, 2010), postnatal growth is characterised by an increase in length, cross-sectional area and size of the myonuclear domain of each individual myofibre (Rosser *et al.*, 2002; White *et al.*, 2010; Bachman *et al.*, 2018). Myofibre growth after weaning [by postnatal day (P)21] was believed to be dependent exclusively on fibre hypertrophy, yet more recent studies showed that residual addition of proliferative PAX7+ cells until the pre-pubertal stage sustains growth (Bachman & Chakkalakal, 2022).

Embryonic, fetal, and adult myoblasts differ in appearance in culture, in their response to signalling molecules *in vivo* and *in vitro* (Hauschka, 1974; Biressi, Molinaro & Cossu, 2007a; Hutcheson *et al.*, 2009; Friedrichs *et al.*, 2011; Murphy *et al.*, 2014; Stantzou *et al.*, 2017), their molecular signatures (Biressi *et al.*, 2007b; Mourikis *et al.*, 2012; Alonso-Martin *et al.*, 2016; Tierney *et al.*, 2016; Pietrosevoli *et al.*, 2017; Schaum *et al.*, 2018; Dell'Orso *et al.*, 2019; Giordani *et al.*, 2019; Barruet *et al.*, 2020; Micheli *et al.*, 2020; Rubenstein *et al.*, 2020; Xi *et al.*, 2020; Contreras, Córdova-Casanova & Brandan, 2021a) and engraftment capacity (Sakai *et al.*, 2013; Tierney *et al.*, 2016). Adult MuSCs are also recognised as functionally non-equivalent (Evano & Tajbakhsh, 2018; Relaix *et al.*, 2021). However, it is unclear to what extent heterogeneity of the upstream populations persists and impacts specialisation of nascent myonuclei after fusion. For instance, full reprogramming (Pajak, Mariappan & Wiczorek, 1991) or maintenance of different MyHC programs in distinct nuclear domains (Pin & Merrifield, 1997) have been observed in heterokaryons. *In vivo* studies also showed some discrepancies - myoblasts transferred to embryonic limbs appeared to be committed to specific fibre fates (DiMario, Fernyak & Stockdale, 1993), yet fusion of myoblasts to pre-existing fibres resulted in reprogramming of the nascent myocyte nucleus to the host fibre gene expression program (Hughes & Blau, 1992). This is in keeping with the concept of 'dominant myonuclei', which postulates that nuclei of the resident or 'primary' myoblasts

behave as dominant to convert the subsequent added myonuclei. This scenario was reported in the *Drosophila* larva (Bataillé *et al.*, 2017), where each muscle forms by fusion of a founder cell with multiple fusion-competent myoblasts (Bate, 1990), and the specific muscle morphology arises by the combination of identity transcription factors (iTFs) expressed by the founder cell (Joussineau *et al.*, 2012). In this context, fusion-competent myoblasts can be transcriptionally reprogrammed to a given founder cell identity post-fusion, but depending on the iTF and muscle context, the founder cell nucleus can also maintain a transcriptional program distinct from the other syncytial nuclei during muscle development (Bataillé *et al.*, 2017).

In mammals, the question of whether specific fibre properties are governed by intrinsic myoblast heterogeneity has been hindered by the lack of early markers that unambiguously distinguish temporally emerging populations and their incorporation forming myofibres. Large-scale integration of singlecell (sc) and snRNAseq data from postnatal and adult stages generated a transcriptomic model for postnatal myogenesis, including short-lived states of myogenic commitment and fusion (McKellar *et al.*, 2021). However, the full trajectory from specific progenitors to a certain myonuclear identity could not be reconstructed with this approach. Ideally, combining snRNAseq with barcoding lineage methods during development could offer an opportunity to integrate complementary information on cell lineage, cell state and myonuclear fates without requiring prior knowledge of the marker genes (Wagner & Klein, 2020).

The idea that extrinsic signals can, to some extent, override the intrinsic commitment of myoblasts to specific myofibre programs is supported by heterotopic transplantation studies (Hoh & Hughes, 1988, 1991; Sambasivan *et al.*, 2009; Kang *et al.*, 2010). It is now well established that the muscle microenvironment can indirectly influence the properties of stem cells, progenitors, and myoblasts, along with the temporal dynamics of their transitions

(reviewed by Murphy & Kardon, 2011; Evano & Tajbakhsh, 2018; Mashinchian *et al.*, 2018; Helmbacher & Stricker, 2020; Contreras, Rossi & Theret, 2021*b*). Recent longitudinal scRNAseq and snRNAseq studies revealed simultaneous changes in signatures of skeletal muscle progenitors and adjacent connective tissue (CT) during development (Xi *et al.*, 2020; Lima *et al.*, 2021), and their modes of cell–cell communication in the adult (Wosczyzna *et al.*, 2019; McKellar *et al.*, 2021; Fukada & Uezumi, 2022). Genetic studies demonstrated that muscle CT fibroblasts can extrinsically regulate the positioning of myogenic precursors and instruct the orientation of myofibres and points of attachment to bones (Kardon, Harfe & Tabin, 2003; Hasson *et al.*, 2010; Colasanto *et al.*, 2016; Vallecillo-García *et al.*, 2017; Besse *et al.*, 2020). Certain muscles are embedded in CT of different developmental origins at their ends (Heude *et al.*, 2018; Comai *et al.*, 2020; Kuroda *et al.*, 2021). Interestingly, mesenchymal progenitors can also indirectly affect muscle–nerve interactions (Fukada & Uezumi, 2022). Thus, it tempting to speculate that local differences in CT could confer distinct properties to underlying myonuclei. This can happen either indirectly, *via* cell–cell communication, or directly by contribution of nuclei through fusion, as discussed in the following section.

(2) Other myonuclear sources

While myoblasts are the major source of myonuclei throughout life, two reports identified fibroblast cells that fuse with existing myofibres, mostly at the tips, of growing muscles (Lima *et al.*, 2021; Yaseen *et al.*, 2021) (Fig. 3, Table 2). Remarkably, these cells displayed a hybrid transcriptome profile and BMP signalling, which seems to regulate this fibroblast-to-myoblast conversion (Lima *et al.*, 2021). Moreover, FISH analysis demonstrated that fusion of lateral plate mesoderm-derived fibroblasts to the fibre ends facilitates the introduction of fibroblast-specific transcripts into the elongating myofibres. The resulting hybrid fibre zone enables a

smooth transition from muscle to tendon, which is an essential feature for forming robust MTJs (Yaseen *et al.*, 2021).

Other studies identified interstitial cell populations that fuse to myofibres. For example, mesenchymal *Twist2*⁺ progenitors, which are molecularly and anatomically distinct from PAX7⁺ MuSCs, contribute to type IIB/X myofibres during adulthood and muscle regeneration (Liu *et al.*, 2017a). This specificity is driven by Sema3a–Nrp1 signalling that prevents these progenitors from fusing with IIA/IIX fibres (Li *et al.*, 2019). Similarly, another study identified a *Hoxa11*⁺ cell population that does not express PAX7 and is not capable of forming myotubes autonomously, but that can contribute myonuclei to adult myofibres (Flynn *et al.*, 2023). However, the extent to which these interstitial populations contribute to myonuclear heterogeneity, and if their initial signatures are partially or completely rewired upon fusion, is unknown. Spatial transcriptomics and functional studies might establish more precise links between connective tissue subpopulations and myonuclear diversity in individual myofibres.

IV. COMPARTMENTALISING NUCLEI FOR SPECIFIC FUNCTIONAL ROLES

The impact of extrinsic signals and intercellular communication on the development of specialised myonuclear programs is best exemplified at the level of the NMJ and MTJ.

(1) Neuromuscular junction myonuclei

The NMJ converts electrical impulses generated by the motor neuron into electrical activity in muscle fibres. NMJ myonuclei (NMJm) are a small cluster of 5–10 nuclei in the vicinity of the post-synaptic apparatus and are characterised by a larger and rounder nuclear morphology, enrichment in decondensed chromatin and expression of genes required for neuronal endplate function, such as acetylcholine receptor (AChR) subunits and acetylcholine esterase (AChE)

(Jasmin, Lee & Rotundo, 1993; Chan *et al.*, 1999; Blotnick-Rubin & Anglister, 2018) (Fig. 1).

Innervation and electrical activity are the triggers of the massive spatial myonuclear rearrangements (Grady *et al.*, 2005; Bai *et al.*, 2022) and the progressive segregation of gene expression between synaptic and extra-synaptic myonuclei (reviewed by Ferraro, Molinari & Berghella, 2012; Belotti & Schaeffer, 2020). For instance, compartmentalisation of AchR expression is established through three major steps: (1) initial activation of AchR subunits, including the developmental γ -subunit, by the MRFs along the fibre; (2) down-regulation of AchR subunit gene expression in extrasynaptic nuclei by electrical activity and MRF repression; and (3) clustering of synaptic proteins and of a few muscle nuclei upon the release of several neuronal factors (i.e. AGRIN, NRG, WNT ligands) that activate MUSK, LRP4 and ERBB receptors at the postsynaptic membrane. Sub-synaptic nuclei develop a specific gene expression program driven by the Ets transcription factors *Erm/Etv5* and *Gabp* (known as *Nrf2/E4tf1*) which bind specific N-box motifs in the promoter of synaptic genes and adult AchR subunits (Hippenmeyer *et al.*, 2007; O'Leary *et al.*, 2007) (Table 3). Of note, *Erm* is specifically activated in sub-synaptic nuclei and *Gabp* is expressed and translated along the fibre length, yet it is present in its phosphorylated and active form only close to the NMJ in response to neuronal factors (Fromm & Burden, 2001; Hippenmeyer *et al.*, 2007).

snRNAseq studies brought further insights into the transcriptional profile of these nuclei (Chemello *et al.*, 2020; Kim *et al.*, 2020; Petrany *et al.*, 2020; Santos *et al.*, 2020; Eraslan *et al.*, 2022) which constitute around 0.8% of myonuclei in adult mouse tibialis anterior muscle (Petrany *et al.*, 2020). While the role of *Ufsp1* and *Gramd1b* as novel regulators of the NMJm fate was further confirmed by loss-of-function studies in C2C12 cells, it is unclear whether all the novel NMJm markers are regulated by Ets transcription factors, and what their roles are *in vivo*. A comparison of NMJm in single- *versus* multiple-innervated muscle fibres, such as

extraocular and certain neck muscles (Bendiksen, Dahl & Teig, 1981; Périé, Guily & Sebille, 1999; Lienbacher & Horn, 2012), might also reveal differences in the NMJm contribution to the total myonuclear population, their transcriptome, and insights into how motor unit territories are organised along the fibre length.

While NMJm seem to depend mostly on the establishment of the synapse and thus are dependent on extrinsic factors during development, the identity and/or fusion site of subpopulations of MuSCs could also bias the fate of accreted myonuclei towards a NMJm fate in adulthood (Liu *et al.*, 2015, 2017b; Larouche *et al.*, 2021). Using MuSC lineage tracing with a nuclear reporter in a model that disrupts innervation (sciatic nerve transection), reporter-positive myonuclei were exclusively confined to or near the NMJ. This subpopulation of MuSCs at the NMJ, called synaptic MuSCs (Sy-MuSCs) share a transcriptome signature with NMJm and are molecularly distinct from activated MuSCs involved in global regeneration of myofibres (Larouche *et al.*, 2021). The existence of Sy-MuSCs is also supported by genetic experiments, where ablation of MuSCs impairs the regeneration of NMJs after nerve injury (Liu *et al.*, 2015) and accelerates ageing-related NMJ defects with reduction in the size of post-synaptic myonuclear clusters (Liu *et al.*, 2017b). Notably, a specific myonuclear cluster with NMJ markers was observed already at embryonic day (E)14.5 at the centre of the fibres (Santos *et al.*, 2023). This cluster expressed *Col25a1*, a gene necessary for myoblast fusion and formation of NMJs (Munezane *et al.*, 2019). Further studies are needed to determine if subpopulations of myogenic progenitors specifically contribute to the NMJ during development or alternatively, Sy-MuSCs emerge after the first stable NMJ is set during development.

(2) Myotendinous junction myonuclei

The MTJ is a complex specialised region located at the muscle–tendon interface that represents the primary site of force transmission (Maartens & Brown, 2015; Subramanian & Schilling, 2015). Muscle–tendon connection sites exhibit structural specialisation and express specific extracellular matrix (ECM) and adhesion proteins compared to neighbouring muscle and tendon tissue (Koch *et al.*, 2004; Karlsen *et al.*, 2022) (Fig. 1).

Histological analysis has shown that myonuclei near the MTJ express specific proteins or display an enrichment of proteins present at the whole fibre level (Table 3). seq allowed the identification of a transcriptionally unique population of MTJ myonuclei (MTJm) that account for approximately 4% of all limb muscle myonuclei (Kim *et al.*, 2020; Petraný *et al.*, 2020; Santos *et al.*, 2020). Interestingly, seq identified a specific set of TF motifs, including KLF and SP families, enriched in MTJm suggesting that they might be responsible for the regionalised expression of specific MTJ markers (Santos *et al.*, 2020). Moreover, seq on genetically labelled myonuclei isolated by flow cytometry permitted identification of two MTJm subpopulations in mouse tibialis anterior (Kim *et al.*, 2020); one expressing canonical MTJ markers (MTJ-A), and a novel one (MTJ-B), expressing connective tissue and ECM genes. Other MTJm clusters were found in the mouse soleus and plantaris muscles and may represent differences among muscle groups (Wen *et al.*, 2021). Using seq and spatial transcriptomics of human MTJ, a recent study discovered a novel myonuclear population presenting an intermediate transcriptional profile between muscle body myonuclei and MTJm. This population called ‘transitional MTJm’, expresses cell adhesion molecules such as NCAM1 but lacks the typical MTJ marker COL22A1 (Karlsen *et al.*, 2023).

