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Extraocular muscle stem cells exhibit distinct cellular properties associated with non-muscle molecular signatures

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1 **Abstract**

2 Skeletal muscle stem cells (MuSC) are recognized as functionally heterogeneous. Cranial MuSCs are
3 reported to have greater proliferative and regenerative capacity when compared to the ones in the limb.
4 A comprehensive understanding of the mechanisms underlying this functional heterogeneity is lacking.
5 Here we used clonal analysis, live imaging and scRNA-seq to identify critical features that distinguish
6 extraocular (EOM) from limb muscle stem cell populations. A *Myogenin*^{tdTom} reporter showed that the
7 increased proliferation capacity of EOM MuSCs correlates with deferred differentiation and lower
8 expression of the myogenic commitment gene *Myod*. Unexpectedly, *in vitro* activated EOM MuSCs
9 expressed a large array of extracellular matrix components typical of mesenchymal non-muscle cells.
10 Computational analysis underscored a distinct co-regulatory module, which is absent in limb MuSCs,
11 as driver of these features. The EOM transcription factor network, with *Foxc1* as key player, appears to
12 be hardwired to EOM identity as it persists during growth, disease, and *in vitro* after several passages.
13 Our findings shed light on how high-performing MuSCs regulate myogenic commitment by remodeling
14 of their local environment and adopting properties not generally associated with myogenic cells.
15

16 INTRODUCTION

17 Genetic and transcriptomic studies have shown that the MuSC population in any particular anatomical
18 location is heterogeneous. Certain subsets are more prone to self-renewal or differentiation, differ in
19 transplantation efficiency, as well as their stem cell-niche interactions, metabolism, and resistance to
20 stress upon activation (Barruet et al., 2020; Chakkalakal et al., 2014; Dell'Orso et al., 2019; Dumont et
21 al., 2015; Gayraud-Morel et al., 2012; Hernando-Herraez et al., 2019; Micheli et al., 2020; Morree et al.,
22 2019; Ono et al., 2012; Rocheteau et al., 2012; Scaramozza et al., 2019; Tierney et al., 2018; Vartanian
23 et al., 2019; Yartseva et al., 2020; Yennek et al., 2014). Despite this diversity, MuSCs share common
24 functions that are essential for growth and repair (Lepper, 2011; Murphy, 2011; Sambasivan, 2011).
25 Myogenic commitment and differentiation involving *Myod* and *Myog*, respectively, occur in response to
26 injury or growth factors in culture media following isolation of the *Pax7*-expressing MuSCs (Evano and
27 Tajbakhsh, 2018; Zammit et al., 2004).

28 Unexpectedly, MuSCs in different anatomical locations were found to be programmed with distinct
29 upstream transcription factors (TF) prior to acquiring myogenic identity (Gopalakrishnan et al., 2015;
30 Harel et al., 2009; Kelly et al., 2004; Sambasivan et al., 2009; Tajbakhsh et al., 1997). Extraocular
31 muscles (EOMs) are derived from unsegmented cranial mesoderm and are regulated by distinct
32 transcription factors and signalling molecules compared to the somite-derived limb and trunk muscles
33 (Grimaldi and Tajbakhsh, 2021; Michailovici et al., 2015; Sambasivan et al., 2011). For example, mice
34 lacking the transcription factor *Pitx2* do not form EOMs whereas other cranial and somite derived
35 muscles are unaffected (Diehl et al., 2006; Gage et al., 1999; Zacharias et al., 2011). As some muscle
36 subsets, like the EOMs, are preferentially spared in muscular dystrophies and during ageing (Emery,
37 2002; Formicola et al., 2014; Verma et al., 2017) and their MuSCs are functionally more robust in terms
38 of proliferation and engraftment efficiency (Stuelsatz et al., 2015), intrinsic properties of MuSCs or
39 myofibers might determine their differential sensitivity to disease (Randolph and Pavlath, 2015; Terry
40 et al., 2018). Notably, single-cell transcriptomic analysis identified the thyroid hormone signalling
41 pathway as a key factor preventing senescence entry of EOM MuSCs in dystrophin deficient (DMD) rats
42 (Taglietti et al., 2023). Moreover, profiling of EOM MuSCs after heterotopic transplantation into limb
43 muscle showed that, despite significant transcriptional changes, approximately 10% of EOM-specific
44 genes persisted in the ectopic niche (Evano et al., 2020). This finding suggests that cell autonomous
45 regulation of MuSC properties predominates to a certain extent in EOM. Yet, the existence of deeply-
46 rooted TF gene regulatory networks within MuSC subsets and their role in maintaining anatomically
47 distinct phenotypes remains largely unexplored.

48 Using droplet-based scRNA-seq, we investigated the transcriptional states that govern the
49 outperformance of MuSC subsets. We identified key regulators that confer distinct mesenchymal-like
50 features to activated EOM MuSCs, and extensive expression of extracellular matrix (ECM) components.
51 Activated EOM MuSCs are less prone to differentiation and thus appear to be in a more stem-like state
52 that is maintained *in vitro*, upon passages and *in vivo* during growth, through a specific set of co-
53 regulated transcription factors.

54 RESULTS

55 Temporal heterogeneity in myogenic commitment to differentiation among MuSCs

56 We isolated EOM and *Tibialis anterior* (TA) MuSCs by FACS using *Tg:Pax7-nGFP* mice (Sambasivan
57 et al., 2009) and plated them at the same density to follow up their proliferation and differentiation
58 dynamics (**Figure 1A-B**). Quantification of the number of total and proliferative nuclei (5-Ethynyl-2'-
59 deoxyuridine, EdU uptake, 2h pulse) at D3, D4 and D5 showed a 2.9 to 4.4-fold change increase in
60 EOM compared to TA (**Figure 1C**). However, the percentage of total proliferative cells remained
61 unchanged in both conditions at every time point (**Figure 1D**), arguing that the division probability is
62 equal for EOM and TA. Yet, EOM cultures had twice the percentage of PAX7+ cells and less fusogenic
63 myoblasts compared to TA at later timepoints (D10, **Figure 1E, F**).

64 We then performed clonal analysis of freshly isolated EOM MuSCs (**Suppl Figure 1A**) and showed that
65 they displayed a 7-fold higher clonal capacity (mean 1315 cells/clone) compared to those from TA (186
66 cells/clone) (**Suppl Figure 1B, T1**), in agreement with a previous study (Stuelsatz et al., 2015).
67 Surprisingly, the higher clonogenic properties of EOM persisted even when they were pre-amplified for
68 2 days *in vitro* where they yielded 3656 cells/clone compared to 297 cells/clone for TA (12-fold
69 difference; **Suppl Figure 1B, T2**). Given that EOM and TA MuSCs exhibited a similar proliferative rate
70 in bulk cultures, the clonal data suggests that the greater cellular output of EOM MuSCs is probably due
71 to delayed differentiation. Since *Myog* expression is followed by cell-cycle withdrawal (Andrés and
72 Walsh, 1996; Benavente-Diaz et al., 2021; Guo et al., 1995), a delay in its expression could allow for
73 sustained expansion of EOM MuSCs.

74 To test this hypothesis, we assessed the differentiation dynamics of EOM and TA MuSCs *in vitro* by live
75 imaging using the *Tg:Pax7-nGFP;Myog^{ntdTom}* mouse line (Benavente-Diaz et al., 2021; Sambasivan et
76 al., 2009), which allowed simultaneous isolation of MuSCs and monitoring of the onset of *Myog*
77 expression by a nuclear tdTOMATO (tdTOM) reporter. Myogenic cells were tracked continuously from
78 D3 and tdTOM intensity scored (**Figure 1G**). TA myogenic cells initiated reporter gene expression earlier
79 than EOM cells (**Figure 1H**). The percentage of tdTOM+ cells sharply increased in the TA from 5% at
80 80h (D3+8hours) post-plating to 40% at 96h (D4). Significantly, only ~7% of EOM cells were tdTOM+ by
81 96h *in vitro* (**Figure 1I**). Western Blot (**Suppl Figure 1C, D**) data were in agreement with the live imaging
82 analysis. For instance, expression of the cell cycle inhibitor *p21*, which regulates cell cycle exit (Guo et
83 al., 1995; Zhang et al., 1999), and expression of phosphorylated p38, which promotes MEF2
84 transcriptional activity to initiate differentiation (Rugowska et al., 2021), were significantly downregulated
85 in activated EOM MuSCs compared to TA. RT-qPCR showed higher levels of *Myogenin* and *p21*
86 transcripts in TA compared to EOM MuSCs already at D2 (**Figure 1J**). As such, this data suggests that
87 EOM progenitors are less prone to myogenic commitment.

88 Finally, to test if EOM cells have a global impairment in myogenic differentiation, we used the *Tg:Pax7-*
89 *nGFP;Myog^{ntdTom}* mice to isolate the stem (PAX7+/GFP+) and committed (MYOG+/tdTOM+) fractions

90 after D5 *in vitro* (**Figure 1K, Suppl Figure 1E**). No differences were seen by RT-qPCR among EOM
91 and TA tdTOM+ cells for *Myod*, *Myogenin*, the fusogenic markers *Myomixer* and *Myomaker* (Sampath
92 et al., 2018) and embryonic myosin heavy chain (*Myh3*) indicating that differentiation of EOM cells was
93 not generally impeded (**Figure 1L**). However, plating EOM and TA tdTOM+ myoblasts at high density
94 in differentiation media revealed a lower fusion index and higher percentage of EOM myoblasts
95 incorporating EdU on their last division (**Suppl Figure 1F-H**, (Benavente-Diaz et al., 2021)). Therefore,
96 a general delay in the differentiation of EOM MuSCs appears to contribute to the higher number of
97 myogenic cells (**Suppl Figure 1I**).

98 **Distinct signature of EOM MuSCs upon activation**

99 To understand the phenotypic differences between EOM and TA MuSCs, we performed scRNA-seq of
100 *in vitro* activated MuSCs using the 10x Chromium (**Figure 2A**). Unsupervised clustering divided cells
101 into 2 clusters per sample (**Figure 2B**) that were annotated as progenitors (Prog) or differentiating (Diff)
102 based on myogenic marker expression (**Figure 2B, C**). Notably, EOM progenitors exhibited reduced
103 expression of *Myod* RNA and protein across the entire population at D3 and D5 *in vitro*, thereby
104 suggesting a lower propensity to differentiate (**Figure 2B, Suppl Figure 2A**).

105 Analysis of differentially expressed genes (DEGs) showed that each cell state had a distinct
106 transcriptional pattern (**Figure 2D, Suppl Table 1**). As expected, TA progenitors expressed a defined
107 Hox-signature (Evano et al., 2020) and inhibitors of differentiation (Id1, Id2, (Jen et al., 1992; Kumar et
108 al., 2009)). EOM and TA differentiating cells shared part of their signature (*Myog*, *Mymx*, *Myl4*).
109 However, EOM progenitors displayed markers not been previously noted in MuSCs, such as *Mgp*, *Bgn*,
110 *Col1A2* and *Acta2* (smooth muscle actin) (**Figure 2D**).

111 We then performed reactome analysis of DEGs (**Figure 2E, Suppl Figure 2B, Suppl Table 2**). As
112 suggested by the DEG heatmap, TA progenitors shared common pathways with the differentiating
113 clusters. Multiple pathways involved in ECM organisation characterised EOM progenitors including
114 “ECM proteoglycans”, “crosslinking of collagen fibrils” and “integrin cell surface interactions”. EOM
115 progenitors were also specifically enriched in Pdgfr β , a receptor for platelet-derived growth factor, and
116 proteins related to insulin like growth factor binding and integrin signaling (**Figure 2F, Suppl Figure 2C,**
117 **Suppl Table 3**). Thus, these analyses uncovered a non-canonical signature in EOM progenitors upon
118 activation.