As discussed in Section III.2, fibroblast-to-myofibre fusion is part of the normal developmental program generating syncytial myofibres with hybrid developmental origin at their ends (Lima *et al.*, 2021; Yaseen *et al.*, 2021). Given the overlapping signatures between MTJ-B myonuclei observed in adult muscles (Kim *et al.*, 2020) and contributing to the

muscle tips during embryonic and postnatal development (Lima *et al.*, 2021; Yaseen *et al.*, 2021), it is likely that these are indeed the same nuclei. Yet, it is unclear whether this mechanism contributing to the specialisation of MTJ nuclei is present throughout the body or is restricted to certain muscle groups. As MTJ-B myonuclei have not been observed in every fibre in the limb (Kim *et al.*, 2020), the relevance of this subpopulation needs to be clarified. Moreover, while the markers of the MTJ-A and MTJ-B subpopulations were identified in myonuclei from perinatal, adult, and ageing muscles (Petraný *et al.*, 2020), the mechanisms that allow this junctional myonuclear subpopulation to retain a specific transcriptional program in the syncytium are unclear but might rely on local signalling and mechanical cues. For instance, specific signalling pathways and ECM remodelling (Wang *et al.*, 2010; Kraft-Sheleg *et al.*, 2016) are active at the muscle–tendon interface during development. In addition, MTJm positioning and MTJ perimeter are sensitive to stretching (Perillo & Folker, 2018; Rocha *et al.*, 2021) and muscle loading is required for initiation of ECM deposition at the MTJ during development (Lipp *et al.*, 2023). Thus, it is likely that mechanical cues play a role in initiation and maintenance of transcriptional heterogeneity of MTJm.

(3) Muscle spindles: other compartmentalised myonuclear populations

Muscle spindles are encapsulated mechanosensory organs present in most muscles that inform the central nervous system on changes in muscle length. They consist of specialised muscle fibres, the intrafusal fibres, which are contacted by both efferent and afferent neurons and named nuclear bag (bag1 and bag2) or nuclear chain fibres according to their nuclear arrangements at the central non-contractile part of the fibre (Macefield & Knellwolf, 2018; Kröger & Watkins, 2021). Proteomic and bulk RNAseq analysis of genetically labelled muscle spindles and adjacent extrafusal fibres identified a set of new markers for the different tissues that compose the spindle (Bornstein *et al.*, 2023). Further insights into the structural

proteins of each intrafusal fibre type and their corresponding myonuclear populations was provided by seq on purified spindle myonuclei (Kim *et al.*, 2020). Notably, intrafusal fibres possess similar populations of MTJm and NMJm as extrafusal fibres, although they are innervated by γ and β motor neurons and not α motor neurons (Stifani, 2014; Kröger & Watkins, 2021). In addition, intrafusal fibres contain specialised nuclei that correspond to a sensory NMJ population. These nuclei are marked by *Calcr1*, *Etv4* and *Pdgfra*, and localise in the centre of the fibre under the endplate of the afferent neuron (Kim *et al.*, 2020). Whether this population is present in both bag and chain fibres is unclear. Intriguingly, two populations of chain myonuclei were observed, with one expressing the ‘super-fast’ myosin *Myh13* and the other characterised by the maturation-related transcription factor *Maf*. In extraocular muscles, MHC13 is only found in the central part of the fibre (Briggs & Schachat, 2002). Similar segregation along the chain fibres could explain the presence of these two subpopulations.

(4) Regulation of myonuclear positioning

A regular distribution of nuclei is required for proper muscle function, and this is disturbed in disease and ageing (Cristea *et al.*, 2010; Iyer *et al.*, 2017; Roman & Gomes, 2017; Azevedo & Baylies, 2020; Hansson & Eftestøl, 2023). In vertebrates, myonuclei form clusters underneath the NMJ (Englander & Rubin, 1987; Sanes *et al.*, 1991; Grady *et al.*, 2005) and an increased myonuclear density was observed at the fibre tips (Rosser *et al.*, 2002; Bruusgaard *et al.*, 2003; Kim *et al.*, 2020; Liu *et al.*, 2020). In *Drosophila*, quantitative analysis showed that the post-synaptic myonuclei near the NMJ, and the myonuclei residing near MTJm have different spacing requirements compared to other myonuclei (Perillo & Folker, 2018).

Among other regulators, myonuclear positioning involves specific proteins of the linker of nucleoskeleton and cytoskeleton (LINC) complex that anchor the nuclear lamina to the

cytoskeleton (Crisp *et al.*, 2006). The LINC complex is composed of SUN proteins and KASH proteins (called Nesprins or Syne proteins in vertebrates), with the latter binding actin, microtubule motors and intermediate filaments (reviewed by Chang, Worman & Gundersen, 2015). Notably, overexpression of the C-terminal fragment (containing the KASH domain) of either *Syne-1* or *Syne-2* partially damages the anchorage of synaptic nuclei (Grady *et al.*, 2005; Zhang *et al.*, 2007). Instead, deletion of the KASH domain of *Syne-1*, but not of *Syne-2*, completely abolishes the formation of NMJm clusters and disrupts the organisation of non-synaptic nuclei in skeletal muscle (Zhang *et al.*, 2007). *Sun1* and *Sun2* function redundantly in the anchorage of myonuclei, including synaptic and non-synaptic nuclei (Lei *et al.*, 2009). A muscle-specific isoform of the cardiac islet-1 interaction protein (CIP), which mediates the connection between the LINC complex and the microtubule cytoskeleton, also plays an important role for proper myonuclear alignment and positioning within myofibres, including those of the NMJm and MTJm (Liu *et al.*, 2020). Importantly, loss of *Cip* and *Sun* genes leads to profound myonuclear positioning defects and severe muscular dystrophy (Meinke *et al.*, 2014; Liu *et al.*, 2020).

Altogether, while several proteins have been identified as regulators of NMJm and MTJm positioning and clustering, only few are selectively expressed in those locations. Thus, deeper analysis of seq data sets might provide insights into novel proteins or molecular mechanisms essential for myonuclear positioning at junctional sites. Moreover, seq at the initiation of muscle contraction and the use of mechanosensors could help to understand how stretching is translated into gene expression changes at the junctional sites.

(5) Are all myonuclei specialised? Insights from myofibre body nuclei

Over two decades ago it was suggested that nuclei producing muscle-specific transcripts clustered together and formed functional domains (Newlands *et al.*, 1998). However, it was

unclear if this reflected actual divergent myonuclear states or if loci alternate between states of transcriptional activity and inactivity. seq studies started to expose the extent of transcriptional diversity of myonuclei within the myofibre body, which do not seem to have a preferred localisation within myofibres. These novel subpopulations have been generally named after their main differentially expressed transcript (Fig. 1, Table 1).

The *Enah*⁺ myonuclear subpopulation, is characterised by an increased expression of genes encoding scaffolding proteins and it is predominantly observed at P21, after the main nuclear accretion phase (Petrany *et al.*, 2020). This subpopulation, also called sarcomere assembly myonuclei, does not express adult myosin isoforms, but instead expresses genes involved in myofibrillogenesis (such as *FilaminC*) and *Myh9* and *Myh10* (encoding for NMHC-IIA and B respectively), which serve as scaffolds for mature myosin assembly (Sanger *et al.*, 2006; Vicente-Manzanares *et al.*, 2009; Wang *et al.*, 2018). A similar subpopulation of *Titin*⁺ myonuclei co-expressing *Runx1*, *Dysferlin*, *FilaminC*, and *Myh9* was identified in adult limb muscles, yet it did not form a separate subcluster (Petrany *et al.*, 2020; Santos *et al.*, 2020).

As *FilaminC* is involved in early stages of myofibrillar remodelling and repair, this population could correspond to myonuclei in areas where myofibre repair occurs upon microlesions during homeostasis (Leber *et al.*, 2016; Barthélémy *et al.*, 2018; Roman *et al.*, 2021; Roman & Muñoz-Cánoves, 2022). Notably, seq identified a higher fraction of nuclei expressing the repair signature in ageing mice (Petrany *et al.*, 2020), suggesting that sarcomeres are damaged under basal conditions. In parallel, this study also identified a specific myonuclear cluster characterised by expression of *Ampd3*⁺, *Atrogin*, neuromuscular markers and genes associated with apoptosis and proteasomal degradation (Petrany *et al.*, 2020), which probably represent the dysfunctional denervated state (Larsson *et al.*, 2019).

The *Rian*⁺ or *Meg3*⁺ population is characterised by the expression of maternally imprinted long non-coding RNAs (lncRNAs) from the *Dkl1–Dio3* genomic region or ‘Callipyge’ (Kim

et al., 2020; Wen *et al.*, 2021). As *Dlk1*, the main coding gene of this cluster, is required for muscle development, regeneration, and hypertrophy (Waddell *et al.*, 2010; Andersen *et al.*, 2013), these myonuclei could exert a key function during fibre type transitions. Several other myofibre body nuclei subpopulations have been described (e.g. a *Gssos2+*, *Bcl2+* and *Suz12+* subpopulation), but their functional roles are unclear (Kim *et al.*, 2020) (Fig. 1, Table 1).

Notably, considering the observed disparity between transcriptomes and proteomes (Liu, Beyer & Aebersold, 2016; Moritz *et al.*, 2019), future studies should address the relevance of the above-mentioned subpopulations at the protein level. Also, functional studies will be required to assess their physiological role and their temporal emergence during development or postnatal life. In addition, as the above-mentioned seq studies focused mainly on limb muscles, it is possible that myofibre body nuclei subpopulations could be even more diverse in other anatomical locations. For instance, it is unclear whether the existence of different upstream TFs governing the entry and progression in the myogenic program at different locations (reviewed in Grimaldi & Tajbakhsh, 2021) could impinge on the number and relevance of myonuclear subpopulations on individual fibres. Whether specific myonuclear subpopulations exist in muscles with continuous myonuclear turnover (McLoon *et al.*, 2004; Keefe *et al.*, 2015; Pawlikowski *et al.*, 2015; Randolph *et al.*, 2015) is also unknown.

However, unravelling the signature of newly fused and pre-existing nuclei in muscles with continuous turnover or during postnatal growth has faced challenges due to the lack of spatial information of seq approaches and faithful genetic reporters showing limited diffusion of the reporter protein into other nuclei within the shared cytoplasm (Masschelein *et al.*, 2020).

Substantial progress has been achieved by the development of a recombination-independent lineage-tracing approach, where the transcription of the nuclear reporter in MuSCs is temporally regulated but retains a long-lived H2B-GFP reporter protein that is kept as the

myogenic progenitor fuses into the syncytium (Sun *et al.*, 2023). The use of this reporter in combination with nuclear bulk and seq allowed the elucidation of the signature of newly fused myonuclei in growing muscles, which was characterised by elevated expression of *H19* and *Igf2*, *Myomaker* and proteins associated with plasma membrane remodelling (Sun *et al.*, 2023). This subpopulation, called DevMyonuclei, was clearly distinct from the pre-existing nuclei carrying a more mature muscle signature and was largely absent when myoblast fusion was impaired.

(6) Changes in myonuclear subpopulations upon stress

Skeletal muscle exhibits a great capacity to adapt to different stresses through changes in protein synthesis and degradation, metabolic reprogramming, and tissue remodelling. Denervation induces pronounced muscle atrophy and is relevant to clinical conditions including neurodegenerative diseases and age-related muscle atrophy (Burns *et al.*, 2012). Using a sciatic nerve transection or compression as permanent or reversible models of denervation in limb muscles, sn-RNAseq and spatial transcriptomics identified robust transcriptional reprogramming with the upregulation of atrophy-related genes including *Atrogin*, *Runx1*, and *Gadd45a* in type IIb myonuclei (D'Ercole *et al.*, 2022; Lin *et al.*, 2022). Interestingly, in the reversible denervation model, expression of *Atrogin* returned to control levels by 30 days when innervation is restored, but expression of metabolic genes did not fully recover by this time (D'Ercole *et al.*, 2022). Surprisingly, these studies did not report significant changes in the NMJm both in terms of proportion and general NMJm profile, including expression of *Ache* and *Colq* (D'Ercole *et al.*, 2022; Lin *et al.*, 2022). By contrast, global RNAseq of synaptic and non-synaptic areas, where non-muscle cells may be sequenced along with myonuclei, showed that, already at 3 days post-denervation, the global transcriptional diversity between synaptic and non-synaptic areas was greatly diminished (Bai

et al., 2022). This is in line with the observation that denervation and ageing lead to increased levels of NMJ-specific mRNAs in non-synaptic regions (Goldman & Staple, 1989; Li, Xiong & Mei, 2016; Jing *et al.*, 2021). As contradictory results were observed regarding NMJm number changes following different stresses (Bai *et al.*, 2022; Ruiz *et al.*, 2023), these results should be interpreted with caution as various morphological or genetic markers need to be used to identify NMJm.

Following synergist ablation surgery and muscle overload (MOV) in mouse plantaris muscle, bulk myonuclear RNAseq revealed an induction of genes related to ECM remodelling, together with *c-Myc*, *Ankrd1* and *Runx1* (Murach *et al.*, 2022; Sun *et al.*, 2023). To determine whether this MOV signature was arising from newly fused and pre-existing myonuclei, bulk myonuclear and sn-RNAseq were combined with lineage tracing and sm-FISH (Sun *et al.*, 2023). This study highlighted two novel myonuclear subpopulations in MOV, corresponding to recently fused nuclei expressing *H19*, *Igf2*, *Runx1*, developmental myosins and *Tnnt1/2*, as well as the enrichment of the sarcomere assembly state (i.e. *Atf3*, *Enah*, *Ankrd1* and *FilaminC*) in pre-existing nuclei (Sun *et al.*, 2023). Given that newly fused nuclei seem to be more prominent at the myofibre centre and *Ankrd1* is a marker of MTJm (Baumeister, Arber & Caroni, 1997; Kim *et al.*, 2020; Petrany *et al.*, 2020; Santos *et al.*, 2020) that increases markedly under conditions of mechanical stress (Barash *et al.*, 2004; Pijl *et al.*, 2021), its upregulation might reflect changes in pre-existing nuclei at the MTJ or a more global myonuclear response to overload. sm-FISH on isolated fibres upon MOV could help distinguish between these possibilities.

Finally, sn-RNAseq on MuSC-depleted muscles subjected to training identified a subpopulation of ‘cryptic myonuclei’ with an elusive transcription profile, co-expressing genes characteristic of fibroadipogenic progenitors, together with type I and type II myonuclei (Wen *et al.*, 2021). Cryptic myonuclei were suggested to represent a subset of resident

myonuclei that lose their canonical transcriptional identity as a compensatory mechanism in the absence of MuSCs (Wen *et al.*, 2021).

(7) Changes in myonuclear subpopulations in disease conditions

Pathological conditions can strongly affect myonuclear specialisation. Duchenne muscular dystrophy (DMD) is a fatal muscle disorder caused by germline mutations in the *ystrophin* gene, which is part of a multimeric protein complex that connects the myofibre cytoskeleton to the extracellular matrix (Wallace & McNally, 2009). DMD is characterised by cycles of degeneration and regeneration of multinucleated myofibres and pathological activation of a variety of other muscle-associated cell types (Kharraz *et al.*, 2014; Duan *et al.*, 2021). sn-RNAseq in two different DMD mouse models identified novel myonuclear subpopulations not present in uninjured muscles (Chemello *et al.*, 2020; Kim *et al.*, 2020). A novel model lacking dystrophin exon 51 (Chemello *et al.*, 2020), uncovered the presence of a unique subpopulation of myonuclei in dystrophic fibres that the authors called RegMyon, characterised by expression of genes involved in myogenic differentiation and muscle regeneration. In the widely used *mdx* mice (McGreevy *et al.*, 2015), other subpopulations were scored including dying fibres, which express various non-coding transcripts, and a subpopulation associated with fibre repair (Kim *et al.*, 2020), that expresses *FilaminC*, *Xirp1*, *Nrap* and thus partially overlaps with the sarcomere assembly state described in late postnatal development and ageing muscle (Petraný *et al.*, 2020). The extent of overlap between RegMyon and the fibre repair subpopulation, or whether they constitute distinctive entities remains unclear. Functional studies of these specific myonuclear subpopulations observed in DMD models could provide insights into the molecular pathophysiology of muscle disease and their potential use as biomarkers of disease progression.

Further, in the Δ Ex51 model of DMD, components of the protein ubiquitination pathway were enriched in almost all the myonuclear types including NMJm and MTJm (Chemello *et al.*, 2020). In the *mdx* model instead, coexpression of several NMJm and MTJm markers were lost, suggesting that junctional nuclei are not properly maintained (Kim *et al.*, 2020).

Altogether, while the analyses of the two DMD models do not fully overlap regarding specific subpopulations and their respective signatures, they likely reflect the various changes at the NMJ (reviewed by Ng & Ljubicic, 2020) and MTJ (Law & Tidball, 1993; Martinez *et al.*, 2021) observed in DMD patients and *mdx* mice. Finally, an exon-skipping approach on *mdx* mice revealed a partial rescue of MuSC and myonuclear programs, however, the phenotypes of the NMJm and MTJm were not fully characterised in this model (Scripture-Adams *et al.*, 2022).

Defects in NMJm have been also detected in other myopathies and during ageing. In autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD), mutations in LMNA, which encodes Lamin A and C nuclear lamina proteins, lead to mislocalisation of NMJm nuclear proteins, reduction in NMJm number and an increase in histone acetylation, which is a hallmark of denervation (Méjat *et al.*, 2009).

Collectively, these studies revealed a multifaceted response at the single-nucleus level in disease and ageing conditions and hold promise for the development of novel therapeutic interventions.

V. COORDINATION OF THE MYOSIN HEAVY CHAIN PROFILE AT THE SINGLE-FIBRE LEVEL

Until recently, the classification of myofibres into distinct profiles (slow oxidative, fast oxidative, fast glycolytic) was only possible at the whole-fibre scale and relied on a combination of metabolic and contractile markers (Schiaffino *et al.*, 2015). MyHCs are the

main determinants of the contraction velocity and have been used extensively as a proxy to profile the global fibre properties. Adult MyHCs proteins can be subdivided from slower to faster isoforms according to the following continuum: MyHC I (*Myh7* gene) \Leftrightarrow IIA (*Myh2* gene) \Leftrightarrow IIX (*Myh1* gene) \Leftrightarrow IIB (*Myh4* gene). Two specific developmental fast myosins, *Myh3* (embryonic) and *Myh8* (perinatal) are not normally expressed in adult muscle, except during regeneration upon injury or in disease (Sartore, Gorza & Schiaffino, 1982; Schiaffino *et al.*, 1986; Schiaffino & Reggiani, 2011). Recently, a combination of sn-ATACseq, Chip-seq and 4C-seq experiments in adult fast and slow skeletal muscles identified a 42-kb open chromatin region interacting with fast myosin (*Myh2*, *Myh1* and *Myh4*) promoters by DNA looping, and responsible for the switch between different fast isoforms within limb muscles (Santos *et al.*, 2022) (Fig. 4A).

sn-RNAseq studies allowed establishing a correspondence between *Myh* gene expression and specific metabolic genes at the nuclear scale (Kim *et al.*, 2020; Petrany *et al.*, 2020; Santos *et al.*, 2020; D'Ercole *et al.*, 2022). While the vast majority of adult limb myonuclei are 'pure' and express only one *Myh* gene in a coordinated manner (Santos *et al.*, 2020), multiple MyHC proteins isoforms are present within so-called 'hybrid fibres' (Medler, 2019) (Fig. 4B, C). Hybrid fibres can be observed either transiently, as intermediates of fibre-type transitions associated with skeletal muscle development (Rosser *et al.*, 2000; Rosser & Bandman, 2003), ageing (Andersen, Terzis & Kryger, 1999; Andersen, 2003), and regeneration (Sartore *et al.*, 1982) or be present constitutively in certain muscle groups (Peuker & Pette, 1997; Zhou, Liu & Kaminski, 2010; Zhang & Gould, 2017; Medler, 2019). In the mouse, constitutive hybrid fibres represent around 25% of the fibres in normal adult muscles (Medler, 2019) and provide a functional continuum of fibre phenotypes between 'pure' MyHC profiles (Reiser *et al.*, 1985, 1988). At the nuclear scale, hybrid fibres could result from two mechanisms: (1) each nucleus expressing a single *Myh* isoform, with all nuclei within the length of a fibre not

expressing the same isoform; or (2) some nuclei expressing multiple *Myh* isoforms at the same time. A recent study using intronic smRNA-FISH probes for *Myh* mRNA suggested that both mechanisms can occur physiologically in limb muscles (Santos *et al.*, 2020).

Interestingly, some cranial muscles also present hybrid fibres and express embryonic myosins as well as unique isoforms such as MyHC6, 13, 14, 15, and 16 that are not present in trunk muscles (Rossi *et al.*, 2010; Zhou *et al.*, 2010; Schiaffino & Reggiani, 2011; Medler, 2019; Hoh, 2020). Whether non-canonical myosins in cranial muscles also display coordinated expression among myonuclei remains an open question.

Another important issue is how *Myh* coordination is achieved within the syncytium. In regard to temporal *Myh* coordination, a sequential transition from embryonic to neonatal and to adult *Myh* isoforms occurs in skeletal muscles during mouse postnatal development (Rosser *et al.*, 2000; Agbulut *et al.*, 2003). sm-FISH with intronic probes showed that the majority of myonuclei start to express a single adult fast *Myh* gene from fetal development in agreement with the future adult pattern (Santos *et al.*, 2020). A combined single-nucleus multiome atlas of mouse limb skeletal muscle revealed a TF switch that would enable coordinated *Myh* transitions during pre/postnatal development (Santos *et al.*, 2023). This involves *Myogenin*, *Tead4*, and *Klf5* that cooperate synergistically to activate the expression of muscle genes in developing myofibres and *Maf*, a basic domain/leucine zipper transcription factor that activates fast mature muscle gene expression (Sadaki *et al.*, 2023; Santos *et al.*, 2023). The TF switch was proposed to be triggered by electrical activity and calcium signalling during muscle fibre maturation (Santos *et al.*, 2023).

The other aspect related to coordination is the establishment and maintenance of a specific *Myh* pattern among nuclei of a single fibre. Given that fibre type is dependent, at least in part, on the intrinsic properties of the progenitors that contributed to their generation (Pin *et al.*, 2002; Kalhovde *et al.*, 2005; Biressi & Rando, 2010; Gundersen, 2011; Keenan & Currie,

2019), different mechanisms could be responsible for the establishment and maintenance of *Myh* coordination in single fibres. For instance, ‘preprogrammed’ myogenic stem cells could fuse homotypically and activate a single *Myh* gene along the myofibre, and under the influence of environmental signals. By using live imaging in the zebrafish (*Danio rerio*) somite, a recent study proposed another model in which slow muscles guide fast myocytes by funnelling them close together, thus enhancing fusion probability among them (Mendieta-Serrano *et al.*, 2022). Alternatively, as discussed in Section III.1, founder myonuclei could reprogram newly fused myonuclei and induce coordination of expression between nuclei of the same fibre as seen in *Drosophila* (Bataillé *et al.*, 2017). This scenario is supported by recent work showing that newly fused and pre-existing myonuclei reciprocally influence each other to establish the myofibre syncytium and respond to adaptations in adult muscle (Sun *et al.*, 2023). Future work must be directed towards unravelling the mechanisms of *Myh* coordination at the tissue level.

sn-RNAseq studies showed that MTJm and NMJm express different *Myh* isoforms, as do myofibre body nuclei do (Chemello *et al.*, 2020; Kim *et al.*, 2020; Petrany *et al.*, 2020; Santos *et al.*, 2020). Upon denervation, loss of coordination between myofibre body nuclei and MTJm is also observed (Dos Santos, 2020). *Myh3* mRNA is strongly enriched at the level of the NMJ and MTJ during prenatal and early postnatal development (Santos *et al.*, 2020). Thus, it is possible that *Myh3* is transiently activated in these regions during growth or reflects newly accreted myonuclei in these regions. As genetic studies have shown that *Myh3* mediates non-cell-autonomous effects on myoblasts (Agarwal *et al.*, 2020), *Myh3* expression at the fibre ends could be involved in driving fusion of LPM-derived fibroblasts at the level of the MTJ. Alternatively, *Myh3* may exert mechanotransduction functions at this level through YAP signalling (Bharadwaj *et al.*, 2023). The relevance of these different scenarios requires further investigation *in vivo*.

Several factors are known to influence *Myh* expression at the whole-fibre level (Izumo, Nadal-Ginard & Mahdavi, 1986; Rosser *et al.*, 2000; Gundersen, 2011; Schiaffino & Reggiani, 2011; Rowan *et al.*, 2012; Blaauw, Schiaffino & Reggiani, 2013). Among them, differences in frequency and temporal firing pattern of neurons are the main determinants of fibre-type-specific gene-expression programs (Schiaffino, Sandri & Murgia, 2007). Upon denervation of fast muscles, an increase in the number of de-coordinated hybrid fibres is observed together with a shift of *Myh* expression towards the slower isoform (Santos *et al.*, 2020). Coordination of fast *Myh* seems to be dependent on SIX homeoproteins, which are known regulators of fast *Myh* genes (Grifone *et al.*, 2004; Sakakibara *et al.*, 2014, 2016; Maire *et al.*, 2020). However, less studied are the factors that explain divergent *Myh* profiles of a subset of myonuclei within the same fibre. Interestingly, intronic smRNA-FISH showed that hybrid myonuclei expressed adjacent isoforms in the glycolytic-to-oxidative spectrum (Santos *et al.*, 2020). This is in line with the demonstration that a combination of NFAT isoforms led to the transition between adjacent *Myh* isoforms (Calabria *et al.*, 2009). Thus, a plausible explanation for the existence of hybrid myonuclei could rely in part on variable protein import rates among myonuclei (Abbott *et al.*, 1998; Cutler *et al.*, 2017), which notably affect NFAT transcription factors (Medler, 2019). Whether Ca^{2+} concentrations and intracellular signalling vary locally along the fibre length are other important issues to understand *Myh* patterns in hybrid fibres.

VI. MYONUCLEAR ALIGNMENT AND FUSION SITE AS SOURCES OF HETEROGENEITY

As myonuclei in regenerating myofibres are not transcriptionally equivalent (Newlands *et al.*, 1998; Kim *et al.*, 2020; McKellar *et al.*, 2021, 2022), one possibility that remains to be tested is whether transcriptional heterogeneity reflects actual positioning within the fibres.

During the early phase of myofibre regeneration, the ‘ghost fibre’ (remnant of damaged fibres), serves as a scaffold for repositioning myonuclei into long centralised linear arrays (nuclear chains), in clusters, as well as in a peripheral position (Vracko & Benditt, 1972; Wada, Katsuta & Soya, 2008; Webster *et al.*, 2016; Pizza & Buckley, 2023). Myonuclei are initially distributed randomly under the myofibre basal lamina and chains form secondarily to the rapid onset of myoblast-to-myoblast fusions (Collins *et al.*, 2023). Peripheral nuclei arise instead from myoblasts fusing to regenerating myofibres at later time points (Collins *et al.*, 2023). EdU-uptake experiments are in agreement with the differential timing of incorporation of central *versus* peripheral nuclei (Cutler *et al.*, 2022; Collins *et al.*, 2023). By detection of nascent mRNA, central nuclear chains were shown to display higher collective transcriptional activity than peripheral or clustered myonuclei during regeneration (Buckley, Nestor-Kalinoski & Pizza, 2022). Central positioning might be the consequence of nuclei becoming constrained because their path towards the periphery is blocked by the high rate of myofibrilogenesis (Collins *et al.*, 2023; Pizza & Buckley, 2023). However, given that centralised chains of myonuclei persist in regenerated myofibres for months and indelibly mark regenerated myofibres (Collins *et al.*, 2023; Pizza & Buckley, 2023), more work is required to underscore the long-term transcriptional consequences of this central positioning and whether this reflects ongoing aspects of myofibre maturation. Central myonuclear alignment also has been observed following training induced hypertrophy. Using a doxycycline-dependent HSA-GFP reporter mouse and EdU uptake, it was shown that most central nuclei were derived from a recent myoblast fusion event, but about 10% of them were resident nuclei that translocated to the fibre centre (Murach *et al.*, 2020). Whether these two populations of centred myonuclei are transcriptionally distinct is unclear.