119 **Closer molecular overlap of EOM MuSCs across cell states**

120 We then compared the signature of EOM and TA myogenic progenitors *in vitro* with the quiescent
121 counterparts *in vivo* by scRNA-seq immediately after isolation by FACS (**Figure 3A, B**). While both
122 populations displayed consistent expression of MuSC markers, *Myod* expression was restricted to TA
123 MuSCs (**Figure 3C, D**), suggesting an earlier activation in response to the isolation procedure (Brink et
124 al., 2017; Machado et al., 2017; Velthoven et al., 2017). Differentiation markers like *Myog* were absent

125 in both populations (**Figure 3C, D**). Visualization of the top 25 most variably expressed genes
126 highlighted distinct transcriptional programs of EOM and TA quiescent clusters (**Figure 3E, Suppl Table**
127 **4**). Moreover, microarray analysis showed that EOM quiescent MuSCs also stand apart from those in
128 other cranial-mesoderm derived muscles, such as the esophagus and masseter (**Suppl Figure 3A**).

129 Interestingly, we found several conserved genes between the quiescent and activated sc-RNAseq
130 states at each anatomical location, while others were unique to the quiescent or activated cell states
131 (**Figure 3F-I**). EOM genes unique to the quiescent state included *Tshr*, encoding the thyroid-stimulating
132 hormone receptor, previously recognized in bulk RNAseq as “EOM-resistant”, i.e. retained following
133 engraftment into the limb (Evano et al., 2020), and shown to prevent senescence in EOM MuSCs of
134 DMD rats (Taglietti et al., 2023). Matrix Gla protein (*Mgp*), a critical regulator of angiogenesis in multiple
135 organs (Kida and Yamaguchi, 2022), was exclusively upregulated in EOM cells upon activation (**Figure**
136 **3H**). Amongst the conserved EOM genes across cell states we identified *Pitx2*, a major upstream
137 regulator of EOM development (Gage et al., 1999), *Fos*, which labels a subset of limb MuSCs with
138 enhanced regenerative capacity (Almada et al., 2021) and *Foxc1*, which is involved in ocular
139 development (Smith et al., 2000) and reported to regulate the balance between myogenic and vascular
140 lineages within somites (Lagha et al., 2009; Mayeuf-Louchart et al., 2016) (**Suppl Figure 3B**). The EOM
141 signature also featured *Igf1bp7*, a specific marker of quiescent MuSCs (Fukada et al., 2007) that is
142 upregulated upon exercise (Chen et al., 2020) (**Figure 3F, Suppl Figure 3B, C**). Additionally, the EOM
143 common signature encompassed several ECM components and regulators (e.g. *Bgn*, *Loxl1*, *Col1a2*,
144 *Col6a1*), TFs associated with fibrosis and connective tissue development (e.g. *Foxp1* (Grimaldi et al.,
145 2022; Shao and Wei, 2018), *Egr1* (Havis and Duprez, 2020)) and *Acta2* (alpha smooth muscle actin), a
146 marker of smooth muscle, fibroadipogenic progenitors (Joe et al., 2010; Uezumi et al., 2010) and smooth
147 muscle-mesenchymal cells (SMMCs, (Giordani et al., 2019)) (**Figure 3F, H**).

148 In the TA, *Lbx1*, *Vgll2* and *Hox* genes were found as common signature throughout cell states (**Figure**
149 **3G,I; Suppl Figure 3B, C**). *Lbx1* is a homeobox transcription factor required for the migration of
150 myogenic progenitor cells to the limbs (Brohmann et al., 2000; Gross et al., 2000) and *Vgll2* (previously
151 Vito-1) is a key cofactor of the myogenic differentiation program (Günther et al., 2004; Maeda et al.,
152 2002). *Lbx1*, *Vgll2* and several *Hox* genes had been already identified in the limb both at the MuSC level
153 and in entire muscles in the adult (Evano et al., 2020; Honda et al., 2017; Terry et al., 2018) (**Figure 3I**).

154 Altogether, our analysis revealed a closer molecular overlap for EOM MuSCs across cell states
155 (quiescence vs *in vitro* activation), including several TFs and ECM markers that were distinct from TA
156 MuSCs.

157 **Activation of EOM MuSCs involves extensive ECM remodelling**

158 Given the well-established role of ECM synthesis and remodelling on MuSC proliferation and self-
159 renewal (Baghdadi et al., 2018; Rayagiri et al., 2018; Tierney et al., 2016; Yin et al., 2013), we examined
160 these features using Matrisome database (Matrisome DB), which compiles *in silico* and experimental

161 data on ECM constituents (Naba et al., 2015). We identified components of the Matrisome DB present
162 in our single-cell dataset and created a global score for ECM component expression (**Figure 4A**) (see
163 Methods). Significantly, the EOM progenitor cluster had the highest matrisome score (**Figure 4A**) and
164 expressed the highest number of genes for each matrisome category (**Figure 4B**).

165 We then validated some matrisome candidate genes by immunofluorescence or Western blot on *in vitro*
166 activated EOM and TA MuSCs (**Figure 4C-D**). EOM progenitors expressed higher levels of Fibronectin
167 (FN1), which promotes MuSC expansion in a cell autonomous manner (Bentzinger et al., 2013),
168 Collagen I (COL1), a major component of the fibrotic ECM (Dulauroy et al., 2012) shown to suppress
169 differentiation of C2C12 cells (Alexakis et al., 2007) and Collagen IV (COL4), which is secreted by
170 MuSCs as well as myoblasts and fibroblasts in culture (Baghdadi et al., 2018; Kühl et al., 1984) (**Figure**
171 **4C**). In addition, EOM progenitors also displayed differential expression of PDGFR β , a gene identified
172 on the EOM molecular functions (**Suppl Figure 2C**). PDGFR β is a tyrosine-kinase receptor commonly
173 expressed by mesenchymal cells and pericytes (Hellström et al., 1999; Levéen et al., 1994; Soriano,
174 1994) (**Figure 4C**). Interestingly, Pdgfr β and Acta2 are markers of Smooth Muscle-Mesenchymal Cells
175 (SMMCs), a novel Itga7+ Vcam- Pdgfr β + Acta2+ cell subpopulation present in adult muscle that exhibits
176 myogenic potential and promotes MuSC engraftment following transplantation (Giordani et al., 2019).

177 Western blot analysis confirmed that EOM progenitors produce higher levels of Caveolin1 (CAV1) and
178 CAVIN1 (**Figure 4D**), which are co-expressed in caveolae and downregulated upon differentiation of
179 rhabdomyosarcoma cells (Faggi et al., 2015). CAV1 is a marker of quiescent and Pax7+ activated
180 mouse MuSCs (Gnocchi et al., 2009) whereas in human, CAV1+ MuSCs are associated with ECM
181 organization, expression of quiescent markers, and increased engraftment capacity (Barruet et al.,
182 2020). SPARC, MGP and IGFBP7 were also upregulated in EOM activated MuSCs (**Figure 4D**). Their
183 activity appears to be context dependent as they promote or suppress proliferation in different cell types
184 (Ahmad et al., 2017; Artico et al., 2021; Cho et al., 2000; Jing et al., 2019; Li et al., 2020; Melouane et
185 al., 2018). Given the large number of matrisome genes characterising EOM progenitors, we examined
186 the expression of MMP2, a matrix remodelling protein (Gonçalves et al., 2022) whose activation
187 increases the proportion and mobility of Pax7+ cells (Mu et al., 2010). As expected, an enrichment of
188 the active form of MMP2 was observed in EOM progenitors. TA progenitors displayed instead an
189 enrichment of the pro-MMP2 or latent form (**Figure 4D**).

190 In parallel, we assessed to what extent activated EOM MuSCs resemble other cells in skeletal muscle
191 displaying mesenchymal features (**Suppl Figure 4A, Suppl Table 5**). Notably, EOM progenitors
192 displayed a higher score for SMMCs (Giordani et al., 2019), FAPs (Oprescu et al., 2020), myotendinous
193 junction B myonuclei (Kim et al., 2020), Twist2+ cells (Liu et al., 2017), fetal MuSCs (Tierney et al.,
194 2016), developing limb connective tissues (Lima et al., 2021) and the skeletal muscle mesenchyme
195 subpopulation identified in human fetal limb (Xi et al., 2020). In contrast, TA progenitors displayed a
196 higher score for myogenic commitment and differentiation (**Suppl Figure 4A**).

197 Finally, we focused on PDGFR β , for which FACS antibodies exist, to validate the potential link between
198 mesenchymal EOM features and *in vitro* expansion capacity. Flow cytometry analysis corroborated the
199 scRNA-seq and immunofluorescence data, where PDGFR β was enriched in EOM samples (**Suppl**
200 **Figure 4B, C**). To distinguish the functional properties of PDGFR β ⁺ and PDGFR β ⁻ cells, we pulsed
201 them with EdU (**Figure 4G**). Notably, PDGFR β ⁺ cells had a significantly higher proliferative capacity
202 (**Figure 4H-J**). While the percentage of MYOD⁺ cells was unchanged between the two cell populations,
203 MYOG was much more abundant in the PDGFR β ⁻ fraction (**Figure 4K, L**). Therefore, the PDGFR β ⁺
204 myoblasts are characterised by a higher proliferative potential and decreased differentiation status.
205 Altogether, our analysis showed that EOM progenitors have an unusual transcriptome profile, express
206 a wide range of ECM-related factors, and harbour a mesenchymal signature.

207 **EOM transcriptome profile is associated with a unique transcription factor network**

208 To further investigate myoblast heterogeneity, we inferred single cell regulatory networks using
209 pySCENIC (Aibar et al., 2017; Sande et al., 2020) where co-expression patterns and transcription factor
210 binding motifs identify "regulons" (transcription factors and putative targets) (**Figure 5A, Suppl Table**
211 **6**). As expected, regulons associated with myoblast differentiation such as *Myod*, *Myog* and *Mef2* family
212 were specifically active in differentiated cells of EOM and TA (**Figure 5B**). Of note, the top 5 regulons
213 of TA progenitors were also found to be active in EOM progenitors, whereas the top 5 regulons of EOM
214 progenitors were unique to this cluster (**Figure 5B**). EOM progenitors displayed unique regulons
215 involved in connective tissue and ECM remodelling including *Egr1* (Havis and Duprez, 2020) and
216 *Creb3l1*, a downstream effector of thyroid hormone signaling (García et al., 2017; Sampieri et al., 2019).
217 Top regulons of EOM progenitors are also involved in cell proliferation (*Foxc1* (Yang et al., 2017), *Sox4*
218 (Moreno, 2019), *Fos* (Almada et al., 2021), *Klf6* (Dionyssiou et al., 2013), *Ebf1* (Györy et al., 2012)),
219 commitment into endothelial and smooth muscle fates (*Foxc1* (Han et al., 2017; Mayeuf-Louchart et al.,
220 2016; Whitesell et al., 2019; Yang et al., 2017)) as well as differentiation into mesenchymal lineages
221 (*Ebf1*, (El-Magd et al., 2021; Jimenez et al., 2007; Pagani et al., 2021). Of note, some EOM regulons
222 were already enriched in quiescent EOM MuSCs, and their expression was upregulated upon activation
223 (**Suppl Figure 5A, B**).

224 We then built a network restricted to transcription factors (**Figure 5C**), where each node (circle) is an
225 active transcription factor and each edge (distance between nodes) is an inferred regulation between 2
226 transcription factors (Grimaldi et al., 2022). When placed in a force-directed environment (see Methods),
227 these nodes aggregated based on the number of shared edges thereby highlighting associated and co-
228 regulating transcription factor modules. Strikingly, the transcription factors of the most specific regulons
229 of each cluster preferentially organised as tightly related modules (**Figure 5C**). In agreement with our
230 previous analyses, known co-regulating transcription factors in differentiated cells (*Myod*, *Myog*, *Mef2a*,
231 *Mef2c*, *Myf6*) formed a tight module (**Figure 5C**). The TA module was composed of genes required for
232 limb embryonic development (*Hox* genes, *Lbx1*) (Gross et al., 2000; Swinehart et al., 2013), where
233 *Hoxa11*, is a determinant of embryonic limb identity (Zakany and Duboule, 2007), and *HoxA* and *HoxC*

234 clusters are signatures of adult TA MuSCs (Evano et al., 2020; Yoshioka et al., 2021) (**Figure 5C, Suppl**
235 **Figure 5B**).