Could the fusion site also be a source of heterogeneity of myofibre body nuclei? The existence of Sy-MuSCs and fibroblasts giving rise to MTJm established a solid link between

fusion site and transcriptional heterogeneity. Concerning myofibre body nuclei, evidence is less compelling. Myonuclear addition during development was reported to occur mainly at the myofibre ends (Zhang & McLennan, 1995) and to result in higher myonuclear density (Rosser *et al.*, 2000, 2002). During postnatal growth and in stretched muscles, sarcomeres appear to be serially added at the myofibre tips (Kruse *et al.*, 2021). However, regions of higher myonuclear density or containing higher numbers of recently fused nuclei were not observed (White *et al.*, 2010; Sun *et al.*, 2023), implying that myonuclei were probably not added preferentially to a particular region of the myofibre. Given these non-overlapping results, sm-FISH in isolated myofibres during growth or spatial transcriptomics might help determine whether the sarcomere assembly myonuclear state that has been observed by sn-RNAseq studies (Petrany *et al.*, 2020; Santos *et al.*, 2020) displays a particular localisation in the fibre.

VII. TRANSPORT AND COMMUNICATION WITHIN THE SYNCYTIIUM

(1) Role of the cytoskeleton and nuclear import pathways

An important question relates to how myonuclei communicate within a large cytoplasmic space while maintaining specialisation and coordination. From the perspective of sn-RNAseq experiments showing the existence of different myonuclear subpopulations, compartmentalisation within the syncytium implies that the distribution of genetic products is limited. Pioneer studies showed that myonuclear mRNAs remain mainly proximal to their nucleus of origin, yet proteins could be distributed broadly in the cytoplasm, cell membrane and among adjacent nuclei (Ralston & Hall, 1989*a,b*, 1992; Hall & Ralston, 1989). In myofibres, besides the transcriptional territories originally defined around myonuclei (Ralston & Hall, 1992; Ralston, McLaren & Horowitz, 1997) and enrichment at the MTJ and NMJ (Dix & Eisenberg, 1990; Sanes *et al.*, 1991), various other mRNA localisation patterns have been described, at the subsarcolemmal level (Mitsui *et al.*, 1997; Nissinen *et al.*, 2004;

Nevalainen, Kaakinen & Metsikkö, 2013), in the myofibre core (Dix & Eisenberg, 1990; Shoemaker, Ryan & Lieber, 1999) and associated with the cytoskeleton (Pomeroy *et al.*, 1991; Cripe, Morris & Fulton, 1993; Mitsui *et al.*, 1994; Nissinen *et al.*, 2004). mRNA distribution in myotubes was proposed to rely on ribosome availability, on mRNA targeting to particular subcellular locations, and on limited diffusion of mRNA and proteins due to steric hindrance (Pavlati *et al.*, 1989; Ralston & Hall, 1992; Gramolini *et al.*, 1997; Gramolini, Bélanger & Jasmin, 2001; Ralston *et al.*, 1997; Papadopoulos, Jürgens & Gros, 2000). However, a major limitation of these studies was the small number of transcripts or proteins analysed at the same time and the lack of tools to track them faithfully within the syncytium. Recently, genetically encoded fluorescent reporters, single-molecule resolution imaging, multiplexing sm-FISH and image-analysis pipelines have addressed some of these questions (reviewed by Bagley *et al.*, 2023). In *Drosophila* embryos, live imaging of GFP-tagged mRNAs combined with fluorescence loss in photobleaching (FLIP) experiments, exposed myonuclear domains, and showed that movement of mRNAs within the domains is rapid, yet exchange between the domains is relatively slow (Gemert *et al.*, 2009). High-resolution sm-FISH in C2C12-derived myotubes and isolated myofibres showed that microtubules are the driving matrix for distributing mRNA away from the myonuclear source (Denes, Kelley & Wang, 2021; Pinheiro *et al.*, 2021). The factors influencing mRNA transport remain debated as transcript length (Pinheiro *et al.*, 2021) or expression levels (Denes *et al.*, 2021) were correlated with mRNA spreading. Different types of microtubules seem to be involved in this process, whereby nest-like microtubules around NMJs might restrict mobility of RNAs produced in postsynaptic nuclei and grid-like microtubules in the extra-synaptic regions may promote efficient dispersion of RNAs (Denes *et al.*, 2021). Interestingly, sm-FISH on differentiating C2C12 cultures showed that passive diffusion of mRNAs is restricted to proliferating myoblasts and that myotube maturation imposes an absolute reliance on

microtubules to disperse mRNAs (Denes *et al.*, 2021). While considerable differences in transport might exist between myofibres and immature C2C12 myotubes, a key role for microtubule-dependent mRNA distribution is highlighted by its requirement for efficient protein translation in myofibres and cardiomyocytes (Denes *et al.*, 2021; Scarborough *et al.*, 2021). Given that increased microtubule density was observed in *mdx* mice (Khairallah *et al.*, 2012), it will be important to assess whether differences in mRNA distribution along the myofibre can contribute to the physiopathology of DMD.

How nuclear proteins that are produced in a shared cytoplasm become sequestered to specific myonuclei is equally complex. Apart from microtubule-dependent protein transport (Pizon *et al.*, 2005; Wang *et al.*, 2013), the spatial and temporal modulation of nuclear import pathways was proposed as mechanism for transport of specific proteins between myonuclei and protein regionalisation within myofibres (Cutler *et al.*, 2017). Using a mosaic transfection model to create myotubes that contained a single myonucleus expressing a fluorescent reporter, it was possible to predict how far a nuclear protein would travel based on its size, nuclear import rates that function as molecular sinks and ability to diffuse across the nuclear pore (Taylor-Weiner *et al.*, 2020). Notably, myotube thickening *in vitro* mimicking muscle hypertrophic conditions extended the propagation of large nuclear proteins (Taylor-Weiner *et al.*, 2020). Yet, it is unclear whether these mechanisms play a role in the regulated transport of transcription factors between myonuclei for the establishment or maintenance of myonuclear specialisation *in vivo*.

(2) Myonuclear crosstalk in adaptations

The clearest functional examples of communication within the syncytium are related to local fibre repair and hypertrophy. Local microinjuries, such as those provoked by exercise rely on myofibre self-repair without the involvement of MuSCs (Roman & Muñoz-Cánoves, 2022).

Time-lapse imaging and optogenetic methods to generate local laser-damaged myofibres showed that a subset of pre-existing myonuclei migrate towards the injury site using microtubules and dynein. Migrating myonuclei alter their transcriptome to a gene-expression profile similar to the repair signature observed in sn-RNAseq studies (Petraný *et al.*, 2020; Santos *et al.*, 2020) to deliver mRNA locally for cell repair (Roman *et al.*, 2021). The selective response of a defined group of myonuclei was shown to be dependent on the proximity to the injured myofibre membrane and calcium signaling (Roman *et al.*, 2021). During postnatal myogenesis and upon muscle loading, myofibres undergo sustained hypertrophy due to a progressive increase in the number and size of myofibrils (Pizza & Buckley, 2023). However, how myofibres decide either to add a new myonucleus or to regulate nuclei that are already present in the myofibre is unclear. Thus, a long-standing question pertains to how pre-existing nuclei communicate among themselves and/or with nascently fused nuclei to maintain a functional myofibre unit under different adaptations. In developing muscle fibres, maximal growth rates were proportional to DNA content (i.e. nuclei number), allowing fibres with more nuclei to grow faster than fibres with fewer nuclei (Hansson *et al.*, 2020). Blocking the fusion capacity of myogenic cells during the first weeks of postnatal development showed an intrinsic need for additional myonuclei to achieve the normal radial myofibre size (Bachman *et al.*, 2018; Cramer *et al.*, 2020) but it revealed also the capacity of pre-existing myonuclei to compensate transcriptionally and support growth (Cramer *et al.*, 2020). Titration of myonuclear numbers during postnatal growth uncovered an inherent ‘reserve capacity’ that can be elicited in myonuclei during development that is determined by the number of nuclei in the myofibre: myofibres with fewer myonuclei exhibit higher flexibility regarding their ability to increase mRNA concentrations (Cramer *et al.*, 2020). Heterokaryon experiments showed that both DNA content and cellular volume are integrated to produce the appropriate amount of RNA for a given size, and most likely

through sequestration of a factor involved in transcription to DNA (Padovan-Merhar *et al.*, 2015). Thus, the inverse relationship between number of nuclei and mRNA output could be caused by competition among nuclei for essential factors or negative cross-regulation among myonuclei (Cramer *et al.*, 2020). During adult muscle hypertrophy, a fusion blockage results in a lack of growth (Egner *et al.*, 2013; Goh & Millay, 2017; Goh *et al.*, 2019; Englund *et al.*, 2020*a,b*), pointing to limited flexibility of the resident myonuclei that were added during development to increase mRNA concentration and sustain hypertrophic growth. Of note, using a nuclear lineage-tracing model that distinguishes newly fused from pre-existing myonuclei, and fusion-deficient mice, a recent study further showed that: (1) the transcriptional trajectory of newly fused nuclei is impacted by the syncytial environment; and (2) myonuclear accretion is needed for pre-existing myonuclei to respond appropriately respond to an overload stimulus (Sun *et al.*, 2023). While newly fused myonuclei contribute unique transcripts, they appear to be needed to coordinate transcription of pre-existing myonuclei (Englund *et al.*, 2020*a*; Sun *et al.*, 2023).

VIII. IMPLICATIONS OF NUCLEAR COMMUNICATION IN PATHOLOGY AND THERAPEUTIC CONDITIONS

The importance of understanding intracellular myofibre communication is highlighted in pathological and therapeutic conditions. In case of DMD (an X-linked recessive disorder), females are generally asymptomatic given that 50% of all myonuclei still express the native transcript due to random X-chromosome inactivation. Nevertheless, muscle weakness is observed in cases of skewed X-inactivation where more than half of the myonuclei express the mutant transcript (Viggiano *et al.*, 2016).

Injection of normal myoblasts into diseased muscle to form ‘heterokaryons’ was proposed as a therapeutic strategy assuming that fusion of a fraction of normal nuclei could rescue

membrane stability of the entire fibre (Chaudhari, Delay & Beam, 1989; Karpati *et al.*, 1989; Partridge *et al.*, 1989; Gussoni *et al.*, 1992). While this concept is still of relevance today (Negroni *et al.*, 2016; Biressi, Filareto & Rando, 2020; Olson, 2021), the success of this approach relies on: (1) replacing enough nuclei; and (2) redistribution of the protein encoded by the wildtype nuclei along the fibre. Of note, dystrophin protein does not appear to be restricted to nuclear domains in heterokaryons *in vitro* (Huard *et al.*, 1991; Denetclaw *et al.*, 1993) but the extent of diffusion of the protein upon transplant of wildtype cells into *Dystrophin* deficient mice is debated (Gussoni, Blau & Kunkel, 1997; Kinoshita *et al.*, 1998; Blaveri *et al.*, 1999; Chretien *et al.*, 2005). Moreover, while these studies assumed that only one transplanted myoblast had fused per fibre when assessing at the extent of *Dystrophin* recovery, multiple fusions could not be excluded. More conclusive evidence was provided by a novel fluorescently tagged *Dystrophin* (*Dmd*^{EGFP}) mouse model (Petkova *et al.*, 2016). In heterozygous mutant female mice, the reporter showed DYSTROPHIN distribution to be compartmentalised, being restricted to myonuclear-defined sarcolemmal territories extending ~80 µm, called ‘basal sarcolemmal *Dystrophin* units’, that lack lateral mobility (Morin *et al.*, 2023) (Fig. 5A). This contrasts with experiments in embryonic zebrafish, where overexpressed human DYSTROPHIN-EGFP protein distributed into three distinct pools (Bajanca *et al.*, 2015).

It has been estimated that restoring the *Dystrophin* reading frame in only 1 out of 30 nuclei would be sufficient to ensure presence of the protein on the complete sarcolemma and would thus be therapeutic (Iyombe-Engembe *et al.*, 2016). CRISPR/Cas9-mediated genome-editing experiments showed that a partial correction of mutant *Dmd* alleles was sufficient to allow homogeneous *Dystrophin* expression in most myofibres, comparable to levels in wildtype mice, with improved histopathological hallmarks of the disease (reviewed by Olson, 2021). By contrast, when CRISPR-mediated correction was induced intramuscularly in adult

Dmd^{EGFP} mice, gene editing restored mosaic *Dystrophin* domains (Morin *et al.*, 2023) (Fig. 5A). Therefore, while CRISPR-mediated repair in a subset of myonuclei holds therapeutic promise, it is unclear what is the minimal number of nuclei requiring repair. Rates of myonuclear turnover and changes in myonuclear numbers during growth (as treatment before onset of symptoms is preferable) should be also considered (Morgan & Muntoni, 2021). In this light, a deeper understanding of mRNA and protein pharmacodynamics is still needed for therapy.

Multinucleation can also lead to the propagation of deleterious molecules.

Facioscapulohumeral dystrophy (FSHD) is characterised by overexpression of full-length *DUX4* mRNA, which is normally silenced in most somatic tissues (Lemmers *et al.*, 2010; Snider *et al.*, 2010). Given that full length *DUX4* mRNA is detectable in only a few patient myoblasts (~1/1000; Snider *et al.*, 2010) and myotubes (Tassin *et al.*, 2013), this raised the question of how a rare protein could cause muscle defects. The role of internuclear communication in the disease appears evident as overexpressed *DUX4* is detected in a gradient in consecutive myonuclei of individual myotubes *in vitro* (Tassin *et al.*, 2013) and *DUX4* produced in one nucleus can diffuse/be transported to adjacent nuclei (Block *et al.*, 2013) (Fig. 5B). Notably, while transcript expression is restricted to only 1–2 nuclei per myotube, expression of downstream target genes is observed throughout the myotube, a phenomenon related to *DUX4* diffusion and signal amplification by downstream target transcription factors, such as *DUXA* (Jiang *et al.*, 2020). In addition, sn-RNAseq identified two populations of FSHD myotube nuclei with distinct *DUX4* and target gene expression levels (FSHD-Hi and FSHD-low) that could reflect potentially different pathological states of patient myotubes (Jiang *et al.*, 2020). Moreover, *DUX4* and target gene expression were found to be sometimes discordant, raising the possibility that the deleterious effects may persist even after *DUX4* transcripts are no longer detectable (Jiang *et al.*, 2020; Chau *et al.*,

2021). These aspects have been recently modelled *in silico* and permit investigation of the consequences of anti-DUX4 therapy (Cowley).