236 The EOM module included *Foxc1*, *Egr1*, *Creb3l1*, *Dmrta2*, *Sox4*, *Fos* and *Egr1* together with *Pax7* and
237 *Hes1* which support MuSC quiescence (Baghdadi et al., 2018; Mourikis et al., 2012; Olguin and Olwin,
238 2004; Relaix and Zammit, 2012). However, this network persists in proliferating EOM progenitors as
239 assessed by the higher levels of PAX7, CCND1 (Cyclin D1), together with EOM TFs (FOXC1, EBF1
240 and CREB3L1) at the protein level (**Suppl Figure 5C, D**). Although *Fos* and *Egr1* act as stress
241 signatures following tissue dissociation (Machado et al., 2021) in our dataset, the expression of these
242 genes (i.e. the StressIndex) correlated with *Pax7* expression and anti-correlated with *Myod* (**Suppl**
243 **Figure 5E, F**). Regression of the StressIndex or removal of these genes did not alter the general aspect
244 of the data (**Suppl Figure 5G**), pointing at a role for these genes also in the EOM progenitor
245 maintenance.

246 **EOM features are present during the growth phase *in vivo* and retained upon passages *in vitro***

247 Given that EOM MuSCs possess an unusual transcriptomic state and delayed myogenic commitment,
248 we examined these features following passage of MuSCs isolated from *Tg:Pax7-nGFP;Myog^{ntdTom}* *in*
249 *vitro* (**Figure 6A, Suppl Figure 6A, B**). While the total cell number was reduced with passages for both
250 EOM and TA (**Suppl Fig 6A, B**), the normalised cellular output was consistently higher for the EOM and
251 correlated with a lower tdTOM/GFP ratio (**Figure 6B, C**). Real-time qPCR and protein analysis showed
252 that expression of *Pax7* and *Hey1*, a bHLH transcription factor that is required for MuSC maintenance
253 (Noguchi et al., 2019), EOM specific regulon TFs (*Foxc1*, *Sox4*, *Ebf1*, *Creb3l1*) and genes identified by
254 the matrisome or molecular functions (*Bgn*, *Sparc*, *Igfbp2*, *Igfbp7*, *Pdgfrb*) were all retained or even
255 increased in whole EOM cell populations (mix of GFP+ and tdTOM+ cells) after several passages
256 (Figure 6D-G), and specifically in the GFP+ fraction (**Suppl Figure 6C-E**). These results show that EOM
257 MuSCs cells retain a cell autonomous non-canonical signature that is hard-wired even after extended
258 cell culture and that is not present in the TA.

259

260 Next, we assessed whether EOM progenitor features were already present in their activated state *in*
261 *vivo*, for instance, in fetal development and postnatal stages, where extensive muscle growth and
262 myogenic cell expansion occurs (Gattazzo et al., 2020a; Relaix and Zammit, 2012) or if these features
263 were only acquired in adulthood upon reactivation from the quiescent state *in vitro*. First, we isolated
264 EOM and TA MuSCs at E18.5 and P21 and showed that here too, EOM cells were less differentiated
265 upon activation *in vitro* (**Suppl Figure 7A**). Then, we examined activated MuSCs directly *in vivo* by RT-
266 qPCR. Given that GFP protein persists during myogenic commitment (Sambasivan et al., 2009) and this
267 might introduce a bias in the initial populations, we isolated by GFP+/tdTOM- fractions from *Tg:Pax7-*
268 *nGFP;Myog^{ntdTom}* mice at P7-P10 (**Figure 7A, Suppl Figure 7B**). Similarly, to the *in vitro* sc-RNAseq
269 data, RT-qPCR revealed significantly lower levels of *Myogenin* and higher transcript levels for EOM
270 specific regulon TFs (*Foxc1*, *Ebf1*, *Sox4*, *Creb3l1*) and matrisome components on the activated EOM
271 progenitors (**Figure 7B**). Together with our live-imaging (Figure 1G-I), FACS (Suppl Figure 1E) and sc-

272 RNAseq data (Figure 2), this analysis suggests that EOM MuSCs repress myogenic commitment and
273 appear to maintain a more "stem-like" state upon activation, and this property is conserved from
274 development to adulthood.

275 **Foxc1 marks the EOM MuSC lineage and plays a role in progenitor cell maintenance**

276 We then focused on *Foxc1* for further analysis as it is one of the top regulons and DEG of the activated
277 and quiescent sc-RNAseq dataset (**Figure 5 A, B, Suppl Figure 3B, Suppl Figure 5B, D**) and we
278 previously identified *Foxc1* as a DEG and top regulon of EOM progenitors in the early embryo
279 (E11.5,(Grimaldi et al., 2022)). Notably, bulk RNAseq (Terry et al., 2018) also showed higher *Foxc1*
280 expression in entire EOMs compared to other adult muscle groups (**Suppl Fig 7C**). As such, *Foxc1* is
281 a good candidate for determining EOM properties throughout the myogenic lineage continuum and
282 across developmental states.

283 First, we validated FOXC1 protein expression *in vivo* and *in vitro*. As expected, immunostaining on tissue
284 sections of E12.5 *Myf5^{Cre};R26^{tdTom}* embryos showed expression of FOXC1 in 54.7% of EOM myogenic
285 progenitors but not in limb, back, nor masseter muscles (**Suppl Figure 7D, E**). Analysis of
286 *Pax7^{CreERT2};R26^{tdTom}* mice showed FOXC1 expression in postnatal (P10) and adult EOM MuSCs (**Figure**
287 **7C-E**) but not in the TA muscles. FOXC1 was strongly expressed in adult EOM MuSC cultures (**Suppl**
288 **Figure 7F-H**). FOXC1 was expressed in about 75% of PAX7+ cells at D5, expression was progressively
289 lost during differentiation and the majority of the FOXC1+ cells incorporated EdU at every time point
290 (**Suppl Figure 7I, J**). Thus, differences in *Foxc1* expression between the EOM and TA appear to arise
291 during development and persist upon activation.

292 As differences in *Myogenin* mRNA between EOM and TA were already evident at D2 following *in vitro*
293 activation (**Figure 1J**), we used this early timepoint to functionally validate a potential role for *Foxc1* in
294 progenitor maintenance. Thus, we silenced *Foxc1* using siRNA in EOM MuSCs right after FACS (**Suppl**
295 **Figure 7K**). The total cell number and percentage of PAX7+ cells were not changed 2 days after
296 silencing, despite effective downregulation of FOXC1 at the protein level (**Suppl Figure 7L-L''**) and
297 transcript level (**Suppl Figure 7M**). Yet, a 2.7-fold increase in *Myogenin* expression was detected by
298 RT-qPCR following silencing (**Suppl Figure 7M'**). Given that the effect of siRNAs was only transient,
299 we transduced EOM activated MuSCs with lentiviruses expressing different short-hairpin RNAs (shRNA)
300 against *Foxc1* to be able to examine later timepoints (**Suppl Figure 7N**). Immunofluorescence and EdU
301 uptake at D5 showed an efficient depletion of FOXC1 protein, and concomitant severe reduction in the
302 total cell number and percentage of EdU+ cells (**Suppl Figure 7O-P**). While lower number of myotubes
303 were formed in shRNA FOXC1 TA cells, the fusion index was not significantly different (**Suppl Figure**
304 **7Q, R**).

305

306 To assess if *Foxc1* would confer some EOM features to TA, we overexpressed this gene in activated
307 TA MuSCs cells (**Figure 7F**) using gain-of-function (GOF) FOXC1 lentiviruses carrying mCherry as
308 reporter that allow to re-isolate the transduced cells (**Figure 7G, H**). Notably, there was robust

309 upregulation of FOXC1 (15-30x) at D5 (**Figure 7I, J, K', N**) and many *Foxc1* direct targets (**Suppl Table**
310 **7**), including matrisome components (e.g. *Sparc*, *Pdgfr β* , *Fbn1*) and other EOM specific regulon TFs
311 (e.g. *Ebf1*, *Creb3l1*, *Egr1*). Properties of re-isolated GOF cells (**Figure 7K-Q**) included a larger cellular
312 and nuclear area (**Figure 7K, K', O**) and less fusogenic potential (**Figure 7L, M**). Unexpectedly, FOXC1
313 overexpression also resulted in downregulation of *Pax7* and *Myod*, less cells/well and reduced EdU
314 uptake (**Figure 7J, P, Q**). These phenotypes might be the result of strong overexpression and cells
315 acquiring a strong mesenchymal character. We thus investigated the FOXC1 GOF condition in “mixed
316 cultures” which contain transduced (FOXC1+mCherry+) and non-transduced (FOXC1-mCherry-) TA
317 cells (**Figure 7F, R-Y**). This strategy allowed us to assess the effect of secreted factors produced by
318 FOXC1+ cells on non-transduced cells. As expected for mixed cultures, FOXC1 fluorescence intensity
319 showed a bimodal distribution, with one population being as negative control and the other expressing
320 high levels of the protein (**Figure 7R**). Interestingly, the total number of nuclei in the mixed GOF wells
321 was higher than controls (**Figure 7T**), which contrasts with the result in pure GOF cultures (**Figure 7P**).
322 Thus, we evaluated the individual cellular properties of transduced and non transduced cells in the
323 mixed GOF cultures. While FOXC1+ cells showed higher nuclear area and reduced EdU uptake as in
324 pure GOF cultures, overexpression of FOXC1 led to an augmentation in both the number and
325 percentage of proliferating cells within the non-transduced population (compare FOXC1+/-
326 subpopulations in **Figure 7S, U** with **Figure 7O, Q**).

327

328 Finally, we performed live imaging on FOXC1 GOF mixed cultures (**Figure 7F**). While starting with
329 control and GOF cultures with similar transduction efficiencies (88% and 80% mCherry+ cells at 12h,
330 respectively) (**Figure 7V**), we found that the density of mCherry+ cells in GOF wells was reduced by
331 half of that of control wells by 48h (**Figure 7X, Movie 1 and 2**), with a concomitant increase in the density
332 of mCherry- cells (**Figure 7Y, Movie 1 and 2**).

333 Therefore, our data suggest a role for the FOXC1-induced secretome in cell proliferation of adjacent
334 cells (**Figure 7Z**). Importantly, overexpression of *Creb3l1*, another EOM regulon TF, in TA MuSCs
335 (**Suppl Figure 7S**) did not show alterations in *Pax7*, *Myod*, most matrisome genes examined, nor the
336 cellular properties (**Suppl Figure 7S-Z**). Altogether, our findings support the notion that physiological
337 levels of *Foxc1* in EOM MuSCs may allow their maintenance in a progenitor like state, likely through
338 secreted factors.

339 **Transcription dynamics expose EOM and TA disparities in progenitor-state maintenance**

340 We then performed more detailed bioinformatic analysis of the matrisome by correlating the number of
341 matrisome-driving regulons between EOM and TA upon activation (**Figure 8A-C, Suppl Table 8**). EOM
342 MuSCs consistently regulated a higher number of matrisome genes than TA cells (**Figure 8A**). The ratio
343 of the number of regulations of matrisome genes between EOM and TA activated MuSCs showed that
344 the difference was maximal when considering the top 5 regulons, including as expected *Foxc1* together
345 with *Alx4*, *Dmrta2*, *Zmiz1* and *Fos* (**Figure 8B, C**). Interestingly, *Foxc1*, *Dmrta2* and *Fos* were also active
346 regulons in quiescence, with slight disparities between EOM and TA (**Suppl Table 9**).

347 Next, we set out to determine whether matrix genes underlie the transition towards progenitors and
348 committed cells during activation using scVelo (Bergen et al., 2020). This method computes local
349 changes in the relative number of unspliced/spliced variants (Manno et al., 2018) and identifies
350 candidate "driver genes", i.e. most transcriptionally dynamic and responsible for the inferred velocity
351 (**Figure 8D, E**). Two distinct velocity streams stood out in both datasets, towards differentiation
352 (*Myog*^{High}) and towards a progenitor-state (*Pax7*^{High}). Strikingly, a larger fraction of EOM cells appeared
353 to transition towards a progenitor state compared to TA (**Figure 8D, E**). These trajectories did not appear
354 to be specifically correlated with cell cycle phases (**Suppl Figure 8 A, B**), which was shown to influence
355 transcriptomic data in some cases (McDavid et al., 2016). Hence, the velocity streams observed are
356 most likely reflect transitions between distinct cell states instead of the cell cycle progression of a
357 homogeneous cell state.

358 Using scVelo built-in functions, we extracted the top driver genes underlying the velocity towards the
359 progenitor state in both datasets (**Figure 8F-G, Suppl Table 10, 11**). Out of the top 100 driver genes,
360 30 were common to both datasets, including *Col5a1*, which plays a critical role in maintenance of
361 quiescence (Baghdadi et al., 2018) (**Figure 8F,G**). Interestingly, several matrisome components
362 including *, other collagens, and *Igfbp7* (Insulin growth factor binding protein 7), were unique to EOM.
363 Of note, IGFs bind and regulate insulin-like growth factors (IGFs) thereby repressing myogenic
364 differentiation but they also have IGF-independent activity (Clemmons, 1997; Engert et al., 1996; Jin et
365 al., 2020). GO molecular functions associated with these driver genes during myogenic progenitor
366 maintenance showed that this transition was characterised by active upregulation of ECM components
367 specifically in EOM, in agreement with our previous results (**Suppl Figure 8C**).*

368 We then performed conditioned media (CM) exchange experiments to gain functional insights into the
369 role of EOM secreted factors by culturing hindlimb cells in presence of EOM-CM, TA-CM or control
370 media for 3 days (**Figure 8H**). In agreement with the FOXC1 GOF experiments (**Figure 7R-Z**) EOM-CM
371 induced a ~5x increase in the total number of nuclei/well, whereas no significant differences were
372 observed with TA-CM and control media (**Figure 8I**). Further, the percentage of EdU+ cells remained
373 unchanged (**Figure 8J**) suggesting that EOM secreted factors may delay differentiation.

374 To assess if mesenchymal features of EOM cells were recapitulated *in vivo*, we asked whether bipotent
375 myogenic progenitors that we previously identified in EOM to give rise to myogenic and connective
376 tissue cells during embryogenesis (Grimaldi et al., 2022) persisted postnatally. Examination of
377 *Myf5^{nlacZ};Pdgfra^{H2BGFP}* mice, where *Myf5^{nlacZ}* allows tracing myogenic progenitors and progeny, and GFP
378 labels mesenchymal connective tissue cells, confirmed the presence of β gal+/GFP+ cells in the EOM
379 but not in masseter and tongue muscles in the head (**Figure 8K, L**). Surprisingly, analysis of EOM and
380 TA MuSCs and fibroadipogenic cells (FAPs) identified a hybrid population positive for both Sca-1 (FAPS
381 marker, (Joe et al., 2010)) and GFP, only in EOM samples (8% of total GFP+ population; **Suppl Figure**
382 **8D-F**). We then isolated FAPS, MuSCs, and the SCA-1+GFP+ cells from the EOM, and performed RT-
383 qPCR, for *Pdgfra*, *Pdgfrb* and *Col1a2* (**Suppl Figure 8G**). Notably, the Sca-1+GFP+ progenitor
384 population showed an intermediate pattern of expression of these matrisome genes between that of

385 MuSCs and FAPS, suggesting a mesenchymal signature that exclusively belongs to EOM MuSCs
386 subsets. Finally, differential gene expression analysis of a publicly available sc-RNAseq dataset filtering
387 on our EOM-specific regulon TFs, highlighted *Egr1* and *Foxc1* as upregulated in dystrophic EOM (**Suppl**
388 **Figure 8H**), a condition where EOM MuSCs were described to be proliferative (Taglietti et al., 2023).
389 Altogether, this *in vivo* data corroborated the results obtained by bioinformatic analysis on our *in vitro*
390 activated MuSC dataset.

391 **DISCUSSION**

392 An unusual feature of skeletal muscle stem cells is their reliance on distinct gene regulatory networks
393 (GRNs) in different anatomical locations. Most studies on myogenesis have focused on trunk and limb
394 muscles and only a handful of transcription factors and signalling pathways have been identified as
395 hallmarks of specific muscle groups. Here, we used multiple approaches and identify unusual features
396 and specific GRNs that functionally distinguish EOM from TA MuSCs.

397 **EOM MuSCs are more refractory to *in vitro* differentiation**

398 By monitoring *Myog* and *Myod* expression, we showed that EOM MuSCs have a lower propensity to
399 differentiate following activation and persist as a proliferative population. Given the pivotal role of *Myod*
400 in commitment and differentiation (Vicente-García et al., 2022), the lower levels of *Myod* transcript and
401 protein in activated EOM MuSCs even upon upregulation of the *Myog*^{tdTom} reporter is intriguing and
402 deserves further investigation. As foetal and early postnatal EOM MuSCs are also refractory to
403 differentiation, we propose that this property might be hardwired by unique GRNs that are retained
404 throughout development and adulthood. Notably, trunk foetal MuSCs were shown to be more resistant
405 to myogenic progression upon *in vitro* expansion and contribute more efficiently upon transplantation than
406 the adult counterparts (Sakai et al., 2013; Tierney et al., 2016). In addition, limb MuSCs cells isolated at
407 birth displayed prolonged expansion and delayed fusion compared with those in the adult (Gattazzo et
408 al., 2020b). Similarly, EOM MuSCs showed a reduced fusion index compared to those in adult limb.
409 Altogether our data suggest that EOM MuSCs retain features of respective foetal and neonatal
410 myogenic cells.

411 Major obstacles for cell-based therapies are the large cell numbers required for transplantation and the
412 fact that *ex vivo* amplification of somite-derived MuSCs leads to a drastic decline in regenerative
413 potential due to commitment to differentiation (Briggs and Morgan, 2013; Ikemoto et al., 2007; Montarras
414 et al., 2005). While significant progress has been made (Charville et al., 2015; Ishii et al., 2018; L'honoré
415 et al., 2018; Xie et al., 2021), the identification of factors that regulate cell fate decisions in distinct MuSC
416 populations is another resource for advancing knowledge in this context.

417 **EOM progenitors exhibit a mesenchymal genetic signature upon activation**

418 scRNA-seq analyses provided some insights into the transcriptional landscape regulating MuSC
419 quiescence, activation and self-renewal in somite-derived muscles (Dell'Orso et al., 2019; Hernando-

420 Herraiez et al., 2019; Machado et al., 2021; Micheli et al., 2020; Yartseva et al., 2020). For cranial
421 MuSCs, our scRNA-seq analysis of activated EOM and TA MuSCs showed distinct transcriptional
422 profiles that divided myoblasts into two subpopulations: those that resembled progenitors and those
423 differentiating. The EOM progenitor population was characterised by higher *Pax7*, reminiscent of *in vitro*
424 reserve cells (Laumonier et al., 2017; Yoshida et al., 1998; Zammit et al., 2004) and ontology analysis
425 of its DEGs showed an enrichment in ECM organisation processes and *Pdgf* signalling.

426 Transcriptomic analysis of trunk MuSCs highlighted a dynamic profile of *Pdgf* ligands and receptors
427 during myogenesis (Contreras et al., 2021). Treatment with NOTCH and PDGFR β ligands (DLL4 and
428 PDGF-BB respectively) enhanced migration, expression of stem cell markers and perivascular-like
429 features in MuSCs (Gerli et al., 2019) and embryonic myoblasts (Cappellari et al., 2013). Notably also,
430 heterogeneity of activated MuSCs was noted during regeneration, including a transitional Notch2-high
431 state and an ECM-high state that regulates self-renewal (Yartseva et al., 2020). As PDGFR β and several
432 Notch pathway components (e.g. Notch1, Notch3, Hey1) are co-expressed in activated EOM
433 progenitors, and at significantly higher levels than in TA muscle, we speculate that cross-talk between
434 these pathways could take place in this subpopulation.

435 **Putative role of ECM secretion and remodelling by activated EOM MuSCs**

436 Self-renewal in the muscle lineage is dependent on cell-autonomous expression, deposition, and
437 remodelling of ECM components such as Fibronectin (Bentzinger et al., 2013; Lukjanenko et al., 2016;
438 Tierney et al., 2016), Col VI (Urciuolo et al., 2013) and Laminin- α 1 (Rayagiri et al., 2018). EOM
439 progenitors relate more to fetal MuSCs with respect to enrichment in matrisome components such as
440 Fn1, Fbn1, Vcam and collagens (Tierney et al., 2016). Notably, an extensive and more complex ECM
441 is observed in EOMs *in vivo* (McLoon et al., 2018). Therefore, we hypothesize that *in vitro* activated
442 EOM MuSCs secrete high amounts of ECM and self-autonomously maintain stemness when removed
443 from their niche. For instance, FAPs exert a supportive role in myogenesis by secreted matrix and
444 cytokine components (Joe et al., 2010; Kotsaris et al., 2023; Murphy et al., 2011; Uezumi et al., 2014).
445 Myogenic cells expressing mesenchymal markers were also reported in human embryos and from
446 human iPSCs *in vitro* (Xi et al., 2020). In the EOM, this plasticity was observed *in vivo*, where EOM
447 myogenic progenitors transition towards non-myogenic cell fates (Grimaldi et al., 2022). Here, we have
448 extended these observations to the early postnatal period, where considerable proliferation and fusion
449 occurs. We did so by using using *Pdgfra*^{H2BGFP}; *Myf5*^{nlacZ} mice and the identification of a subpopulation
450 of EOM GFP+ cells co-expressing the mesenchymal stem cell marker SCA-1 *in vivo*, which is normally
451 used as a negative marker of MuSCs (Liu et al., 2015).

452 **A unique network of transcription factors maintains EOM progenitors**

453 Most head regulon genes that we identified in EOM progenitors are not typical myogenic TFs. One of
454 the most active regulons was *Foxc1*, a pro-mitogenic factor in cancer (Yang et al., 2017) and driver of
455 endothelial/smooth muscle fates (Lagha et al., 2009; Mayeuf-Louchart et al., 2016; Whitesell et al.,

456 2019). Another top regulon was *Egr1*, which promotes expression of many ECM-related genes (Gaut et
457 al., 2016; Milet et al., 2017). Interestingly, *Foxc1* and *Ebf1* are active transcription factors underlying the
458 transition of myogenic towards non-myogenic cell fates in embryonic EOM (Grimaldi et al., 2022). As
459 *Foxc1* is expressed in quiescent and activated EOM MuSCs, this gene might reinforce a
460 progenitor/stem-cell identity throughout the lineage. Interestingly, FOXC1 overexpression in TA MuSCs
461 resulted in upregulation of *Egr1* and downregulation of *Ebf1* in support our bioinformatic analysis
462 indicating that these TFs form a co-regulatory module.