Myotonic dystrophy (MD) 1 is caused by expansion of CTG trinucleotide repeats in the 3' untranslated region of the *DMPK* gene (Mahadevan *et al.*, 1992). Expanded CUG toxic transcripts form RNA foci in the nucleus and perturb the function of RNA-binding proteins and several downstream events (reviewed by Braz *et al.*, 2018). Transplantation of wildtype myogenic progenitors into an MD1 mouse model upon injury resulted in hybrid myofibres containing pathogenic RNA foci inside donor-derived nuclei (Mondragon-Gonzalez *et al.*, 2019), suggesting that the deleterious RNA was exported within the syncytium and disrupted alternative splicing upon import into donor-derived myonuclei (Mondragon-Gonzalez *et al.*, 2019) (Fig. 5C). However, in the context of FSHD cell therapy, engraftment of mouse myogenic progenitors still promoted muscle regeneration in an environment undergoing bursts of DUX4 expression (Azzag *et al.*, 2022). These examples highlight the importance of a better understanding of internuclear communication for therapeutic benefits.

IX. LIMITATIONS OF CURRENT APPROACHES

While sn-RNAseq has contributed to major advances in the field, there are several important limitations.

One potential disadvantage is that only nuclear transcripts are assessed, with a concurrent enrichment of nuclear-resident transcripts such as lncRNAs and pre-mRNAs (Zeng *et al.*, 2016; Hurni *et al.*, 2022). This is of relevance because factors such as transcript length, intron size, splicing dynamics or the presence of factors inducing nuclear mRNA retention can greatly influence the ratio of nuclear *versus* cytoplasmic mRNA localisation (Carmo-Fonseca & Kirchhausen, 2014; Akef, Lee & Palazzo, 2015; Hurni *et al.*, 2022). Moreover, while sequencing depth (i.e. number of reads/nuclei) is often used as a metric for the quality of a

data set, the computational pipelines, and parameters of the analysis (i.e. threshold used for definition of gene detection, filtering and noise-removal strategies; Heumos *et al.*, 2023) should not be overlooked as they can vastly affect the final UMAP/tSNE plots and the resulting number of subpopulations.

Sampling method is another source of discrepancy for identifying subpopulations. Given the large size of myofibres, and that specialised nuclei comprise <1% of all myonuclei for rare subpopulations, the risk of sampling bias is significant. For instance, as muscle spindles are rare, with 1–2 spindles present per TA section of adult mice (Cheret *et al.*, 2013), this myonuclear subpopulation was not detected by most sn-RNAseq studies due to the overwhelming number of nuclei derived from the rest of the myofibres. This problem was overcome by using *Calb1-Cre* specifically to isolate muscle spindle nuclei thereby identifying different myonuclear subtypes (Kim *et al.*, 2020). Similarly, the transitional-MTJm subpopulation was only identified when biopsies at the MTJ level were used (Karlsen *et al.*, 2023).

Spatial information is also lost in sn-RNAseq experiments as cells or nuclei are removed from their native tissue environment where specific cell–cell communication networks are established. In addition, as nuclei are extracted without a fibre identifier, this restricts the inference of myonuclear subpopulations that contribute to a particular fibre. Thus, it is essential that results from sn-RNAseq studies are rigorously validated by sm-FISH or other *in situ* methods in isolated fibres and tissue sections to confirm the relevance of the subpopulations and their anatomical disposition (Kim *et al.*, 2020; Santos *et al.*, 2020). Some of these issues could be solved by performing sn-RNAseq on individual myofibres, as demonstrated with ATACseq (Sahinyan *et al.*, 2021) or using spatial transcriptomic technologies on tissue sections (Williams *et al.*, 2022a). Moreover, this technology can provide a direct correlation between tissue architecture and gene expression to expand our

understanding of how each tissue unit responds in a physiopathological context. The first spatial RNAseq data set of regenerating murine skeletal muscle using the Visium platform succeeded in identifying potential interactions between cell types that are spatially co-occurrent during the injury response (McKellar *et al.*, 2021). Similarly, morphofunctional domains in muscle and their response to tenotomy (Ruoss *et al.*, 2022) and reversible nerve damage (D'Ercole *et al.*, 2022) were reported. Further, combining spatial transcriptomics antibody, and metabolic fibre stainings, permitted unbiased identification of functional anatomical clusters in healthy and denervated muscle (D'Ercole *et al.*, 2022). Mapping of sn-RNAseq specific subclusters into spatial transcriptomic data successfully identified distinct myonuclear profiles of human MTJ (Karlsen *et al.*, 2023). Further, while spatial RNAseq methods were restricted to capturing only polyadenylated transcripts, the addition of an enzymatic *in situ* polyadenylation step of RNA has enabled the detection of the full spectrum of RNA present in skeletal muscle during regeneration (McKellar *et al.*, 2022). Yet, a major issue with a widely used spatial transcriptome technology (i.e. Visium), is the resolution size in xy [spot size 55 μm in diameter (Moses & Pachter, 2021) *versus* $\sim 10 \mu\text{m}$ myonuclear length (Watkins & Cuien, 1988)] and the low detection of nascent mRNAs (McKellar *et al.*, 2022). Thus, a one-to-one match association of transcript to myonuclei of origin cannot be performed in this case.

X. PERSPECTIVES

Modelling adult myogenesis *in vitro* has been a major limitation to understanding myonuclear coordination and specialisation (reviewed by Bagley *et al.*, 2023). Nevertheless, the optimisation of protocols to generate skeletal muscle *in vitro* from iPSCs allows recapitulation of many aspects of *bona fide* skeletal myogenesis (reviewed in Chal & Pourquié, 2017). Notably, these protocols result in the formation of long and highly organised myofibrils that

undergo the stage-specific transitions of MyHC isoforms (embryonic, fetal, perinatal/fast) (Chal *et al.*, 2015), a phenotype that is not yet achieved by differentiation of MuSCs or myogenic cell lines (such as C2C12) *in vitro*. While iPSC-derived skeletal myotubes can harbour a postsynaptic element and extremities reminiscent of MTJs and show an autonomous multi-scale self-organisation in the absence of external cues (Chal & Pourquié, 2017; Mao *et al.*, 2022), the degree of heterogeneity of the respective myonuclei in the absence of the *in vivo* positional cues remains unresolved. Interestingly, sc-RNAseq of human iPSCs subjected to myogenic differentiation protocols identified significant numbers of non-myogenic populations (Xi *et al.*, 2020). Here, the comparison of sn-RNAseq data sets from *in vivo* and *in vitro* heterogeneous cell cultures could serve as a resource to advance our knowledge of human myogenesis. Moreover, it could help identify cell–cell communication networks between myonuclei and non-myogenic cell types with the final aim to generate more mature myofibres *in vitro*. In this respect, great advances have been achieved using three-dimensional (3D)-myotube culture methods (Afshar *et al.*, 2020; Jalal, Dastidar & Tedesco, 2021; Jacques *et al.*, 2022) and 3D human neuromuscular co-cultures that recapitulate early NMJ synaptogenesis (Bakooshli *et al.*, 2019). The use of micromoulded or ultrasoft gelatin hydrogels designed to generate more mature myotubes from C2C12 cells (Denes *et al.*, 2019; Jensen *et al.*, 2020) might also help move the field forward. These models might be useful for revisiting issues related to molecular diffusion and transport in C2C12 myotubes, where key diffusion barriers (e.g. sarcomere, endoplasmic reticulum structure) are not properly formed. Until recently, cell atlases primarily relied on fresh tissue samples from a single organ or tissue. Major advantages of sn-RNAseq include its application to fresh and snap-frozen archived tissues and the ability to capture cell types that do not survive long dissociation procedures (Mendelev *et al.*, 2022). In addition, the first sn-RNAseq human atlas of eight tissue types including skeletal, cardiac, and smooth muscle became available recently

(Eraslan *et al.*, 2022). This study provided a first basis for understanding cross-tissue and cross-individual variation of cellular phenotypes in relation to disease-associated genetic variation. Yet, a major limitation of these studies, and a major challenge for a future exhaustive human cell atlas, resides in the representation of all muscle groups to evaluate the relevance of previously identified myonuclear subpopulations in individual muscle groups, and the location of the muscle biopsy which can influence the number of subpopulations scored. This limitation is further reflected by the very small overlap in the signature of human (Karlsen *et al.*, 2023) and all the rodent MTJ clusters (Santos *et al.*, 2020; Kim *et al.*, 2020; Petransy *et al.*, 2020; Wen *et al.*, 2021) which investigated different limb muscle groups. Moreover, recent insights on hybrid fibres, the existence of specific myonuclear programs at the level of the NMJ, MTJ and among myonuclei within the myofibre body, underscore the need to reconsider the fibre as a whole when analysing patient muscle biopsies (Cotta *et al.*, 2021). As such, the discovery of intra-fibre heterogeneity is of relevance in pathologies and may impact current diagnostic methods for muscle diseases.

In the future, integration of multimodal and multi-omic data, such as the combination of barcode-based lineage-tracing methods, live imaging, sn-RNAseq and spatial transcriptomics could allow the creation of high-dimensional fate maps, where nuclear identity, state, lineage, and behaviour can be probed at any stage (VanHorn & Morris, 2020). Moreover, single-cell genome-wide chromosome conformation capture (Hi-C) (Chi *et al.*, 2022), which allows the definition of units of chromatin domains [known as topological-associated domains (TADs)] at the single-nucleus level, could bring new insights of modules of transcriptional regulation in subpopulations of specialised and global myofibre nuclei.

Single-cell genomics is also a powerful tool to build comprehensive catalogues of cell types and their associated gene regulatory programs in model and non-model organisms (reviewed in Sachkova & Burkhardt, 2019; Tanay & Seb -Pedr s, 2021). Myonuclear heterogeneity

could constitute one of the molecular bases of anatomical and evolutionary differences that are observed between muscle groups. How specific subpopulations emerged, and how they could impinge on whole-fibre properties remains understudied. The existence of specific myonuclear subpopulations could contribute to unique regenerative mechanisms relying on myofibre differentiation in certain species (Baghdadi & Tajbakhsh, 2018) or to specific sparing of certain muscle groups in disease (Mercuri, Bönnemann & Muntoni, 2019).

XI. CONCLUSIONS

(1) Until recently, most studies focused on differences between fibres, whereas little attention was given to intra-fibre heterogeneity. Single-nucleus RNAseq, single-molecule FISH, and spatial transcriptomic approaches opened new avenues for defining myonuclear heterogeneity.

(2) The identification of regionalised myonuclear programs underscores the need to reconsider myofibre subdomains operating in parallel during development, homostasis, disease, and ageing.

(3) In this review, we focused on the question of myonuclear heterogeneity, and how it is established and maintained. We propose a model in which several mechanisms would be responsible for myonuclear specialisation (Fig. 6). These include location within the syncytia and associated proximal signalling from adjacent cell types, dynamics of mRNA and protein transport within the syncytium, contribution of myonuclei by specific fusogenic subpopulations and myonuclear positioning.

XII. ACKNOWLEDGEMENTS

Work in S.T.'s laboratory is supported by funding from Institut Pasteur, Laboratoire d'Excellence Revive, Agence Nationale de la Recherche (ANR-10-LABX-73 to S.T. and ANR-21-CE13-0005 MUSE to G.E.C.), and ERC Advanced Grant 101055234 (to S.T.), Association Française contre les Myopathies (Grant #20510 to S.T. and #23201 to G.E.C.), and the Centre National de la Recherche Scientifique (CNRS).

XIII. AUTHOR CONTRIBUTIONS

A.K. and G.E.C. searched the literature and drafted the manuscript. All authors read, commented on, and approved the final version. Drawings for Figs 1 and 5 are by Hannah Franjou and other figures were assembled using BioRender. We thank the anonymous reviewers for carefully reading our manuscript and their insightful comments and suggestions.

REFERENCES

Fig. 1. Myonuclear specialisation within the myofibre syncytium. Neuromuscular junction myonuclei (NMJm), myotendinous junction myonuclei (MTJm) and myonuclei within the myofibre body display specialised gene expression programs. These specialised programs partly rely on the existence of fusogenic populations, i.e. lateral plate mesoderm-derived fibroblasts at the MTJ, and synaptic muscle stem cells (Sy-MuSCs) at the NMJ. Changes in myonuclear subpopulations in myofibre body myonuclei upon stress and disease are highlighted. Following massive injury or microlesions of the myofibre membrane, different mechanisms are deployed to repair the tissue. DMD, Duchenne muscular dystrophy; ECM, extracellular matrix; FAP, fibro–adipogenic progenitor; mRNA; messenger RNA; MyHC, myosin heavy chain; P, post-natal day.

Fig. 2. Novel technologies to study myonuclear heterogeneity. (A) Scheme of a single-nucleus RNA-sequencing (snRNA-seq) pipeline using a droplet-based system. (B) Visualisation of myofibre compartmentalisation by single-molecule fluorescent *in situ* hybridisation (FISH). At post-natal day (P) 5 *Myh8* (red) shows ubiquitous distribution along myofibres while *Myh3* (green) is enriched at the myotendinous junction (MTJ) and neuromuscular junction (NMJ). In adult single myofibres, *Ache* (acetylcholinesterase) and *Tigd4* respectively label NMJ myonuclei and MTJ myonuclei. Images from Kann & Krauss (2019) (adult NMJ), Kim *et al.* (2020) (adult MTJ) and Dos Santos *et al.* (2020) (P5).