463 As predicted by our analysis *in silico*, FOXC1 overexpression in TA MuSCs upregulated several EOM
464 matrisome components and downregulated *Myod*, in accordance with a previous study (Wright et al.,
465 2021), thereby promoting a less committed state. Given that *Pitx2* appears as a target of *Foxc1* in our
466 studies (Suppl Table 7) and its overexpression promotes MuSC proliferation and enhances the
467 regenerative potential of dystrophic MuSCs (Vallejo et al., 2018), we expected an increase in the nuclear
468 output when overexpressed in TA MuSCs. Yet, we observed the opposite phenotype in pure TA GOF
469 cultures, thereby raising several possibilities. First, it was reported that sustained expression of FOXC1
470 can induce stem cell quiescence in the skin (Lay et al., 2016; Wang et al., 2016). Alternatively, a strong
471 upregulation of FOXC1 might have induced an irreversible drift towards a mesenchymal phenotype in
472 hindlimb MuSCs as denoted by the reduced expression of *Pax7*. Yet, our mixed GOF cultures also
473 suggest that the FOXC1-dependent secretome can promote proliferation of adjacent non-transduced
474 cells. Finally, as *Foxc1* is part of a co-regulatory module in the EOM its singular overexpression might
475 not be sufficient to confer the full EOM phenotype to limb cells. Future studies should assess the role of
476 this TF in EOM progenitor emergence and disease.

477 **Conclusion**

478 Using in-depth bioinformatic analysis, *in vitro* approaches, and analysis of expression patterns *in vivo*,
479 we propose a model where the outperformance of EOM MuSCs depends on the expression of a tightly
480 associated module of transcription factors regulating a distinct pattern of ECM-remodelling factors, cell
481 receptors and growth factor binding proteins. These components define the pace at which EOM MuSCs
482 progress through the myogenic lineage and maintenance of a stem-like population. As such, our study
483 lays the groundwork for elucidating the mechanisms of selective sparing of muscle groups in dystrophic
484 disease by providing information on a unique core GRN within this muscle group.

485 **Materials and methods**

486

487 **Animal care**

488 Animals were handled according to national and European Community guidelines and an ethics
489 committee of the Institut Pasteur (CETEA) in France approved protocols. Except when indicated
490 otherwise, males and females of 2-4 months were used. *Tg:Pax7-nGFP* (Sambasivan et al., 2009),
491 *Myf5^{Cre}* (Halder et al., 2007), *Myf5^{nlacZ}* (Tajbakhsh et al., 1996) *Myog^{ntdTom}* (Benavente-Diaz et al., 2021),

492 *Pdgfra*^{H2BGFP} (Hamilton et al., 2003), *Pax7*^{CreERT2} (Mathew et al., 2011) and *R26*^{tdTom} (Madisen et al.,
493 2009) mouse lines were maintained in a C57Bl/6Jrj background. To induce recombination of
494 *Pax7*^{CreERT2};*R26*^{tdTom} mice a 20 mg/ml stock solution of Tamoxifen was prepared in 5% ethanol and 95%
495 sunflower seed oil by thorough resuspension with rocking at 4 °C. For adult mice, 2 mg of tamoxifen
496 (Sigma #T5648) were administered by gavage during 5 consecutive days and animals sacrificed 5 days
497 later. To induce recombination of pups, the Tamoxifen stock solution was diluted to 15mg/ml with
498 sunflower seed oil and 0.15 mg were administered daily by subcutaneous injection between P4 and P6
499 daily. Pups were sacrificed at P10 by decapitation and adult mice by cervical dislocation.

500

501 **Muscle stem cell culture, treatment and transfection**

502 MuSC isolation was performed as described (Gayraud-Morel et al., 2017) with some modifications
503 indicated in Supplemental Methods. Cells isolated by FACS were cytopun (3 min at 50g at RT) and
504 plated onto Matrigel® (Corning, 354248) coated dishes and cultured in growth media at 3% O₂, 5%
505 CO₂, 37°C for the indicated time. Half volume of media was changed every second or third day. To
506 assess proliferation, cells were pulsed with 10⁻⁶ M of EdU (ThermoFisher, C10640) in cell culture media
507 for 2h prior to fixation. To induce myogenic differentiation and fusion, myoblasts were plated at high
508 density (33,000 in Figure 7M, Suppl Figure 7R, Z or 75,000 cells/cm² in Suppl Figure 1H) onto Matrigel-
509 coated plates in the growth medium. Once adherent, cells were changed to differentiation medium
510 (DMEM with 5% serum and 1% P/S).

511 Conditioned media (CM) experiment were performed isolating EOM and TA MuSCs by FACS based on
512 GFP fluorescence from *Tg:Pax7-nGFP* mice, and collecting the media 3 days post plating. Hindlimb
513 MuSCs were cultured in growth media (control) or treated with CM from day 1 to day 4 post plating
514 before analysis.

515 For loss of function experiments, freshly isolated satellite cells from *Tg:Pax7-nGFP* mice were
516 transfected in suspension immediately after FACS with the ON-TARGET plus SMARTpool against
517 FOXC1 (Dharmacon, L-047399-01-0005) or Scramble (Dharmacon, ON-TARGETplus Non-targeting
518 Control siRNA, D0018100205) at 200 nM final concentration using Lipofectamine 3000 (ThermoFisher,
519 L3000001) in Opti-MEM (Fisher Scientific, 11564506) as described by the manufacturer. Briefly, a pre-
520 mix of siRNA/Optimem (1.5ul /20ul) and Lipofectamine3000/Optimem (0.3ul / 20ul) were incubated
521 separately 5 min RT, mixed at 1:1 ratio and incubated 15 min more at RT. 2.10⁴ cells in 40ul of Optimem
522 were incubated with an equal volume of the transfection mix for 2h at 3% O₂, 5% CO₂, 37°C in
523 Eppendorf tubes whose caps had had been punctured with a needle to allow gas exchange. Two hours
524 after transfection, three volumes of fresh growth medium were added and cells were plated at 10k/cm²
525 in Matrigel coated wells containing growth media. Two days upon transfection wells were processed for
526 immunostaining or RNA collected with Tryzol as above. Details on immunostaining, RT-qPCR and
527 Western Blot are described in Supplemental Methods.

528

529 **Lentivirus transduction for gain and loss of function experiments**

530 For GOF assays, in-lab made lentiviral viruses (control (PLVX-CAG-P2A-mCherry) and Foxc1-mCherry
531 (PLVX-CAG-Foxc1-P2A-mCherry), CAG promoter) and viruses produced by VectorBuilder

532 (<https://en.vectorbuilder.com/>) (control (pLVX-EF1A>mCherry), Foxc1-mCherry (pLVX-EF1A>mFoxc1-
533 T2A-mCherry), Creb3l1-mCherry (pLVX-EF1A>mCreb3l1-T2A-mCherry), EF1a promoter) were used.
534 T2A or P2A causes co-translational cleavage of the encoded polypeptide (Supplementary Table 12).
535 GOF assays of FOXC1 with in-lab or vector builder made viruses were used interchangeably as they
536 gave comparable results. For LOF assays, three different Foxc1-shRNA vectors were tested with
537 identical results (U6 promoter for shRNA and hPGK for mCherry reporter).

538
539 Freshly isolated satellite cells from *Tg:Pax7-nGFP* mice were plated in matrigel coated wells, cultured
540 overnight and transduced at an MOI (multiplicity of infection) of 100 in 45µl (for 96 well plates) or 125µl
541 (for 48 well plates) of MuSC media containing 5 µg/ml of polybrene. After 4h incubation at 37°C, cells
542 were washed three times with media and cultured for 4 more days in GM prior to fixation for
543 immunostaining, protein or RNA collection, or re-sorting of mCherry+ when needed.

544

545 **Image analysis**

546 For cells grown on 96 wells, images from the Opera Phenix high-content microscope were quantified
547 using Harmony software and a semi-automated pipeline. Nuclei were detected based on Hoechst signal
548 and mean intensity of fluorescence were automatically quantified on the nuclear region for nuclear
549 markers. Number of nuclei positive for a certain nuclear marker were scored on a manually defined
550 threshold. For cells grown on IBIDI 8 well plates, czi images were manually quantified in a blinded
551 manner using the point toll counter in Fiji (Schindelin et al., 2012) or using the TrackMate7 pipeline
552 (Ershov et al., 2022).

553 Immunostaining on ECM markers on cells were scanned using either the Opera Phoenix high-content
554 microscope (Perkin Elmer) or Zeiss LSM800 microscope with ZEN software. Imaging of tissue sections
555 was done on a Zeiss LSM800 microscope or a Ti2E Spinning disk (Nikon).

556 The fusion index was calculated as the fraction of nuclei contained within MF20+ myotubes, which had
557 two or more nuclei, as compared to the number of total nuclei within each image.

558 Figures were assembled in Adobe Photoshop, Illustrator and InDesign (Adobe Systems).

559

560 **Time lapse microscopy**

561 MuSC were plated on a microscopy culture chamber (IBIDI, 80826) or 96 wells of TC-Treated Black
562 µCLEAR plates (Greiner Bio-One 655090) and cultured in growth media. The plate was incubated at
563 37°C, 5% CO₂, and 3% O₂ (Zeiss, Pecon). A Zeiss Observer.Z1 connected to a Plan-Apochromat
564 20x/0.8 M27 objective and Hamamatsu Orca Flash 4 camera piloted with Zen (Zeiss) was used. After
565 activation for 3 days *in vitro*, EOM and TA cells were imaged every 12 min for ~48h. Individual cells
566 were tracked and tdTOM intensity was scored at selected time points. Tracking of *Myog*^{tdTom} cells was
567 performed using the Manual Tracking feature of the TrackMate plug-in (Tinevez et al., 2017) in Fiji.

568 For lentivirus live imaging experiments, detection of total cell numbers and mCherry positive cells was
569 done with a custom-made pipeline. To detect cell contours from the bright field images, we retrained the
570 « livecell » model of CellPose with CellPose 2.0 (Pachitariu and Stringer, 2022). The retraining dataset
571 was created from small crops of the full data with low density and high density regions and the different

572 cell phenotypes. To homogenize the data, preprocessing of the bright field channel was applied to all the
573 data: we enhanced the contrast locally with CLAHE (Contrast Limited Adaptive Histogram Equalization)
574 in Fiji (with a blocksize of 127, default parameters) and normalized the intensity (for each frame). To
575 assess which cells were infected by the virus (mCherry+), we measured the mean mCherry fluorescent
576 intensity in each cell normalised by the local mean fluorescent intensity in the well (by averaging the
577 fluorescent intensity with a radius around 300 μm). This allowed to compensate for differences in
578 illumination even within the same acquisition but can slightly under-estimate the relative intensity of cells
579 in very dense region. Finally, the mCherry intensity channel was thresholded after a background removal
580 with Fiji Triangle threshold method calculated with the histogram of the 4 time frames together.
581 The retrained models were then runned on the full data (cropped regions or full wells) to detect the cell
582 contours through the Fiji plugin Trackmate-CellPose (Ershov et al., 2022) which allowed us to directly
583 access measurements of the cells (« spots » table in TrackMate).

584

585 **Data analysis and statistics**

586 Data analysis, statistics and visualisations were performed using Prism (Graphpad Software) or using
587 R (Team, 2014) and the package ggplot2 (Wickham, 2009). Tests are described on Figure legends.
588 Information related to microarray data analysis and sc-RNAseq of DMD rat EOM are described in
589 Supplemental Methods.

590

591 **scRNAseq data generation**

592 MuSCs were isolated on BD FACSAria™ III based on GFP fluorescence and cell viability from *Tg:Pax7-*
593 *nGFP mice* (Sambasivan et al., 2009). Quiescent MuSCs were manually counted using a
594 hemocytometer and immediately processed for scRNA-seq. For activated samples, MuSCs were
595 cultured *in vitro* as described above for four days. Activated MuSCs were subsequently trypsinized and
596 washed in DMEM/F12 2% FBS. Live cells were re-sorted, manually counted using a hemocytometer
597 and processed for scRNA-seq.