Fig. 3. Muscle stem cell (MuSC)-independent sources of myonuclei. (A) Lateral plate mesoderm (LPM)-derived fibroblasts contribute to myotendinous junction (MTJ) myonuclei, while *Twist2*⁺ and *Hoxa11*⁺ interstitial cells contribute to myonuclei of the myofibre body. (B) Scheme of quail-into-chicken limb LPM grafts. (B') A high density of quail nuclei of LPM origin (red) is observed inside Myosins⁺ fibres (green) at the muscle tips. (C) *Hoxa11*^{CreERT2}:*R26*^{tdTom} lineage tracing in adult limb muscles initially labels *Hox11a*⁺ interstitial cells (4 days post-Tamoxifen induction) which give rise to an increasing number of tdTom⁺ myofibres over time, upon fusion. ECU, extensor carpi ulnaris. (D) *Hoxa11*^{CreERT2}:*R26*^{tdTom} lineage tracing in adult limb muscles shows contribution of *Twist2*⁺ cells to type IIb/x myofibres upon fusion. G/P, gastrocnemius/plantaris. Images adapted from Lima *et al.* (2021) (B'), Flynn *et al.* (2023) (C) and Liu *et al.* (2017) (D).

Fig. 4. Coordination of *Myh* gene expression in myofibres. (A) Scheme of mouse fast *Myh* locus (fMyh), where a fMyh super enhancer (Enh) controls the fast *Myh* genes (*Myh2*, *Myh1*, *Myh4*), *Myh8* and probably also *Myh13*. (B) Single-molecule fluorescent *in situ* hybridisation (FISH) on an isolated fibre from the extensor digitorum longus shows coordination of *Myh* messenger RNA (mRNA) along the fibre length. *Myh4* (red) is the only fast *Myh* produced in a mono-/biallelic fashion. (C) Scheme of pure or hybrid fibres showing coordinated or

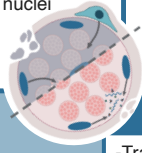
uncoordinated *Myh* expression. Top and middle, generic limb muscle fibres. Bottom, extraocular muscles. In extraocular muscles, *Myh13* is only present in the central part of the fiber, around the neuronal endplate, while *Myh3* is expressed in the periphery. Note that myonuclei in extraocular muscles might express other *Myh* genes in addition to these two. Adapted from Dos Santos *et al.* (2022).

Fig. 5. Diseases and therapies related to myonuclear communication. (A) Myonuclear heterogeneity in *dystrophin* expression in Duchenne muscular dystrophy (DMD). In DMD heterozygote females (XX), dystrophin is present in a segmented pattern at the myofibre membrane (cyan), in so-called basal sarcolemmal dystrophin units (BSDUs), due to random X-inactivation (Morin *et al.*, 2023). All male (XY) myonuclei express the *DMD* mutation. Myonuclei corrected by gene or cell therapies restore dystrophin function in part of the fibre (green). (B) Facioscapulohumeral muscular dystrophy (FSHD) myonuclear propagation of DUX4 proteins. DUX4 proteins produced by a small number of myonuclei diffuse in the fibre and are imported into adjacent nuclei where they activate DUX4-target genes, including DUXA, that leads to amplification of deleterious processes. (C) Myotonic dystrophy 1 (MD1) myonuclei with diffusible *DMPK* RNA foci. Newly accreted myonuclei (green) get RNA foci imported. mRNA, messenger RNA.

Fig. 6. Environmental and cellular factors contributing to myonuclear heterogeneity. ECM, extracellular matrix; MuSC, muscle stem cell; mRNA, messenger RNA; expⁿ, expression.

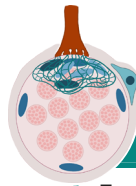
Severe injury

- Myonuclei added by MuSC fusion
- Transcription related to myonuclear position (central arrays, peripheral nuclei and clusters)



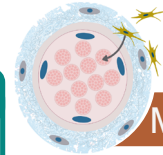
Microlesions

- Transcription of scaffolding genes : *FilaminC*, *Enah*, *Runx1*, *Myh9*
- Migration of proximal myonuclei to the site of damage to deliver mRNAs
- Dependence on microtubules, dynein and Ca²⁺ signalling



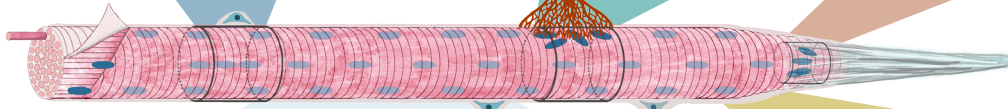
NMJm

- Transcription of post-synaptic genes: *AchR*, *Ache*, *Chrna1*, *Musk*, *ColQ*, *Utrophin*, *Etv4/5*, *Gabp*, *Ufsp1*
- Dependence on paracrine signalling
- Smaller myonuclear domain
- Contribution by specialised synaptic MuSCs (Sy-MuSCs)
- Density altered in DMD
- Signature altered upon denervation
- Dense/nest-like microtubule network



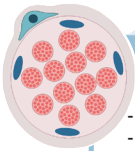
MTJm

- Transcription of MTJ markers: *Col22a1*, *Ankrd1*, *Tigd4*, *Itgb1*, *Slc24a2*, *Ebf1*, *Pdgfrb*, collagens
- MTJ-A and MTJ-B
- Smaller myonuclear domain
- Contribution by fibroblasts (MTJ-B)
- Sensitive to stretching and immobilisation



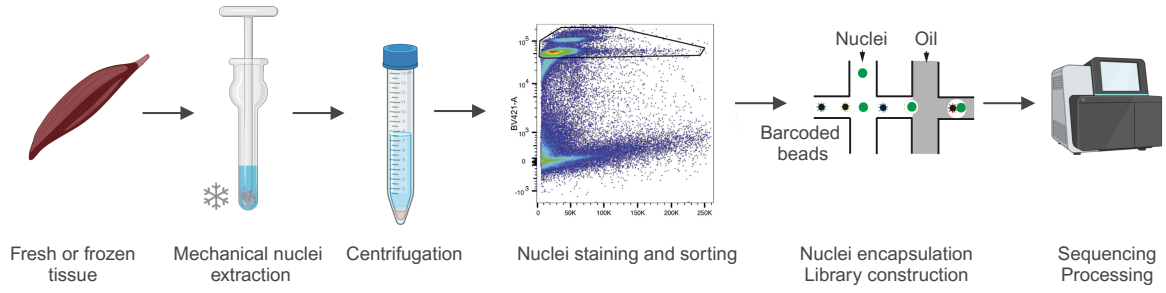
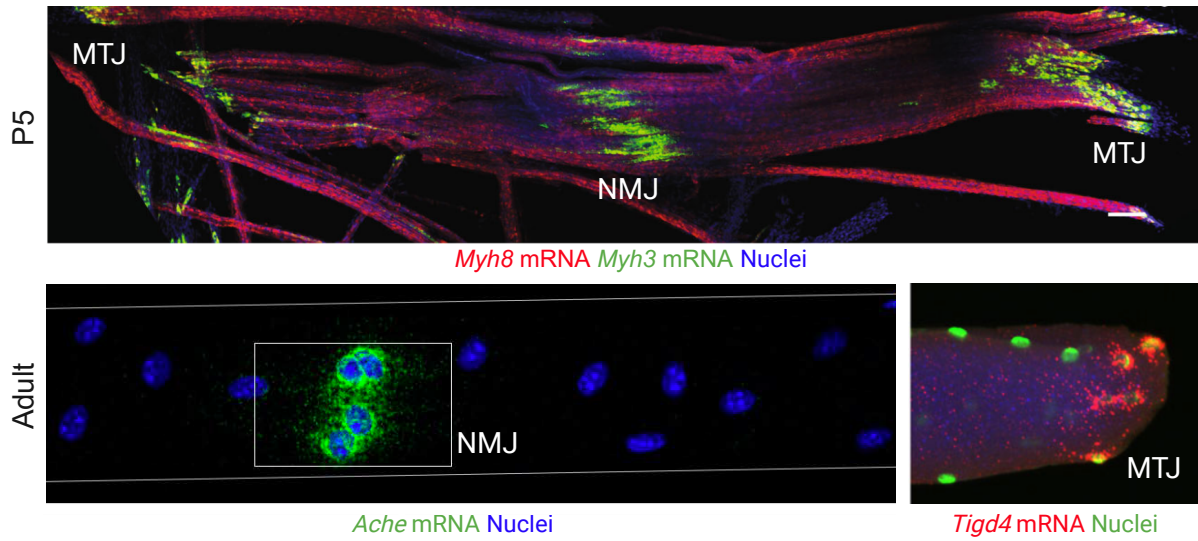
Transitional MTJm

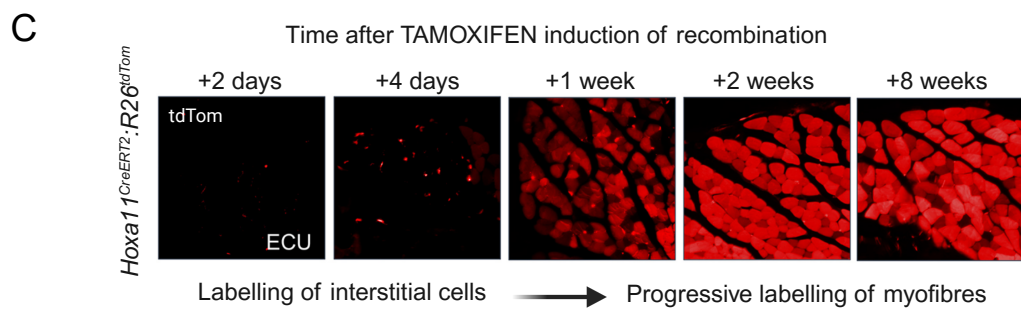
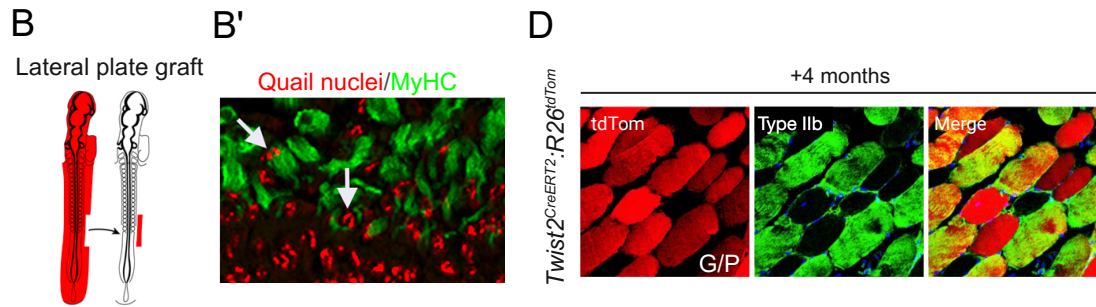
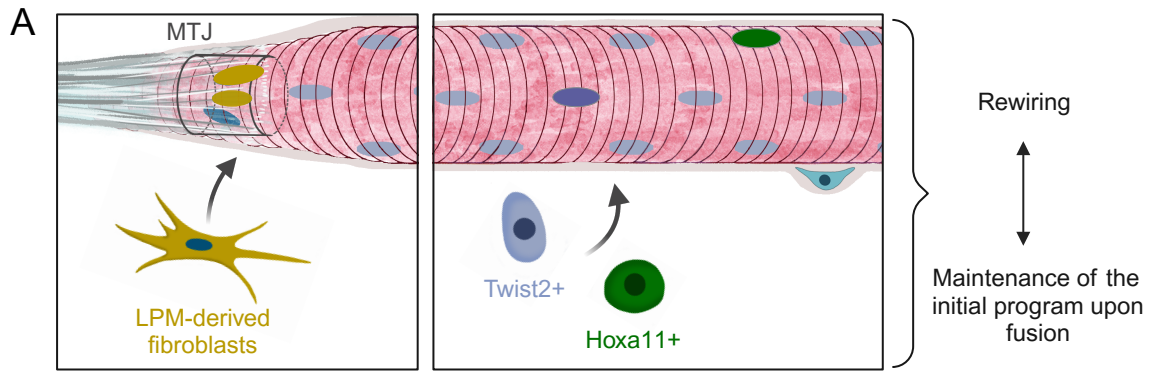
- Transcription of some MTJ markers: *Ncam1*, *Ablim1*
- Absence of *Col22a1*

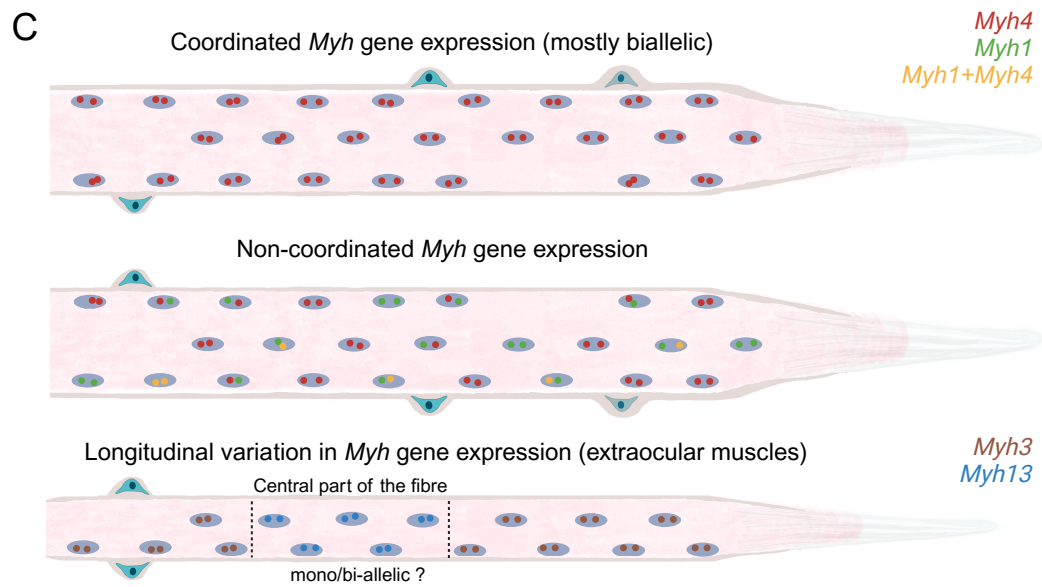
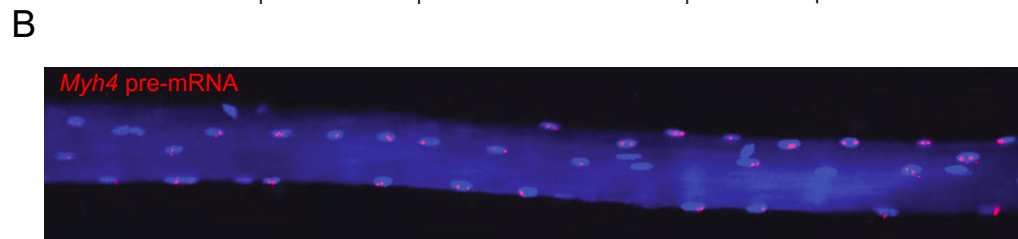
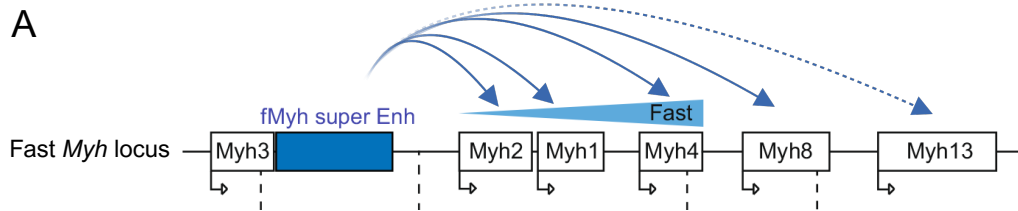


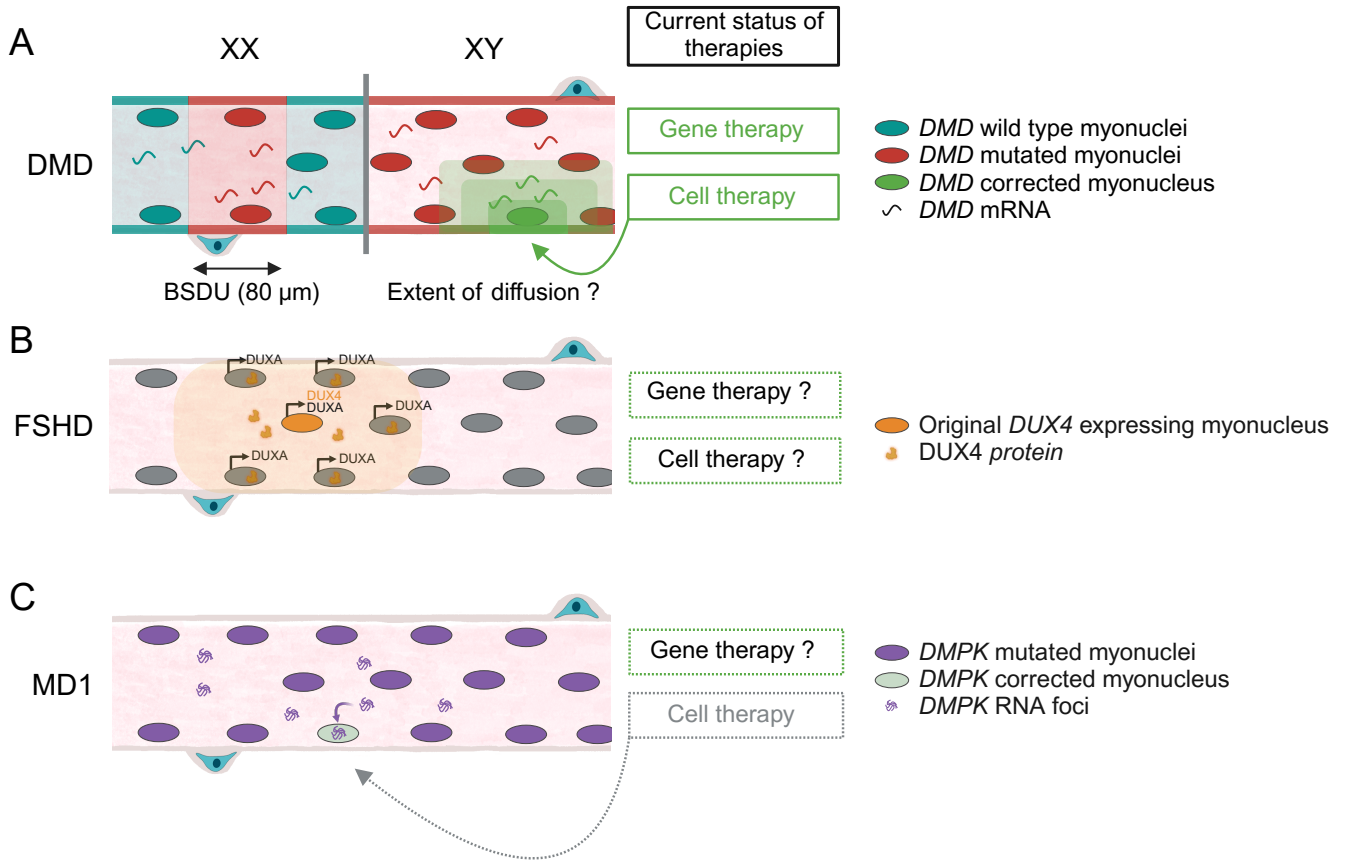
Myofiber body myonuclei

- Grid-like microtubules that may promote efficient mRNA dispersion
- Responsible for the metabolic and contractile profile
 - **pure fibres**: single MyHC, coordinated expression along fibre length, most muscles
 - **constitutive hybrid**: multiple MyHC, uncoordinated expression, specific muscles and intrafusal fibres
 - **transient hybrid**: multiple MyHC, development, denervation, disease, regeneration
- Myonuclear subpopulations:
 - **Sarcomere assembly/Enah+**: scaffolding genes (*FilaminC*, *Nrap*, *Runx1*, *Myh9/10*), mainly observed at P21 and ageing
 - **Rian+/Meg3+**: maternally imprinted, potential role for fibre type transitions
 - **Gsox2+**: sarcoplasmic reticulum genes • **Bcl2+**: steroid signalling genes • **Suz12+**: epigenomic regulation
- Myonuclear changes upon stress:
 - **Denervation**: atrophic signature (*Atrogin*, *Runx1*, *Gadd45a*)
 - **Mechanical overload**: ECM remodelling genes, *Ankrd1*, *Runx1*, *c-Myc*, newly accreted myonuclei express *Myh3* and *H19*
 - **Training + MuSC-depletion**: appearance of 'cryptic' myonuclei with a mixed FAPs/myonuclei profile
- Myonuclear changes in disease:
 - **DMD mouse models**:
 - *mdx*: fibre repair (*Xirp1*, *FilaminC*, *Hspb7*, *Nrap* and *Klh40*), dying fibres, ↓ NMJm and MTJ-B markers
 - ΔEx51: RegMyon (*Xirp1*, *Runx1*, *Myh3*, *Myh8*, *Mymk*), ↑ proteasome genes of myonuclei and NMJ/MTJm
 - **ageing**: ↑ *Ampd3*, *Atrogin*, apoptosis and proteasome genes (mouse), ↓ *FOXO3* (non-human primate)

A**Single-nucleus omics****B****Single-molecule FISH**







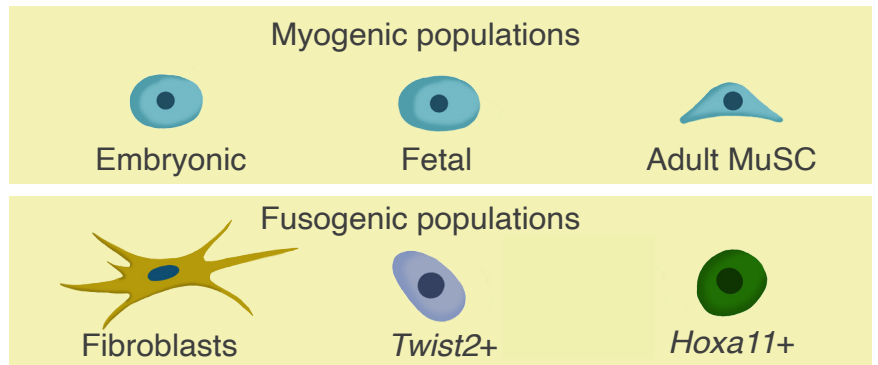
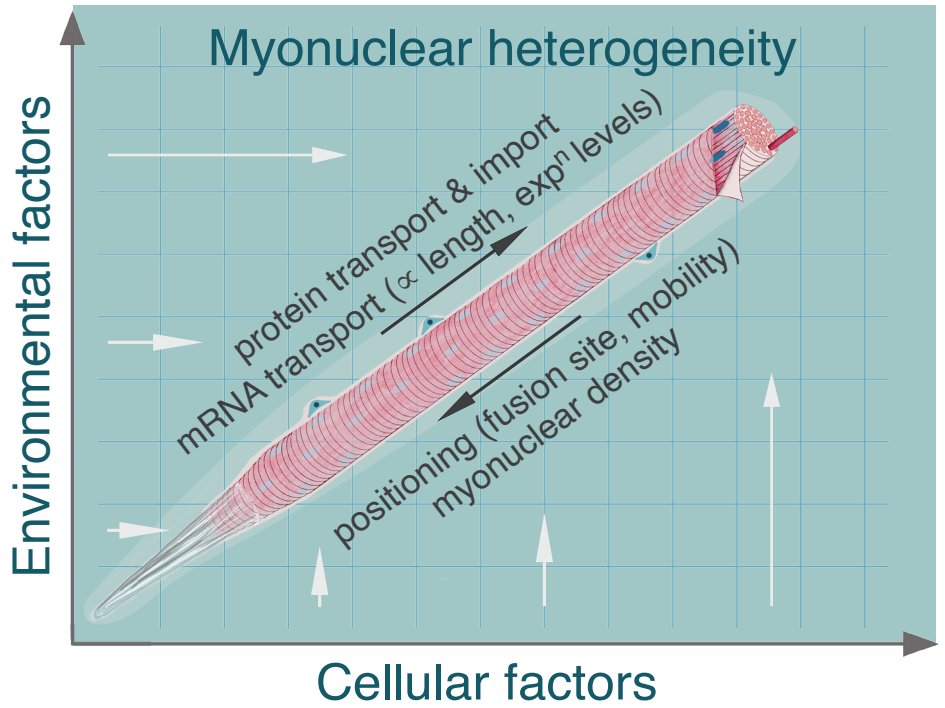
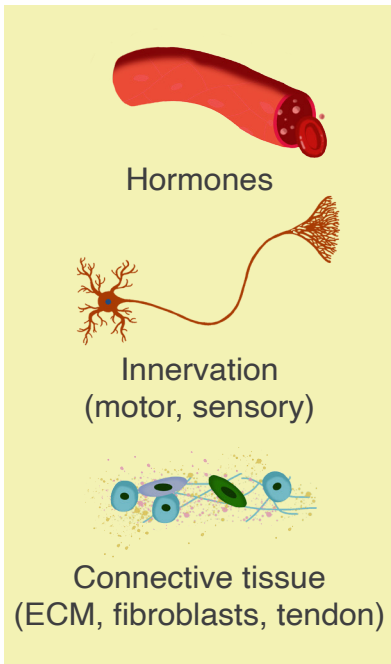


Table 1. Summary of single-nucleus RNA-sequencing (snRNA-seq) studies on skeletal muscles highlighting myonuclear heterogeneity.

Context	Organism /Strain	Sex	Age	Tissue	Platform	Main identified myonuclear subpopulations	Findings related to myonuclear heterogeneity	References
undifferentiated vs differentiated myoblasts	human Hu5/KD3 cells	female	44 years	subcutaneous abdominal muscle	Fluidigm C1	N/A	<ul style="list-style-type: none"> • snRNA-seq facilitates detection of lncRNAs enriched in the nucleus • Mononucleated cells that failed to undergo cell fusion show a mesenchymal-like state 	Zeng <i>et al.</i> (2016)
FSHD mutation vs wild type	human	N/A	N/A	quadriceps, tibialis anterior (fresh)	Fluidigm C1	<i>DUX4</i> ⁺ FSHD-high FSHD-low	<ul style="list-style-type: none"> • <i>DUX4</i> is only transcribed in a small subset of nuclei • Myonuclei subpopulations express different levels of FSHD-induced genes 	Jiang <i>et al.</i> (2020)
fast vs slow muscles	C57BL/6J mouse	female	6–8 weeks	tibialis anterior, gastrocnemius, soleus, plantaris, extensor digitorum longus, quadriceps (fresh)	10x Genomics GEX	<i>Myh7</i> ⁺ <i>Myh2/Myh1</i> ⁺ <i>Myh4</i> ⁺ MTJ NMJ Unidentified (<i>FilaminC</i> ⁺)	<ul style="list-style-type: none"> • Most myonuclei express only one isoform of <i>Myh</i> but mixed <i>Myh</i> expression can be observed • <i>Myh</i> expression is correlated with the expression of specific metabolic and sarcomeric genes • <i>Myh</i> coordination depends on innervation and Six proteins for pure fast muscles • Unidentified myonuclei population expresses sarcomere assembly genes 	Dos Santos <i>et al.</i> (2020)
ΔEx51 DMD model vs wild type	C57BL/6J mouse	N/A	4 weeks	tibialis anterior (fresh)	10x Genomics GEX	<i>Myh2</i> ⁺ <i>Myh1</i> ⁺ <i>Myh1/Myh4</i> ⁺ <i>Myh4</i> ⁺ NMJ MTJ RegMyon (<i>Myh3</i> ⁺)	<ul style="list-style-type: none"> • Myonuclei in dystrophic mice show reduced transcription of sarcomeric genes • ‘Regenerative myonuclei’ population present in the ΔEx51 DMD model 	Chemello <i>et al.</i> (2020)
4-week cycle training; MuSC depleted vs control	<i>Pax7</i> ^{CreERT2} : <i>R26</i> ^{DTA} mouse	female	6 months	soleus (fresh)	10x Genomics GEX	N/A	<ul style="list-style-type: none"> • Threshold of myonuclear accretion reached after 4 weeks of training in control condition 	Englund <i>et al.</i> (2020)