598 Prior to scRNAseq, RNA integrity was assessed using Agilent Bioanalyzer 2100 to validate the isolation
599 protocol (RIN>8 was considered acceptable). 10X Genomics Chromium microfluidic chips were loaded
600 with around 9000 and cDNA libraries were generated following manufacturer's protocol. Concentrations
601 and fragment sizes were determined using Agilent Bioanalyzer and Invitrogen Qubit. cDNA libraries
602 were sequenced using NextSeq 500 and High Output v2.5 (75 cycles) kits. Count matrices were
603 subsequently generated following 10X Genomics Cell Ranger pipeline.

604 Following normalisation and quality control, we obtained an average of 5792 ± 1415 cells/condition.

605

606 **Seurat preprocessing**

607 scRNAseq datasets were processed using Seurat (<https://satijalab.org/seurat/>) (Butler et al., 2018).
608 Cells with more than 10% of mitochondrial gene fraction were discarded. 4000-5000 genes were
609 detected on average across all 4 datasets. Dimensionality reduction and UMAPs were generated
610 following Seurat workflow. The top 100 DEGs were determined using Seurat "FindAllMarkers" function
611 with default parameters. When processed independently (scvelo), the datasets were first regressed on

612 cell cycle genes, mitochondrial fraction, number of genes, number of UMI following Seurat dedicated
613 vignette, and doublets were removed using DoubletFinder v3 (McGinnis et al., 2019). A "StressIndex"
614 score was generated for each cell based on the list of stress genes previously reported (Machado et al.,
615 2021) using the "AddModule" Seurat function. 94 out of 98 genes were detected in the combined
616 datasets. UMAPs were generated after 1. StressIndex regression, and 2. after complete removal of the
617 detected stress genes from the gene expression matrix before normalization. In both cases, the overall
618 aspect of the UMAP did not change significantly (**Figure S5**). Although immeasurable confounding
619 effects of cell stress following isolation cannot be ruled out, we reasoned that our datasets did not show
620 a significant effect of stress with respect to the conclusions of our study.

621

622 **Matrisome analysis**

623 After subsetting for the features of the Matrisome database (Naba et al., 2015) present in our single-cell
624 dataset, the matrisome score was calculated by assessing the overall expression of its constituents
625 using the "AddModuleScore" function from Seurat (Butler et al., 2018).

626

627 **RNA velocity and driver genes**

628 Scvelo was used to calculate RNA velocities (Bergen et al., 2020). Unspliced and spliced transcript
629 matrices were generated using velocity (Manno et al., 2018) command line function. Seurat-generated
630 filtering, annotations and cell-embeddings (UMAP, tSNE, PCA) were then added to the outputted
631 objects. These datasets were then processed following scvelo online guide and documentation. Velocity
632 was calculated based on the dynamical model (using *scv.tl.recover_dynamics(adata)*, and
633 *scv.tl.velocity(adata, mode='dynamical')*) and differential kinetics calculations were added to the model
634 (using *scv.tl.velocity(adata, diff_kinetics=True)*). Specific driver genes were identified by determining the
635 top likelihood genes in the selected cluster. The lists of top 100 drivers for EOM and TA progenitors are
636 given in Supplemental Tables 10 and 11.

637

638 **Gene regulatory network inference and transcription factor modules**

639 Gene regulatory networks were inferred using pySCENIC (Aibar et al., 2017; Sande et al., 2020). This
640 algorithm regroups sets of correlated genes into regulons (i.e. a transcription factor and its targets)
641 based on binding motifs and co-expression patterns. The top 35 regulons for each cluster was
642 determined using scanpy "scanpy.tl.rank_genes_groups" function (method=t-test). Note that this
643 function can yield less than 35 results depending on the cluster. UMAP and heatmap were generated
644 using regulon AUC matrix (Area Under Curve) which refers to the activity level of each regulon in a given
645 cell. Visualizations were performed using scanpy (Wolf et al., 2018). The outputted list of each regulon
646 and their targets was subsequently used to create a transcription factor network. To do so, only genes
647 that are regulons themselves were kept. This results in a visual representation where each node is an
648 active transcription factor and each edge is an inferred regulation between 2 transcription factors. When
649 placed in a force-directed environment, these nodes aggregate based on the number of shared edges.
650 This operation greatly reduced the number of genes involved, while highlighting co-regulating
651 transcriptional modules. Visualization of this network was performed in a force-directed graph using

652 Gephi “Force-Atlas2” algorithm (<https://gephi.org/>). Of note, a force-directed graph is a type of
653 visualization technique where nodes are positioned based on the principles of physics that assign forces
654 among the set of edges and the set of nodes. Spring like attractive forces are used to attract pairs of
655 edges towards each other (connected nodes) while repulsive forces, like those of electrically charged
656 particles, are used to separate all pairs of nodes. In the equilibrium state for this system, the edges tend
657 to have uniform length (because of the spring forces), and nodes that are not connected by an edge
658 tend to be drawn further apart (because of the electrical repulsion).

659

660 **Gene ontology analysis**

661 Gene ontology analyses were performed on top 100 markers (obtained from Seurat function
662 FindAllMarkers) or on top 100 driver genes (obtained from scvelo), using Cluego (Bindea et al., 2009).
663 “GO Molecular Pathway”, and “REACTOME pathways” were used independently to identify common
664 and unique pathways involved in each dataset. In all analyses, an enrichment/depletion two-sided
665 hypergeometric test was performed and p-values were corrected using the Bonferroni step down
666 method. Only the pathways with a p-value lower than 0.05 were displayed.

667

668 **Literature scores**

669 The scores for SMMCs (Giordani et al., 2019), FAPs (Oprescu et al., 2020), myotendinous junction B
670 myonuclei (Kim et al., 2020), Twist2+ population(Liu et al., 2017), fetal MuSCs (Tierney et al., 2016),
671 developing limb connective tissues (Lima et al., 2021) and the human skeletal muscle mesenchyme (Xi
672 et al., 2020) were calculated by assessing the overall expression of the markers of each population
673 (Supplemental Table 5) using the "AddModuleScore" function from Seurat (Butler et al., 2018).

674

675 **Code availability and Data availability**

676 The code that was used to generate the TF network is available at this address:
677 <https://github.com/TajbakhshLab/TFnetwork>. scRNAseq datasets are available in open access on
678 DRYAD at the following address: (to be updated if manuscript accepted). Raw data scRNAseq
679 sequencing data have been deposited in the NCBI Gene Expression Omnibus database with the
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681

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695

696 **Author contributions**

697 MBD, GC, and ST conceived the study. DDG, MM, MDB, PTL and GC performed experiments. PTL,
698 SG, MK and BE contributed to *in vitro* experiments and image acquisition. AG, MBD and DDG performed
699 sc-RNAseq. AG and MBD run the bioinformatic analysis with help from SM and VL. JYT and GL
700 performed image analysis. AG, MBD and GC wrote the manuscript with input from DDG, MM and ST.
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1130 **FIGURE LEGENDS**

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Figure 1. Functional differences between EOM and TA MuSCs following activation.

- A. Experimental scheme. Adult MuSCs isolated from *Tg:Pax7-nGFP* mice were plated in growth media (GM) and pulsed with EdU before fixation and immunostaining at Day (D) 3, D4, D5 and D10.
 - B. Immunofluorescence for MF20, PAX7 and EdU detection as per in A. Scale bar 100 μ m.
 - C-D. Bar plots of total number of nuclei and EdU+ nuclei represented as EOM/TA fold change (C) and percentage of EdU+ cells/total number of nuclei at D3, D4, D5. N>3 independent experiments with n \geq 3 mice per experiment.
 - E. Quantification of the percentage of Pax7+ cells/total number of nuclei at D10. n \geq 3 mice.
 - F. Quantification of the Fusion Index at D10. n=4 mice.
 - G. Experimental scheme. Live imaging of adult EOM and TA MuSCs from *Tg:Pax7-nGFP;Myog^{ntdTom}* from D3 *in vitro*.
 - H. Representative overlaid DIC and red fluorescence channel images at selected time points. Scale bar 25 μ m.
 - I. Percentage of tdTom+ cells over total cell number at each timepoint. n=4 mice.
 - J. RT-qPCR on *in vitro* activated adult EOM and TA MuSCs for *Pax7*, *Myod*, *Myogenin* and *p21* normalized to *Rpl13*. n=8 mice.
 - K. Experimental scheme. Adult MuSCs from *Tg:Pax7-nGFP;Myog^{ntdTom}* mice were cultured for 5 days and re-sorted based on GFP and tdTom fluorescence for further analysis.
 - L. RT-qPCR for key myogenic markers of cells isolated as per in K. n=4 mice.
- Data presented as mean \pm SEM. Two-tailed unpaired Student's t-test; ns, non-significant, p-value>0.05, * p-value < 0.05, ** p-value<0.01, *** p-value < 0.005, **** p-value<0.0001. GM, growth media; DM, differentiation media.

1157 **Figure 2. Single cell transcriptome signatures of activated EOM and TA MuSCs.**

- A. sc-RNAseq pipeline for *in vitro* activated adult MuSCs.
- B. UMAP visualization of EOM progenitors, EOM differentiating, TA progenitors, and TA differentiating clusters (left). Violin plots of the expression of myogenic markers *Pax7* (progenitor), *Myod* (committed) and *Myog* (differentiating) in each cluster (right).
- C. Expression plots of myogenic markers.
- D. Heatmap representing top differentially expressed genes in each cluster and expression levels across all cells.
- E-F. Reactome pathway (E) and GO Molecular function (F) network analysis on top 100 DEGs of each cluster. Pie charts represent relative contribution of each cluster to this ontology term.

1168 **Figure 3. Molecular signature of EOM and TA quiescent MuSCs.**

- A. sc-RNAseq pipeline for quiescent adult MuSCs.
- B. UMAP visualization of EOM and TA quiescent clusters.
- C-D. Violin plot (C) and expression plot (D) of *Myf5*, *Pax7*, *Myod* and *Myog*.
- E. Heatmap representing top differentially expressed genes in each cluster and expression levels across all cells.
- F-G. Venn diagrams of the overlapping differentially expressed genes between quiescent and global *in vitro* activated datasets for EOM (F) and TA (G).
- H-I. Expression plots of selected EOM (H) and TA (I) markers exclusive to Quiescence (Unique Q), common to quiescent and activated states (Conserved) and exclusive to activation (Unique Act) from the analysis in F-G.

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1180 **Figure 4. EOM MuSC activation is accompanied by ECM remodelling.**

- A. Violin plots of matrisome scores for each cluster of the *in vitro* activated sc-RNAseq dataset.
- B. Dot plot visualisation of differentially expressed matrisome components. ECM, extracellular matrix; A-P, affiliated proteins; PG, proteoglycans.
- C. Immunofluorescence for Fibronectin (FN1), Collagen I (COL1), Collagen IV (COLIV) and PDGFR β . Representative of N>2 different experiments. Scale bar 25 μ m.
- D. Western blot showing expression of CAV1, CAVIN1, MGP, MMP2, SPARC, IGFBP7 and H3 for normalization. Cells from n=3 mice pooled per lane.
- E-F. Violin plot (E) and UMAP (F) of *Pdgfrb* expression on *in vitro* activated sc-RNAseq dataset.