							<ul style="list-style-type: none"> • MuSC depletion affects myonuclei transcriptome upon training 	
injured (7- and 14-days post-injury) vs control; <i>mdx</i> DMD model; muscle spindles	C57BL/6J mouse	N/A	2.5 months	tibialis anterior (fresh)	CEL-Seq2	Body myonuclei NMJ MTJ (A/B) <i>Bcl2</i> ⁺ <i>Gssos2</i> ⁺ <i>Rian</i> ⁺ <i>Suz12</i> ⁺ Perimysial (<i>Muc13</i> ⁺) Repair (<i>FilaminC</i> ⁺)	<ul style="list-style-type: none"> • Two populations of MTJ myonuclei (A/B) • Identification of additional myonuclear populations: <i>Bcl2</i>⁺ (steroid signalling), <i>Gssos2</i>⁺ (ER and Golgi trafficking), <i>Rian</i>⁺ (lncRNA targeting mitochondrial proteins), <i>Suz12</i>⁺ (polycomb complex) • ‘Repair’ myonuclei population present in the <i>mdx</i> DMD model • New myonuclear subpopulations in muscle spindles 	Kim <i>et al.</i> (2020)
post-natal development and ageing	C57BL/6J mouse	male	P10, P21, 5 months, 24 months, 30 months	tibialis anterior, soleus (fresh)	10x Genomics GEX	<i>Myh7</i> ⁺ <i>Myh2</i> ⁺ <i>Myh1</i> ⁺ <i>Myh4</i> ⁺ NMJ MTJ <i>Ampd3</i> ⁺ <i>Ckm/Coll1a1</i> ⁺ <i>Enah/FilaminC</i> ⁺ <i>Nr4a3</i> ⁺	<ul style="list-style-type: none"> • Myonuclear subpopulations proportions change during development/ageing • Identification of additional myonuclear populations: <i>Ampd3</i>⁺ (denervation genes), <i>Ckm/Coll1a1</i>⁺ (matrix remodelling), <i>Enah</i>⁺ (sarcomere assembly, enriched in P21 and aged muscles), <i>Nr4a3</i>⁺ (metabolic adaptation) 	Petrany <i>et al.</i> (2020)
stress induced by dissociation vs control; injury with BaCl ₂ vs control	C57BL/6J mouse	female	8 weeks	tibialis anterior, gastrocnemius, soleus, plantaris, and extensor digitorum longus (fresh)	10x Genomics GEX	N/A	<ul style="list-style-type: none"> • Identification of stress signature upon dissociation and muscle injury 	Machado <i>et al.</i> (2021)
sedentary vs 4-week cycle training (4h and 24h post- last round of exercise); MuSC depleted vs control	<i>Pax7</i> ^{CreERT2} ; <i>R26</i> ^{DTA} ; <i>HSA-rtTA/TRE-H2B-GFP</i> mouse	female	6 months	plantaris, soleus (fresh)	10x Genomics GEX	<i>Myh7</i> ⁺ <i>Myh2</i> ⁺ <i>Myh2/Myh1</i> ⁺ <i>Myh1</i> ⁺ <i>Myh4</i> ⁺ NMJ MTJ	<ul style="list-style-type: none"> • Disappearance of <i>Myh4</i>⁺ myonuclei in soleus upon training • Low abundance of <i>Myh3</i>⁺ myonuclei after cycle training • ‘Cryptic’ myonuclei population, with a mixed FAPs-myonuclei 	Wen <i>et al.</i> (2021)

						<i>Rian+</i> <i>Myh3+</i> Cryptic (<i>Lmod2+</i>)	signature, present in the absence of MuSC	
multi-omics	human, rat	N/A	N/A	quadriceps femoris muscle group, gastrocnemius (frozen)	10x Genomics GEX, ATAC-seq & multiome	<i>MYH7+</i> <i>MYH1+</i>	<ul style="list-style-type: none"> Correspondence of single nucleotide polymorphism and specific muscle cell types 	Orchard <i>et al.</i> (2021)
slow vs fast muscles	C57BL/6J mouse	female	6–8 weeks	quadriceps, soleus (fresh)	10x Genomics ATAC-seq	<i>Myh7+</i> <i>Myh2+</i> <i>Myh1+</i> <i>Myh4+</i>	<ul style="list-style-type: none"> Active competition of fast <i>Myh</i> genes promoters for a common super-enhancer may coordinate myonuclei <i>Myh</i> expression 	Dos Santos <i>et al.</i> (2022)
snRNA-seq atlas	human	male and female	N/A	gastrocnemius, oesophagus (frozen)	10x Genomics GEX	<i>MYH7+</i> <i>MYH2+</i> NMJ Cytoplasmic	<ul style="list-style-type: none"> Identification of monogenic muscle disease genes related to specific myonuclear populations (slow, fast, NMJ) Myonuclei population containing high levels of mature/mitochondrial transcripts 	Eraslan <i>et al.</i> (2022)
sciatic nerve transection vs contralateral control (2 weeks post-denervation)	C57BL/6J mouse	male	12 weeks	gastrocnemius (fresh)	10x Genomics GEX	<i>Myh7+</i> <i>Myh2+</i> <i>Myh2/Myh1+</i> <i>Myh1+</i> <i>Myh1/Myh4+</i> <i>Myh4+</i> NMJ MTJ	<ul style="list-style-type: none"> <i>Myh4+</i> (2) myonuclei proportion increases with denervation 	Lin <i>et al.</i> (2022)
<i>mdx</i> DMD model vs <i>mdx</i> e23AON treated vs wild type	C57BL/6J mouse, human	male	32–36 weeks	tibialis anterior, vastus lateralis (frozen)	10x Genomics GEX	<i>Myh2+</i> <i>Myh1+</i> <i>Myh4+</i> Partly mature Immature NMJ MTJ <i>Tcap</i> -high/ <i>Dmd</i> -low	<ul style="list-style-type: none"> Wild type-like profile of myofibres after exon-skipping <i>Tcap</i>-high myonuclei population enriched in the <i>mdx</i> DMD model 	Scripture-Adams <i>et al.</i> (2022)

young vs old (sarcopenic and non-sarcopenic)	human	male and female	18–30 years, 65–85 years	vastus lateralis (fresh)	10x Genomics GEX	<i>MYH7+</i> <i>MYH2/MYH1+</i> Senescent (<i>CDKN1A/MYH8+</i>)	<ul style="list-style-type: none"> Ageing leads to upregulation of <i>PDKA</i> and <i>HLA</i> genes and downregulation of <i>IGF1</i> and <i>MYH2</i> in <i>MYH2/MYH1+</i> myonuclei Senescent myonuclei population observed only in aged muscles 	Perez <i>et al.</i> (2022)
young vs old	cynomolgus monkeys	N/A	4-6 years, 18–21 years	quadriceps (frozen)	10x Genomics GEX	<i>MYH7+</i> <i>MYH2+</i> <i>MYH1+</i> NMJ	<ul style="list-style-type: none"> Myonuclei populations are differentially affected by ageing Global protective effect of <i>FOXO3</i> against ageing 	Jing <i>et al.</i> (2023)
myotendinous junction	human	male	20-44 years	semitendinosus muscle extremity (frozen)	10x Genomics GEX	<i>MYH7+</i> <i>MYH2+</i> <i>MYH2/MYH1+</i> <i>MYH1+</i> Fast MTJ Slow MTJ Fast transitional MTJ Slow transitional MTJ	<ul style="list-style-type: none"> Transitional MTJ myonuclei express some MTJ markers but not <i>COL22A1</i> MTJ populations might express different <i>MYH</i> genes from the body myonuclei depending on the fibre type (fast/slow) 	Karlsen <i>et al.</i> (2023)
embryonic and post-natal development	C57BL/6J mouse	male and female	E14.5, E18.5, P5, 8 weeks	hindlimb skeletal muscles (frozen)	10x Genomics multiome	N/A	<ul style="list-style-type: none"> Myonuclei express distinct signatures depending on the developmental stage 	Dos Santos <i>et al.</i> (2023)
peripheral artery disease	human	male and female	adult	gastrocnemius (frozen)	10x Genomics GEX	<i>MYH7+</i> <i>MYH2/MYH1+</i> Hybrid I-II NMJ Regenerating myonuclei (<i>MYH3/RUNX1+</i>)	<ul style="list-style-type: none"> Myonuclei from PAD patients express genes related to hypoxia and atrophy Increased proportion of ‘regenerating myonuclei’ in PAD patients 	Pass <i>et al.</i> (2023)
myonuclear accretion during development and muscle overload	C57BL/6J mouse	N/A	P10, 3 months, 5 months	plantaris (fresh)	10x Genomics GEX	<i>Myh7+</i> <i>Myh2+</i> <i>Myh1+</i> <i>Myh4+</i> Dev (<i>Myog+</i>) <i>Atf3+</i> (<i>FilaminC+</i>)	<ul style="list-style-type: none"> Partially shared signature of recently fused myonuclei during development (Dev) and after muscle overload 	Sun <i>et al.</i> (2023)

						Type A/B (<i>Myh3+</i>)	<ul style="list-style-type: none"> Differences between myonuclei accreted into pre-existing (type A) or <i>de novo</i> fibres (type B) 	
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AON, anti-sense oligonucleotide;
 DMD, Duchenne muscular dystrophy;
 E, embryonic day;
 ER, endoplasmic reticulum;
 FAP, fibro-adipogenic progenitor;
 FSHD, facioscapulohumeral muscular dystrophy;
 GEX, gene expression;
 lncRNA, long non-coding RNA;
 MTJ, myotendinous junction;
 MuSC, muscle stem cell;
 N/A, not available;
 NMJ, neuromuscular junction;
 P, post-natal day;
 PAD, peripheral arterial disease;
 snRNA-seq, single-nucleus RNA-sequencing;

Table 3. Canonical and novel myotendinous junction myonuclei (MTJm) and neuromuscular junction myonuclei (NMJm) markers.

Canonical NMJm markers	References
<i>Ache</i>	Chan <i>et al.</i> (1999)
<i>Utrophin</i>	Gramolini <i>et al.</i> (1997); Khurana <i>et al.</i> (1991)
<i>Musk</i>	Lacazette <i>et al.</i> (2003)
<i>ColQ</i>	Ohno <i>et al.</i> (1998)
<i>N-CAM, 43k-rapsyn, S-laminin</i>	Moscoso <i>et al.</i> (1995)
<i>Nestin</i>	Carlsson <i>et al.</i> (1999)
Novel NMJm markers identified by snRNA-seq	References
<i>Ablim2, Ano4, Etv4, Gramd1b, Lrfn5, Phldb2, Prkar1a, Ufsp1, Vav3</i>	Chemello <i>et al.</i> (2020); Kim <i>et al.</i> (2020); Petrany <i>et al.</i> (2020); Dos Santos <i>et al.</i> (2020)

Canonical MTJm markers	References
<i>Col22a1</i>	Koch <i>et al.</i> (2004); Charvet <i>et al.</i> (2013)
<i>Ankrd1</i>	Baumeister <i>et al.</i> (1997)
<i>Loxl3</i>	Kraft-Sheleg <i>et al.</i> (2016)
<i>Nestin</i>	Carlsson <i>et al.</i> (1999)
Enrichment in dystrophin, talin and desmin (compared to the myofibre body)	Samitt & Bonilla (1990); Bassett <i>et al.</i> (2003); Morin <i>et al.</i> (2023); Tidball <i>et al.</i> (1986); Tidball (1992)
Increased MyHC production and myofibrillogenesis upon stretching	Dix & Eisenberg (1990)
Novel MTJm markers identified by snRNA-seq	References
MTJ-A: <i>Ablim1, Adamts20, Col24a1, Frasl, Itgb1, Maml2, Pelil, Tigd4, Slc24a2, Sorbs2</i>	Chemello <i>et al.</i> (2020); Kim <i>et al.</i> (2020); Petrany <i>et al.</i> (2020); Dos Santos <i>et al.</i> (2020); Wen <i>et al.</i> (2021)
MTJ-B: <i>Col1a2, Col6a1, Col6a3, Ebf1, Fst1l, Pdgfrb</i>	Kim <i>et al.</i> (2020); Petrany <i>et al.</i> (2020)

MTJ, myotendinous junction;
 MTJm, myotendinous junction myonuclei;
 MyHC, myosin heavy chain;
 NMJm, neuromuscular junction myonuclei;
 snRNA-seq, single-nucleus RNA-sequencing

Table 2. Characteristics of recently identified fusogenic populations.

Model used	Specific contribution	Stage of contribution			Notes	References
		Development	Postnatal/adult	Regeneration		
Chick/Quail Chimeras	MTJm	✓	?	?	Fibroblast-to-myoblast conversion promoted by BMP signalling	De Lima <i>et al.</i> (2021)
<i>Osr1^{CreERT2}</i> , <i>Scx-Cre</i>	Mostly MTJm	✓	?	?	Nuclei also seen at the muscle belly	De Lima <i>et al.</i> (2021)
<i>Prx1^{Cre}</i>	MTJm	✓	?	?	<i>Lox13</i> is transcribed by fibroblasts but the enzymatic activity localises at muscle tips upon fusion	Yaseen <i>et al.</i> (2021)
<i>Twist2^{CreERT2}</i>	Type IIb/x myofibres	✗	✓	✓	<i>Twist2</i> ⁺ cells secrete Neuropilin1; chemorepulsion of type I and IIa myofibres that express Semaphorin3a	Liu <i>et al.</i> (2017a)
<i>Hoxa11^{CreERT2}</i>	All fibre types	✗	✓	Minimal	Signature of <i>Hoxa11</i> ⁺ cells largely overlaps with <i>Twist2</i> ⁺ cells	Flynn <i>et al.</i> (2023)

BMP, bone morphogenetic protein

MTJm, myotendinous junction myonuclei;