- 1189 G. Experimental scheme. MuSCs from adult *Tg:Pax7-nGFP* mice were activated *in vitro*, re-
 1190 sorted based on their PDGFR β expression and plated for further analysis.
 1191 H. Representative images of PDGFR β positive and negative EOM MuSC fractions stained for
 1192 MYOD, MYOGENIN and EdU as per in G. Scale bar 50 μ m.
 1193 I-L. Quantification of number of cells/cm² (I) and percentage of EdU⁺ (J), MYOD⁺ (K) and MYOG⁺
 1194 (L) cells. n=3 mice.
 1195 Data presented as mean \pm SEM. Two-tailed unpaired Student's t-test; ns, non-significant, p-
 1196 value>0.05, ** p-value<0.01, *** p-value < 0.005.
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1198 **Figure 5. Distinct gene regulatory networks underlie EOM and TA activation dynamics.**

- 1199 A. Top 4 regulon activity for each cluster of the *in vitro* activated adult sc-RNaseq dataset
 1200 overlaid onto UMAP representation.
 1201 B. Heatmap of Top 7 regulons in each cluster with activity level in each cell. Myod1_(+) appears
 1202 twice as it is a regulon of both EOM Diff and TA Diff subpopulations.
 1203 C. Transcription factor network highlighting top regulons of each cluster as well as common
 1204 regulons (35 regulons maximum). Proximity of the nodes in the network indicates a higher
 1205 number of shared edges, highlighting core modules.
 1206

1207 **Figure 6. EOM properties are retained despite several passages *in vitro*.**

- 1208 A. Scheme of isolation and passages of EOM and TA adult MuSCs. Cells from *Tg:Pax7-*
 1209 *nGFP;Myog^{ntdTom}* were cultured for 3 days (Act) and the entire wells passaged (P1, P2) every
 1210 3 days.
 1211 B. Normalized cell number ratios from experiment in A. Total cell numbers per well were counted
 1212 at day 3 (D3), days 5 (D5) and upon passages (P) and normalized on the averaged EOM cell
 1213 number in all wells at each time point. n=3 mice.
 1214 C. Ratio of tdTom⁺/GFP⁺ cells per time point obtained through FACS analysis of *Tg:Pax7-*
 1215 *nGFP;Myog^{ntdTom}* EOM and TA adult MuSCs at D5, P1 and P2. n \geq 3 mice.
 1216 D. RT-qPCR at D3 (Act, Activated), P1 and P2 on whole cell populations for key myogenic
 1217 markers, regulon TFs and matrisome genes identified in EOM progenitors isolated from adult
 1218 *Tg:Pax7-nGFP* mice. n=4 mice.
 1219 E-F. Western blot for TFs (E) and matrisome genes (F) on total P2 population from EOM and TA
 1220 MuSCs isolated from adult *Tg:Pax7-nGFP* mice. ACTIN and TUBULIN were used for
 1221 normalization of protein loading. n=3 mice.
 1222 G. Densitometric analysis of the Western Blots in E-F.
 1223 Data presented as mean \pm SEM. Two-tailed unpaired Student's t-test; ns, non-significant, p-
 1224 value>0.05, * p-value < 0.05, ** p-value<0.01, *** p-value < 0.005.
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1227 **Figure 7. EOM activated features are present during postnatal growth and can be modulated by**
 1228 **FOXC1.**

- 1229 A. Scheme of isolation of EOM and TA MuSCs from *Tg:Pax7-nGFP;Myog^{ntdTom}* from postnatal
 1230 day (P)7-P10 mice.
 1231 B. RT-qPCR on GFP⁺/tdTom⁻ cells. n>3 mice.
 1232 C-D. Immunostaining for FOXC1, tdTom (TOM) and DYSTROPHIN on cryosections from EOM
 1233 and TA muscles isolated from P10 (C) and adult (D) *Pax7^{CreERT2};R26^{tdTom}* mice. White
 1234 arrowheads point to PAX7+FOXC1+ cells. Black arrowheads point to PAX7+FOXC1-cells.
 1235 Scale bars 50 μ m.
 1236 E. Quantification of the experiment in D. n=4 mice.
 1237 F. Scheme of lentivirus transduction for FOXC1 gain of function (GOF) experiments on adult TA
 1238 MuSCs. The transduced cells (mCherry+) were re-isolated by FACS or directly analysed in
 1239 mixed cultures.
 1240 G-H. FACS plots of adult *Tg:Pax7-nGFP* TA MuSCs transduced with control (G) or GOF FOXC1
 1241 (H) virus at D5. Both viruses carry an mCherry reporter.
 1242 I. Western blot of transduced adult TA MuSCs for mCherry and Foxc1. VINCULIN was used for
 1243 normalisation of protein loading. n \geq 3 mice.
 1244 J. RT-qPCR of transduced adult TA MuSCs for Myogenic Markers, Regulon Transcription factors
 1245 (TFs) and key ECM proteins and regulators identified in EOM progenitors. n \geq 3 mice.
 1246 K-K'. Immunostaining of transduced adult TA MuSCs for FOXC1, EdU and Cell Mask Blue (CM-
 1247 Blue, cytoplasmic stain). Scale bar 100 μ m.

1248 L. Immunostaining of transduced adult TA MuSCs grown in differentiation media for 48h and
 1249 stained for MF20. Scale bar 100 μ m.
 1250 M. Quantification of the Fusion Index for the images in L. n=4 mice.
 1251 N-U. Quantification of cellular properties of adult TA MuSCs in mixed cultures containing
 1252 transduced and non-transduced cells. (N, R) Violin plots showing the single cell distribution of
 1253 FOXC1 fluorescence intensity. Bar plots of quantification of nucleus area (O, S), total number
 1254 of nuclei (P, T) and percentage of EdU+ nuclei (Q, U). For N-Q n \geq 4 mice, R-U n \geq 3 mice.
 1255 Dashed bar in P indicates total output in the mixed culture irrespective of FOXC1 expression.
 1256 V-Y. Quantification of live imaging of adult TA MuSCs in mixed cultures containing transduced
 1257 and non-transduced cells. Brightfield and red fluorescent channel images were acquired at 4
 1258 different timepoints (+12, +24, +36, +48 hours) post transduction. (V-Y) Quantification of the
 1259 relative abundance of mCherry+ and mCherry- cells (V) at 12h time point (t) and cell density
 1260 (V,W) at all timepoints from movie images. n=3 mice.
 1261 Z. Proposed model of the role of FOXC1 upon overexpression in TA MuSCs (TA GOF with re-
 1262 sorting; TA GOF w/o re-sorting) and endogenously in EOM MuSCs.
 1263 Data presented as mean \pm SEM. Two-tailed unpaired Student's t-test except in S,U where p values
 1264 were obtained using one-way ANOVA with Tukey's post hoc; ns, non-significant, p-value>0.05, * p-
 1265 value < 0.05, ** p-value<0.01, *** p-value < 0.005, **** p-value<0.0001. GM, growth media; DM,
 1266 differentiation media.
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1270 **Figure 8. RNA velocity reveals distinct population kinetics and potential key regulators of**
 1271 **progenitor maintenance.**

1272 A. Number of regulatory links between regulons and matrisome genes depending on the number
 1273 of top regulons in EOM and TA *in vitro* activated sc-RNAseq datasets.
 1274 B. Ratio of number of regulations of matrisome genes between EOM and TA adult *in vitro*
 1275 activated MuSCs. Maximal difference for top 5 first regulons.
 1276 C. Heatmap of top regulons in global *in vitro* activated sc-RNAseq dataset with activity level in
 1277 each cell.
 1278 D-E. Velocity streams overlaid onto a UMAP representation for EOM (D) and TA (E), along with
 1279 expression patterns of *Myog* and *Pax7* on *in vitro* activated adult sc-RNAseq dataset.
 1280 F-G. Heatmap of driver gene expression from the velocity streams in D-E.
 1281 H. Experimental scheme. MuSCs isolated from adult *Tg:Pax7-nGFP* mice were cultured for 3
 1282 days to obtain EOM and TA conditioned media (CM). Hindlimb MuSCs were left untreated
 1283 (control) or treated with CM from D1 to D4 post plating and pulsed with EdU 2h before fixation.
 1284 I-J. Quantification of number of nuclei normalised on initial cell number (I) and percentage of
 1285 EdU+ nuclei (J) of hindlimb MuSCs treated as per in H. N \geq 2 independent experiments with
 1286 n \geq 2 mice each. Different symbol per experiment.
 1287 K. Immunostaining of *Myf5^{nlacZ};Pdgfra^{H2BGFP}* P3 mouse head cryosections for β -galactosidase (β -
 1288 gal) and GFP. Scale bar 500 μ m. Higher magnifications in K'-K''. Scale bar 100 μ m.
 1289 L. Quantification of the number of β -gal+ GFP+ cells per mm² in head muscles. n=3 mice.
 1290 Data presented as mean \pm SEM. p values were obtained using one-way ANOVA with Tukey's post
 1291 hoc; ns, non-significant, p-value>0.05, ** p-value<0.01, *** p-value < 0.005, **** p-value<0.0001.
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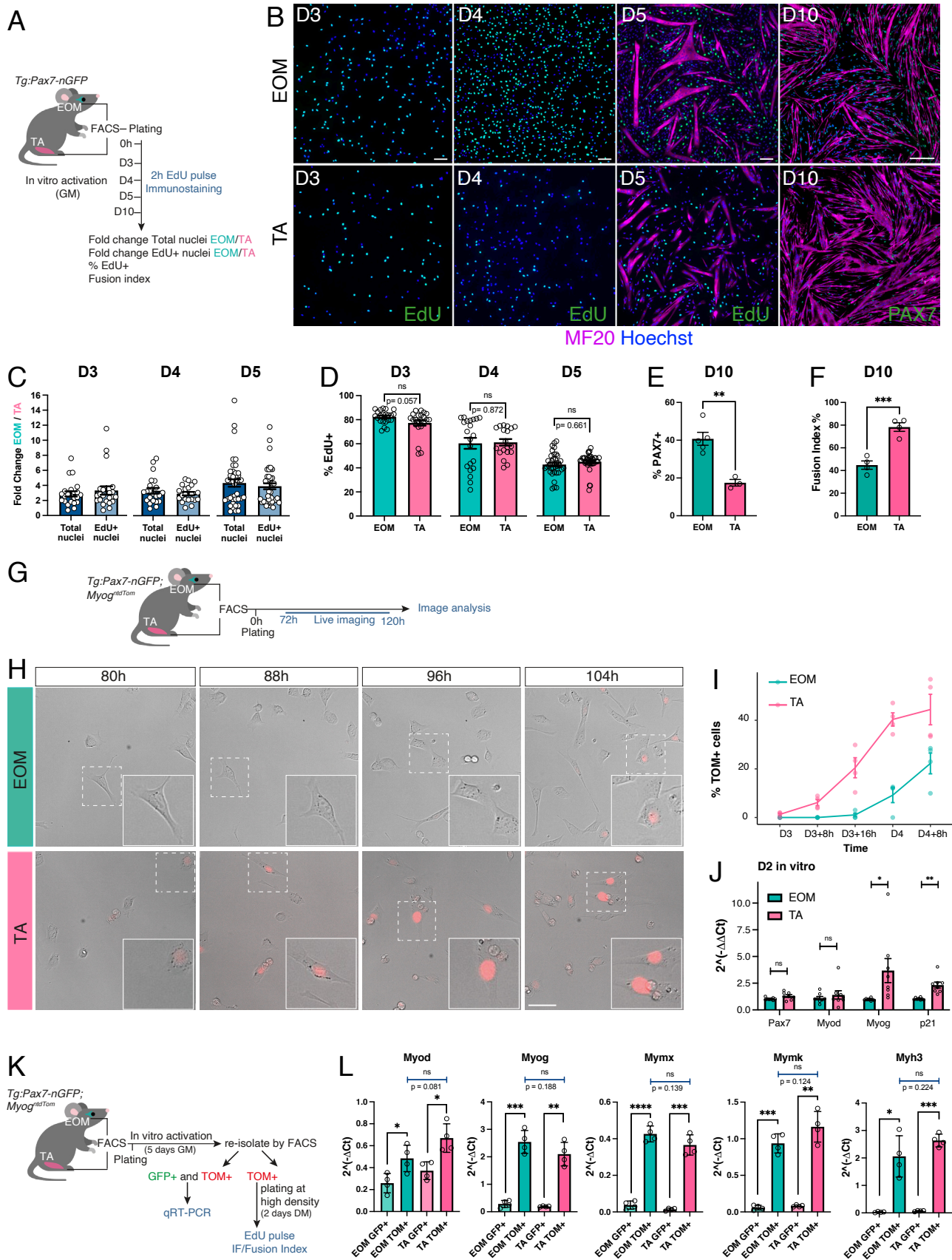


Figure 2

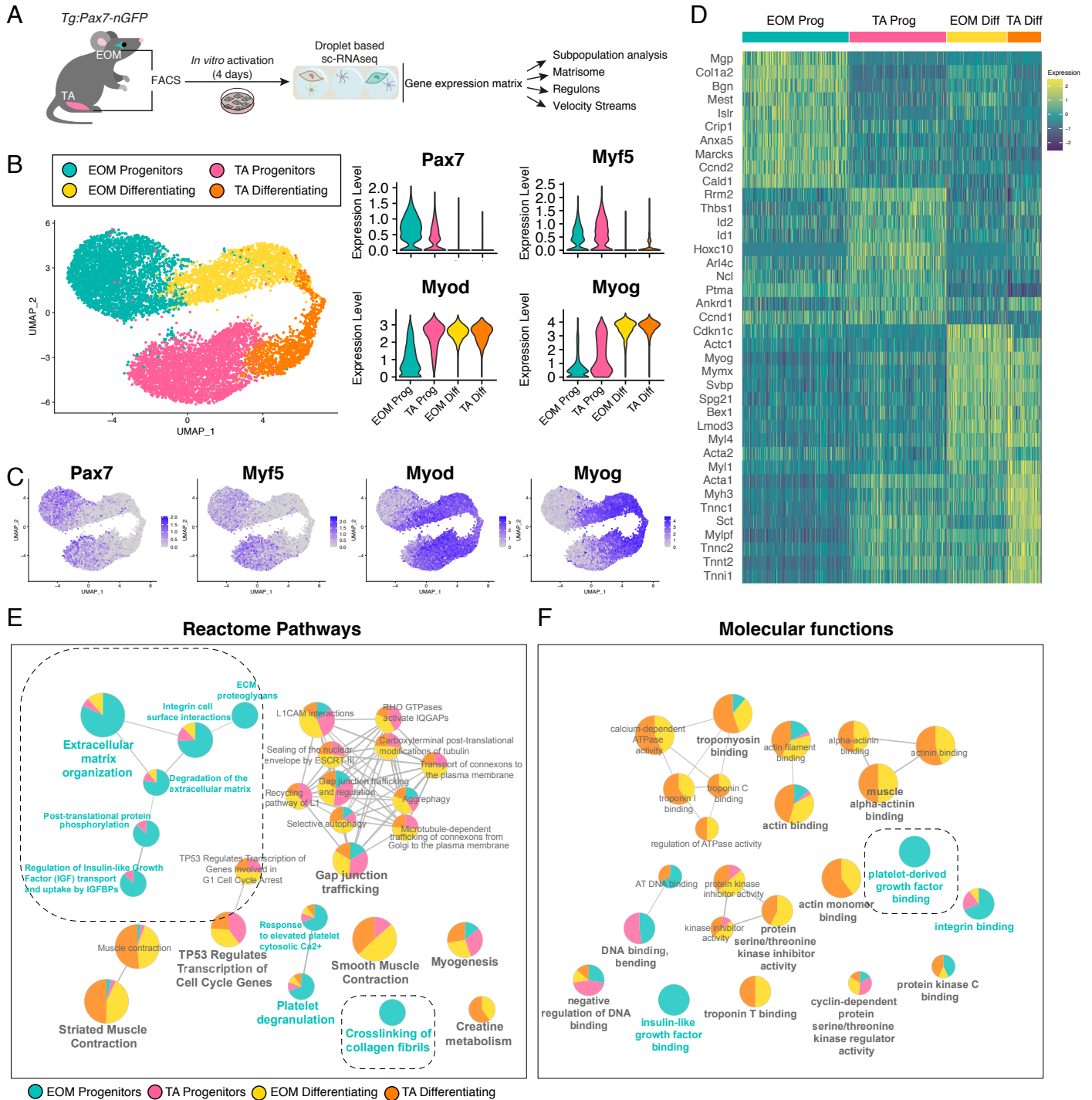
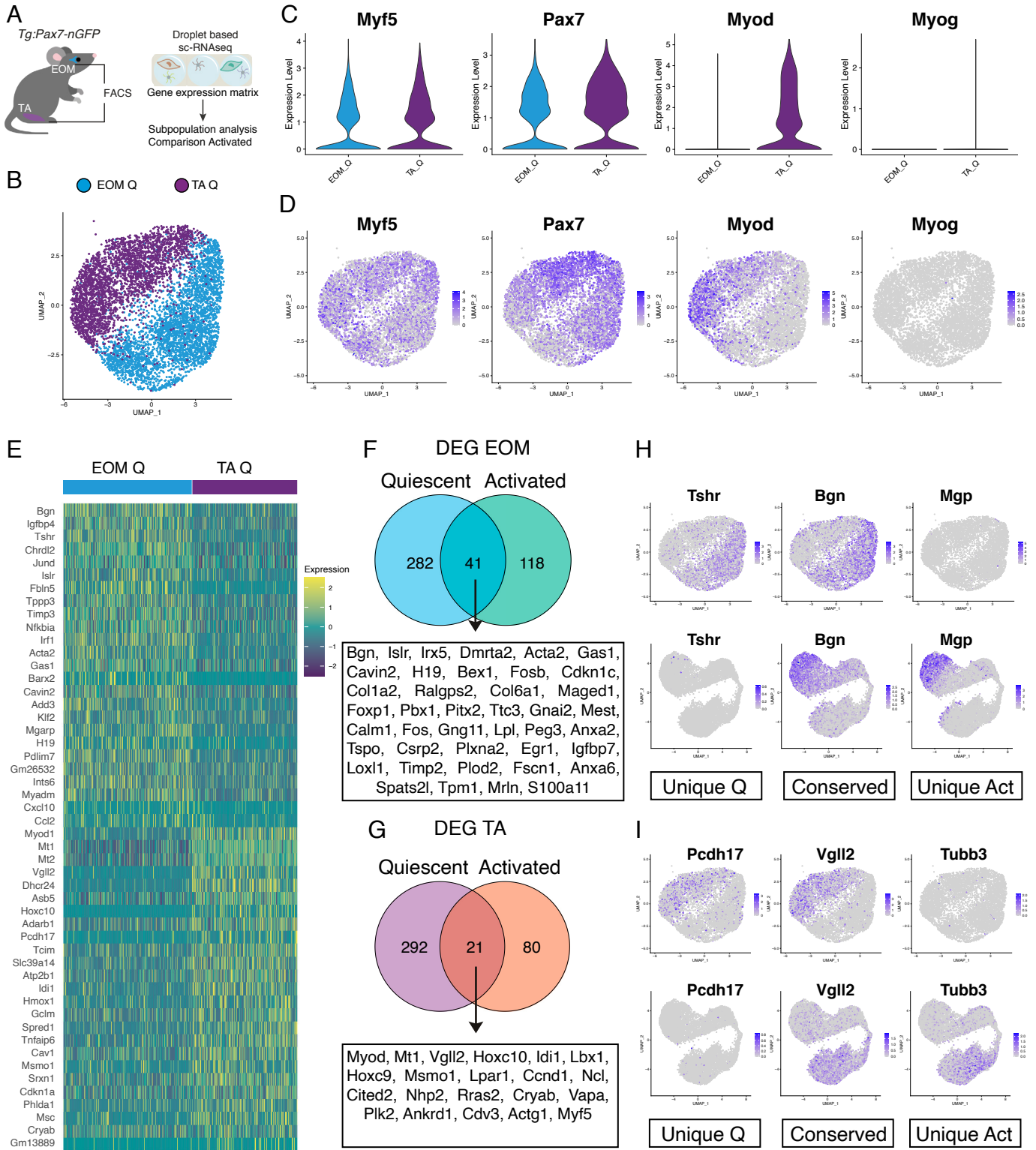


Figure 3



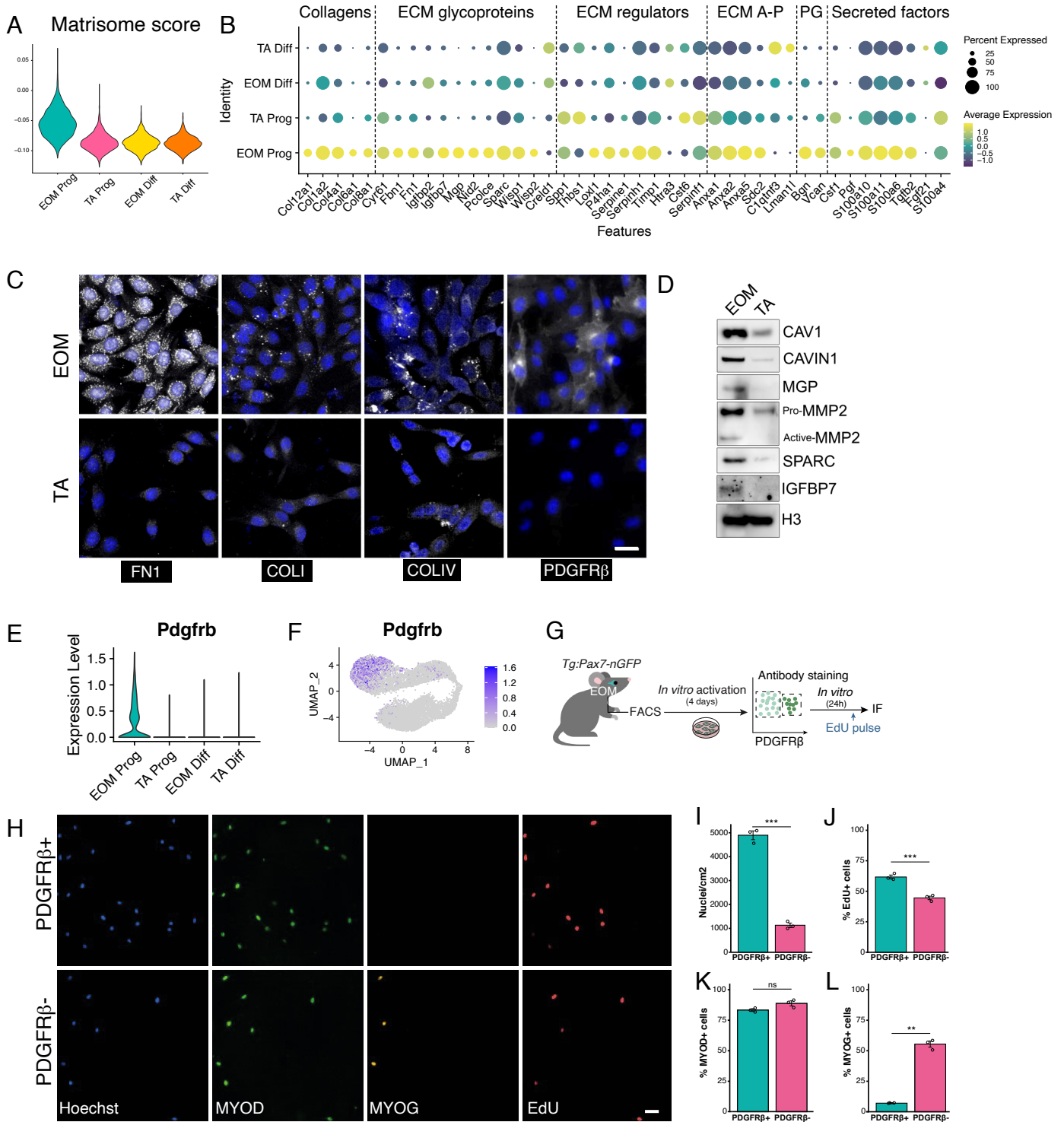


Figure 5

